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日中笹川医学奨学金制度
第４０期（学位取得コース）

報　　告　　書

2018 年 4 月～2020 年 3 月

公益財団法人　日中医学協会

目　次

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

Supported by 日本財団 THE NIPPON FOUNDATION

第 40 期　研究者番号：G4001　作成日　：2020 年 3 月 5 日

氏　名	鄭 衛青	ZHENG　WEIQING	性別	M	生年月日　1980. 12. 06
所属機関（役職）	南昌市疾病預防控制中心　消毒与病媒生物防制科（検験技師　）				
研究先（指導教官）	帯広畜産大学　原虫病研究センター（　玄 学南　教授(センター長)）				
研究テーマ	中国におけるマダニ媒介感染症の疫学調査と有効な駆除法の開発				
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	当該研究者はこの 2 年間、中国（江西省、黒竜江省）におけるマダニとマダニ媒介感染症に関する疫学調査とマダニの病原体媒介機構に関する研究を行い、下記のような成果が得られた。 1)　江西省に分布しているマダニを採集し、種の同定を行ったところ、計 15 種類のマダニ種を特定した。 2)　江西省に分布しているマダニおよび動物からバベシア属、ボレリア属、リケッチア属、アナプラズマ属などマダニ媒介病原体を検出し、これらの病原体が当該地域において、人や動物に健康被害を与える可能性を示唆した。 3)　黒竜江省から採集したマダニを実験動物に吸血させた後に、病原体の検査を行ったところ、3 種類のバベシア属原虫の分離に成功した。 4)　上記で特定した主要種であるフタトゲチマダニにおいて、ポーリン分子、VDAC 分子などがバベシア属原虫の媒介に関与していることを突き止めた。	
総合評価	【良かった点】 当該研究者の所属研究機関の所在地を含む中国の二つの地域におけるマダニとマダニ媒介感染症の流行実態解明の成果は、今後これらの地域におけるマダニ媒介感染症対策を講ずる上で貴重な基礎データになると期待される。また、マダニの病原体媒介機構解明の成果は、将来マダニ媒介感染症制御法の確立につながるものと期待される。 【改善すべき点】 今後、マダニとマダニ媒介感染症に関して、もっと広い視野で情報収集を行い、グローバル視点でマダニとマダニ媒介感染症制御戦略を構築できる研究者に成長してほしい。 【今後の展望】 マダニとマダニ媒介感染症の研究分野において、新進気鋭の若手研究者として、日本と中国の関連研究者の間の相互交流の架け橋になって活躍できると思われる。	
学位取得見込	当初予定より少々遅れているが、2020 年度中の取得は十分期待できる。	
評価者（指導教官名）	玄 学南	

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：G4001　　作成日：2020年3月2日

氏名	ZHENG　WEIQING	鄭 衛青	性別	M	生年月日　1980.12.06
所属機関(役職)	南昌市疾病予防管理センター　消毒与病媒生物防制科　（リサーチアシスタント）				
研究先（指導教官）	帯広畜産大学　原虫病研究センター　（玄 学南　教授(センター長)）				
研究テーマ	中国におけるマダニとマダニ媒介感染症の疫学調査と有効な駆除法の開発 Epidemiological study on ticks and tick-borne diseases in China and establishment of their control measures				
専攻種別	論文博士	☑	課程博士	□	

1．研究概要（1）

1）目的（Goal）

Tick and tick-borne pathogen investigation in Jiangxi and isolation of tick-borne *Babesia* in *Ixodes persulcatus* from Heilongjiang are still less performed. Therefore, this study focused on the survey of ticks and tick-borne pathogens including *Babesia* , in two regions. The dominant tick in Jiangxi was used for the important pathogen infection and differentially expressed genes were screened using suppression subtractive hybridization technology (SSH). Finally, the expression profiles of important tick genes were analyzed and furthermore functions of these genes were assessed with RNA interference.

2）戦略（Approach）

Ticks were collected from breeding sites and host animals, and then subjected to morphological and molecular identification. DNA extracted from ticks and SCID mice challenged with field-collected *Ixodes persulcatus* were detected for pathogen presence with conventional PCR and sequencing. In addition, blood smear method was also used to detect SCID mice for *Babesia* infection. The differentially expressed genes in the dominant tick infected with the important pathogen were screened with SSH technology. The expression profiles and functions of screened genes were analyzed with real-time PCR and RNAi, respectively.

3）材料と方法（Materials and methods）

Tick population characterization

Sample collection

We planned 12 investigation sites in Jiangxi province, southeastern China and three investigation sites in Heilongjiang province, northeastern China. For each investigation site in Jiangxi province, tick collection was performed by flagging vegetation and picking up ticks from host animals. For the investigation sites in Heilongjiang, the ticks were collected by flagging vegetation.

Tick identification

Ticks were identified to species level according to taxonomic keys compiled by Sun Yi and Xu Rongman and molecular verification with DNA marker COI.

Ticks in habitats

Tick distribution patterns in habitats were expressed as numbers of ticks at different developmental stages.

Ticks on host animals

Infestation rate was calculated as infested hosts divided by total ticks for specific tick species, and difference in infestation rate was evaluated by Chi square. $P<0.05$ indicates that difference in infestation rate of host animals is significant. Ticks/hosts was denoted as tick density. I also calculated tick composition as specific ticks in total ticks.

Tick-borne pathogens in ticks

Tick population

Ticks were identified to species level and developmental stages.

Tick-borne pathogens in ticks from Jiangxi

Tick-borne pathogens were detected in ticks from Jiangxi by PCR of genes of tick-borne pathogens. Amplicons were sent to Shanghai Sangon Biotechnology for sequencing, and pathogen verification was conducted by alignment with candidate sequence deposited in GenBank.

Tick-borne pathogens in ticks from Heilongjiang

Every mouse was loaded with 10 field-collected *I. persulcatus* ticks from Heilongjiang province and the presence of *Babesia* were determined by blood smear stained with Giemsa and PCR followed by sequencing each day 5 days post tick attachment. Meanwhile, the engorged ticks were used to detect *Babesia* presence with conventional PCR and sequencing.

1．研究概要（2）

Porin expression profiles in *Haemaphysalis longicornis*

The expression levels of *porin* in nymphs with *B. microti* infection or non-infection, and non-feeding, partial feeding or engorgement were determined with real-time PCR. The functions of tick *porin* in blood feeding, *Babesia* infection, and relation with mitochondria-related apoptosis were also assessed with RNAi technology, conventional and real-time PCR methods.

Identification of differentially expressed genes in *H. longicornis* infected with *B. microti*

Differentially expressed genes in *H. longicornis* in response to infection with *B. microti* Gray strain were identified by using SSH procedure, and the results of the SSH studies were verified by real-time PCR. Functional analyses were conducted on selected genes by RNA interference in *H. longicornis* ticks.

4）実験結果（Results）

Tick populations in Jiangxi

Fifteen tick species were found, including *H . longicornis* , *Rhipicephalus sanguineus sensu lato* , *Haemaphysalis yeni* , *Haemaphysalis kitaokai, Ixodes sinensis, Dermacentor auratus, Haemaphysalis campanulata, Haemaphysalis flava, Haemaphysalis doenitzi, Haemaphysalis hystricis, Rhipicephalus haemaphysaloides, Rhipicephalus microplus, Ixodes granulatus, Amblyomma testudinarium* and *Ixodes acuminatus. H. longicornis* was the most frequently collected species and widely distributed tick species of the total collection ticks (in 11 sampling sites), and had a broad host range.

Tick-borne pathogens in Jiangxi

Tick-borne pathogens in ticks included *Babesia* spp. in *H. flava* and *R. microplus* , *Borrelia yangtzensis* in *I. granulatus, Rickettsia raoultii* or *Rickettsia slovaca* related genospecies in *H. longicornis* and *H. flava, Hepatozoon canis* or *Hepatozoon felis* related genospecies in *H. longicornis.*

Tick-borne babesias in Heilongjiang

Three *Babesia* species including *Babesia bigemina* , *Babesia divergens* and *Babesia venatorum* were detected in *I. persulcatus* ticks and SCID mice that ticks were fed on, and one mouse was coinfected with two *Babesia* species, namely *B. bigemina* and *B. venatorum* . The total infection rate of *Babesia* parasites for SCID mice with tick infestation was 14.55%.

Porin* expression profiles in *H. longicornis* infected with *B. microti

In the nymphal stage, there was no difference in *porin* expression levels between unfed and partially fed ticks, mRNA expression maintained similar levels at 0-2 days after engorgement (dAE), and then significantly increased at 3dAE. *Cytc* is an apoptogenic factor related to *porin* -interfered apoptosis and its mRNA levels were upregulated in nymphs at 3dAE in contrast to nymphs at 2dAE. Non-significant differences in *porin* mRNA transcripts were seen between infected and uninfected nymphs, and the gene was not significantly differentially expressed in nymphs during *B. microti* infection. Especially, the highest *B. microti* burden negatively affected *porin* mRNA levels in both nymphs and female adults. *Porin* knockdown affected body weight and *Babesia* infection levels, and significantly downregulated the expression level of *Cytc* in *H. longicornis* female ticks.

***H. longicornis* genes that elicit responses associated with *B. microti* infection**

The differentially expressed genes in infected ticks were enriched with SSH technique and cloned into *E. coli* .Three hundred and two, and one hundred twelve clones were randomly selected for sequencing and analyzed in forward and reverse subtracted SSH cDNA library related to *Babesia* infection, respectively. Gene ontology assignments, and sequence analyses of tick sequences in forward subtracted SSH cDNA library showed that 14 genes were annotated as response to stimulus or/and immune system process, and 10 genes had the larger number of standardized sequences per kilobase (SPK). Subsequent real-time PCR detection informed us of that eight genes including those encoding for *Obg-like ATPase 1* (*OLA1*), *Calreticulin* (*CRT*), *vitellogenin 1* (*Vg1*) and *Vg2* were over-expressed in fed ticks while mRNA levels of three genes were down-regulated in fed ticks. Compared to uninfected ticks, infected ticks had six up-regulated genes, including *OLA1* , *CRT* and *Vg2* , and three less represented genes. Functional analysis of over-expressed genes in *Babesia* -infected fed ticks by RNA interference showed that knockdown of *CRT* and *Vg1* significantly reduced engorged female weight, while knockdown of *OLA1* and *Vg2* significantly shortened blood feeding period in *B. microti* -infected ticks. *Vg2* knockdown reduced pathogen infection level by 51% when compared with controls.

1．研究概要（3）

5）考察（Discussion）

Zoogeographically, China is divided into Palaearctic and Oriental Realm, and has abundant tick species which form approximately 1/8 of tick species worldwide [1]. *H. longicornis* is a predominant tick species in many regions of China, including Niaoning, Henan, Hubei, Shandong and Jiangxi [2-6], and can vector variety of tick-borne pathogens like *Rickettsia* , *Babesia* , *Borrelia* and some human pathogenic viruses [7,8]. *Babesia* species are a group of tick-borne protozoa prevalent in China, found in southwestern, central and northeastern China, including Jiangxi province [7,9]. Here, we detected *Babesia vogeli* and other unidentified *Babesia* spp. in ticks from Jiangxi [10], and isolated *B. bigemina* , *B. divergens* and *B. venatorum* in *I. persulcatus* from Helongjiang. *B. microti* and its related genospecies are another *Babesia* species frequently determined in China, ranging from north to south and from west to east [7,11]. In the present research, I exploited interface of *H. longicornis* and *B. microti* Gray strain, and screened important tick proteins having roles in affecting tick-borne pathogen infection in ticks. Various studies on pathogen-tick interaction showed that ticks can activate immune response to combat against infection when they are infected by pathogen. The tick innate immune related molecules comprise defensins, immunophlin and tick antimicrobial peptides (AMPs), such as longicin, microplusin, proteins related with lipid recognition, proteases and protease inhibitors, longipain, and leucine-rich repeat domain-containing protein (LRR) [12]. Meanwhile, pathogen could modulate tick molecules for infection, The spirochete also enhances expression of a gene encoding for a 15 kDa feeding-induced salivary gland (salp15), which interacts with *Borrelia burgdorferi* by binding to OspC [13]. On the other hand, pathogen might induce cytoskeletal rearrangement to maintain infection, for example, by altering the ratio between monomeric globular G actin and filamentous F actin of *Ixodes scapularis* to facilitate *Anaplasma phagocytophilum* infection, or by up-regulation of spectrin alpha chain or Alpha-fodrin of *I. scapularis* in response to *A. phagocytaphilum* infection [14]. Additionally, *A. phagacytaphilum* may benefit from the tick cells ability to limit pathogen infection through phosphoenolpyruvate carboxykinase (PEPCK) and voltage-dependent anion channel (VDAC) inhibition that leads to the inhibition of cell apoptosis for increasing infection in tick cells [14-17]. My data showed that VDAC was less expressed in *B. microti* -infected engorged female *H. langicarnis* ticks. The results based on *H. longicarnis* -*B. micrati* infection model suggested that OLA1, CRT and vitellogenin family protein such as Vg1, Vg2 may have important roles in blood feeding and *Babesia* infection in *H. longicornis* female ticks.

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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	First molecular evidence of *Anaplasma phagocytophilum* in rodent populations of Nanchang, China					
掲載誌名 Published journal	Japanese Journal of Infectious Diseases					
	2018 年 2 月	71 巻（号）	129 頁 ～ 133 頁		言語 Language	English
第 1 著者名 First author	Weiqing Zheng	第 2 著者名 Second author	Yangqing Liu	第 3 著者名 Third author	Huiying Tao	
その他著者名 Other authors	Zifen Li, Xuenan Xuan, Xiaoqing Liu, Paul Franck Adjou Moumouni, Yayun Wu, Wenqing Liu, Haiying Chen					
論文名 2 Title	Tick-borne pathogens in ixodid ticks from Poyang Lake Region, Southeastern China					
掲載誌名 Published journal	Korean Journal of Parasitology					
	2018 年 12 月	56（6） 巻（号）	589 頁 ～ 596 頁		言語 Language	English
第 1 著者名 First author	Weiqing Zheng	第 2 著者名 Second author	Xuenan Xuan	第 3 著者名 Third author	Renlong Fu	
その他著者名 Other authors	Huiying Tao, Yangqing Liu,Xiaoqing Liu, Dongmei Li, Hongmei Ma, Haiying Chen					
論文名 3 Title	Preliminary investigation of ixodid ticks in Jiangxi Province of Eastern China					
掲載誌名 Published journal	Experimental and Applied Acarology					
	2019 年 1 月	77(1) 巻（号）	93 頁 ～ 104 頁		言語 Language	English
第 1 著者名 First author	Weiqing Zheng	第 2 著者名 Second author	Xuenan Xuan	第 3 著者名 Third author	Renlong Fu	
その他著者名 Other authors	Huiying Tao, Rongman Xu, Yangqing Liu,Xiaoqing Liu, Jiafu Jiang, Haixia Wu, Hongmei Ma, Yi Sun, Haiying Chen					
論文名 4 Title	*Porin* expression profiles in *Haemaphysalis longicornis* infected with *Babesia microti*					
掲載誌名 Published journal	Frontiers in Physiology					
	年 月	巻（号）	頁 ～ 頁		言語 Language	English
第 1 著者名 First author	Weiqing Zheng	第 2 著者名 Second author	Rika Umemiya-Shirafuji	第 3 著者名 Third author	Qian Zhang	
その他著者名 Other authors	Kiyoshi Okado, Paul Franck Adjou Moumouni, Hiroshi Suzuki, Haiying Chen, Mingming Liu, Xuenan Xue					
論文名 5 Title	*Haemaphysalis longicornis* genes that elicit responses associated with *Babesia microti* infection					
掲載誌名 Published journal	Parasitology Research					
	年 月	巻（号）	頁 ～ 頁		言語 Language	English
第 1 著者名 First author	Weiqing Zheng	第 2 著者名 Second author	Rika Umemiya-Shirafuji	第 3 著者名 Third author	Shengen Chen	
その他著者名 Other authors	Kiyoshi Okado, Paul Franck Adjou Moumouni, Hiroshi Suzuki, Haiying Chen, Mingming Liu, Xuenan Xue					

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 meetings.

学会名 Conference	National Research Center for Protozoan Diseases seminar
演　題 Topic	The role of several tick genes in acquisition of *Babesia microti* in *Haemaphysalis longicornis* ticks
開催日 date	2018 年 7 月 26 日　開催地 venue Obihiro, Hokkaido
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	National Research Center for Protozoan Diseases seminar
演　題 Topic	Characterization of *porin* gene in *Haemaphysalis longicornis* and its role played in tick biology and *Babesia* infection
開催日 date	2019 年 7 月 23 日　開催地 venue Obihiro, Hokkaido
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	China-Japan joint seminar
演　題 Topic	Transcriptional responses in *Haemaphysalis longicornis* ticks infected with *Babesia microti* Gray strain
開催日 date	2019 年 9 月 24 日　開催地 venue Obihiro, Hokkaido
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	National Research Center for Protozoan Diseases seminar
演　題 Topic	*Haemaphysalis longicornis* genes that elicit responses associated with *Babesia microti* infection
開催日 date	2020 年 2 月 20 日　開催地 venue Obihiro, Hokkaido
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 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	Jiangxi Provincial Department of Science and Techology
助成金名称 Grant name	Key research project of Jiangxi province (20192BBH180013)
受給期間 Supported period	2019 年 6 月 ～ 2020 年 12 月
受給額 Amount received	470,000 円
受給実績 Receipt record	■ 有　□ 無
助成機関名称 Funding agency	Jiangxi Provincial Department of Science and Techology
助成金名称 Grant name	Key research project of Jiangxi province (20161BBG70005)
受給期間 Supported period	2016 年 6 月 ～ 2019 年 6 月
受給額 Amount received	830,000 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	
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 country	
出願内容（概要） Application contents			

9．その他 Others

指導責任者（署名）

Jpn. J. Infect. Dis., 71, 129–133, 2018

Original Article

First Molecular Evidence of *Anaplasma phagocytophilum* in Rodent Populations of Nanchang, China

Weiqing Zheng[1,4], Yangqing Liu[1], Huiying Tao[1], Zifen Li[1], Xuenan Xuan[4], Xiaoqing Liu[1], Paul Franck Adjou Moumouni[4], Yayun Wu[2], Wenqing Liu[3], and Haiying Chen[1]*

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SUMMARY: In this study, systematic surveillance of rodent populations in Nanchang of China and determination of *Anaplasma phagocytophilum* infection in rodents were performed. Between 2011 and 2015, 110,084 rodent snap traps were set in 4 counties and in the city center of Nanchang, China. Finally, 942 rodents were captured, with a relative density of 0.86%. The densities varied considerably by geographical area with Anyi being the most rodent-infested County. Frequently captured rodents were sewer rats (*Rattus norvegicus*), house mice (*Mus musculus*), and *Rattus flavipectus*. The *Anaplasma* genera were investigated by PCR in 19 live rodents trapped by welded cages in Anyi, 6 rodents were assessed as positive based on amplification of 16S rRNA. Sequence analysis revealed 3 variants of *A. phagocytophilum* in Nanchang. PCR analysis of the *gltA* (citrate synthase) gene found 1 sample that was positive for *A. phagocytophilum* infection. The sequence of *A. phagocytophilum gltA* gene formed a clade with and showed 99% identity to *A. phagocytophilum* that has been previously described in rodents from South-Eastern China. Taken together, our research indicated that commensal rodents are potential hosts for *A. phagocytophilum* and controlling the rodent population may facilitate subsequent prevention of human granulocytic anaplasmosis in Nanchang, China, in the future.

INTRODUCTION

Rodents are important destructive animals globally that compete with humans for food, particularly through pre-harvest damage to rice crops. Some rodents occur commensally in human residential areas, easily contaminating the human environment by the production of metabolic excrement, faeces, and urine. Important pathogens like hemorrhagic fever with renal syndrome virus can be transmitted directly to humans by contact with objects and inhalation of air contaminated by rodents. Other pathogens, like *Borrelia* spirochetes, can indirectly infect humans through the bites caused by ectoparasites, such as lice, fleas, and ticks on the rodents. The research mentioned above was reviewed and documented in previous works (1,2).

