日中笹川医学奨学金制度（学位取得コース）評価書
論文博士：指導教官用

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第 40 期　研究者番号：G4007　作成日：2020 年 3 月 10 日

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研究テーマ	腹腔鏡トレーニングシステムと消化管改良再建技術の連携により、完全腹腔鏡下幽門側胃切除術の有効性及び安全性についての分析				
専攻種別	☑ 論文博士		☐ 課程博士		

研究者評価（指導教官記入欄）

成績状況	優 良 可 不可	取得単位数 取得単位数／取得すべき単位総数
学生本人が行った研究の概要	完全腹腔鏡下幽門側胃切除術（TLDG）は複雑な手術である。その中にはリンパ節郭清術及び消化管再建は手術の難所で肝心なプロセスとなる。外科医の若手医師がいち早くこの手術を取得するために適切な、効率的な、安全な腹腔鏡トレーニングシステムの構築が必要と考えられる。本研究は、手術後の短期効果の分析により、TLDG 手術に適切な腹腔鏡トレーニングシステムが検討された。Augmented rectangle technique（ART）による Billroth-I 法で吻合した TLDG 手術を受けた 92 名の胃癌患者の分析が行われた。若手医師全員は腹腔鏡トレーニングを受け、手術の全過程をリンパ節郭清と消化管再建に分けて分析された。若手医師と内視鏡外科専門医の初期手術の結果比較を通じて、若手医師の手術の信頼性と安全性の分析が行われた。5 人の若手医師が合計 52 例（56.5%）、2 人の内視鏡外科専門医が合計 40 例（43.5%）の TLDG 手術実績を比較して、深達度とステージの差異を除いて、臨床病理学上の差異はみられなかった。内視鏡外科専門医より若手医師のほうがより多くの D2 胃切除手術を行い、総手術時間も長かった。胃の小彎側に沿ってリンパ節郭清と Billroth I 再建の手術時間、術後合併症に関して、2 組の間には相違がみられなかった。若手医師の学習曲線から見ると、平均的に 5 回の TLDG 手術以降、安定期に入るという結果になる。我々の研究結果はシステム化の腹腔鏡トレーニングの重要性を提示している。腹腔鏡手術手順の標準化及び簡単な ART 消化管再建術を採用することは、若手医師が安全かつ有効的に TLDG を行うのに役立つと考えられる。	
総合評価	【良かった点】 非常に研究熱心で、留学中に英語論文5本を作成また臨床でも多くの手術を積極的に参加。 【改善すべき点】 なし	

	【今後の展望】 当分野の非常勤の研究協力員として継続の予定。
学位取得見込	2020年4月 学位取得確定
	評価者（指導教官名） 福永 聡 印

日中笹川医学奨学金制度(学位取得コース)報告書
研究者用

Supported by 日本財団 THE NIPPON FOUNDATION

第40期　　研究者番号：　G4007　　作成日：2020年3月9日

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研究テーマ	腹腔鏡トレーニングシステムと消化管改良再建技術の連携により、完全腹腔鏡下幽門側胃切除術の有効性及び安全性についての分析 Effectiveness And Safety Of A Laparoscopic Training System Combined With Modified Reconstruction Techniques For Total Laparoscopic Distal Gastrectomy				
専攻種別	論文博士	☑	課程博士	□	

１．研究概要（1）

1）目的（Goal）

Total laparoscopic distal gastrectomy (TLDG) is increasing due to some advantages over open surgery, which has generated interest in all gastrointenstinal (GI) surgeons. However, TLDG is technically demanding, especially for the procedures of lymphadenectomy and GI reconstruction. During the course of training, trainee surgeons have less chances to perform open gastrectomy compared with that of senior surgeons.

2）戦略（Approach）

The characters, less chances to perform open gastrectomy and higher technical demands, make laparoscopic training procedure for young surgeons differ from what surgeon pioneer experienced previously. Appropriate and efficient training system suitable for current situation need to be urgently established.

3）材料と方法（Materials and methods）

3.1 Patients

We retrospectively studied patients with gastric cancer, who underwent TLDG plus Billroth I reconstruction at Juntendo University Hospital, Tokyo, Japan, from June 2016 and June 2019. Clinical, surgical, and pathological data of these patients were collected and analyzed.

3.2 Laparoscopic techniques

Laparoscopic gastrectomy was performed using a five trocar system. LN dissection was done according to Japanese gastric cancer treatment guidelines[3]. Dissection was conducted in the following order: infrapyloric LNs (No.6), suprapyloric LNs (No.5), great curvature LNs (No.4, or plus 12a), suprapancreatic LNs (No.8a, 7, 9, or plus 11p), and along lesser curvature LNs (No. 1, 3).

3.3 Billroth I reconstruction using augmented rectangle technique

For those needing Billroth I reconstruction, the augmented rectangle technique (ART) is applied,and all the procedures were created laparoscopically. The operator performed this technique on the left side of the patient. Three automatic laparoscopic linear staplers were used to create the gastroduodenostomy. The duodenum was transected from the greater curvature to less curvature.

3.4 Trainer and trainees

Seven operators were involved in this study. There were two trainers and five trainees. Two trainers were Endoscopic Surgical Skill Qualification System for gastric cancer accredited surgeons. Trainees had at least 7 years' experience as a surgeon after graduation.

3.5 Education system for laparoscopic gastrectomy

Our training system covers four parts: understanding of anatomy and standard procedures, practicing basic laparoscopic skills, performing simple laparoscopic surgery and providing focal points during laparoscopic gastrectomy.

3.6 Learning curve of the trainees

Two variables, operation time and intraoperative estimated blood loss, from patients who underwent TLDG by trainees were used to define the learning curve. Variables in each group were calculated as mean ± SD and then compared with that of those performed by the trainer surgeons.

3.7 Statistical analysis

Continuous data are presented as median and ranges. Independent-sample t test was used to analyze continuous data, and X2 or Fisher's exact tests was used to assess differences in categorical data. Statistical analysis was performed using the SPSS statistical software program (version 23). A $p < 0.05$ was considered significant.

１．研究概要（2）

4）実験結果（Results）

Five trainees performed a total of 52 TLDG (56.5 %), while 40 TLDG were conducted by the two trainers (43.5 %). Except for depth of invasion and pathology stage, there were no difference in patient clinicopathological characteristics. Trainers performed more D2 gastrectomies than trainees. The total operation time was significantly longer in the trainees. The time of along less curvature lymph nodes dissection and Billroth I reconstruction were similar between the two groups. No difference was found in postoperative complications between two groups. The learning curve of the trainees plateaued after 5 TLDG cases.

5）考察（Discussion）

Preparing trainees with a laparoscopic view of surgical anatomy, standard operative procedures and practice in essential laparoscopic skills enabled trainees to perform TLDG safely and feasibly. Making laparoscopic procedure standard and using the easy reconstruction method are useful in the success of the training system.

6）参考文献（References）

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2. 執筆論文 Publication of thesis ※記載した論文を添付してください。Attach all of the papers listed below.

論文名 1 Title	Internet videos and colorectal cancer in mainland China: a content analysis					
掲載誌名 Published journal	Mini-invasive Surgery					
	2018 年 12 月	18(129) 巻（号）	1 頁 ～ 6 頁	言語 Language	英語	
第1著者名 First author	張 順	第2著者名 Second author	Yao Yang	第3著者名 Third author	Dongyi Yan	
その他著者名 Other authors	Biao Yuan, Xiaohua Jiang, Chun Song					
論文名 2 Title	Current status of technique for Billroth-I anastomosis in totally laparoscopic distal gastrectomy for gastric cancer					
掲載誌名 Published journal	Mini-invasive Surgery					
	2019 年 1 月	3(2) 巻（号）	頁 ～ 頁	言語 Language	英語	
第1著者名 First author	張 順	第2著者名 Second author	福永 哲	第3著者名 Third author		
その他著者名 Other authors						
論文名 3 Title	Current surgical treatment of esophagogastric junction adenocarcinoma					
掲載誌名 Published journal	World Journal of Gastrointestinal Oncology					
	2019 年 8 月	11(8) 巻（号）	567 頁 ～ 578 頁	言語 Language	英語	
第1著者名 First author	張 順	第2著者名 Second author	折田 創	第3著者名 Third author	福永 哲	
その他著者名 Other authors						
論文名 4 Title	Sensitization of Gastric Cancer Cells to Irinotecan by p53 Activation					
掲載誌名 Published journal	BPB Reports					
	201 年9 12 月	2(6) 巻（号）	130 頁 ～ 133 頁	言語 La nguag	英語	
第1著者名 First author	張 順	第2著者名 Second author	小平 佳典	第3著者名 Third author	折田 創	
その他著者名 Other authors	伊志嶺 百々子, 小林 敏之, Sharlyn Mae Buendia Chua, 中岡 博史, 井ノ上 逸朗, 樋野 興夫, 横溝 岳彦, 福永 哲, 李 賢哲					
論文名 5 Title	Concerns of quality, utility, and reliability of laparoscopic gastrectomy for gastric cancer in public video sharing platform					
掲載誌名 Published journal	Annals of Translational Medicine					
	2020 年 月	8 巻（号）	1 頁 ～ 10 頁	言語 Language	英語	
第1著者名 First author	張 順	第2著者名 Second author	福永 哲	第3著者名 Third author	岡 伸一	
その他著者名 Other authors	折田 創, 加治 早苗, 夕部 由規謙, 山内 卓, 小平 佳典, 顕川 博芸					
論文名 6 Title	Effectiveness And Safety Of A Laparoscopic Training System Combined With Modified Reconstruction Techniques For Total Laparoscopic Distal Gastrectomy					
掲載誌名 Published journal	World Journal of Gastroenterology					
	2020 年 3 月	29（12） 巻（号）	頁 ～ 頁	言語 Language	英語	
第1著者名 First author	張 順	第2著者名 Second author	折田 創	第3著者名 Third author	顕川 博芸	
その他著者名 Other authors	松井 亮太, 山内 卓, 夕部 由規謙, 加治 早苗, 高橋 徹, 岡 伸一, 稲木 紀幸, 福永 哲					

3. 学会発表 Conference presentation ※筆頭演者として総会・国際学会を含む主な学会で発表したものを記載し

※Describe your presentation as the principal presenter in major academic meetings including general meetings or i

学会名 Conference			
演 題 Topic			
開催日 date	年 月 日	開催地 venue	
形式 method	□ 口頭発表 Oral □ ポスター発表 Poste	言語 Language	□ 日本語 ☑ 英語 □ 中国語
共同演者名 Co-presenter			

学会名 Conference			
演 題 Topic			
開催日 date	年 月 日	開催地 venue	
形式 method	□ 口頭発表 Oral □ ポスター発表 Poste	言語 Language	□ 日本語 ☑ 英語 □ 中国語
共同演者名 Co-presenter			

学会名 Conference			
演 題 Topic			
開催日 date	年 月 日	開催地 venue	
形式 method	□ 口頭発表 Oral □ ポスター発表 Poste	言語 Language	□ 日本語 ☑ 英語 □ 中国語
共同演者名 Co-presenter			

学会名 Conference			
演 題 Topic			
開催日 date	年 月 日	開催地 venue	
形式 method	□ 口頭発表 Oral □ ポスター発表 Poste	言語 Language	□ 日本語 □ 英語 □ 中国語
共同演者名 Co-presenter			

4. 受賞（研究業績）Award (Research achievement)

名 称 Award name					
	国名 Country name		受賞年 Year of	年 月	
名 称 Award name					
	国名 Country name		受賞年 Year of	年 月	

研究者番号：G4007

5．本研究テーマに関わる他の研究助成金受給 Other research grants concerned with your re

受給実績 Receipt record	■ 有　□ 無
助成機関名称 Funding agency	中国国家自然科学基金委員会
助成金名称 Grant name	中国国家自然科学基金(No. 81700452)
受給期間 Supported period	2018 年 1 月 ～ 2020 年 12 月
受給額 Amount received	3,500,000 円

6．他の奨学金受給 Another awarded scholarship

受給実績 Receipt record	□ 有　□ 無
助成機関名称 Funding agency	
奨学金名称 Scholarship name	
受給期間 Supported period	年 月 ～ 年 月
受給額 Amount received	円

7．研究活動に関する報道発表 Press release concerned with your research activities

※記載した記事を添付してください。 Attach a copy of the article described below

報道発表 Press release	■ 有　□ 無	発表年月日 Date of release	2019年4月5日
発表機関 Released medium	順天堂大学シミュレーションセンター		
発表形式 Release method	・新聞　・雑誌　■Web site　・記者発表　・その他（　　）		
発表タイトル Released title	【センター情報】ドイツ・シャリテ医科大学のセンター見学		

8．本研究テーマに関する特許出願予定 Patent application concerned with your research th

出願予定 Scheduled	□ 有　□ 無	出願国 Application	
出願内容（概要） Application contents			

9．その他 Others

指導責任者（署名） 福永 哲

BMC Medical Informatics and Decision Making

RESEARCH ARTICLE Open Access

Internet videos and colorectal cancer in mainland China: a content analysis

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Abstract

Background: Colorectal cancer incidence and mortality have been increasing in China and as one of the most important health problems facing the nation. Adequate dissemination of correct information about colorectal cancer could help in reducing cancer-related morbidity and mortality. This study aims to assess the completeness and reliability of colorectal cancer-related information available on the video website of Youku in mainland China.

Methods: Youku (https://www.youku.com/) was searched on September 15, 2016 for the search terms colorectal cancer. Only Chinese videos were included. Two reviewers independently evaluate the videos for characteristics, information source and usefulness. Content was analysed under six categories (aetiology, anatomy, symptoms, preventions, treatments and prognosis). Completeness was evaluated with a checklist developed by the researchers. Any discrepancies were resolved by consensuses. SPSS software was used to analyze data.

Results: There were 242 videos with relevant information about colorectal cancer. The type of source were as follows: independent users, 118 (49%); health information web sites, 60 (25%); medical doctors, 31 (13%); news network, 22 (9%); and hospital/university, 11 (4%). In all, 57% of videos had useful information about colorectal cancer, 21% were misleading. Videos posted by medical doctors ($P = 0.021$) and health information web sites ($p = 0.039$) were less incomplete than videos by independent users. Of the Traditional Chinese medicine (TCM) videos, 97 (76%) had information about treatments of colorectal cancer. 30% TCM videos contain misleading information, whose misleading rate was higher than total's (21%).

Conclusions: The colorectal cancer videos in mainland China represented by Youku varied base on ownership and content and information incompleteness were fairly high. It is necessary that professionals adapt to the advanced technology and think useful methods to solve the variable quality of information of internet video websites in mainland China.

Keywords: Colorectal cancer, Internet, Youku, Mainland China

Background

Cancer incidence and mortality have been increasing in China and have created a significant number of health concerns [1]. Colorectal cancer ranks the fifth most commonly diagnosed cancer among male and female in China [2]. The ratio of estimated new colorectal cancer mortality incidence is 50.8% in China for 2015 [2] compared with 36.3% in the United States for 2016 [3]. This considerably higher ratio means cancer prevention and control in China lags behind some Western countries.

Up to 31 December 2016, it was reported that 731 million Chinese internet users, and more than 695 million people were using mobile devices to quick browse online information. Over 570 million online video users accounted for three-quarters of total internet users [4]. Health and medical treatment has been the most popular science topics in mainland China [5]. Freely available video websites, such as YouTube, are popular sources of information dissemination with more than 100 million viewers every day [6]; however, YouTube is blocked in China because of Chinese internet censorship.

Chinese people prefer online video websites, such as Yoku, iQiyi, Sohu Tv or Tencent Video. Youku is the most popular source of video blogs and short original

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videos uploaded by individuals in mainland China [7]. Youku initially emphasized user-generated content. The average number of daily video views was 1.18 billion [8]. The number of monthly active users was over 500 million, and 60% of audiences were male [7]. Youku features the same kinds of videos on YouTube and is considered the largest Chinese video broadcast site. Similar to YouTube, the posted videos are not peer control, could be uploaded from different sources and are likely to be of variable quality [9].

Many studies reported that video broadcast sites have positive and negative effects on health information dissemination. Some videos can provide useful resources for knowledge and were used by medical students as a learning resource [10, 11]. Videos may promote misleading information, such as disparaging vaccinations [9] and describing ineffective or potentially dangerous natural therapies for gallstone disease [12]. Not only were audience attempting therapies that may be harmful, but they were not going in for accurate therapy, which can lead to other complications.

The use of video broadcasting sites as a source of information in disease areas, especially in colorectal cancer in mainland China, has not been evaluated. Thus, the present study aimed at evaluating the completeness and reliability of Chinese-language colorectal cancer-related information available on the video website of Youku in mainland China; assess the overall quality of online information on colorectal cancer; and share our thoughts on important future directions for managing information about colorectal cancer on websites of mainland China.

Method

We searched Youku (www.youku.com) on November 15, 2016 to locate video clips containing relevant information about colorectal cancer in human patients. The keyword "colorectal cancer" was used to identify related video clips. Videos that were duplicated, not in Chinese and not directly related to the investigated condition were excluded.

We included all unique videos with Chinese language content that contained any message about human colorectal cancer. All videos were downloaded and saved. We assessed each video according to the following characteristics: duration, ownership, number of views, video quality, and colorectal cancer content. Ownership was classified by medical doctor, hospital/university, news network, health information website or independent user.

All videos were viewed and analysed for content by 2 reviewers, and disagreements were resolved by an arbitrator. All researchers had medical background and specialized in gastrointestinal surgery. All researchers had finished their respective residencies at general hospital and had enough experience in the diagnosis and management of colorectal cancer. The reviewers classified the videos as useful, misleading or useless, as defined by the following: **useful**—containing scientifically correct information about any aspect of the disease: symptoms, treatment, and prevention; **misleading—** containing scientifically unproven information; **useless**—without containing the any aspect of colorectal cancer or addressing personal experience. If the video included trustable and misleading information at the same time, the videos were categorized as "misleading".

We assessed the quality of each video using a completeness score (Table 1). Two reviewers viewed each video in all content areas (aetiology, anatomy, symptoms, preventions, treatments and prognosis). At present, no validated tool for this purpose exists in the literature. Any disagreements were resolved with consensuses.

Traditional Chinese medicine (TCM) has been is deeply embedded in the populations of China and applied to the prevention and treatment of various diseases from ancient times until now. TCM is promoted and institutionalized by the Chinese government, has spread to more than 100 countries and has grown into an international industry [13]. For this reason, we also analysed TCM content regarding colorectal cancer in our study. Inter-observer agreement was evaluated with a kappa coefficient. Differences between groups were compared with a one-way ANOVA. Data analysis was performed with SPSS Version 16 Software. If the p-value is less than 0.05, the result was considered to be significant.

Table 1 Completeness checklist

Content	Description
Aetiology	Precancerous lesion
	Heredity
	Eating habits
Anatomy	–
Symptoms	Stool change
	Altered bowel habits
	Abdominal pain
	Abdominal mass
	Systemic symptoms
Preventions	Screening
	Daily habits
Treatments	Surgery
	Chemotherapy
	Radiotherapy
	Traditional Chinese medicine
Prognosis	TNM stage
	Perioperative treatments
	Others

Result

A Youku search revealed 348 videos for colorectal cancer. Videos were removed for a variety of reasons (Table 2). Video duplication and not being in Chinese were the two main reasons. Of the 348 videos screened, 242 videos met the inclusion criteria.

Ownership

A total of 49% of the videos were posted on the website by independent users. Health information websites were responsible for uploading 25% of the total videos. The videos contributed by medical doctors were only 13% but higher than other owner videos by max viewership and mean viewership (Table 3). This difference among groups was statistically significant ($p < 0.05$).

Information reliability

The 242 included videos were classified as useful (136 [57%]), misleading (51 [21%]), and useless (55 [22%]) according to medical content (Table 4). The kappa coefficient statistics of agreement of these videos was 0.88.

The number of videos containing misleading information was 51. A large part (41 [80%]) were amateur videos about personal experiences and emotions. The mean duration of the videos was 4.0 min with no significant differences between useful and misleading videos or between useful and irrelevant videos ($p < 0.05$).

Content

Useful videos were analysed based on the information they contained. In all of the categories, treatments were the most frequently covered topic (70%), followed in descending order by symptoms (33%), prognosis (26%), anatomy (20%), preventions (15%) and aetiology (11%). Table 5 shows the information completeness scores. Videos by medical doctors ($p = 0.021$) and health information websites ($p = 0.039$) sources were significantly more complete than those posted by independent users.

Traditional Chinese medicine

There were 128 videos containing TCM from diagnosis to treatment. Of the TCM videos, 97 (76%) had information about treatments of colorectal cancer. Among these videos, 10 included TCM and Western medicine at the same time. The information reliability is shown in Table 6. Medical doctors and university provided more reliable information than others ($p < 0.05$). A total of 30% TCM videos contain misleading information, and this misleading rate was higher than the totals (21%). Among the videos containing both TCM and Western medicine, the misleading rate was as high as 90%. Most of the videos exaggerated the actual effect of TCM and understated therapies, except for the health information websites mean viewership (798: 895). The other sources' mean viewership in TCM were higher than those containing both TCM and non-TCM videos.

Table 2 Reasons for excluding videos

Reason for exclusion	No.
No audio	1
No video	2
Not in Chinese	15
Not related to subject	3
Duplicate	85
Total exclusions	106

Discussion

Colorectal cancer ranks as the fifth leading cause of cancer death among both male and female in mainland China. Because the population of China accounts for one fifth of the global world, colorectal cancer cases in China account for 22% of all newly diagnosed cases and 27% of all deaths from worldwide [14]. The effectiveness of prevention, early detection, and management of colorectal cancer is not only important for China but also for the world.

Internet video websites can provide useful diagnostic, treatment and preventative medical services information. Previous research has evaluated YouTube as an important source of information on disease topics [15]. Although YouTube is blocked due to many reasons in mainland China, there are many similar internet video websites delivering the same functionality, such as Youku. To the best of our knowledge, no study has been performed to assess the accuracy and usefulness of internet videos as a source of healthy information for colorectal cancer in mainland China.

In this study, we selected Youku.com as the target video website, which is ranked the largest Chinese video broadcast site. The website of Youku not only focuses on professionally produced videos but also emphasizes user-generated content. The monthly unique visitors of Youku were 2,6376,000,000 according to the data of October in 2016 [7].

Our study demonstrates approximately 242 videos addressing colorectal cancer were provided by different sources. Independents users represent the greatest number of sources. The content was mainly about personal experiences in surgical procedures or hospital stays. Our results also show that Chinese medical doctors and health related institutions comprising 17% of colorectal cancer videos do not pay sufficient attention to the platform for the distribution of information. Doctors in china frequently experience work overload, tend to work overtime and experience energy deficiencies, which seem to be one of reasons for this phenomenon [16]. The videos that were viewed most often were the videos posted

Table 3 Sources and classification of detected videos

Source	Total videos	Max viewership	Min viewership	Mean viewership
Independent user	118	53,455	8	10,621
Health information web site	60	60,234	167	15,390
Medical doctor	31	61,132	12,583	23,893
Hospital/University	11	20,343	670	8783
News network	22	57,890	4791	9321

by doctors followed by health information websites. This indicates that people are more interested in a professionals experience regarding disease rather than their peers.

As the content of most videos often lacks peer or institutional quality review, many may not be subject to quality controls and may not be evidence-based; thus, it is not surprising that a majority of this content is misleading or irrelevant. According to previous studies, the dissemination of inaccurate information by video websites differs from diseases. A total of 56.5% of the video information on cholecystolithiasis [11], 16.2% on H1N1 influenza [17] and 1.6% on acute appendicitis in children [18] on YouTube were misleading. In our study, it was demonstrated that only one-fifth of website videos contain no scientifically oriented information. Only 36% of the independent users videos reviewed were considered to be useful compared with 90% useful doctors' videos.

The most commonly watched videos from independent users were those that contained misleading information, while the lowest number of views were from medical doctors and health information websites. These results also indicated that effective regulatory measures are needed to control scientifically accredited information. If misleading videos were less viewed by audiences, the harm might be reduced.

Regarding videos addressing colorectal cancer, it is highly difficult for laypeople or patients to distinguish between useful videos or those containing no accurate information. Our result indicates that an important element to assess the reliability of videos regarding colorectal cancer may be the ownership. If academic institutions represent the source, such as hospital/university or medical doctors, the videos may be regarded to be trustworthy on the basis of content [19]. The result is similar to those of other studies conducted outside of mainland China [15, 16].

We found that the average completeness scores were only 18% with a combination of aetiology, anatomy, symptoms, preventions, treatments and prognosis. Most of the included website videos only contained one of the above-mentioned categories. In all of the categories, treatments were the most frequently covered topic (70%). It is unlikely to expect all videos to comprehensively cover all aspects of colorectal cancer; therefore, it should be deemed that some videos, whilst incomplete, do contain precise and valuable content. Our results indicated that videos from medical doctors and health related institutions have significantly higher completeness scores than those posted by independent users. This result may suggest that videos posted by layperson mainly aim a more social goal and videos posted by health and medical organizations commonly take a more educational purpose. The study indicated that professionals should utilize their expertise and contribute to more high-quality videos for patients as information sources in mainland China.

When video contents were analyzed, the most universal topic were the treatment aspects of the colorectal cancer. This finding may indicate that most publishers thought that treatment factors are the most important component of colorectal cancer. Surgery, chemotherapy and radiotherapy have been the mainstay of colorectal cancer treatment. Approximately 70% of videos contained one of the above subjects. As the country of origin and application of TCM, China has a unique TCM theoretical system and effective treatment methods. In mainland China, TCM has been recognized as additional

Table 4 Sources and classification of detected videos

Ownership	Total Videos	Useful (Mv)	Misleading (Mv)	Useless (Mv)
Independent users	118	43 (7689)	41 (18123)	34 (5283)
Health information web site	60	48 (17517)	4 (895)	8 (9876)
Medical doctors	31	28 (24588)	1 (12583)	2 (19812)
Hospital/University	11	11 (8783)	0 (–)	0 (–)
News network	22	6 (7302)	6 (4567)	11 (12583)
Mean duration (min ± SD)	4.0 ± 2.3	5.5 ± 3.7	4.3 ± 2.1	3.7 ± 3.1

Abbreviation: *SD*, standard deviation; Mean viewership: *Mv*

Table 5 Completeness score

Completeness score	No	Mean ± SD
Aetiology	15	1.53 ± 0.51
Anatomy	27	–
Symptoms	43	2.77 ± 1.23
preventions	21	1.24 ± 0.44
Treatments	95	2.07 ± 0.83
Prognosis	35	1.77 ± 0.69
Total (max = 17)	136	3.07 ± 1.94

treatment methods for colorectal cancer [20]. Our study shows that approximately 128 videos were about the anticancer properties of traditional Chinese medicine.

In oncology, TCM is believed to have great healing properties such as exerting specific anticancer activity or chemosensitisation to help in the individualization of anticancer treatment [21, 22]. Chinese cancer patients frequently believe that herbs of TCM can help them against suffering from complications and to live well. Doctors trained in Western medicine published fewer videos than doctors trained in Chinese medicine. However, 30% of TCM videos contained misleading information that exaggerated actual effects and propaganda error messages, such as curing colorectal cancer. The highest total and misleading number of videos were posted by independent users. The meanest viewership was also from independent users. The misleading rate was higher than total misleading rate (21%). There have been a large number of controlled clinical studies published in Chinese literature, but high-level evidence for the clinical efficacy of TCM is still lacking [23]. Mistakes were often found in professorial papers and in internet videos.

Colorectal cancer is characterized by high prevalence, a long asymptomatic period and eminently treatable precancerous lesions which, taken together, suggests that screening is a prudent option in mainland China [24]. For this reason, facilitating the earlier diagnosis of colorectal cancer may have a more immediate impact on the existing cancer burden in mainland China. A total of 21 videos contained colorectal cancer screening, which represented only 15% of all useful videos. Almost all screening videos address the importance of a Faecal Occult Blood Test, digital rectal exam, and colorectaloscopy.

Despite the rising colorectal cancer incidence, public awareness is still low in mainland China. Chinese internet websites, such as Youku, provide a different medium to disseminate colorectal cancer information to the public by video instead of written text. The written healthy information is commonly at a considerably higher reading level for Chinese patients. This video-based information source can help them and their caregivers get better understanding. Use of the internet for colorectal cancer information is likely to increase. It is necessary that professional individuals and academic institutions adapt to the advanced technology and think useful methods to solve the variable quality of information uploaded on internet video websites in mainland China. To maximize the potential of video-based information and minimize the quantity misleading or unhelpful information, multilateral efforts between doctors, governments and websites are needed.

Limitations

First, the main bias of our study was the subjectivity of judgement. There were no validated tools for assessing video data. Therefore, our classification method was subjective. However, the kappa statistic indicated quite high agreement between two reviewers. Second, there was no website, such as YouTube, with a clearly dominant position in China. Selecting only one Chinese video website's data may lead to some bias. Youku was the most popular website and had the largest audience in China. Youku in mainland China may still reflect the reliability of information available on video websites. Third, our results comprise a snapshot of information distribution to illustrate the quality of internet video at one point in time in China mainland, and these results may change according to the videos that can be added or removed with time.

Table 6 Treatments of Traditional Chinese medicine

	Total videos	Useful (Mv)	Misleading (Mv)	Useless (Mv)
Independent users	62	23 (13532)	35 (20021)	4 (6577)
Health information web site	24	16 (23122)	3 (798)	5 (1201)
Medical doctors	7	7 (27349)	0 (–)	0 (–)
Hospital/University	1	1 (13653)	0 (–)	0 (–)
News network	3	1 (12021)	0 (–)	2 (13216)

Abbreviation: Mean viewership: *Mv*

Conclusions

Colorectal cancer videos represented by Youku in mainland China varied significantly by ownership and content and information incompleteness were fairly high. It is necessary that professionals adapt to the advanced technology and think useful methods to solve the variable quality of information uploaded on internet video websites in mainland China.

Abbreviation

TCM: Traditional Chinese medicine

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Availability of data and materials

The datasets used and/or analysed during this study are available from the corresponding author upon reasonable request. All the video were from Youku (www.youku.com) on November 15, 2016. Because everyday many new videos can be uploaded in the internet. Maybe now the number of videos have been changed in the website.

Authors' contributions

SZ, CS and XHJ developed the idea for the paper and led the development of the paper. SZ and YY conducted the data searches in Internet. SZ, DYY and BY extracted relevant data and analysis data. XHJ, CS and YY critically reviewed the manuscript for important intellectual content. SZ did the structure and writed the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study did not require approval by the local Research Ethics Board as it involved publicly available data only.

Consent for publication

Not applicable.

Competing interests

The authors' declare that they have no competing interests.

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Mini-invasive Surgery

Review

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Current status of technique for Billroth-I anastomosis in totally laparoscopic distal gastrectomy for gastric cancer

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Abstract

Several reconstruction techniques are possible after totally laparoscopic distal radical gastrectomy. An optimal technique of digestive tract reconstruction after distal gastrectomy has not yet been established. The ideal reconstruction should be not only for doctors but also for patients. Alimentary intake, satisfactory nutritional status and easy performing should be all considered. The aim of the study was to describe the different Billroth-I reconstruction techniques that can be proposed after totally laparoscopic distal radical gastrectomy.

Keywords: Billroth-I anastomosis, totally laparoscopic distal gastrectomy, gastric cancer

INTRODUCTION

In 1994, Kitano firstly reported the technique for laparoscopy assisted Billroth-I (B-I)[1]. Since then, the use of laparoscopic treatments for gastric cancer is increasing due to the advantages of improving patients' quality of life. The new technologies and improved techniques have allowed laparoscopy gastrectomy to expand its indications and also to use this treatment for more complex cases. Japan Society of Endoscopic Surgery (JSES) conducted national survey every 2 years and indicated the percentage of laparoscopic procedures for gastric cancer was increasing. According to the 12th JSES survey, laparoscopic distal gastrectomy (LDG) was the most commonly performed type of laparoscopic gastrectomy[2].

In initial series for LDG, the majority of anastomoses were performed by laparoscopy assisted procedures.

The assisted procedures needed a mini-laparotomy incision of 60-70 mm in length made on the epigastrium[3]. But this procedure was not always easy to do, especially on patients with a small remnant stomach or obese patients with thick abdominal walls[4]. Anastomosis in such restricted space was usually difficult. With the accumulation of laparoscopic surgery experience and the development of laparoscopic devices, the gastrointestinal reconstruction now can be completed laparoscopically. Furthermore, unnecessary manipulations and the incision made on the epigastrium can be avoided.

The ideal reconstruction should be not only for doctors but also for patients. Alimentary intake, satisfactory nutritional status and easy performing should be all considered[5]. The B-I anastomosis is preferred by many doctors. It is said that the B-I anastomosis is simple and can provide a physiological route for food digestion and absorb without the need for an intestinal bypass or blind loop. Until now, various intracorporeal B-I anastomosis techniques were reported. In this article, we will review theses reconstruction methods.

HAND-SEWN ANASTOMOSIS IN INTRACORPOREAL B-I RECONSTRUCTION

After the accumulation of operative experience, some experienced surgeons had also presented intracorporeal hand-sewn techniques.

Takiguchi *et al.*[6] firstly reported B-I intracorporeal hand-sewn anastomosis in 2003. In his study, the Albert-Lembert method was used for the laparoscopic hand-sewing procedure and the anastomosis time was 90 min. Due to the complexity of the procedure and large amount of time required for anastomosis, it seemed that the hand-sewn anastomosis was not widely performed.

After almost 10 years, Matsuo *et al.*[7] reported another study about hand-sewn B-I anastomosis. They performed hand-sewn gastroduodenal anastomosis in 18 cases. The mean time of B-I anastomosis was 64.6 min. Matsuo *et al.*[7] described that 3-0 absorbing thread was placed in the lesser curvature as a supporting thread. A seromyotomy of the stomach was performed at the posterior wall. Both the remmant stomach and the duodenum's seromuscular layer were discontinuously sutured by extracorporeal knot-typing method. The lumen was opened with the stomach and the duodenum in a fixed status. The thread of the anchor suture was lifted upward to the abdominal wall. After that all layers of the stomach and the duodenum at the posterior wall were continuously sutured. The authors believed that hand-sewn anastomosis had some advantages. Hand-sewn sutures were not affected by the degree of freedom of the duodenum. Because staplers were not used, the anastomosis area was soft and highly flexible. The hand-sewn anastomosis was economical due to that less staplers were used.

CIRCULAR STAPLER USED IN INTRACORPOREAL B-I RECONSTRUCTION

In the open surgery, circular stapler is well applied as a standardized reconstruction method of gastroduodenostomy. However, when it was attempted laparoscopically, the situation was often the opposite.

Uyama *et al.*[8] firstly described intracorporeal B-I reconstruction using a circular stapling device and introduced one case in 1995. The method was defined by the same anatomic parameters as for the open B-I. After that, Moriya *et al.*[9] and Mayers and Orebaugh[10] also reported B-I gastroduodenostomy with a circular stapler device. Both techniques were complicated and difficult to operate, and especially at the left subcostal area where an extended incision was needed. The extra incision spoiled the merit of minimally invasive surgery.

There are 2 major difficulties when circular stapler is applied in laparoscopic gastroduodenostomy: the first is the lack of a safe and fast intracorporeal purse-string suture technique and the second is the difficulty

in manipulating the stapler and the stomach in a narrow abdominal cavity. In order to enable the anvil placement into the dudenum, many strategies were applied, such as a triple stapling procedure[8] and the use of the natural pyloric ring with endo-looping of the duodenum[9]. Some techniques usually used in esophagoenteral anastomosis were also reported, such as using specially modified laparoscopic purse-string instrument[11] and opening the lumen and applying manual purse-string suture[12]. However, there are still many difficulties to be overcome.

Kim *et al.*[13] reported a method which seemed to be quick and economical. The atraumatic clamps were used to prevent slippage of the duodenum which was cut with ultrasonic shear instead of linear stapler. After that, a seromuscular suture was done around the duodenal outer layer along the clamp. Omori *el al.*[14] reported a method like reverse puncture technique used in total gastrectomy. The anvil secured with vicryl suture was inserted into the duodenum through semicircumferential duodenotomy. The needle was advanced to the anterior duodenal wall and then the duodenum was staple-transected. Finally, the center rod penetrated the duodenal wall. In this method the need for purse-string suture placement was totally eliminated.

Although the skill inserting anvil head in the duodenal stump can be improved, laparoscopically inserting the circular stapler into the remnant stomach was not always easy. After removing two-thirds to three-quarters of the stomach, the small remnant stomach was usually so small that it was difficult to insert the stapler, even from the epigastric region. Sometime it was very difficult to form a straight line among the duodenum, remnant stomach and the circular stapler from the umbilical wound. Omori *el al.*[15] described a novel method to insert the circular stapler to connect the anvil head. Firstly, the anvil head was passed through the posterior gastric wall with laparoscopic endloop, which can make the duodenum and remnant stomach form a straight line. Secondly anterior gastric suture was used to exteriorize the anvil shaft partly from the gastrotomy. And then the anvil shaft was advanced into the remnant stomach to make the anvil and the stapler join tighter.

LINEAR STAPLER INTRACORPOREAL B-I RECONSTRUCTION

Delta-shaped anastomosis and modified delta-shaped anastomosis

With the development of laparoscopic instruments and the continuous accumulation of surgical experience in recent years, linear stapler intracorporeal gastrointestinal anastomosis techniques have been developed.

Kanaya *et al.*[16] firstly reported a anastomosis method which used only laparoscopic linear staplers in the hope of overcoming the drawbacks of extracorporeal reconstruction. The method named delta-shaped anastomosis (DA) was a modified intracorporeal B-I reconstruction which was soon promoted. The emergence of the DA method made intracorporeal gastroduodenostomy possible, which greatly promoted the development of totally laparoscopic distal gastrectomy (TLDG). Utilization DA method allows gastroduodenal anastomosis with a diameter of at least 30 mm while avoiding stricture. Kanaya *et al.*[17] analyzed the result of initial 100 procedures and showed that the mean time of the anastomosis was 13 min and the rate of anastomosis related complications was rare in 2011.

But some surgeons worry about the blood supply affected during cutting, which would result in leakages ranging from 0.42% to 8.5%[17-20] and anatomical distortion which exist in twisting around the anastomosis[21]. In order to overcome the twisting around the anastomosis, some modified delta-shaped techniques were studied.

Huang *et al.*[22] reported modified DA in 2014. This was different from the conventional DA in closing the common stab incision of stomach and duodenum. In order to avoid the poor blood supply of the duodenum, the duodenal cutting was totally resected. The appearance of the anastomoses was changed

from two intersections to only one as an inverted T-shape, which could decrease the anastomotic weak point. They reported comparable postoperative outcomes and showed that modified DA was technically safe and feasible[23] in another study.

After DA, many surgeons develop many other anastomosis methods based on linear stapler.

Triangulating stapling technique

Tanimura *et al.*[24] described the triangulating stapling technique based on a linear stapling device in 2008. The mean anastomotic time was 35 min. In this method, the duodenum can be transected in any direction, and by forming a triangle, the anastomosis lumen is made wide with no ischemic areas. Both stumps of duodenum and remnant stomach were opened fitting their caliber, the gastroduodenostomy linear stapler in the posterior wall and 2 everted sutures in the anterior wall with linear staplers. Before each direction anastomosis, both duodenum and remnant stomach were elevated ventrally with 3 stay sutures. But there are still some problems about this method. There were some differences between the stomach and duodenum in terms of lumen size, wall thickness, and wall extensibility. The first introverted anastomosis, which forms the base of the triangle, was cumbersome once all of the staple lines on the stomach and duodenum had been cut off.

Book-binding technique

Ikeda *el al.*[25] described the book-binding technique using linear stapler in 2012. The mean anastomotic time was 34 min. In their method, the duodenum was transected form the greater curvature side to the lesser curvature side. Small openings are made in the remnant stomach and duodenal stumps just wide enough to insert one of the jaws of the linear stapler. After the first stapling, there were three staple lines including those from the transection of the stomach and duodenum, which ran in parallel to the anterior wall. To prevent the formation of ischemic areas, a large opening was created on the anterior wall by transecting the entire duodenal stump and one-third of the gastric stump together with the anterior wall of the first anastomosis line. The anterior hole was then fired by linear stapler twice to close the large opening. Because a large opening was created on the anterior wall by transecting tissue and anastomosis line, maybe some tension was generated after the anterior hole was closed by the linear stapler. Further studies need to be done.

Linear-shaped gastroduodenostomy

Byun *et al.*[26] developed a linear-shaper gastroduodenostomy method by which the appearance of anastomosis was completed inverted T-shaped in 2009. Duodenum was transected from the greater curvature side to less side. One incision was done in the greater curvature of remnant stomach at the point 60 mm apart from the resected line. The other incision was done on the superior edge of the duodenal transection line. After creating the c anastomosis lumen, the common entry incision was closed by laparoscopic linear staplers. Finally, the greater curvature of stomach and the antero-superior of duodenum were perpendicular. By using this method, the rotation duodenum and remnant stomach was not needed which can reduce the risk of poor vascular supply. In their study, there were less bile reflux, gastritis degree and residual food grade compared to DA anastomosis in 6 months after surgery.

Augmented rectangle technique

In our group, we developed a method named augmented rectangle technique (ART) anastomosis. Three automatic laparoscopic linear staplers were used to create the gastroduodenostomy and the anastomotic opening was wide and less likely to become stenosed or twisted[27]. This method was easy and time-saving. We performed 160 LDG operations using this technique from December 2013 to August 2017. There were no postoperative complications associated with the reconstruction, such as anastomotic leakage, hemorrhage or stenosis. In the ART method, the duodenum was transected form the greater curvature side to less side. Small incisions were made in duodenal stumps and the greater curvature of remnant stomach in order to insert the jaws of the linear stapler. After inserting the stapler, the lesser curvature

Table 1. Summary of different methods applied in intracorporeal Billroth-I reconstruction

Year	Author	No.	Age	Method	Anastomotic time (min)	Operative time (min)	Blood loss	Postoperative stay (d)	Anastomosis-related complations
				Hand-sewn anastomosis in intracorporeal B-I reconstruction					
2003	Takiguchi *et al.*[6]	1	50	Hand-sewn	90	420	NS	7	0
2012	Matsuo *et al.*[7]	18	NS	Hand-sewn	64.6	NS	53.1 ± 91	21.7	0
				Circular stapler used in intracorporeal B-I reconstruction					
1995	Uyama *et al.*[8]	1	56	CS	NS	318	NS	14	0
2012	Kim *et al.*[13]	23	60.3 ± 11.3	CS	43.3 ± 15.4	209.7 ± 49.9	72.6 ± 47.9	7.7 ± 2.3	0
2012	Omori *et al.*[15]	20	NS	CS	NS	279	NS	9	0
				Linear stapler intracorporeal B-I reconstruction					
2011	Kanaya *et al.*[17]	100	65.5 ± 9.3	DA	13.0 ± 3.9	239.2 ± 53.2	92.6 ± 89.7	16.7 ± 13.8	1 (anastomotic leak)
2014	Okabe *et al.*[20]	185	NS	DA	NS	283	NS	NS	5 (anastomotic leak) 3 (delayed gastric emptying)
2011	Noshiro *et al.*[19]	71	70 ± 10	DA	NS	260 ± 56	63 ± 79	NS	6 (anastomotic leak)
2014	Huang *et al.*[22]	102	60 ± 12	Modified DA	12.2 ± 4.2	150.6 ± 30.2	48.2 ± 33.2	12.0 ± 6.5	2 (anastomotic leak)
2008	Tanimura *et al.*[24]	196	NS	TST	28 ± 4	249 ± 38	NS	NS	1 (anastomotic leak)
2013	Ikeda *et al.*[25]	9	59.3	BBT	34 ± 7	255 ± 13	50 ± 66	14.2 ± 2.3	0
2016	Byun *et al.*[26]	190	57.2 ± 12.5	LSGD	NS	147.9 ± 49.4	97.3 ± 95.7	6.8 ± 3.1	2 (anastomotic stenosis)
2018	Fukunaga *et al.*[27]	160	69.5 ± 10	ART	NS	227 ± 75	47.3 ± 50	12 ± 5	0

CS: Circular stapler; DA: delta-shaped anastomosis; TST: triangulating stapling technique; BBT: book-binding technique; LGSD: linear-shaped gastroduodenostomy; ART: augmented rectangle technique; NS: not stated

end of the duodenal stump was rotated externally by 90°. After the initial suturing between the remnant stomach and the duodenum, the two sides (posterior wall and cranial wall), the posterior wall and caudal wall, form a V-shape. A 30 mm linear stapler was applied to close the insertion holes up to the closest side of the duodenal resection margin. After gastric and duodenal resection margins were ensured to be close together, the 60 mm laparoscopic linear stapler was used to transect the duodenal resection margin to create the margin. After the above steps, all the previous linear staplers were removed from duodenal resection margin.

Thanks to the elimination of the stay sutures in the anastomosis site, the risk of leakage of the intestinal contents into the peritoneal cavity can be reduced with a result of reduced incidence of peritoneal abscess[28,29]. Removing the staple line of the duodenal stump without creating a T-shaped anastomotic region can avoid postoperative stenosis. The ART can create larger 4-sided anastomosis diameters than 3-sided ones, without worrying about whether the width of the opening will be reduced by the final stapling.

APPLICATION OF BARBED SUTURE IN INTRACORPOREAL ANASTOMOSES

Intracorporeal suturing and knot typing in some B-I anastomosis were time-consuming and tedious and especially these procedures were the last steps to do in LDG. But various devices have been developed to simplify the placement of intracorporeal sutures, and barbed suture is one such device. Using the barber suture could reduce the number of knot typing, the suturing efficiency and reduce the cost of intracorporeal reconstruction with staplers[30]. Lee *et al.*[30] used barber sutures to close entry hole in 354 patients instead of staplers with a result of minimizing the suturing time. There were no patients who needed to be converted to usual sutures or mechanical closure with staplers and only one patient presented with postoperative anastomotic bleeding.