Human granulocytic anaplasmosis (HGA) is considered a rodent-borne disease caused by *Anaplasma phagocytophilum*, which is an emerging obligate intercellular bacterial pathogen (3). HGA is increasingly recognized as an important and frequent cause of fever worldwide (4). Anaplasmosis was first recognized as a disease of humans in the United States in the mid-1990s. Cases of anaplasmosis have generally increased from 350 cases in 2000 to 1,163 cases in 2009. The number of reported cases increased by 52% between 2009 and 2010 (5). By the end of 2015, more than 100 HGA cases had been reported in Mainland China, covering the north and south of the Yangtze River. The cases were mainly distributed in Shandong and Beijing (6). In 2010, an HGA case was reported in a healthy young man from Nanchang of Jiangxi Province, China. The patient noticed an unknown arthropod bite one day before the initiation of fever, and died 10 days later (unpublished data). Clinical manifestations in humans range from mild self-limiting febrile illness to fatal infections. The most frequent manifestations in patients are non-specific influenza-like symptoms with fever, headache, myalgias, and malaise. Few patients have arthralgia or involvement of the gastrointestinal tract, respiratory tract, liver, or the central nervous system (4).

A. phagocytophilum appears to be a generalist, infecting a wide range of animals, such as humans, domestic animals, and wildlife, including rodents, throughout the world (7–10). Previous studies showed that various field and domestic animals are considered as important reservoirs of *A. phagocytophilum*. Roe deer play a key role in the endemic cycles of the pathogen in Germany (11). Dogs from both urban and rural habitats in Brazil were found to be positive for *A. phagocytophilum* (12). In addition, goats and sheep were highly infected with *A. phagocytophilum* in China (9). In Nanchang, however, a previous examination could not detect the presence of *A. phagocytophilum*. In the survey, 328 dogs and goats were found to be negative for *A. phagocytophilum* infection (unpublished data), suggesting that domes-

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tic animals might have a limited role as hosts for *A. phagocytophilum* in Nanchang.

Rodents are common in both urban and rural human residential areas of Nanchang city (13). Furthermore, they can cause certain rodent-borne diseases, such as hemorrhagic fever with renal syndrome and leptospirosis (12,13). However, little information on the systematic statistics of the rodent population and the presence of *A. phagocytophilum* in commensal rodents is available. The objective of this study was to survey a wide range of commensal rodent populations monthly, covering all regions of Nanchang, between the period of 2011 and 2015. The highest rodent-infested county was then selected to monitor whether *A. phagocytophilum* infection could be found in rodents.

MATERIALS AND METHODS

Commensal rodent surveillance: Rodent population densities were determined using snap traps at 58 sites in Nanchang city from 2011 to 2015. The sites were distributed in 4 counties and the city center, and were randomly selected with regard to land use and geographic location (Fig. 1A). Rodent snap traps, with fried bread sticks as bait, were set in the evening and collected in the next morning. A total of 61,554, 12,088, 12,209, 12,352, and 11,881 traps were placed in the city center, Xinjian County, Nanchang County, Anyi County, and Jinxian County, respectively. Traps were spaced at approximately 5-meter intervals in the vertical direction and at 25-meter intervals in the lateral direction. All rodents captured were measured, weighed, examined for sex and developmental condition, and, finally, morphologically identified. The rodent population density was calculated according to the following equation:

$$R(\%) = \frac{N_r}{N_e} \times 100$$

Where: R = rodent population density:
N_r = number of traps with captured rodents:
N_e = number of effective traps collected; effective traps indicating non-exciting traps and the traps with rodents.

Occurrence of *A. phagocytophilum* in commensal rodents: Live rodents were trapped using welded cages in Anyi, the county with the highest rodent density in Nanchang city. Rodent capture sites were localized in 3 residential areas near 2 brick kiln industries, and a pig breeding base without optimal hygienic conditions (Fig. 1B). Rodents were captured from late March to early April of 2016. Traps were spaced 10 m apart along lines, with bait and were checked in the morning at 24 h and 48 h after setup. Captured rodents were characterized and morphologically identified before blood collection. Blood samples were collected through incision of the femoral artery and were then transferred to EDTA-treated tubes. All samples were stored at –80°C until analyses.

Genomic DNA was extracted from a volume of 200 µl of whole blood using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. One hundred micro-liter eluted DNA samples (30–90 ng/µl) were harvested and stored at –30°C for subsequent PCR analysis. DNA concentrations and purities were determined with a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The samples with A_{260}/A_{280} of 1.8–2.0 ratio and a concentration greater than 20 ng/µl were used in the analysis. The presence of *Anaplasma* genus DNA was examined by a semi-nested PCR assay targeting 16S rRNA as previously described (14). DNA of *A. phagocytophilum* was detected by a nested PCR based on the *gltA* (citrate synthase) gene, according to a previous description (15).

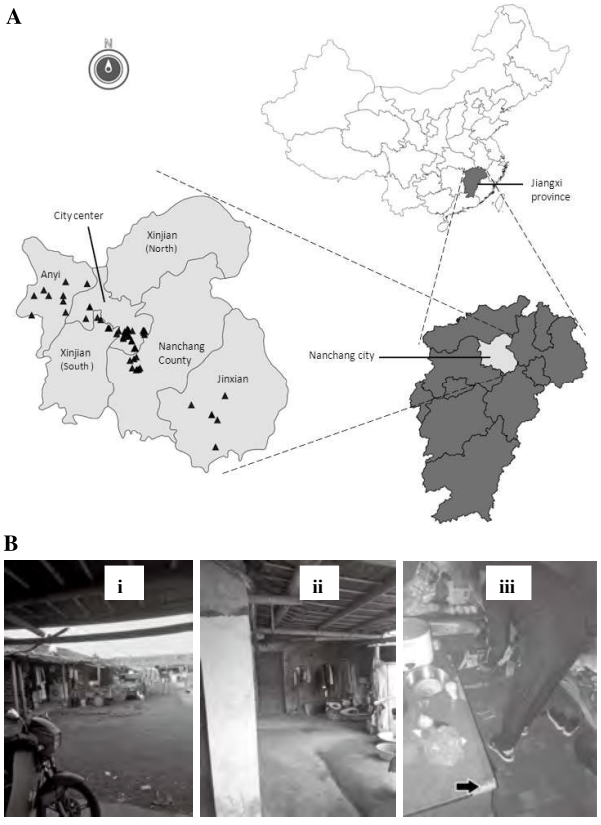

Fig. 1. (A) Investigation sites. Filled triangles on the map show 58 rodent surveillance sites. (B) Rodent capture site. (i) Full view of a residential area; (ii) Outside view of a house; (iii) Indoor view of a room, household items were in chaotic arrangement. Rodent faeces and bites in food and household items, such as tables and shoes, were observed here and there. A black arrow with white margin shows a rodent gnaw mark on a table.

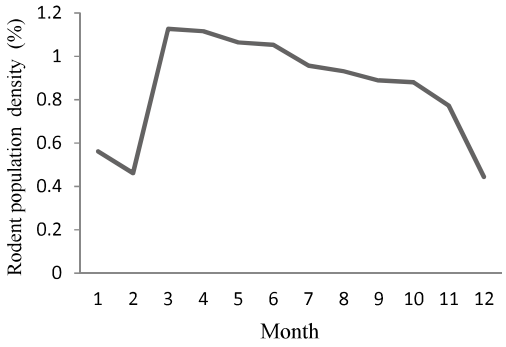

Fig. 2. The fluctuation of rodent population density in Nanchang.

Samples positive for *Anaplasma* genus (*phagocytophilum*) infection were randomly selected for cloning and sequencing of amplicons. For each specimen, at least 5 clones confirmed positive by colony PCR were multiplied and purified using a NucleoSpin Plasmid Quick Pure (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed using ABI BigDye Terminator Kit v3.1 (Applied Biosystems, Foster City, CA, USA). DNA fragment sequences were determined using an ABI PRISM 3100 DNA sequencer (Applied Biosystems). Identities and similarities of the nucleotide sequences were analyzed using BLASTN online at GenBank. The SeqMan and MEGA 6 programs were used to build the alignment of all selected sequences, and then a neighbor-joining phylogenetic tree was constructed using the MEGA 6 program. The confidence of internal branches was estimated by bootstrapping with 1,000 replications.

Statistical analysis: The results were analyzed by using the χ^2 test in IBM SPSS Statistics ver. 20 (Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Rodent population density in Nanchang between 2011 and 2015: Over the five-year trapping period, 944 (0.86%) rodents were caught at 58 sites in Nanchang. The overall male-to-female sex ratio was 1:1.02 (467:477), and the rodent population density did not differ significantly between sexes ($\chi^2 = 0.105$, df = 1, $P > 0.05$). However, the rodent population density was significantly higher between 2011 and 2013 (0.92% to 0.99%) than from 2014 to 2015 (0.68% to 0.73%) ($\chi^2 = 22.14$, df = 4, $P < 0.001$; Table 1). The rodent population density showed a peak in March–April, followed by a gradual decline from May to a low level in October. This was followed by a substantial decrease in density from October to the lowest level in December (Fig. 2).

Rodents captured included sewer rats (*Rattus norvegicus*), house mice (*Mus musculus*), and *Rattus flavipectus*. The most frequently captured rodents were sewer rats, accounting for 34.75% (328 individuals); house mice consisted of 33.47% (316 individuals). 28.07% (265 rodents) of captures were *R. flavipectus*. Some rarely seen small wild mammals were also caught around residences during the trapping period, for example, rodents (*Apodemus agrarius* and *Rattus losea*) and the insectivore *Suncus murinus*. Rodent populations were at the highest density (3.65%) in Anyi County of Nanchang city. Anyi County had three-fold higher rodent population density than Nanchang County, which had the second highest (0.99%) rodent population density at the county level across Nanchang city. Sewer rats in Anyi County had undergone large population increases between 2012 and 2015 and consequently became the

Table 1. Average annual rodent density and composition at the county level in Nanchang city, China, between 2011 and 2015

Location	Species	2011	2012	2013	2014	2015	Whole period
City center	Sewer rats	0.21 (26)[1)]	0.36 (44)	0.36 (45)	0.17 (21)	0.24 (30)	0.27 (166)
	Rattus flavipectus	0.12 (15)	0.04 (5)	0.07 (9)	0.05 (6)	0.12 (15)	0.08 (50)
	House mice	0.08 (10)	0.03 (4)	0.04 (5)	0.03 (4)	0.05 (6)	0.05 (29)
	Other	0.04 (5)	0.02 (2)	0.02 (2)	0.00 (0)	0.00 (0)	0.01 (9)
	subtotal	0.46 (56)	0.45 (55)	0.49 (61)	0.26 (31)	0.41 (51)	0.41 (254)
Xinjian County	Sewer rats	0.16 (4)	0.28 (7)	0.12 (3)	0.13 (3)	0.20 (5)	0.18 (22)
	Rattus flavipectus	0.21 (5)	0.16 (4)	0.29 (7)	0.22 (5)	0.16 (4)	0.21 (25)
	House mice	0.45 (11)	0.52 (13)	0.41 (10)	0.04 (1)	0.16 (4)	0.32 (39)
	Other	0.29 (7)	0.00 (0)	0.00 (0)	0.09 (2)	0.04 (1)	0.08 (10)
	subtotal	1.11 (27)	0.97 (24)	0.81 (20)	0.49 (11)	0.57 (14)	0.79 (96)
Nanchang County	Sewer rats	0.16 (4)	0.28 (7)	0.04 (1)	0.00 (0)	0.13 (3)	0.12 (15)
	Rattus flavipectus	0.24 (6)	0.16 (4)	0.16 (4)	0.25 (6)	0.04 (1)	0.17 (21)
	House mice	0.73 (18)	1.19 (30)	0.83 (21)	0.25 (6)	0.39 (9)	0.69 (84)
	Other	0.00 (0)	0.00 (0)	0.00 (0)	0.04 (1)	0.00 (0)	0.01 (1)
	subtotal	1.13 (28)	1.63 (41)	1.03 (26)	0.54 (13)	0.57 (13)	0.99 (121)
Anyi County	Sewer rats	0.52 (13)	0.28 (7)	0.49 (12)	1.05 (26)	2.42 (60)	0.96 (118)
	Rattus flavipectus	1.17 (29)	2.03 (50)	1.91 (47)	0.97 (24)	0.40 (10)	1.30 (160)
	House mice	1.49 (37)	1.63 (40)	1.71 (42)	1.46 (36)	0.16 (4)	1.29 (159)
	Other	0.04 (1)	0.04 (1)	0.16 (4)	0.04 (1)	0.28 (7)	0.11 (14)
	subtotal	3.22 (80)	3.98 (98)	4.28 (105)	3.52 (87)	3.27 (81)	3.65 (451)
Jinxian County	Sewer rats	0.09 (2)	0.00 (0)	0.00 (0)	0.22 (5)	0.00 (0)	0.06 (7)
	Rattus flavipectus	0.17 (4)	0.00 (0)	0.16 (4)	0.00 (0)	0.04 (1)	0.08 (9)
	House mice	0.17 (4)	0.04 (1)	0.00 (0)	0.00 (0)	0.00 (0)	0.04 (5)
	Other	0.04 (1)	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	0.01 (1)
	subtotal	0.47 (11)	0.04 (1)	0.16 (4)	0.22 (5)	0.04 (1)	0.19 (22)
Nanchang city	Sewer rats	0.22 (49)	0.29 (65)	0.27 (61)	0.26 (55)	0.45 (98)	0.30 (328)
	Rattus flavipectus	0.27 (59)	0.28 (63)	0.32 (71)	0.19 (41)	0.14 (31)	0.24 (265)
	House mice	0.36 (80)	0.40 (88)	0.35 (78)	0.22 (47)	0.10 (23)	0.29 (316)
	Other	0.06 (14)	0.01 (3)	0.03 (6)	0.02 (4)	0.04 (8)	0.03 (35)
	Total	0.92 (202)	0.99 (219)	0.97 (216)	0.68 (147)	0.73 (160)	0.86 (944)

[1)]: Numbers before the round brackets represent rodent density (in %), and numbers in the parentheses are the total number of species-specific rodents in different years.

dominant rodent species in 2015 (Table 1).

Occurrence of *A. phagocytophilum* in rodents: A total of 19 live rodents were captured including 17 sewer rats, 1 *R. flavipectus*, and 1 home mouse in Anyi County. The distribution of rodents according to sex, stage, and infection status is shown in Table 2. The *Anaplasma* genus was detected in 6 (31.58%) rodents based on 16S rRNA PCR, and in 1 (5.26%) rodent by *gltA* amplification.

Four sewer rats and 1 *R. flavipectus* that were positive for *Anaplasma* genus infection by nested PCR of the partial 16S rRNA sequence were selected for sequence analyses. A 335-base pair nucleotide sequence was obtained from 5 individual clones of each sample. These sequences showed 94.53–99.40% identity with the published sequences of the HGA agent (KT454992).

Table 2. Presence of *A. phagocytophilum* in live rodent captures by using wire cages

No.	Species	Sex	DS	Result
1	SR	F	adult	
2	SR	F	adult	
3	SR	M	adult	
4	SR	M	adult	++ KY024480, KX757024[1)]
5	SR	M	adult	
6	SR	F	immature	
7	HM	M	adult	
8	SR	M	adult	
9	SR	F	immature	
10	SR	F	immature	
11	SR	F	adult	+ KX757023
12	SR	M	adult	+ KX757023
13	SR	M	adult	+
14	SR	F	adult	
15	SR	M	adult	+ KX757023
16	SR	M	adult	
17	SR	F	adult	
18	SR	M	adult	
19	Rf	M	adult	+ KY024479

DS, developmental stage; SR, sewer rat; HM, house mouse; Rf, *Rattus flavipectus*; F, female; M, male; +, the rodents found to be positive for *Anaplasma phagocytophilum* based on the amplification of 16S rRNA and DNA sequencing. ++, the rodent found to be positive based on both 16S rRNA and *gltA* PCR. Letter/number combinations are accession No. of target 16S rRNA and *gltA* sequence deposited in GenBank.

1): The former is accession No. of *gltA* sequence, and the latter is accession No. of 16S rRNA.

Sequence analysis of the 25 plasmid clones revealed 3 variants of *A. phagocytophilum*. One (KX757023) was found only in sewer rats and was highly identical to the sequence from the HGA agent, with only 2 nucleotide differences at positions of 773 and 899 and formed a clade with the sequence of the HGA agent in the phylogenetic tree (Fig. 3). In the phylogenetic tree, the other variants (KY024479 and KY024480) formed distinct branches. These variants differed from the HGA agent by 7 and 18 base-pairs, respectively. The sequences of the 3 variants obtained in the study were not 100% identical to the sequences from non-rodent hosts (HQ872465, KU321304, and KX115422), or even from rodent hosts (HM439430 and KC470064) in China. However, the fragments (HM439430 and KC470064) from rodents in China fully constructed a subclade (Fig. 3).

Five clones of the *A. phagocytophilum gltA* sequence were obtained from a sewer rat in our study. Four clones (numbered 1, 3, 4, and 5) had identical sequences to each other and differed from clone 2 by a single nucleotide mutation. Here, we used clone 1 to represent *A. phagocytophilum gltA* sequence (KX757024) extracted from the sewer rat. Our sequence was well separated from other *A. phagocytophilum* corresponding sequences from dog (KP861637) and sheep (KP861639) hosts, and tick (KP861638 and KP276595) vectors in the phylogenetic tree. However, the sequence (KX757024) shared a clade with *A. phagocytophilum* previously described in rodents from south-eastern China (DQ458809) and showed 99% identity to each other (Fig. 4).

DISCUSSION

Rodents are suspected to act as natural reservoirs for *A. phagocytophilum*. The prevalence of *A. phagocytophilum* was detected in *Apodemus sylvaticus*, *Apodemus flavicollis*, and *Myodes glareolus* in France (7). The first wild animals observed to be positive for *A. phagocytophilum* in east China were *Apodemus peninsulae*, *A. agrarius*, and *Eutamias sibiricus* (15). *A. agrarius* was subsequently found to be infected with *A. phagocytophilum* in west China (9). This evidence appeared to support the role of the genus *Apodemus* as reservoirs in the natural life cycle of *A. phagocytophilum*.

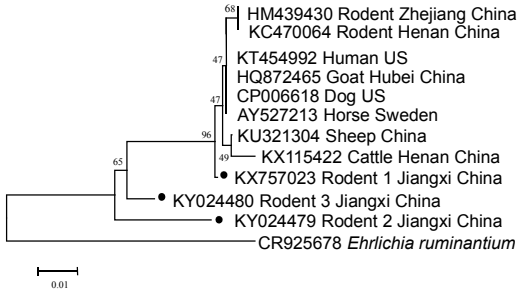

Fig. 3. Phylogenetic analysis based on *Anaplasma* genus 16S rRNA fragment. ●, sequences were obtained in this study. The *Ehrlichia ruminantium* homolog gene (CR925678) was used as the outgroup. The number of each branch was provided as bootstrap value at the node.

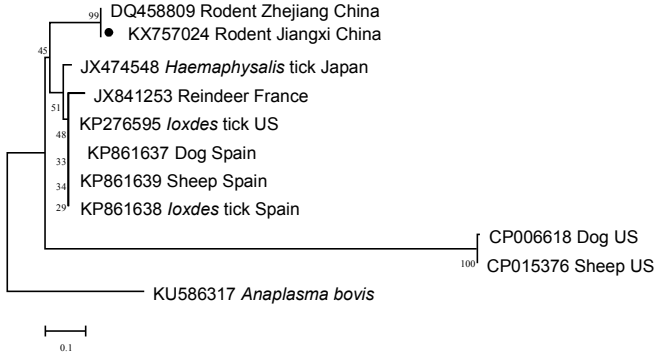

Fig. 4. Phylogenetic analysis based on *A. phagocytophlium gltA* fragment.●, sequence was obtained in this study. The *Anaplasma bovis* homolog gene (KU586317) was used as the outgroup. The number of each branch was provided as bootstrap value at the node.