CONCLUSION

Several reconstruction techniques are possible after TLDG [Table 1]. The best reconstruction is the one, that simplifies the technique, maintains satisfactory nutritional status and quality of life while keeping

postoperative morbidity as low as possible. We believe that the new technologies and improved techniques will bring more benefits to patients and doctors.

DECLARATIONS

Authors' contributions

Study's conception and design: Zhang S, Fukunaga T
Writing the paper: Zhang S
Provided administrative, technical, and material support: Fukunaga T

Availability of data and materials

Not applicable.

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Not applicable.

Consent for publication

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REVIEW

Current surgical treatment of esophagogastric junction adenocarcinoma

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Abstract

The incidence of esophagogastric junction (EGJ) adenocarcinoma has shown an upward trend over the past several decades worldwide. In this article, we review previous studies and aimed to provide an update on the factors related to the surgical treatment of EGJ adenocarcinoma. The Siewert classification has implications for lymph node spread and is the most commonly used classification. Different types of EGJ cancer have different incidences of mediastinal and abdominal lymph node metastases, and different surgical approaches have unique advantages and disadvantages. Minimally invasive surgeries have been increasingly applied in clinical practice and show comparable oncologic outcomes. Endoscopic resection may be a good therapy for early EGJ cancer. Additionally, there is still a great need for well-designed, large RCTs to forward our knowledge on the surgical treatment of EGJ cancer.

Key words: Esophagogastric junction cancer; Surgery; Lymph nodes; Siewert classification

Core tip: This is a review article on the current strategies for the surgical management of esophagogastric junction (EGJ) cancer. This article covers the different aspects related with the surgical treatment of EGJ cancer and provides comparison between different modalities discussed.

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INTRODUCTION

Gastrointestinal (GI) cancers are aggressive diseases, accounting for more than one-fourth of the newly diagnosed cancers worldwide (more than 4 million new cases per year). Among the GI cancers, the esophagogastric junction, or esophagogastric junction (EGJ), is a special anatomical site with a remarkably high risk of adenocarcinoma. The incidence of EGJ adenocarcinoma has shown an upward trend over the past several decades both in the West and East[1-3]. Due to its location between the esophagus and stomach, some investigators regard EGJ cancer as an entity separate from esophageal and gastric cancers. There has been much debate as to the pathogeny, diagnosis, classification, and optimal therapy for EGJ cancer, and the debate continues[4].

The definition of the location of the EGJ by endoscopy or upper GI radiography and its appearance on histopathology are different. The EGJ or Z-line is theoretically defined as the histological transition from the squamocolumnar junction between the esophagus and stomach. Actually, this transition does not occur exactly in the anatomical transition between the esophagus and stomach[5]. In clinical practice, the EGJ is defined by the proximal margin of the longitudinal folds of the stomach transformed by the tubular esophagus.

In this article, we review previous studies and aimed to provide an update on the different aspects related to the surgical treatment of EGJ cancer.

EGJ CANCER CLASSIFICATION

To improve the diagnosis and to allow the comparison of treatment results, Siewert and coworkers developed a system that separated EGJ tumors into three subtypes based purely on the macroscopic location of the tumor epicenter[6] (Table 1). Type I tumors are with an epicenter 1-5 cm above the EGJ; type II: Those within 1 cm above and 2 cm below the EGJ; and type III: Those 2-5 cm below the EGJ. The Siewert classification has practical implications for lymph node spread and is the most commonly used classification. The aim of the Siewert classification is not only for prognosis but also for therapeutic decision-making.

In the current (8th) edition of the TNM classification of malignant tumors, EGJ adenocarcinoma was redefined. Tumor epicenters within 2 cm proximal or distal to the EGJ are staged as esophageal adenocarcinomas, and those whose epicenters are more than 2 cm distal from the EGJ are staged as gastric cancer. The TNM classification also indicated that using the genetic signature of EGJ cancers may identify the cell of origin for cancer staging more accurately than the gross location of the tumor[7,8]. Cancer genetics will be included in the next (9th) edition staging of EGJ cancers.

Japanese gastric cancer treatment guidelines define EGJ cancer as a tumor (≤ 4 cm diameter) with an epicenter located within 2 cm of the EGJ, whether adenocarcinoma or squamous cell carcinoma. The Japanese classification was based on retrospective data from 3177 patients operated on between 2001 and 2010 from 273 institutions[9]. Siewert type III and part of Siewert type I tumors are not covered by the Japanese classification.

THE IMPORTANCE OF THE PRECISE LOCALIZATION OF TUMORS

EGJ cancers have unique characteristics that make the risk of lymph node (LN) metastasis high, and both the mediastinal and abdominal fields are the main lymphatic drainage areas. The surgical approach and type of lymphadenectomy have a close relationship with LN metastasis. The pattern of LN spread is also closely related to the location of the EGJ tumor. To develop the optimal treatment for EGJ cancers, it is important to identify the exact tumor location and estimate the exact length and depth of esophageal and gastric invasion preoperatively.

Table 1 Different classification of esophagogastric junction cancer

System	Classification	Description
Siewert classification	Type I	1-5 cm above the EGJ
	Type II	Within 1 cm above and 2 cm below the EGJ
	Type III	2-5 cm below the EGJ
AJCC/UICC TNM	Esophageal adenocarcinomas	Within 2 cm proximal or distal to the EGJ
	Gastric cancer	More than 2 cm distal from the EGJ
Japanese classification	-	A tumor (≤ 4 cm diameter) with an epicenter locating within 2 cm of the EGJ, whether adenocarcinoma or squamous cell carcinoma

EGJ: Esophagogastric junction; AJCC: American Joint Committee on Cancer; UICC: Union for International Cancer Control.

The precise localization of tumors can be frequently difficult to assess through endoscopic ultrasound (EUS) and computed tomography (CT), which are thought to be the best techniques currently available. This is particularly problematic for Siewert II type cancer. EUS precisely localizes tumors only 66% of the time, and CT precisely localizes tumors 57% of the time, compared to final operative pathology[10].

LYMPH NODE METASTASES ACCORDING TO THE SIEWERT CLASSIFICATION

EGJ cancers have unique characteristics, and lymphatic drainage occurs in both the mediastinal and abdominal areas. Adequate LN lymphadenectomy is an important key to oncologically successful surgical resection. The incidence of LN metastases increases with the depth of tumor infiltration, but LN location depends on the tumor location. Siewert's group reported the incidence of lymph node metastasis based on 1602 consecutive surgical patients[11]. Type II and type III cancers showed a higher risk of LN metastases. The incidence of metastasis was 51.9%, 65.2%, and 77.8% for type I, type II, and type III, respectively. Studies from Japan report that the incidence of metastasis was 64.1% and 75% for type II and III, respectively[12]. The data were based on 126 patients who underwent curative resection.

LN METASTASES IN TYPE I EGJ CANCER

Type I EGJ cancers metastasize to lower mediastinal LNs, and 15% metastasize to upper mediastinal LNs. Paracardial regions and lower posterior mediastinal LNs are the most frequently observed locations in type I cancers[11]. More recent studies from Japan yielded similar results. LNs, including nos. 1, 2, 3a, and 7, had a frequent incidence of metastasis[13], while other LNs were rarely involved. Therefore, total gastrectomy for type I cancer is not routine due to the extremely rare risk of LN metastases in the lower perigastric LNs. A surgical approach allowing both upper perigastric and mediastinal lymphadenectomy would be suitable for type I cancer.

LN METASTASES IN TYPE II EGJ CANCER

Most studies focus on Siewert type II cancer, since it is considered the true EGJ tumor, and the characteristics of metastases to mediastinal LNs remain debatable. The extent of lymph node dissection determines the surgical field and the type of surgery. In particular, it has an important influence on the topic of the transabdominal approach due to the potential risk of leaving positive nodes in the mediastinal region. Twelve percent of LN metastases involve lower mediastinal regions among surgical patients reported by Siewert's group. They also indicated that as the location of the tumor approaches the gastric side, the incidence of mediastinal LN metastases gradually decreases, while the incidence of abdominal LN metastases increases[11].

Many other studies have indicated that the location of mediastinal LN metastases is closely related to the distance from the EGJ to the tumor. A Japanese multicenter study retrospectively analyzed 315 pT2-4 Siewert II patients who received R0 or R1 resection. The results showed that the incidence of metastasis or recurrence was 4%,

7%, and 11% in the upper, middle, and lower mediastinal LNs, respectively. Among 315 patients in the study by Kurokawa *et al*[14], 176 underwent LN dissection in the lower mediastinal region, and the metastasis rate in the lower mediastinal nodes was 17.6%. In 139 other patients who did not undergo dissection, the researchers described a long follow-up period. The recurrence rate among these 139 patients was 3.6%. Therefore, the researchers combined metastasis with recurrence to determine the final overall rate of metastasis or recurrence, which was 11.4%. We should recognize that recurrence does not always reflect metastasis at the time of surgery. This point was the limitation of their study. It also revealed that the length of esophageal invasion correlated with the number and location of mediastinal LN metastases. The incidence of metastasis was much higher when the length of esophageal invasion was > 3 cm for the upper or middle mediastinal nodes and > 2 cm for the lower mediastinal nodes[14]. The authors indicated that based on this result, if esophageal invasion of > 3 cm is noted, the upper and middle mediastinal LNs should be harvested. A systematic review reported that the frequency of LN metastasis in the lower mediastinal stations ranged from 7.5 to 23.8%, whereas patients with upper mediastinal node involvement had a frequency of LN metastasis below 4%[15].

Several retrospective studies of abdominal LN metastasis in type II cancer were performed in Japan[12,13,16-19]. Fujitani *et al*[16], Yoshikawa *et al*[13] and Yamashita *et al*[17] all reported that the incidence of metastasis was especially low in the lower perigastric LNs (nos. 4d-6), whereas it was higher in the upper half of perigastric LNs (nos. 1, 2, and 3) and the second-tier LNs (nos. 7, 9, and 11). LN nos. 1 and 3 had the highest metastasis incidence (up to 39.1%)[12], and that in the celiac axis around the splenic artery and the splenic hilum was less than 10%[12,18]. However, if the distance from the EGJ to the distal end of the tumor was more than 5 cm, the LN metastasis incidence at the greater curvature (nos. 4sa, 4sb, 4d, and 6) or antrum was as high as 20%[19]. These results may indicate that harvesting the perigastric nodes of the lower half of the stomach is not beneficial if the distance from the EGJ to the anal edge of the tumor is greater than 5 cm.

Taken together, these results show that type II cancers mainly metastasize to the abdominal LNs around the stomach. The lower mediastinal compartment is the most common site of mediastinal LN metastases. Esophagectomy with proximal gastrectomy might be enough in type II cancer; however, it is better that the lower mediastinal compartment be routinely sampled during the operation. An accurate preoperative evaluation of the length of esophageal invasion is therefore essential, as it can be used as a reference point for mediastinal LN metastases.

LN METASTASES IN TYPE III EGJ CANCER

Regarding type III cancer, perigastric LNs are the most common metastasis areas, with approximately 2% to 18% of them having simultaneous positive mediastinal nodes[20-23]. Among the perigastric LNs, nos. 4sa, 4sb, 4d, 8a, 9, and 11p show a high risk of metastasis, whereas LN nos. 1, 2, 3, and 7 do not[20,24]. Although the incidence of LN no. 10 metastasis ranges from 10%-20%, there is no survival benefit associated with adding a splenectomy to a D2 lymphadenectomy[25,26]. It is recommended that the splenectomy be performed only to obtain R0 resection[27,28]. Notably, a splenic hilar lymphadenectomy is technically difficult and quite sophisticated due to the deeply located operative field, limited space, and tortuous and variant vessels at this site. With the accumulation of experience, new technological emergences and new surgical energy instruments, this procedure has gradually become possible.

Taken together, these results indicate that total gastrectomy should be conducted for type III cancers to obtain enough LNs, but splenectomy is not routine only to obtain R0 resection.

TUMOR SIZE AND INVASION WITH LN METASTASIS

The depth of tumor invasion is another factor that is significantly correlated with the presence of distal positive nodes[29], with an incidence of ≥ 60% in T2 and ≥ 85% in T3-4 patients[20,21]. It was also reported that tumor size is a predictor of LN metastasis, especially in large tumors (> 4 cm)[30].

LYMPHADENECTOMY AND PROGNOSIS

LN metastasis is also an indicator of prognosis. The highest risk factor is the number

of metastatic LNs ≥ 7[15,31]. Locoregional LN involvement is associated with improved survival compared with para-aortal or other distant LNs[15]. In a systematic review including 2252 type II cancer patients, ≥ 7 metastatic LNs (N3) indicated much worse survival (2.0%-17.4%) compared to no LN metastasis (up to 82.7%)[15]. Whether a more extensive lymphadenectomy in EGJ cancer is correlated with survival benefits has not been determined. Extended dissection might improve the prognosis, but the morbidity and mortality rates might also increase. In particular, some studies from the West have shown no superior survival rates compared with the East when using a more extended lymphadenectomy[31-34]. A multicenter retrospective study from the United States indicated that the number of LNs harvested was an independent predictor for survival after surgery. The authors concluded that a minimum of 23 regional LNs harvested can offer a survival benefit[35]. A cohort study of 262 pN0 type II patients from China also confirmed this conclusion. The researchers indicated that more than 15 LNs were recommended for patients undergoing curative resection[36]. Whether a more extensive lymphadenectomy in EGJ cancer can provide more survival benefit was recently challenged. A Dutch study found no benefit from an extended lymphadenectomy for type II disease[37]. A study from the United Kingdom (n = 606)[38] and another recent retrospective cohort study from Denmark (n = 510)[31] also showed no significant difference in survival between the extended and the less extended lymphadenectomy.

Therefore, although LN metastasis puts a patient at high risk and is considered an indicator of a poor prognosis, existing evidence does not support the benefits of an extensive lymphadenectomy. Moderately extensive lymph node removal may be enough to maximize the outcomes after EGJ cancer surgery.

PROXIMAL RESECTION MARGIN

The definition of R0 resection for EGJ is important. Feith e*t al*[11] retrospectively analyzed 1602 patients and found that the 5-year survival rate was 43.2% for a negative margin versus 11% for a positive margin. However, the optimal extent of esophagus resection required for the prevention of recurrence and longer survival remains controversial[18,39-42]. Ito *et al*[43] advocated the proximal gross margin length of at least 6 cm in patients with Siewert type II/III EGJ cancers, while Mariette *et al*[39] advocated that 8 cm is necessary to prevent local recurrence.

A longer proximal margin length can ensure a negative margin, but it can also increase the operation difficulty. An increasing number of studies have indicated that a shorter proximal resection length may prove to be an adequate oncologic margin. Barbour *et al*[40] reported that 5 cm of a grossly normal *in vivo* (approximately 3.8 cm *ex vivo*) proximal esophagus was associated with improved survival for patients (≥ T2 and ≤ 6 positive lymph nodes) with Siewert types I/II/III. There were 58 patients with more than 6 positive LNs. However, both univariate and multivariable analyses showed that the proximal margin carried no prognostic significance for these patients. Mine *et al*[18] reported another study of an even shorter proximal margin in Siewert type II and III patients who received a transhiatal (TH) total gastrectomy. They indicated improved survival with a proximal resection margin of 3.0 cm *in vivo* (approximately 2.0 cm *ex vivo*)[18]. Feng *et al*[42] found that the proximal margin length had no relationship with the survival of patients with Siewert type II/III EGJ cancers. They concluded that a negative proximal margin may be sufficient during the surgical resection of Siewert type II/III tumors[42]. A similar result was reported from the United States Gastric Cancer Collaborative[44]. The authors found that the proximal margin length was not associated with local recurrence or overall survival. They suggested that achieving a specific proximal margin distance should be abandoned.

In conclusion, there is a trend that a shorter proximal resection margin is being adopted in clinical practice due to similar oncology outcomes. Surgery is much easier if the distal esophagus can be dissected through a transabdominal approach rather than a transthoracic approach in an attempt to pursue a longer proximal margin.

SURGERY CHOICE ACCORDING TO THE SIEWERT CLASSIFICATION

The key factors to a successful oncologic surgery are as follows: curative R0 resection, adequate LN dissection, and the minimization of surgical morbidity. An esophagogastrectomy with a moderate, adequate lymphadenectomy is still considered the standard surgical strategy for EGJ cancer, although there are some differences according to Siewert types.

Because type I cancers arise from the distal esophagus, most experts and guidelines recommend that they be treated surgically as esophageal cancer, with an esophagogastrectomy plus both mediastinal and upper perigastric LN resection. For type II cancers, some individuals recommend an esophagectomy with a proximal gastrectomy, which allows the dissection of both the abdominal and mediastinal LNs. Others advocate for a total gastrectomy and extended lymph node dissection with a TH approach into the posterior mediastinum[45]. For type III cancers, an esophagogastrectomy includes a total gastrectomy plus a distal esophagectomy via laparotomy, by which the diaphragm is opened. The final anastomosis site is in the distal part of the thoracic cavity. GI anastomosis is commonly an esophagojejunostomy with a Roux-en-Y reconstruction[46]. However, there is still no consensus as to which surgical approach is suitable for an esophagogastrectomy. To summarize, there are three main approaches for EGJ cancer resection - all are based on the Siewert classification (Table 2): (1) The right transthoracic (RT) approach (the 2-step Ivor-Lewis approach or the 3-step McKeown approach); (2) The left transthoracic (LT) approach; and (3) The TH approach. Every approach has potential advantages and disadvantages.

The transthoracic approach is usually performed with a laparotomy plus a thoracotomy and sometimes with a cervical incision, allowing exploration of the entire mediastinum. The final anastomosis is performed in the intrathoracic area (Ivor Lewis approach) or the cervical area (McKeown approach). The potential advantages of the RT approach are as follows: (1) There is a sufficient distance of the proximal resection margin even in advanced EGJ cancers with extensive esophageal invasion; and (2) It allows the exposure to the entire mediastinum to harvest even the upper mediastinal LN. This procedure may especially benefit advanced-stage patients with long esophageal invasion. Due to the low rate of invaded upper mediastinal LNs, the Ivor Lewis approach without upper mediastinal LN dissection is usually performed in Western countries[47]. The LT consisting of the left thoracoabdominal (LTA) approach and left thoracophrenolaparotomy is not commonly used, although it has the following advantages: (1) A sufficient proximal margin can be ensured; (2) Body position change is not needed during the operation; and (3) The surgical procedure around the esophageal hiatus is easy to perform under direct visualization. TH esophagectomy is usually performed through a laparotomy with a cervical incision, without a thoracotomy. Surgical stress, particularly respiratory damage, is the main disadvantage of a thoracotomy. The TH approach consisting of the TH surgical operation from the abdomen to the lower mediastinum minimizes such disadvantages due to the avoidance of a thoracotomy. Changes in body position are also not needed during the TH operation.

TH is inappropriate for esophageal cancer due to limited periesophageal LN harvesting. However, many studies on esophageal cancer have demonstrated no significant survival advantage for more radical surgery[48], and TH can be used to treat esophageal cancer, with similar OS and even less morbidity[49]. Regarding EGJ cancers, few studies comparing TH and the transthoracic approach have been reported.

Two randomized controlled trials comparing transthoracic with TH esophagectomy were performed in the West and East[50,51]. The Dutch phase III clinical trial (n = 205) compared RT with TH in patients with type I or type II EGJ cancer. The RT group did not achieve a survival benefit but instead exhibited higher postoperative morbidity[50]. In a subgroup analysis, the 5-year OS rate was similar between RT and TH for patients with type II cancer but higher following RT than TH for patients with type I cancer[37]. The authors concluded that RT may be recommended only for patients with type I tumors and not type II tumors. The Japanese phase III trial (n = 67) compared oncologic outcomes between LTA and TH in patients with type II or type III EGJ cancer. However, due to limited efficacious resection, the trial was stopped at the first interim analysis[51]. After 10 years of follow-up, the LTA achieved no benefits in OS or DFS and did not reduce the cancer recurrence rate in LNs. However, the LTA was associated with higher morbidity and mortality[52]. Based on these results, the researchers suggested that the LTA be avoided as a surgical therapy for adenocarcinoma of the EGJ or the gastric cardia. In Japan, the consensus is that Siewert type II and type III cancers should be treated by an abdominal, TH approach with en bloc lower mediastinal dissection with a length of esophageal invasion ≤ 3 cm.

A United Kingdom cohort study (n = 664) found no differences between TH and transthoracic approaches regarding survival or tumor recurrence in patients with esophageal or EGJ cancer[32]. Yan *et al*[53] conducted a systematic review of 2202 patients to compare the clinical outcomes between TH and open thoracic esophagectomy in EGJ cancer. The TH group showed decreased hospitalization, operation time, and blood loss, with less LN dissection. The complication and survival rates were not different between these approaches. A subtype analysis showed no

Table 2 Different approach for esophagogastric junction cancer

Approach	Surgical technique	Procedure	Disadvantage
RT	Ivor Lewis	Midline laparotomy	Limited proximal margin
			Requirement of body position change
			Surgical stress is significant
	Mckeown	Right thoracotomy	Increased risk for recurrent laryngeal nerve injury
		Midline laparotomy	Surgical stress is significant
		Left cervical	
LT	LTA	Left thoracotomy extended to upper midline laparotomy	No middle or upper thoracic lymphadenectomy
			Surgical stress is significant
	Left thoracophrenolaparotomy	Transdiaphragmatic thoracotomy	No middle or upper thoracic lymphadenectomy
		Midline laparotomy	Surgical stress is significant
TH	-	Midline laparotomy	Limited proximal margin
		Left cervical	Surgical view of the lower mediastinum is poor
			No middle or upper thoracic lymphadenectomy
TG	-	Midline laparotomy	Limited proximal margin
			No thoracic lymphadenectomy

RT: Right Transthoracic; LT: Left Transthoracic; TH: transhiatal; TG: Total Gastrectomy.

significant differences according to the Siewert type[53]. Omloo *et al*[37] compared the transthoracic and TH approaches for esophagectomy and found that the TH approach was associated with a lower morbidity; however, better medium-term survival with transthoracic esophagectomy was observed in two subgroups: patients with type I AEG and those with ≤ 8 metastatic nodes.

Taken together, existing evidence does not support one technique over the other regarding oncological outcomes. Future large RCTs are still needed to examine these techniques and their effects on long-term OS.

MINIMALLY INVASIVE SURGERIES FOR EGJ CANCER

Minimally invasive surgeries are the gold standard in many fields of surgery. The first minimally invasive esophagectomy was described by Cuschieri *et al*[54] in 1993, and after one year, Kitano *et al*[55] reported the first minimally invasive gastrectomy. Since then, the techniques for gastric cancer have evolved from laparoscopic assisted to total laparoscopic surgery, and the techniques for esophagectomy have also evolved from hybrid approaches to an entirely minimally invasive manner. Both minimally invasive surgeries show similar surgical and oncological outcomes compared with open surgeries, especially in early-stage patients. Zhou *et al*[56] conducted a systematic review of minimally invasive esophagectomy approaches for esophageal or EGJ cancer. The review that included 1 RCT and 47 observational studies indicated that minimally invasive procedures (n = 4509) have lower pulmonary complications compared with open surgery (n = 6347). There were no differences in anastomotic leak or gastric tip necrosis between the two groups[56]. However, in the minimally invasive procedures group, the authors included not only total minimally invasive procedures but also thoracoscopy-assisted or hybrid procedures.

For type I and II cancers, there are different minimally invasive techniques according to transthoracic or TH approaches compared to open surgery. Usually, the minimally invasive Ivor-Lewis technique is the main choice, although intrathoracic anastomosis is sometimes difficult. The operation starts with a laparoscopy with a proximal gastrectomy plus a lymphadenectomy. Then, the operation is followed by a right thoracoscopy, including esophagus mobilization and a mediastinal lymphadenectomy between the area from the carina to the azygos vein. The gastric tube is pulled into the thorax through the hiatus to create an intrathoracic anastomosis. The anastomosis methods include end-to-side anastomosis with a manual or circular stapler (with or without an OrVil device)[57] and side-to-side

anastomosis with a linear stapler (with or without barbed sutures)[58].

The minimally invasive McKeown procedure commences with a right thoracoscopy followed by esophagus dissection and a mediastinal lymphadenectomy, which are similar to the previous description of the Ivor Lewis technique. Subsequently, the patient's position is changed to a supine position, and then a laparoscopic gastrectomy with a lymphadenectomy is performed. The formation of the gastric tube is also similar to that descried in the Ivor Lewis technique. After the laparoscopy, a left cervical incision is made, and the divided esophagus is anastomosed with a gastric tube manually using end-to-end anastomosis[59]. van Workum *et al*[60] conducted a systematic review (n = 1681) to compare the totally minimally invasive McKeown and Ivor Lewis technique used for esophageal and EGJ cancers. The Ivor Lewis group showed decreased RLN trauma, hospitalization, and blood loss compared to the McKeown group, while the anastomotic leakage rate was not different[60]. It is noteworthy that the evidence is limited, and all included studies were cohort studies with a moderate risk of bias. It is still uncertain which minimally invasive technique is suitable. The Netherlands is now performing the first randomized controlled trial containing 200 patients between minimally invasive McKeown and Ivor Lewis approaches. This clinical trial is powered for finding differences in morbidity, the severity of complications and quality of life[61]. The minimally invasive TH procedure consists of a laparoscopy and a left cervical incision followed by a gastrectomy plus a lymphadenectomy and TH dissection of the distal esophagus through a laparoscopy. The gastric tube is created extracorporeally and then pulled into the cervical area where the anastomosis is made[62].

For type III cancers, a laparoscopic gastrectomy is the main choice. A total D2 gastrectomy is performed, and the duodenum is divided using a liner stapler. The diaphragm is opened, and the distal esophagus is mobilized. Only the distal periesophageal LNs are resected, and then the vagal nerves and esophagus above the cancer are transected. Because of the limited size of the hiatus, the OrVil® (Medtronic, Inc., Minneapolis, MN, United States) is usually used to perform the end-side esophagojejunostomy anastomosis[63].

In conclusion, there is still no agreement about the ideal type of minimally invasive surgery, and existing evidence does not support that one technique is much better than the other. Many anastomotic methods can be adopted, such as manual, circular stapler, linear stapler, and even robot-assisted anastomoses. Large randomized controlled trials are still needed to test which minimally invasive technique is most suitable for EGJ cancer.

ENDOSCOPIC RESECTION FOR EARLY EGJ CANCER

Endoscopic resection (ER), including endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), is used to remove superficial neoplasms from the GI tract[64,65]. However, the curative resection criteria, particularly for type II cancers, differ between esophageal cancer and gastric cancer, since the rate of LN metastasis is different[66].

The indications for ER in early EGJ adenocarcinoma are also under study. A Japanese multicenter study retrospectively analyzed 458 esophageal or EGJ adenocarcinoma patients who received surgical or ER treatment. Lymphovascular involvement, a poorly differentiated tumor, and lesion size > 30 mm were independent risk factors for metastasis. Mucosal and submucosal cancers with invasion of less than 500 μm without the abovementioned risk factors may also be suitable for ER[67].

Favorable oncological results were also reported in several studies. A systematic review analyzed 359 early EGJ adenocarcinoma patients who received ESD treatment. More than 20% of tumors were reported to have deep submucosal invasion (> 500 mm from the muscularis mucosa). The en bloc resection and complete resection rates were 98.6% and 87%, respectively. Patients with curative resection showed no local recurrence or distant metastases[68]. A Korean retrospective study demonstrated similar 5-year OS rates between ESD and surgery (93.9% *vs* 97.3%, respectively, P = 0.37). Local recurrence and cancer-related deaths were not observed[69]. Recently, a retrospective study from 13 centers in Japan reported the long-term outcomes of ER for EGJ adenocarcinoma. The 5-year cumulative incidences of local recurrence were 13% for EMR and 0.5% for ESD. In this study, patients were classified into 2 groups based on the risk of metastasis according to the histologic features. Patients at a low risk for metastasis were defined as those with mucosal cancer without LVI and a poorly differentiated component or those with a cancer with an SM depth ≤ 500 μm without LVI, without a poorly differentiated component, and measuring ≤ 30 mm.

High-risk patients were defined as those with mucosal and SM EGJ (except for low-risk criteria). According to the abovementioned risk factors for LN metastasis, there were 277 patients in the low-risk group and 95 patients in the high-risk group. The 5-year OS rates of the low-risk group, the high-risk group with additional treatment, and the high-risk group without additional treatment were 93.9%, 77.7%, and 81.6%, respectively. The authors concluded that patients with a low risk for LN metastasis may obtain favorable long-term outcomes after ER treatment[70].

Therefore, ER may be a good therapy for early-stage (intramucosal) EGJ cancer. Not all patients with early EGJ cancer can be treated with ER. The incidence of metastasis should be understood, and a confirmation of the indication would maximize the benefits of ER for early EGJ cancer. However, RCTs are needed to inform the benefits and harms of ER therapy for early EGJ cancer.

CONCLUSION

The incidence of EGJ cancer is increasing. Tumor location is an important factor in determining the optimal surgical therapy for EGJ. The Siewert classification has implications for lymph node spread and is the most commonly used classification. Different types of EGJ cancer have different incidences of mediastinal and abdominal LN metastases, and different surgical approaches have unique advantages and disadvantages. The length of the tumor and the depth of tumor invasion should also be considered when deciding the proper surgical technique. An extensive lymphadenectomy may not provide additional benefits. Minimally invasive surgeries are increasingly applied in clinical practice and show comparable oncologic outcomes. ER may be a good therapy for early EGJ cancer. Additionally, there is still a great need for well-designed large RCTs to forward our knowledge in the surgical treatment of EGJ cancer.

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Report

Sensitization of Gastric Cancer Cells to Irinotecan by p53 Activation

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Irinotecan (camptothecin-11 [CPT-11]) is a topoisomerase I inhibitor that has been used in the treatment of a wide spectrum of cancers including gastric cancer. Recent reports suggest that the expression of CES2, a serine hydrolase that converts irinotecan to its active compound SN-38, is regulated by the tumor-suppressor p53. In this study, we investigated whether irinotecan acted synergistically with a p53 activator nutlin-3a in human gastric cancer cells. Nutlin-3a treatment enhanced the expression of CES2 in gastric cancer cell lines with wild-type p53. However, this effect was not observed in cells with non-functional p53. Irinotecan showed synergistic antitumor effects in combination with nutlin-3a in gastric cancer cells with wild-type p53, whereas the survival of cells with non-functional p53 was not significantly affected by the presence of nutlin-3a. These results provide evidence that p53 activation can enhance the antitumor effect of irinotecan or other anticancer prodrugs activated by CES2 in gastric cancer cells through upregulation of CES2 expression.

Key words CES2, gastric cancer, irinotecan, p53, nutlin-3a

INTRODUCTION

Gastric cancer is relatively prevalent malignancy and ranks the fifth most commonly diagnosed malignancy and the third in cancer-related death worldwide.[1)] Gastric cancer is generally asymptomatic in early stages and has progressed to an advanced unresectable stage by the time of presentation.[2)] The prognosis of patients with gastric cancer remains extremely poor.[3)] Even patients with resectable tumor usually have a high rate of local recurrence and distant relapse.[4)] The standard palliative treatment for patients with advanced gastric cancer is chemotherapy, which both controls tumor-related symptoms and improves overall survival. Clinical trials have shown the survival benefit of irinotecan (camptothecin-11 [CPT-11]) in advanced gastric cancer as second-line chemotherapy.[5)]

Irinotecan is an anticancer drug that is used for the treatment of a wide spectrum of cancers including gastrointestinal cancer. Irinotecan is a prodrug and converted to its active compound 7-ethyl-10-hydroxy-camptothecin (SN-38) by the carboxylesterase CES2.[6)] However, the expression of CES2 is frequently downregulated in many types of cancers including gastric cancer,[6)] which may affect the therapeutic efficacy of irinotecan. Recent studies have indicated that CES2 can be transcriptionally activated by p53, a tumor suppressor that controls the transcription of plethora of genes in response to cellular stresses such as DNA damage, oxidative stress, and hypoxia.[7)] Therefore, it is conceivable that activation of p53 could sensitize gastric cancer cells to irinotecan by upregulating CES2 expression.

In this study, using various cell lines of human gastric cancer (Table 1), we provide evidence that irinotecan exerts a synergistic antitumor effect in combination with a p53 activator in human gastric cancer cells. We used nutlin-3a as a p53 activator, which inhibits binding of the E3 ubiquitin ligase MDM2 to p53 and thereby directly activates p53 signaling without genotoxic side effects.[13)] Nutlin-3a treatment enhanced CES2 expression in gastric cancer cells and sensitized these cells to irinotecan in a p53-dependent manner. Our results highlight the importance of *TP53* gene status and the combination use of p53-activating drugs for the efficacy of irinotecan and other antitumor prodrugs that are activated by CES2 in human gastric cancer.

MATERIALS AND METHODS

Cell Lines and Reagents The human gastric cancer cell lines MKN1, MKN7, MKN74 and NUGC4 were obtained from the RIKEN BRC Cell Bank (Tsukuba-shi, Ibaraki, Japan). TMK1 cells were provided by Hiroshima University (Hiroshima-shi, Hiroshima, Japan). NUGC3 cells were provided by the Health Science Research Resources Bank (Sennan-shi, Osaka, Japan). AGS cells were from the American Type Culture Collection (ATCC). The cells were cultured at 37°C and 5% CO_2 in RPMI 1640 medium (Wako) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Nutlin-3a was purchased from AdooQ BioScience. All

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[1] The first two authors equally contributed to this study.

Table 1. Human Gastric Cancer Cell Lines Used in This Study

Name	Histological type[a]	Origin	*TP53* Status	*TP53* Mutation found at				Reference
				cDNA description	Exon	Codon	Amino acid change	
AGS	as	Stomach	wt	-	-	-	-	8
NUGC4	sig	metastasis (LN)	wt	-	-	-	-	8
MKN74	tub2	metastasis (Liver)	wt	-	-	-	-	9
NUGC3	por	metastasis (Brachialis muscle)	mt	c.659A>G	6	220	Tyr to Cys	8
MKN1	por	metastasis (LN)	mt	c.428T>C	5	143	Val to Ala	10
MKN7	tub1	metastasis (LN)	mt	c.832C>T	8	278	Pro to Ser	11
	tub1	metastasis (LN)	mt	c.751A>C	7	251	Ile to Leu	12
TMK1	por	metastasis (Liver)	mt	c.517G>A	5	173	Val to Met	10

[a]According to the Japanese Classification of Gastric Carcinoma. por, poorly differentiated adenocarcinoma; as, adenosquamous carcinoma; tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; sig, signet ring cell carcinoma; LN, lymph node; wt, wild type; mt, mutant.

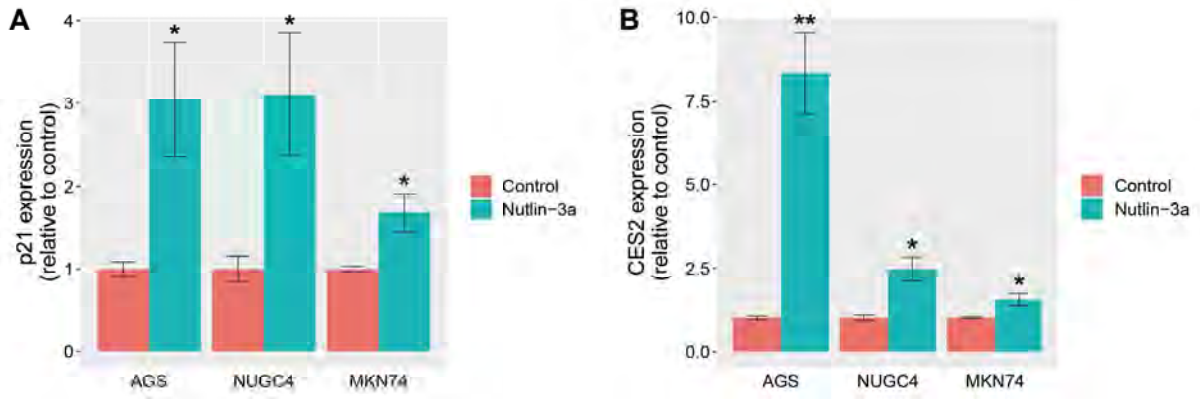

Fig. 1. Upregulation of CES2 Expression by Nutlin-3a in Gastric Cancer Cells with Wild-type p53.

Human gastric cancer cell lines with wild-type p53 (AGS, NUGC4, and MKN74) were treated with 5 μM nutlin-3a for 24 h. The expression of p21 (A) and CES2 (B) was quantified by real-time reverse transcriptase PCR. GAPDH was used as the reference gene. Data represent the mean values ± SEM (three independent experiments). *$p < 0$ 05; **$p < 0$ 01. An unpaired two-tailed *t*-test was used.

reagents were dissolved in sterile dimethyl sulfoxide (DMSO) to make 100 mmol/l (mM) stock. The cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and incubated for 24 h. The cells were then treated with nutlin-3a for another 24 h. The cells were washed twice with PBS and harvested by scraping.

Real-Time Reverse Transcriptase PCR Total RNA from cultured cell lines was extracted using the FastGene RNA Basic kit (Nippon Genetics Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Semiquantitative real-time PCR was performed using the Luna Universal One-Step RT-qPCR Kit (New England BioLabs) on a LightCycler 96 (Roche) in duplicate. The gene expression of target genes was normalized to GAPDH by the differences in Ct values, and then these values were used to calculate the relative mRNA expression levels with the $2^{-\Delta\Delta Ct}$ method. The primer sequences for the genes were as previously described.[14]

Cell Viability Assay Cell viability was determined by XTT (Cell Proliferation Kit II) assay (Roche). Briefly, cells were plated in triplicate into 96-well plates and cultured for 24 h, and then incubated with irinotecan at different concentrations in the presence or absence of 5 μM nutlin-3a for 24 h. The medium was then replaced with fresh medium and the cells were incubated with the XTT reagent for 3 h. The absorbance at 450 nm (reference wavelength at 660 nm) was measured with an iMark Microplate Absorbance Reader (Bio-Rad). Best-fit IC50 values were calculated with Prism 7 (GraphPad Software Inc., San Diego, CA) and compared by an extra sum-of-square *F* test.

RESULTS AND DISCUSSION

CES2 Expression was Upregulated by p53 Activation in Gastric Cancer Cells We first asked whether p53 activation enhanced CES2 expression in gastric cancer cells. Human gastric cancer cell lines with wild-type p53 (AGS, NUGC4, and MKN74) (Table 1) were treated with the p53 activator nutlin-3a. The expression of p21, a major downstream target of p53,[15] was upregulated by nutlin-3a treatment in all p53 wild-type cell lines tested, demonstrating activation of the p53 pathway in these cells (Fig. 1A). We also observed significant upregulation of CES2 expression in these cells (Fig. 1B). In contrast, nutlin-3a treatment did not significantly affect the expression of these genes in gastric cancer cells with p53 mutation (Table 1, Fig. 2). These results indicate that nutlin-3a enhances CES2 expression by activating functional p53 in human gastric cancer cells.

Synergistic Antitumor Effects of Irinotecan and Nutlin-3a in p53 Wild-Type Cells We next investigated whether nutlin-3a treatment sensitized gastric cancer cells to irinotecan, an anticancer prodrug that is converted by CES2 to its active compound SN-38. We treated two p53 wild-type cell lines (AGS and NUGC4) and two cell lines with non-functional p53 (NUGC3 and TMK1) with various concentrations of iri-

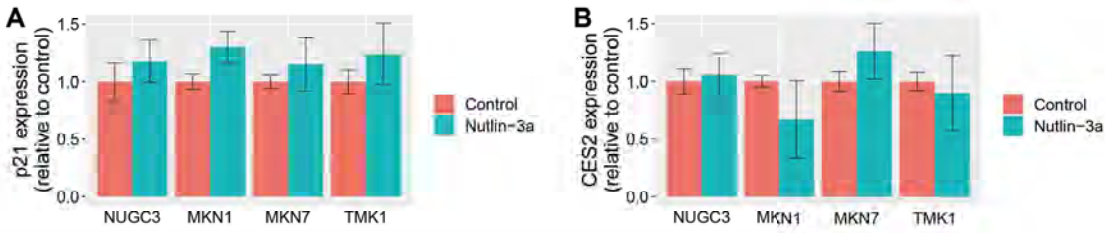

Fig. 2. Effects of Nutlin-3a on CES2 Expression in Gastric Cancer Cells with p53 Mutation.

Human gastric cancer cell lines with p53 mutation (NUGC3, MKN1, MKN7, and TMK1) were treated with 5 μM nutlin-3a for 24 h. The expression of p21 (A) and CES2 (B) was quantified by real-time reverse transcriptase PCR. GAPDH was used as the reference gene. Data represent the mean values ± SEM (three independent experiments). There was no significance between control and nutlin-3a. An unpaired two-tailed *t*-test was used.

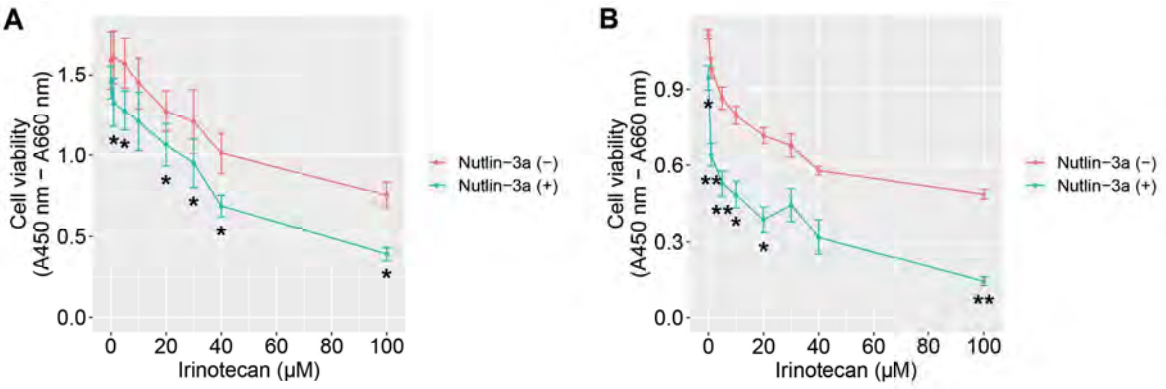

Fig. 3. Synergistic Effects of Nutlin-3a and Irinotecan in Gastric Cancer Cells with Wild-type p53.

AGS (A) and NUGC4 (B) cells were treated with various concentrations of irinotecan in the presence or absence of 5 μM nutlin-3a for 24 h. The cell viability was determined by XTT assay. Data represent the mean values ± SEM (three independent experiments). *$p < 0\ 05$; **$p < 0\ 01$. A paired two-tailed *t*-test was used.

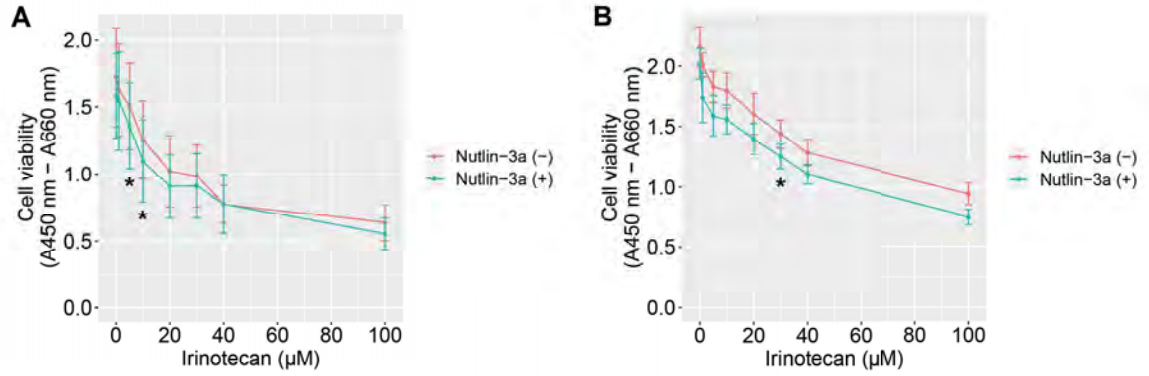

Fig. 4. Effects of Nutlin-3a and Irinotecan in Gastric Cancer Cells with p53 Mutation.

NUGC3 (A) and TMK1 (B) cells were treated with various concentrations of irinotecan in the presence or absence of 5 μM nutlin-3a for 24 h. The cell viability was determined by XTT assay. Data represent the mean values ± SEM (three independent experiments). *$p < 0\ 05$. A paired two-tailed *t*-test was used.

notecan with or without nutlin-3a. In p53 wild-type AGS and NUGC4 cells, the cell viability was not significantly or only slightly affected by single treatment with nutlin-3a, respectively (Fig. 3A and 3B). We observed strong synergistic effects of irinotecan and nutlin-3a in AGS and NUGC4 cells (Fig. 3A and 3B). The IC50 value of irinotecan was decreased by nutlin-3a by 2-fold, from 82.94 μM (95% CI [confidence interval]: 71.47–99.43 μM) to 42.94 μM (95% CI: 35.99–52.86 μM) ($p < 0.0001$) in AGS cells, and 7-fold from 59.59 μM (95% CI: 47.99–78.01 μM) to 8.731 μM (95% CI: 5.058–13.52 μM) ($p < 0.0001$) in NUGC4 cells, respectively. In contrast, nutlin-3a had almost no effect on the sensitivity to irinotecan in cells with non-functional p53 (Fig. 4A and 4B; the IC50 value from 38.69 μM [95% CI: 32.35–47.51 μM] to 35.79 μM [95% CI: 29.19–45.23 μM] in NUGC3 cells [$p = 0.5735$], from 74.25 μM [95% CI: 62.01–93.09 μM] to 56.71 μM [95% CI: 43.49–81.59 μM] in TMK1 cells [$p = 0.1328$]). These results suggest that p53 activation in gastric cancer cells leads to increased conversion of irinotecan to its active compound and thereby enhances the sensitivity to irinotecan.

Although a recent study has shown the survival benefit of irinotecan monotherapy as third-line or later treatment

in advanced gastric cancer,[16] irinotecan has been mostly used in combination with other anticancer drugs such as 5-fluorouracil (5-FU), which also activates p53.[17] Thus, the beneficial effects of these regimens in gastric cancer may be in part attributed to activation of p53 and upregulation of CES2, leading to efficient conversion of irinotecan. In this context, we used nutlin-3a to investigate the role of p53 because it directly activates p53 signaling pathway without untoward genotoxic side effects that may compromise the interpretation of the results. Consequently, we found that nutlin-3a upregulated CES2 expression only in gastric cancer cells with functional p53. Thus, p53 plays an important role in regulating CES2 expression in gastric cancer cells. p53 signaling is frequently dysregulated in many types of cancer including gastric cancer. Indeed, a genomic analysis of gastric adenocarcinomas has found p53 to be the most frequently mutated gene, accounting for 46% of total tumors.[18] In addition, irinotecan exhibits gastrointestinal toxicity and often causes severe diarrhea. Thus, understanding *TP53* gene status of gastric cancer may be useful to predict the efficacy of irinotecan-containing regimens.

In addition to irinotecan, several other anticancer prodrugs are also activated by CES2.[19–21] Capecitabine is an orally administered prodrug of 5-FU, which is effective and well tolerated in the treatment of gastric cancer. Various capecitabine-based chemotherapies have been shown to extend survival in advanced gastric cancer.[22] LY2334737 is an oral prodrug of the clinically efficacious anticancer agent gemcitabine. Gemcitabine is widely used in the treatment of pancreatic cancer[23] and advanced gastric cancer.[24] CES2 also converts Pentyl PABC-Doxaz to the active compound doxazolidine, a formaldehyde conjugate of doxorubicin that exhibits enhanced toxicity against a wide variety of tumor cell lines including cell lines resistant to doxorubicin.[25] Thus, the sensitivity of gastric cancer cells to these prodrugs may also be enhanced by p53 activation.

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Conflict of interest The authors declare no conflict of interest.

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Original Article

Concerns of quality, utility, and reliability of laparoscopic gastrectomy for gastric cancer in public video sharing platform

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Background: The rapid expansion of laparoscopic gastrectomy (LG) for gastric cancer has generated interest among surgeons. The adequate dissemination of correct information about such advanced laparoscopic surgery can certainly be useful for surgeons and trainees. Online video resources such as YouTube are frequently used for education. This study aimed to evaluate the quality, utility, and completeness of LG videos for gastric cancer on the video website YouTube.

Methods: The terms "laparoscopic gastrectomy" and "gastric cancer" were searched on YouTube on August 16, 2019. The first 100 videos in three sorting categories (website's default setting, view count, and length of duration) were checked by two experienced surgeons. The popularity was evaluated with the video power index (VPI). The reliability was measured using the *Journal of American Medical Association* (*JAMA*) benchmark criteria. The educational value and completeness were evaluated with a checklist developed by the researchers.

Results: A total of 102 videos were analyzed. Laparoscopic distal gastrectomy (LDG) and laparoscopic total gastrectomy were the most frequently recorded techniques. Lymph node (LN) dissection was the most frequently covered topic (89.2%), followed in descending order by GI reconstruction (87.3%). The mean VPI, JAMA benchmark score and completeness score of all videos were 2.63, 1.94 and 8.53, respectively. The types of sources were as follows: private users, 73 (71.6%); academic institutions, 20 (19.6%); and others, 9 (8%). A total of 97 videos with an identifiable primary surgeon originated from eighteen different countries.

Conclusions: Laparoscopic videos represented by YouTube represent a useful and appropriate educational tool. However, the quality of videos varied, and the level of information incompleteness was fairly high due to insufficient reviews. The role of private uploaders and academic institutions in surgical education cannot be overestimated. It is necessary that surgeon trainers and surgical educators critically analyze the quality of video content and exercise responsibility in directing trainee surgeons. In the current era, it is best for trainees to search for peer-reviewed content.

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Introduction

Gastric cancer is a relatively prevalent malignancy and ranks fifth in diagnosed cancers and third in cancer-related deaths in the world (1). In 1994, Kitano first reported the technique for laparoscopic distal gastrectomy (LDG) (2). Since then, the use of laparoscopic gastrectomy (LG) for gastric cancer has increased due to its multiple advantages of improving patients' quality of life over open surgery. The rapid expansion of LG has generated interest among surgeons. Performing advanced laparoscopic surgery such as LG normally requires higher technique skills and a lengthy period of learning. Based on recent concerns, new learning tools are now required to overcome the constraints related to laparoscopic education. In contrast to open surgery, laparoscopy videos can provide surgical trainees with operating procedures and essential information on anatomy and operation technique. Operative videos are undoubtedly a useful and appropriate training tool for laparoscopic surgery.

Since 2005, YouTube has become the most visited video broadcast site on the internet. Over 1.9 billion logged-in users visit YouTube each month, and people watch over a billion hours of video and generate billions of views every day (3). Given that the online videos can be accessible anytime and anywhere on laptops, tablets, and smartphones, YouTube was considered a platform to provide medical information and education (4,5). YouTube was the most commonly used video platform by trainees, with 95% of surgical residents using YouTube to prepare for surgery (5). However, videos can be uploaded from different sources and may be of varying quality. The videos posted on YouTube are not peer-reviewed and are normally ranked according to popularity, view counts, comments and user history, which are not valid criteria for videos with educational purposes.

Some studies have evaluated YouTube as a source of medical information. However, until now, no information is known about the characteristics of existing YouTube videos focusing on LG. The purpose of the present study was to evaluate the completeness and quality of LG videos for gastric cancer on the public video platform YouTube and to share our thoughts on important future directions for managing surgical videos about LG for gastric cancer.

Methods

We searched YouTube (www.youtube.com) on August 16, 2019 to locate video clips that included relevant information about LG for gastric cancer. The terms "laparoscopic gastrectomy" and "gastric cancer" were used to identify related video clips.

We performed our search and sorted the results according to three setting categories. The results were sorted by the website's default setting and view count separately. We also used length of duration to filter videos longer than 20 minutes, assuming that longer videos may have better educational value. The first 100 videos of each set of sorted results were gathered and analyzed.

Each video was viewed and analyzed for content. Any disagreements were resolved with consensus. Two experienced surgeons in the field of laparoscopic surgery reviewed videos together. Videos without demonstrations of laparoscopic technique (i.e., animations, lectures, patient experiences, news) were excluded.

We analyzed each video according to the following characteristics: length of duration, video provider, year of upload, number of views, video resolution, voice commentary, likes, and dislikes. Video provider was classified as academic, private, and other.

Video Power Index (VPI)

To evaluate the popularity of the videos, we use the "Video Power Index" (VPI) to assess both the view and the like ratio of the videos. The VPI was calculated as follows: first, calculate the like ratio (like*100/[like+dislike]) and the view ratio (number of views/days); then, the VPI is equal to the like ratio*view ratio/100 (6,7).

Journal of American Medical Association benchmark criteria

The *Journal of American Medical Association* (*JAMA*) benchmark criteria was used to evaluate the basic quality and reliability of the videos (6,7). The criteria consists of a 4-item (authorship, attribution, disclosure and currency) rating scale (*Table 1*). By assigning 1 point for the presence of each criterion, the total *JAMA* benchmark score was calculated (8).

Completeness scores for LG

At present, no validated tool for this purpose exists in the literature to provide a specific assessment of analyzed LG for gastric cancer-related videos. For a more detailed evaluation of the quality of videos, we used a completeness

Table 1 The *Journal of American Medical Association* (*JAMA*) benchmark criteria

Criteria	Description
Authorship	Authors and contributors, their affiliations, and relevant credentials should be provided
Attribution	References and sources for all content should be listed clearly, and all relevant copyright information noted
Disclosure	Web site "ownership" should be prominently and fully disclosed, as should any sponsorship, advertising, underwriting, commercial funding
Currency	Dates that content was posted and updated should be indicated

Table 2 Completeness checklist

Contents	Score
Videos resolution	
High definition	1
Preoperative evaluation	
Age, Gender, BMI	1
Extent of gastrectomy	1
Extent of LNs dissection	1
GI reconstruction method	1
Procedure description	
Words or voice comments	1
During surgery	
Port location	1
LNs dissection	
Infrapyloric LNs	1
Suprapyloric LNs	1
Great Curvature LNs	1
Suprapancreatic LNs	1
Less Curvature LNs	1
GI reconstruction	
Stomach resection	1
Anastomosis	1
After surgery	
Pathology stage	1
Surgical outcomes	1

BMI, body mass index; LNs, lymph nodes; GI, gastrointestinal.

score that we developed (*Table 2*). The lymph node (LN) dissection was defined as the area of LNs that needed to be harvested when LG, including but was not limited to laparoscopic part gastrectomy (LPG), LDG, laparoscopic total gastrectomy (LTG), or laparoscopic proximal gastrectomy (LPG), was performed. The anatomical definitions of LN stations were as follows: infrapyloric LNs (No. 6), suprapyloric LNs (No. 5), along greater curvature LNs (No. 4, or plus 12a for LDG; No. 4, 2 or plus 12a for LTG), suprapancreatic LNs (No. 8a, 7, 9, or plus 11p/d), and along lesser curvature LNs (No. 1, 3).

Statistical analysis

Statistical analysis was performed with SPSS Version 23 software. Data were summarized as frequencies (n) and percentages (%) for categorical variables and means or medians (standard deviations or ranges) for continuous and ordinal variables, respectively. Internal consistency between reviewers was evaluated with a kappa coefficient. The one-way ANOVA was used to compare the differences between the groups. A P value less than 0.05 was considered significant.

Results

Video selection process

In three search categories, we retrieved the first 100 videos. Some videos were duplicated in three different search categories. After duplicate videos were removed, 202 videos were selected for evaluation. Of the 202 videos screened, 102 videos met the inclusion criteria. The video selection process is shown in *Figure 1*.

Video characteristics

The characteristics of the analyzed videos are shown in *Table 3*. The mean length of duration for the videos was 5.1 minutes (range, 1.1–306.5), and the mean view count was 1,071 (range, 22–30,230). The mean video age was 2.9 years (range, 6 days to 10.2 years). Audio commentary was present in 33 (32.4%) videos, among which English accounted for 72.7%, followed by Bulgarian, accounting for 18.2%. High definition (HD) resolution was provided in 53 (52%) videos, most of which were created within the past 4 years. According to the video source, 71.6% of the videos were posted on YouTube by private users. Academic institutions

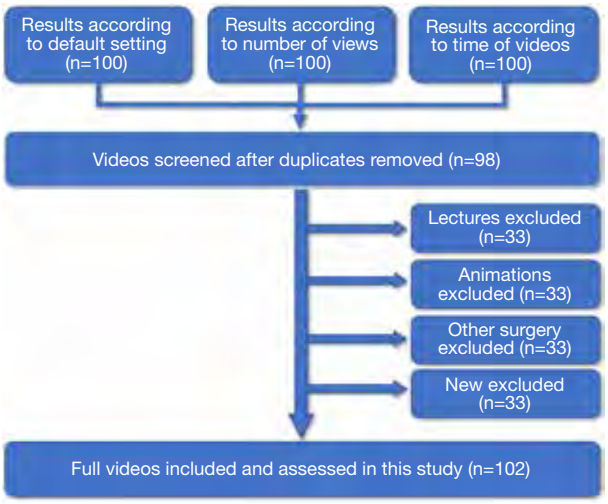

Figure 1 Flow diagram of video selection.

were responsible for uploading 19.6% of the total videos. The videos contributed by other sources, such as commercial companies, comprised only 8.8% of all videos.

VPI

The mean VPI score of all videos were 2.63 (range, 0 to 30.67). There were no differences based on whether the videos were sorted by source or content.

JAMA benchmark scores

The videos had a mean *JAMA* benchmark score of 1.94 (range, 1 to 4). There were 17 videos with 1 point, 75 videos with 2 points, 9 videos with 3 points and only 1 video with the full 4 points.

Laparoscopic technique content

In the evaluation based on surgical procedures, 49 (48%) surgical procedures were LDG, 49 (48%) were LTG, two were LPG and the remaining procedures could not be identified. *Table 4* shows the exact contents of LDG and LTG. The extent of LN dissection was identified in 64 of the 102 (62.7%) videos. Among the 64 videos, there were 59 for D2 gastrectomy. Among the LG procedures, LN dissection was the most frequently covered topic (89.2%), followed in descending order by GI reconstruction (87.3%). Twenty-four full-time videos without editing contain all technique details of LN dissection and GI reconstruction.

Table 3 Included videos characters

Category	Number/median
Video source	
Academic institutions	20 (19.6%)
Private users	73 (71.6%)
Others	8 (8.8%)
Cancer stage	
Early stage	23 (22.5%)
Advanced stage	36 (35.3%)
Unclassified	43 (42.2%)
Gastrectomy	
Laparoscopic distal gastrectomy	49 (48.0%)
Laparoscopic total gastrectomy	49 (48.0%)
Laparoscopic proximal gastrectomy	2 (2.0%)
Unknown	2 (2.0%)
Lymphadenectomy	
D1	3 (2.8%)
D1+	2 (2.0%)
D2	59 (57.1%)
D2+	1 (0.9%)
Unclassified	38 (37.2%)
Origin	
North America	16 (15.7%)
Central and South America	2 (2.0%)
Europe	54 (53.0%)
South East	8 (7.8%)
East Asia and Pacific	14 (13.7%)
Middle East and Africa	3 (2.8%)
Unknown	5 (5.0%)
Videos resolution	
Low definition	48 (47.1%)
High definition	54 (52.9%)
Video editing	
Edited	70 (68.6%)
Non-edited	32 (31.4%)
Video characteristics	
Number of days online	1,089 [6–3,710]
Number of views	1,071 [22–30,230]
Number of likes	6 [0–99]
Number of dislikes	0 [0–12]
Video duration	15.1 [1.1–306.5]

Data are expressed as the number of cases (percentage) or median [range].

Table 4 Descriptive data of the videos

XXXX	LDG (n=49)	LTG (n=49)	P value
Number of views	4,972 [77–30,230]	2,907 [22–16,281]	0.400
Technique description			0.049
Yes	15 (30.6%)	24 (49.0%)	
No	34 (69.4%)	25 (51.0%)	
Lymphadenectomy			0.460
D1	2	1	
D1+	1	0	
D2	27	27	
D2+	0	1	
Unclassified	13	17	
No contents	6	2	
Anastomosis			
Billroth-I	5	–	–
Billroth-II	17	–	
Roux-en-Y	13	45	
Overlap	–	19	
Orvil™	–	19	
Hand sewn	–	3	
Reverse puncture	–	2	
No contents	13	6	
Information on scores			
VPI	3.23±6.56	2.03±2.71	0.241
JAMAS	1.92±0.57	1.96±0.54	0.717
Completeness	8.84±3.29	9.73±2.32	0.123

LDG, laparoscopic distal gastrectomy; LTG, laparoscopic total gastrectomy.

Table 5 Completeness score

Completeness score	Number	Mean ± SD
Videos resolution	54	0.54±0.50
Surgical information	94	2.00±0.99
Procedure description	42	0.41±0.49
Laparoscopic techniques	102	5.64±2.19
Port location	30	0.29±0.46
LNs dissection	91	3.72±1.82
GI reconstruction	89	1.63±0.67
Surgical results	31	0.48±0.78
Total (max =16)	102	8.53±2.85

LNs, lymph nodes; GI, gastrointestinal.

The integrity of the video content was analyzed based on the surgical information completeness scores. *Table 5* shows the information completeness scores.

Video sources

A total of 71.6% of the videos were posted on the YouTube by private users. Academic institutions were responsible for uploading 19.6% of the total videos (*Table 6*). Other sources, such as commercial companies, accounted for 8.8% of the videos. A total of 97 (95.1%) videos with an identifiable primary surgeon originated from eighteen different countries, most often from Bulgaria and the United States. Academic institutions (rather than other sources) tended to add demonstrations for each surgical procedure. The videos from academic institutions and private users were significantly more complete than those posted by other sources.

Discussion

During the past decade, many randomized controlled trials have confirmed that LG is safe and feasible and has many benefits, especially in postoperative recovery, compared with open gastrectomy. The percentage of laparoscopic procedures for gastric cancer is increasing, especially in the Asian region (9). Increasing interest in the LG procedure has forced people to obtain information over the internet (*Figure 2*). LG for gastric cancer normally includes complicated procedures and demands delicate and precise techniques. During surgical education, obvious difficulties may be associated with learning and practicing the theoretical and applied aspects of such minimally invasive techniques compared with traditional open gastrectomy. Trainee surgeons interested in such techniques can learn from training boxes, courses, conferences and even from online websites.

The incidence of gastric cancer is variable by region and culture. The incidence rate is markedly elevated in East Asia (1). The distribution of countries producing the analyzed videos differed from the distribution of countries based on gastric cancer incidence. More than 70% of videos came from Europe and Americas. However, only 13.7% of

Table 6 Sources and classification of detected videos

XXXX	Academic (n=20)	Private (n=73)	Others (n=9)	P value
Number of views	4,953 [256–30,230]	3,948 [54–16,281]	1,334 [64–4,526]	0.751
Technique description				0.000
Yes	16 (80%)	24 (32.9%)	2 (22.2%)	
No	4 (20%)	49 (67.1%)	7 (77.8%)	
Information on scores				
VPI	2.05±2.29	2.91±5.65	1.70±2.37	0.665
JAMAS	1.10±0.45	1.96±0.51	1.44±0.73	0.008
Completeness	9.60±3.02	9.53±2.53	5.67±3.46	0.001

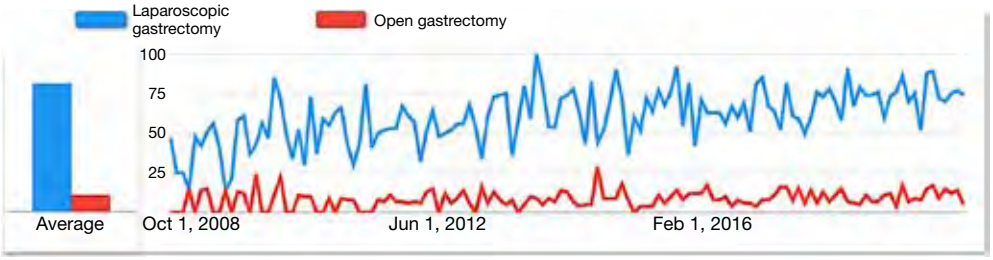

Figure 2 Increase in search keyword laparoscopic gastrectomy on Internet compared with open gastrectomy (https://trends.google.com).

videos came from East Asia. One reason for this may be that users in East Asia favored uploaded surgical videos using their native language (instead of English). Medical doctors in this region, who account for the majority of video sources, do not have time to pay sufficient attention to the platform because they frequently experience work overload and energy deficiencies (10-12), which seem to be another reason for this phenomenon.

The demonstration of each surgical procedure is critical because it is sometimes difficult for medical students or trainee surgeons to distinguish important anatomical structures and key steps of the surgical procedure from videos. Forty-one videos (40%) included descriptions of surgical procedures, whether through the use of text, voice, or a combination with pictures. Academic institutions had the largest proportion (80%) of technique descriptions compared with other sources. The results indicated that academic institutions may upload videos on YouTube primarily for educational purposes.

According to previous studies, the proportion of videos with HD resolution is usually less than 50% (13,14). Our results showed that approximately 52% of videos had HD resolution. However, we believe that the number of HD videos is still insufficient due to the complexity of the anatomical structures involved in LG and the dedicated skills required for appropriate technique. Excessive compression of image resolution can distort the view and reduce image quality to clinically unacceptable levels, which makes it difficult to distinguish important elements of the procedure.

When content was analyzed, videos on LDG and LTG each accounted for 48% of the total number of videos. LDG is the most frequently performed surgery among LG and is normally the first technique for trainee surgeons to learn. Three types of GI reconstruction are usually applied: gastroduodenostomy (Billroth I), loop gastrojejunostomy (Billroth II), and Roux-en-Y gastrojejunostomy. There is still no consensus regarding the best reconstruction method after distal gastrectomy (15,16). The choice is usually dependent on tumor location and surgeons' preferences (17). Our results showed that Roux-en-Y was the most highly reconstructed method, followed by Billroth II. Roux-en-Y in LDG is a more complicated procedure than Billroth I or Billroth

Table 7 Other English video-based education resources as an alternative to YouTube

Quality video-based education resource	Cost	Video platform	Website address
WebSurg e-surgical reference	Free	Private platform	http://www.websurg.com
American College of Surgeons (ACS) video library	Charge	Private platform	https://cine-med.com/acsonline
Society of American Gastrointestinal and Endoscopic Surgeons (SAGES) video library	Free	Based on YouTube	https://www.sages.org/video
Surgical Council on Resident Education (SCORE)	Charge	Private platform	https://portal.surgicalcore.org
MedTube	Free	Private platform	https://medtube.net

II because it has two anastomoses: jejunojejunostomy and gastroduodenostomy. With the improvement of laparoscopic instruments and the continuous accumulation of surgical experience, the procedures can be performed for total laparoscopy. Our results showed that approximately 57.1% jejunojejunostomy was performed intracorporeally in videos with procedures of Roux-en-Y reconstruction. However, all gastroduodenostomy was performed by linear stapler intracorporeally. The proportion of LTG videos was 48% (the same as the proportion of LDG videos), which may indicate that most publishers thought that laparoscopy may be acceptable for total gastrectomy. The laparoscopic technique has been gradually adopted for total gastrectomy (18). However, LTG is still considered a very demanding procedure due to technical difficulties, especially esophago-jejunal reconstruction. The placement of anvil and intracorporeal purse-string sutures and anvil placement are technical challenges. The OrVil™ method by circular stapler and the overlap method by linear stapler are the most commonly used techniques for esophagojejunostomy. Our results showed that the OrVil™ method was applied in 42% of the videos, and the overlap method was also applied in 42% of the videos. There is still controversy about the reconstruction method (19). OrVil™ can reduce the difficulty of anvil placement but not tension through the use of a circular stapler. However, circular staplers are difficult to use, especially in obese patients with restricted space. The overlap method can overcome such an obstacle but requires a sophisticated suturing technique.

Our study demonstrated that 102 videos about LG were provided by different sources. Private users represent the greatest number of sources, followed by academic institutions. The video source may be related to the video quality, authenticity, and reliability. Academic institutions were considered to provide higher quality and valuable videos (20,21). Most likely because of the professional field of LG, private uploaders in our study were usually surgeons who were proficient in the treatment of gastric cancer. The completeness scores did not differ between academic institutions and private users. To assess the popularity of videos about LG, we applied the VPI score. Our results show that private uploaders have a higher VPI. Watching operations performed by different surgeon pioneers around the world is an invaluable supplement to traditional education methods for laparoscopic surgery. However, we should note that surgeons may upload their laparoscopic videos on YouTube for reasons other than educational purposes. The total average VPI of the included videos was much lower than that of other kinds of surgical videos on YouTube (7,22). One reason may be that people prefer to watch and learn simpler surgeries, such as laparoscopic cholecystectomy, on YouTube. The quality of laparoscopic videos varies and may limit the validity of the education provided. Because the validated scoring systems that are available do not reflect LG, we included a completeness score for LG. We found that the average completeness scores were only 54.1%, with a combination of image quality, surgical information, procedure description, laparoscopic techniques and outcomes. Some videos only focused on part of the content of LG, such as the techniques of lymphadenectomy or reconstruction. It is unlikely to expect all videos to comprehensively cover all aspects of LG; it should be deemed that some videos, while incomplete, do contain precise and valuable content.

There is little doubt that YouTube is the largest video site in the world. However, there are now many other platforms that can provide educational surgical videos, both free-access and pay-per-view (*Table* 7). The videos are usually posted after academic review and quality control; a typical example is WebSurg. There were 29 videos about LG for gastric cancer by the end of September 2019. All the videos have voice commentaries and descriptions for each

surgical procedure. The earliest videos of LG for gastric cancer were uploaded prior to those on YouTube (*Table S1*). These websites can be used as an alternative to YouTube.

The importance of online media in laparoscopic education cannot be overestimated. Surgeon trainers have been previously suggested that trainees should be taught "not what to know, but rather…how to acquire information, discriminate and make the right choice in the present moment" (23). This concept was expressed 15 years ago when the internet was not yet widely popular worldwide. However, it still seems to be representative today. It is necessary for trainer surgeons and surgical educators to exercise responsibility in directing trainee surgeons to use reliable resources.

There are limitations to our study. First, this study only comprises a snapshot of information when the study data were collected, and results may change due to new videos being added or removed with time. Second, we only selected YouTube to analyze laparoscopic videos, given that the website is reported as the largest and most popular video source. There may also be other surgical video websites organized by academic societies or commercial organizations.

Conclusions

Surgical videos about LG were largely provided by professionals, such as those in academic institutions and surgeons. The role of private uploaders and academic institutions in surgical education cannot be overestimated. The most covered techniques were LDG and LTG, which indicates that YouTube can serve as a useful and appropriate educational tool. However, the quality of videos varied, and the information incompleteness was fairly high due to insufficient reviews. It is necessary for trainer surgeons and surgical educators to critically analyze the quality of video content and to exercise responsibility in guiding trainee surgeons. In the current era, it is best for trainees to search for peer-reviewed content.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study did not require approval by the local Research Ethics Board as it involved publicly available data only.

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Supplementary

Table S1 LG videos on platform of WebSurg

Category	Number/median
Gastrectomy	
LDG	11 (37.9%)
LTG	16 (55.2%)
LAPPG	2 (6.9%)
Lymphadenectomy	
D1	1 (3.5%)
D1+	4 (13.8%)
D2	11 (37.9%)
Unclassified	13 (44.8%)
Country	
Korea	7 (24.2%)
Italy	5 (17.3%)
France	4 (13.9%)
Spain	3 (10.4%)
Japan	2 (6.9%)
Argentina	2 (6.9%)
Belgium	1 (3.4%)
Brazil	1 (3.4%)
Portugal	1 (3.4%)
United of Kingdom	1 (3.4%)
Ireland	1 (3.4%)
Switzerland	1 (3.4%)
Videos resolution	
Low definition	3 (10.3%)
High definition	26 (89.7%)
Video editing	
Edited	29 (100%)
Technique description	
Voice commentary	29 (100%)
Video characteristics	
Number of days online	2,293 [70–4,900]
Number of views	3,953 [1,202–10,745]
Number of likes	119 [5–427]
Number of dislikes	–
Video duration	17.3 (6.7-306.5)

Data are expressed as the number of cases (percentage) or median (range). LG, laparoscopic gastrectomy; LDG, laparoscopic distal gastrectomy; LTG, laparoscopic total gastrectomy; LAPPG, laparoscopy-assisted pylorus-preserving gastrectomy.

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Retrospective Study

Effectiveness and safety of a laparoscopic training system combined with modified reconstruction techniques for total laparoscopic distal gastrectomy

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Abstract

BACKGROUND

Total laparoscopic distal gastrectomy (TLDG) is increasing due to some advantages over open surgery, which has generated interest in all gastrointenstinal (GI) surgeons. However, TLDG is technically demanding, especially for the procedures of lymphadenectomy and GI reconstruction. During the course of training, trainee surgeons have less chances to perform open gastrectomy compared with that of senior surgeons.

AIM

To evaluate an appropriate, efficient and safe laparoscopic training procedures suitable for trainee surgeons

METHODS

Ninety-two consecutive patients with gastric cancer who underwent TLDG plus Billroth I reconstruction using an augmented rectangle technique and involving trainees were reviewed. The trainees were taught a laparoscopic view of surgical anatomy, standard operative procedures and practiced essential laparoscopic skills. The TLDG procedure was divided into regional lymph nodes dissections and GI reconstruction for analyzing trainee skills Early surgical outcomes were compared between trainees and trainers to clarify the feasibility and safety of TLDG performed by trainees. Learning curves were used to assess the utility of our training system.

RESULTS

Five trainees performed a total of 52 TLDGs (56.5 %), while 40 TLDGs were conducted by two trainers (43.5 %). Except for depth of invasion and pathologic stage, there were no difference in clinicopathological characteristics. Trainers performed more D2 gastrectomies than trainees. The total operation time was significantly longer in the trainee group. The spent during the lesser curvature lymph node dissection and the Billroth I reconstruction were similar between the two groups. No difference was found in postoperative complications

between two groups. The learning curve of the trainees plateaued after 5 TLDG cases.

CONCLUSION

Preparing trainees with a laparoscopic view of surgical anatomy, standard operative procedures and practice in essential laparoscopic skills enabled trainees to perform TLDG safely and feasibly.

Key words: Gastric cancer; Total laparoscopic gastrectomy; Education system; Trainees; Augmented rectangle technique; Standard procedure

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Core tip: The rapid expansion of total laparoscopic distal gastrectomy has led to concern about education for young surgeons. The characters, less chances to perform open gastrectomy and higher technical demands, make laparoscopic training procedure for young surgeons differ from what surgeon pioneer experienced previously. We introduced our laparoscopic training system. Making laparoscopic procedure standard and using the easy reconstruction method are useful in the success of the training system.

INTRODUCTION

Laparoscopic assisted distal gastrectomy (LADG) was first reported by Kitano in 1991[1]. Since then, the use of laparoscopic surgery has rapidly become popular due to improving patients' quality of life and improving efficacy outcomes. The Japan Society of Endoscopic Surgery (JSES) performs a national survey every 2 years which indicates that number of laparoscopic procedures for gastric cancer is increasing. According to the 13th JSES survey, laparoscopic distal gastrectomy accounted for the highest proportion of laparoscopic gastrectomies[2]. Nevertheless, laparoscopic distal gastrectomy involves technically complex elements and requires dedicated skills especially in the procedures of lymphadenectomy and gastrointestinal (GI) reconstruction. Adequate harvesting of lymph nodes (LNs) is necessary for the quality of gastrectomy and now is mentioned in most gastric cancer guidelines[3]. GI reconstructions were initially performed extracorporeally by laparoscopy assisted procedures. However, it is sometimes difficult in patients with a small remnant stomach or in obese patients with thick abdominal walls[4]. With the development of laparoscopic devices and improvement of the anastomosis method, the reconstruction procedures can be completed laparoscopically[5].

The rapid expansion of laparoscopic surgery has led to concern about education for young surgeons. Experienced surgeons learned, developed, and introduced laparoscopic gastrectomy after mastering conventional open surgery. However, training and learning may differ for young surgeons who have less experience with open surgery[6]. The feasibility of laparoscopic gastrectomy operated by trainees is still debatable. To our knowledge, there are few studies describing the safety of LADG performed by trainee surgeons, and even fewer studies on total laparoscopic distal gastrectomy (TLDG).

Our department was founded in May 2015 and mainly focuses on minimally invasive surgery. One experienced laparoscopic surgeon started performing laparoscopic gastrectomy in April 2004. About 100 cases were conducted yearly. TLDG is the standard procedure for distal gastrectomy. For those needing Billroth I reconstruction, the augmented rectangle technique (ART) is applied[7].

We established an education system for TLDG to help young surgeons master the technique quickly.

This study reports the technical feasibility and short-term surgical outcomes of TLDG combined with modified reconstruction techniques performed by trainee gastric surgeons using our training system

MATERIALS AND METHODS

Patients

We retrospectively studied patients with gastric cancer, who underwent TLDG plus Billroth I reconstruction at Juntendo University Hospital, Tokyo, Japan, from June 2016 and June 2019. Clinical, surgical, and pathological data of these patients were collected and analyzed. The clinicopathological variables included age, gender, body mass index, American Society of Anesthesiologists physical status classification, medical history, pathological record, and duration of postoperative hospital stay. The surgical variables included operation time, LN dissection time, estimated blood loss and number of harvested LNs. Histological results were described according to Japanese Classification of Gastric Carcinoma[8]. Intraoperative and postoperative complications were stratified using the Clavien–Dindo classification system[9].

Laparoscopic techniques

Laparoscopic gastrectomy was performed using a five trocar system. LN dissection was done according to Japanese gastric cancer treatment guidelines[3]. Dissection was conducted in the following order: infrapyloric LNs (No.6), suprapyloric LNs (No.5), great curvature LNs (No.4, or plus 12a), suprapancreatic LNs (No.8a, 7, 9, or plus 11p), and along lesser curvature LNs (No. 1, 3). The operator stood on the left side of the patient for infrapyloric LN dissection and on the right side for other LNs dissection. Concomitant cholecystectomy was performed during the operation for patients with symptomatic gallbladder stones. Concomitant appendectomy was performed for patients with recurrent appendicitis.

Billroth I reconstruction using ART

ART was applied for Billroth I reconstruction, and all the procedures were created laparoscopically. The operator performed this technique on the left side of the patient. Three automatic laparoscopic linear staplers were used to create the gastroduodenostomy. The duodenum was transected from the greater curvature to less curvature. Small incisions were made on the greater curvature side, for each of the duodenal stumps and the remnant stomach. One jaw of the stapler was pressed against the posterior wall of the stomach 2 cm away from the gastric resection margin, and then the remnant stomach was rotated clockwise to the duodenal side. The duodenal stump was inserted by another jaw of stapler and then rotated externally by 90°. After the initial suturing between the remnant stomach and the duodenum, the posterior wall and caudal wall formed a V-shape. A 30 mm linear stapler was then applied to close the insertion holes up to the closest side of the duodenal resection margin, creating the third side of a rectangle. After gastric and duodenal resection margins were ensured to be close together, the 60 mm linear stapler was used to transect the duodenal resection margin to create the fourth side of the rectangle. After the above steps, all the previous linear staplers were removed from duodenal resection margin and the augmented rectangular gastroduodenal anastomosis was completed.

Trainer and trainees

Seven operators were involved in this study. There were two trainers and five trainees. Two trainers were Endoscopic Surgical Skill Qualification System for gastric cancer accredited surgeons. Trainees had at least 7 years' experience as a surgeon after graduation. The surgical outcomes of five trainees who had performed more than five TLDG procedures were compared with the other two trainers.

Education system for laparoscopic gastrectomy

Trainees received systematic education about laparoscopic gastrectomy, in four components. (Figure 1)

The first point: Understanding the anatomy and standard procedures of TLDG. (1) Study basic theoretical knowledge of vascular and lymphatic drainage anatomy especially in laparoscopy; and (2) watch non-edited operative video operated by trainer as well as trainees' videos repeatedly.

The second point: Master and improve the basic laparoscopic skills. (1) Develop hand-eye coordination, practice laparoscopic knot-tying and suturing techniques using training box; (2) strengthen basic skills using computer simulator with programs for laparoscopic surgery; and (3) participate in training sessions, such as hands-on training using porcine laboratory training organized by the Department of Minimally Invasive Surgery of Juntendo University Hospital, and educational seminars organized by the Juntendo University Medical Technology and Simulation Center and other organizations.

The third point: Experiences during laparoscopic surgery. (1) Operate with simple laparoscopic surgery such as laparoscopic cholecystectomy, and laparoscopic partial gastrectomy; and (2) be a scope operator and then an assistant to understand the standard procedure of TLDG.

The fourth point: Receive direction during real TLDG. During the trainees performing the TLDG, the trainer surgeon was usually the first assistant to give guidance.

Learning curve of the trainees

Two variables, operation time and intraoperative estimated blood loss, from patients who underwent TLDG by trainees were used to define the learning curve. Variables in each group were calculated as mean ± SD and then compared with that of those performed by the trainer surgeons. Continuous curves were plotted for each variable to identify any plateau effect. Plateau was defined as variable with < 5% change. The patient number at which a < 5% change occurred within variable gave the minimum number of cases needed to reach the learning curve for that variable.

Statistical analysis

Continuous data are presented as median and ranges. Independent-sample *t* test was used to analyze continuous data, and χ^2 or Fisher's exact tests was used to assess differences in categorical data. Statistical analysis was performed using the SPSS statistical software program (version 23). A $P < 0.05$ was considered significant.

RESULT

Ratio of the operator cases of TLDG by the trainees

A total of 92 patients received TLDG with ART between June 2016 and June 2019. Among them, 52 patients were operated by the trainees group while the remaining 40 patients were operated by trainers group (Figure 2). Compared with trainers, trainees performed more than 50% of the TLDG except for the first year.

Clinicopathological characteristics of the patients

Patient clinicopathological characteristics are summarized in Table 1. There were no significant differences between the two groups in patient characteristics, including age, sex, body mass index, American society of anesthesiologists status, and pathology staging. The trainers' group tended to perform operations for patients with higher depth of invasion ($P = 0.004$) and higher pathology stage ($P = 0.017$).

Surgical outcomes

The surgical outcomes, including intraoperative blood loss, and harvested number of LNs, were not significantly different between trainees and trainers group (Table 2). The trainers group performed more D2 gastrectomies than trainees ($P = 0.034$). The operation time was significantly longer in the trainees group compared with the trainers group ($P = 0.002$). No patient required conversion to open gastrectomy in either group. The postoperative stay was almost equivalent. The results of lymphadenectomy and GI reconstruction time are shown in Table 3. There were significant differences between the groups in the infrapyloric, suprapyloric, greater curvature, and suprapancreatic LN

dissection times. The times for lesser curvature LN dissection and GI reconstruction were similar between the two groups.

Postoperative complications

Four patients in the trainee group (7.7%) and two patients in the trainers' group (5%) had complications (Table 4). The most frequent complication was intra-abdominal abscess (3.8%) in the trainee group. No complication needed surgical intervention. There was no mortality associated with surgery in both groups.

Learning curve of the trainees

Among the 52 patients resected by trainees, the mean value of operation time is shown in Figure 3. The average operating time decreased from 315 min in cohort 1 min to 253 in cohort 10. The average operation time for the trainees plateaued at around 260 min after 5 cases. There was less than 5% change in average operation time after cohort 5 up to cohort 10, but still a big gap compared with that of the trainers. The average operative blood loss was similar for the two groups.

DISCUSSION

Gastric cancer ranks the fifth most common cancer and the third in cancer-related death worldwide with highest incidence rate in Eastern Asia[10]. Radical resection is the only curative modality for patients with resectable gastric cancer. Introduction of laparoscopic gastrectomy has shown promising results in early gastric cancer[11] and even comparable outcomes in advanced gastric cancer[12,13] when compared with open surgery. Laparoscopic gastrectomy has therefore rapidly gained popularity in the world. With the developments in anastomosis devices and modification in anastomotic techniques[5,14], more and more cases can be performed by total laparoscopic gastrectomy[2]. Intracorporeal GI reconstruction showed some benefits especially in the setting of narrow spaces in obese patients and small remnant stomachs from high location of tumor. Total laparoscopic gastrectomy generates interest and desire

not only in experienced surgeons but also in trainee surgeons. In this context, many efforts for research and education on laparoscopic surgery have been made. The JSES established Endoscopic Surgical Skill Qualification System and provides the educational environment for the training of qualified surgeons[15]. Some high-volume centers also reported their experiences of educating young surgeons on LADG[16-19]. However, most studies mixed different kinds of gastrectomy and even different reconstruction methods, which may cause bias of the results. In order to limit the influence of different techniques on results, we only focus on the most performed TLDG using the same surgical procedures and reconstruction methods for each patient in this study.

Our department was founded in 2015, and laparoscopic surgeries represent most of our surgeries. More than 90% of gastrectomies were performed by laparoscopy, and most GI reconstructions are done intracorporeally. The advantage of our volume is more opportunities for trainee surgeons to perform such surgeries. However, shortcomings of this are also evident in less opportunity to learn open surgery and higher technical skills. These characteristics make laparoscopic training procedures for young surgeons different from what experience surgeons experienced previously, placing higher educational and technical demands for residents[20]. Appropriate and efficient training systems suitable for the current situation need to be urgently established. One experienced surgeon in our department has performed laparoscopic surgery since 2004 and been concentrating on laparoscopic training[16,21]. When starting LG in our newly founded department, an educational and training system for young surgeon was set up at the same time.

Our training system covers four parts: understanding of anatomy and standard procedures, practicing basic laparoscopic skills, performing simple laparoscopic surgery and providing focal points during laparoscopic gastrectomy. It is useful to use a dry box to help trainees practice laparoscopic suturing techniques and improve hand-eye coordination[19,22]. However, the camera in the box is usually fixed to a particular point, which is different from practical surgery. In order to create a more realistic laparoscopic environment, we also use computer simulators to train young surgeons. Computer

simulators with laparoscopic programs and magnetic feedback systems can strengthen trainees' basic skills and surgical training more than a traditional dry box. Using a video recording system and online video websites, trainees can watch other surgeons' operative videos before operating and can analyze each step of their own surgery repeatedly after operation. Before becoming an operator, experiences gained from watching LG videos to operate simple laparoscopic surgeries, from being scope assistant to the first assistant helps trainees master laparoscopy-specific anatomical views, acquire skills of handling of laparoscopic energy devices and cooperating closely with other surgeons. Trainers play an important role, especially during the trainees' actual operation. In our department, trainers usually worked as the first assistant when trainees performed TLDG. Trainers could not help the trainee's procedures since the assistant's hands were always occupied for exposing the operative field of vision[16]. But as the first assistant trainers could directly provide direction, give confidence and control the quality of surgery.

The technically challenging component of TLDG for trainees mainly centers on lymphadenectomy and GI reconstruction. Trainees performed TLDG with longer time compared trainers. After splitting the whole procedure of TLDG into lymphadenectomy for each station and GI reconstruction, we found that lymphadenectomy in the infra-pyloric and supra-pancreatic areas took longer in the trainees group. However, reconstruction time was similar between the two groups. Standardizing operative procedures is useful for surgical education[22-24]. Paying more attention to the details of lymphadenectomy could improve surgical efficiency, reduce unnecessary injury and result in less bleeding. In our department, D1+ LN dissection is normally performed in the following order: "No. 6 → No. 5 (plus 12a in D2) → No. 4sa, 4d → No. 8a, 7, 9 (plus 11p in D2) → No. 3, 1" The total procedure is just like page-turning, which may make a good field of vision and avoid repeated clamping of the diseased gastric wall. Our results show there is a positive relationship between infra-pyloric and supra-pancreatic lymphadenectomy time. LN dissection in infra-pyloric and supra-pancreatic areas requires delicate manipulations especially in obesity patients. Much more adipose tissues make it difficult to identify the

correct anatomical planes. We developed an intracorporeal reconstruction technique named ART which is easily performed[7]. Two 60 mm and one 30 mm laparoscopic linear staplers are used to create a larger 4-sided anastomosis. Stay sutures are canceled and less (even no) intersection angle sutures are needed, which makes the technique easy and time-saving especially for trainees. Our results showed that the reconstruction time of trainees is similar to that of trainers. No anastomosis-related complications were found between the two groups. These results may indicate that this reconstruction method is easy and safe to perform for young surgeons.

In this study, we compared early outcomes of TLDG between trainees and trainers to clarify whether our training system was useful in maintaining the quality of trainees' operations. The number of harvested LNs and intraoperative bleeding were not significantly different between the two groups. The incidence of postoperative complications was similar between trainees and trainers groups. These results indicated that TLDG performed by trainees is safe and feasible. Some studies indicated pancreatic fistula occurred much more in trainees surgeries[17,23,24]. However, our results showed the incidence of pancreatic fistula was similar between the two groups. Much attention was paid to the dissection of the infra-pyloric and supra-pancreatic LNs. During operations we normally compress the adjacent tissues at the inferior border of the pancreas, instead of directly touching the pancreas itself. The postoperative hospital stays are relatively long compared with other reported studies. One reason is that more older patients were included in our series[7].

The trainees in our department have less chance to perform open gastrectomies. However, the learning curve for TLDG showed that the average operative time of the trainees reached a plateau after 5 cases, especially in older age and higher stage patients, compared with other studies[17,23]. The learning curve showed no difference in blood loss in the two groups. All the results may support that our educational and training system may enable trainees to quickly learn to perform TLDG. The influence of patient selection on the learning curve should also be evaluated. In our department, trainees usually

performed surgery for lower stage gastric cancer versus trainers. Careful patient selection for trainees might be one important factor for a successful initial experience with TLDG. We should take cognizance of the situation clearly that there is still a big gap between the two groups after trainees reaching the plateau, which may be accounted for the technical complexity of lymphadenectomy needing much longer learning time.

There are some limitations in our study. Its retrospective nature may induce some bias. Because of the length of follow up, our study did not provide enough data to show conclusions about oncologic safety and long-term outcomes. LNs dissection number may be prognostic factor for survival of patients. In our study, harvested LNs number more than 15 reach in 97.8% patients. We still need follow up these patients. The number of patients in our study is limited. But the study interval was shorter than other studies and only focused on TLDG with same procedure and reconstruction method. These may reduce the impacts of surgical techniques.

In conclusion, trainees can perform TLDG safely and feasibly after receiving systemic training. Making laparoscopic procedure standard and using easy reconstruction method are useful in the success of the training system.

ARTICLE HIGHLIGHTS

Research background

Total laparoscopic distal gastrectomy (TLDG) which involved technically complex elements and requires dedicated skills generates interest and desire not only in surgeon pioneers but also in trainee surgeons. The rapid expansion of TLDG has led to concern about education for young surgeons.

Research motivation

The characters, less chances to perform open gastrectomy and higher technical demands, make laparoscopic training procedure for young surgeons differ from what surgeon pioneer experienced previously. Appropriate and efficient training system suitable for current situation need to be urgently established.

Research objectives

The patients underwent TLDG plus Billroth I reconstruction from June 2016 and June 2019. Clinical, surgical, and pathological data of these patients were collected and analyzed.

Research methods

This study assesses our laparoscopic training system for TLDG based on short-term surgical outcomes. We reviewed ninety-two consecutive patients with gastric cancer who underwent TLDG plus Billroth I reconstruction using augmented rectangle technique. The trainees were required to receive systemic laparoscopic training. The total procedure of TLDG was divided into different regional lymph nodes dissection and gastrointenstinal reconstruction for analyzing. Early surgical outcomes were compared between trainees and trainers to clarify the feasibility and safety of TLDG performed by trainees.