The fragment of 16S rRNA from *A. phagocytophilum* was detected in blood samples from 6 rodents by semi-nested PCR and DNA sequence analysis. However, the *gltA* gene of *A. phagocytophilum* was determined in only 1 rodent. The difference in detection rates of the same samples was likely explained by the failure of our primers to bind to highly variable regions of *gltA* gene. A recent study in France (7) showed that 6.6–22.8% of rodents can carry *A. phagocytophilum* bacteria, similar to those (5.26% for *gltA* amplification and 31.57% for 16S rRNA PCR and DNA sequencing) described in our study.

Molecular typing based on single nucleotide polymorphisms (SNPs) in genes is the most frequently used method to analyze *A. phagocytophilum* genetic diversity. The most intensively used markers are the *groESL* operon, 16S rRNA locus, and the *ankA* and *msp2* genes. One previous study of species-specific 16S rRNA in the USA isolated the human pathogenic variant AP-ha (EP-ha, *Ehrlichia phagocytophila*-human agent) from other non-pathogenic variants (16). In Europe, the use of the species-specific 16S rRNA also clearly discriminated variants infecting red deer from those infecting roe deer (17,18). However, the conclusions were challenged by other studies in which this marker showed poor resolution and did not confirm any host species segregation for *A. phagocytophilum* variants (19). Finally, species-specific 16S rRNA data is unable to distinguish variants according to their geographical origins (20). In the present study, in Anyi County, we used PCR amplification of genus-specific 16S rRNA fragments extracted from individuals of different rodent species and DNA sequencing to classify *A. phagocytophilum* into 3 variants. Despite the identical geographical origins of the microorganisms in the same host species, they were dispersedly distributed in the phylogenetic tree.

The *gltA* gene encodes citrate synthase which catalyzes the condensation of acetyl coenzyme A with oxaloacetate to form citrate and has been assumed to be an important control point for determining the metabolic rate of the cell. The *A. phagocytophilum gltA* gene has multiple gene polymorphisms. *A. phagocytophilum* isolated from different dog samples showed diverse SNPs in the *gltA* gene and could be sub-grouped by alpha and beta variants (21). Analysis of the *gltA* sequences shows that our strain, in combination with that of a rodent from south-eastern China, was unique compared with all other variants, branching out from the group of sequences isolated from dog and sheep hosts in Spain, and tick vectors in the USA and Spain. Our study indicated that polymorphisms in the *A. phagocytophilum gltA* gene are probably related to the hosts and their geographical distribution.

Taken together, our results suggested that commensal rodents are the reservoirs for *A. phagocytophilum*, and controlling the rodent population wound make a great contribution to preventing HGA in Nanchang, China.

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Conflict of interest None to declare.

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▣ ORIGINAL ARTICLE

Tick-Borne Pathogens in Ixodid Ticks from Poyang Lake Region, Southeastern China

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Abstract: Ticks are the vectors of various pathogens, threatening human health and animal production across the globe. Here, for the first time we detected *Ricketssia* spp., *Borrelia* spp. and protozoan in ticks from Poyang Lake region in Jiangxi Province of eastern China. In 3 habitat categories and on 12 host species, 311 ticks from 11 species were collected. *Haemaphysalis longicornis* was the predominant species, accounting for 55.63%, followed by *Rhipicephalus microplus, Haemaphysalis flava* and *Ixodes granulatus*. Of the collected ticks, 7.07% were positive for tick-borne pathogens, and *H. longicornis* and *H. flava* were found to be co-infected with *Ricketssia* spp. and protozoan. *H. flava* was the most detected positive for tick-borne pathogens, whereas *H. longicornis* had the lowest infection rate, and the difference in infection rates between tick species was significant ($\chi^2=61.24$, $P<0.001$). Furthermore, adult ticks demonstrated remarkably greater infection rate than immature ticks ($\chi^2=10.12$, $P=0.018$), meanwhile ticks on Erinaceidae showed significantly higher positivity than ticks collected on other host species ($\chi^2=108.44$, $P<0.001$). Genetic fragment sequencing and analyses showed at least 4 pathogen species presence in ticks, namely *Borrelia yangtzensis, Rickettsia slovaca* or *Rickettsia raoultii* related genospecies, *Babesia vogeli* and *Hepatozoon canis* or *Hepatozoon felis* related genospecies. The finding indicates that the abundant ticks can carry diverse pathogens in Poyang Lake region, and pathogen infection is highly related to species, vertebrate hosts and life stages of ticks.

Key words: Tick-borne pathogens (TBPs), tick, epidemiology, risk factors, Poyang Lake region

INTRODUCTIOIN

Ticks, a group of specialized obligate hemophagous ectoparasites, parasitize abundant host species and are the vectors of wide range of pathogens of veterinary and public health importance [1-6]. Recently, they are considered to occupy the second place after mosquitoes as vectors of human infectious diseases in the world. As of May 31 2015, there were at least 5,568 cases of human tick-borne diseases reported around China, including large number of patients with Lyme diseases and newly emerging severe fever with thrombocytopenia syndrome [1].

China has the complex distributions and the great diversity of tick species because of its diverse ecological habitats. Ticks in China were reported to be carriers of various human pathogens including protozoans and bacterium like *Borrelia* spp. and *Richettsia* spp. [1,7,8]. Poyang Lake region, belonging to Jiangxi (a province of southeastern China), has already recorded sporadic human tick-borne diseases and at least 13 tick species. Our previous work detected some tick-borne pathogens in a few kinds of hosts, such as rodents and dogs in Poyang Lake region [4-6]. However, knowledge on tick-borne pathogens in tick vectors in this region is limited. Therefore, in this study we showed evidence to illustrate the distribution of pathogens comprising *Borrelia* spp., *Rickettsia* spp., and protozoa in tick vectors from Poyang Lake region in Jiangxi, and elucidated its relation with tick species, developmental stage, host and vegetation. The results will be a basis for future epidemiological studies and risk assessment of human tick-borne pathogens in Poyang Lake region.

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MATERIAL AND METHODS

Study area

The study had been conducted for 3 years (2013-2015) in Poyang Lake region of Jiangxi Province, southeastern China, which has altitudes higher than 35 m and lower than 190 m above sea level. This area experiences a subtropical climate with over than 1,000 mm of annual rainfall, -10˚C of maximum low temperature and 40˚C of maximum high temperature. Temperatures usually vary from 10 to 37˚C between May and October when tick populations are active. Types of vegetation cover include mixed broadleaf and coniferous woodland and grassland (Table 1). We selected 12 counties in Poyang lake region as investigation sites (Table 1).

Tick collection and identification

Ticks in vegetation covers were collected by flagging or dragging both at ground level and over and through the vegetation with a cotton cloth (100×60 cm). Each site was visited at least 3 times to cover all of 3 categories of habitats (grassland, woodland, and shrubs). Each habitat category was selected to cover a 900-m^2 area with many animal trails and tracks. Ticks were removed from the cotton cloth every 2 minutes. Ticks parasitizing hosts were collected from 24 villages and 12 wild animal markets. In villages, domestic animals and fowls were restricted by owners for sampling. In markets, wild animal bodies were employed for tick collection. Rodentia around villages were trapped using peanut baited rodent traps for tick examination. All the procedures were carried out according to ethical guidelines for the use of animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Animal experiment access num: 28-100). The information regarding all of the collected specimens, including their location, vegetation type, host, number of ticks collected from the body of each animal and the date of collection, were recorded. Ticks were collected from the entire body of each host into separate sample bottle containing 70% ethanol. Standard taxonomic keys were used to morphologically identify adults [9]. Larvae and nymphs were identified individually based on molecular methods [10]. The specimens were kept in 70% ethanol and used for further molecular identification and detection of tick-borne pathogens.

Table 1. Location and vegetation type of 12 plots sampled in this study

Location	Geographic coordinates	Vegetation type	Year surveyed
Anyi	N 28.6173°, W 115.5423°	G, S, W	2014, 2015
Wanli	N 28.8400°, W 115.7589°	W	2014
Xinjian	N 28.9800°, W 115.9154°	G	2014
Qingyunpu	N 28.6389°, W 115.9127°	G	2013, 2014
Duchang	N 29.2542°, W 116.1946°	W	2015
Hukou	N 29.7469°, W 116.2330°	W	2015
Wuning	N 29.2574°, W 115.0986°	G, S	2015
Poyang	N 29.0000°, W 116.6730°	G	2015
Wannian	N 28.6899°, W 116.9728°	W	2015
Wuyuan	N 29.2709°, W 117.75793°	G, S, W	2015
Yichun city	N 27.5914°, W 114.3252°	G, S, W	2015
Xingan	N 27.7327°, W 115.3791°	G, S, W	2015

W, woodland; S, shrubs; G, grassland.

DNA isolation

Tick specimens immersed in 70% ethanol were air dried, and then rinsed in sterile water for 3 times. After rinsed in sterile phosphate-buffered saline, ticks were dried on sterile filter paper in a biosafety hood, and individually ground in sterile tubes. DNA was extracted using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The genomic DNA was stored at 4˚C until used as a template in PCR assays.

Pathogen identification

A total of 3 groups of pathogens were assayed: *Borrelia* spp., *Rickettsia* spp. and protozoa. A conventional PCR was performed with a set of primers (forward: 5′-ACATATTCAGATGCAGACAGAGGT-3′, reverse: 5′-GCAATCATAGCCATTGCAGATTGT-3′) designed to amplify the 665-bp flagellin gene of *Borrelia* spp. For citrate synthase encoding gene (*glt*A), a primer set of primer 1 (5′-GCAAGTATCGGTGAGGATGTAAT-3′) and primer 2 (5′-GCTTCCTTAAAATTCAATAAATCAGGAT-3′) was used and expected to yield a 401-bp fragment depending on the *Rickettsia* spp. For amplification of 209-214 bp fragment of 18S ribosomal RNA (rRNA) in the protozoa, a set of primers (forward: 5′-GCATTTAGCGATGGACCATTCAAG-3′, reverse: 5′-CCTGTATTGTTATTTCTTGTCACTACCTC-3′) was designed for PCR. PCR reagents were used as recommended by the manufacturer (Takara Bio Inc., Dalian, China). The amplification for *flagellin* gene included 5 min pre-denaturation at 94˚C followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 1 min, and final extension at 72˚C for 7 min. The amplifications for 18S rRNA gene in the protozoa and *glt*A gene in the *Rickettsia* spp. were performed under the

same conditions as *flagellin* gene except the extension at 72˚C for 45 sec for the protozoa and the annealing at 50˚C for 30 sec. Positive samples were sequenced to identify potential microbial species with a resemblance to known species based on by an online software (http://www.bioinformatics.org/sms2/ident_sim.html).

Sequence analysis

All obtained sequences were assembled and edited by using SeqMan software. We compared them with sequences in the GenBank database. We performed multiple sequence alignments by using the ClustalX program. Phylogenetic trees were constructed by using the Neighbor-Joining (NJ) algorithm in the MEGA v.7.0.26 software. Support for the tree nodes was calculated with 1,000 bootstrap replicates.

Data analyses

All the raw data were collated in Excel spreadsheets. The differences in infection rates of ticks at species levels, at developmental stages, on hosts, in habitat categories, and the difference in infection rates of ticks collected in vegetation covers and on hosts were evaluated using Chi square (http://quantpsy.org). In the 2×2 case of the chi-square test of independence, if expected frequencies is less than 5, Yates' correction is employed [11].

RESULTS

Tick samples

A total of 311 ticks belonging to 5 genera and 11 species were collected from 5 species of domestic animals (*Canis familiaris, Capra aegagrus hircus, Bos* spp., *Bubalus bubalis*, and *Equus ferus*), 5 species of wild animals (*Lepus sinensis*, Erinaceidae, *Apodemus agrarius, Rattus norvegicus, Rattus rattoides*), a species of bird (*Phasianus colchicus*) and a species of chicken (*Gallus gallus domesticus*), in 2 kinds of vegetation types from 12 locations in Poyang Lake region (Table 1). *L. sinensis* harbored abundant

Table 2. Summary of species and number of ticks collected from hosts and by flagging over vegetation cover

Vegetation covers/Animal hosts	Tick species	No. of ticks collected			Density[a]
		L	N	A	
Woodland (a=800 m^2)	*Haemaphysalis longicornis*	0	0	2M	0.0038
	Dermacentor auratus	0	0	1M	
Grassland (a=800 m^2)	*H. longicornis*	3	34	5M6F	0.06
Subtotal (a=1,600 m^2)	-	3	34	8M6F	0.032
Canis familiaris (n=24)	*H. longicornis*	0	5	4M25F	1.79
	R. sanguineus	0	0	1M8F	
Capra aegagrus hircus (n=44)	*H. longicornis*	0	2	0	0.80
	Haemaphysalis flava	2	2	3M1F	
	Rhipicephalus microplus	0	8	6M11F	
Bos spp. (n=13)	*H. longicornis*	1	8	0	4.31
	R. microplus	0	20	8M19F	
Bubalus bubalis (n=7)	*H. longicornis*	0	0	1F	1
	R. microplus	0	0	5F	
	Amblyomma testudinarium	0	0	1F	
Phasianus colchicus (n=5)	*Haemaphysalis phasiana*	0	5	0	1
Lepus sinensis (n=22)	*H. longicornis*	57	12	5M1F	3.77
	Ixodes acuminatus	0	0	1F	
	Ixodes sinensis	0	0	2M2F	
	Rhipicephalus haemaphysaloides	3	0	0	
Erinaceidae (n=3)	*H. flava*	0	1	3M9F	4.33
Apodemus agrarius (n=206)	*Ixodes granulatus*	0	0	1M4F	0.02
Rattus norvegicus (n=95)	*I. granulatus*	0	0	3M1F	0.04
Rattus rattoides (n=8)	*I. granulatus*	0	5	2M	0.88
Equus ferus (n=6)	*H. longicornis*	0	0	1F	0.17
Gallus gallus domesticus (n=30)	*H. longicornis*	1	0	0	0.03
Subtotal (n=463)	-	64	68	38M90F	0.56
Total (n=463; a=1,600 m^2)	-	67	102	46M96F	-

L, larvae; N, nymph; A, adult; M, male; F, female.
[a]Tick population density is denoted as ticks/hosts for ticks on hosts, ticks/m^2 for ticks collected from vegetation covers.

ticks such as *Haemaphysalis longicornis, Ixodes acuminatus, Ixodes sinensis* and *Rhipicephalus haemaphysaloides*, with the third highest tick population density of 3.77 ticks per a host. Hosts with the first highest and second highest tick loads were Erinaceidae (4.33 ticks per host) and *Bos* spp. (4.31 ticks per host), respectively. Other hosts with higher tick abundance were *B. bubalis* and *C. aegagrus hircus*, harboring 3 tick species. Sixty-seven female (14.79%) and 102 male (30.87%) adult ticks were obtained. Sixty-seven larvae and 102 nymphs accounted for 21.54% and 32.80% of the total number of ticks collected respectively. Of the 11 tick species collected, 3 species belonged to the genus *Haemaphysalis*, 3 species belonged to the genus *Rhipicephalus*, 3 species belonged to *Ixodes*, 1 species belonged to *Dermacentor*, and 1 other species belonged to the genus *Amblyomma*. The most abundant species was *H. longicornis* (55.63%), found in a kind of vegetation cover and infesting the most diverse host species (7 species). Three other common species included *H. flava*, *R. microplus* and *I. granulatus* (Tables 2, 3).

Pathogen infections in ticks

Protozoa, *Borrelia* spp. and *Rickettsia* spp. were detected in 4 tick species. Overall, 7.07% of ticks were tested positive for at least 1 pathogen. In detail, 2.31% of *H. longicornis* were detected positive for *Rickettsia* spp., or/and Protozoa, 18.75% of *I. granulatus* for *Borrelia* spp., 52.38% of *H. flava* for protozoa or/and *Rickettsia* spp. and 5.19% of *R. microplus* for protozoa. Infection rate in *H. flava* was significantly greater than that in *H. longicornis* ($\chi^2 = 61.24$, $P < 0.001$). Coinfection with protozoa and *Rickettsia* were found in *H. longicornis* and *H. flava*, with coinfection rate of 0.58% and 47.62%, respectively. There was no positive samples found in 7 tick species (*H. phasiana, I. acuminatus, R. sanguineus, R. haemaphysaloides, I. sinensis, A. testudinarium* and *D. auratus*) (Table 3).

The effect of risk factors on the pathogen distribution

The overall prevalence of pathogens in larvae, nymphs, male, and female ticks were 1.49%, 2.94%, 10.87%, and 13.54%, respectively. There was major difference in the prevalence of these pathogens between immatures (larvae and nymphs) and matures (males and females) ($\chi^2 = 10.12$, $P = 0.018$). However, there was no significant difference in prevalence of these pathogens in ticks among host species and vegetation, although the positive rate of pathogens in ticks collected from hosts was approximately 2 times more than that collected by flagging over vegetation ($\chi^2 = 0.44$, $P = 0.51$). Prevalence of these pathogens in ticks collected from *Canis familiaris, C. aegagrus hircus*, Muridae and Erinaceidae were 4.65%, 11.43%, 18.75%, and 84.62%, respectively, and ticks on Erinaceidae were at significantly higher risk for pathogen infection compared to ticks on other hosts ($\chi^2 = 108.44$, $P < 0.001$). There was no positive ticks found on other host species. Prevalence of these pathogens in ticks in grasslands and woodland were 2.08% and 0, respectively, and there was on significant difference ($\chi^2 = 1.37$, $P = 0.24$) (Table 4).