Research results

Five trainees performed a total of 52 TLDG (56.5 %), while 40 TLDG were conducted by the two trainers (43.5 %). Except for depth of invasion and pathology stage, there were no difference in patient clinicopathological characteristics. Trainers performed more D2 gastrectomies than trainees. The total operation time was significantly longer in the trainees. The time of along less curvature lymph nodes dissection and Billroth I reconstruction were similar between the two groups. No difference was found in postoperative complications between two groups. The learning curve of the trainees plateaued after 5 TLDG cases.

Research conclusions

Preparing trainees with a laparoscopic view of surgical anatomy, standard operative procedures and practice in essential laparoscopic skills enabled trainees to perform TLDG safely and feasibly.

Research perspectives

Making laparoscopic procedure standard and using the easy reconstruction method are useful in the success of the training system.

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Footnotes

Institutional review board statement: This study was reviewed and approved by the Institutional Review Committee of Juntendo University Hospital.

Informed constent statement: Patients were not required to give informed consent to the study because our study was done retrospectively. Data for study were obtained after each patient agreed to treatment.

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Figure Legends

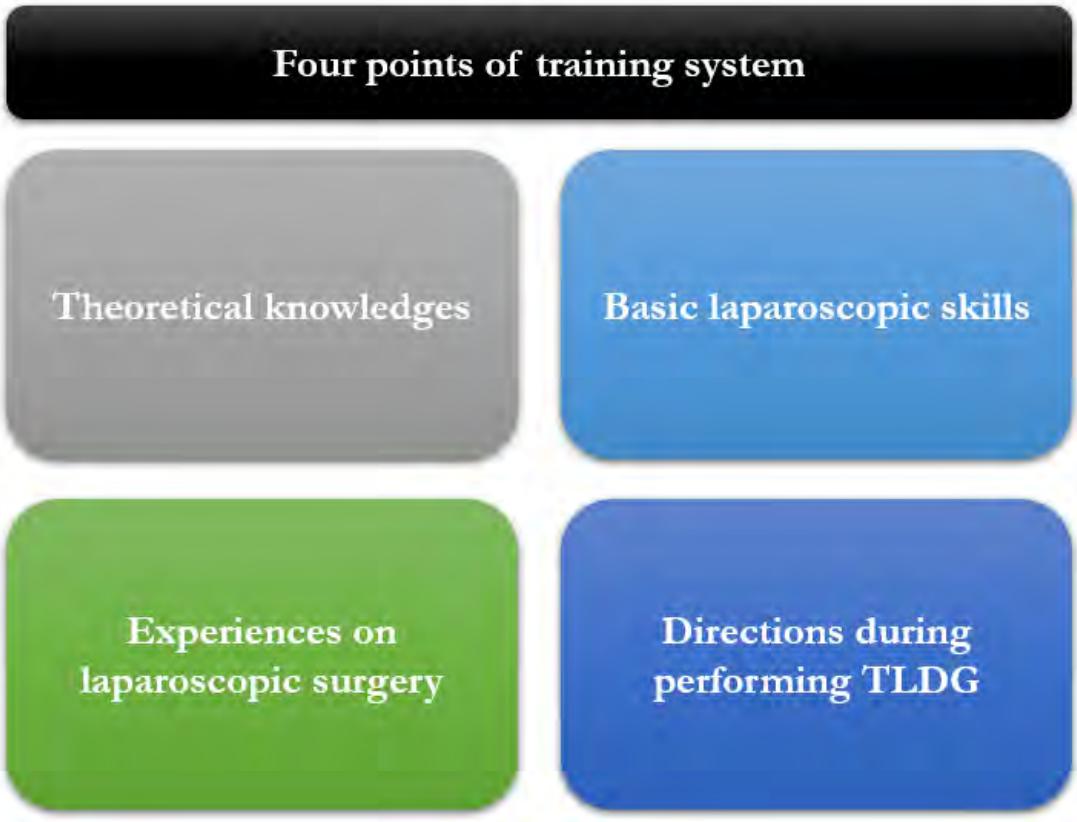

Figure 1 Four points of our training system. TLDG: Total laparoscopic distal gastrectomy.

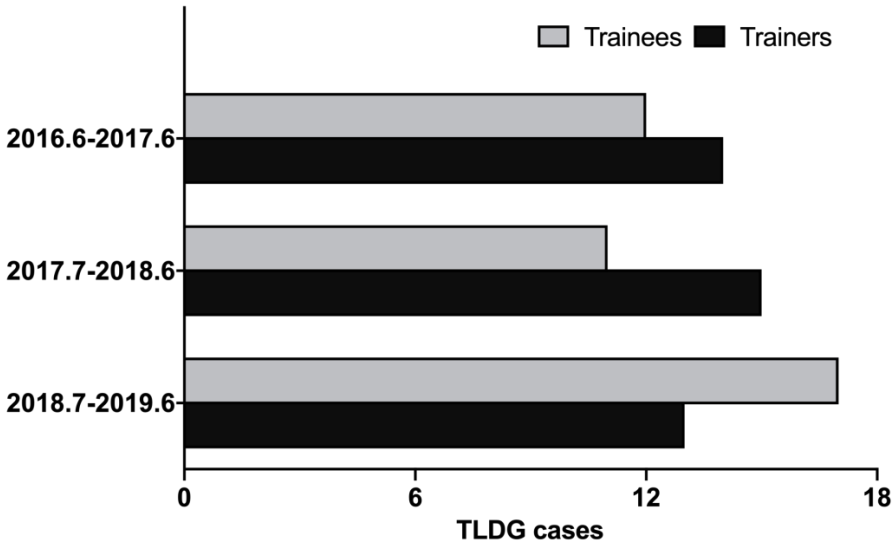

Figure 2 The number of patients who underwent total laparoscopic distal gastrectomy in our department each year. TLDG: Total laparoscopic distal gastrectomy.

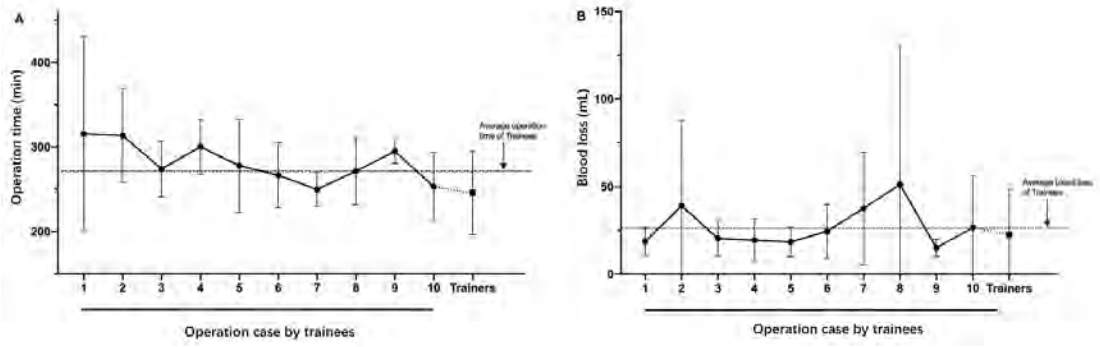

Figure 3 Learning curve of trainees. A: Average operation time for total laparoscopic distal gastrectomy (TLDG) performed by the trainees was compared among cases and that of trainers. After starting TLDG as an operator, the average operative time reached a plateau after 5 cases; B: Average blood loss for TLDG performed by the trainees was compared among cases and that of trainers.

Table 1 Patients' clinicopathological characteristics

Characteristics	Trainee surgeon	Trainer surgeon	*P* value
Age (yr)			
Median	68.5 (37-83)	69.6 (42-90)	0.630
< 80	44(84.6%)	31 (77.5%)	0.794
≥ 80	8 (15.4%)	9 (22.5%)	
Sex			
Male	32 (61.5%)	23 (57.5%)	0.830
Female	20 (38.5%)	17 (42.5%)	
BMI (kg/m)			
Median	22.01 (14.98-36.00)	23.11 (18.67-32.56)	0.145
< 25	43 (82.7%)	30 (75%)	0.440
≥ 25	9 (17.3%)	10 (25%)	
ASA			
1	20 (38.5%)	13 (32.5%)	0.793
2	29 (55.8%)	24 (60.0%)	
3	3 (5.7%)	3 (7.5%)	
Previous abdominal surgery			
Yes	14(26.9%)	8 (20%)	0.472
No	38 (73.1%)	32 (80%)	
pT			
T1	42 (80.8%)	22 (55.0%)	0.004
T2	3 (5.8%)	5 (12.5%)	
T3	6 (11.5%)	4 (10%)	
T4	1 (1.9%)	9 (22.5%)	
pStage			
IA	35 (67.3%)	17 (42.5%)	0.017
IB	7 (13.5%)	6 (15%)	
IIA	5 (9.6%)	3 (7.5%)	
IIB	4 (7.7%)	3 (7.5%)	

IIIA	1 (1.9%)	8 (20%)
IIIB	0	1 (2.5%)
IIIC	0	2 (5%)

Data are expressed as the median (range) or number of patients. BMI: Body mass index; ASA: American society of anesthesiologists.

Table 2 Surgical outcomes

Items	Trainee surgeon	Trainer surgeon	*P* value
LN dissection			
D1+	43 (82.7%)	25 (62.5%)	0.034
D2	9 (17.3%)	15 (37.5%)	
Combined organ resection	4 (7.7%)	2 (5%)	0.568
Cholecystectomy	4	0	
Appendicectomy	0	1	
Colectomy	0	1	
Blood loss	26 (5-170)	23 (3-125)	0.566
Conversion to open procedure	0	0	
Operation time (min)			
Median (range)	270 (199-512)	239 (154-375)	0.002
Harvested LNs (number)			
Median (range)	39 (14-86)	39 (14-70)	0.989
Postoperative hospital stays (d)			
Median (range)	13.38 (7-60)	12.70 (7-27)	0.720

Data are expressed as the median (range) or number of patients. LN: Lymph node.

Table 3 Lymphadenectomy and reconstruction outcome

Items	Trainee surgeon	Trainer surgeon	*P* value
lymphadenectomy			
Infrapyloric LNs	58.8 (27-135)	42.0 (19-85)	0.004
Suprapyloric LNs	18.8 (4-40)	10.6 (3-24)	0.001
Great Curvature LNs	17.7 (8-34)	12.3 (6-32)	0.004
Suprapancreatic LNs	41.0 (23-82)	28.4 (17-51)	0.001
Along lesser curvature LNs	16.6 (7-36)	14.1 (7-34)	0.213
GI reconstruction	19.0 (11-37)	18.9 (11-39)	0.988

Data are expressed as the median (range). LN: Lymph node; GI: Gastrointestinal.

Table 4 Postoperative complications

Items	Trainee surgeon	Trainer surgeon	*P* value
Anastomotic leakage	0	0	1.000
Anastomotic bleeding	1 (1.9%)	0	0.497
Anastomotic stnenosis	0	0	1.000
Intra-abdominal abscess	2 (3.8%)	1 (2.4%)	0.683
Pancreatic fistula	1 (1.9%)	1 (2.4%)	1.000
Ileus	0	0	1.000
Mortality	0	0	1.000

Data are expressed as number of patients. Clavien–Dindo grade 2 or higher.

【センター情報】ドイツ・シャリテ医科大学のセンター見学

Recent News

2019年4月5日

4月4日(木)に順天堂大学との協定校であるドイツ・シャリテ医科大学のルッツ シュタイナー国際課長らが大学を表敬訪問され、施設見学の一環としてシミュレーションセンターを見学されました。

福永副センター長に代わり、消化器・低侵襲外科の張医師にシミュレーションセンターを案内していただきました。

シュタイナー国際課長らには、実際にアンギオVRシミュレーター、ラパロVRシミュレーター、腹腔鏡ドライボックスなどの各シミュレーターを体験していただき、これらを含むシミュレーターを使用した学生教育を行っていること、そのほかにも医師や看護師、臨床研修医が施設を利用しトレーニングを行っていること、などを説明しました。

センター基本情報

営業時間：8:00 ～ 17:00
休業日：日曜日、祝日、創立記念日（5月15日）、年末年始（12月29日～1月3日）

お問合わせ
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学校法人 順天堂　〒113-8421　文京区本郷2丁目1番1号　大代表：03-3813-3111

Retrospective Study

Effectiveness and safety of a laparoscopic training system combined with modified reconstruction techniques for total laparoscopic distal gastrectomy

Zhang S *et al*. Education of laparoscopic gastrectomy for trainees

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Author contributions: All authors helped to perform the research; Zhang S and Orita H contributed to manuscript writing, performing procedures, and data analysis; Egawa H, Matsui R, Yube Y, Kaji S, Takahashi T and Oka S contributed to performing procedures, and data analysis; Egawa H and Matsui R contributed to writing the manuscript; Zhang S, Yamauchi S and Inaki N contributed to data analysis and statistical review; Orita H and Fukunaga T contributed to writing the manuscript, and drafting the conception and design of this work.

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Abstract

BACKGROUND

Total laparoscopic distal gastrectomy (TLDG) is increasing due to some advantages over open surgery, which has generated interest in all gastrointenstinal (GI) surgeons. However, TLDG is technically demanding, especially for the procedures of lymphadenectomy and GI reconstruction. During the course of training, trainee surgeons have less chances to perform open gastrectomy compared with that of senior surgeons.

AIM

To evaluate an appropriate, efficient and safe laparoscopic training procedures suitable for trainee surgeons

METHODS

Ninety-two consecutive patients with gastric cancer who underwent TLDG plus Billroth I reconstruction using an augmented rectangle technique and involving trainees were reviewed. The trainees were taught a laparoscopic view of surgical anatomy, standard operative procedures and practiced essential laparoscopic skills. The TLDG procedure was divided into regional lymph nodes dissections and GI reconstruction for analyzing trainee skills Early surgical outcomes were compared between trainees and trainers to clarify the feasibility and safety of TLDG performed by trainees. Learning curves were used to assess the utility of our training system.

RESULTS

Five trainees performed a total of 52 TLDGs (56.5 %), while 40 TLDGs were conducted by two trainers (43.5 %). Except for depth of invasion and pathologic stage, there were no difference in clinicopathological characteristics. Trainers performed more D2 gastrectomies than trainees. The total operation time was significantly longer in the trainee group. The spent during the lesser curvature lymph node dissection and the Billroth I reconstruction were similar between the two groups. No difference was found in postoperative

complications between two groups. The learning curve of the trainees plateaued after 5 TLDG cases.

CONCLUSION

Preparing trainees with a laparoscopic view of surgical anatomy, standard operative procedures and practice in essential laparoscopic skills enabled trainees to perform TLDG safely and feasibly.

Key words: Gastric cancer; Total laparoscopic gastrectomy; Education system; Trainees; Augmented rectangle technique; Standard procedure

Zhang S, Orita H, Egawa H, Matsui R, Yamauchi S, Yube Y, Kaji S, Takahashi T, Oka S, Inaki N, Fukunaga T. Effectiveness and safety of a laparoscopic training system combined with modified reconstruction techniques for total laparoscopic distal gastrectomy. *World J Gastroenterol* 2020; In press

Core tip: The rapid expansion of total laparoscopic distal gastrectomy has led to concern about education for young surgeons. The characters, less chances to perform open gastrectomy and higher technical demands, make laparoscopic training procedure for young surgeons differ from what surgeon pioneer experienced previously. We introduced our laparoscopic training system. Making laparoscopic procedure standard and using the easy reconstruction method are useful in the success of the training system.

INTRODUCTION

Laparoscopic assisted distal gastrectomy (LADG) was first reported by Kitano in 1991[1]. Since then, the use of laparoscopic surgery has rapidly become popular due to improving patients' quality of life and improving efficacy outcomes. The Japan Society of Endoscopic Surgery (JSES) performs a national survey every 2 years which indicates that number of laparoscopic procedures for gastric cancer is increasing. According to the 13th JSES survey, laparoscopic distal gastrectomy accounted for the highest proportion of laparoscopic gastrectomies[2]. Nevertheless, laparoscopic distal gastrectomy involves technically complex elements and requires dedicated skills especially in the procedures of lymphadenectomy and gastrointestinal (GI) reconstruction. Adequate harvesting of lymph nodes (LNs) is necessary for the quality of gastrectomy and now is mentioned in most gastric cancer guidelines[3]. GI reconstructions were initially performed extracorporeally by laparoscopy assisted procedures. However, it is sometimes difficult in patients with a small remnant stomach or in obese patients with thick abdominal walls[4]. With the development of laparoscopic devices and improvement of the anastomosis method, the reconstruction procedures can be completed laparoscopically[5].

The rapid expansion of laparoscopic surgery has led to concern about education for young surgeons. Experienced surgeons learned, developed, and introduced laparoscopic gastrectomy after mastering conventional open surgery. However, training and learning may differ for young surgeons who have less experience with open surgery[6]. The feasibility of laparoscopic gastrectomy operated by trainees is still debatable. To our knowledge, there are few studies describing the safety of LADG performed by trainee surgeons, and even fewer studies on total laparoscopic distal gastrectomy (TLDG).

Our department was founded in May 2015 and mainly focuses on minimally invasive surgery. One experienced laparoscopic surgeon started performing laparoscopic gastrectomy in April 2004. About 100 cases were conducted yearly. TLDG is the standard procedure for distal gastrectomy. For those needing Billroth I reconstruction, the augmented rectangle technique

(ART) is applied[7]. We established an education system for TLDG to help young surgeons master the technique quickly.

This study reports the technical feasibility and short-term surgical outcomes of TLDG combined with modified reconstruction techniques performed by trainee gastric surgeons using our training system

MATERIALS AND METHODS

Patients

We retrospectively studied patients with gastric cancer, who underwent TLDG plus Billroth I reconstruction at Juntendo University Hospital, Tokyo, Japan, from June 2016 and June 2019. Clinical, surgical, and pathological data of these patients were collected and analyzed. The clinicopathological variables included age, gender, body mass index, American Society of Anesthesiologists physical status classification, medical history, pathological record, and duration of postoperative hospital stay. The surgical variables included operation time, LN dissection time, estimated blood loss and number of harvested LNs. Histological results were described according to Japanese Classification of Gastric Carcinoma[8]. Intraoperative and postoperative complications were stratified using the Clavien–Dindo classification system[9].

Laparoscopic techniques

Laparoscopic gastrectomy was performed using a five trocar system. LN dissection was done according to Japanese gastric cancer treatment guidelines[3]. Dissection was conducted in the following order: infrapyloric LNs (No.6), suprapyloric LNs (No.5), great curvature LNs (No.4, or plus 12a), suprapancreatic LNs (No.8a, 7, 9, or plus 11p), and along lesser curvature LNs (No. 1, 3). The operator stood on the left side of the patient for infrapyloric LN dissection and on the right side for other LNs dissection. Concomitant cholecystectomy was performed during the operation for patients with symptomatic gallbladder stones. Concomitant appendectomy was performed for patients with recurrent appendicitis.

Billroth I reconstruction using ART

ART was applied for Billroth I reconstruction, and all the procedures were created laparoscopically. The operator performed this technique on the left side of the patient. Three automatic laparoscopic linear staplers were used to create the gastroduodenostomy. The duodenum was transected from the greater curvature to less curvature. Small incisions were made on the greater curvature side, for each of the duodenal stumps and the remnant stomach. One jaw of the stapler was pressed against the posterior wall of the stomach 2 cm away from the gastric resection margin, and then the remnant stomach was rotated clockwise to the duodenal side. The duodenal stump was inserted by another jaw of stapler and then rotated externally by 90°. After the initial suturing between the remnant stomach and the duodenum, the posterior wall and caudal wall formed a V-shape. A 30 mm linear stapler was then applied to close the insertion holes up to the closest side of the duodenal resection margin, creating the third side of a rectangle. After gastric and duodenal resection margins were ensured to be close together, the 60 mm linear stapler was used to transect the duodenal resection margin to create the fourth side of the rectangle. After the above steps, all the previous linear staplers were removed from duodenal resection margin and the augmented rectangular gastroduodenal anastomosis was completed.

Trainer and trainees

Seven operators were involved in this study. There were two trainers and five trainees. Two trainers were Endoscopic Surgical Skill Qualification System for gastric cancer accredited surgeons. Trainees had at least 7 years' experience as a surgeon after graduation. The surgical outcomes of five trainees who had performed more than five TLDG procedures were compared with the other two trainers.

Education system for laparoscopic gastrectomy

Trainees received systematic education about laparoscopic gastrectomy, in four components. (Figure 1)

The first point: Understanding the anatomy and standard procedures of TLDG. (1) Study basic theoretical knowledge of vascular and lymphatic drainage anatomy especially in laparoscopy; and (2) watch non-edited operative video operated by trainer as well as trainees' videos repeatedly.

The second point: Master and improve the basic laparoscopic skills. (1) Develop hand-eye coordination, practice laparoscopic knot-tying and suturing techniques using training box; (2) strengthen basic skills using computer simulator with programs for laparoscopic surgery; and (3) participate in training sessions, such as hands-on training using porcine laboratory training organized by the Department of Minimally Invasive Surgery of Juntendo University Hospital, and educational seminars organized by the Juntendo University Medical Technology and Simulation Center and other organizations.

The third point: Experiences during laparoscopic surgery. (1) Operate with simple laparoscopic surgery such as laparoscopic cholecystectomy, and laparoscopic partial gastrectomy; and (2) be a scope operator and then an assistant to understand the standard procedure of TLDG.

The fourth point: Receive direction during real TLDG. During the trainees performing the TLDG, the trainer surgeon was usually the first assistant to give guidance.

Learning curve of the trainees

Two variables, operation time and intraoperative estimated blood loss, from patients who underwent TLDG by trainees were used to define the learning curve. Variables in each group were calculated as mean ± SD and then compared with that of those performed by the trainer surgeons. Continuous curves were plotted for each variable to identify any plateau effect. Plateau was defined as variable with < 5% change. The patient number at which a < 5% change occurred within variable gave the minimum number of cases needed to reach the learning curve for that variable.

Statistical analysis

Continuous data are presented as median and ranges. Independent-sample *t* test was used to analyze continuous data, and χ^2 or Fisher's exact tests was used to assess differences in categorical data. Statistical analysis was performed using the SPSS statistical software program (version 23). A $P < 0.05$ was considered significant.

RESULT

Ratio of the operator cases of TLDG by the trainees

A total of 92 patients received TLDG with ART between June 2016 and June 2019. Among them, 52 patients were operated by the trainees group while the remaining 40 patients were operated by trainers group (Figure 2). Compared with trainers, trainees performed more than 50% of the TLDG except for the first year.

Clinicopathological characteristics of the patients

Patient clinicopathological characteristics are summarized in Table 1. There were no significant differences between the two groups in patient characteristics, including age, sex, body mass index, American society of anesthesiologists status, and pathology staging. The trainers' group tended to perform operations for patients with higher depth of invasion ($P = 0.004$) and higher pathology stage ($P = 0.017$).

Surgical outcomes

The surgical outcomes, including intraoperative blood loss, and harvested number of LNs, were not significantly different between trainees and trainers group (Table 2). The trainers group performed more D2 gastrectomies than trainees ($P = 0.034$). The operation time was significantly longer in the trainees group compared with the trainers group ($P = 0.002$). No patient required conversion to open gastrectomy in either group. The postoperative stay was almost equivalent. The results of lymphadenectomy and GI

reconstruction time are shown in Table 3. There were significant differences between the groups in the infrapyloric, suprapyloric, greater curvature, and suprapancreatic LN dissection times. The times for lesser curvature LN dissection and GI reconstruction were similar between the two groups.

Postoperative complications

Four patients in the trainee group (7.7%) and two patients in the trainers' group (5%) had complications (Table 4). The most frequent complication was intra-abdominal abscess (3.8%) in the trainee group. No complication needed surgical intervention. There was no mortality associated with surgery in both groups.

Learning curve of the trainees

Among the 52 patients resected by trainees, the mean value of operation time is shown in Figure 3. The average operating time decreased from 315 min in cohort 1 min to 253 in cohort 10. The average operation time for the trainees plateaued at around 260 min after 5 cases. There was less than 5% change in average operation time after cohort 5 up to cohort 10, but still a big gap compared with that of the trainers. The average operative blood loss was similar for the two groups.

DISCUSSION

Gastric cancer ranks the fifth most common cancer and the third in cancer-related death worldwide with highest incidence rate in Eastern Asia[10]. Radical resection is the only curative modality for patients with resectable gastric cancer. Introduction of laparoscopic gastrectomy has shown promising results in early gastric cancer[11] and even comparable outcomes in advanced gastric cancer[12,13] when compared with open surgery. Laparoscopic gastrectomy has therefore rapidly gained popularity in the world. With the developments in anastomosis devices and modification in anastomotic techniques[5,14], more and more cases can be performed by total laparoscopic gastrectomy[2]. Intracorporeal GI reconstruction showed some benefits

especially in the setting of narrow spaces in obese patients and small remnant stomachs from high location of tumor. Total laparoscopic gastrectomy generates interest and desire not only in experienced surgeons but also in trainee surgeons. In this context, many efforts for research and education on laparoscopic surgery have been made. The JSES established Endoscopic Surgical Skill Qualification System and provides the educational environment for the training of qualified surgeons[15]. Some high-volume centers also reported their experiences of educating young surgeons on LADG[16-19]. However, most studies mixed different kinds of gastrectomy and even different reconstruction methods, which may cause bias of the results. In order to limit the influence of different techniques on results, we only focus on the most performed TLDG using the same surgical procedures and reconstruction methods for each patient in this study.

Our department was founded in 2015, and laparoscopic surgeries represent most of our surgeries. More than 90% of gastrectomies were performed by laparoscopy, and most GI reconstructions are done intracorporeally. The advantage of our volume is more opportunities for trainee surgeons to perform such surgeries. However, shortcomings of this are also evident in less opportunity to learn open surgery and higher technical skills. These characteristics make laparoscopic training procedures for young surgeons different from what experience surgeons experienced previously, placing higher educational and technical demands for residents[20]. Appropriate and efficient training systems suitable for the current situation need to be urgently established. One experienced surgeon in our department has performed laparoscopic surgery since 2004 and been concentrating on laparoscopic training[16,21]. When starting LG in our newly founded department, an educational and training system for young surgeon was set up at the same time.

Our training system covers four parts: understanding of anatomy and standard procedures, practicing basic laparoscopic skills, performing simple laparoscopic surgery and providing focal points during laparoscopic gastrectomy. It is useful to use a dry box to help trainees practice laparoscopic

suturing techniques and improve hand-eye coordination[19,22]. However, the camera in the box is usually fixed to a particular point, which is different from practical surgery. In order to create a more realistic laparoscopic environment, we also use computer simulators to train young surgeons. Computer simulators with laparoscopic programs and magnetic feedback systems can strengthen trainees' basic skills and surgical training more than a traditional dry box. Using a video recording system and online video websites, trainees can watch other surgeons' operative videos before operating and can analyze each step of their own surgery repeatedly after operation. Before becoming an operator, experiences gained from watching LG videos to operate simple laparoscopic surgeries, from being scope assistant to the first assistant helps trainees master laparoscopy-specific anatomical views, acquire skills of handling of laparoscopic energy devices and cooperating closely with other surgeons. Trainers play an important role, especially during the trainees' actual operation. In our department, trainers usually worked as the first assistant when trainees performed TLDG. Trainers could not help the trainee's procedures since the assistant's hands were always occupied for exposing the operative field of vision[16]. But as the first assistant trainers could directly provide direction, give confidence and control the quality of surgery.

The technically challenging component of TLDG for trainees mainly centers on lymphadenectomy and GI reconstruction. Trainees performed TLDG with longer time compared trainers. After splitting the whole procedure of TLDG into lymphadenectomy for each station and GI reconstruction, we found that lymphadenectomy in the infra-pyloric and supra-pancreatic areas took longer in the trainees group. However, reconstruction time was similar between the two groups. Standardizing operative procedures is useful for surgical education[22-24]. Paying more attention to the details of lymphadenectomy could improve surgical efficiency, reduce unnecessary injury and result in less bleeding. In our department, D1+ LN dissection is normally performed in the following order: "No. 6 → No. 5 (plus 12a in D2) → No. 4sa, 4d → No. 8a, 7, 9 (plus 11p in D2) → No. 3, 1" The total procedure is just like page-turning,

which may make a good field of vision and avoid repeated clamping of the diseased gastric wall. Our results show there is a positive relationship between infra-pyloric and supra-pancreatic lymphadenectomy time. LN dissection in infra-pyloric and supra-pancreatic areas requires delicate manipulations especially in obesity patients. Much more adipose tissues make it difficult to identify the correct anatomical planes. We developed an intracorporeal reconstruction technique named ART which is easily performed[7]. Two 60 mm and one 30 mm laparoscopic linear staplers are used to create a larger 4-sided anastomosis. Stay sutures are canceled and less (even no) intersection angle sutures are needed, which makes the technique easy and time-saving especially for trainees. Our results showed that the reconstruction time of trainees is similar to that of trainers. No anastomosis-related complications were found between the two groups. These results may indicate that this reconstruction method is easy and safe to perform for young surgeons.

In this study, we compared early outcomes of TLDG between trainees and trainers to clarify whether our training system was useful in maintaining the quality of trainees' operations. The number of harvested LNs and intraoperative bleeding were not significantly different between the two groups. The incidence of postoperative complications was similar between trainees and trainers groups. These results indicated that TLDG performed by trainees is safe and feasible. Some studies indicated pancreatic fistula occurred much more in trainees surgeries[17,23,24]. However, our results showed the incidence of pancreatic fistula was similar between the two groups. Much attention was paid to the dissection of the infra-pyloric and supra-pancreatic LNs. During operations we normally compress the adjacent tissues at the inferior border of the pancreas, instead of directly touching the pancreas itself. The postoperative hospital stays are relatively long compared with other reported studies. One reason is that more older patients were included in our series[7].

The trainees in our department have less chance to perform open gastrectomies. However, the learning curve for TLDG showed that the

average operative time of the trainees reached a plateau after 5 cases, especially in older age and higher stage patients, compared with other studies[17,23]. The learning curve showed no difference in blood loss in the two groups. All the results may support that our educational and training system may enable trainees to quickly learn to perform TLDG. The influence of patient selection on the learning curve should also be evaluated. In our department, trainees usually performed surgery for lower stage gastric cancer versus trainers. Careful patient selection for trainees might be one important factor for a successful initial experience with TLDG. We should take cognizance of the situation clearly that there is still a big gap between the two groups after trainees reaching the plateau, which may be accounted for the technical complexity of lymphadenectomy needing much longer learning time.

There are some limitations in our study. Its retrospective nature may induce some bias. Because of the length of follow up, our study did not provide enough data to show conclusions about oncologic safety and long-term outcomes. LNs dissection number may be prognostic factor for survival of patients. In our study, harvested LNs number more than 15 reach in 97.8% patients. We still need follow up these patients. The number of patients in our study is limited. But the study interval was shorter than other studies and only focused on TLDG with same procedure and reconstruction method. These may reduce the impacts of surgical techniques.

In conclusion, trainees can perform TLDG safely and feasibly after receiving systemic training. Making laparoscopic procedure standard and using easy reconstruction method are useful in the success of the training system.

ARTICLE HIGHLIGHTS

Research background

Total laparoscopic distal gastrectomy (TLDG) which involved technically complex elements and requires dedicated skills generates interest and desire

not only in surgeon pioneers but also in trainee surgeons. The rapid expansion of TLDG has led to concern about education for young surgeons.

Research motivation

The characters, less chances to perform open gastrectomy and higher technical demands, make laparoscopic training procedure for young surgeons differ from what surgeon pioneer experienced previously. Appropriate and efficient training system suitable for current situation need to be urgently established.

Research objectives

The patients underwent TLDG plus Billroth I reconstruction from June 2016 and June 2019. Clinical, surgical, and pathological data of these patients were collected and analyzed.

Research methods

This study assesses our laparoscopic training system for TLDG based on short-term surgical outcomes. We reviewed ninety-two consecutive patients with gastric cancer who underwent TLDG plus Billroth I reconstruction using augmented rectangle technique. The trainees were required to receive systemic laparoscopic training. The total procedure of TLDG was divided into different regional lymph nodes dissection and gastrointenstinal reconstruction for analyzing. Early surgical outcomes were compared between trainees and trainers to clarify the feasibility and safety of TLDG performed by trainees.

Research results

Five trainees performed a total of 52 TLDG (56.5 %), while 40 TLDG were conducted by the two trainers (43.5 %). Except for depth of invasion and pathology stage, there were no difference in patient clinicopathological characteristics. Trainers performed more D2 gastrectomies than trainees. The total operation time was significantly longer in the trainees. The time of along

less curvature lymph nodes dissection and Billroth I reconstruction were similar between the two groups. No difference was found in postoperative complications between two groups. The learning curve of the trainees plateaued after 5 TLDG cases.

Research conclusions

Preparing trainees with a laparoscopic view of surgical anatomy, standard operative procedures and practice in essential laparoscopic skills enabled trainees to perform TLDG safely and feasibly.

Research perspectives

Making laparoscopic procedure standard and using the easy reconstruction method are useful in the success of the training system.

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Footnotes

Institutional review board statement: This study was reviewed and approved by the Institutional Review Committee of Juntendo University Hospital.

Informed constent statement: Patients were not required to give informed consent to the study because our study was done retrospectively. Data for study were obtained after each patient agreed to treatment.

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Figure Legends

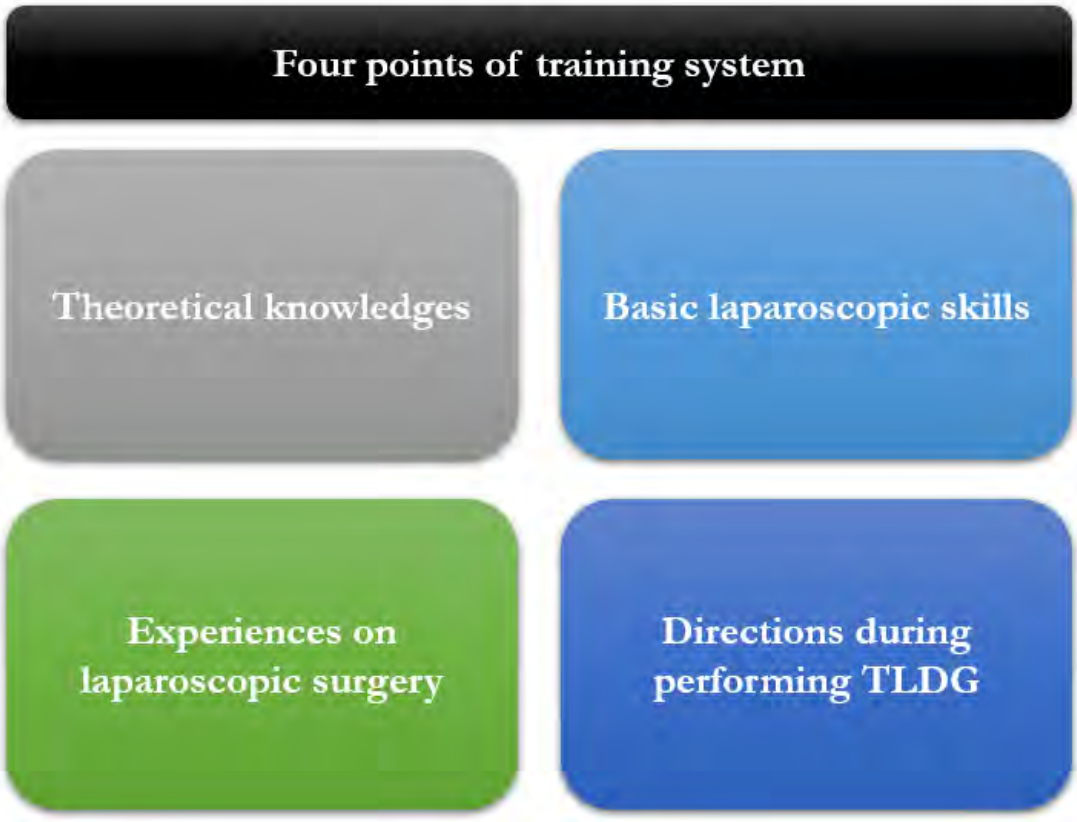

Figure 1 Four points of our training system. TLDG: Total laparoscopic distal gastrectomy.

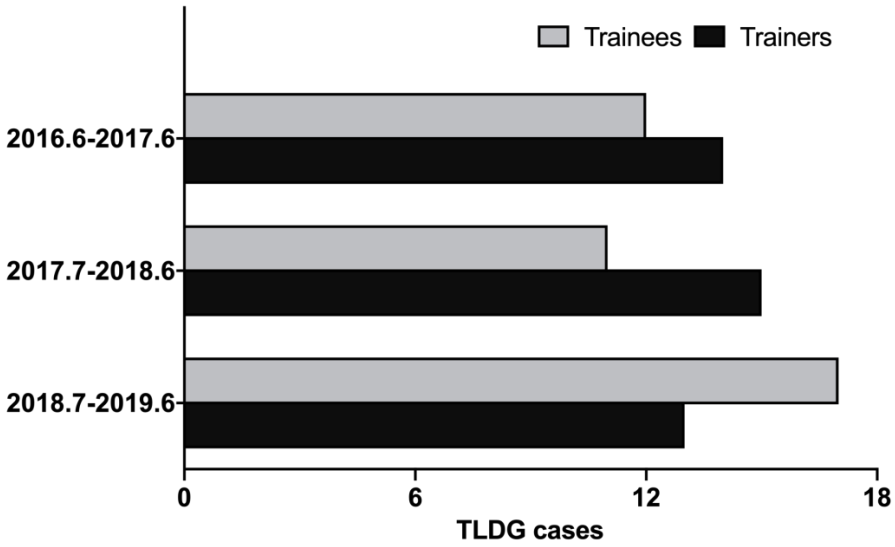

Figure 2 The number of patients who underwent total laparoscopic distal gastrectomy in our department each year. TLDG: Total laparoscopic distal gastrectomy.

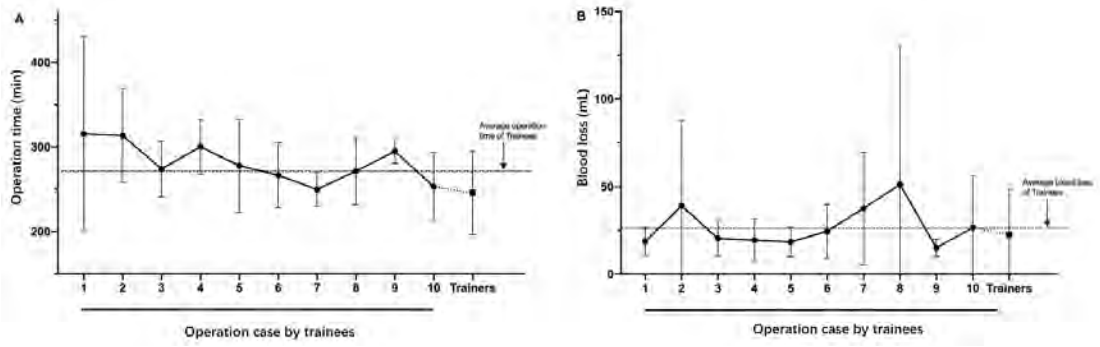

Figure 3 Learning curve of trainees. A: Average operation time for total laparoscopic distal gastrectomy (TLDG) performed by the trainees was compared among cases and that of trainers. After starting TLDG as an operator, the average operative time reached a plateau after 5 cases; B: Average blood loss for TLDG performed by the trainees was compared among cases and that of trainers.

Table 1 Patients' clinicopathological characteristics

Characteristics	Trainee surgeon	Trainer surgeon	*P* value
Age (yr)			
Median	68.5 (37-83)	69.6 (42-90)	0.630
< 80	44(84.6%)	31 (77.5%)	0.794
≥ 80	8 (15.4%)	9 (22.5%)	
Sex			
Male	32 (61.5%)	23 (57.5%)	0.830
Female	20 (38.5%)	17 (42.5%)	
BMI (kg/m)			
Median	22.01 (14.98-36.00)	23.11 (18.67-32.56)	0.145
< 25	43 (82.7%)	30 (75%)	0.440
≥ 25	9 (17.3%)	10 (25%)	
ASA			
1	20 (38.5%)	13 (32.5%)	0.793
2	29 (55.8%)	24 (60.0%)	
3	3 (5.7%)	3 (7.5%)	
Previous abdominal surgery			
Yes	14(26.9%)	8 (20%)	0.472
No	38 (73.1%)	32 (80%)	
pT			
T1	42 (80.8%)	22 (55.0%)	0.004
T2	3 (5.8%)	5 (12.5%)	
T3	6 (11.5%)	4 (10%)	
T4	1 (1.9%)	9 (22.5%)	
pStage			
IA	35 (67.3%)	17 (42.5%)	0.017
IB	7 (13.5%)	6 (15%)	
IIA	5 (9.6%)	3 (7.5%)	
IIB	4 (7.7%)	3 (7.5%)	
IIIA	1 (1.9%)	8 (20%)	

IIIB	0	1 (2.5%)
IIIC	0	2 (5%)

Data are expressed as the median (range) or number of patients. BMI: Body mass index; ASA: American society of anesthesiologists.

Table 2 Surgical outcomes

Items	Trainee surgeon	Trainer surgeon	*P* value
LN dissection			
D1+	43 (82.7%)	25 (62.5%)	0.034
D2	9 (17.3%)	15 (37.5%)	
Combined organ resection	4 (7.7%)	2 (5%)	0.568
Cholecystectomy	4	0	
Appendicectomy	0	1	
Colectomy	0	1	
Blood loss	26 (5-170)	23 (3-125)	0.566
Conversion to open procedure	0	0	
Operation time (min)			
Median (range)	270 (199-512)	239 (154-375)	0.002
Harvested LNs (number)			
Median (range)	39 (14-86)	39 (14-70)	0.989
Postoperative hospital stays (d)			
Median (range)	13.38 (7-60)	12.70 (7-27)	0.720

Data are expressed as the median (range) or number of patients. LN: Lymph node.

Table 3 Lymphadenectomy and reconstruction outcome

Items	Trainee surgeon	Trainer surgeon	*P* value
lymphadenectomy			
Infrapyloric LNs	58.8 (27-135)	42.0 (19-85)	0.004
Suprapyloric LNs	18.8 (4-40)	10.6 (3-24)	0.001
Great Curvature LNs	17.7 (8-34)	12.3 (6-32)	0.004
Suprapancreatic LNs	41.0 (23-82)	28.4 (17-51)	0.001
Along lesser curvature LNs	16.6 (7-36)	14.1 (7-34)	0.213
GI reconstruction	19.0 (11-37)	18.9 (11-39)	0.988

Data are expressed as the median (range). LN: Lymph node; GI: Gastrointestinal.

Table 4 Postoperative complications

Items	Trainee surgeon	Trainer surgeon	*P* value
Anastomotic leakage	0	0	1.000
Anastomotic bleeding	1 (1.9%)	0	0.497
Anastomotic stnenosis	0	0	1.000
Intra-abdominal abscess	2 (3.8%)	1 (2.4%)	0.683
Pancreatic fistula	1 (1.9%)	1 (2.4%)	1.000
Ileus	0	0	1.000
Mortality	0	0	1.000

Data are expressed as number of patients. Clavien–Dindo grade 2 or higher.

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専攻種別	☑論文博士		☐課程博士		

研究者評価（指導教官記入欄）

成績状況	優	取得単位数 取得単位数／取得すべき単位総数
学生本人が行った研究の概要	Tリンパ性白血病や自己免疫疾患など、種々の難治疾患にT細胞の異常が関わっている。これらの疾患における異常なT細胞の増殖や病態を抑える薬物として副腎皮質ステロイド薬（GC）があるが、その多彩な副作用やGC耐性の発現が治療を困難なものにしている。許氏は、主成分が中国や日本で臨床に用いられている生薬に注目して、GCのT細胞抑制作用に対するこれら薬物の併用効果を検討した。検討した生薬由来の薬物は、中国防已由来のテトランドリン、セファランチン、および日本防已由来のシノメニンである。許氏は、これら３種の化合物がいずれもGCのヒトT細胞白血病増殖抑制作用や活性化ヒトTリンパ球増殖抑制作用を相乗的に増強することを、初めて明らかとした。またその作用機序として、アポトーシス誘導作用、P糖タンパク質抑制作用、細胞周期修飾作用、MAPK抑制作用、GC受容体の核への移行増強作用等にも基づくことを明らかとした。	
総合評価	【良かった点】 難治疾患治療薬候補化合物としての生薬由来成分の可能性を示すとともに、その作用機序を分子レベルで多角的に検討し明らかにした点が評価に値する。また本研究結果は、４報の欧文論文として発表しており、さらに本研究内容は学位論文としてまとめ上げ、これにより許氏は本年３月に博士（薬学）号を授与される予定である。GC とセファランチンの相乗効果については種々の自己免疫疾患の治療に応用可能であるため、現在特許出願中である。 【改善すべき点】本学における研究は、当初の予定通りほぼ終了しており、改善すべき点は特にない。強いて言えば、研究成果の臨床応用に向けた今後の臨床試験等の研究計画が未完である点が挙げられる。 【今後の展望】許氏は、４月には武漢に帰国予定であるが、その後も日中連携研究を続け、本研究成果の臨床応用に向けた取り組みを行っていきたい。具体的には、まず武漢においては種々の免疫関連腎疾患患者に対するテトランドリンあるいはセファランチンとGCとの併用効果を検証していく。また本学においては、姉妹校である東京医科大学の各科医師らとの共同研究により、臨床応用のための基盤研究と臨床試験を実施していく方針である。	
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評価者（指導教官名）平野 俊彦

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研究テーマ	ヒト末梢血リンパ球に対する生薬成分の効果 Immunosuppressive Efficacies of Herbal Compounds on Human Peripheral Blood Lymphocytes				
専攻種別	論文博士	☑	課程博士	☐	

1．研究概要（1）

1）目的（Goal）

Tetrandrine (TET) and cepharanthine (CEP) are two bisbenzylisoquinoline alkaloids isolated from the traditional herbs. Recent molecular investigations firmly supported that TET or CEP would be a potential candidate for cancer chemotherapy. Prognosis of patients with glucocorticoid resistant T cell acute lymphoblastic leukemia (T-ALL) remains poor; here we examined the anti-T-ALL effects of TET and CEP and the underlying mechanism.

2）戦略（Approach）

Glucocorticoid resistant human leukemia Jurkat T cell line was used to examine the effects of TET and CEP in vitro.

3）材料と方法（Materials and methods）

Cell viability measured by WST-8 assay. Jurkat T cells at a cell density of 1.5×10^5 cells/mL were seeded in 96-well plates. TET or CEP was subsequently added into the corresponding wells to adjust the final concentrations of 3, 5, 10 and 15 μM. The cells were incubated with ethanol as a control. After 48 h treatment at 37℃, 10 μL of WST-8 solution was added to each well, followed by additional 3 h incubation. Optical density value was measured at 450 nm absorbance (ref. 650 nm).

Assessment of apoptosis. Jurkat T cells were seeded in 24-well plates and treated with serial concentrations of TET or CEP (0.3, 3, 5, 10 and 15 μM). 10 nM of CPT group was set up as a positive control for apoptotic cells. After 48 h incubation at 37℃, cells were harvested and washed by PBS twice. Then, the cells were co-stained using FITC Annexin V Apoptosis Detection Kit (BD PharmingenTM). Fluorescence of the cells was immediately determined by a flow cytometer (FACSCanto™ II, BD Biosciences, CA, US).

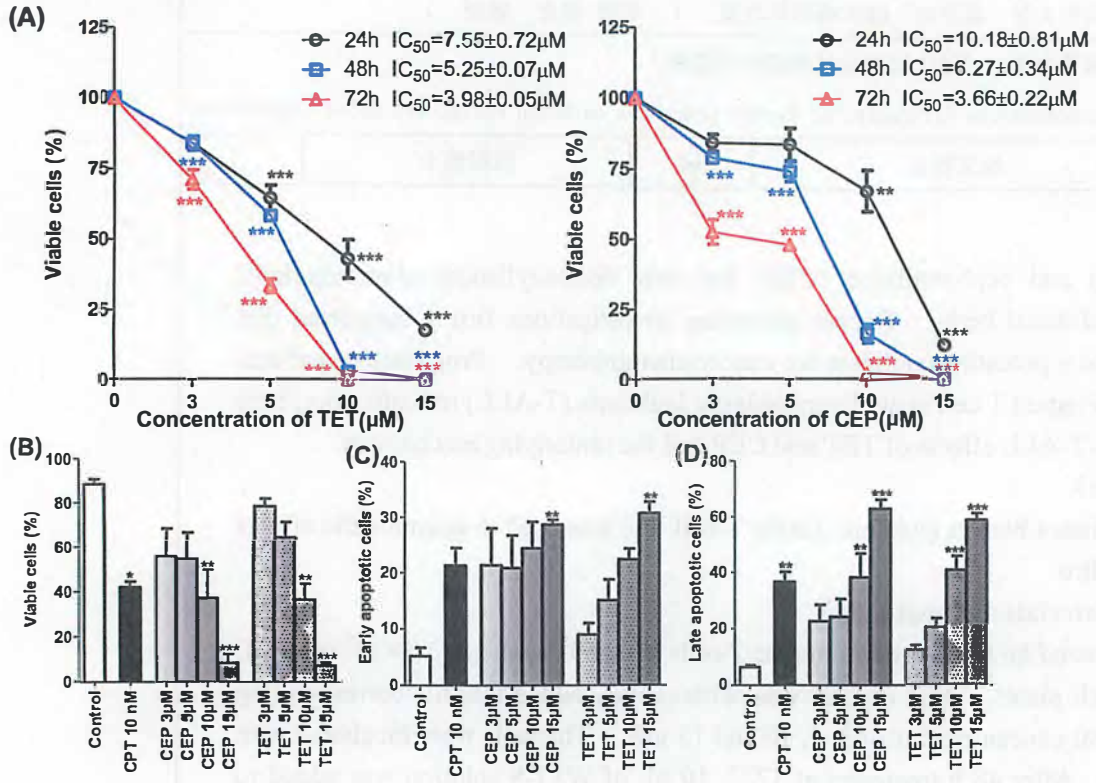
Western blot analysis. Whole cell protein was extracted by RIPA buffer. Western blot was performed using standard procedures. Membranes were incubated with primary antibodies against individual proteins overnight at 4℃ followed by an appropriate secondary antibody for 1 h at room temperature. The membranes were then analyzed in a luminescent image analyzer (Fujifilm; LAS-3000; Fujifilm, Tokyo, Japan).

4）実験結果（Results）

The result of cell viability was provided in **Fig. 1A**. Both CEP and TET inhibited the proliferation of Jurkat T cells significantly in a dose- and time-dependent manner [1-2]. Percentages of apoptotic cells after treated by TET or CEP were summarized in Fig. B-D. 15 μM of CEP and TET showed the strongest cytotoxic effects, and the

1．研究概要（2）

mean±SD values of viable cells were 8.4±6.9 and 7.4±2.4%, respectively ($P<0.001$, **Fig. B**). Consequently, the percentages of the early and the late apoptotic cells increased significantly after treating with 10 nM CPT or higher doses of TET and CEP (**Fig. B-D**).



Further investigations showed that TET or CEP not only upregulated the expression of initiator caspases such as caspase-8 and 9, but also increased the expression of effector caspases such as caspase-3 and 6. As the important markers of apoptosis, p53 and Bax were both upregulated by the treatment of TET and CEP. However, TET and CEP paradoxically increased the expression of anti-apoptotic proteins such as Bcl-2 and Mcl-1, and activated the survival protein NF-κB, leading to high expression of p-NF-κB. Cell cycle arrest at S phase accompanied by increase in the amounts of cyclin A2 and cyclin B1, and decrease in cylcin D1 amount in cells treated with TET or CEP will be another possible mechanism. During the process of apoptosis in Jurkat T cells, treatment with TET or CEP also increased the phosphorylation of JNK and p38. The PI3K/Akt/mTOR signaling pathway modification appears to play significant role in the Jurkat T cell apoptosis induced by TET or CEP. Moreover, TET and CEP seemed to downregulate the expressions of p-PI3K and mTOR in an independent way from Akt, since these two drugs strongly stimulated the p-Akt expression.

5）考察（Discussion）

These results provide fundamental insights into the clinical application of TET or CEP for the treatment of patients with relapsed T-ALL.

6）参考文献（References）

[1] Chen, J. Suzuki, H. Zhou, YW. *et al.* 2001. *J Cell Biochem.* **82**(2): 200-214.

[2]. Liou, JT. Lin, CS. Liao YC. *et al.* 2017. *Acta Pharmacol Sin.* **38**(8): 1171-1183.

2. 執筆論文 Publication of thesis ※記載した論文を添付してください。Attach all of the papers listed below.

論文名 1 Title	Plant - derived alkaloid sinomenine potentiates glucocorticoid pharmacodynamics in mitogen - activated human peripheral blood mononuclear cells by regulating the translocation of glucocorticoid receptor					
掲載誌名 Published journal	Phytotherapy Research					
	2019 年 1 月	33(1) 巻(号)	187 頁 ～ 196 頁	言語 Language	English	
第1著者名 First author	Wencheng Xu	第2著者名 Second author	Xiaoqin Wang	第3著者名 Third author	Yuanchao Tu	
その他著者名 Other authors	Hiroshi Masaki, Sachiko Tanaka, Kenji Onda, Kentaro Sugiyama, Haruki Yamada, Toshihiko Hirano					
論文名 2 Title	Tetrandrine and cepharanthine induce apoptosis through caspase cascade regulation, cell cycle arrest, MAPK activation and PI3K/Akt/mTOR signal modification in glucocorticoid resistant human leukemia Jurkat T cells					
掲載誌名 Published journal	Chemico-Biological Interactions					
	2019 年 9 月	310 巻(号)	108726 頁 ～ 頁	言語 Language	English	
第1著者名 First author	Wencheng Xu	第2著者名 Second author	Xiaoqin Wang	第3著者名 Third author	Yuanchao Tu	
その他著者名 Other authors	Hiroshi Masaki, Sachiko Tanaka, Kenji Onda, Kentaro Sugiyama, Haruki Yamada, Toshihiko Hirano					
論文名 3 Title	Vitamin K2 immunosuppressive effect on pediatric patients with atopic dermatitis					
掲載誌名 Published journal	Pediatrics International					
	2019 年 12 月	61(12) 巻(号)	1188 頁 ～ 1195 頁	言語 Language	English	
第1著者名 First author	Wencheng Xu	第2著者名 Second author	Kehan Meng	第3著者名 Third author	Hongguang Wu	
その他著者名 Other authors	Taro Miura, Shunsuke Suzuki, Masako Chiyotanda, Sachiko Tanaka, Kentaro Sugiyama, Hisashi Kawashima, Toshihiko Hirano					
論文名 4 Title	Tetrandrine recovers the impaired glucocorticoid receptor translocation via inhibiting P-glycoprotein in daunorubicin-resistant human T lymphoblastoid leukemia cells					
掲載誌名 Published journal	投稿中					
	年 月	巻(号)	頁 ～ 頁	言語 Language		
第1著者名 First author	Wencheng Xu	第2著者名 Second author	Xiaoqin Wang	第3著者名 Third author	Shuhe Chen	
その他著者名 Other authors	Yuanchao Tu, Hongguang Wu, Sachiko Tanaka, Kenji Onda, Kentaro Sugiyama, Haruki Yamada, Toshihiko Hirano					
論文名 5 Title	Low-dose methylprednisolone synergistically enhance the cytotoxic efficacy of tetrandrine on glucocorticoid resistant T lymphoblastoid leukemia MOLT-4 cells					
掲載誌名 Published journal	投稿中					
	年 月	巻(号)	頁 ～ 頁	言語 Language		
第1著者名 First author		第2著者名 Second author		第3著者名 Third author		
その他著者名 Other authors						

3. 学会発表 Conference presentation ※筆頭演者として総会・国際学会を含む主な学会で発表したものを

※Describe your presentation as the principal presenter in major academic meetings including general meeting

学会名 Conference	The 18th conference of Japanese Society of Clinical TCM and the Joint Meeting of Chinese - Japanese TCM		
演 題 Topic	Tetrandrine and cepharanthine induce apoptosis through caspase cascade regulation, cell cycle arrest, MAPK activation and PI3K/Akt/mTOR signal modification in glucocorticoid resistant human leukemia Jurkat T cells		
開催日 date	2019 年 9 月 14 日	開催地 venue	Peking
形式 method	☑ 口頭発表 Oral ☐ ポスター発表 Post	言語 Language	☐ 日本語 ☑ 英語 ☐ 中国語
共同演者名 Co-presenter			
学会名 Conference			
演 題 Topic			
開催日 date	年 月 日	開催地 venue	
形式 method	☐ 口頭発表 Oral ☐ ポスター発表 Post	言語 Language	☐ 日本語 ☐ 英語 ☐ 中国語
共同演者名 Co-presenter			
学会名 Conference			
演 題 Topic			
開催日 date	年 月 日	開催地 venue	
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学会名 Conference			
演 題 Topic			
開催日 date	年 月 日	開催地 venue	
形式 method	☐ 口頭発表 Oral ☐ ポスター発表 Post	言語 Language	☐ 日本語 ☐ 英語 ☐ 中国語
共同演者名 Co-presenter			

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名 称 Award name			
	国名 Country	受賞年 Year of	年 月
名 称 Award name			
	国名 Country	受賞年 Year of	年 月

5．本研究テーマに関わる他の研究助成金受給 Other research grants concerned with your resa:

受給実績 Receipt record	☐ 有　☐ 無
助成機関名称 Funding agency	
助成金名称 Grant name	
受給期間 Supported	年　月 ～　年　月
受給額 Amount received	円
受給実績 Receipt record	☐ 有　☐ 無
助成機関名称 Funding agency	
助成金名称 Grant name	
受給期間 Supported	年　月 ～　年　月
受給額 Amount received	円

6．他の奨学金受給 Another awarded scholarship

受給実績 Receipt record	■ 有　☐ 無
助成機関名称 Funding agency	China Scholarship Council
奨学金名称 Scholarship	State Scholarship Fund
受給期間 Supported	2018 年 12 月 ～ 2019 年 11 月
受給額 Amount received	160,000 円

7．研究活動に関する報道発表 Press release concerned with your research activities

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報道発表 Press release	☐ 有　☐ 無	発表年月日 Date of release	
発表機関 Released medium			
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発表タイトル Released title			

8．本研究テーマに関する特許出願予定 Patent application concerned with your research theme

出願予定 Scheduled	■ 有　☐ 無	出願国 Application	Japan
出願内容（概要） Application contents	植物由来アルカロイドを含有する医薬組成物		

9．その他 Others

指導責任者（署名） 平野俊彦

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RESEARCH ARTICLE

WILEY

Plant-derived alkaloid sinomenine potentiates glucocorticoid pharmacodynamics in mitogen-activated human peripheral blood mononuclear cells by regulating the translocation of glucocorticoid receptor

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Sinomenine has been used as an antirheumatic drug in China. Glucocorticoid combined with sinomenine could be an alternative therapeutic approach. In this study, we evaluated the sinomenine potential effect on glucocorticoid pharmacodynamics in vitro using a human peripheral blood mononuclear cell (PBMC) culture system. We also disclosed the possible action mechanism of sinomenine with a focus on P-glycoprotein function and glucocorticoid receptor (GR) translocation into nucleus. The median (range) of methylprednisolone IC_{50} values against the PBMC proliferation was 3.18 (0.45–6.81) ng/mL, whereas the median (range) IC_{50} values of methylprednisolone combined with 0.03, 0.3, 3, and 30 μM sinomenine were 1.85 (0.05–5.15), 0.83 (0.10–3.90), 0.56(0.09–1.62), and 0.59(0.05–1.30) ng/mL, respectively. Sinomenine significantly decreased the IC_{50} values of methylprednisolone and enhanced the immunosuppressive effect of methylprednisolone ($p < 0.05$). Sinomenine alone regulated the GR translocation in both Jurkat T cells and normal human PBMCs, and the combination of sinomenine and methylprednisolone showed stronger GR-modulatory activity than methylprednisolone alone. Thus, the additive effect of sinomenine to promote the methylprednisolone immunosuppressive efficacy was suggested to be related to nuclear GR-translocation. However, sinomenine did not significantly inhibit the P-glycoprotein function in the activated PBMCs, suggesting that sinomenine's additive effect seemed to be unrelated with the P-glycoprotein inhibition.

KEYWORDS

glucocorticoid receptor, immunosuppressive effect, methylprednisolone, peripheral blood mononuclear cells, P-glycoprotein, sinomenine

1 | INTRODUCTION

Sinomenine (SN), the main active alkaloid of herbal plant *Sinomenium acutum* (Thunb.), has been used to treat patients with rheumatoid arthritis (RA) in China since 1980s (Liu, Resch, & Kaever, 1994; Yamasaki, 1976). Synergistic effects of SN in combination with the immunosuppressive drugs tacrolimus and mycophenolic acid were confirmed in vitro (Vieregge, Resch, & Kaever, 1999). Therefore, immunosuppressive drugs combined with SN became to be an alternative therapeutic approach for RA patients in China.

P-glycoprotein (P-gp) is known to be localized on the immune cell membrane and acts as "efflux pumps," lowing the intracellular

concentration of many lipophilic drugs and thus leading to glucocorticoid (GC) resistant (Garcia-Carrasco et al., 2015). Persistent expression of P-gp was identified on human peripheral blood lymphocytes (PBMCs) of GC resistant patients with systemic lupus erythematosus (Kansal, Tripathi, Rai, & Agarwal, 2016). GC resistant RA patients also companied with higher percentage of P-gp expression (Maillefert et al., 1996). SN was reported to down-regulate P-gp expression and inhibit the efflux function of P-gp in multidrug resistant bladder cancer cell model 253 J/DOX (Chen et al., 2014). Whether therapeutic dose of SN can suppress the function of P-gp in PBMCs, to potentiate immunosuppressive effect of GC is essential to be clarified.

An immune-cell culture system in vitro using the mitogen-activated PBMCs appeared to be able to simulate the immune network in vivo (Xu, Meng, Kusano, et al., 2017; Xu, Meng, Tu, et al., 2017). Hence, in the present study, we evaluated the potential additive effect of SN on GC pharmacodynamics in vitro to suppress the proliferation of T-cell mitogen activated PBMCs with a focus on P-gp function.

GCs mediate their effects by binding on the glucocorticoid receptor (GR), an intracellular hormone receptor, which in absence of a stimulus remain inactive in the cytoplasm. After GC binding, GRs undergo conformational change and translocate into nucleus where they homodimerize and act as a transcriptional regulator of many target genes by binding on GC response elements. Consequently, the GC-GR homodimer shows strong anti-inflammatory and immunosuppressive potencies (Onda et al., 2006; Panagiotou, Mihailidou, Brauhli, Katsarou, & Moutsatsou, 2018). To explain the additional effects of SN on GC pharmacodynamics, we investigated SN on GR expression levels and GR subcellular localization. Comparing with the T-cell mitogen activated PBMCs, human leukemic Jurkat T-cells were easier to obtain with higher established reliability and thus chosen for this investigation (Panagiotou et al., 2018), in addition to PBMCs.

2 | MATERIALS AND METHODS

2.1 | Reagents

Roswell Park Memorial Institute (RPMI) 1640 and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, United States). Concanavalin A was purchased from Seikagaku Kogyo Co., Tokyo, Japan. SN (purity: more than 98%, biological source: *S. acutum*) and verapamil were purchased from Sigma Aldrich (St. Louis, MO, United States). SN was dissolved in ethanol (Wako Pure Chemical Industries, Ltd., Japan) at a concentration of 15 mM, filtered through a 0.2 μm membrane filter (Advantec Co., Japan) then stored at 4°C until use. The working concentrations were prepared by dilution with ethanol. Methylprednisolone (MP) was provided from Sigma Aldrich (St. Louis, MO, United States), dissolved in ethanol, and then stored at 4°C until use. The cell proliferation WST-8 assay kits were obtained from Dojindo Molecular Technologies, Inc., Japan. FITC mouse antihuman CD4 and APC Mouse Anti-Human CD8 were obtained from BD Biosciences, San Jose, CA, United States. GR antibody (G-5) was provided by Santa Cruz Biotechnology, INC (dilution 1:1000, # sc-393232). β-actin was purchased from Proteintech Group (dilution 1:5000, # 66009–1-Ig). Anti-TATA binding p antibody was obtained from Abcam (dilution 1:1000, # ab818). All other reagents were of the highest quality available from commercial vendors.

2.2 | Subjects

The present study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The study was approved by the Ethical Committee of Tokyo University of Pharmacy and Life Sciences, and written informed consent was obtained from all healthy subjects included in the study. The study included seven healthy subjects (four males and three females with a mean age of 26.8 years). These subjects had neither a history of immunological disorders nor a history of taking immunosuppressive drugs including GC.

2.3 | Isolation and culture of PBMCs and evaluation of drug effects in vitro

Twenty milliliters of venous blood were taken from healthy subjects between 9:00 and 11:00 in the morning and heparinized. This 20 mL sample size was the smallest possible to examine the effects of MP in the presence or absence of SN. The heparinized blood was loaded on 4 mL of Ficoll–Hypaque (Nakarai Co., Japan), and centrifuged at 1,300 g for 20 min. PBMCs were separated and suspended with RPMI 1640 medium containing 10% FBS, 100,000 IU/L penicillin, and 100 mg/L streptomycin to a final density of 1×10^6 cells/mL as we described previously (Xu, Meng, Kusano, et al., 2017).

One hundred ninety-four microliters of this cell suspension were loaded into wells of a 96-well plate. Concanavalin A, as a T-cell mitogen, was added to each well to a final concentration of 5 μg/mL. Subsequently, 2 μL of an ethanol solution containing MP were added to give final concentrations of 0.05, 0.5, 5, and 50 ng/mL. To examine the additional effect of SN combined with MP, 4 μL of an ethanol solution containing SN were subsequently added to give final concentrations of 0.03, 0.3, 3, 30, and 300 μM, respectively. Six microliters of ethanol were added to the control wells. The plate was incubated for 96 h in 5% CO_2 at 37°C. After the culture, 10 μL of WST-8 assay reagent solution were added to each well, and the plate was incubated for another 3 h. The PBMC proliferation was determined by measuring the optical density at 450 nm absorbance (ref. 650 nm). PBMCs proliferation was calculated by (Test-Blank)/(Control-Blank) × 100%. IC_{50} values of MP were obtained by GraphPad Prism 5. The culture wells of blank group included RPMI 1640 only. The culture system of test group included concanavalin A, SN, MP, or their combination and PBMC suspension. The culture wells of control group included concanavalin A, ethanol, and PBMC suspension (Xu, Meng, Tu, et al., 2017).

2.4 | Functional assays for P-glycoprotein in PBMCs

PBMCs were loaded with a final concentration of 2 μM of Rhodamine 123 (Rh123) and incubated for 10 min in 5% CO_2 at 37°C. Verapamil was used as a P-gp specific competitive inhibitor. Then, Rh123-treated cells were resuspended in a Rh123-free complete media with or without 0.03, 0.3, 3, 30, or 300 μM SN, combining with or without 0.5 ng/mL MP, 0.5 ng/mL MP, and 5 μM verapamil at 37°C for 180 min. After the efflux period, cells were washed twice with ice-cold PBS/10%FBS

and were then stained with FITC mouse anti-human CD4 and APC Mouse Anti-Human CD8. After staining, the cells were washed twice and then resuspended in PBS and kept on ice in the dark until analysis by FACSCanto™ II as we described before (BD Biosciences, San Jose, CA) (Xu, Meng, Tu, et al., 2017).

2.5 | Jurkat cell culture

Human leukemic Jurkat T-cells were purchased from ATCC and cultured in RPMI 1640 medium containing 10% FBS, 100,000 IU/L penicillin and 100 mg/L streptomycin, at 37°C in a humidified atmosphere of 5% CO_2. In order to prepare cells for Western blot analysis and immunofluorescence, cells were cultured with different concentrations of SN (0.03, 0.1, 0.3, 1, and 3 μM) and/or MP (5 ng/mL).

2.6 | Western blot analysis

PBMCs and Jurkat cells were pre-treated with SN for 24 h and harvested in 2 h after MP treatment. Cytoplasmic and nuclear proteins were extracted using the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, United States) according to the manufacturer's instruction. Protein concentration was quantified by Pierce BCA Protein Assay Kit (Thermo Scientific). Cytoplasmic and nuclear extractions were subsequently separated by SDS-polyacrylamide gel electrophoresis and then electrotransferred to hydrophobic polyvinylidene fluoride membrane (Immobilon-P; Merk Millipore, Darmstadt, Germany). Membranes were blocked with 5% skimmed milk for 1 h and washed with Tris-buffered saline/0.1% Tween-20 subsequently. Then, the membranes were incubated with primary antibodies against individual proteins overnight at 4°C. After triple washing with TBST, the membranes were continued to incubate with secondary antibody (Anti-mouse IgG, HRP-linked, #7076, Cell Signaling Technology, Inc.) at a dilution of 1:1000 for 1 h at room temperature. After triple washing with TBST, the signals were detected with an ECL or ECL Prime Western Blotting detection kit (GE Healthcare) in a luminescent image analyzer (Fujifilm; LAS-3000; Fujifilm, Tokyo, Japan). The images were subsequently quantitatively analyzed by ImageJ software (version 1.52e, National Institutes of Health, United States; http://imagej.nih.gov/ij).

2.7 | Immunofluorescence

In order to confirm the effect of SN on GR subcellular compartmentalization, Jurkat cells were subjected to immunofluorescence after treatment with SN and/or MP. Briefly, Jurkat cells were treated with SN for 24 h and then cultured with MP for another 2 h. Cells were harvested and fixed with 4% paraformaldehyde. After washing with PBS, cells were blocked for 1 h in PBS/3% BSA/0.5% triton-X at room temperature and incubated with primary GR antibody (1:100; G-5, sc-393232, Santa Cruz, CA, United States) overnight at 4°C. After washing with PBS, cells were continued to incubate with second goat anti-mouse antibody (1:500; Alexa Fluor 488 goat anti-mouse IgG/IgM, # A-10680, Invitrogen) for 1 h at room temperature, followed by 10 min nuclear staining with Hoechst 33342 (#17535, AAT Bioquest, Inc, CA, United States). Images were captured with a fluorescence microscope (Biozero BZ-8000 Series, Keyence, Japan). Signals were quantified using ImageJ program as mentioned above. A same size area was selected and the signal was measured in each nucleus, giving the intensity in arbitrary unit.

2.8 | Statistical analysis

Differences in the percentages of PBMC-proliferation, IC_{50} values of drugs, Rh123 accumulated amounts between controls and the cell fractions treated with serial concentrations of drugs, and protein expression levels were analyzed with Bonferroni tests. Expression and subcellular localization of GR in Jurkat cells after treatment with MP and/or SN were also analyzed with Bonferroni tests. Subcellular localization of GR in Jurkat cells following treatment with MP and/or SN and expression and subcellular localization of GR in PBMCs after treatment with MP and/or SN were performed using Dunnett's Multiple Comparison Test. These analyses were performed with GraphPad PRISM 5.0 (GraphPad Software Inc., San Diego, CA). In each case, two-sided p values (<0.05) were considered to be significant.

3 | RESULTS

3.1 | Effects of sinomenine in the presence or absence of methylprednisolone on mitogen-activated proliferation of PBMCs

The effects of SN on cell proliferation of PBMCs activated with a T-cell mitogen, concanavalin A, were examined in vitro. The results were presented in Figure 1A. SN at concentrations of 0.03–3 μM showed a little inhibitory effects, but SN at concentrations ≥30 μM exhibited strong suppressive effects on the activated proliferation of PBMCs ($p < 0.001$). The median (range) of SN IC_{50} values against the proliferation of PBMCs was 38.4 (2.51–187.00) μM.

Then, we continued to examine the additive efficacy of SN with MP to suppress the proliferation of mitogen-activated PBMCs of healthy subjects. The median (range) of MP IC_{50} values was 3.18 (0.45–6.81) ng/mL, whereas the median (range) IC_{50} values of the drug combined with 0.03, 0.3, 3, and 30 μM of SN decreased dose dependently to 1.85 (0.05–5.15), 0.83 (0.10–3.90), 0.56 (0.09–1.62), and 0.59 (0.05–1.30) ng/mL, respectively (Figure 1b). The additive effect of 30 μM SN compared with the suppressive effect of MP alone was statistically significant ($p < 0.05$) (Figure 1b).

3.2 | Effects of sinomenine in the presence or absence of methylprednisolone on P-glycoprotein function of PBMCs

Next, we investigated whether SN suppressed the function of P-gp in PBMCs to enhance the GC immunosuppressive effect. SN at 0.03 to 30 μM did not show any inhibitory ability on the efflux function of $CD4^+$, $CD8^+$ T cells, or lymphocytes. Three hundred micrometer of SN tended to suppress the P-gp function of these immune cells, but the effect was not significant (Figure 2a). Similar tendency was observed on the combination of MP and SN (Figure 2b). In contrast, verapamil at 5 μM significantly inhibited the efflux potencies of these cells ($p < 0.05$) (Figures 2a and b).

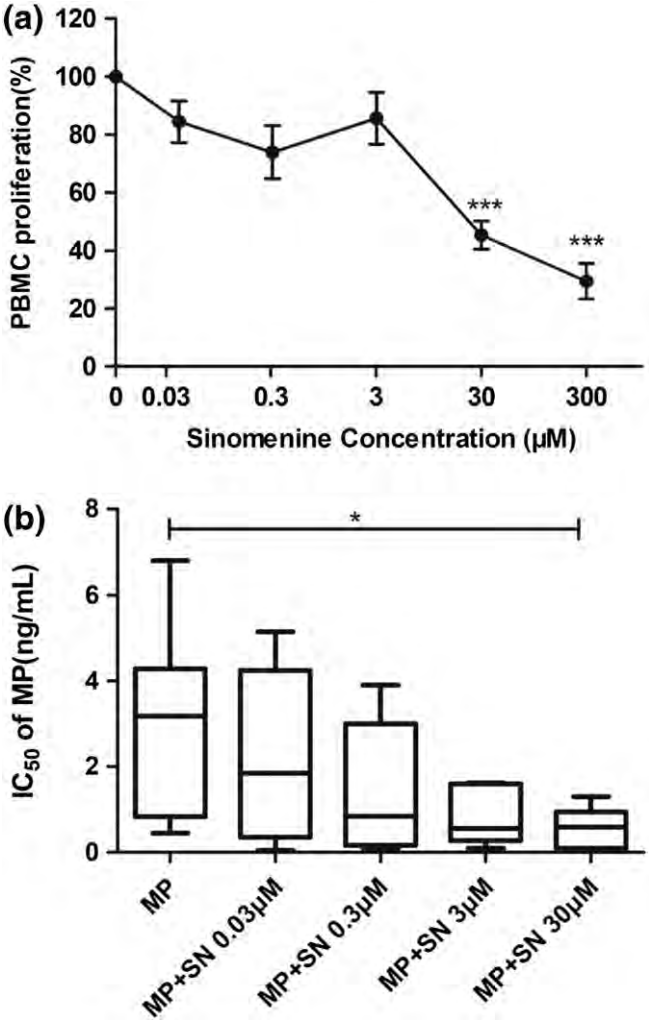

FIGURE 1 Effects of methylprednisolone (MP) in the presence or absence of sinomenine (SN) on proliferation of human peripheral blood mononuclear cells (PBMCs) activated with concanavalin A. Cell proliferation was determined by WST-8 assay. PBMC proliferation was estimated from the formula (Test-Blank)/(Control-Blank) × 100% (PBMC proliferation%). The IC_{50} values were calculated by GraphPad Prism 5. (a) Effects of SN on the proliferation of PBMCs. (b) IC_{50} values of methylprednisolone in the presence or absence of SN on the PBMC proliferation. The data were expressed as means ± S.E.M. Statistical analyses were performed using Bonferroni's multiple comparison tests, $^{*}p<0.05$ and $^{***}p<0.001$. ($n = 6$)

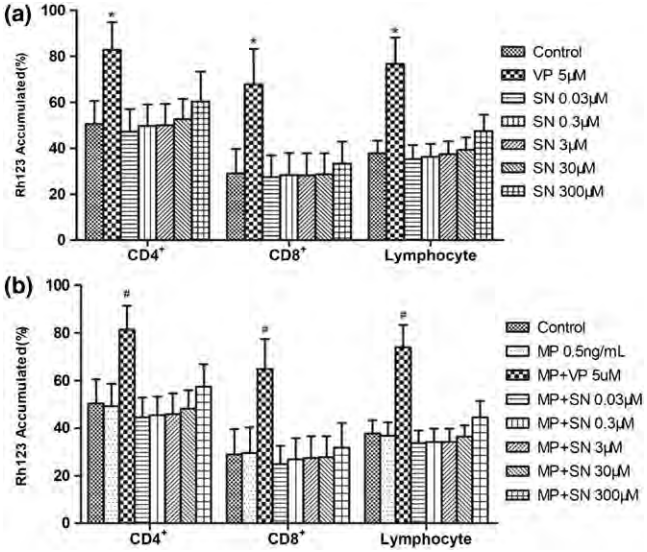

FIGURE 2 Rhodamine 123 (Rh123) accumulation in the human peripheral blood mononuclear cells (PBMCs) in the presence of sinomenine (SN). PBMCs were incubated with 2 μM Rh123 to uptake the fluorescent dye for 10 min. Subsequently, the cells were washed and cultured with or without drugs to efflux intracellular dye for 180 min. (a) Effects of SN, as compared with verapamil (VP), the prototype P-glycoprotein blocker, on P-glycoprotein function of PBMCs. PBMCs were incubated with 0.03–300 μM of SN for the efflux period, and the Rh123 accumulation was measured by flow cytometry. (b) P-glycoprotein inhibitory effect of SN in the presence of methylprednisolone (MP). Rh123 mean fluorescence intensities were detected by flow cytometry analysis, and Rh123 accumulated (%) was calculated by the formula: Dye accumulated/Dye uptaken × 100 (%). The results were expressed as means ± S.E.M. Statistical analyses were performed using Bonferroni's multiple comparison tests. $^{*}p<0.05$ as compared with the group without drugs. $^{\#}p<0.05$ as compared with the group treated with methylprednisolone alone. ($n = 3$)

3.3 | Effects of sinomenine in the presence methylprednisolone on GR expression level and GR subcellular localization of Jurkat cells

To further investigate the underlying mechanism of the additional effect of SN on GC, the GR expression levels and GR subcellular localization were evaluated. Nuclear and cytoplasmic extractions were subjected to Western blot analysis, respectively. As shown in Figure 3a, the levels of GR expression in nucleus was upregulated by 5 ng/mL of MP in Jurkat cells, and this GR upregulation effect of MP was significantly potentiated by 0.3 ($p < 0.05$), 1 ($p < 0.001$), and 3 μM ($p < 0.001$) of SN in a dose-dependent manner. Meanwhile, SN itself also seemed to increase the GR expression in the nucleus of Jurkat cells clearly, although these effects were negatively correlated to the dose of SN (dose range 0.3–3 μM) and the data were not significant. However, the levels of GR expression in cytoplasm were not dramatically changed by MP and/or SN (Figure 3b).

Subsequently, GR subcellular localization of Jurkat cells was analyzed by using the data of Figure 3a and 3b. As shown in Figure 3c, after 2 h treatment by MP, GR was translocated into nucleus, and thus, the ratio of GR distribution in nucleus and cytoplasm was changed by MP treatment. However the influences of SN in combination with MP on GR distribution were obvious, comparing with the MP alone. Therefore, the combination of MP and SN changed the ratio of GR distribution in nucleus and cytoplasm more strongly ($p < 0.01$).

3.4 | Effects of sinomenine alone on GR expression level and GR subcellular localization of Jurkat cells

As mentioned above, SN alone (0.3–3 μM) showed a tendency to upregulate GR expression levels in the nucleus of Jurkat cells, while in a negative dose-dependent manner. To confirm the effect of SN itself on GR translocation, enlarged range doses of SN (0.03–3 μM) were chosen to investigate (Figure 4). SN increased the GR expression in the nucleus dose-dependently. Among the five experimental doses of SN, 0.3 μM of SN showed the strongest effect to upregulate the

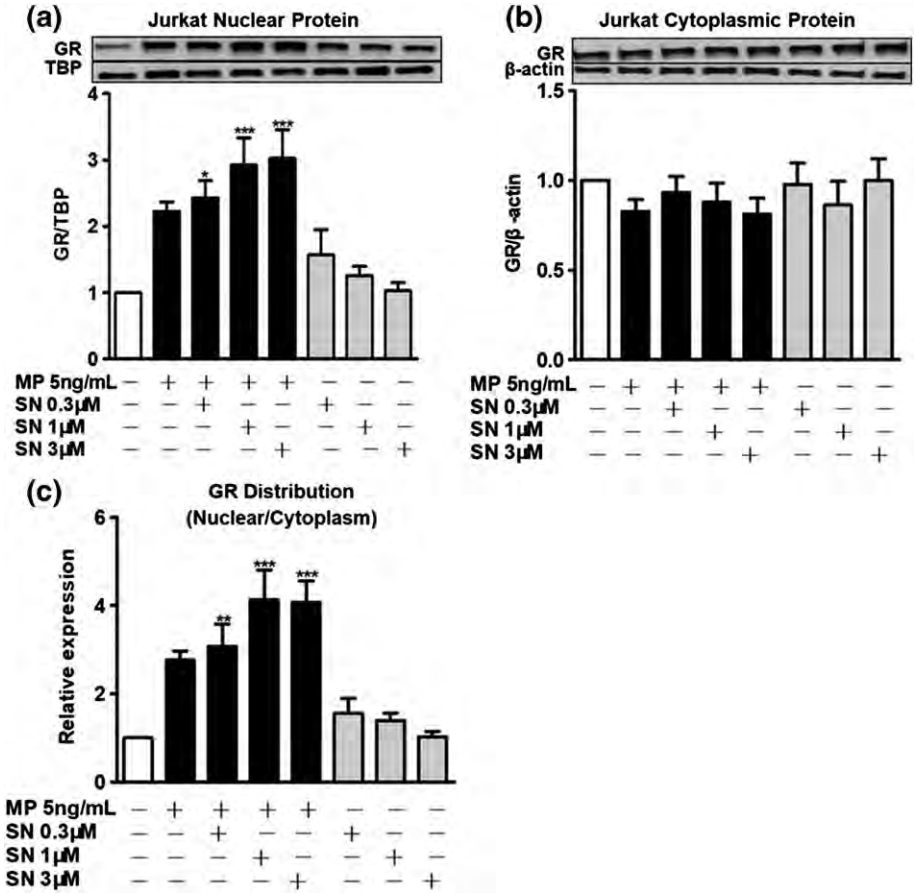

FIGURE 3 Expression and subcellular localization of glucocorticoid receptor (GR) in Jurkat cells after treatment with methylprednisolone (MP) and/or sinomenine (SN). Western blot analysis and densitometric quantification of total GR in nuclear (a) and cytoplasmic (b) extracts of Jurkat cells. Distribution of GR in nuclear/cytoplasmic fractions (c). Cells were pretreated with sinomenine for 24 h and harvested in 2 h after methylprednisolone treatment. Nuclear protein extracts were normalized to TATA-binding protein (TBP) and cytoplasmic extracts to β-actin. Data represented the mean ± S.E.M. of seven independent experiments. Statistical analyses were performed using Bonferroni's multiple comparison tests, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, as compared with the control group, respectively

nuclear GR expression level ($p < 0.01$, Figure 4a). All the experimental doses of SN had little effects on the cytoplasmic GR expression, although larger doses (≥1 μM) showed tendency to increase GR expression in cytoplasm (Figure 4b). Thus, the ratio of GR distribution in nucleus and cytoplasm changed by SN in a dose-dependent manner (Figure 4c).

3.5 | Effects of sinomenine in the presence or absence of methylprednisolone on GR subcellular compartmentalization using immunofluorescence in Jurkat cells

To provide morphologic evidence of cells, we further checked the subcellular distribution of GR in Jurkat cells after treatment with SN and/or MP by immunofluorescence. As shown in Figure 5a, treatment with MP (5 ng/mL) facilitated the nuclear translocation of GR. Similar results were observed after incubation of Jurkat cells with SN (0.03–3 μM, Figure 5a). Moreover, SN at 0.3 ($p < 0.05$), 1 ($p < 0.05$), and 3 μM ($p < 0.01$) significantly enhanced the translocation of GR in nucleus caused by MP in Jurkat cells (Figure 5b). These observations were in agreement with the results from Western blot (Figures 3 and 4).

3.6 | Effects of sinomenine in the presence or absence of methylprednisolone on GR expression level and GR subcellular localization of PBMCs

To confirm the modulation effects of SN and MP on the GR translocation, PBMCs obtained from three healthy subjects were fractionated, and GR expression levels were detected by Western blot. Figure 6a showed GR protein in the nucleus of PBMCs treated by the agents. 0.3 and 3 μM of SN tended to stimulate the GR expression in nucleus. Meanwhile, 3 μM of SN influenced the effect of MP (5 ng/mL) on nuclear GR expression slightly. However, in Figure 6b, the level of GR expression decreased by the combination of SN (3 μM) and MP (5 ng/mL) significantly ($p < 0.05$). The combination of MP and SN tended to change the distribution more largely (Figure 6c), which meant that SN could affect the GR subcellular localization of PBMCs.

4 | DISCUSSION

Comparing with isolated T cells, mitogen-activated PBMCs were much closer to the human immune network in vivo. Thus, PBMC culture

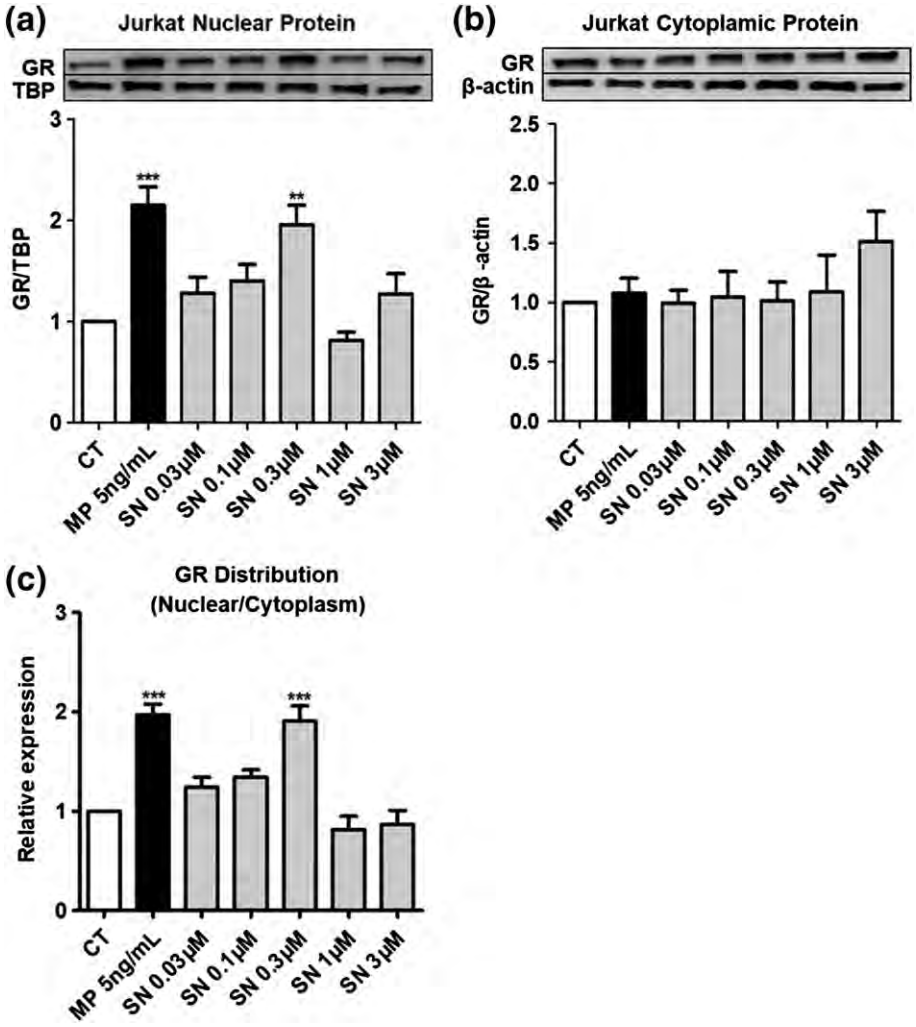

FIGURE 4 Expression and subcellular localization of glucocorticoid receptor (GR) in Jurkat cells after treatment with sinomenine (SN) alone. Western blot analysis and densitometric quantification of total GR in nuclear (a) and cytoplasmic (b) extracts of Jurkat cells. Distribution of GR in nuclear/cytoplasmic fractions (c). Cells were pretreated with sinomenine for 24 h and methylprednisolone (MP) for 2 h. Nuclear protein extracts were normalized to TATA-binding protein (TBP) and cytoplasmic extracts to β-actin. Data represented the mean ± S.E.M. of seven independent experiments. Statistical analyses were performed using Bonferroni's multiple comparison tests, $^{**}p < 0.01$ and $^{***}p < 0.001$, as compared with the control group, respectively

procedure was chosen to examine the SN potential effect on GC immunosuppressive efficacy (Xu, Meng, Tu, et al., 2017). SN at 0.03–3 μM tended to suppress the proliferation of PBMCs. The additional function of these concentrations of SN also tended to enhance the immunosuppressive efficacy of MP to decrease the IC_{50} values (Figures 1a and b). SN at ≥30 μM significantly inhibited the PBMC proliferation (Figure 1a); meanwhile, the agent potentiated the GC pharmacodynamics significantly (Figure 1b). Our findings in vitro may partially explain why GC combined with SN has been used for the treatment of RA patients in China, especially for those who were suffering from refractory RA.

A lot of studies reported that SN had anticancer function in breast and many other cancers due to its suppressive effects on cell proliferation and apoptosis promotion effects (Lu et al., 2013; Song et al., 2015; Song et al., 2018). To exclude a related cytotoxic effect of SN on human T cells, the SN effects on Jurkat T cells were examined by the WST-8 procedures in vitro. All the experimental concentrations of SN, including the highest one 300 μM, did not show any cytotoxic function in Jurkat T cells (data not shown).