Pathogen identification and sequence analyses

Further sequencing and sequence alignment showed that 1 *Borrelia* species (*Borrelia yangtzensis*), 2 protozoan species (*Babesia vogeli* and *Hepatozoon canis* or *Hepatozoon felis* related geospecies), and 1 *Rickettsia* species (*Rickettsia slovaca* or *Rickettsia raoultii* related genospecies) were successfully sequenced from 4 tick species. The 665-base pair sequence of *Borrelia* spp. flagellin gene (MG717513) yielded in the study was 99.21-99.37% identical to other 2 sequences of MG717514 and MG717515 pro-

Table 3. Pathogen infection rates in ticks collected in Poyang Lake region

Tick species (number collected)	*Borrelia*	*Rickettsia*	Protozoa	Protozoa+ *Rickettsia*	Infection rate (%)	χ^2	*P*-value
Haemaphysalis longicornis (n=173)	0	4 (2.31)	1 (0.58)	1 (0.58)	4 (2.31)	61.24	<0.001
Ixodes granulatus (n=16)	3 (18.75)	0	0	0	3 (18.75)		
Haemaphysalis flava (n=21)	0	11 (52.38)	10 (47.62)	10 (47.62)	11 (52.38)		
Rhipicephalus microplus (n=77)	0	0	4 (5.19)	0	4 (5.19)		
Haemaphysalis phasiana (n=5)	0	0	0	0			
Ixodes acuminatus (n=1)	0	0	0	0			
Rhipicephalus sanguineus (n=9)	0	0	0	0			
Rhipicephalus haemaphysaloides (n=3)	0	0	0	0			
Ixodes sinensis (n=4)	0	0	0	0			
Amblyomma testudinarium (n=1)	0	0	0	0			
Dermacentor auratus (n=1)	0	0	0	0			

duced in the study. When compared to other fragments deposited in GenBank, MG717513 showed 98.73-98.89% identity to *B. yangtzensis* (EU135599, EU135601, and EU135602), 98.57-98.73% identity to *Borrelia valaisiana* (AB022134 and AB022135), and 95.25% identity to *Borrelia burgdorferi* sensu lato (X75202, X63413, and D63364). Therefore, 3 individuals of *Borrelia* spp. in the study were identified as *B. yangtzensis* or *B. yangtzensis*-related species. In *Rickettsia* spp., the 401 base-pair sequence of *glt*A gene (MG717516) obtained in a *H. longicornis* tick collected in grassland was 100% identical to the sequences of *glt*A gene isolated from 2 *H. longicornis* ticks (MG717517 and MG717523) on *C. familiaris* and 10 *H. flava* ticks on Erinaceidae (MG717518-MG717522, MG717524-MG717528), and 96.26% identical to the sequence in a *H. flava* tick on Erinaceidae (MG717529) (Table 5; Fig. 1). Our 13 sequences (MG717516-MG717528) showed 99.75% identity to the sequences of *R. raoultii* (MF002517) and *R. slovaca* (MF002529) deposited in GenBank, in addition, 1 remaining sequence (MG717529) presented 96.01% identity to *R. raoultii* and *R. slovaca*. The *Rickettsia* spp. pathogens in the study were identified as *R. raoultii* or *R. slovaca* related genospecies. Of 15 protozoa-positive specimens for amplification of 209-214 base-pair 18S ribosomal RNA by means of PCR method, 2 specimens were successfully sequenced (Table 5). The closest matches of 209 base-pair 18S ribosomal RNA of protozoa in our study were *B. vogeli* isolated in dogs from Jiangsu, China (MG586235, 100%), Serbia (KY747491, 100%), and Argentina (KY290978, 99%), and in *R. sanguineus* from India (MG050159, 100%) and from Australia (MG758132, 100%), in *Haemaphysalis concinna* from Czech Republic (KX8 57477, 100%). The 214 base-pair 18S ribosomal RNA of protozoa (MG675579) isolat-

Table 4. Comparison of difference of collected ticks and positive rates of pathogens among ticks by life stage, host species and vegetation type

Group		Sampled ticks	Positive ticks		χ^2	*P*-value
			No.	%		
Life stage	Larvae	67	1	1.49	10.12	0.018
	Nymph	102	3	2.94		
	Male	46	5	10.87		
	Female	96	13	13.54		
Vegetation type	Grassland	48	2	4.17	1.37	0.24
	woodland	3	0	0.00		
Host	Muridae	16	3	18.75	108.44	<0.001
	Canis familiaris	43	2	4.65		
	Capra aegagrus hircus	35	4	11.43		
	Lepus sinensis	83	0	0.00		
	Erinaceidae	13	11	84.62		
	Bubalus bubalis	7	0	0.00		
	Bos spp.	56	0	0.00		
	Phasianus colchicus	5	0	0.00		
Vegetation vs host	Vegetation	51	2	3.92	0.44	0.51
	Host	260	20	7.69		

Table 5. Pathogens in ticks collected from different hosts in different locations

Pathogens		Ticks species (No. positive)	Host species	Sampling site	GenBank accession No.
Borrelia	*B. yangtzensis*	*I. granulatus* (1♂1♀)	*R. norvegicus*	Anyi	MG717514-MG717515
		I. granulatus (1N)	*R. rattoides*	Anyi	MG717513
Rickettsia	*R. raoultii* or *R. slovaca* related genospecies	*H. longicornis* (2♀)	*Canis familiaris*	Xinjian, Poyang	MG717517, MG717523
		H. longicornis (1♀)	Grassland	Qingyunpu	MG717516
		H. flava (1N3♂1♀)	Erinaceidae	Hukou	MG717518-MG717522
		H. flava (1♂5♀)	Erinaceidae	Hukou	MG717524- MG717529
	Rickettsia sp.	*H. longicornis* (1L)	Grassland	Qingyunpu	-
Protozoa	*Babesia vogeli*	*H. flava* (1♂)	Erinaceidae	Hukou	MG675580
	Babesia sp.	*H. flava* (9♀)	Erinaceidae	Hukou	-
		R. microplus (1N3♀)	*Capra aegagrus hircus*	Yichun	-
	Hepatozoon canis or *Hepatozoon felis* related genospecies	*H. longicornis* (1♀)	Grassland	Qingyunpu	MG675579

ed in *H. longicornis* from grassland in the study showed 94.86% to *H. canis* (MG917719 and MG209594) and *H. felis* (KU232308), 92.99% identity to *Hepatozoon ursi* (KU232308), hence we proposed the protozoan as *H. canis* or *H. felis* related genospecies. *R. slovaca* or *R. raoultii* related genospecies was most frequently identified (14 times, 3 from the tick *H. longicornis*, 11 times from the tick *H. flava*), followed by *B. yangtzensis* (triple from *I. granulatus*). The other 2 protozoan species were detected only once. Twenty one of the 33 detections of pathogens were on *H. flava* collected from Erinaceidae (Table 5).

For phylogenetic analyses, 3 sequences of *B. yangtzensis flagellin* gene obtained from *I. granulatus* belonged to the same cluster where they shared with the strain QLZSP, QSYSP, and QTMP2 of *B. yangtzensis* and strain CKA3a and CMN1b of *B. valaisiana* (Table 5; Fig. 2). The sequences of *R. raoultii* or *R. slovaca* related *Rickettsia* spp. (MG717516-MG7175129) were clustered with those of *R. raoultii* (MF002517) and *R. slovaca* (MF002529) (Fig. 2).

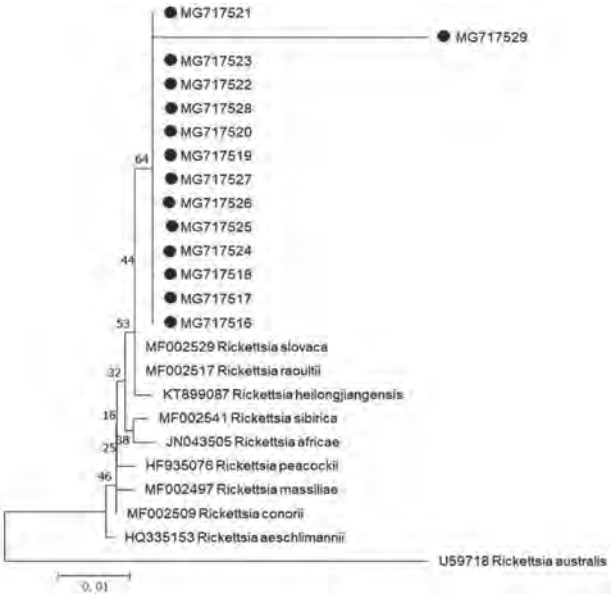

Fig. 1. Phylogenetic tree of *Rickettsia* spp. based on *glt*A gene. The trees were calculated by the neighbor-joining method using MEGA v.7.0.26 software. Values of the bootstrap support of the particular branching calculated for 1,000 replicated are indicated at the nodes. The variant sequences obtained from GenBank are designated by accession number and species. *Rickettsia australis* is used as outgroup. (●) denotes sequences of *R. slovaca* or *R. raoultii* related genospecies obtained in the study.

DISCUSSION

In Poyang Lake region, the common animals and birds with potential for tick parasitism and easy to contact human were *C. familiaris, C. aegagrus hircus, A. agrarius, R. norvegicus, G. gallus domesticus*, and *L. sinensis*, accounting for over 90% of hosts captured. Rodents like *A. agrarius* and *R. norvegicus* were trapped with large number, but a few ticks were found, whereas *B. yangtzensis* was occasionally detected in ticks removed from the rodents. *B. yangtzensis*, a *Borrelia* species in the *B. burg-*

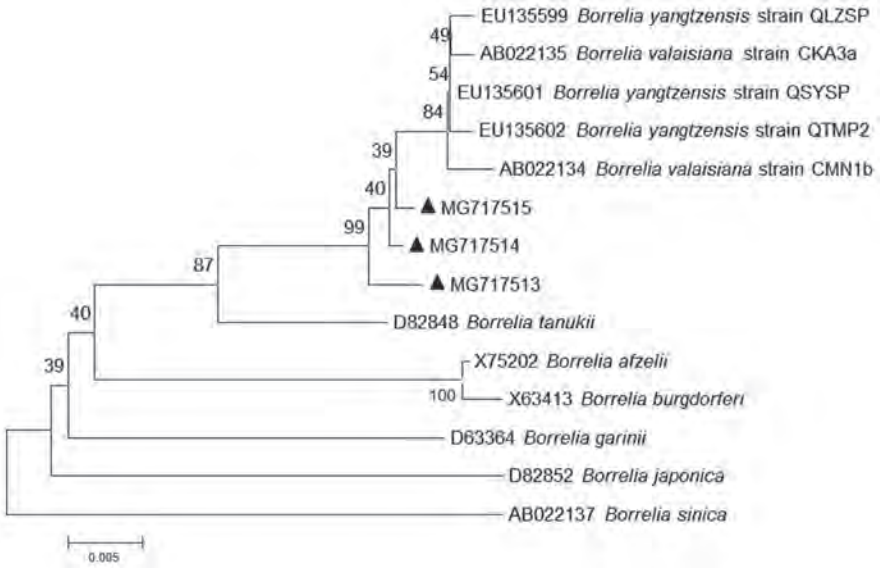

Fig. 2. Molecular phylogenetic tree of the *Borrelia* agent. The aligned nucleotide sequence of flagellin gene was subjected to analysis. Bootstrap 1,000 replicates are showed at the nodes. Scale bars indicate nucleotide substitutions per sites. *Borrelia sinica* is used as outgroups. (▲) prior to accession numbers are the sequences in the study.

dorferi complex was originally discovered in Chinese Yangtze River Valley region in 2015, and it was reported in *H. longicornis* and *I. granulatus* ticks from small mammals in China and isolated in rodents or shrews in Japan [12]. However, *B. yangtzensis* was not detected in *H. longicornis* albeit greater than 50% ticks collected in the study were *H. longicornis*. The reason, we guessed, might be that *H. longicornis* was not an efficient vectors of *B. yangtzensis*, hence the pathogen was rarely presented in the ticks. Poyang Lake region belongs to part of Yangtze River Valley region, and has similar distribution pattern of ticks and tick related small mammals to other parts of Yangtze River Valley region, therefore *B. yangtzensis* can also be found in *I. granulatus* collected in rodents in our study. The sequences of *flagellin* gene in *B. yangtzensis* in the study showed higher identity to *B. valaisiana* than to other known Lyme Borreliosis group spirochaete species, which was in agreement with the previous study [12].

Despite some *L. sinensis* were majorly tick infested, pathogens were not found in those ticks. We had 3 Erinaceidae hosts, and found diverse pathogens like *R. slovoca* or *R. raoultii*-like genospcies and *Babesia* spp. in attached ticks with high infection rate. Ticks on Erinaceidae might serve as vectors within Erinaceidae populations in this region, thus readily leading to high infection rate. This increases the chance that ticks transport pathogens from a natural hedgehog cycle to other hosts, including humans [13]. *C. familiaris*, usually functioning as a guard dog and a pet in investigated sites, were closely related to human, furthermore, some ticks on dogs in our study were positive for *R. slovoca* or *R. raoultii* related genospcies which is likely considered as human pathogen. Dogs, incidental hosts for the agent of spotted fever group, can become infected by a bite of ixodid ticks, and then transmit the pathogens to human [14]. Therefore, people should avoid contact with such dogs and ticks.

In this study, 11 tick species were collected, with *H. longicornis* acting as the predominant species, and other common ticks included *H. flava*, *R. microplus* and *I. granulatus*. These common tick species were also reported in other subtropical regions of China like Zhejiang and Hubei [15]. Our findings indicated that *H. flava* and *H. longicornis* were the ticks frequently detected positive for presence of *R. raoultii* or *R. slovoca* related genospecies. *R. raoultii* and *R. slovoca* were reported as human pathogenic agents [3,16,17]. Previous researches showed that *R. raoultii* had been reported in northern regions of China [3,7], and *R. slovaca* recorded in Europe and Xinjiang, China [7,16,17]. Although natural infection with tick-borne pathogens occurs [1], other tick species like *R. sanguineus, R. haemaphysaloides, I. sinensis*, and *A. testudinarium* were tested negative for *Borrelia* spp., *Rickettsia* spp. and protozoa infection in our study. The possible reason might be because of a few numbers of ticks collected and thus decreasing the probability of pathogen detection.

Compared to immature ticks, mature ticks tended to pathogen infection, furthermore, we found that females had comparable positivity rate with males. In contrast, a study conducted in Europe showed higher pathogen infection rate in immatures than matures [18]. Our study demonstrated that relatively high infection rate were determined in adult ticks collected from hedgehogs. The result suggests hedgehogs functioning as important pathogen reservoirs, and corresponds with a previous indication that several species of birds played a role as Lyme disease spirochetal reservoirs infective to ticks [18]. Therefore, in some cases, positivity rate is not depended by tick developmental stage but by which reservoir hosts that ticks attach to. For vegetation types, grassland sheltered more ticks than woodland and shrubs, and there were some ticks infected with *R. slovaca* or *R. raoultii* related genospecies in grassland. However, non-infected ticks were found in woodland, in contrast to more than 6 tick-borne pathogens infection in ticks from French suburban woodland [19]. Workers and visitors for travelling in the field should pay more attention to questing ticks in grassland in prevention of occurrence of tick-borne diseases. In addition, people in this region should keep a distance from hosts with tick infestation, especially the hosts with high risk for human tick-borne pathogens including hedgehogs, dogs and rodents.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Preliminary investigation of ixodid ticks in Jiangxi Province of Eastern China

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Abstract
In recent years, a large effort has been made for tick surveys for public health importance around China, especially after outbreaks of severe fever with thrombocytopenia syndrome (SFTS) occurred in humans in 2009. In this paper, the preliminary species composition and population distribution of ticks in Jiangxi Province of Eastern China is reported. Ticks were collected in three habitats (grassland, shrubs and woodland) and from nine host groups in 12 sampling sites throughout Jiangxi Province between 2011 and 2018. Six tick species including *Haemaphysalis longicornis, Rhipicephalus sanguineus* sensu lato, *Haemaphysalis yeni, Haemaphysalis kitaoka, Ixodes sinensis* and *Dermacentor auratus* were collected from the vegetation. *Haemaphysalis longicornis* was most abundant tick species, accounting for 90.6% of the total ticks. *Haemaphysalis yeni* and *H. kitaoka* were newly recorded tick species in Jiangxi Province. Tick presence was remarkably greater in grassland (89.4%) than in woodland (9.4%) and shrubs (1.2%), and nymphs (68.2%) and larvae (19.1%) were more frequently found than adult females (6.6%) and males (6.0%). On hosts, a total of 1513 ticks, from 13 species and four genera, were collected. These were *H. longicornis, Haemaphysalis campanulata, Haemaphysalis flava, Haemaphysalis phasiana, H. yeni, H. kitaoka, Haemaphysalis hystricis, R. sanguineus* (s.l.), *Rhipicephalus haemaphysaloides, Rhipicephalus microplus*, *Ixodes granulatus, I. sinensis* and *Amblyomma testudinarium. Amblyomma testudinarium* was a newly recorded tick species in Jiangxi Province. Based on this investigation, *H. longicornis* was the most frequently collected species (30.5%) and widely distributed tick species of the total collection ticks (in 11 sampling sites). *Haemaphysalis longicornis* had a broad host range and its presence (hosts with at least one tick) was significantly greater on *Lepus sinensis* (33.3%) than on *Canis familiaris* (2.3%) ($\chi^2 = 23.68$, $p = 0.0013$). In addition, the number of *H. longicornis* collected on *L. sinensis* (64.0%) was higher than on other host groups. Of all ticks collected on hosts, different developmental stages were obtained, which included 347 larvae (22.9%), 249 nymphs (16.5%), 404 adult males (26.7%) and 513 females (33.9%) and sex distribution was relatively uniform. These data indicate that a broad range of tick species is widely distributed throughout Jiangxi Province in Eastern China.

Keywords Species composition · Population distribution · Ixodid ticks · Jiangxi · Eastern China

Extended author information available on the last page of the article

Introduction

Ticks can transmit a great variety of pathogenic micro-organisms including protozoan, viral and bacterial agents (Zheng et al. 2017a, b, 2018; Wu et al. 2013). They are surpassed only by mosquitoes as arthropod vectors for transmitting human diseases and have gained enormous notoriety for affecting animal production in the world (Goodman et al. 2005). Most ticks falling into two families: the hard ticks (Ixodidae) and the soft ticks (Argasidae), are widely distributed in five continents around the globe. The world's hard ticks comprises 244 species in the genus *Ixodes*, 167 species in the genus *Haemaphysalis*, 132 species in the genus *Amblylomma*, and 148 species in the genera A*nomalohimalaya, Bothriocroton, Cosmiomma*, *Dermacentor, Hyalomma, Margaropus, Nosomma, Rhipicentor* and *Rhipicephalus* in the family Ixodidae (Guglielmone and Nava 2014).

Zoogeographically, China is divided into Palaearctic and Oriental Realm, and has abundant tick species which form approximately 1/8 of tick species worldwide. As of 2009, Chen et al. reported that hard tick fauna of this area involving 104 species in the following genera: *Amblyomma* (8 species), *Anomalohimalaya* (2 species), *Dermacentor* (12 species), *Haemaphysalis* (44 species), *Hyalomma* (6 species), *Ixodes* (24species) and *Rhipicephalus* (8 species) (Chen et al. 2010). To date, 15 hard tick species are recognized in Jiangxi Province (Xu et al. 2016, 2017; Zheng et al. 2011; Chen et al. 2010; Liu et al. 2013), which is less than one-third of tick species reported in Fujian Province bordered by Jiangxi (Chen et al. 2010). There appears to be incomplete investigation in some areas of Jiangxi Province. Thus a further investigation is needed in these areas. In the present study, we preliminarily investigated species composition and the distribution of hard ticks from wide range of hosts and three types of habitats in whole region of Jiangxi Province of Eastern China.

Methods and materials

Investigation site

We established 12 sampling sites in nine counties and three cities in Jiangxi Province, including Hukou, Wuning, Duchang, Jing'an, Nanchang, Wuyuan, Poyang, Wannian, Xingan, Yichun, Xinyu and An'yuan from 2011 to 2018 (Fig. 1). Jiangxi experiences a subtropical climate with over than 1000 mm of annual rainfall, − 5 °C of maximum low temperature and 38 °C of maximum high temperature. Types of vegetation cover include mixed broadleaf and coniferous woodland, shrubs and grassland. Woodland mainly involves the trees from the family of Pinaceae, Taxodiaceae Fagaceae, Theaceae, Lauraceae and Hamamelidaceae, and other trees such as *Sassafras tsumu* and *Acer* spp. are occasionally observed. The predominant shrub species are *Quercus fabri, Castanea sequinii, Adinandra millettii, Lindera aggergata, Vaccinium bracteatum, Actinidia chinensis, Dalbergia hupeana, Rhus chinensis*, and *Rhododendron simsii* in Shrubs. Grassland is dominated by *Eleusine indica, Conyza Canadensis, Cynodon dactylon, Setaria viridis, Avena fatua, Artemisia argyi, Imperata cylin drical, Humulus scandens, Erodium stephanianum* and *Galium aparine*.

Fig. 1 Map of Jiangxi Province. Stars indicate investigation sites in the study. Hukou county (HK), Wuning county (WUN), Duchang county (DC), Wuyuan county (WY), Jing'an county (JA), Poyang county (PY), Nanchang city (NC), Wannian county (WAN), Yichun city (YC), Xinyu city (XY), Xingan county (XG), Anyuan county (AY)

Tick collection

Tick investigations in vegetation covers and on hosts were both conducted 8 times (one time a year during 2011–2018) in Nanchang, 2 times (one time in the year 2015 and other time in the year 2018) in Yichun, once in the remaining places (the year 2015 or 2018).

Each investigation of ticks in vegetation covers involved three categories of habitats (grassland, woodland, and shrubs), and each habitat category was selected in a 900-m^2 area with many animal trails and tracks. Ticks were collected by flagging or dragging both at ground level and over and through the vegetation and were checked every 2 min; Ticks from hosts were collected from two villages and one wild animal market in each investigation. In villages, domestic animals and fowls were restricted by owners for sampling. In markets, wild animal bodies were employed for tick collection. Rodentia around villages were trapped using peanut baited rodent traps for tick examination. All the procedures were carried out according to ethical guidelines for the use of animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Animal experiment access num: 28–100). The information regarding all of the collected specimens, including their location, vegetation type, host, number of ticks collected from the body of each animal and the date of collection, were recorded. Tick were collected from the entire body of each host into separate sample bottle containing 70% ethanol.