Some GC-resistant refractory RA patients were known to be accompanied with higher P-gp expression on peripheral lymphocytes (Maillefert et al., 1996). Our data suggested that the additional function of SN to enhance GC immunosuppressive efficacy seemed not to be related with P-gp target of PBMCs because we did not observe the inhibitor capacity of SN (<300 μM) on the P-gp of PBMCs. Although SN (≥300 μM) tended to suppress the efflux function of P-gp on $CD4^+$, $CD8^+$ T cells, and lymphocytes (Figure 2), the pharmacokinetic parameters of SN tablet in PBMCs of healthy volunteers suggested that the serum concentration of SN could not accumulate with such higher concentration because the C_{max} of SN was 246.604 ± 71.165 ng/mL after taking 80 mg SN tablet once (Yan et al., 1997). Chen et al. reported that SN at 100 or 200 μg/mL exerted significant suppressive effects on P-gp of human bladder cancer 253 J/DOX cells (Chen et al., 2014). However, we did not

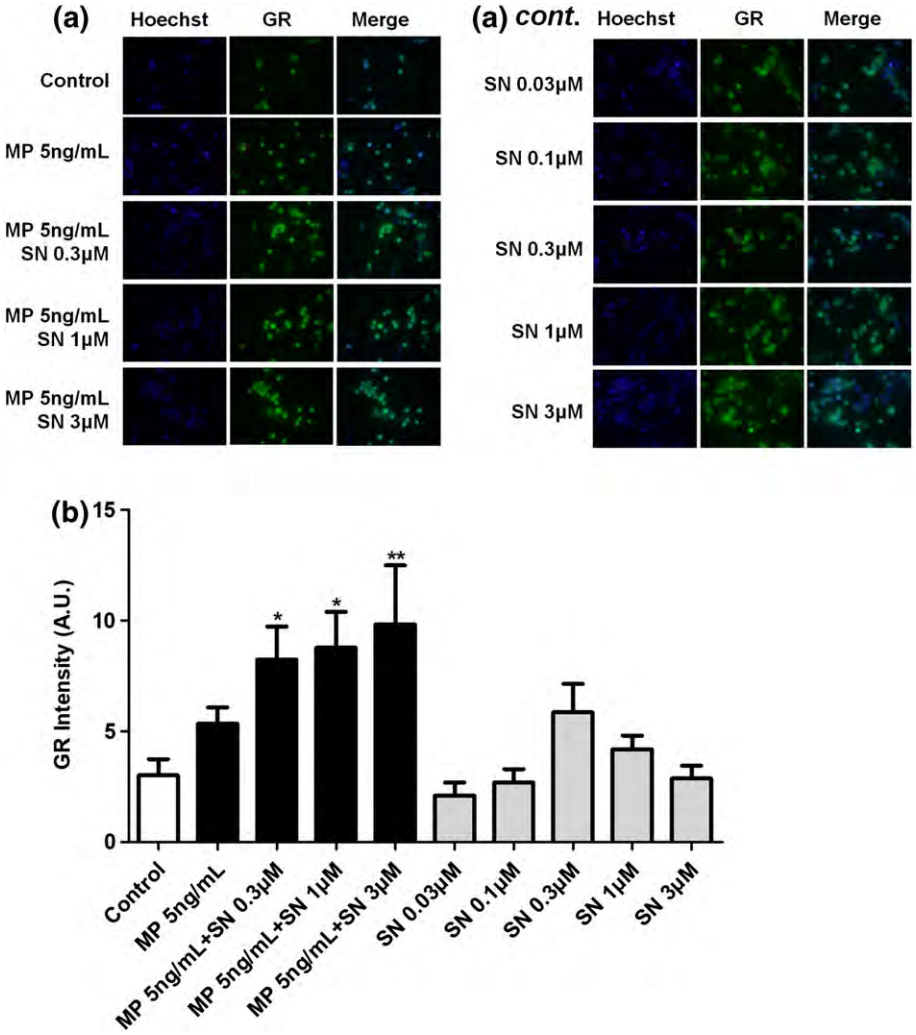

FIGURE 5 Subcellular localization of glucocorticoid receptor (GR) in Jurkat cells following treatment with methylprednisolone (MP) and/or sinomenine (SN). Cells were pretreated with sinomenine for 24 h and harvested in 2 h after methylprednisolone treatment. After washing, the cells were fixed and incubated with GR antibody, following with Alexa Fluor 488 goat anti-mouse IgG/IgM. Images were examined by a fluorescence microscope and the representative result of three independent experiments was shown as in Figure (a). Signals were quantified using ImageJ program, giving the intensity in arbitrary unit (b). GR intensity was expressed as means ± S.E.M. of three independent experiments and analyzed by using Dunnett's Multiple Comparison Test, $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with the control group, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

obtain the similar results when using the human PBMC culture system, which suggested that P-gp inhibitory function of SN was a cell-type specific effect.

To elucidate the mechanism of the additional function of SN on MP pharmacodynamics, we continued to examine the effects of SN in the presence or absence of MP on GR expression level and GR subcellular localization by using Jurkat cells. SN alone at 0.03–3 μM increased the GR expression in nucleus, showing the maximum efficacy of the agent at these concentrations (Figure 4). In a recent study, Li et al. reported that 1 μM of SN was enough to stimulate the phosphorylation of the mitogen-activated protein kinase p-38, JNK, and ERK on human breast cancer cell lines MDA-MB-231 and MCF-7 (Li et al., 2014). However, lots of researches disclosed that GRs might be phosphorylated by these kinases that alter their binding affinity for GC, stability, translocation to the nucleus, binding to DNA, and interaction with other proteins, such as transcription factors and molecular chaperones (Barnes & Adcock, 2009; Hirano, 2007; Weigel & Moore, 2007). The above information may partially explain the phenomenon that SN at concentrations more than 1 μM showed even lower efficacy on the GR translocation.

However, 1 or 3 μM SN combined with MP exhibited larger efficacy to potentiate the GR translocation (Figure 3), which was in accordance with the tendency of the agents on PBMC pharmacodynamics (Figure 1b). The information hinted that some other mechanisms were existed in the additional effect of SN at concentrations of 1–3 μM. One of the possible reasons was that higher doses of SN showed mild effect to increase the amount of GR, resulting in enhanced ability of GR translocation in Jurkat cytoplasm (Figure 4b), and thus the GR expression in nucleus increased. On the other hand, 1 μM SN was reported to prevent IL-1β-induced p-NF-κB p65 expression in human fibroblast-like synoviocytes (Yao, Zhao, Zhao, & Cai, 2017). SN significantly decreased the expression of p-NF-κB p65 in the cardiac tissue of diabetic rats in vivo (Jiang, Tong, Zhang, Liu, & Wang, 2017). Activation and/or increase in number of NF-κB might result in attenuation

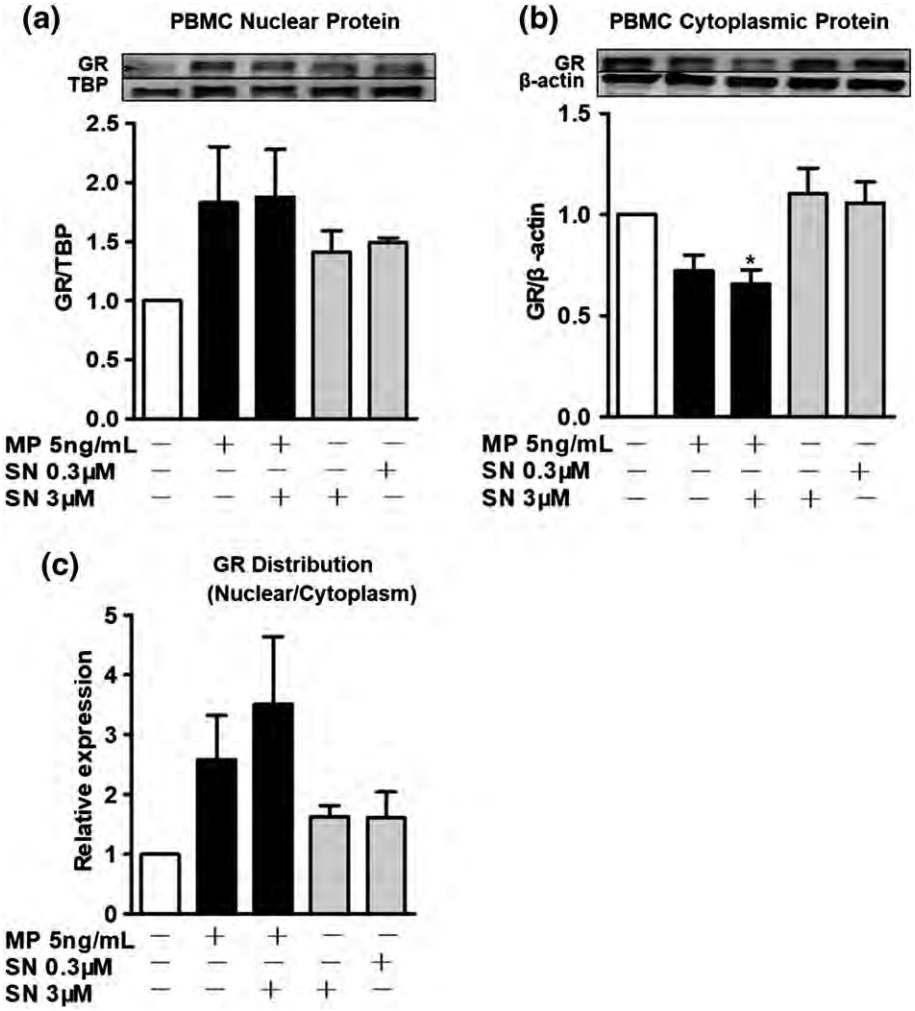

FIGURE 6 Expression and subcellular localization of glucocorticoid receptor (GR) in peripheral blood mononuclear cells (PBMCs) after treatment with methylprednisolone (MP) and/or sinomenine (SN). Western blot analysis and densitometric quantification of total GR in nuclear (a) and cytoplasmic (b) extracts of PBMCs. Distribution of GR in nuclear/cytoplasmic fractions (c). Cells were pretreated with sinomenine for 24 h and harvested in 2 h after methylprednisolone treatment. Nuclear protein extracts were normalized to TATA-binding protein (TBP) and cytoplasmic extracts to β-actin. Data represented the mean ± S.E.M. of more than three experiments. Statistical analyses were performed using Dunnett's Multiple Comparison Test, $^*p < 0.05$ as compared with the control group

of GC efficacy because proinflammatory transcription factors could interact with activated GC-GR complex in nucleus (Barnes & Adcock, 2009; Hirano, 2007). Therefore, the additional effect of SN on GR translocation might be related to the regulatory activity of SN on NF-κB signaling pathway. In agreement with our result of Western blot, morphologic evidences of immunofluorescence provided visual images, as shown in Figure 5.

Considering the differences of Jurkat T cells and PBMCs, we further investigated the GR-mediated effects of SN by using PBMCs. SN was confirmed to regulate the translocation of GR in PBMC culture system. SN alone seemed to stimulate the expression of GR in nucleus (Figure 6a) and SN potentiated MP-mediated moving of GR in cytoplasm of PBMC significantly (Figure 6b). All these findings in PBMCs were consistent with the results obtained from Jurkat cells. According to our best knowledge, it was the first time to report that *N*-Me morphinan alkaloid SN regulated GC receptor translocation in PBMCs and Jurkat T cells. In our experiment, we also certified that GR amounts in the nucleus of PBMCs was less abundant than that in Jurkat T cells because the signals of GR bands in Figure 6a were much weaker than those in Figure 3a and Figure 4a.

Low- to medium-dose GC had been shown to have not only anti-inflammatory but also modifying properties in RA. However, numerous fears about GC adverse events such as osteoporosis, glucose intolerance, and cardiovascular disease had arisen in RA. In fact, these serious side effects might be largely related to high-dose GC treatment (Santiago & da Silva, 2014). In contrast, two meta-analysis reports showed that SN alone or in combination with methotrexate was widely used for treating RA in China with better clinical efficiency and fewer adverse events (Chen, Huang, Huang, Chu, & Yan, 2015; Liu et al., 2016). Due to the higher tolerability and safety combined with the results of the present research, SN might be a proper agent to maintain the low-dose GC immunosuppressive efficacy and thus decrease the incidence rate of GC adverse events.

In conclusion, our study provided strong evidence that plant-derived alkaloid SN potentiates the GC pharmacodynamics significantly. However, the additional effect of SN had no relation to the

P-gp mediated efflux function because P-gp inhibitory function of SN was not observed in PBMC culture system. To disclose the underlying mechanism of SN actions, we found that SN itself regulates the GR translocation in both Jurkat T cells and normal human PBMCs, and the combination of SN and MP showed stronger GR-modulatory activity. These effects of SN were suggested to be beneficial for treatment of RA patients, and thus GC combined with SN would be an alternative and reasonable therapeutic approach for RA.

ACKNOWLEDGEMENTS

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CONFLICT OF INTERESTS

None.

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Original Article

Vitamin K_2 immunosuppressive effect on pediatric patients with atopic dermatitis

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Abstract ***Background*:** Over 20 kinds of steroids, tacrolimus ointments, and cyclosporine capsules are usually recommended for the treatment of atopic dermatitis (AD), depending on the symptoms of patients. However, several side effects sometimes occur with the extensive use of these agents for the treatment of pediatric AD patients. The purpose of this study was to explore whether vitamin K_2 could be a new immunosuppressive candidate for pediatric patients with AD.

***Methods*:** The immunosuppressive efficacy of vitamin K_2 was evaluated through a cell-culture procedure using mitogen-activated peripheral blood mononuclear cells (PBMCs) obtained from pediatric AD patients.

***Results*:** The mean (SD) IC_{50} value of vitamin K_2 for the proliferation of concanavalin A-activated PBMCs was 15.37 (30.05) μmol/L, while the value for tacrolimus was 0.10 (0.28) ng/mL (0.12 (0.35) nmol/L). There was a significant correlation between the IC_{50} values for vitamin K_2 and those for tacrolimus ($P = 0.0001$, $r = 0.8871$). However, there was no significant correlation between the IC_{50} values of vitamin K_2 and those of cyclosporine A or methylprednisolone. A significant correlation between the IC_{50} values of vitamin K_2 or tacrolimus and blood eosinophil counts ($P = 0.0099$, $r = 0.7086$ and $P = 0.0032$, $r = 0.7722$, respectively) was observed.

***Conclusion*:** Vitamin K_2-inhibited T-cell mitogen stimulated proliferation of PBMCs from pediatric AD patients in a dose-dependent manner. The PBMCs from pediatric AD patients were more sensitive to the immunosuppressive efficacy of vitamin K_2 than the PBMCs from healthy subjects. The individual immunosuppressive pharmacological efficacy of vitamin K_2 and of tacrolimus could be inferred from the blood eosinophil count of pediatric AD patients.

Key words immunosuppressive efficacy, pediatric atopic dermatitis, peripheral blood mononuclear cells, vitamin K_2.

Atopic dermatitis (AD) is clinically characterized by chronic, pruritic eczematous skin lesions, and is accompanied by elevated serum concentrations of immunoglobulin E.[1] Aberrations in the immune regulatory system are considered to be related to the pathogenesis of the disease.[2] It has been reported that 10% to 20% of children and teenagers were affected by AD in Europe,[3] whereas the prevalence in Japan was 11.2%.[4] Immunosuppressive drugs, including more than 20 kinds of steroids or tacrolimus ointments, are usually recommended for the treatment of these patients based on the appropriate diagnosis and evaluation of cutaneous symptoms.[5] As a categorized calcineurin inhibitor, tacrolimus ointment is the first choice for treatment of AD patients.[6] Oral cyclosporine is another, which is used for adult AD patients in Japan. However, side effects of tacrolimus ointment and cyclosporine capsules were recorded in 70% of the patients.[7] The risk-benefit balance of oral steroids has also been difficult to evaluate because of the systemic adverse effects, especially in children, and the rebound phenomenon following treatment cessation.[8] It has thus been a dilemma for pediatricians to choose the suitable immunosuppressant for AD patients.[9]

Vitamin K_2, also known as menaquinone-4, administered at a pharmacological dosage of 45 mg/day, has been used for the treatment of osteoporosis in Japan.[10] Our previous work demonstrated that vitamin K_2 suppressed the proliferation of T-cell mitogen-activated peripheral blood mononuclear cells (PBMCs) of healthy subjects *in vitro*.[9] Moreover, vitamin K_2 reduced the percentage of $CD4^+$ and $CD4^+CD25^+$ cells in the activated PBMCs of pediatric AD patients, and the amount of IL-2 produced from the cells. These results suggested that vitamin K_2 could modulate T-cell function in the PBMCs of pediatric AD patients.[9]

In the present study, we evaluated the immunosuppressive properties of vitamin K_2 on the proliferation of T-cell

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mitogen-activated PBMCs obtained from pediatric AD patients and related the drug efficacy to the clinical characteristics or laboratory data of the patients. To characterize the clinical application of vitamin K_2 to AD further, we also compared the pharmacological efficacy of vitamin K_2 on the activated PBMCs of pediatric AD patients with that of cyclosporine A, tacrolimus, and methylprednisolone.

Materials and Methods

Reagents

RPMI 1640 and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). Concanavalin A was purchased from Seikagaku Kogyo Co., Tokyo, Japan. Cyclosporine A was obtained by Wako Chemicals, Co. (Tokyo, Japan). Tacrolimus was obtained by Toronto Research Chemicals Inc. (Toronto, Canada). Methylprednisolone and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St Louis, MO, USA). Vitamin K_2 was provided by Wako Chemicals, Co. (Tokyo, Japan). Lymphocyte separation solution was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of the highest quality available from commercial vendors.

Subjects

The present study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The study was approved by the institutional ethics committee for studies in humans in both Tokyo Medical University Hospital and the Tokyo University of Pharmacy and Life Sciences. A total of 12 AD patients and 12 healthy subjects were enrolled in this study. All pediatric atopic dermatitis patients (two females and 10 males with a mean age of 6.0 $\pm$ 3.8 years) and healthy subjects (six female and six males with a mean age of 26.8 $\pm$ 2.2 years) gave their written informed consent for blood donation. Healthy subjects had no history of immunological disorders or of taking immunosuppressive drugs. Basic characteristics of the patients are shown in Table 1, including gender, age, atopic dermatitis score (SCORAD), blood eosinophil count, serum total IgE, specific IgE in serum (*Dermatophagoides pteronyssinus*), thymus activation-regulated chemokine (TARC), and stimulation index.

Isolation of PBMCs and PBMC culture

Heparinized venous blood (20 mL) was taken from each healthy subject and patient. Peripheral blood mononuclear cells were separated and suspended with RPMI 1640 medium containing 10% fetal bovine serum, 100 000 IU/L penicillin, and 100 mg/L streptomycin to a final density of 1×10^6 cells/mL as described previously,[11–13] and 196 μL of the PBMC suspension, as prepared above, was placed into each well of a non-treated 96-well microplate. Concanavalin A, as a T-cell mitogen, was added to each well to a final concentration of 5.0 μg/mL. Subsequently, 4 μL of ethanol solution containing methylprednisolone or cyclosporine A was added to give a serial concentration of 0.01, 0.1, 1, 10, 100, 1,000, and 10 000 ng/mL, respectively. In the case of tacrolimus, 4 μL of ethanol solution was added to give final concentrations of 0.01, 0.1, 1, 10, 100, and 1,000 ng/mL. For vitamin K_2, 4 μL of ethanol solution were added to give final concentrations of 0.1, 1, 10, and 100 μmol/L. The same volume of ethanol was added to the control wells (vehicle group). The plate was incubated for 96 h in an atmosphere of 5% CO_2 at 37 °C.

Evaluation of immunosuppressant pharmacological efficacy with MTT assay

After 96 h of culture, 10 μL of 5 mg/mL MTT solution dissolved in saline was added to each well and then the cultures were re-incubated under 5% CO_2 at 37 °C for 4 h.[14–16] The plates were centrifuged at $375 \times g$ for 5 min to precipitate cells and formazan produced by growing cells. Aliquots of the supernatant were removed from each well and dimethyl sulfoxide was added. The absorbance was read with a microplate reader at 550 nm. Dose-response curves were plotted, and the IC_{50} values of each immunosuppressive drug were calculated.

Statistical analysis

Differences in the MTT values for PBMC proliferation between the pediatric AD patients and healthy subjects were measured using the Mann–Whitney test. The correlation between the IC_{50} values of immunosuppressive drugs and the basic characteristics of pediatric AD patients were analyzed using Pearson's test. Differences in median values for the drug IC_{50} or stimulation indices between any two subject groups were analyzed with Mann–Whitney *U*-tests. The MTT values in PBMCs cultured in the presence of vitamin K_2 were compared with those in PBMCs cultured in the absence of drug (control), and the differences were analyzed by Dunnett's multiple comparison test. These analyses were performed with GraphPad PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA). In each case, two-sided P values <0.05 were considered to be significant.

Results

Effects of cyclosporine A, tacrolimus, and methylprednisolone on PBMCs of pediatric AD patients and healthy subjects

Typical dose-response curves of cyclosporine A, tacrolimus, and methylprednisolone on the PBMC proliferation are presented in Figure 1a. The mean (SD) values obtained with the MTT assay in PBMCs cultured in the presence of these drugs, and concanavalin A, are shown in Figure 1b,c,d. The mean (SD) of IC_{50} values of cyclosporine A, tacrolimus, and methylprednisolone in the PBMCs of pediatric AD patients

Table 1 Characteristics and laboratory data of pediatric patients with atopic dermatitis (n = 12)

Case	Gender	Age (years)	SCORAD	Blood eosinophil count (/μL)	Serum total IgE (U/mL)	Specific IgE in serum (Dermatophagoides pteronyssinus) (U_A/mL)	TARC (pg/mL)	Stimulation index†
1	M	5.4	33.5	460	1,210	98.8	1,933	4.22
2	M	9.4	10.5	625	3,850	100	341	2.60
3	M	1.5	20	272	148	0.1	401	6.41
4	M	3.2	15	40	323	0.1	564	7.63
5	M	4.8	40	391	1,020	100	598	3.93
6	F	2.1	32.5	1120	1,010	100	965	2.06
7	M	2.8	10	456	374	69.3	-	5.21
8	M	12.2	10	606	1,450	98.9	455	2.70
9	F	10	23	511	703	100	278	3.47
10	M	3.7	-	181	644	83.3	-	2.36
11	M	12.8	-	118	585	10.0	331	4.78
12	M	4.4	13.5	266	94	25.4	702	4.17
Mean ± SD		6.0 ± 3.8	20.8 ± 10.5	421 ± 276	951 ± 963	65.5 ± 41.4	657 ± 468	4.13 ± 1.62
Median		4.6	17.5	424	674	91.1	510	4.05
Minimum		1.5	10	40	94	0.1	278	2.06
Maximum		12.8	40	1,120	3,850	100	1,933	7.63

Abbreviated symbols: SCORAD, scoring of atopic dermatitis; TARC, thymus and activation-regulated chemokine.
†Peripheral blood mononuclear cells BMC proliferation ratio between cells cultured in the presence and absence of concanavalin A (Materials and Methods).

were 2.00 (1.19), 0.10 (0.28), and 2.75 (3.83) ng/mL, respectively, whereas the mean (SD) IC_{50} values of these immunosuppressive drugs in PBMCs of healthy subjects were 206.40 (408.19), 0.19 (0.27), and 42.21 (57.20) ng/mL, respectively (Table 2). The IC_{50} values of cyclosporine A (P = 0.0005) and methylprednisolone (P = 0.0024) in the PBMCs of pediatric AD patients were significantly lower than those in the PBMCs of healthy subjects (Fig. 2).

Effects of vitamin K_2 on PBMCs of pediatric AD patients and healthy subjects

The effects of vitamin K_2 against the concanavalin A-stimulated proliferation of PBMCs of the AD patients and the healthy subjects are presented in Figure 3. Vitamin K_2 significantly suppressed PBMC proliferation at 10 μmol/L. Vitamin K_2 at 100 μmol/L showed the strongest anti-proliferative effect on the PBMC proliferation in both pediatric AD patients and healthy subjects (Fig. 3a) ($P < 0.01$). The mean (SD) IC_{50} values of vitamin K_2 on the proliferation of PBMCs of these two groups were 15.37 (30.05) and 20.67 (25.87) μmol/L, respectively, and the values in PBMCs of the patients were significantly lower than those in the PBMCs of the healthy subjects (P = 0.0179) (Table 2, Fig. 3b).

Relationship between IC_{50} values of vitamin K_2 and immunosuppressive drugs on PBMCs of pediatric AD patients

To evaluate the relative anti-proliferative efficacy on the PBMCs of pediatric AD patients of vitamin K_2 and immunosuppressive drugs, we examined the correlation between the IC_{50} values of vitamin K_2 and those of three typical immunosuppressive drugs using Pearson's coefficient of correlation (Fig. 4). There was a significant correlation between the IC_{50} values of vitamin K_2 and those of tacrolimus (Fig. 4b, P = 0.0001, r = 0.8871). There were no significant correlations between the IC_{50} values of vitamin K_2 and those of cyclosporine A or methylprednisolone (Fig. 4a,c).

Relationship between the PBMC-suppressive potencies of the drugs and clinical parameters of pediatric AD patients

To examine the relationship between the drug effects on PBMCs and characteristics or laboratory data of the pediatric AD patients, the IC_{50} values of vitamin K_2, cyclosporine A, tacrolimus, and methylprednisolone were analyzed for their relationship with each of seven patient factors: age, stimulation index, atopic dermatitis scores (SCORAD), blood eosinophil counts, serum total IgE concentrations, specific IgE in serum (*Dermatophagoides pteronyssinus*) concentrations, and thymus and activation-regulated chemokine concentrations (TARC) (Table 1). There was a significant correlation between the IC_{50} values of vitamin K_2 and blood eosinophil counts (P = 0.0099, r = 0.7086). The IC_{50} values of tacrolimus and blood eosinophil counts were also significantly correlated (P = 0.0032, r = 0.7722) (Table 3). However, no correlation was observed between the IC_{50} values of these drugs and other characteristics or the laboratory data of the patients.

Discussion

Our results showed that vitamin K_2 inhibited the proliferation of PBMCs of both pediatric AD patients and healthy subjects in a dose-dependent way (Fig. 3a). Considering the

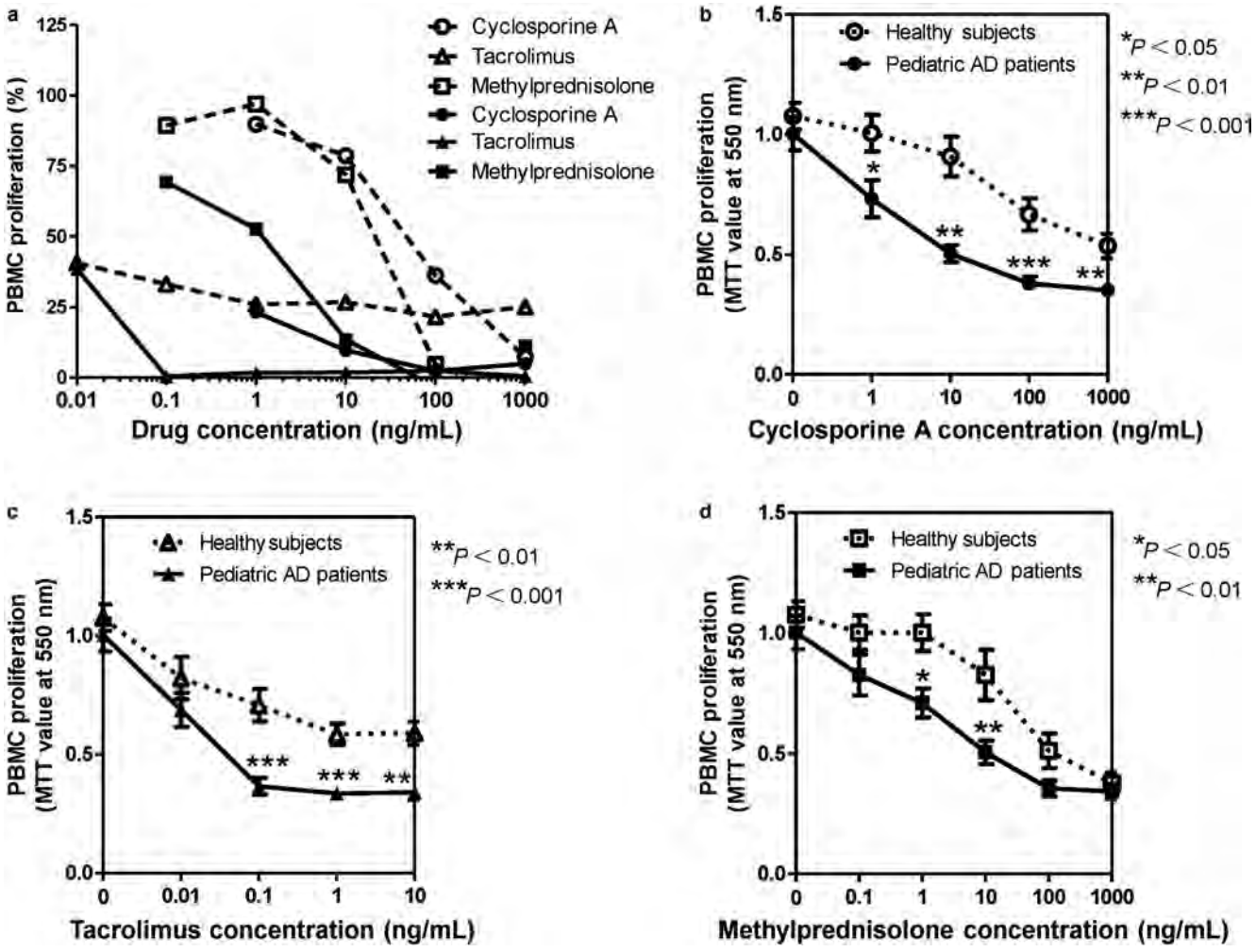

Fig. 1 Effects of cyclosporine A, tacrolimus, and methylprednisolone on the proliferation of mitogen-activated PBMCs of pediatric AD patients and healthy subjects. (a) Typical dose-response curves of cyclosporine A (○), tacrolimus (△) and methylprednisolone (□) on proliferation of concanavalin A-stimulated PBMCs of one pediatric AD patient (full line with closed symbol) and one healthy subject (dotted line with open symbol). (b–d) Comparison of the MTT values of PBMC proliferation between the pediatric AD patients (full line with closed symbol) and healthy subjects (dotted line with open symbol). Statistical analyses were performed using the Mann–Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, as compared to the values of healthy subjects at the same concentration.

immunosuppressive efficacy of vitamin K_3 and K_5, as we reported previously,[16] naphthoquinone skeleton is suggested to be an important chemical unit to develop new immunosuppressants with fewer side effects, especially for pediatric patients with immunological diseases, such as AD.[9]

According to our present data, pediatric AD patients were likely to be more sensitive towards vitamin K_2 and the immunosuppressive drugs such as cyclosporine A and methylprednisolone (Figs 2 and 3b). The mean (SD) ages of the AD patients we examined in this study were significantly lower than that of the healthy subjects, and thus the difference in ages should be taken into account to evaluate the individual sensitivity of PBMCs to the immunosuppressive agents in pediatric patients.[17] In this regard, however, the IC_{50} values of the immunosuppressive drugs or vitamin K_2 were not significantly correlated with ages (Table 3), suggesting that the PBMC sensitivity to the immunosuppressive efficacy of these agents was not age dependent. Considering the effect of tacrolimus, external use of vitamin K_2 would be an alternative strategy to manage pediatric AD, as a strong correlation between IC_{50} values of vitamin K_2 and tacrolimus on the PBMCs of AD patients was observed ($P = 0.0001$, $r = 0.8871$; Fig. 4b).

Eosinophilia is a typical feature of many AD patients. As eosinophilia changes more rapidly than serum IgE concentration, blood eosinophil count has been reported to be an index to assess changes in the AD condition.[5] The present study also revealed that there was a strong and significant correlation between the blood eosinophil counts of the pediatric AD patients and the individual pharmacodynamics of tacrolimus and vitamin K_2 on PBMCs (Table 3). Comparing with cyclosporine A and steroids, these data suggested that it is of

Table 2 IC_{50} values of drugs on concanavalin A-stimulated proliferation of PBMCs of pediatric patients with atopic dermatitis (n = 12) and healthy subjects (n = 12)

Case	Pediatric patients with atopic dermatitis				Healthy subjects			
	IC_{50} values of cyclosporine A (ng/mL)	IC_{50} values of tacrolimus (ng/mL)	IC_{50} values of methylprednisolone (ng/mL)	IC_{50} values of vitamin K2 (μmol/L)	IC_{50} values of cyclosporine A (ng/mL)	IC_{50} values of tacrolimus (ng/mL)	IC_{50} values of methylprednisolone (ng/mL)	IC_{50} values of vitamin K2 (μmol/L)
1	3.70	0.02	0.77	50.04	1,461.80	0.62	140.26	19.41
2	1.00	0.01	0.53	0.10	332.97	0.01	83.06	5.48
3	2.16	0.02	0.10	0.10	46.90	0.01	21.22	100.00
4	1.00	0.01	1.17	1.33	151.80	0.01	10.86	24.27
5	2.47	0.04	6.86	3.01	6.85	0.01	18.12	17.85
6	0.01	1.00	2.00	100.00	15.12	0.01	2.83	4.05
7	1.64	0.02	1.83	5.29	15.73	0.04	4.25	11.77
8	1.47	0.03	4.44	4.13	0.01	0.01	0.42	12.01
9	3.06	0.02	1.96	3.14	85.48	0.34	7.38	8.51
10	1.00	0.01	0.10	2.56	68.51	0.28	166.36	20.96
11	3.93	0.01	0.10	13.86	56.27	0.14	5.79	18.20
12	2.55	0.01	13.10	0.90	235.41	0.81	46.00	5.51
Mean ± SD	2.00 ± 1.19	0.10 ± 0.28	2.75 ± 3.83	15.37 ± 30.05	206.40 ± 408.19	0.19 ± 0.27	42.21 ± 57.20	20.67 ± 25.87
Median	1.90	0.02	1.50	3.08	62.39	0.03	14.49	14.93
Minimum	0.01	0.01	0.10	0.10	0.01	0.01	0.42	4.05
Maximum	3.93	1.00	13.10	100.00	1,461.79	0.81	166.36	100.00

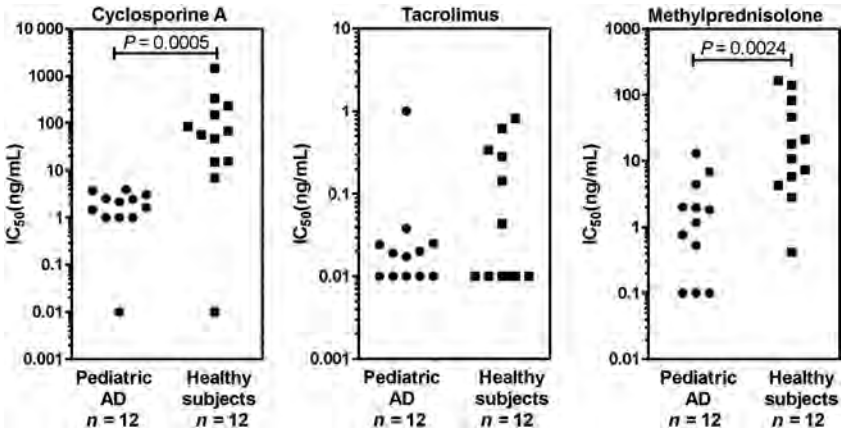

Fig. 2 Comparison of the IC_{50} values of cyclosporine A, tacrolimus, and methylprednisolone after the stimulation by concanavalin A between the PBMCs of the pediatric AD patients and the PBMCs of healthy subjects. The data were analyzed with the Mann–Whitney test.

value to pay more attention to the relationship between the therapeutic dose of tacrolimus and blood eosinophil count in patients, together with the clinical symptoms of AD. The relationship between the immunosuppressive effect of vitamin K_2 and eosinophil count is another point that should be addressed.

T cells and T-cell-derived cytokines partly contribute to the initiation of AD pathophysiology.[1] The skin lesions of AD patients are infiltrated by activated T cells. Then eosinophils and antigen-presenting Langerhans cells bind and present IgE-complexed allergens on their surface. The activation of peripheral blood T cells preferentially secretes Th 2 cytokines and helps B cells to produce large amounts of IgE, which is associated with this skin disease.[18] Inhibiting T-cell proliferation and T-cell-derived cytokine secretion would therefore be helpful in improving the AD symptoms of patients. The results of MTT assays showed that 10 μmol/L of vitamin K_2 inhibited the proliferation of PBMCs of pediatric patients (Fig. 3a). This finding is consistent with the results in Myneni *et al.*[19] One possible mechanism is that vitamin K_2 inhibits the activation of NF-κB through the repression of IKKα/β phosphorylation because NF-κB plays a critical role in T-cell development.[20,21] Our previous finding also showed that vitamin K_2 could change the balance of Th1 and Th2 in the PBMCs of pediatric AD patients *in vitro*.[9] Specifically, the ratio of IL-4/IL2 decreased from 3.4 to 0.7, whereas the ratio of IL-4/IFN-γ increased from 0.001 to 0.0025 after treatment by 100 μmol/L of vitamin K_2.[9] The median (range) of vitamin K_2 IC_{50} values

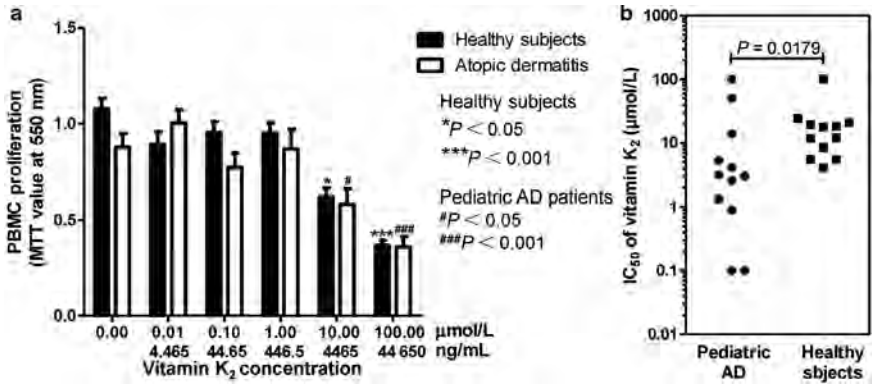

Fig. 3 Effects of vitamin K_2 on proliferation of PBMCs of pediatric AD patients and healthy subjects. Cell proliferation was determined by MTT assay. (a) Inhibition of PBMCs by vitamin K_2 (black-filled column, healthy subjects; white-filled column, pediatric AD patients). The data were expressed as means ± SEM and analyzed by Dunnett's multiple comparison test. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ (pediatric AD patients) and $*P < 0.05$, $***P < 0.001$ (healthy subjects), as compared to each control groups respectively. (b) Comparison of the IC_{50} values of vitamin K_2 between pediatric AD patients and healthy subjects. The data were analyzed with the Mann–Whitney test. ($n = 12$.)

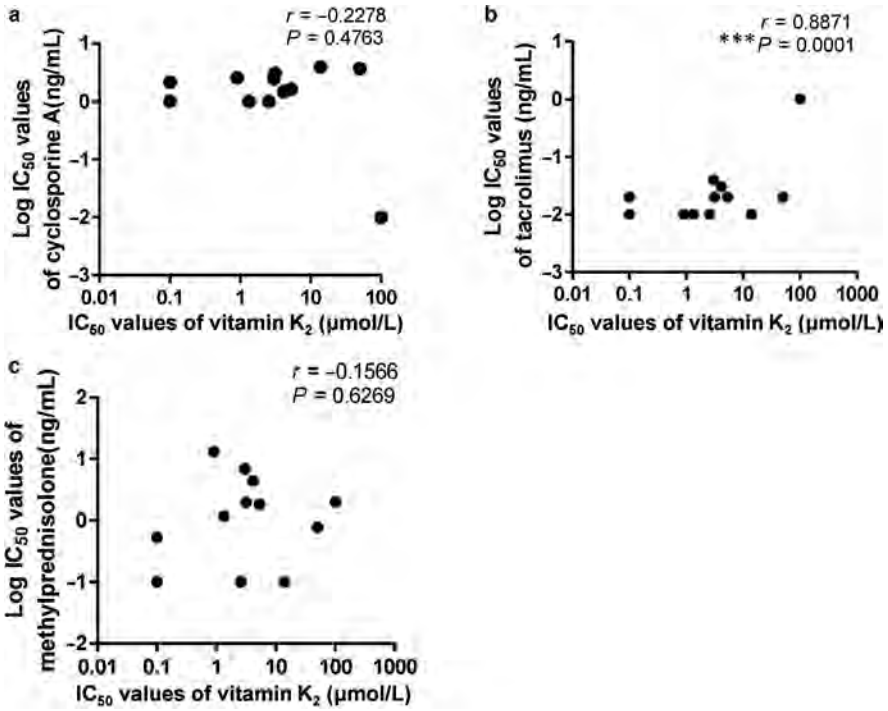

Fig. 4 Relationship between the IC_{50} values for cyclosporine A (a), tacrolimus (b) or methylprednisolone (c) and the IC_{50} values of vitamin K_2 in concanavalin A-stimulated PBMCs of pediatric AD patients. The data were analyzed with the Pearson's coefficient of correlation test.

against the proliferation of pediatric AD patient PBMCs was 3.08 (0.10 ~ 100.00) µmol/L (Table 2). According to the clinical investigation, 45 mg/day of vitamin K_2 taken orally could achieve maximum blood concentration of 40–50 µmol/L.[19] In addition to oral administration, it is also possible for vitamin K_2 to provide pediatric AD patients with topical treatment because vitamin K_2 has the suitable lipid-water partition coefficient. From the viewpoint of controlling T-cell proliferation and cytokine production, vitamin K_2 is a potential immunosuppressant for AD patients.

Peripheral blood mononuclear cells include T lymphocytes, B lymphocytes, and other mononuclear immune cells. Concanavalin A is a non-specific mitogen, and most T lymphocytes will be stimulated by this mitogen. On the other hand, comparing with concanavalin A, anti-CD3 antibody appears to be more suitable for analyzing the T cell signaling pathway

Table 3 Relationship between IC_{50} values of drugs on PBMC proliferation and subject-related factors as analyzed by Pearson's coefficient of correlation test. Pediatric patients with atopic dermatitis ($n = 12$). Healthy subjects ($n = 12$)

Drugs	Statistical Parameter	Patient-related factors							Healthy subject-related factors	
		Age	Stimulation index	SCORAD	Blood eosinophil count	Serum total IgE	Specific IgE in serum (Dermatophagoides pteronyssinus)	TARC	Age	Stimulation index
Vitamin K_2	*r*	−0.2454	−0.3510	0.5357	0.7086	0.03025	0.3246	0.6048	−0.3240	0.2607
	P	0.4421	0.2632	0.1105	0.0099*	0.9256	0.3033	0.0640	0.3042	0.4132
Cyclosporine A	*r*	0.4104	0.2388	0.2861	−0.4304	−0.2451	−0.1848	0.1779	−0.07552	−0.3786
	P	0.1852	0.4548	0.4229	0.1625	0.4427	0.5653	0.6230	0.8156	0.2249
Tacrolimus	*r*	−0.3085	−0.3902	0.3885	0.7722	0.01813	0.2664	0.2217	0.2524	−0.1911
	P	0.3293	0.2098	0.2673	0.0032*	0.9554	0.4026	0.5382	0.4287	0.5519
Methylprednisolone	*r*	−0.06087	−0.1003	−0.01623	−0.01125	−0.2149	−0.02163	−0.01011	0.3519	−0.3254
	P	0.8509	0.7564	0.9654	0.9723	0.5023	0.9468	0.9779	0.2620	0.3020

Abbreviated symbols: SCORAD, scoring of atopic dermatitis; TARC, thymus and activation-regulated chemokine; stimulation index represents the PBMC proliferation ratio between cells cultured in the presence and absence of concanavalin A. (See Materials and Methods.)

*$P < 0.01$.

because it stimulates T cell antigen specially. In the present study, concanavalin A-stimulated PBMCs were used to evaluate the pharmacological parameters of immunosuppressive drugs *in vitro* because this PBMC culture procedure can simulate the complicated immune system *in vivo* in several autoimmune disorders. The results obtained *in vitro* by the use of this PBMC culture system stimulated by concanavalin A were significantly correlated with the clinical efficacy of these drugs in our previous studies.[22–25] However, we look forward to new reports of animal experiments, which may reveal anti-AD effects of vitamin K_2 *in vivo*.

Conclusions

Vitamin K_2 inhibited T-cell mitogen-stimulated proliferation of PBMCs from pediatric AD patients in a dose-dependent way. The PBMCs from pediatric AD patients were more sensitive to the immunosuppressive efficacy of vitamin K_2 than the PBMCs from healthy subjects. The immunosuppressive pharmacological efficacy of vitamin K_2 and tacrolimus could be inferred from the blood eosinophil counts of the pediatric AD patients.

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Disclosure

The authors declare no conflict of interest.

Author contributions

K.S. designed and directed the study. W.X. and K.M. performed the experiments and wrote the manuscript. T.M., S.S., M.C., and H.K. collected clinical data and provided the patients' blood samples. H.W. and S.T. gave technical support. T.H. gave conceptual advice. All of the authors critically reviewed and approved the final manuscript.

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Tetrandrine and cepharanthine induce apoptosis through caspase cascade regulation, cell cycle arrest, MAPK activation and PI3K/Akt/mTOR signal modification in glucocorticoid resistant human leukemia Jurkat T cells

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Apoptosis

ABSTRACT

Tetrandrine (TET) and cepharanthine (CEP) are two bisbenzylisoquinoline alkaloids isolated from the traditional herbs. Recent molecular investigations firmly supported that TET or CEP would be a potential candidate for cancer chemotherapy. Prognosis of patients with glucocorticoid resistant T cell acute lymphoblastic leukemia (T-ALL) remains poor; here we examined the anti-T-ALL effects of TET and CEP and the underlying mechanism by using the glucocorticoid resistant human leukemia Jurkat T cell line *in vitro*. TET and CEP significantly inhibited cell viabilities and induced apoptosis in dose- and time-dependent manner. Further investigations showed that TET or CEP not only upregulated the expression of initiator caspases such as caspase-8 and 9, but also increased the expression of effector caspases such as caspase-3 and 6. As the important markers of apoptosis, p53 and Bax were both upregulated by the treatment of TET and CEP. However, TET and CEP paradoxically increased the expression of anti-apoptotic proteins such as Bcl-2 and Mcl-1, and activated the survival protein NF-κB, leading to high expression of p–NF–κB. Cell cycle arrest at S phase accompanied by increase in the amounts of cyclin A2 and cyclin B1, and decrease in cylcin D1 amount in cells treated with TET or CEP will be another possible mechanism. During the process of apoptosis in Jurkat T cells, treatment with TET or CEP also increased the phosphorylation of JNK and p38. The PI3K/Akt/mTOR signaling pathway modification appears to play significant role in the Jurkat T cell apoptosis induced by TET or CEP. Moreover, TET and CEP seemed to down-regulate the expressions of p-PI3K and mTOR in an independent way from Akt, since these two drugs strongly stimulated the p-Akt expression. These results provide fundamental insights into the clinical application of TET or CEP for the treatment of patients with relapsed T-ALL.

1. Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous hematologic disease characterized by the proliferation of immature lymphoid cells in the bone marrow, peripheral blood, and other organs [1]. Approximately 75–80% of ALL develop in children [2]. Optimal use of antileukemic drugs and improved supportive care in contemporary clinical trials have improved the 5-year survival rate of childhood ALL above 85% in developed countries [3]. However, the majority (~80%) of relapses occurs within 2 years of diagnosis in T cell ALL (T-ALL), and the prognosis after relapse is dismal, with a 5-year survival of less than 10% [4].

Tetrandrine (TET, Fig. 1A), isolated from *Stephania tetrandra* S. Moore, is a bisbenzylisoquinoline alkaloid and approved for treating patients with silicosis and rheumatic arthritis in China [5]. Our previous study revealed that TET inhibited the proliferation of T-cell

Abbreviations: T-ALL, T cell acute lymphoblastic leukemia; WST-8, Tetrazolium salt; FBS, Fetal bovine serum; TBST, Tris buffered saline Tween 20; MAPK, Mitogen-activated protein kinases; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Mcl-1, Myeloid cell leukemia 1; NF-κB, Nuclear factor kappa light chain enhancer of activated B cells; PI3K, Phosphatidylinositol 3-kinase; mTOR, Mammalian target of rapamycin

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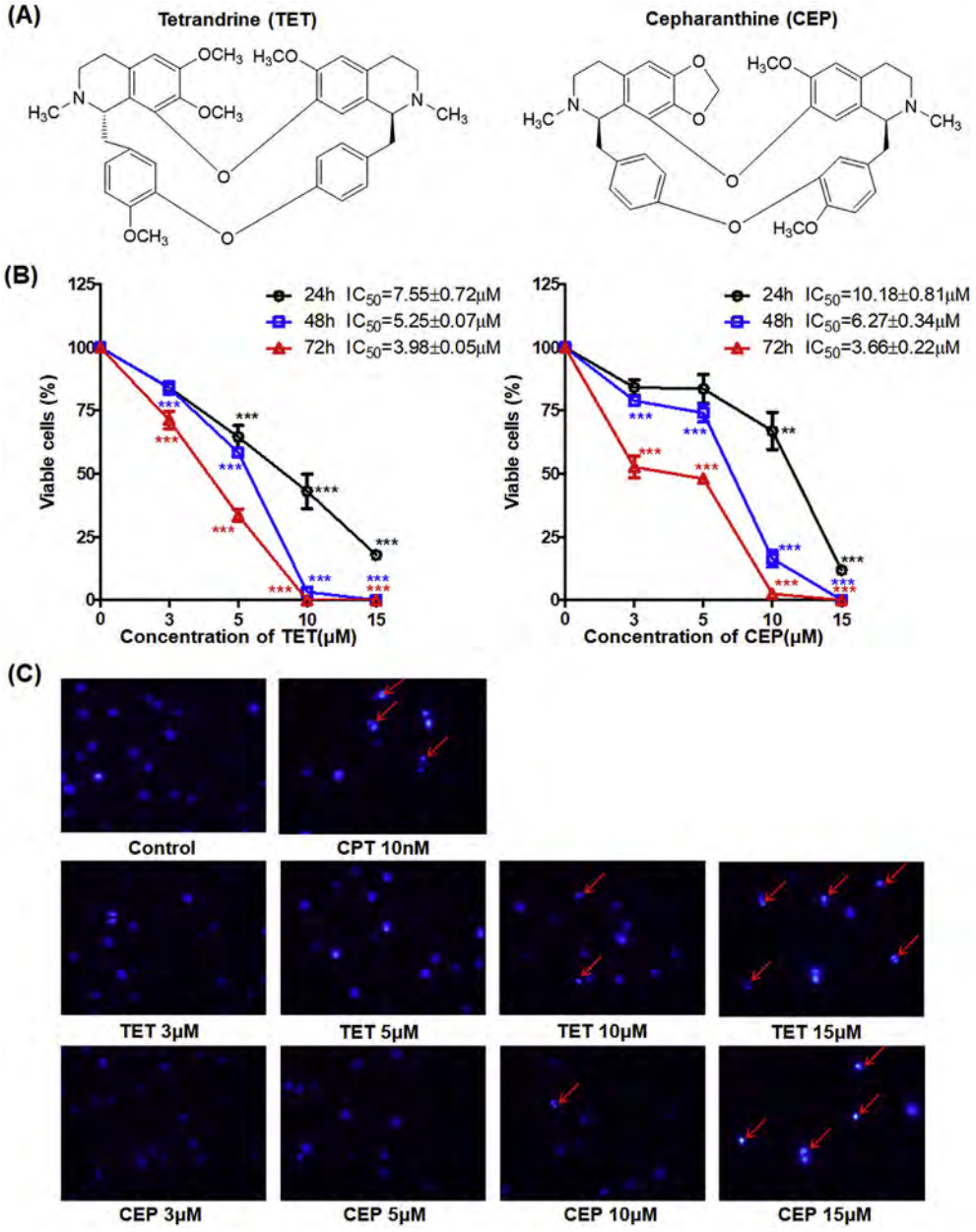

Fig. 1. Effects of tetrandrine (TET) and cepharanthine (CEP) on the viability of Jurkat T cells. (A) Chemical structures of TET and CEP. (B) Jurkat T cells were treated with various concentrations (3, 5, 10, 15 μM) of TET (left) or CEP (right) for 24 h (○), 48 h (□) and 72 h (△), respectively, and the cell viability was assessed by WST-8 assay. All of the data were expressed as the mean ± S.E.M. (n = 3). Asterisks indicate significant differences between the control and the drug treatment groups ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). (C) Morphologic change of nuclei in drug-treated Jurkat T cells. Cells were incubated with drugs for 48 h followed by Hoechst 33,342 staining. Camptothecin (CPT) group was set up as a positive control. Representative result of three independent experiments was shown as in the figure. Abnormal nuclei were indicated by red arrows.

mitogen-activated human peripheral blood mononuclear cells and enhanced the glucocorticoid pharmacodynamics by inhibiting mitogen-activated protein kinase (MAPK) [5,6]. Meanwhile, recent investigations firmly supported that TET would be a potential candidate for cancer chemotherapy [7,8]. TET upregulated the caspase cascade protein (cleaved PARP, cleaved caspase-3 and cleaved caspase-9) and inhibited the phosphorylation of Akt/mTOR, resulted in significant apoptosis on human gastric cancer cell [9]. TET also inhibited pancreatic cancer tumor growth by downregulating Skp2, an E3 ligase specific for degradation of $p27^{Kip1}$ during the cell cycle, and indirectly impaired the activities of CDK4/6 to halt deregulated cell cycle [10]. Moreover, one of the clinical trials for TET showed that TET, in combination with traditional chemotherapy drugs, had potential-reversing effect for the treatment of refractory and relapsed acute myelogenous leukemia [11]. The above information hints that TET could inhibit the proliferation of both T cells and cancer cells. Sharing the similar chemical skeleton with TET, cepharanthine (CEP, Fig. 1A) is another bisbenzylisoquinoline alkaloid isolated from the plant *Stephania cepharantha* Hayata [12]. From these points of view, we are encouraged to investigate the anti-cancer effects of TET and CEP on human T-lymphoblastic leukemia cells having features of both T cells and cancer cells.

Since poor response to glucocorticoid is one of the strongest predictors of adverse outcome for the treatment of childhood T-ALL [13], glucocorticoid resistant human leukemia Jurkat T cell line [14], a well-established T-ALL tumor line from the peripheral blood of a 14-year-old boy [15], was used in the present research to examine potential efficacies of TET and CEP, and their underlying action mechanisms focusing on caspase cascade, cell cycle, MAPK and PI3K/Akt/mTOR signaling pathway.

2. Materials and methods

2.1. Drugs and reagents

RPMI 1640 and FBS were purchased from Gibco BRL (Grand Island, NY, USA). TET and CEP were obtained from Sigma Aldrich and Cayman Chemical Company respectively. Camptothecin (CPT) was provided by

Wako Pure Chemical Industries, Ltd., Japan. Phospho–NF–κB (dilution 1:1000, #sc-136,548) and β-actin (dilution 1:5000, #66009-1-Ig) antibodies were purchased from Santa Cruz Biotechnology and Proteintech Group respectively. Primary antibodies against Cyclin A2 (dilution 1:2000, #4656), Cyclin B1 (dilution 1:2000, #4135), Bcl-XL (dilution 1:1000, #2764), Mcl-1 (dilution 1:1000, #5453), Caspase-3/6/7/8/9 (dilution 1:1000, #9665/9762/12,827/9746/9508), Lamin A/C (dilution 1:2000, #4777), PARP (dilution 1:1000, #9542), and Phospho-PI3K (dilution 1:1000, #4228) were purchased from Cell Signaling Technology, Inc. Anti mTOR antibody was obtained from BioLegend (dilution 1:2000, #659201). Anti-topoisomerase 1 antibody was provided by Bioss Antibodies (dilution 1:1000, #bsm-51396 M). Primary antibodies against Bcl-2 (dilution 1:400, #NBP2-34391), Bax (dilution 1:400, #NBP2-32809), p53 (dilution 1:400, #NBP2-44982), Cyclin D1 (dilution 1:400, #NBP2-32840) and Akt1 p Ser 473 (dilution 1:250, #NB100-56749) were purchased from Novus Biologicals, LLC. ERK (0.2 μg/mL, #AF1576), p-ERK (0.1 μg/mL, #AF1018), JNK (0.2 μg/mL, #AF1387), p-JNK(0.5 μg/mL, #AF1205), p38 (0.5 μg/mL, #AF8691) and p-p38 (1 μg/mL, #MAB8691) were provided by R&D Systems, Inc. All other reagents were of the highest quality available from commercial vendors.

2.2. Cell cultures

Human leukemic Jurkat T-cells were grown in RPMI 1640 medium supplemented with 10% FBS, 100,000 IU/L penicillin and 100 mg/L streptomycin at 37 °C in the presence of 5% CO_2 as we described before [16].

2.3. Cell viability measured by WST-8 assay

Cellular proliferation was assessed using WST-8 assay. Jurkat T cells at a cell density of 1.5×10^5 cells/mL were seeded in 96-well plates. TET or CEP was subsequently added into the corresponding wells to adjust the final concentrations of 3, 5, 10 and 15 μM. The cells were incubated with ethanol as a control. After 48 h treatment at 37 °C, 10 μL of WST-8 solution was added to each well, followed by additional 3 h incubation. Optical density value was measured at 450 nm absorbance (ref. 650 nm). Percentages of cell viability in the reagent-treated group, as compared to control (ethanol-treated group), were presented [17].

2.4. Hoechst 33,342 fluorescent staining

In order to observe the influence of drugs on the morphology of Jurkat T cells, cells were treated with TET or CEP for 48 h and then harvested to stain with Hoechest 33,342 (#17535, AAT Bioquest, Inc, CA, USA). Briefly, after collecting, cells were fixed with 4% paraformaldehyde, followed with PBS washing. Then, the cells were continued to incubate with Hoechst 33,342 for 10 min at room temperature. The images of nuclear alteration-related apoptosis were captured with a fluorescence microscope (Biozero BZ-8000 Series, Keyence, Japan) [16].

2.5. Assessment of apoptosis

Jurkat T cells were seeded in 24-well plates and treated with serial concentrations of TET or CEP (0.3, 3, 5, 10 and 15 μM). 10 nM of CPT group was set up as a positive control for apoptotic cells. After 48 h incubation at 37 °C, cells were harvested and washed by PBS twice. Then, the cells were co-stained using FITC Annexin V Apoptosis Detection Kit (BD Pharmingen™). Fluorescence of the cells was immediately determined by a flow cytometer (FACSCanto™ II, BD Biosciences, CA, US) [17].

2.6. Cell cycle analysis

Jurkat T cells were seeded in 6-well plates and incubated with RPMI 1640 medium containing blank solvent, CPT (10 nM), CEP (5, 10 and 15 μM) and TET (5, 10 and 15 μM) for 48 h, respectively. Then, the cells were harvested and washed with PBS, and subsequently fixed in 70% ethanol at 4 °C for 1 h, and stained with PI (50 μg/mL, #P4864, Sigma Chemical Co.) and RNase A solution (0.25 mg/mL, #R5500, Sigma Chemical Co.). DNA content of cells was determined by flow cytometry (FACSCanto™ II, BD Biosciences, CA, US). Data were analyzed by ModFit LT™ (Version 3.1, Verity Software House, Topsham, ME, USA) [17].

2.7. Western blot analysis

Whole cell protein was extracted by RIPA buffer containing Protease and Phosphatase Inhibitor (#A32961, Thermo Scientific). Western blot was performed using standard procedures, as we described previously [16]. Membranes were incubated with primary antibodies against individual proteins overnight at 4 °C followed by an appropriate secondary antibody (Anti-mouse IgG, HRP-linked, #7076, Cell Signaling Technology, Inc.) at a dilution of 1:1000 for 1 h at room temperature. After incubation with regents of an ECL or ECL Prime Western Blotting detection kit (#RPN2109 and RPN2232, GE Healthcare), the membranes were then analyzed in a luminescent image analyzer (Fujifilm; LAS-3000; Fujifilm, Tokyo, Japan). Quantitative densitometry data of the images were evaluated by ImageJ software (version 1.52e, National Institutes of Health, USA; http://imagej.nih.gov/ij).

2.8. Statistical analysis

Differences in the percentages of viable cells, early or late apoptotic cells and percentage of cells in cell-cycle phases were analyzed with Bonferroni Multiple Comparison Tests. Statistical analyses for the expressions several proteins were performed using Dunnett's Multiple Comparison Test. These analyses were processed by using GraphPad PRISM 5.0 (GraphPad Software Inc., San Diego, CA). In each case, two-sided p values < 0.05 were considered to be significant.

3. Results

3.1. TET and CEP inhibit the proliferation of Jurkat T cells and change the cell morphology

To investigate the cytotoxicity of TET and CEP against glucocorticoid resistant human leukemia cells, Jurkat T cells, were exposed to serial concentrations of TET and CEP (3, 5, 10 and 15 μM). The result of cell viability was provided in Fig. 1B. Both CEP and TET inhibited the proliferation of Jurkat T cells significantly in a dose- and time-dependent manner. After the cells were stained by Hoechst 33,342, the cell number decreased obviously in the cells treated by TET or CEP at concentrations of 10 and 15 μM compared with control group (Fig. 1C). Images from the fluorescence microscope also showed that 10 nM of camptothecin (CPT) used as a positive drug, 10 and 15 μM of TET and CEP largely changed the cell morphology with abnormal nuclear of karyorrhexis, chromatin condensation and fragmentation (Fig. 1C).

3.2. TET and CEP induce apoptosis in Jurkat T cells

To explore whether apoptosis was involved in the cytotoxicity of TET and CEP, Annexin V-FITC/PI dual staining assay was performed. Percentages of apoptotic cells were summarized in Fig. 2B–D and typical dot-plot diagram was shown as Fig. 2A. Comparing with control group, the percentage of viable cells treated by 10 nM CPT was 42.1 ± 4.4% (mean ± SD, $P < 0.05$, Fig. 2B). Both TET and CEP inhibited the cell growth dose-dependently. 15 μM of CEP and TET

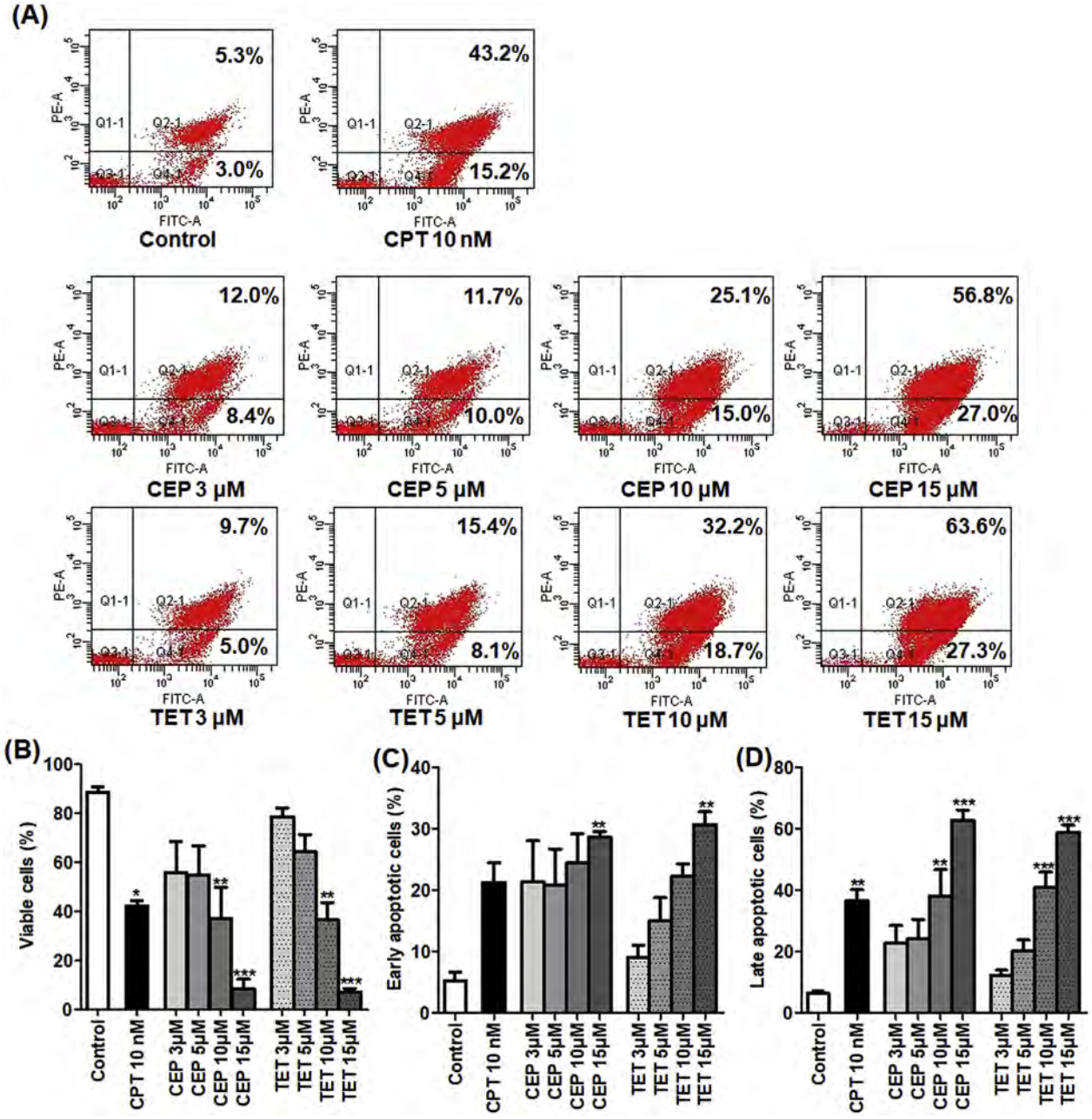

Fig. 2. Tetrandrine (TET) and cepharanthine (CEP) induces apoptosis of Jurkat T cells. (A) A dot-plot analysis of Jurkat T cells treated with TET or CEP. Cells were treated with TET or CEP (3, 5, 10, 15 μM) for 48 h and, then, stained with Annexin V/PI, following with detection by flow cytometer. Camptothecin (CPT) group was set up as a positive control. Cells were classified into four groups based on different quadrants: viable cells (Q3-1, Annexin V^-/PI^-), early apoptotic cells (Q4-1, Annexin V^+/PI^-), late apoptotic cells (Q2-1, Annexin V^+/PI^+) and necrotic cells (Q1-1, Annexin V^-/PI^+). (B) Viable cells were decreased by the treatment with TET or CEP in a dose-dependent manner. Early apoptosis (C) and late apoptosis (D) were induced by both CPT and TET or CEP significantly ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). Data represented the mean ± S.E.M. of three independent experiments.

showed the strongest cytotoxic effects, and the mean ± SD values of viable cells were 8.4 ± 6.9 and 7.4 ± 2.4%, respectively ($P < 0.001$, Fig. 2B). Consequently, the percentages of the early and the late apoptotic cells increased significantly after treating with 10 nM CPT or higher doses of TET and CEP (Fig. 2C and D).

3.3. TET and CEP regulate the expression of proteins related to caspase cascades

To further investigate the mechanism underlying the TET or CEP-induced apoptosis, the expressions of proteins related to caspase cascades were examined. Complicated regulation networks were divided into apoptotic markers and anti-apoptotic factors.

Apoptotic markers in the cells, such as caspase-3/6/8/9, p53 and Bax, were upregulated by TET or CEP (Fig. 3A–I). As shown in Fig. 3A, the expression levels of caspase-3 significantly increased following treatment with 10 and 15 μM TET ($P < 0.01$). Meanwhile, TET and CEP tended to increase the expression of caspase-8 dose-dependently (Figs. 3D), and 15 μM of TET showed a significant up-regulation ($P < 0.05$). A similar increasing tendency was observed in the expressions of caspase-9 and caspase-6 after the cells were exposed to CEP or TET (Fig. 3B and E). However, the drug treatment seemed to show little influence on the expression of caspase-7 (Fig. 3C). CPT at 10 nM did not upregulate the expression of caspase-3/6/7/8/9, whereas CPT

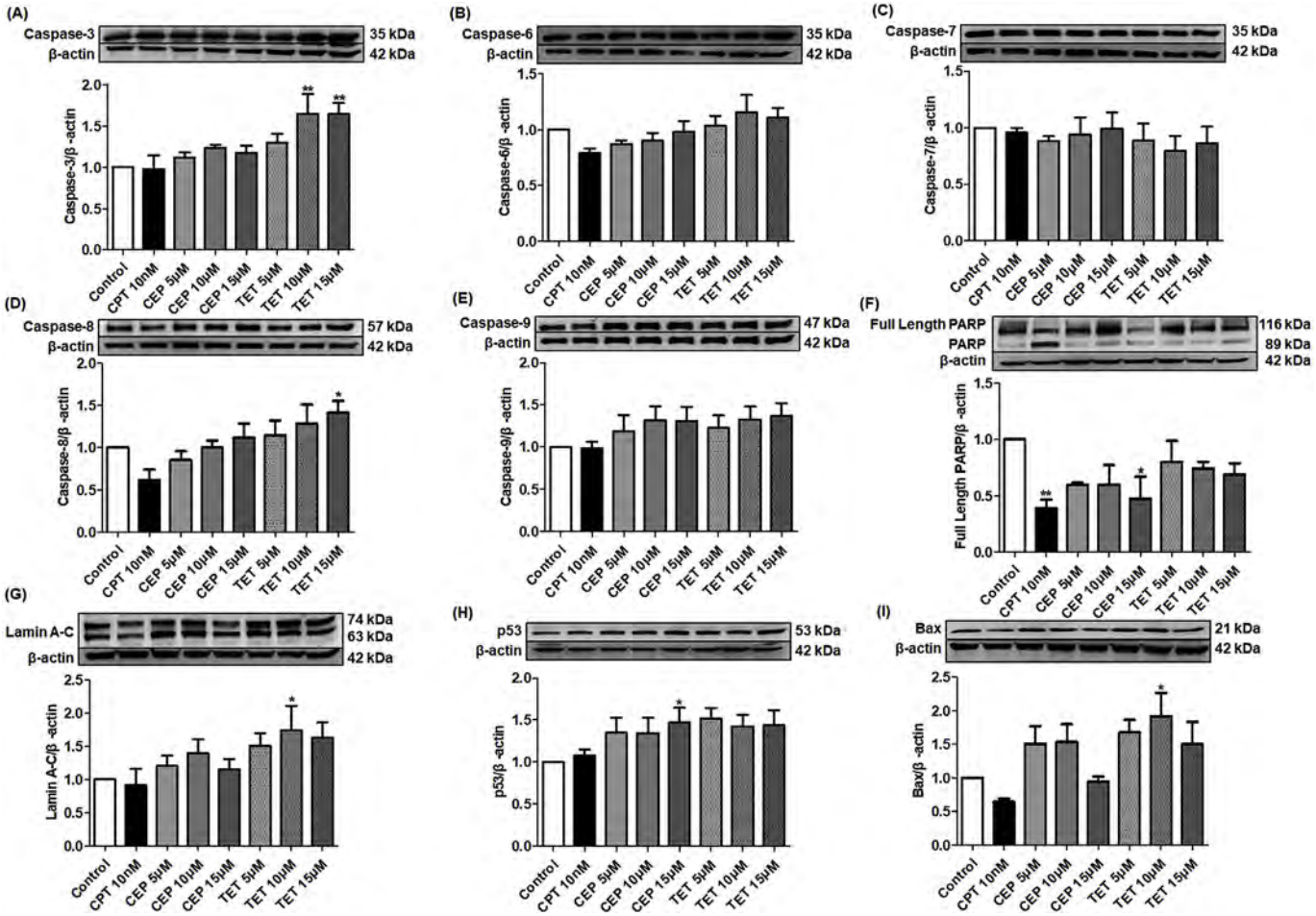

Fig. 3. Effects of tetrandrine (TET) and cepharanthine (CEP) on the expression levels of pro-apoptotic protein in caspase cascades. Jurkat T cells were treated with different concentrations of TET or CEP (5, 10 and 15 μM) and camptothecin (CPT, 10 nM) for 72 h. The cell lysates were examined by Western blot. More than three independent experiments were carried out and representative results were shown in the figures. β-actin was used as internal control. Asterisks indicate significant differences between the control and the drug treatment groups. ($^{*}P < 0.05$ and $^{**}P < 0.01$).

treatment induced the formation of cleaved PARP (89 kDa), which leading to a significant downregulation of full length PARP (Fig. 3F). 15 μM of CEP also downregulated the expression of full length PARP significantly ($P < 0.05$), but we did not observe the cleavage type (Fig. 3F). Whereas, the effects of TET on full length PARP were not statistically significant (Fig. 3F). As shown in Fig. 3G and I, Lamin A-C and Bax were influenced by the treatment of TET or CEP, and 10 μM of TET maximally increased the expression levels of these two pro-apoptotic proteins significantly ($P < 0.05$, Fig. 3G and I). Furthermore, CEP treatment stimulated the expression of p53 in a dose-dependent manner, and 15 μM of CEP significantly increased the amount of p53 ($P < 0.05$, Fig. 3H). TET at 5–15 μM tended to stimulate the expression of p53, though the effects were not statistically significantly (Fig. 3H).

Paradoxically, anti-apoptotic factors, such as Bcl-2, Mcl-1 and p–NF–κB, were also enhanced by the treatment with CPT, TET and CEP (Fig. 4A–D). As shown in Fig. 4A, TET or CEP dose-dependently increased the expression of Bcl-2, and 15 μM of TET showed a significant increase in the amount of this protein ($P < 0.05$). Meanwhile, both TET and CEP largely upregulated the expression levels of Mcl-1 and p–NF–κB significantly in a dose-dependent manner (Fig. 4C and D). None of the three agents changed the expression of Bcl-XL (Fig. 4B).

3.4. TET and CEP trigger the cell cycle arrest in Jurkat T cells

To evaluate whether TET or CEP mediated inhibition of cell growth correlated with cell cycle arrest, Jurkat T cells were treated with serial concentrations of TET or CEP (5, 10 and 15 μM). As shown in Fig. 5A–B, 10 nM of CPT as a positive control, largely increased cell population at S phase ($P < 0.001$), followed with a significant decrease of cell population at G_0/G_1 phase ($P < 0.001$). Interestingly, TET or CEP showed similar results that they triggered cell cycle arrest and leaded the cell growth to stop at S phase in a dose-dependent manner, and thus decreasing the percentage of cells at G_0/G_1 phase. None of these three agents influenced the population of cells at G_2/M phase largely.

3.5. TET and CEP regulate the expression of cell cycle-related proteins

We continued to examine the influences of TET and CEP on cell cycle-related proteins. As shown in Fig. 6B and 10 nM of CPT significantly increased the expression level of cyclin B1 ($P < 0.01$), and TET or CEP showed the similar regulation effect in a dose-dependent manner. All of these three drugs enhanced the expression of cyclin A2, and 15 μM of TET possessed the strongest efficacy ($P < 0.05$, Fig. 6A). In contrast, cyclin D1 protein expression was effectively downregulated by TET or CEP (Fig. 6C).

3.6. TET and CEP activate MAPK

MAPK activation maintains the proliferation of T cells. To investigate the molecular basis for the effects of TET and CEP against Jurkat T cell growth, we examined the influence of TET or CEP on MAPK activation. As shown in Fig. 7A, both TET and CEP apparently

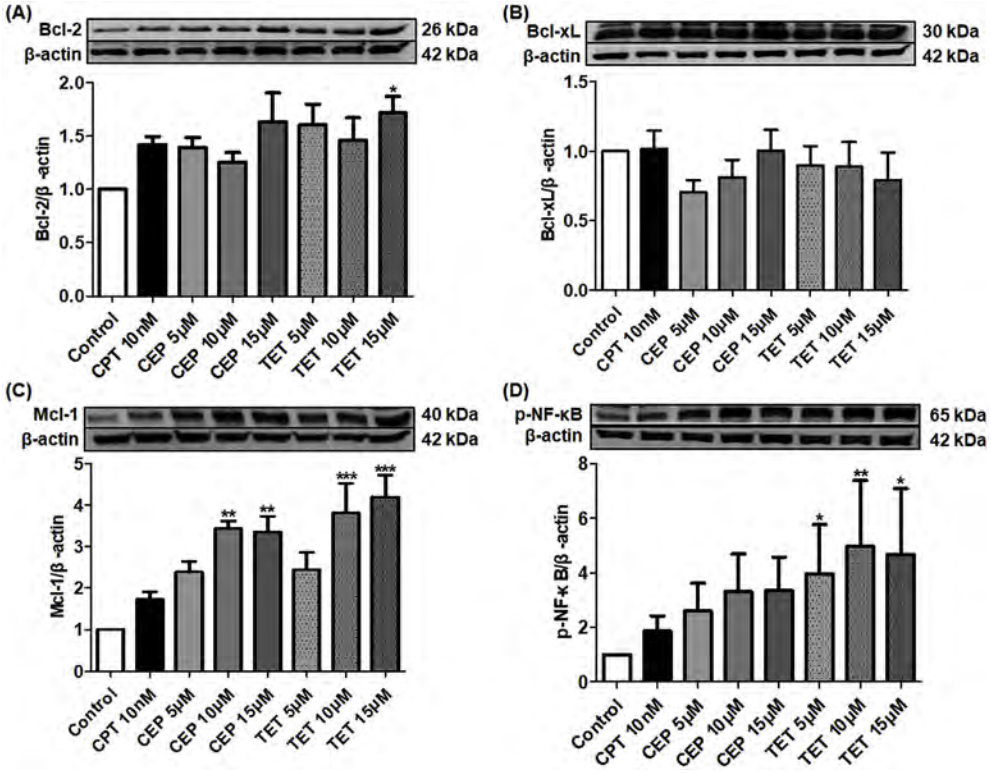

Fig. 4. Effects of tetrandrine (TET) and cepharanthine (CEP) on the expression levels of anti-apoptotic protein in caspase cascades. Jurkat T cells were treated with different concentrations of TET or CEP (5, 10 and 15 μM) and camptothecin (CPT, 10 nM) for 72 h. The cell lysates were examined by Western blot. More than three independent experiments were carried out and representative results were shown in the figures. β-actin was used as internal control. Asterisks indicate significant differences between the control and the drug treatment groups. ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

stimulated the phosphorylation of p38 in a dose-dependent manner (Fig. 7A). 15 μM of CEP changed the ratio of p-p38 and p38 significantly ($P < 0.05$). As shown in Fig. 7B and 10 nM of CPT significantly increased the phosphorylation of JNK ($P < 0.05$), and TET or CEP also tended to increase the phosphorylation of JNK dose-dependently, though the effects were not significant. However, none of these three drugs significantly influenced the expression of p-ERK (Fig. 7C).

3.7. TET and CEP modify PI3K/Akt/mTOR signaling pathway

To further investigate the molecular mechanism underlying the inhibitory effects of TET and CEP on cell survival and growth in the Jurkat T cells, we evaluated the effect of TET or CEP on PI3K/Akt/mTOR signaling pathway. As shown in Fig. 8A and 10 nM of CPT inhibited the expression of p-PI3K significantly ($P < 0.05$). Similarly, both TET and CEP appeared to decrease the p-PI3K expression dose-dependently (Fig. 8A). All of these three agents largely downregulated the expression of mTOR, and TET or CEP showed the inhibitory effect in a dose-dependent manner (Fig. 8C). However, CPT and TET or CEP paradoxically increased the expression of p-Akt1, and the upregulating effects of 10 and 15 μM TET were statistically significant ($P < 0.01$ and 0.05, respectively).

4. Discussion

The WST-8 assay data suggested that 10 and 15 μM TET or CEP inhibited the proliferation of Jurkat T cells significantly (Fig. 1B), and the inhibitory tendency was consistent with the results obtained from the flow cytometer analysis by use of Annexin-V and PI staining (Fig. 2B). Images obtained from fluorescence microscopy revealed that the cell morphology was largely changed to show karyorrhexis, chromatin condensation and fragmentation (Fig. 1C), which indicated that cell apoptosis seriously occurred. This finding was also certified by the results of apoptosis analysis in Fig. 2C and D. Both TET and CEP showed cytotoxic effect on Jurkat T cells by inducing apoptosis, which were consistent with the observations of previous reports [18,19].

Caspase cascades are pivotal components of apoptosis. Caspases are expressed in cells as inactive zymogens, which are also known as procaspases and are activated *via* proteolytic cleavage. Wu et al. revealed that CEP treatment induced activation of capase-3/8/9 accompanied by cleavage of PARP in Jurkat T cells [19]. CEP-induced apoptosis was completely blocked by a caspase inhibitor Z-VAD-fmk [19]. Similarly, TET-induced apoptotic DNA damage in T cells requires activated caspase-3, and this effect of TET could be also inhibited by caspase inhibitors Z-VAD-fmk and Z-DEAD-fmk [20]. Thus, the present study continuously observed the effects of TET and CEP on procaspases and other caspase cascades. Our present investigation showed that TET or CEP not only upregulated the expression of initiator caspases such as caspase-8 and 9 (Fig. 3D and E), but also increased the expression of effector caspases such as caspase-3 and 6 (Fig. 3A and B). However, TET or CEP seemed to show little effect on caspase-7 (Fig. 3C). Compared with CPT, TET or CEP treatment did not largely stimulate the cleavage of PARP, but downregulated the expression amount of full length PARP (Fig. 3F). The above different phenomenon would be related to the different activities of CPT, TET and CEP on caspase cascades, since CPT had almost no effects on the expression of caspase-3/6/7/8/9 (Fig. 3A–E). Lamins and p53, as the downstream proteins of caspase cascades, were also influenced by the treatment of TET and CEP (Fig. 3 G and H). However, apoptosis induced by these alkaloids did not require functional p53 since Jurkat T cells have a mutated p53 [21,22]. Bax is one of the members of Bcl-2 family, which induces apoptosis through mitochondrial stress [23–25]. TET or CEP strongly stimulated the expression of Bax at 5 and 10 μM, while they decreased the expression at 15 μM (Fig. 3I). Excessive stimulation of p-Akt1 by 15 μM TET or CEP, as shown in Fig. 8B, may account for the paradoxical results, since Akt inhibits a conformational change in the pro-apoptotic Bax protein and its translocation into mitochondria [26]. However, all of these three agents seem to enhance the expression of survival protein, p–NF–κB, and anti-apoptotic proteins of Bcl-2 family such as Bcl-2 and Mcl-1 (Fig. 4A–D). However, our analysis using the Annexin V staining (Fig. 2) showed that very high percentage of cells underwent apoptosis. The paradoxical phenomenon suggested that both caspase-

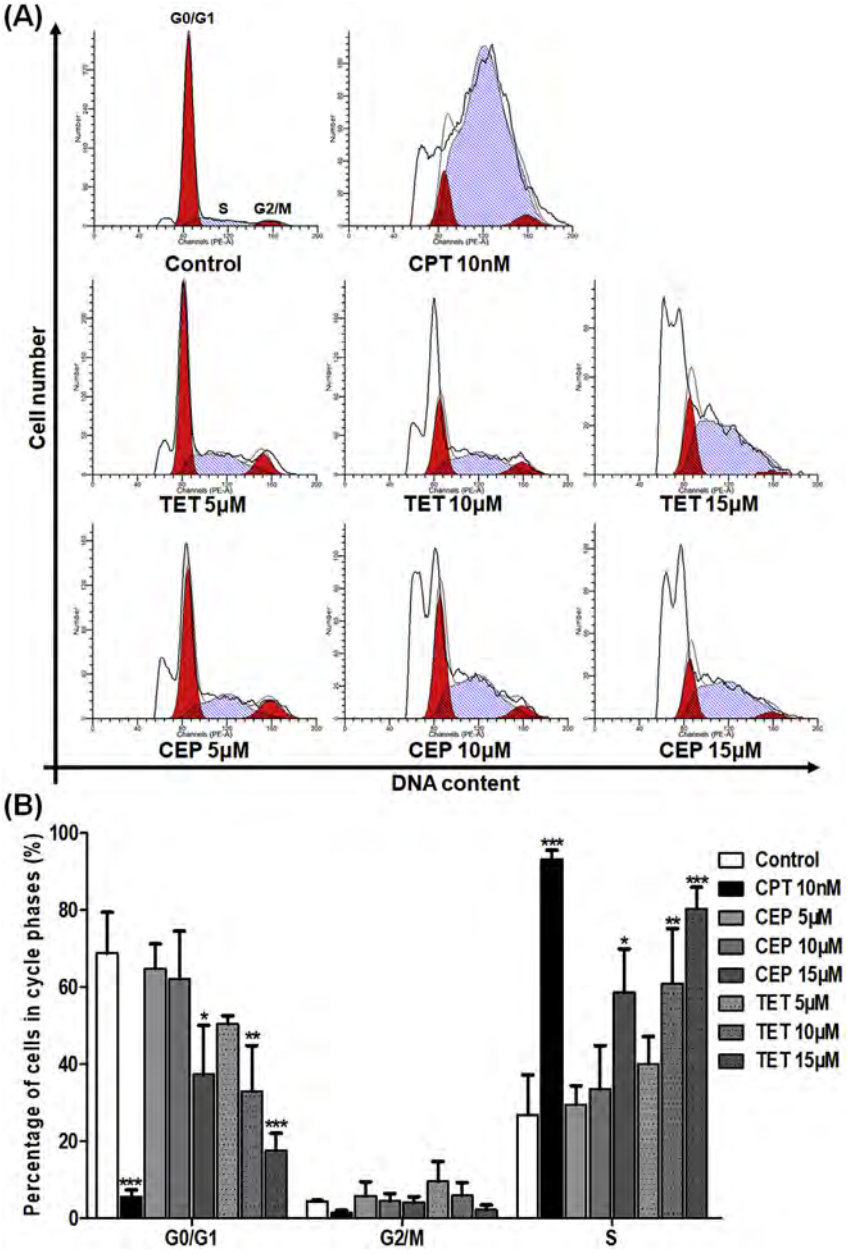

Fig. 5. Tetrandrine (TET) and cepharanthine (CEP) triggers cell cycle in Jurkat T cells. (A) Cells were treated with serial concentration of TET and CEP (5, 10 and 15 μM) for 48 h, respectively. 10 nM of camptothecin (CPT) was used as a positive control. The peaks marked in the figure represent G0/G1, S and G2/M phases in the cell cycle, respectively. (B) Percentages of cell numbers in the cell cycle after 48 h of the agent treatment. All data were expressed as the mean ± S.E.M. of three independent experiments. Asterisks indicate significant differences between the control and the drug treatment groups. ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

independent and dependent apoptotic signaling pathways are implicated in the action of TET or CEP, and this proposed explanation was consistent with the finding of Lai et al. [20].

Similar to the most of common human tumors, cell cycle arrest would be an important way to regulate T-ALL cell growth and proliferation [17]. In the present study, we found that CPT, TET and CEP largely arrested the cell cycle progression at S phase in a dose-dependent manner, accompanied by a significant decrease of cell population at G_0/G_1 phase (Fig. 5A and B). S phase is a crucial event in the cell cycle that allows for proper replication of DNA without accumulating genetic abnormalities [27]. Topoisomerase I relaxes the DNA supercoil form during the DNA replication process, and we found that CPT significantly inhibited the expression of topoisomerase I in Jurkat T cells (data not shown). Our present data suggested that CPT caused DNA damage and activated the cell cycle checkpoint with a cell cycle arrest at S phase, as has been suggested by other researchers [28]. In contrast, TET or CEP, sharing the similar cell cycle arrest with CPT, did not influence the expression of topoisomerase I in Jurkat T cell nucleus (data not shown). As we have known, transition from one cell cycle phase to another occurs in an orderly fashion and is regulated by different cellular proteins, such as cyclin A/B/D [29]. Further investigation revealed that both CPT and TET or CEP upregulated the expressions of cyclin A2 and B1 but downregulated the expression of cyclin D1, which might contribute to their similar effects on cell cycle arrest (Fig. 6A–C).

Activated mitogen-activated protein kinase (MAPK) has been reported to play a major role in promoting and maintaining T lymphocyte populations [30,31]. Unlike the normal T cells, activated MAPK may contribute to the apoptotic process of malignant Jurkat T cells. As shown in Fig. 7A, p38 was activated by the treatment with TET or CEP apparently and dose-dependently, which was accompanied by the

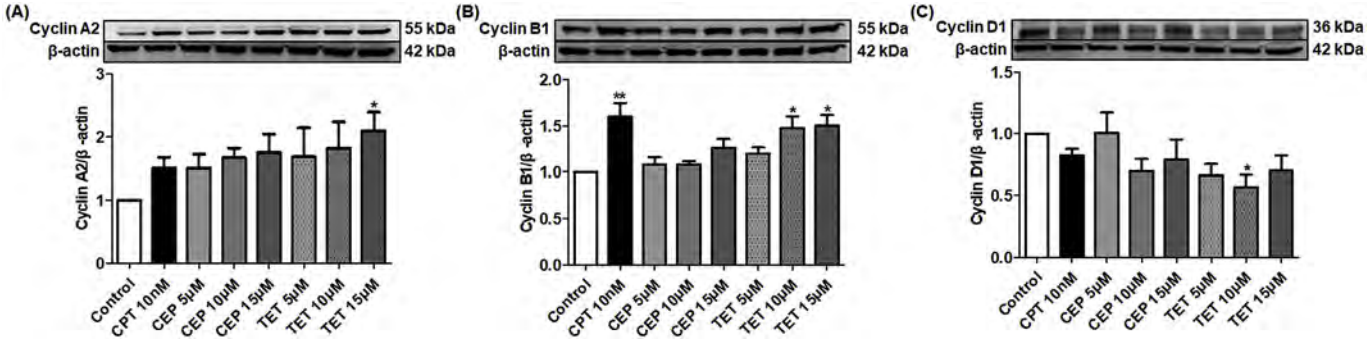

Fig. 6. Effects of tetrandrine (TET) and cepharanthine (CEP) on cell cycle regulators in Jurkat T cells. Cells were treated with different concentrations of TET or CEP (5, 10 and 15 μM) and camptothecin (CPT, 10 nM) for 72 h. The cell lysates were examined by Western blot. More than three independent experiments were carried out and representative results were shown in the figures. β-actin was used as internal control. Asterisks indicate significant differences between the control and the drug treatment groups. ($^{*}P < 0.05$).

induction of apoptosis. This finding was consistent with that of the previous study [32]. Comparing with TET or CEP, 10 nM of CPT strongly stimulated the phosphorylation of JNK (Fig. 7C). It was reported that JNK mediated phosphorylation of anti-apoptotic proteins Bcl-2/Bcl-xL, which change the mitochondrial membrane potential to release cytochrome C. Then caspase-9 is activated, following with caspase-3 activation. Finally, JNK sensitizes cancer cells to genotoxic stress-induced cell death [33]. However, all of the three agents rarely

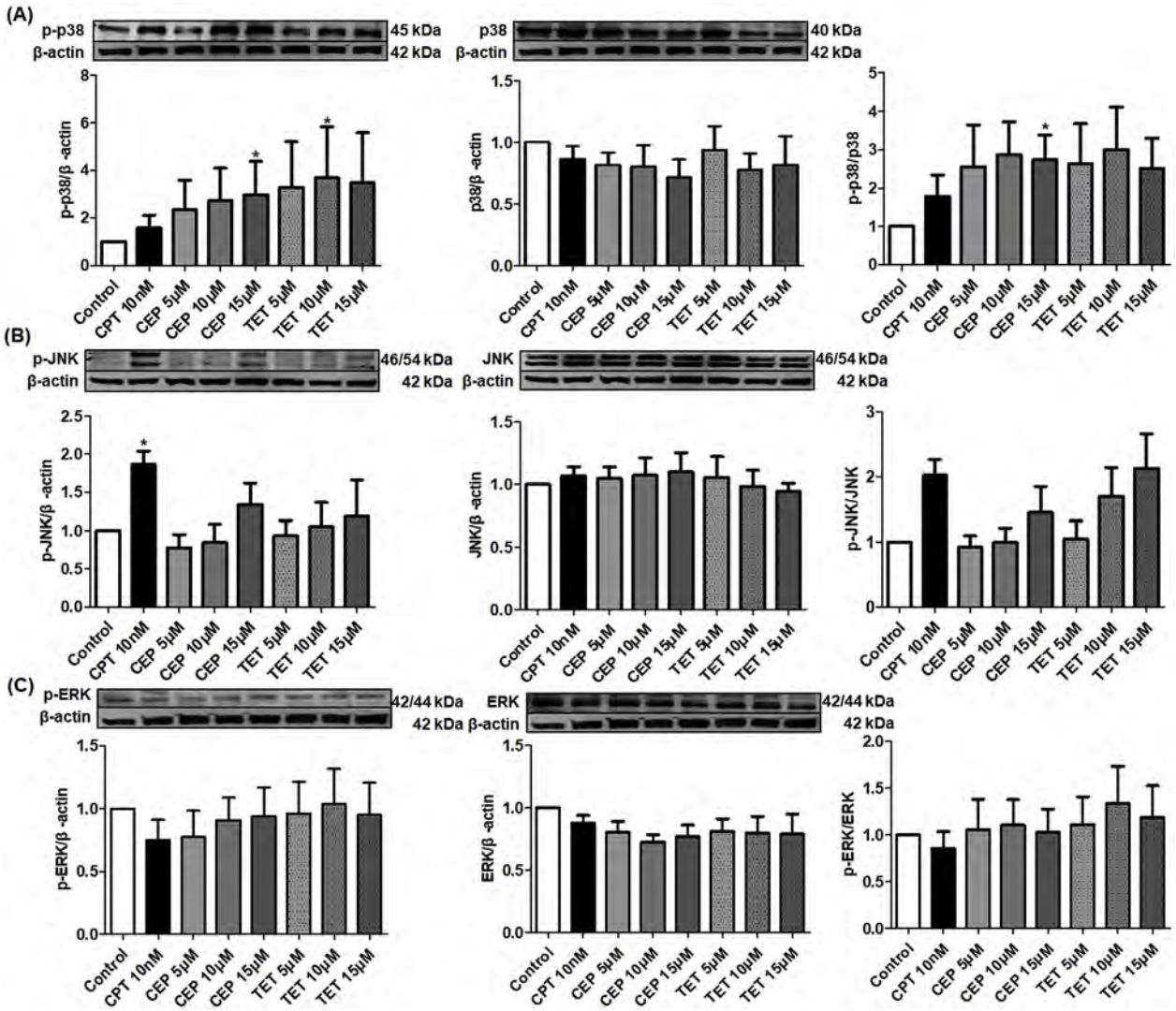

Fig. 7. Effects of tetrandrine (TET) and cepharanthine (CEP) on the expression levels of mitogen-activated protein kinase proteins. Cells were treated with different concentrations of TET or CEP (5, 10 and 15 μM) and camptothecin (CPT, 10 nM) for 24 h. The cell lysates were examined by Western blot. More than three independent experiments were carried out and representative results were shown in the figures. β-actin was used as internal control. Asterisks indicate significant differences between the control and the drug treatment groups. ($^{*}P < 0.05$).

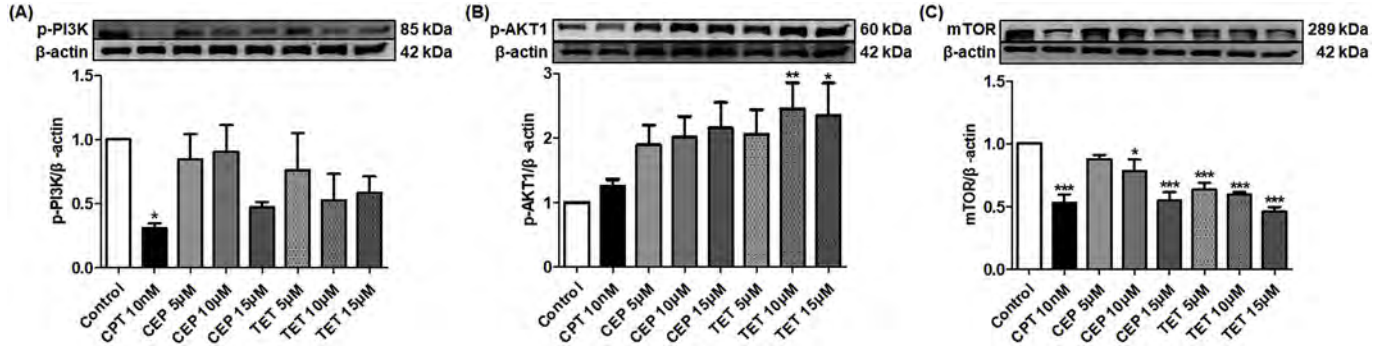

Fig. 8. Effects of tetrandrine (TET) and cepharanthine (CEP) on the expression levels of proteins in the PI3K/Akt/mTOR signaling pathway. Cells were treated with different concentrations of TET or CEP (5, 10 and 15 μM) and camptothecin (CPT, 10 nM) for 72 h. Western blot assays were carried out to examine the effects of TET or CEP on the expressions of key proteins p-PI3K (A), p-Akt (B) and mTOR (C). β-actin was used as internal control. All images are representative of three independent experiments. Asterisks indicate significant differences between the control and the drug treatment groups. ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

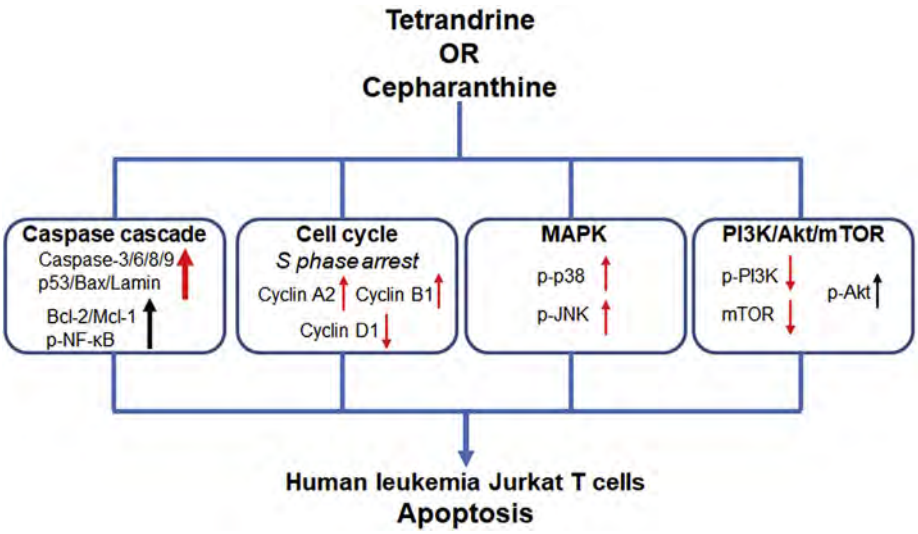

Fig. 9. Possible action mechanism of tetrandrine (TET) and cepharanthine (CEP) to induce apoptosis in glucocorticoid resistant human leukemia Jurkat T cells. TET or CEP not only upregulates the expression of initiator caspases such as caspase-8 and 9, but also increases the expression of effector caspases such as caspase-3 and 6. As the important markers of apoptosis, p53 and Bax are both upregulated by the treatment with TET and CEP. However, TET and CEP paradoxically increase the expression of anti-apoptotic proteins, such as Bcl-2 or Mcl-1 and activate the survival protein NF-κB, leading to high expression of p–NF–κB. S phase arrest in Jurkat T cells triggering by TET or CEP is another possible mechanism, following with increase in cyclin A2, cyclin B1 and decrease in cylcin D1. During the process of apoptosis in Jurkat T cells, treatment with TET or CEP also increases the phosphorylation of JNK and p38, which belong to the family of mitogen-activated protein kinase proteins. PI3K/Akt/mTOR signaling pathway modification play significant role in the apoptosis of Jurkat T cells which is induced by TET or CEP. Moreover, TET and CEP seem to downregulate the expressions of p-PI3K and mTOR in an independent way on Akt, since these two drugs stimulates the expression of p-Akt strongly.

influenced on the activation of ERK (Fig. 7C).

PI3K/Akt/mTOR signaling pathway controls multiple cellular responses, including metabolic regulation, cell growth, and survival [34]. In human T-ALL, constitutive activation of the PI3K/Akt/mTOR signal transduction pathway is achieved by deletions or mutations targeting PTEN in about 15% of cases [34–36]. Our data showed that both CPT and TET or CEP inhibited the expression amounts of p-PI3K and mTOR, whereas the treatment by these agents resulted in the high expression of p-Akt1 paradoxically (Fig. 8A–C). Although Akt was viewed as a major downstream effector of PI3K, at least in physiological processes, several studies suggested that PI3K and Akt act independently in cancers [37]. While PI3K is a major regulator of Akt activation in response to a variety of ligands, recent studies highlighted that diverse groups of tyrosine (Ack1/TNK2, Src, PTK6) and serine/threonine (TBK1, IKBKE, DNAPKcs) kinases also activate Akt directly to promote growth, proliferation and oncogenic transformation [38]. Thus, TET and CEP seemed to regulate the expressions of p-PI3K and mTOR in an independent way on Akt. On the other hand, downregulation of mTOR expression always associated with the activation of autophagy, which may maintain the tumor homeostasis and lead to drug resistance. Wong et al. revealed that TET could induce autophagic cell death in mTOR-dependent way in MCF-7 cells [39]. Subsequently, TET was also reported to suppress proliferation and induce autophagy in MDA-MB-231 cells by inhibiting the PI3K/AKT/mTOR pathway [40]. Moreover, autophagy induction enhances TET-induced apoptosis *via* the AMPK/mTOR pathway in human bladder cancer cells [41]. Although all these findings supported that autophagy induction by TET or CEP may lead to tumor suppression, the anti-cancer properties and molecular mechanisms of TET or CEP are likely to be cell-type specific and remain to be further investigated.

TET and CEP, bisbenzylisoquinoline alkaloids isolated from the traditional herbs, are commercially distributed in China and Japan respectively, for more than 20 years, which certifies the safety of these two compounds [5,12]. Meanwhile, with the advantage of high tolerance and prominent P-glycoprotein inhibitory effect, TET has been registered as CBT-1® in USA and was studied in the clinical trials in association with doxorubicin for treating patients with advanced solid tumors [42]. The present study showed the apoptotic effects of TET and CEP induced by interactional signaling pathways of caspase cascade, cell cycle, MAPK and PI3K/Akt/mTOR signaling pathway in Jurkat T cells. However, these signaling also control the proliferation, survival and death of normal human immune cells. Previous studies already revealed that the potential of this class of compounds also leads to immunosuppression/toxicity on normal human T cells [20,43–45]. Moreover, our current study has confirmed that μM of TET or CEP suppressed the proliferation of mitogen-activated human peripheral blood mononuclear cells of healthy subjects and rheumatoid arthritis patients (data not shown). Thus, it is inevitable clinically to keep balance of the immunosuppressive and the anti-T-ALL effects of these compounds. New functional derivatives of TET or CEP with higher

selectivity on pathological T cells deserve to be developed in the future.

5. Conclusion

From this work, we can conclude that bisbenzylisoquinoline alkaloids represented by TET and CEP show cytotoxic effect on glucocorticoid resistant human leukemia Jurkat T cells by inducing apoptosis through caspase cascade regulation, cell cycle arrest, MAPK activation and PI3K/Akt/mTOR signal modification (Fig. 9).

Declaration of conflicting interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2019.108726.

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専攻種別	□論文博士	☑課程博士			

研究者評価（指導教官記入欄）

成績状況	(優) 良 可 不可 学業成績係数=	取得単位数 5／30
学生本人が行った研究の概要	李さんが行っている研究は、漢方医学科を受診した患者を対象に、当科で行っている漢方治療による望診上の診断方法を検証し、望診の概念を発展させることを目的とする。治療した前後の皮膚の微小循環の変化と肌のキメの変化、Numerical Rating Scale（NRS）（NRS：痛みの程度を数字で測定する方法）による改善度を評価検討する事で、漢方医学の有効性を科学的に検討する。	
総合評価	【良かった点】 1. 李さんは意志が強くて、ポジティブな思考を持って、研究成果を上げるためさまざまな方法に果敢に取り組む。 2. 李さんは研究に対して、その学問における重要課題を見抜く能力や発想力に優れ、研究成果を取得する事だけではなく、研究成果を臨床で応用するという最終的な目標を持ち、研究活動をしている。	
	【改善すべき点】 1. 大勢の人の前だと極度に緊張することである。もっと多くの会議で発言するなど経験を積む必要がある。 2. 自分の思っていることを上手に言葉にすることが少し苦手であり、遠慮なく自分の考えを伝えることも必要である。	
	【今後の展望】 今回の研究は当科へ受診した患者すべてを対象としているが、今後は患者を病気別または漢方医学の「症」別で募集し、漢方治療後の望診上の変化の規律を分析し、望診の概念を臨床で広く発展させることに努力する。	
学位取得見込	李さんはもう 1 篇の英語論文を掲載した。今は第 2 篇を発表できるように頑張っている。今行っている研究も順調に進めているから、2 年間の奨学金支援終了後 3 年以内に日本の博士学位を取得することが可能である。	

評価者（指導教官名）小川恵子 印

日中笹川医学奨学金制度(学位取得コース)報告書
研究者用

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研究テーマ	画像解析技術を用いた人体における漢方薬の評価				
専攻種別	論文博士	□	課程博士	☑	

1. 研究概要(1)

1)目的(Goal)

本研究では、主観的な尺度と客観的な測定方法を用いて、漢方治療による治療効果と望診上の改善を評価することを目的とする。

2)戦略(Approach)

漢方医学は、約1500年の長きにわたって日本人の健康を支えてきた日本の伝統医学である。[1]現在、日本では漢方医学(湯液・鍼灸)が広く用いられている。特に漢方薬は医療保険制度の中で医師が処方しており、漢方医学科でも年々患者数は増加している。

漢方医学は、患者の病状(訴え)や体質を重視し、その結果から処方する。湯液は1剤に複数の有効成分が含まれているため、多様な症状に効くのが大きな特徴である。また、鍼灸は人体の気の流れを整えることができる。湯液と鍼灸を併用すると、湯液と鍼灸それぞれの特徴を合わせて治療をすることで効率が上がり、相乗効果が得られる。しかも、我々が以前行った研究では、入院中の患者を対象に漢方治療(湯液・鍼灸)による治療を行い、高い効果を実感している。[2-4]

漢方の診察は望診・聞診・問診・切診の「四診」を用いて行う。その中の望診は視覚により、患者の全身状態、顔色、舌色、舌質、分泌物等を見て患者の状態を判断する主観的な診察法である。漢方の理論では、人体の気血の盛衰が顔面に現れる、したがって、顔面を望診することは全身の気血の状態を知ることにつながる。望診は医師の主観的な感覚に基づいて判断することである、客観化させる必要があると思う。そして、本研究では、当科における患者への漢方治療前後に皮膚の微小循環の変化と肌のキメの変化、Numerical Rating Scale(NRS)(NRS:痛みの程度を数字で選択する方法)による改善度を評価検討する事で、漢方治療法の有効性を数値化し、望診の概念を発展させ、客観化させる。

3)材料と方法(Materials and methods)

①研究方法

金沢大学附属病院漢方医学科を受診した患者カルテの内容(・診療記録・診療経過・使用薬剤名(処方内容)など)を使用し、患者への漢方医学(鍼灸及び湯液)併用治療の使用頻度や、どのような主訴に対して使用しているかの調査、通常の診察に伴って撮影する皮膚画像を用いて、MATLABを使って、短直線マッチング法(皮膚の凹凸を線上に画像修正することでキメを評価する方法)[5, 6]で解析し、皮膚微小循環の改善[7]とNRSによる改善度の評価による評価を行う。

②研究対象者の選定方針

(1)適格基準

金沢大学漢方医学科を受診した患者

(2)除外基準

特記事項なし

1. 研究概要(2)
(3)目標数:150名
金沢大学漢方医学科の診療を受ける患者は年間約100名であり、2021年までに150名の患者数が見込まれる。

③研究実施期間
研究実施期間:倫理承認日～2021年5月31日(西暦)
研究対象期間:倫理承認日～2021年3月31日
解析期間　:2020年4月1日～2021年5月31日

④観察・検査・報告項目
・カルテの内容:診療記録(視診、聴診、嗅い、問診、触診、腹診、舌診、脈診、疼痛NRS、倦怠感NRS、唾液アミラーゼ活性・診療経過・使用薬剤名(処方内容)など)
・患者への漢方医学(鍼灸及び湯液)併用治療の使用頻度や、どのような主訴に対して使用しているか
・経過中の血液検査の結果
・LEA.O2Cで測定する皮膚表層約2㎜と8㎜の血流量、血流速度、酸素とヘモグロビン値
・カメラで皮膚を撮影、得た画像を短直線マッチング法で解析した結果

⑤統計的事項
解析項目
患者カルテの内容(・診療記録・診療経過・使用薬剤名(処方内容)など)
LEA.O2Cで測定する皮膚表層約2㎜と8㎜の血流量、血流速度、酸素とヘモグロビン値
短直線マッチング法で解析した皮溝の面積率、皮溝の平均太さ、短直線の傾きの標準偏差
解析方法
主に観察研究であるため、同一個体の前後比較、もしくは、多変量解析を行う。

4)実験結果(Results)
現在は、実験のデータを纏めて、分析中である。

5)考察(Discussion)
2019年4月から2020年3月までは83人を募集した。今の段階では、その83人を症状別でデータを分析し、規律を発見する。次の段階では、病気別または症別で患者を募集する予定である。

6)参考文献(References)
[1]安井廣迪, 医学生のための漢方医学.千葉.東洋学術出版社.2008:2-7.
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[3] Keiko Ogawa, et al. " Optical Examination of the Efficacy of Contact Needle Therapy for Chemotherapy-Induced Peripheral Neuropathy: Integration of the Inspection of Kampo Therapy and the Color Spectrum Information " The Journal of Alternative and Complementary Medicine, vol. 20, no. 5, pp. 45-46.
[4] Keiko Ogawa, et al. "A case of extensive pharyngeal vascular malformation successfully treated with Kampo medicine." Auris Nasus Larynx,vol.45,no.1,pp.190-193.
[5] Hiroshi Kobayashi, et al. "Proposal of Quantitative Index of Skin Texture by the Image Processing and Its Practical Application" Transactions of the JSME, vol. 76, no. 764, pp. 138-145.
[6] Mihiro Uchida, Rina Akaho, et al. "Image-based Non-contact Monitoring of Skin Texture Changed by P5loerection for Emotion Estimation" February 15, 2018, 5th Symposium of the color of Digital Imaging in Biomedicine.
[7]Stefan Beckert, DRMED, MD, Maria B. Witte, MD, et al."The Impact of the Micro-Lightguide O2C for the Quantification of Tissue Ischemia in Diabetic Foot Ulcers"Diabetes Care, vol. 27, no. 12, pp. 2863-2867.

2. 執筆論文 Publication of thesis ※記載した論文を添付してください。Attach all of the papers listed below.

論文名 1 Title	The effect of different types of acupuncture manipulations on shoulder pain and cardiovascular dynamics						
掲載誌名 Published journal	Artificial Life and Robotics						
	2019 年 7 月	24 巻(号)	520 頁 ～ 526 頁		言語 Language	English	
第1著者名 First author	Hongyang Li	第2著者名 Second author	Norio Tomita	第3著者名 Third author	Mako Iwahashi		
その他著者名 Other authors	Junsuke Alimitsu, Kanji Kawasaki, Akiko Shirai, Keiko Ogawa-Ochiai						
論文名 2 Title							
掲載誌名 Published journal							
	年 月	巻(号)	頁 ～ 頁		言語 Language		
第1著者名 First author		第2著者名 Second author		第3著者名 Third author			
その他著者名 Other authors							
論文名 3 Title							
掲載誌名 Published journal							
	年 月	巻(号)	頁 ～ 頁		言語 Language		
第1著者名 First author		第2著者名 Second author		第3著者名 Third author			
その他著者名 Other authors							
論文名 4 Title							
掲載誌名 Published journal							
	年 月	巻(号)	頁 ～ 頁		言語 Language		
第1著者名 First author		第2著者名 Second author		第3著者名 Third author			
その他著者名 Other authors							
論文名 5 Title							
掲載誌名 Published journal							
	年 月	巻(号)	頁 ～ 頁		言語 Language		
第1著者名 First author		第2著者名 Second author		第3著者名 Third author			
その他著者名 Other authors							

3. 学会発表 Conference presentation ※筆頭演者として総会・国際学会を含む主な学会で発表したものを記載してください.

※Describe your presentation as the principal presenter in major academic meetings including general meetings or international meeting

学会名 Conference	The 24th International Symposium on Artificial life and Robotics				
演 題 Topic	The effect of different types of acupuncture manipulations on shoulder pain and cardiovascular dynamics				
開催日 date	2019 年 1 月 24 日	開催地 venue	日本 別府		
形式 method	☑ 口頭発表 Oral ☐ ポスター発表 Poster	言語 Language	☐ 日本語	☑ 英語	☐ 中国語
共同演者名 Co-presenter	Norio Tomita, Mako Iwahashi, Kanji Kawasaki, Akiko Shirai, Keiko Ogawa				
学会名 Conference	第68回(公社)全日本鍼灸学会学術大会愛知大会				
演 題 Topic	鍼手技の違いが肩こり及び心循環動態へ及ぼす影響				
開催日 date	2019 年 5 月 12 日	開催地 venue	日本 名古屋		
形式 method	☐ 口頭発表 Oral ☑ ポスター発表 Poster	言語 Language	☑ 日本語	☐ 英語	☐ 中国語
共同演者名 Co-presenter	富田 紀男、津田 昌樹、川崎 寛二、岩橋 麻子、白井 明子、有光 潤介、小川 恵子				
学会名 Conference					
演 題 Topic					
開催日 date	年 月 日	開催地 venue			
形式 method	☐ 口頭発表 Oral ☐ ポスター発表 Poster	言語 Language	☐ 日本語	☐ 英語	☐ 中国語
共同演者名 Co-presenter					
学会名 Conference					
演 題 Topic					
開催日 date	年 月 日	開催地 venue			
形式 method	☐ 口頭発表 Oral ☐ ポスター発表 Poster	言語 Language	☐ 日本語	☐ 英語	☐ 中国語
共同演者名 Co-presenter					

4. 受賞(研究業績) Award (Research achievement)

名 称 Award name				
国名 Country		受賞年 Year of award	年	月
名 称 Award name				
国名 Country		受賞年 Year of award	年	月

5. 本研究テーマに関わる他の研究助成金受給 Other research grants concerned with your resarch theme

受給実績 Receipt record	□ 有 ■ 無
助成機関名称 Funding agency	
助成金名称 Grant name	
受給期間 Supported period	年 月 ～ 年 月
受給額 Amount received	円
受給実績 Receipt record	□ 有 □ 無
助成機関名称 Funding agency	
助成金名称 Grant name	
受給期間 Supported period	年 月 ～ 年 月
受給額 Amount received	円

6. 他の奨学金受給 Another awarded scholarship

受給実績 Receipt record	□ 有 ■ 無
助成機関名称 Funding agency	
奨学金名称 Scholarship name	
受給期間 Supported period	年 月 ～ 年 月
受給額 Amount received	円

7. 研究活動に関する報道発表 Press release concerned with your research activities

※記載した記事を添付してください。Attach a copy of the article described below

報道発表 Press release	□ 有 ■ 無	発表年月日 Date of release	
発表機関 Released medium			
発表形式 Release method	・新聞 ・雑誌 ・Web site ・記者発表 ・その他（ ）		
発表タイトル Released title			

8. 本研究テーマに関する特許出願予定 Patent application concerned with your research theme

出願予定 Scheduled	□ 有 ■ 無	出願国 Application	
出願内容（概要） Application contents			

9. その他 Others

指導責任者（署名） 小川恵子 ㊞

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ORIGINAL ARTICLE

The effect of different types of acupuncture manipulations on shoulder pain and cardiovascular circulation dynamics

Hongyang Li[1] · Norio Tomita[1] · Mako Iwahashi[1] · Junsuke Arimitsu[1] · Kanji Kawasaki[1] · Akiko Shirai[1] · Keiko Ogawa-Ochiai[1]

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Abstract
This study is to compare the effect of contacting needle technique (CNT) and insertion needle technique (INT) on cardiovascular dynamics and visual analogue scale (VAS) in patients with shoulder pain. A total of 11 patients (9 females, 2 males, average age 32.27) were recruited and divided into two groups (CNT group and INT group). The treatment was performed once a week and a total of 4 weeks. The changes in cardiovascular circulation dynamics were detected at baseline, during the treatment and after the treatment. Pain was assessed before and after acupuncture therapy. There was significant difference in VAS within each group. There was no significant difference between the two groups on CO, SV, BPs, BPd and VAS, and had significant difference on PR ($P < 0.05$). PR significantly decreased in both groups, the rate of decrease was significantly higher in the CNT group than that in the INT group.

Keywords Shoulder pain · Acupuncture · Contacting needle technique

1 Introduction

Shoulder pain is the third most common musculoskeletal pain [1]. The annual prevalence of shoulder complaint is reported to be between 41.2 and 48.4, and it is higher in women than that of men [2]. Shoulder pain is caused by several factors,including physical, psychological, lifestyle and cognitive factors [3]. The patients with shoulder pain may present with chronic pain in their shoulders and sometimes show limited activities, and their daily life and capacity for work can also be affected, so their quality of life can substantially decrease.

Treatment of shoulder pain generally involves life style changes, medication and surgery. In the recent years, acupuncture is being frequently used as a complementary and alternative medicine in the world. In 1996, the World Health Organization (WHO) provided 64 suitable diseases that can be treated with acupuncture and chronic pain in the musculoskeletal system (neck, shoulder, spine, knee, etc.) was one of them [4].And both basic and clinical researches have shown that acupuncture therapy was useful in treating many painful diseases, and more often was used to treat pain in clinical practice. The mechanism of acupuncture analgesia is still not clear, however, many researches conducted on this subject have demonstrated that acupuncture can increase blood circulation [5], stimulate the nervous system and release neuropeptides [6].

But what we always mentioned about acupuncture was the insertion needle technique (INT) using stainless steel filiform needles. Contacting needle technique (CNT) is one of the traditional Japanese acupuncture methods, which was developed by Bunkei Ono. It is considered to be much safer and well tolerated than INT, because the needles doesn't have to be inserted but only pressed on the acupuncture points (Fig. 1.). It focuses on the flow of Qi and the entire condition of patients. The stimulation of CNT is not too strong but the curative effect is accurate [7, 8].

Results from Chiu's research suggested that acupuncture is effective in treating hypertension patients [9]. In healthy adults, acupuncture can also reduce systolic blood pressure (SBP), heart rate (HR), and rate pressure products (RPP).

This work was presented in part at the 24th International Symposium on Artificial Life and Robotics, Beppu, Oita, January 23–25, 2019.

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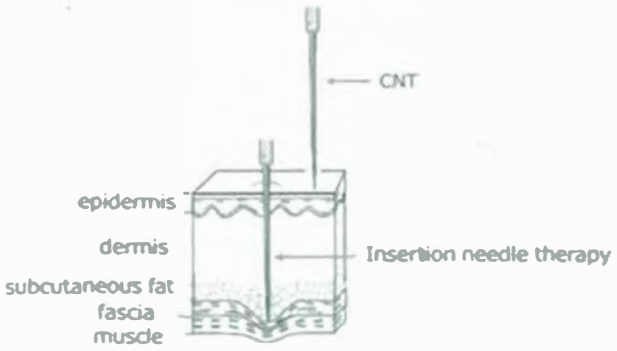

Fig. 1 The difference between INT and CNT

Therefore, acupuncture therapy can be effective in treating cardiovascular disorders [10]. Thus, numerous studies have shown that acupuncture cures the cardiovascular diseases by influencing the cardiovascular circulation dynamics [9], but INT was used in all these studies. To our knowledge, there are no researches on the difference between these two methods (INT and CNT) and its safety on the cardiovascular system.

In this study, we tried to compare the curative effect and cardiovascular safety of INT and CNT, by observing the different changes in the visual analogue scale (VAS) and cardiovascular circulation dynamics between INT and CNT in patients with shoulder pain.

2 Materials and methods

2.1 Subjects

A total of 11 patients who had shoulder pain without cardiovascular diseases were recruited in this study. All patients agreed to receive acupuncture treatment in the form of INT or CNT. The patients were divided into two groups (CNT group and INT group) according to the Kampo diagnosis. Five patients (four females, one male, mean age 35) received INT, and six patients (five females, one male, mean age 30) received CNT.

The study was approved by the ethical committee of Kanazawa University, school of Meidicine and all patients gave informed consent. The study was started in January 2017 and completed in May 2017.

2.2 Acupuncture therapy

Acupuncture manipulations of CNT and INT were applied to LI 14, LI10, GB34, BL60 (both sides), and acupuncture points were determined based on "WHO Standard Acupuncture Point Location in the Western Pacific Regions".

After the patients had rested for 15 min in both groups, they received the acupuncture treatment for 3 min. In the CNT group, disposable sterile silver needles (0.16×24 mm, Asahi, Japan) were used and it was 20 s stimulation per acupuncture point without insertion. In the INT group, disposable stainless needles (0.18×40 mm, Seirin, Japan) were used. The needles were inserted into the skin and muscles to the depth of 10–15 mm and retained for 3 min per acupuncture point.

The acupuncture therapy was once a week and for 4 weeks (a total of 4 treatments). Acupuncture was performed in all cases by the same acupuncturist.

2.3 Outcome measurement

The primary outcome was the change in the VAS score and cardiovascular circulation dynamics.

Cardiovascular circulation dynamics were performed using the ClearSight system (Edwards Lifesciences Corp, Irvine, CA, USA). The ClearSight system is a device comprised of the EV1000 clinical platform and the ClearSight finger cuff [11], and used for measuring arterial blood pressure and cardiac output continuously and noninvasively through finger-cuffed technology [12, 13].The figure cuff of the ClearSight system was placed on the patient's middle finger of right hand, and diastolic blood pressure (BPd), systolic blood pressure (BPs), cardiac output(CO), pulse rate (PR) and stroke volume (SV) were measured.

With the patient in the supine position for 15 min, baseline measurement of cardiovascular circulation dynamics was continued for 2 min period before acupuncture treatment, after that measurement continued for 3 min during treatment, and then measurement continued for 2 min after treatment.

The shoulder pain was assessed before and after acupuncture therapy, using a VAS score.

2.4 Statistical analysis

Statistical analyses were performed using EZR (Easy R) [14] software (version 3.5.1) by a statistician blinded to the participant allocation. Demographic variables of the age, weight, height and body surface area (BSA) in each group were compared using two-sample independent t test and for categorical variables (sex) the Chi square test was used. Means ± standard deviation (SD) were used to represent data. Changes in BPd, BPs, CO, PR, and SV in the two groups over time were analyzed with a two-factor repeated-measures analysis of variance (ANOVA) with "treatment" (between INT and CNT) and "time" (before, during and after treatment) considered as the variables. And a two-sample independent t test was performed to

compare the changes of VAS scores between the two groups. Then, a paired-sample t test was performed to compare the changes in VAS scores within each group. The level of significance was set at $P < 0.05$.

3 Result

3.1 Demographic variables

Participants' demographic variables are presented in Table 1. Eleven participants were involved in the study, two (18.18%) of the participants were male and nine (81.82%) were female. Age, height, weight, BSA and sex did not show significant differences among the two groups ($P > 0.05$).

Table 1 Characteristics of the participants (mean ± SD)

	n	Mean ± SD	F	P value
Age (years)				
INT	5	35 ± 10		
CNT	6	30 ± 8.36	0.267	0.618
Height (cm)				
INT	5	162.8 ± 6.53		
CNT	6	158.83 ± 7.52	0.274	0.614
Weight (kg)				
INT	5	63.6 ± 7.86		
CNT	6	52.17 ± 7.13	0.094	0.766
BSA (m^2)				
INT	5	1.68 ± 0.13		
CNT	6	1.52 ± 0.12	0.07	0.798
Sex				
INT	5	1M/4F		
CNT	6	1M/5F		0.887

INT insertion needle technique, *CNT* contacting needle technique, *BSA* body surface area

3.2 The effect of INT and CNT on VAS

The VAS scores declined after the treatment. In the INT group, VAS scores significantly decreased after the third time and the fourth time treatment ($P < 0.05$). In the CNT group, VAS scores significantly decreased after every treatment ($P < 0.05$). But, there was no significant difference in the change of VAS scores among the two groups ($P > 0.05$). The results are shown in Table 2 and Fig. 2.

3.3 The effect of INT and CNT on cardiovascular circulation dynamics

Cardiovascular circulation dynamics parameters generated by the EV1000 before, during and after treatment are shown in Table 3.

There was no significant difference between CNT group and INT group in CO, SV, BPs and BPd, but showed a significant difference in PR ($P < 0.05$).

The result of CO is presented in Fig. 3 and Table 3. The CO decreased during the treatment and then slightly increased after treatment in both the groups, but there was no statistically significant difference between the two groups ($P > 0.05$).

The result of SV is presented in Fig. 4 and Table 3. The SV slightly decreased after treatment in both groups, but showed no significant difference between the two groups ($P > 0.05$).

The result of PR is presented in Fig. 5 and Table 3. The PR significantly decreased in both groups, the rate of decrease was significantly higher in the CNT group (from 63.29 ± 7.7 to 59.25 ± 6.29) than in the INT group (from 70.10 ± 13.95 to 68.20 ± 11.65) ($P < 0.05$), and the rate of

Table 2 Mean VAS scores of the two groups before and after each treatment (mean ± SD)

	Therapy 1	Therapy 2	Therapy 3	Therapy 4
INT				
Pre-stim	66.8 ± 10.62	73.6 ± 13.58	68.8 ± 19.74	70.0 ± 18.07
Post-stim	57.6 ± 16.06	51.4 ± 26.32	52.6 ± 26.67*	49.6 ± 27.91*
Change	9.2 ± 9.18	22.2 ± 24.06	16.2 ± 12.91	22.2 ± 24.07
CNT				
Pre-stim	62.83 ± 12.78	55.67 ± 27.23	50.67 ± 28.15	56.67 ± 22.61
Post-stim	38.67 ± 24.36*	34.5 ± 20.77*	31.83 ± 19.74*	32.83 ± 20.93*
Change	24.17 ± 13.92	21.17 ± 18.56	18.83 ± 12.86	23.83 ± 7.88

Pre-stim: at the end of pre-stimulation period of 15 min rest (for 2 min)

Post-stim: 5 min after the stimulation period (for 2 min)

Change = pre–post

*$P < 0.05$, compared with pre-stim

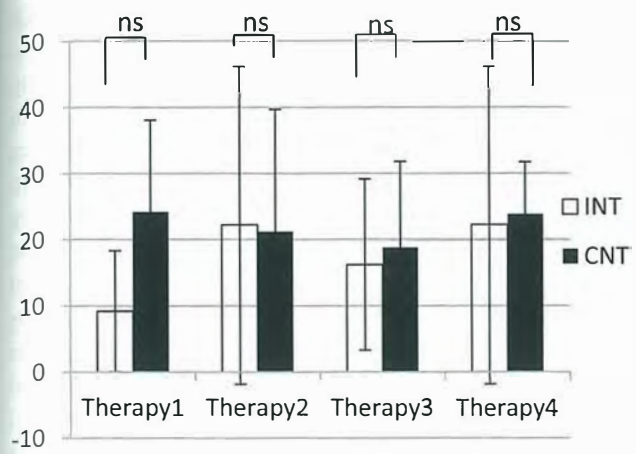

Fig. 2 The change in visual analogue scale (VAS) before and after the acupuncture treatment. *D* values of pre-stim and post-stim were used to represent data

Table 3 Mean values of CO, SV, PR, BPs and BPd throughout the treatment (mean ± SD)

	Pre-stim	During-stim	Post-stim
CO (l/min)			
INT	6.81 ± 0.80	6.45 ± 0.65	6.51 ± 0.65
CNT	5.82 ± 1.04	5.43 ± 0.85	5.65 ± 0.88
SV (ml/b)			
INT	98.85 ± 13.03	97.45 ± 12.89	97.30 ± 13.32
CNT	92.08 ± 9.71	91.79 ± 9.69	91.08 ± 8.23
PR (bpm)			
INT	70.10 ± 13.95	68.20 ± 11.65	69.00 ± 12.10
CNT	63.29 ± 7.70	59.25 ± 6.29	62.04 ± 7.09
BPs (mmHg)			
INT	112.55 ± 14.46	111.2 ± 13.16	112.95 ± 12.81
CNT	107.70 ± 7.40	107.46 ± 7.74	108.50 ± 7.31
BPd (mmHg)			
INT	62.75 ± 6.53	62.75 ± 5.97	63.60 ± 5.56
CNT	59.79 ± 4.75	58.46 ± 4.51	59.96 ± 4.16

Pre-stim: at the end of pre-stimulation period of 15 min rest (for 2 min)

During-stim: the whole stimulation period (for 3 min)

Post-stim: 5 min after the stimulation period (for 2 min)

decrease during treatment was significantly higher than that after the treatment in CNT group than that in INT group.

The BPs and BPd slightly changed during treatment and then increased. But no statistically significant differences in BPs and BPd during and after treatment between the two groups were observed (Figs. 6, 7, Table 3).

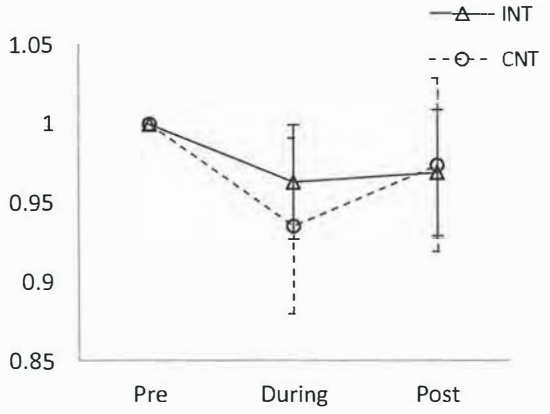

Fig. 3 The change in CO before, during and after the two kinds of manipulation. Let the baseline (before) equal 1, during/baseline, and after/baseline were used to represent data

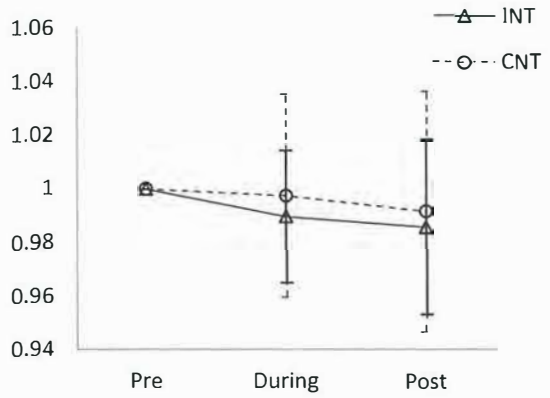

Fig. 4 The change in SV before, during and after the two kinds of manipulation. Let the baseline (before) equal 1, during/baseline, and after/baseline were used to represent data

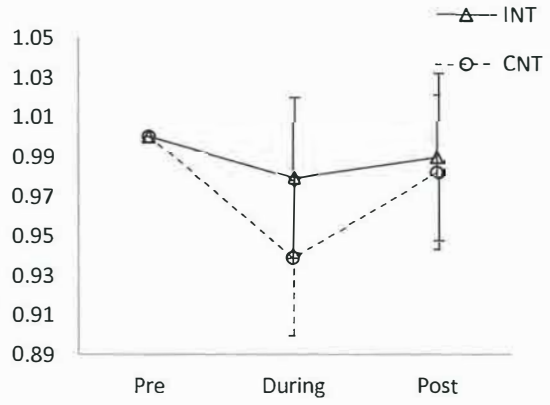

Fig. 5 The change in PR before, during and after the two kinds of manipulation. Let the baseline (before)equal 1, during/baseline, and after/baseline were used to represent data

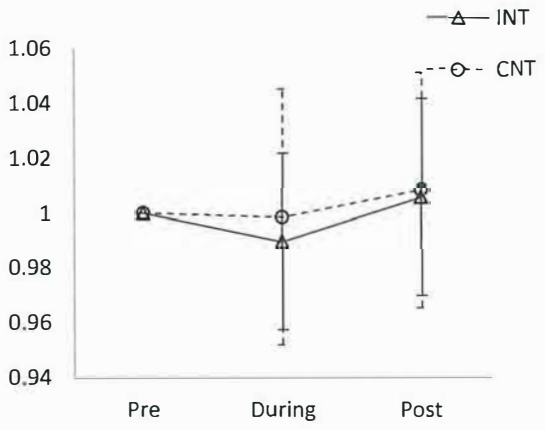

Fig. 6 The change in BPs before, during and after the two kinds of manipulation. Let the baline (before) equal 1, during/baseline, and after/baseline were used to represent data

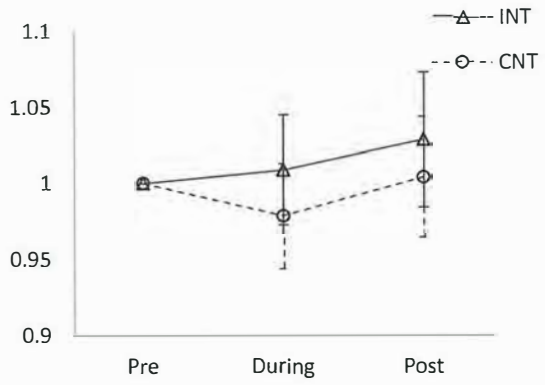

Fig. 7 The change in BPd before, during and after the two kinds of manipulations. Let the baseline (before) equal 1, during/baseline, and after/baseline were used to represent data

4 Discussion

In this study, significant improvement in pain was found in both the groups after the acupuncture treatment. The acupuncture analgesic effect on the shoulder pain subjects has been confirmed by many previous researches [15, 16]. Although the mechanism of acupuncture analgesic has not been schematized, it is clear that it helps with the stimulation of nervous system, immune system, the release of neurotransmitters and hormones. The acupuncture analgesic effect is an explanation for "acupuncture is a small stimulation that can induce a large response" [17]. As for the theory of traditional Japanese medicine, Qi and blood are two vital materials of life, because Qi flows through the body day and night, and when the Qi is blocked, diseases appear. Acupuncture is a treatment method that be used to regulate the flow of Qi, and Qi will circulate the blood, which is known as "Qi promoting blood circulation".

In this research, we found that VAS score of CNT group significantly decreased after the first time treatment, but in INT group it significantly decreased after the third time, so it seems that the analgesic effect of CNT is faster than the INT. In our opinion, when patients who never or rarely have received acupuncture treatment receive INT for the first time, they may feel nervous, on the contrary, may feel relaxed about CNT. It is emphasized in The Yellow Emperor's Inner Classic (Huangdineijing) "When treated with acupuncture, the acupuncturists should take care of the patients' spirit first".

PR significantly decreased in both groups. Similar results were reported by many previous researches (involving both healthy subjects and patients), and this has shown that acupuncture may be able to change the balance of autonomic nervous system, as well as reduce PR [18–22]. At the same time, there are also various views concerning the mechanism of the PR reduction by acupuncture treatment. Some researches indicated that the accentuation of parasympathetic activity played a role in acupuncture treatment [18], while other researches indicated that both accentuation of parasympathetic activity and suppression of sympathetic activity played a role in acupuncture treatment [19, 20]. According to the report of Kazushi Nishijo et al. [19], the decreased response of heart rate following acupuncture was blocked by the administration of atropine and propranolol, indicating that the increase in cardiac vagal activity and decrease in cardiac sympathetic activity can be contributed to the response to decrease in heart rate during acupuncture [18]. So it is already known that acupuncture treatment may induce transitory induction in pulse/heart rate. And, a change in sympathetic nerve and parasympathetic nerve (cardiac vagal) can also be aroused by the acupuncture treatment.

However, the above findings are based on observations in INT subjects. This study also investigated a larger reduction in PR in the CNT group during the treatment. There are a few studies that show the mechanism of CNT. We have known that the heart rate variability (HRV) shows the autonomic balance, and the sympathetic nervous activities are reflected in the low frequency (LF), while the parasympathetic nervous activities are reflected in the low frequency (LF) components of HRV. In the report of Kouki Kurita et al. [23], they calculated the HRV before and after the CNT, and they found the LF/HF values decreased in all participants, indicating that the autonomic balance changed and participants relaxed during the CNT. And in our previous research, we found that CNT can improve peripheral blood flow [8], and it is an effective method to treat CIPN. Although only a few studies have confirmed that CNT does have a definite effect [7, 8], we think that knowledge about the mechanism of CNT is insufficient, and to clarify the

mechanism of the PR reduction by CNT, in future studies, we should focus on the change in the nervous system with CNT using participants.

In this research, CO, SV and BP slightly changed in both groups, but did not show any significant differences between the two groups, which shows that the influence of the two manipulations on subjects without cardiovascular diseases is extremely slight, therefore the two kinds of manipulations are similarly safe. And we also found that the analgesic effect of CNT is faster than INT. The CNT, using the acupuncture needles that need not be inserted into the skin, will make patients feel relaxed and find it acceptable and it can also reduce the risk of infection, so CNT might be considered to be one of the effective treatment methods and may be safer and well tolerated than INT.

We think that there are also three main limitations in this study: no randomized methods, small sample size and no blank control group. For the sake of patients' health, we did not use any random methods, and the division of participants was according to the Kampo diagnosis, so it must have clear limitations. And we recruited 11 subjects into two groups in this study, so the sample size in each group was small, and the results might have been different if we had recruited more subjects. Furthermore, we did not use a blank control group, so we do not know if these changes will also be seen when we do nothing in participants. However, the results of this study are similar to the results of the previous studies. In addition, this study shows that the CNT is an effective and safe therapeutic method for the treatment of shoulder pain, and may be useful for future studies in patients with cardiovascular diseases.

5 Conclusion

In conclusion, the main findings from this study were that INT and CNT both can relieve the pain of shoulder pain patients, and the analgesic effect of CNT maybe faster than INT; the two methods can induce transitory reduction in heart/pulse rate, and the change in CNT during the treatment is significantly higher than that of INT. CNT is one of the effective treatment methods and may be safer than INT.

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