Tick count and species identification

After transferred to the laboratory, ticks immersed in 70% ethanol were counted and identified to species level by morphology according to taxonomic keys (Walker et al. 2003; Estrada-peña et al. 2004; Teng 1978). For damaged samples or immature ticks of different species which cannot be distinguished morphologically, polymerase chain reaction (PCR) amplification of 680 base-pair sequence of cytochrome oxidase subunit 1 (COI) gene and sequencing methods were used for molecular identification (Lv et al. 2014). Briefly, DNA was extracted with Dneasy® Blood and Tissue Kit (Qiagen, Germany) from a damaged tick, larvae or nymph. The partial fragment of the cytochrome c oxidase subunit I (COI) gene was amplified by polymerase chain reaction (PCR) with primers COI-F and COI-R. Fifty micro-liter DNA amplified with EX-Taq polymerase (Takara, Dalian, China) from all taxa was sent to Shanghai Sangon Biotech for sequencing from both 3′ and 5′ terminals of sequences. The sequences obtained from the sequencing company were discarded if spectrum of many nucleotide peaks were undistinguished and overlapped each other, or sequence length was <200 bp. Screened sequences were aligned with the sequences deposited in GenBank on the website of National Center for Biotechnology (NCBI). The sequences were identified according to taxonomic names assigned to reference sequence in GenBank. In the study, 18 sequences were obtained and deposited in GenBank under accession number MG721036-MG721053. The fragments MG721036-MG721044 were identified as *Haemaphysalis longicornis*, fragments MG721045, MG721046 and MG721051 as *Ixodes granulatus*, fragment MG721047 as *Dermacentor auratus*, fragments MG721048-MG721050, and MG721053 as *Rhipicephalus microplus*, and fragment MG721052 as *Haemaphysalis flava*.

Data analyses

All the raw data were collated in Excel spreadsheets. The differences in infestation rate of ticks on hosts and prevalence of species specific ticks on hosts were evaluated using χ^2 tests (http://quantpsy.org). In the 2·2 case of the χ^2 test of independence, if expected frequencies is less than 5, Yates' correction is employed (Preacher 2001).

Results

During the year 2011 and 2018, a total of 680 ticks including 616 *H. longicornis* (90.6%), 46 *Rhipicephalus sanguineus* sensu lato (6.8%), 15 *Haemaphysalis yeni* (2.2%), one *Haemaphysalis kitaoka* (0.1%), one *Ixodes sinensis* (0.1%) and one *D. auratus* (0.1%) were collected from three habitat categories. Of tick species of the total collection ticks in habitats, *H. yeni* and *H. kitaoka* were newly recorded tick species in Jiangxi Province and the specimens were deposited in Medical Insect Museum of Beijing Institute of Microbiology and Epidemiology, Beijing, China. Tick presence was remarkably greater in grassland (89.4%) than in woodland (9.4%) and shrubs (1.2%). Nymphs (68.2%) and larvae (19.1%) had greater numbers than females (6.6%) and males (6.0%) in three habitat categories, and intensity in grassland (nymphs, 68.2%; larvae, 16.9%) was higher than in woodland (nymphs, 0; larvae, 2.2%) and shrubs (nymphs, 0; larvae, 0) (Table 1).

In this study, 2151 individuals of hosts involving 9 groups-Rodentia, *Canis familiaris, Phasianus colchicus*, Erinaceidae, *Lepus sinensis, Bubalus bubalis, Bos* spp., *Capra*

Table 1 Ticks in vegetation types

Habit categories	*R. sanguineus*	*H. longicornis*	*H. yeni*	*H. kitaokai*	*I. sinensis*	*D. auratus*
Grassland	0/0/0/2[a]	464/115/16/10			0/0/0/1	
Woodland	0/0/20/20	0/2/3/2	0/13/2/0	0/0/0/1		0/0/1/0
Shrubs	0/0/0/4	0/0/3/1				
Total	0/0/20/26	464/117/22/13	0/13/2/0	0/0/0/1	0/0/0/1	0/0/1/0

[a]Numbers of ticks are recorded as Larvae/nymphs/males/females

aegagrus hircus and *Ovis aries* were used for tick examination (Table 2). A total of 1513 ticks, from 13 species and four genera, were collected on hosts from 12 sampling sites in Jiangxi. The 13 tick species included *H. longicornis, Haemaphysalis campanulata, H. flava, Haemaphysalis phasiana, H. yeni, H. kitaoka, Haemaphysalis hystricis, R. sanguineus* (s.l.), *Rhipicephalus haemaphysaloides, R. microplus, I. granulatus, I. sinensis* and *Amblyomma testudinarium*. Of tick species of the total ticks collected from hosts, *A. testudinarium* was newly recorded tick species in Jiangxi Province and the specimen was deposited in Medical Insect Museum of Beijing Institute of Microbiology and Epidemiology, Beijing, China. *H. longicornis* was the most frequently collected species (32%) (Fig. 2) and widely distributed tick species of the total collection ticks (in 11 sampling sites) (Table 3). *R. sanguineus* (s.l.) was the second abundant tick species (25%) (Fig. 2) and distributed in three sampling sites (Table 3). *R. microplus* was the second most distributed tick species (in four sampling sites) and the third most frequently observed (Fig. 2; Table 3).

The difference in tick infestation rate between host groups was found to be statistically significant ($p < 0.01$) which was higher in *L. sinensis* (66.7%) and *O. aries* (52.9%) and lower in Rodentia (0.7%). Regarding prevalence of tick species on different hosts, *H. longicornis* was present on *C. familiaris, P. colchicus*, Erinaceidae, *L. sinensis, Bos* spp., *B. bubalis, C. aegagrus hircus* and *O. aries*, and the presence (hosts with at least one tick) was significantly greater on *L. sinensis* (33.3%) than on *C. familiaris* (2.3%) ($\chi^2 = 23.68$, $p = 0.0013$). *Rhipicephalus sanguineus* s.l. presence was significantly higher on *O. aries* (29.4%) than on *C. aegagrus hircus* (1.2%) ($\chi^2 = 17.37$, $p = 0.0038$). *Haemaphysalis flava* was observed on Erinaceidae and *C. aegagrus hircus*, however it significantly preferred to infest Erinaceidae (28.6%) to *C. aegagrus hircus* (1.2%) ($\chi^2 = 7.93$, $p = 0.0049$). We also found that *R. haemaphysaloides* had more likelihood to attach to *L. sinensis* ($\chi^2 = 8.68$, $p = 0.034$), *R. microplus* to *Bos* spp. ($\chi^2 = 7.34$, $p = 0.025$) (Table 2). Both *P. colchicus* (13.9 ticks/individual) and *L. sinensis* (16.2 ticks/individual) were tick infested with higher burden compared with Rodentia (0.1 ticks/individual) and *B. bubalis* (0.4 ticks/individual) (Table 4).

Tick distribution at different developmental stages was roughly uniform, with larvae 22.9%, nymphs 16.5%, females 26.7% and males 33.9%. Of three most abundant tick species, *H. longicornis* larvae had an overwhelming number, accounting for 58.8%, and all of them were collected from *L. sinensis* hosts; Nymphs frequently infested *P. colchicus* and adults were found in greater number on *C. familiaris* and *Bos* spp. However, over than 95.0% *R. sanguineus* s.l. belonged to female and male ticks, most of which were captured on *C. familiaris* and *O. aries*, and *R. microplus* distribution at different developmental stages was relatively uniform (Table 5).

Table 2 Prevalence of tick species on different hosts (%)

Host		Hl	Hc	Hf	Hp	Hy	Hk	Hh	Rs	Rh	Rm	Ig	Is	At	Total
Rodentia (1781)	I	0	0	0	0	0	0	0	0	0	0	13		0	13
	P	0	0	0	0	0	0	0	0	0	0	0.7	0	0	0.7
C. familiaris (171)	I	4	2	0	0	0	0	0	7	2	0	0	0	0	13
	P	2.3	1.2	0	0	0	0	0	4.1	1.2	0	0	0	0	7.6
P. colchicus (7)	I	1	0	0	2	0	0	0	1	0	0	0	0	0	3
	P	14.3	0	0	28.6	0	0	0	14.3	0	0	0	0	0	42.9
Erinaceidae (7)	I	1	1	2	0	0	0	0	0	0	0	0	0	0	3
	P	14.3	14.3	28.6	0	0	0	0	0	0	0	0	0	0	42.9
L. sinensis (21)	I	7	0	0	0	0	0	0	2	3	0	0	2	0	14
	P	33.3	0	0	0	0	0	0	9.5	14.3	0	0	9.5	0	66.7
B. bubalis (14)	I	1	0	0	0	0	0	0	0	0	1		0	1	2
	P	7.1	0	0	0	0	0	0	0	0	7.1	0	0	7.1	14.3
Bos spp. (48)	I	7	0	0	0	0	0	2	3	1	8	1	1	0	20
	P	14.6	0	0	0	0	0	4.2	6.3	2.1	16.7	2.1	2.1	0	41.7
C. aegagrus hircus (85)	I	7	1	1	0	2	1	0	1	2	2	0	1	0	11
	P	8.2	1.2	1.2	0	2.4	1.2	0	1.2	2.4	2.4	0	1.2	0	12.9
O. aries (17)	I	2	0	0	0	2	0	0	5	0	0	0	0	0	9
	P	11.8	0	0	0	11.8	0	0	29.4	0	0	0	0	0	52.9
		$\chi^2=23.68^{※}$ $p=0.0013$	$\chi^2=1.52$ $p=0.47$	$\chi^2=7.93$ $p=0.0049$	/	$\chi^2=1.30$ $p=0.25$	/	/	$\chi^2=17.37$ $p=0.0038$	$\chi^2=8.68$ $p=0.034$	$\chi^2=7.34$ $p=0.025$	$\chi^2=0.05$ $p=0.82$	$\chi^2=2.00$ $p=0.37$	/	$\chi^2=563.91$ $p<0.01$

Numbers in the bracket are hosts examined; I, number of hosts with tick infestation; P, the percentage of hosts with tick infestation and the specific hosts detected; "/" in the table denotes that data is unavailable; ※, In the 2×2 case of the χ^2 test of independence, if expected frequencies is less than 5, Yates' correction is employed. Hl, *H. longicornis*; Hc, *Haemaphysalis campanulata*; Hf, *Haemaphysalis flava*; Hp, *Haemaphysalis phasiana*; Hy, *H. yeni*; Hk, *H. kitaoka*; Hh, *Haemaphysalis hystricis*; Rs, *R. sanguineus* (s.l.); Rh, *Rhipicephalus haemaphysaloides*; Rm, *Rhipicephalus microplus*; Ig, *Ixodes granulatus*; Is, *I. sinensis* and At, *Amblyomma testudinarium*

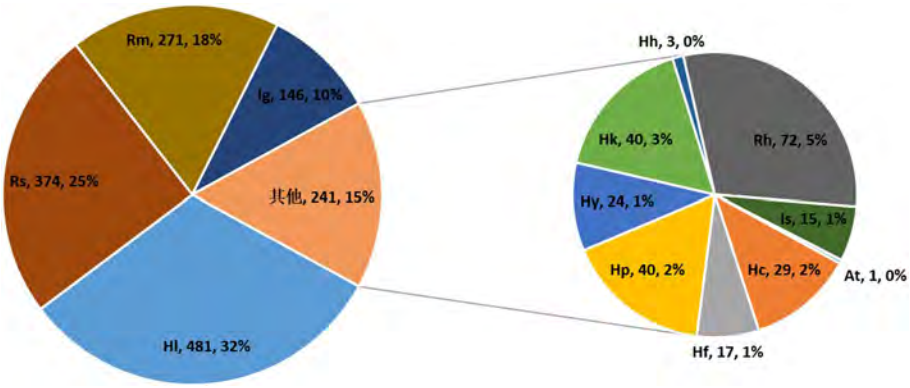

Fig. 2 Species composition of ticks infesting hosts. Hl, *H. longicornis*; Hc, *Haemaphysalis campanulata*; Hf, *Haemaphysalis flava*; Hp, *Haemaphysalis phasiana*; Hy, *H. yeni*; Hk, *H. kitaoka*; Hh, *Haemaphysalis hystricis*; Rs, *R. sanguineus* (s.l.); Rh, *Rhipicephalus haemaphysaloides*; Rm, *Rhipicephalus microplus*; Ig, *Ixodes granulatus*; Is, *I. sinensis* and At, *Amblyomma testudinarium*

Table 3 Geographical distribution of tick species in Jiangxi Province

Locality	Hl	Hc	Hf	Hp	Hy	Hk	Hh	Rs	Rh	Rm	Ig	Is	At	Da
HK			+	+				+						+
WUN	+												+	
DC	+													
WY	+													
JA	+				+	+								
PY	+													
NC	+	+	+	+	+			+	+	+	+	+		
WAN	+							+						
YC	+					+				+	+			
XY	+													
XG	+									+				
AY	+						+			+				
Jiangxi	+	⊞	+	+	⊞	⊞	+	+	+	+	+	+	⊞	+

+, denotes tick species were recognized in the locality. Cross with a box indicates newly recorded tick species in Jiangxi Province

Discussion and conclusion

Haemaphysalis longicornis is a kind of hard tick species, widely distributed in East Asia, Australia and New Zealand, and sometimes can be found in northernmost regions of Primorye (Northeastern USSR) and Hokkaido of Japan (Hoogstraal et al. 1968). In 2010, Chen et al. reported that *H. longicornis* was widely distributed tick species in various regions of China except for Jiangxi Province (Chen et al. 2010). In 2017, Xu et al. informed us of six newly reported tick species of Jiangxi, including *H. longicornis* (Xu et al. 2017). Information about *H. longicornis* presence in Jiangxi Province mirrors either previous incomplete

Table 4 Tick population densities on different hosts

Host	Hosts detected	Ticks on the hosts	Ticks per a host
Rodentia	1781	145	0.1
C. familiaris	171	341	2.0
P. colchicus	7	97	13.9
Erinaceidae	7	29	4.1
L. sinensis	21	341	16.2
B. bubalis	14	6	0.4
Bos spp	48	333	6.9
C. aegagrus hircus	85	129	1.5
O. aries	17	92	5.4
Total	2151	1513	0.7

tick investigation in the region or *H. longicornis* distribution expansion in China. Therefore, we performed the whole region tick investigation to obtain more data on tick species and their distribution in the region. Finally, we found that *H. longicornis*, the dominant species in the region, frequently collected in 11 sampling sites ranging from northern and southern part of Jiangxi Province, and found in three habitat categories and on *L. sinensis*, *P. colchicus*, *Bos* spp. and *C. familiaris* and, occasionally, on *O. aries, C. aegagrus hircus*, Erinaceidae and *B. bubalis*, which is in agreement with a former study conducted in some place of the region (Xu et al. 2017). Our findings are also consistent with what was previously reported in South Korea and Western Japan (Iwakami et al. 2014; Park et al. 2014). *Haemaphysalis longicornis* is easily observed in bush habitat and on cattle host. In our study, a large number of *H. longicornis* ticks were collected by flagging over a grassland of Nanchang, with some bushes growing in the grassland. In addition, we found that *H. longicornis* frequently infested Chinese hares not cattle in large number. Chinese hares, similar to the brown hare in New Zealand (Heath et al. 1987), range extensively and move long distance in open land including grassland, shrubs and forestland to facilitate *H. longicornis* dispersion. Cattle in sampling sites was grazed in good sanitary condition and regularly treated with some acaricides, which majorly reduced possibility of presence of *H. longicornis* on this host. However, above results were based on our primary study and need further verification under investigation of more numerous cattle and hares in the future.

Except *H. longicornis*, there was another abundant tick species *R. sanguineus* s.l. Previous data reported that *R. sanguineus* s.l. was confined to Nanchang, and infested *C. aegagrus hircus* and *L. sinensis* (Xu et al. 2017). Our study verified additional distribution of the species in Hukou and Wannian, its presence in woodland, grassland and shrubs, and more parasitizing *C. familiaris*, *P. colchicus*, *O. aries* and *Bos* spp. Canidae are hosts of all stages of *R. sanguineus* s.l. (Guglielmone et al. 2014), however, we only found female and male *R. sanguineus* s.l. on *C. familiaris*, albeit more than 250 ticks were collected. We mainly investigated tick infestation on *C. familiaris* in May–June in Jiangxi Province, and this term corresponded with mating time of the species in the region.

Previous studies indicate that adults (females and males) of some tick species feed mainly on medium- and large-sized animals, and the most important hosts for immature stages (larvae and nymphs) are small-sized mammals (Yu et al. 2011; Zheng et al. 2012). *L. sinensis*, *C. familiaris*, *P. colchicus*, *O. aries* and *Bos* spp. represent the main hosts for *H. longicornis* and *R. sanguineus* s.l. ticks in the current area investigated. Immatures failed to

Table 5 The compositions of ticks at different developmental stages on hosts

Tick genus	Tick species	Hosts	Larvae	Nymphs	Males	Females
Haemaphysalis	*longicornis*	*C. familiaris*	0 (0)[a]	0 (0)	25 (56.8)	19 (43.2)
		C. aegagrus hircus	0 (0)	10 (40)	5 (20)	10 (40)
		Bos spp.	0 (0)	0 (0)	22 (45.8)	26 (54.2)
		B. bubalis	0 (0)	0 (0)	0 (0)	1 (100)
		L. sinensis	283 (91.9)	13 (4.2)	3 (1.0)	9 (2.9)
		P. colchicus	0 (0)	40 (93.0)	3 (7.0)	0 (0)
		Erinaceidae	0 (0)	5 (100)	0 (0)	0 (0)
		O. aries	0 (0)	0 (0)	6 (85.7)	1 (14.3)
		Sub-total	283 (58.8)	68 (14.1)	64 (13.3)	66 (13.7)
	campanulata	*C. familiaris*	0 (0)	8 (38.1)	2 (9.5)	11 (52.4)
		C. aegagrus hircus	0 (0)	0 (0)	0 (0)	1 (100)
		Erinaceidae	0 (0)	0 (0)	4 (57.14)	3 (42.9)
		Sub-total	0 (0)	8 (27.6)	6 (20.7)	15 (51.7)
	flava	Erinaceidae	0 (0)	0 (0)	7 (41.2)	10 (58.8)
	phasiana	*P. colchicus*	0 (0)	0 (0)	13 (32.5)	27 (67.5)
	yeni	*C. aegagrus hircus*	0 (0)	4 (26.7)	6 (40)	5 (33.3)
		O. aries	0 (0)	0 (0)	0 (0)	9 (100)
		Sub-total	0 (0)	4 (16.7)	6 (25)	14 (58.3)
	kitaoka	Goat	0 (0)	40 (100)	0 (0)	0 (0)
	hystricis	*Bos* spp	0 (0)	0 (0)	2 (66.7)	1 (33.3)
Rhipicephalus	*sanguineus* s.l.	*C. familiaris*	0 (0)	0 (0)	129 (48.5)	137 (51.5)
		C. aegagrus hircus	0 (0)	0 (0)	0 (0)	1 (100)
		Bos spp.	0 (0)	0 (0)	4 (30.77)	9 (69.2)
		L. sinensis	0 (0)	1 (25)	3 (75)	0 (0)
		P. colchicus	14 (100)	0 (0)	0 (0)	0 (0)
		O. aries	0 (0)	0 (0)	48 (63.2)	28 (36.8)
		Sub-total	14 (3.7)	1 (0.3)	184 (49.2)	175 (46.8)
	haemaphysaloides	*C. familiaris*	0 (0)	0 (0)	8 (80)	2 (20)
		C. aegagrus hircus	0 (0)	0 (0)	1 (50)	1 (50)
		Bos spp.	0 (0)	0 (0)	13 (29.6)	31 (70. 5)
		L. sinensis	0 (0)	0 (0)	3 (18.8)	13 (81.3)
		Sub-total	0 (0)	0 (0)	25 (34.7)	47 (65.3)
	microplus	*C. aegagrus hircus*	15 (34.1)	16 (36.4)	7 (15.9)	6 (13.6)
		Bos spp.	24 (10.8)	67 (30.0)	44 (19.7)	88 (39.5)
		B. bubalis	0 (0)	4 (100)	0 (0)	0 (0)
		Sub-total	39 (14.4)	87 (32.1)	51 (18.8)	94 (34.7)
Ixodes	*granulatus*	*Bos* spp.	(0)	(0)	(0)	1 (100)
		Rodentia	11 (7.6)	41 (28.3)	42 (29.0)	51 (35.2)
		Sub-total	11 (7.5)	41 (28.1)	42 (28.8)	52 (35.6)

Table 5 (continued)

Tick genus	Tick species	Hosts	Larvae	Nymphs	Males	Females
	sinensis	*C. aegagrus hircus*	0 (0)	0 (0)	1 (100)	(0)
		Bos spp.	0 (0)	0 (0)	1 (100)	(0)
		L. sinensis	0 (0)	0 (0)	1 (7.69)	12 (92.3)
		Sub-total	0 (0)	0 (0)	3 (20)	12 (80)
Amblyomma	*testudinarium*	*B. bubalis*	0 (0)	0 (0)	1 (100)	0 (0)
Total			347 (22.9)	249 (16.5)	404 (26.7)	513 (33.9)

[a]In parentheses are the percentages of ticks at specific developmental stages

attack large-sized animal like *Bos* spp. Interestingly, overwhelming matures did not attach themselves to small-sized animal like *P. colchicus*. However, both matures and immatures can be simultaneously found on small- and medium-sized animals such as *Lepus sinensis. Haemaphysalis longicornis* and *R. sanguineus* s.l. follow a three-host life cycle. Immature ticks use these hosts only for feeding, while the adults may also apply hosts for seeking a mating partner. The different behavior might lead to more host-specific feeding, obviously adults for large-sized hosts and immatures for small-sized hosts. In addition, adult ticks have larger surface area to volume ratios and are therefore less sensitive to water stress, a major cause of mortality in smaller immature ticks. The difference in desiccation resistance is reflected by the position where adults and immatures quest for a host. Immatures usually stay somewhere near ground where vegetation covers create high humid environment and small-size animals are more available. Whereas, adults find potential hosts in higher vegetation layers, where they may miss small-sized host species (Esser et al. 2016).

Of the selected 12 sampling sites for tick investigation, there were 10 tick species found in Nanchang city, accounting for five-seventh of the total tick species detected. During 2011–2018, we performed the investigations annually in Nanchang, however, twice in Yichun and once in the remaining sampling sites because of lack of human, material and financial support. Lower tick species biodiversity may be explained by incomplete investigation in areas outside Nanchang city. Therefore, sampling effort should be increased for tick surveillance in other regions of Jiangxi Province to enrich the current data on tick species and their distribution in those regions.

Ticks can transmit various pathogen including viruses, protozoan parasites and bacteria. Yu et al. listed 51 important vector hard tick species in China in 2015, including 10 tick species found in this study, such as *I. granulatus, I. sinensis, A. testudinarium, H. longicornis, H. flava, H. kitaokai, H. yeni, D. auratus, R. microplus* and *R. sanguineus* s.l. *Haemaphysalis longicornis* is the vector of New Bunyavirus and *I. granulatus* can transmit *Borrelia burgdorferi*, both of which are greatly important pathogens to human health (Yu et al. 2015). Therefore, inhabitants in the region should reduce exposure to rodent hosts for *I. granulatus* in residential surrounding, and tourists and workers should keep away from hare hosts for *H. longicornis* in the field, in order to achieve the goal of preventing the bite of the two tick species and controlling severe fever with thrombocytopenia syndrome (SFTS) and Lyme disease, which were formerly reported in Jiangxi Province (Fang et al. 2015).

In conclusion, this study has preliminarily shown that like other regions in China, Jiangxi may have abundant tick populations. The results presented in this study highlights

tick species composition and their distribution in Jiangxi through an 8-year-long study. However, more tick investigations are needed in the areas outside Nanchang city for creating a full view of tick population distribution in Jiangxi. Further works regarding tick-borne pathogens detection are recommended in order to better understand the risk posed by the presence of tick populations in Jiangxi to human health and animal production.

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成績状況	(優) 良　可　不可 学業成績係数=	取得単位数 取得単位数 4／取得すべき単位総数 4
学生本人が行った研究の概要	劉氏は、高齢ドライバーの交通事故発生要因を明らかにするため、高齢者のフレイル（虚弱）状態と交通事故発生率の関連性について検討をおこなった。地域在住高齢運転者 349 名（平均年齢 74.4±5.0 歳）を対象として検討した結果、フレイルを有する高齢運転者は身体・認知機能が低い水準にあり、さらには交通事故発生率が有意に高いことが明らかとなった（国際誌へ投稿中）。さらに現在は、地域在住高齢者 4500 名を対象とした郵送調査を完了しており、高齢者のフレイル状態と交通事故発生率との関連について、より強固なエビデンスを報告できるよう検討中である。	
総合評価	【良かった点】 劉氏の研究は、近年社会問題となっている高齢ドライバーの交通事故予防に寄与しうるという点で優れている。さらに、フレイル状態が高齢運転者の交通事故発生に及ぼす影響を明らかにすることができれば、高齢者の重大な健康問題の 1 つであるフレイルを予防することの意義を更に強く主張できるようになる。また、我が国における高齢ドライバーを対象とした研究は未着手領域が多いことから、その先駆け的な研究となりうる可能性を秘めている。 【改善すべき点】 フレイル状態と交通事故発生の関連性について縦断的に検討すること、および、その関連性の間に介在する要因を詳細的に検討することが必要であると考えられる。 【今後の展望】 現在、劉氏は完了した郵送調査のデータを解析中であり、今後国際誌に投稿予定である。さらに、当研究室としては 2020 年度に追跡調査を実施する予定であり、劉氏自身がフレイル状態と交通事故発生の縦断的な関連性を明らかにする。	
学位取得見込	現在、1 編目の原著論文を国際誌へ投稿中であること、さらに博士論文の中核となるデータはすでに取得済みであり、2 編目の原著論文も 2020 年中に投稿を予定していることから、今後 3 年以内に博士（体育科学）の学位を取得できる可能性が高いと考える。	
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研究テーマ	Frailty phenotype associated with traffic crashes among older drivers: A cross-sectional study in rural Japan				

専攻種別	論文博士	□	課程博士	☑

1．研究概要(1)

As the older adult population rapidly increases in Japan, the number of licensed drivers aged 65 and older has reached approximately 18.2 million, which is 51.7% of the aged population. Consequently, although road traffic crashes in Japan have reportedly decreased, the percentage caused by older drivers—especially those aged 75 years and older—rises annually. Therefore, prevention strategies are urgently needed to reduce the number of traffic crashes and protect public safety among the aged population. Another urgent issue associated with the rapidly aging population is the health problem known as frailty. A consensus of medical professionals has defined physical frailty as a medical syndrome featuring diminished strength, endurance, and reduction of physiologic function. Due to multiple possible causes and contributors, physical frailty leads to increased vulnerability of individuals, high risk of dependency on others, and/or death. However, few studies have examined the association between frailty and traffic crashes among older drivers.

1）目的（Goal）

This cross-sectional study aimed to investigate the association between frailty phenotype and self-reported traffic crashes in the past year among Japanese community-dwelling older adults.

2）戦略（Approach）

The present study focused on older drivers living in Kasama (population 74,481, area 240.4 km2, proportion of older adults 31.6% in 2019), a rural area in Japan, to provide some evidence for research related to traffic crashes among Japanese community-dwelling older adults.

3）材料と方法（Materials and methods）

3.1 Participants

This cross-sectional study sampled 429 community-dwelling older adults enrolled in the Kasama Health Checkup for Longevity survey, which has been conducted annually since 2009. Data in the present study were obtained from the 2019 Kasama Health Checkup for Longevity survey. Inclusion criteria were as follows: aged ≥ 65 years, being a resident of Kasama, and not using long-term care insurance. We excluded participants who did not hold a valid driver's license, did not drive a car (n = 73), or drove less than once per week on average (n = 4). We also excluded three participants due to missing data on driving frequency (n = 1) and frailty measure (n = 2). Finally, data from 349 older drivers (81.4%) were analyzed. This study was approved by the Ethical Committee of the University of Tsukuba (Ref No., Tai 30-5). We explained the study's concept to participants and obtained their written informed consent.

3.2 Frailty phenotype

In the present study, five components of frailty phenotype were defined according to Fried's phenotype, using the Japanese version of the Cardiovascular Health Study criteria: (1) weakness, grip strength < 26 kg in men or 18 kg in women; (2) shrinking, answering "yes" to "Have you lost 2 kg or more in the past 6 months;" (3) slowness, gait speed < 1.0 m/s; (4) low physical activity, answering "no" to both "Do you engage in moderate levels of physical exercise or sports aimed at health?" and "Do you engage in low levels of physical exercise aimed at health;" and (5) exhaustion, answering "yes" to "In the past two weeks, have you felt tired without a reason?" Frailty, pre-frailty, and robustness were defined as having scores of 3-5, 1-2, or 0, respectively.

3.3 Traffic crashes and risky driving behaviors

Participants' recent traffic crashes were counted using a self-report yes/no question: "Have you been involved in a traffic crash in the past year when you were the driver?" Another two self-report yes/no questions were used to measure risky driving behaviors: "Have you been stopped by the police for a traffic violation in the past year when you were the driver" and "Have you been involved in a near-miss traffic incident in the past year when you were the driver?"

Physical and cognitive function were evaluated using performance-based measures, the Geriatric Depression Scale (GDS) was used to evaluate depressive symptoms.

3.4 Statistical analysis

All participants were categorized by frailty phenotype as follows: frailty, pre-frailty, and robustness. We performed a one-way analysis of variance for continuous variables, and Chi-squared tests or Fisher's exact tests for qualitative variables, to determine differences in demographics variables, health status, and driving habits across frailty phenotypes. We performed an analysis of covariance (ANCOVA) to assess physical function, cognitive function, depressive symptoms, and risky driving behaviors. Covariates included age, sex, economic status (poor or normal/good), clinical history of low back pain (yes or no), and number of medications. Logistic regression analysis before

4)実験結果(Results)

There were six older drivers (1.7%) categorized as frail, 149 (42.7%) as pre-frail, and 194 (55.6%) as robust. Since only six older drivers were categorized as frail, they were grouped with pre-frail older drivers for comparison against the robust older drivers. There were no significant differences between frailty phenotype and most demographic and health status variables such as age, sex, body mass index, education, living arrangements, smoking, and alcohol consumption. However, pre-frail/frail participants were more likely to have a poorer economic status, more clinical history of low back pain, and use more medications, compared to those who were robust. Additionally, more than 93% of all participants reported limitations on their driving, usually due to a physical problem (pre-frail/frail 84.5%; robust 86.1%) and being unable to drive at night (pre-frail/frail 76.1%; robust 79.4%). However, there were no significant differences between the two groups for other driving habit variables, including driving frequency and distances. Compared to robust participants, pre-frail/frail participants demonstrated poorer physical function, scored lower on total five cognitive functions test (5-Cog), and were more prone to depression. Additionally, pre-frailty/frailty (34.2%) was significantly associated with near-miss traffic incidents, compared to robustness (18.0%). Moreover, 46 participants reported involvement in traffic crashes in the past year (33 of 46 were pre-frail/frail). Before adjustment, pre-frailty/frail participants were found more likely than robust participants to be involved in traffic crashes (OR = 3.77, 95% CI: 1.91-7.45). After adjusting for age, sex, total 5-Cog scores, GDS scores, driving distances, and near-miss traffic incidents, pre-frailty/frailty was still associated with traffic crashes, compared to robustness (OR = 3.74, 95% CI: 1.75-7.96).

5）考察（Discussion）

In the present study, the Japanese version of the Cardiovascular Health Study criteria, based on Fried' s frailty phenotype, was used to measure frailty phenotype, which is considered to be simple and suited to Japanese older adults. A total of 155 older adult (44.4%) participants were categorized as pre-frail/frail (pre-frailty n = 149, 42.7%; frailty n = 6, 1.7%) and 194 were (55.6%) categorized as robust. Logistic regression analysis results of the association between frailty phenotype and traffic crashes showed that pre-frail/frail participants had 3.74 times more traffic crashes in the past year, compared to robust participants, even after adjusting for age, sex, total 5-Cog scores, GDS scores, driving distances, and near-miss traffic incidents. These results are consistent with findings of a previous study which demonstrated that pre-frailty increased the odds of traffic crashes compared to non-frailty (OR = 1.3, 95% CI:1.0-1.8; Man et al., 2019). However, the different odds of traffic crashes between the present study and the previous study (Man et al., 2019) might be explained by the different percentages of participants reporting traffic crashes across frailty phenotypes. In the present study, 6.7% of robust participants and 21.3% of pre-frail/frail participants (pre-frailty 20.8%; frailty 33.3%) had been involved in traffic crashes in the past year, whereas in the previous study, it was 9.7% of non-frail, 12.2% of pre-frail, and 13.6% of frail participants. Thus, the percentages of crash-involved older drivers categorized by pre-frailty or frailty in the present study were much higher than in the previous study, which might have influenced results on the odds of traffic crashes. It is necessary to explain why the difference in percentages of participants reporting traffic crashes appeared across frailty phenotype in the further research. Whether there are significant differences in the comprehensive characteristics of participants between the present and previous study should also be considered, as it may also be due to social differences between older drivers in Japan and in other countries.

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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					
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 2 Title					
掲載誌名 Published journal					
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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					
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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 meetings

学会名 Conference	第73回日本体力医学会大会				
演 題 Topic	複合運動プログラムが地域在住高齢者の認知機能に与える影響：認知機能水準別の検討				
開催日 date	2018 年 9 月 9 日	開催地 venue	日本・福井		
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	藤井啓介，井上大樹，薛載勲，藤井悠也，城賓佳也，大藏倫博				
学会名 Conference	第74回日本体力医学会大会				
演 題 Topic	身体的フレイルを有する高齢運転者の身体・認知機能および交通事故経験率				
開催日 date	2019 年 9 月 21 日	開催地 venue	日本・つくば		
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	藤井悠也，藤井啓介，薛載勲，立岡光臨，大藏倫博				
学会名 Conference	11th IAGG asia/Oceania Regional Congress				
演 題 Topic	Differences Between Frail and Healthy Elder Drivers in the Relationship of Falls to Automobile Accidents in Japan				
開催日 date	2019 年 10 月 24 日	開催地 venue	台北		
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	Fujii Y, Fujii K, Seol JH, Okura T				
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 Year of award	年 月
名 称 Award name				
	国名 Country		受賞 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

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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			
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） 大蔵倫博

日中笹川医学奨学金制度(学位取得コース)評価書
課程博士：指導教官用

Supported by 日本財団 THE NIPPON FOUNDATION

第 40 期　研究者番号：G4003　作成日：2020 年 3 月 日

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研究テーマ	肺がんに対する免疫療法の研究 Immunotherapy of Lung Cancer				
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 取得単位数 24 ／取得すべき単位総数 34
学生本人が行った研究の概要	（1）肺癌免疫療法に関する基礎的研究：マウスに肺癌細胞株 LLC を皮下移植するモデルを用いた基礎研究を行った。LCC 細胞の全エクソーム解析から得られた多数のネオアンチゲン候補の短鎖または長鎖ペプチドによる多種の癌ワクチンならびに免疫チェックポイント阻害薬(anti-PDL1, anti-HTLA-4)の抗癌作用について検討した。得られた結果について２つの国内学会で発表した。(第 23 回日本がん免疫学会総会(2019.8.21 高知市))(第 17 回日本免疫治療学会(2020.2.22 東京都)) （2）当科で手術治療を行った原発性肺癌患者について後方視的観察研究を行った。肺癌術後生存率と、体幹の骨格筋量との関連を調べ、骨格筋量が少ないサルコペニア状態では生命予後が悪いことを明らかにし、下記の論文を著し、さらに２編の論文を投稿中である。 文献： Sun C, Nakajima J, et al. Eur J Cardiothorac Surg 2019 55: 414-420 PMID 30289481	
総合評価	【良かった点】 非常に仕事量の多いマウスを用いた免疫実験を着実にこなされた。また、その合間に臨床観察研究も行い、呼吸器外科関連の一流海外英文論文誌に掲載されたこと 【改善すべき点】 特になし。 【今後の展望】 上記（1）の研究成果を論文化する。内容がよろしければ博士論文として認められる可能性がある。３年目からは貴財団からの助成を受けられないが、引き続き東大で研究に従事する予定	
学位取得見込	通常はあと２年必要。(来年度第３学年だが)特例で大学院３年次に取得できる可能性もある（が稀)。	
評価者（指導教官名） 中島　淳 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：G4003　　作成日：2020年3月9日

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研究先(指導教官)	東京大学大学院　医学系研究科　呼吸器外科　（　中島　淳　教授　）				
研究テーマ	肺がんに対する免疫療法の研究 Immunotherapy of Lung Cancer				
専攻種別	論文博士	☐	課程博士	☑	

1．研究概要（1）

1）目的（Goal）

Immune checkpoint inhibitors are now the mainstay for treatment of lung cancer. However, so-called cold tumors are known to be resistant to therapy. Neoantigens associated with genetic mutations of the tumor have the potential to stimulate a potent and highly specific immune response. To develop effective immunotherapy for cold lung cancer, we examined whether neoantigen-based immunotherapy can develop a robust immune response and display an anti-tumor effect on Lewis lung carcinoma cells (LLC1).

2）戦略（Approach）

Screen immunologic peptides

⬇

Examine anti-tumor effect of peptide candidates

⬇

Develop effective neoantigen-screening strategy

⬇

Explore robust neoantigen-based immunotherapy

3）材料と方法（Materials and methods）

Animal and cell lines

Six-week-old female C57BL/6 mice were purchased from Japan LC (Shizuoka, Japan). Mice were kept in a specific pathogen-free environment. The LLC cells were obtained from the ATCC.

Peptide vaccine

Whole exome sequencing and RNA sequencing were performed in LLC1. Neoantigens were predicted and prioritized by their affinity to MHC class I molecules and their expression. Then, the neoantigen candidates [short peptides (8 to 10mer), or long peptides (21 mer)] were synthesized.

Immunogenicity

Dendritic cells from C57BL/6 bone marrow were cultured for 8 days with GM-CSF and 10% FBS/RPMI and maturated with neoantigen candidates for 4 hours on day 9 before injected with 1*10^6/mouse dendritic cells subcutaneously. Naive mice were vaccinated twice on 2-week intervals, and immune responses were measured with mouse blood and splenocytes using intracellular staining and ELISA after 2 weeks of second vaccination.

Anti-tumor effect

LLC cells (0.5*10^6) were implanted into the right flank of C57BL/6 subcutaneously after twice peptide immunization. Tumor diameter was measured twice weekly and used to calculate tumor volume(mm3) (length*width*height*π/6). Finally, the neoantigen with anti-tumor effect were screened.

１．研究概要（2）

Neoantigen-based immunotherapy

The immune microenvironment of LLC-1 was investigated by immunohistochemical staining, flow cytometry and tumor genomics. With the immune microenvironment knowledge and dynamic change information after treatment, neoantigen-based combinative therapy with immune checkpoint inhibitors and/or immune modulators are conducted.

4）実験結果（Results）

Sixty mutated peptides with netMHC score (IC50) <200 nM and 68 mutated peptides with IC50>200 nM but the ratio of wild to corresponding mutated type peptide in netMHC score>10 were selected as candidate neoantigens. Twenty-five out of 128 short mutated peptides induced peptide-specific CD8+ T cell response to some extent. Immunized mice were then inoculated with LLC1 tumor cells: however, significant antitumor responses were not observed in mice received these single short peptide vaccines.

Next, 25 long peptides (21mer), which incorporated corresponding immunogenic short peptide sequences, were synthesized and used to immunize mice for the evaluation of their antigenicity. DC vaccines pulsed with neoantigen in long peptide format induced both CD4+ and CD8+ T cell response ex vivo. Of them, DC pulsed with long peptide 58, 82 partly inhibited the LLC1 growth in vivo. The anti-tumor effect was further enhanced when PD-L1 and CD38 blockades were administered in mice immunized with DC pulsed with long peptide 82.

5）考察（Discussion）

Our study suggested that the selection or prioritization of neoantigens that can induce anti-tumor response is critical for the development of neoantigen-targeting immunotherapy. A combination of neoantigen vaccines and immune modulators could enhance their anti-tumor activities.

6）参考文献（References）

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[5]Hu Z, Ott PA, Wu CJ. Towards personalized, tumour-specific, therapeutic vaccines for cancer. Nat Rev Immunol 2018;18:168-82.

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	Low truncal muscle area on chest computed tomography: a poor prognostic factor for the cure of early-stage non-small-cell lung cancer					
掲載誌名 Published journal	European Journal of Cardio-Thoracic Surgery					
	2019 年 3 月	55 巻(号)	414 頁 ～ 420 頁	言語 Language	English	
第1著者名 First author	Changbo Sun	第2著者名 Second author	Masaki Anraku	第3著者名 Third author	Takahiro Karasaki	
その他著者名 Other authors	Hideki Kuwano, Kazuhiro Nagayama, Jun-Ichi Nitadori, Masaaki Sato, Jun Nakajima					
論文名 2 Title	Impact of preoperative low pectoralis muscle on the outcome in non-small cell lung cancer (Annals of Thoracic Surgery, submitted)					
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	Peak expiratory flow rate and pectoralis muscle area: two critical factors for preoperative sarcopenia assessment in non-small cell lung cancer (CHEST, submitted)					
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 meetings

学会名 Conference	第112回臨床呼吸生理研究会学術集会				
演 題 Topic	Impact of preoperative pectoralis muscle quantity and density on outcome after complete resectionof non-small cell lung cancer				
開催日 date	2018 年 6 月 20 日	開催地 venue	Tokyo		
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	European Society of Thoracic Surgery Annual Meeting				
演 題 Topic	Assessment of preoperative pectoralis muscle and truncal muscle on chest computed tomography: Towards precise prognostic stratification of resected non-small cell lung cancer				
開催日 date	2019 年 6 月 9 日	開催地 venue	Dublin		
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	第23会日本がん免疫学会総会				
演 題 Topic	The neoantigen landscape of murine lung cancer LLC-1 model				
開催日 date	2019 年 8 月 21 日	開催地 venue	高知市		
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	第17回日本免疫治療学会学術集会				
演 題 Topic	The neoantigen landscape of murine lung cancer LLC-1 model				
開催日 date	2020 年 2 月 22 日	開催地 venue	Tokyo		
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	第112回臨床呼吸生理研究会奨励賞			
	国名 Country	日本	受賞年 Year of award	2018 年 6 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 R eceiptrecord	■ 有　□ 無
助成機関名称 Funding agency	東京大学
助成金名称 Grant name	2019 年度博士課程研究遂行協力制度
受給期間 Supported period	2019 年 8 月 ～ 2020 年 1 月
受給額 Amount received	300,000 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 R eceiptrecord	□ 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 R eleasemethod	・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）　中島 淳

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Low truncal muscle area on chest computed tomography: a poor prognostic factor for the cure of early-stage non-small-cell lung cancer†

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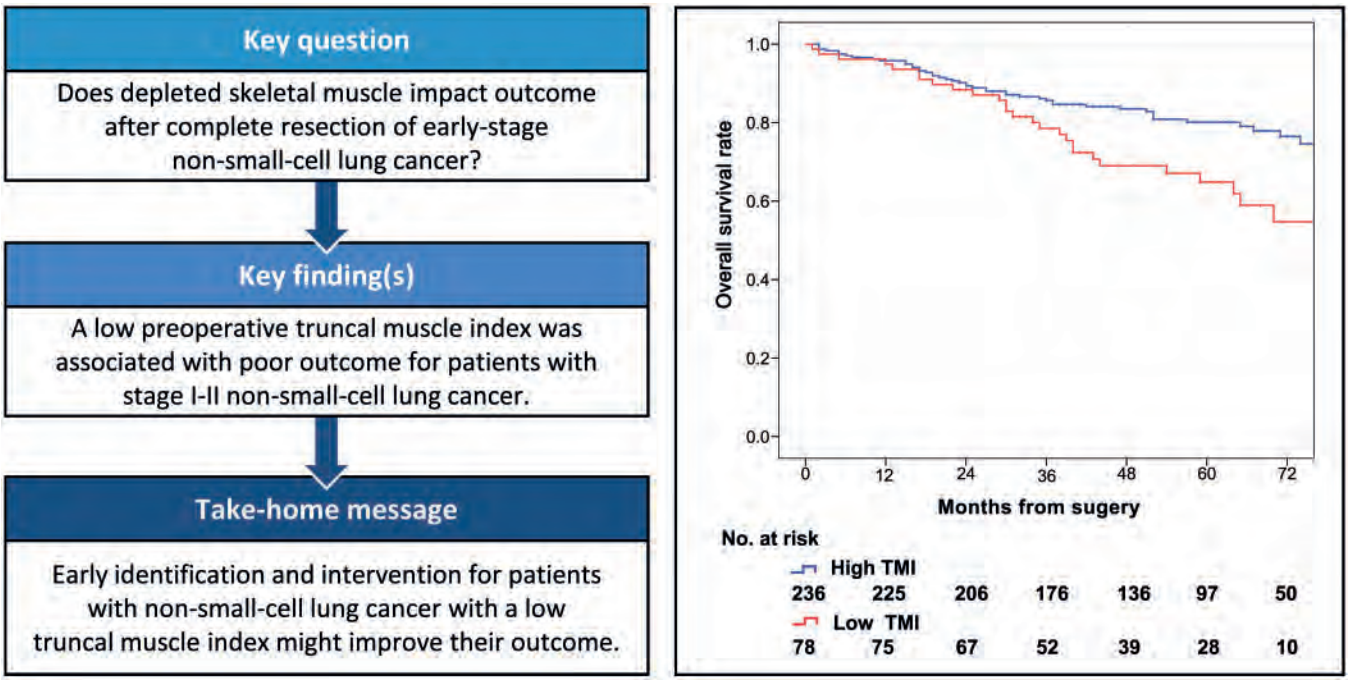

Abstract

OBJECTIVES: Depletion in skeletal muscle is closely associated with limited physical ability and high mortality. In this study, we evaluated the prognostic significance of skeletal muscle depletion in patients with early-stage non-small-cell lung cancer.

METHODS: A retrospective analysis of patients with pathological stages I–II lung cancer, who underwent curative resection between 2009 and 2013, was conducted. The truncal muscle index (TMI) (area/height^2) at the first lumbar vertebral level was measured by preoperative axial computed tomography. Overall survival and recurrence-free survival were compared between the lowest gender-specific quartile of the TMI and the other quartiles.

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RESULTS: A total of 314 subjects were included in the study. The cumulative 5-year recurrence-free and overall survival rates were significantly shorter in patients with lower TMIs (69% vs 83.5%, $P = 0.028$; 64.8% vs 80.1%, $P = 0.003$, respectively). In multivariable models, the TMI was identified as an independent prognostic factor for overall survival ($P = 0.017$, hazard ratio 1.84, 95% confidence interval 1.12–3.05), after adjusting for age, gender, preoperative serum albumin, carcinoembryonic antigen, neutrophil to lymphocyte ratio and pathological stage.

CONCLUSIONS: A low preoperative TMI was associated with a poor postoperative outcome in patients with early-stage non-small-cell lung cancer. This factor may be included in the preoperative assessment of patients, for whom surgical intervention is considered.

Keywords: Skeletal muscle depletion • Truncal muscle index • Early-stage non-small-cell lung cancer • Prognostic factor

INTRODUCTION

Lung cancer is one of the most frequently diagnosed cancers and one of the leading causes of cancer mortality in the world [1]. The overall survival (OS) of patients with early-stage non-small-cell lung cancer (NSCLC; stages I–II) is distinctly better than that of patients with advanced lung cancer. However, the postoperative prognosis is poor for some patients with early-stage NSCLC (stages I–II). It refers to both cancer-specific factors and individual patient characteristics. Poor survival rates due to pathological subtypes or systemic inflammation were reported in patients with early-stage NSCLC (stages I–II) undergoing curative surgery [2–4].

Sarcopenia—the loss of muscle mass and function—has been clinically identified as a poor predictor [5, 6]. Sarcopenia contributes to functional decline, disability, injury and mortality. The link between sarcopenia and poor prognosis was first reported in patients with non-malignant diseases and in geriatric populations. Recently, the clinical importance of sarcopenia has also been increasingly recognized in oncological patients [7, 8]. Low skeletal muscle—a key and objective component of sarcopenia—was investigated, and the results indicated a close association with limited physical ability and high mortality in advanced cancers [9]. However, the correlation between low skeletal muscle and the prognosis of early-stage NSCLC is not well understood.

The present study investigated the truncal muscle area on chest computed tomography (CT) to determine the impact of skeletal muscle mass depletion on the prognosis of patients with stages I–II NSCLC undergoing curative surgery.

MATERIALS AND METHODS

Patients

A retrospective analysis of patients with stages I–II NSCLC who underwent lobectomy and mediastinal lymph node dissection at the University of Tokyo Hospital (Tokyo, Japan) from January 2009 to December 2013 was conducted. Eligible patients had pathological stages I–II NSCLC following surgery. Preoperative (i.e. within 90 days prior to surgery) chest CT images of the study population were available for review (Fig. 1).

Data collection

Data collected from inpatient and outpatient records included demographics [age, gender, body mass index (BMI)], blood count and serum biochemical data for a week prior to the operation [leukocytes, neutrophils, lymphocytes, serum albumin (Alb) and C-reactive protein], tumour-specific data [carcinoembryonic antigen (CEA)], postoperative complications based on the extended Clavien–Dindo classification (see Supplementary Material, Table S1) [10], pathological data [histology and tumour, node and metastasis (TNM) staging according to the 7th UICC-TNM classification] and survival data including recurrence-free survival (RFS) and OS. RFS was defined as the period from the date of surgery to that of first recurrence or death. OS was defined as the period from the date of surgery to that of death (by any cause) or lost follow-up. All patients provided written informed consent prior to the analyses.

Chest CT examinations were performed using a 64-detector CT (Aquilion ONE Vision Edition Aquilion PRIME, Toshiba, Japan or Discovery CT750 HD, General Electric, USA) with a 5-mm slice thickness. The patients were requested to maintain a supine position with raised arms and were asked to hold their breath at deep inspiration during the chest CT examination. The truncal muscle area at the first lumbar vertebral level (L1) was identified on a chest CT scan taken prior to surgery (Fig. 2A). The truncal muscle area, comprising the paraspinal muscles and chest–abdominal wall muscles, was plotted at the transverse process level of L1 [8] (Fig. 2B). The SYNAPSE VINCENT (Fujifilm Medical, Tokyo, Japan) image analysis software was used to define the skeletal muscle area semiautomatically. The skeletal muscle area was identified and quantified based on Hounsfield unit thresholds (−29 to +150) in square millimetres (mm^2) (Fig. 2C).

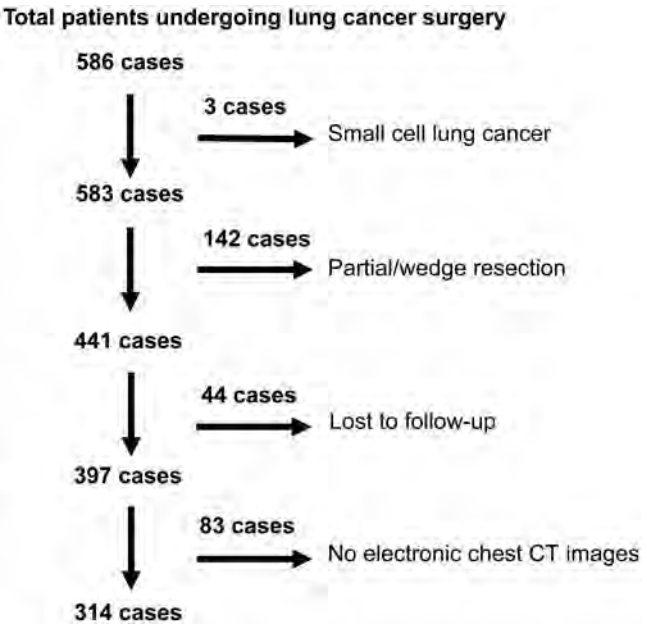

Figure 1: The study cohort. CT: computed tomography.

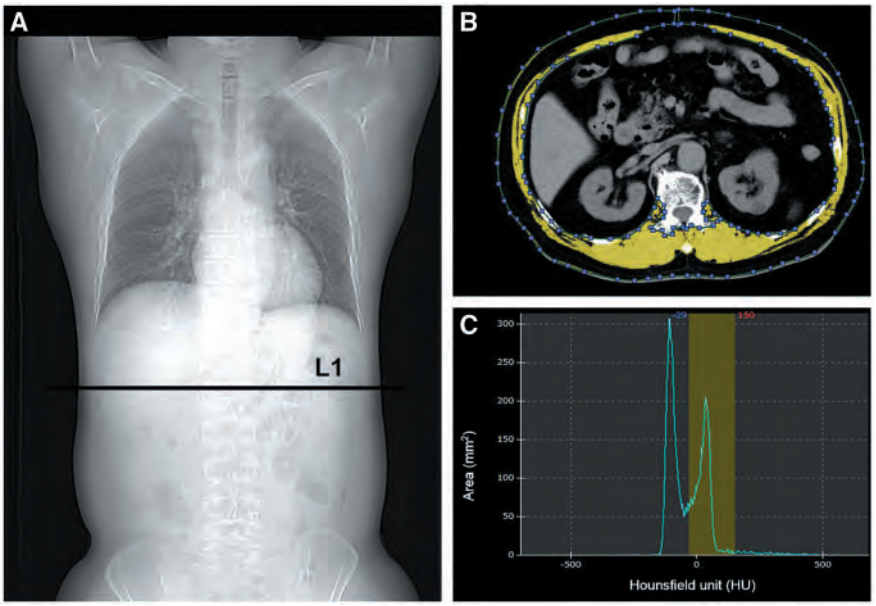

Figure 2: (**A**) Muscle area calculations at the process of the first lumbar vertebra (L1). (**B**) Truncal muscles consist of paraspinal muscles and chest–abdominal wall muscles at L1 (yellow area). (**C**) Truncal muscle area was identified and quantified based on Hounsfield unit thresholds (−29 to +150) in square millimetres (mm^2), with the exception of right wave indicating fat tissue area.

For the calculation of the truncal muscle index (TMI), the muscle area was divided by the square of height (m^2). The lowest quartile cut-off values of the TMI were used to divide patients into the low-TMI group and the high-TMI group in the study. Image analysis was performed without access to information on surgical outcomes to ensure unbiased measurements and calculations.

Statistical analysis

All statistical analyses were performed using the SPSS, version 22.0, software (IBM Inc., Armonk, NY, USA). All data are expressed as the median (interquartile range), with exception of age, which are presented as the mean [±standard deviation (SD)]. Gender and smoking status are showed as categorical data. Differences between groups were analysed using the Mann–Whitney *U*-test for continuous variables and Pearson's χ^2 test for categorical data. RFS and OS curves were plotted using the Kaplan–Meier method, and differences were compared using the log-rank test. Cox regression survival analysis was performed for the following factors: age, smoking history, BMI, Alb, neutrophil to lymphocyte ratio (NLR), CEA, pathological stage (p-TNM) and TMI. Variables with *P*-value <0.05 in the univariable analysis were also used for the multivariable analysis. Differences with *P*-value <0.05 were considered statistically significant.

RESULTS

Patient characteristics

The clinical and pathological characteristics of 314 patients with p-stages I–II NSCLC with a mean age of 68.1 ± 10.6 years included in this study are listed in Table 1. Of them, the majority had adenocarcinoma (227 patients, 72.3%), followed by squamous carcinoma (63 patients, 20.1%), large cell carcinoma (4 patients, 1.2%) and other NSCLCs (20 patients, 6.4%), as confirmed by histology. A total of 59 recurrences and 77 deaths were reported in the follow-up period.

The median TMI for men was 41.2 cm^2/m^2, whereas for women, the value was 33.4 cm^2/m^2. The indices were significantly higher in men than women ($P < 0.001$). The correlation between BMI and TMI was significant (Pearson's $r = 0.574$, $P < 0.001$). The median serum Alb, NLR and CEA were 4 g/dl, 2.2 and 4.3 μg/l, with Alb, NLR, CEA data of 1 patient, 35 patients and 6 patients missing, respectively. Accordingly, 274 patients were analysed in the multivariable analyses.

Clinicopathological factors and survival analysis

The TMI was significantly higher in men than in women ($P < 0.001$). Thus, patients were divided into the low-TMI and high-TMI groups based on the gender-specific lowest quartile cut-off values of the TMI (38 cm^2/m^2 for men and 29.6 cm^2/m^2 for women). As a result, 236 and 78 cases are included in the low-TMI and high-TMI groups, respectively. The comparison of clinicopathological factors between the low-TMI and high-TMI groups is shown in Table 1. Patients with the low TMI had a significantly lower BMI (median 20.1 vs 23 kg/m^2, respectively, $P < 0.001$) and Alb (<4 vs ≥4 g/dl, respectively, $P = 0.046$) than those with the high TMI. Additional factors such as age, smoking history, CEA, NLR, pathology distribution and p-TNM were not significantly different between the low-TMI and high-TMI groups. To identify any factors associated with low TMI, we performed the univariable analyses with age, smoking status, BMI, Alb, CEA, NLR and p-TNM. BMI, NLR and Alb were significant risk factors ($P < 0.001$, $P = 0.007$ and $P = 0.024$, respectively). The factors including BMI, Alb, NLR, age and p-TNM with *P*-value <0.25 in the univariable analyses were evaluated in a multivariable logistic analysis. Only BMI was a significant risk factor for TMI [$P < 0.001$, hazard ratio 1.49, 95% confidence interval (CI) 1.30–1.71].

Table 1: Clinical characteristics of the low TMI and high TMI groups

Category	Low TMI group ($n = 78$)	High TMI group ($n = 236$) (cm^2/m^2)	*P*-value
Gender, *n* (%)			0.89
Male	46 (59.0)	137 (58.1)	
Female	32 (41.0)	99 (41.9)	
Age (years), mean ± SD	72 ± 8.9	67 ± 11	0.073
<65	21 (26.9)	78 (33.1)	
≥65	57 (73.1)	158 (66.9)	
Smoking history, *n* (%)			0.20
Non-smoker	25 (32.1)	95 (40.3)	
Smoker	53 (67.9)	141 (59.7)	
BMI (kg/m^2), median (IQR)	20.1 (18.8–21.6)	23 (21–24.9)	<0.001
<18.5[a]	15 (19.2)	7 (3.0)	
18.5–25[b]	60 (76.9)	173 (73.3)	
≥25.0[c]	3 (3.8)	56 (23.7)	
NLR, median (IQR)	2.4 (1.7–3.6)	2.2 (1.7–3.2)	0.20
≤3	47 (70.1)	153 (72.2)	
>3	20 (29.9)	59 (27.8)	
Alb (g/dl), median (IQR)	4 (3.7–4.2)	4.1 (3.8–4.3)	0.046
<4	37 (48.1)	89 (37.7)	
≥4	40 (51.9)	147 (62.3)	
CEA (μg/l), *n* (%)			0.50
≤5	46 (59.7)	148 (64.1)	
<5	31 (40.3)	83 (35.9)	
Postoperative complication, *n* (%)			0.82
Present	13 (16.7)	42 (17.8)	
Absent	65 (83.3)	194 (82.2)	
Pathology (NSCLC), *n* (%)			0.81
Adenocarcinoma	59 (75.6)	168 (71.2)	
Squamous carcinoma	15 (19.2)	48 (20.3)	
Large cell carcinoma	1 (1.3)	4 (1.7)	
Others	3 (3.8)	16 (6.8)	
p-TNM (7th edition), *n* (%)			0.78
Stage 1	59 (75.6)	195 (82.6)	
Stage 2	19 (24.4)	41 (17.4)	

P is shown as $P < 0.001$ if the actual *P*-value was <0.001.

[a]Underweight.

[b]Normal weight.

[c]Overweight and obesity.

Alb: albumin; BMI: body mass index; CEA: carcinoembryonic antigen; IQR: interquartile range; NLR: neutrophil to lymphocyte ratio; NSCLC: non-small-cell lung cancer; SD: standard deviation; TMI: truncal muscle index (cm^2/m^2); TNM: tumour, node and metastasis.

The RFS and OS Kaplan–Meier curves for patients with the low TMI and high TMI are shown in Fig. 3. RFS was significantly lower in patients with the low TMI compared with that of patients with high TMI (5-year RFS 69% vs 83.5%, respectively, $P = 0.028$, Fig. 3A). Similarly, OS was significantly different between the low-TMI group and high-TMI group (5-year OS 64.8% vs 80.1%, respectively, $P = 0.003$, Fig. 3B). The results of the Cox regression survival analysis of RFS and OS are shown in Tables 2 and 3. The univariable analysis of RFS identified the following significant prognostic factors in patients: smoking history, NLR, Alb, CEA, p-TNM and TMI. However, in the multivariable analysis, only NLR and p-TNM were shown to be the independent prognostic factors for RFS. The univariable analysis of OS indicated that smoking history, NLR, albumin, CEA, p-TNM and TMI were associated with postoperative prognosis. The multivariable analysis demonstrated that the TMI was an independent prognostic factor (hazard ratio 1.84, 95% CI 1.12–3.05; $P = 0.017$), in addition to p-TNM, NLR and Alb.

DISCUSSION

To our knowledge, this is the first study that investigated the impact of skeletal muscle volume represented by truncal muscle cut surface on the outcome of patients with early-stage NSCLC (stages I–II) who underwent curative surgery. The impact of depletion of the cross-sectional truncal muscle area at L1 (TMI) on outcomes was assessed using chest CT. The decrease in the TMI is an independent prognostic factor with an 1.8-fold increased risk of death in patients.

CT, which is routinely performed as a pretreatment staging assessment of patients with cancer, is widely used to evaluate skeletal muscle [11]. The identification and quantification of the skeletal muscle area on CT is recommended due to its precise differentiation between muscle, fat and other tissues. The single cross-sectional area of muscle at the third lumbar vertebra (L3) is referred to as a good modality, as it linearly relates to total body skeletal muscle mass on abdominal CT [12]. However, chest CT rarely extends to the L3 level. The implementation of muscle measurement and further progress in the field of surgical lung cancer care are severely hampered due to the lack of a standardized and efficient approach [13, 14]. In healthy subjects, examination of the muscle area at L1 via chest CT showed high correlation with the total body skeletal muscle mass [15]. As a result, we investigated truncal muscle area on the L1 of chest CT to evaluate the clinical impact on outcome of stages I–II NSCLC patients.

Low truncal muscle is a poor independent prognostic factor after complete resection in patients with early stage NSCLC. Low skeletal muscle, associated with a risk of adverse outcomes such as physical disability, poor quality of life and death, plays an important role in predicting chemotherapeutic toxicity and treatment outcomes in certain advanced cancers [7, 8]. Recently, clinical studies demonstrated that low skeletal muscle prior to surgery negatively impacts survival of patients with resectable gastrointestinal, hepatopancreatobiliary, colorectal and endometrial malignancies [16–19]. However, few studies have focused on the impact of skeletal muscle mass on prognosis in operable NSCLC due to the lack of an appropriate method for the measurement of skeletal muscle using chest CT. Suzuki *et al.* [20] reported that sarcopenia at the L3 level on abdominal CT was associated with poor outcome in a small sample of patients with completely resected early-stage NSCLC. The analysis of the present study indicated that the TMI on chest CT may be a practical and valuable method for the preoperative assessment of skeletal muscle mass in early-stage NSCLC (stages I–II) patients undergoing curative surgery.

It appeared to be multifactorial for the low TMI in early-stage NSCLC patients. It is well acknowledged that the recurrence of NSCLC after complete resection was relatively higher in the first 2 years [2]. The RFS and OS curves of the high- and low-TMI groups in the present study showed no distinct difference in the first 2 years. However, divergences were almost synchronously observed from the third year in both survival curves, although the significance of TMI was not independent in the multivariable

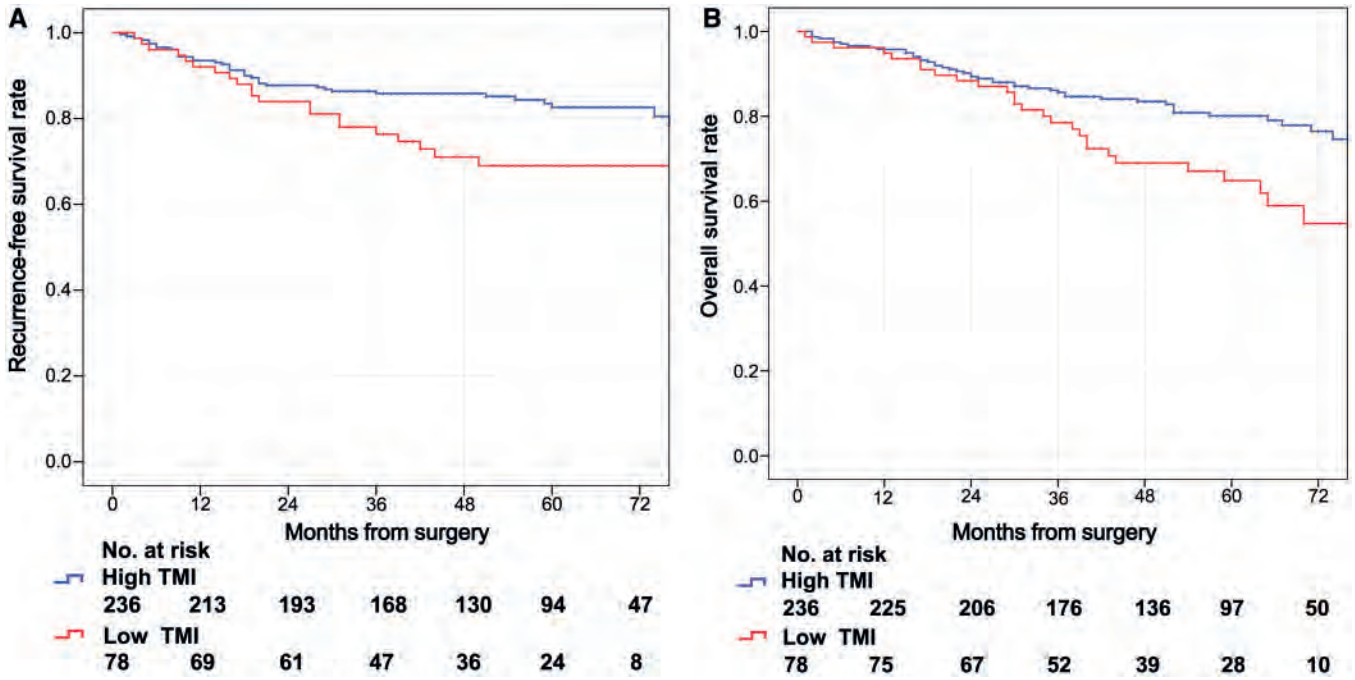

Figure 3: Survival curves of subgroups divided by the lowest quartile and the rest of the TMI. TMI is significantly prognostic for (**A**) recurrence-free survival and (**B**) overall survival. TMI: truncal muscle index.

Table 2: Results of univariable and multivariable analyses of recurrence-free survival ($n = 274$)

Variables	Univariable			Multivariable		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Male gender	1.45	0.85–2.47	0.17			
Age ≥65 years	1.7	0.93–3.10	0.084			
Current/ex-smoker	1.79	1.02–3.16	0.043	1.8	0.97–3.34	0.064
BMI <18.5 kg/m^2	1.51	0.65–3.52	0.34			
NLR >3	2.59	1.51–4.42	0.001	2.08	1.17–3.70	0.013
Alb ≤4	2.11	1.26–3.52	0.004	1.57	0.88–2.81	0.13
CEA >5	1.74	1.04–2.92	0.036	1.09	0.63–1.91	0.76
p-stage II (7th edition)	4.87	2.90–8.17	<0.001	4.09	2.35–7.12	<0.001
Low truncal muscle index	1.81	1.06–3.09	0.029	1.42	0.80–2.52	0.23

Alb: albumin; BMI: body mass index; CEA: carcinoembryonic antigen; CI: confidence interval; HR: hazard ratio; NLR: neutrophil to lymphocyte ratio.

Table 3: Results of univariable and multivariable analyses of overall survival ($n = 274$)

Variables	Univariable			Multivariable		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Male gender	1.77	1.09–2.87	0.021	1.2	0.65–2.21	0.56
Age ≥65 years	1.98	1.14–3.44	0.016	1.16	0.63–2.13	0.64
Smoker	2.22	1.32–3.74	0.003	1.57	0.81–3.04	0.19
BMI <18.5 kg/m^2	1.05	0.42–2.60	0.92			
NLR >3	3.24	2.02–5.21	<0.001	2.35	1.38–4.00	0.002
Alb ≤4	3.03	1.90–4.84	<0.001	2	1.18–3.39	0.01
CEA >5	1.95	1.24–3.08	0.004	1.27	0.78–2.09	0.34
p-stage II (7th edition)	4.01	2.52–6.37	<0.001	3.44	2.08–5.68	<0.001
Low truncal muscle index	2	1.26–3.18	0.002	1.84	1.12–3.05	0.017

Alb: albumin; BMI: body mass index; CEA: carcinoembryonic antigen; CI: confidence interval; HR: hazard ratio; NLR: neutrophil to lymphocyte ratio.

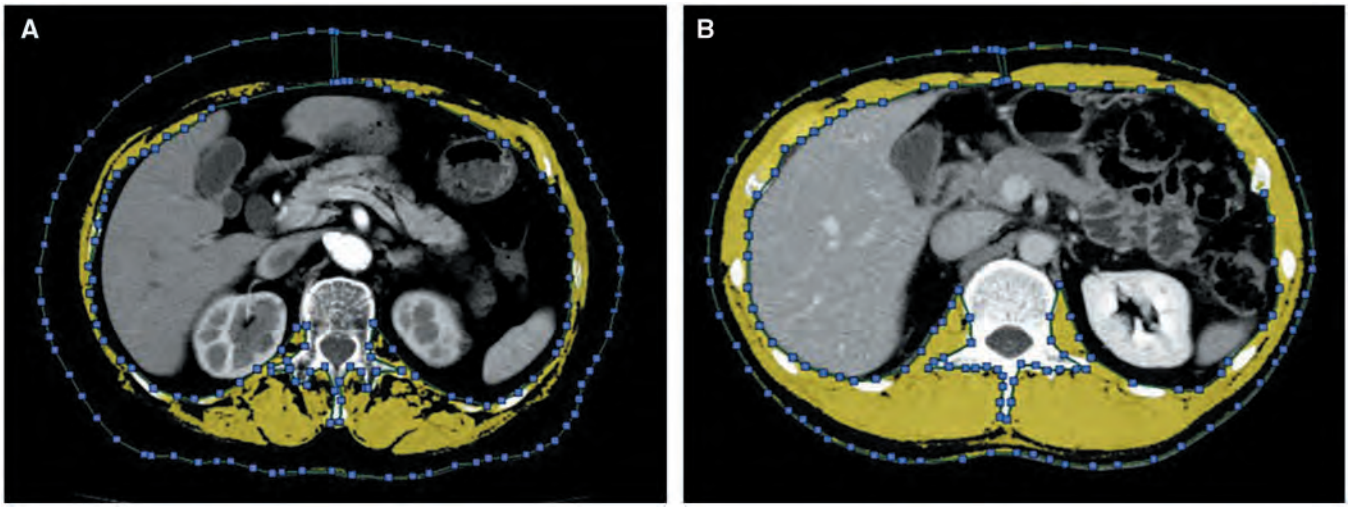

Figure 4: The body mass indices of 2 patients (**A** and **B**) were almost identical (23.2 and 23.5 kg/m^2). However, the muscle areas were different. The truncal muscle indices of these patients were 29 and 48, respectively.

analysis of RFS. Accordingly, low truncal muscle may be caused by tumour-related and non-tumour-related factors such as ageing, metabolic disorder, decreased physical activity, increased risk of cardiovascular disease and therapy-resistant metabolic diseases [9, 21, 22]. This was reflected on poor long-time outcome of patients with low truncal muscle had in early NSCLC.

Some evidence indicated that improving physical activity and nutritional intervention are a promising cancer therapy [23]. However, improving the skeletal muscle prior to surgery was impractical given the urgent need of resection for early NSCLC. The present study showed that low skeletal muscle was a risk factor for prognosis and provided an objective method to evaluate this risk factor at the time of treatment. Comprehensive information with well-planned care could be provided to the patients with poor skeletal muscle prior to surgery. More positive supports such as physical exercise and nutritional intervention for low skeletal muscle patients may be needed even after the treatment, because the survival curves of the low-TMI and high-TMI groups diverged from the third year after the surgery (Fig. 3B). Future prospective studies are needed to clarify whether interventions for increasing skeletal muscle can improve postoperative outcomes in patients with NSCLC.

The relationship between BMI and postoperative outcomes in cancer patients has been a subject of controversy. A previous study reported that increased body weight was associated with increased death rates in all cancers combined [24]. In contrast, obesity in patients with lung cancer has been linked to improved postoperative outcomes in another study [25]. BMI did not show a significant correlation with prognosis, although it was an independent risk factor for TMI in the present study. Instead, body composition (i.e. TMI) was suggested to be a prognostic factor. The difference in the prognostic value of TMI versus BMI may be partly explained by the fact that individuals with similar BMIs may have different body compositions (e.g. more fat or muscle in a patient than in another; Fig. 4) (see Supplementary Material, Fig. S1).

In our study, sex-specific quartile values rather than specific cut-off values were used to define the levels of skeletal muscle depletion. The definition of sarcopenia is an appendicular skeletal muscle index of more than 2 SD below that of healthy adults [5]. However, the actual prevalence of sarcopenia in Japanese patients is still unclear, and the skeletal muscle indices are vary with ethnicity. Therefore, the method of sex-specific quartiles was commonly applied to evaluate the skeletal muscle depletion.

Limitations

The present study is characterized by 2 main limitations. Firstly, this was a retrospective study with a limited patient sample size in a single institution. It is essential that these data are confirmed by large-scale population-based prospective studies. Secondly, this study was based on a single time point of chest CT prior to surgery. The changes in muscle mass postoperatively ought to be evaluated further in future studies. In addition, not only morphological muscle assessment but also sarcopenia-related function evaluation is of interest.

CONCLUSION

In conclusion, the findings of this study demonstrated that truncal skeletal muscle is an independent prognostic factor in patients with stages I–II NSCLC following curative surgery. This factor may be included in the preoperative assessment in patients with early-stage NSCLC, for whom surgical intervention is considered.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *EJCTS* online.

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Conflict of interest: none declared.

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Sarcopenia: an unneglectable nutritional status for patients with surgically treated non-small-cell lung cancer

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Skeletal muscle depletion, also known as sarcopenia, is one of the components of cancer cachexia syndrome, which has been reported to be closely correlated with mobility disorder, disability and even an increased risk of death [1]. In patients with non-small-cell lung cancer (NSCLC), the prevalence of sarcopenia was reported to be as high as 46.8% [2]. It is reported that sarcopenia significantly correlated with the prognosis of patients with advanced lung cancer [3]. However, in patients with surgically treated NSCLC, the prognostic value of sarcopenia has not been fully established due to the fact that only a few relevant studies have been carried out. An interesting study by Sun *et al.* [4], which has been recently published in the *European Journal of Cardio-Thoracic Surgery*, explored the prognostic value of skeletal muscle depletion (sarcopenia) in patients with early-stage NSCLC. By calculating truncal muscle cross-sectional area at the level of the first lumbar vertebra on chest computed tomography normalized by the square of height for defining sarcopenia (cut-off value 38 cm^2/m^2 for men and 29.6 cm^2/m^2 for women), they included a total of 314 patients undergoing lobectomy for stages I–II NSCLC in their analysis (78 sarcopenic patients and 236 non-sarcopenic patients). They found that sarcopenic patients had a significantly lower 5-year overall survival (OS) rate (64.8% vs 80.1%; $P = 0.003$) and 5-year disease-free survival (DFS) rate (69% vs 83.5%; $P = 0.028$) than non-sarcopenic patients and that sarcopenia was an independent predictor of poor OS for patients with early-stage NSCLC after surgical resection [hazard ratio (HR) = 1.84, 95% CI 1.12–3.05; $P = 0.017$). However, sarcopenia was not found to be a significant predictor of poor DFS for patients in the study by Sun *et al.* (HR = 1.42, 95% CI 0.80–2.52; $P = 0.23$). Nevertheless, their study [4] again added to the evidence that sarcopenia was an independent prognostic factor in patients with early-stage NSCLC following curative surgery.

As a matter of fact, in our recent meta-analysis pooling all available studies dated up to July 2018 [5], we included a total of 6 cohort studies consisting of 1213 patients with surgically treated NSCLC (422 sarcopenic patients and 791 non-sarcopenic patients). We also found that sarcopenic patients had a significantly lower 5-year OS rate than those without ($P = 0.008$), and sarcopenia was an independent predictor of poor OS in patients with surgically treated NSCLC (HR = 2.85, 95% CI 1.67–4.86; $P < 0.001$). Moreover, when stratified by the tumour stage, sarcopenia was found to have a significantly negative impact on both 5-year OS and DFS rates of patients with stage I NSCLC, whereas it was not significantly correlated to that of patients with NSCLC of all stages after surgical resection. Taken together, we believe that sarcopenia is a significantly independent predictor of poor prognosis of NSCLC patients after surgical resection, especially in patients with early-stage NSCLC. Moreover, a previous study has also shown that sarcopenia was a risk factor of developing major postoperative complications [6]. Therefore, sarcopenia should remain a non-neglectable nutritional parameter in surgically treated NSCLC patients, which thoracic surgeons should be fully aware of in NSCLC patients intended for surgery during daily practice. As a result, it should be highlighted that routine assessment of sarcopenia should be incorporated into preoperative evaluation and postoperative follow-up of NSCLC patients especially for those at an early stage. Moreover, perioperative correction of the sarcopenic status, a close postoperative follow-up and an optimal adjuvant therapy plan should be highly advocated for sarcopenic NSCLC patients intended for surgery, even if they are at an early stage.

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Reply to Deng *et al.*

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Keywords: Skeletal muscle loss • Truncal muscle index • Non-small-cell lung cancer surgery • Prognosis

We thank Deng *et al.* [1] for their valuable comments on our recently published article entitled 'Low truncal muscle area on chest computed tomography: a poor prognostic factor for the cure of early-stage non-small-cell lung cancer' [2]. As they have pointed out, low preoperative skeletal muscle mass, referred to as sarcopenia, was significantly associated with a negative outcome for patients with surgically treated non-small-cell lung cancer (NSCLC) in the study results.

Identification of patients with muscle loss has become difficult as many cancer patients are overweight or obese. The skeletal muscle determined by preoperative computed tomography (CT) provided an objective method to recognize potential sarcopenia at the time of treatment and could be used to predict the prognosis in patients undergoing complete resection of NSCLC [2, 3]. Comprehensive information and early clinical management, such as better nutrition and rehabilitation, could be applied to patients with low skeletal muscle before surgery. This may be even more necessary after surgery as to the overall survival of patients with low skeletal muscle increasingly worsens from the third year after the surgery [2, 4].

In summary, evaluation of the skeletal muscle mass using CT might be incorporated into the preoperative assessment and postoperative follow-up of patients with NSCLC, even at an early stage of NSCLC, when surgical intervention is considered. Moreover, long-term nutritional management and rehabilitation for patients with low skeletal muscle seem to be important for improving the outcome after complete resection of NSCLC. Prospective studies are needed to determine the role of increasing skeletal muscle in non-small-cell lung cancer.

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Is restricted use of bilateral internal mammary artery grafting strategy ideal for multivessel coronary artery disease?

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Keywords: Bilateral internal mammary arteries • Deep sternal wound infection • Graft failure

I read with interest the article by Gaudino *et al.* [1] on the systematic usage of the bilateral internal mammary artery (BIMA) grafting in a large cohort of unselected, real-world patients. In their metachronous study, the authors reported that the systematic use of BIMA grafting (88.5%) was associated with a higher incidence of adverse events particularly sternal complications (5.5%), perioperative myocardial infarction (3.5%) and graft failure (3%) compared to the control patients where the BIMA grafting strategy was used in 44%. This may be one of the earliest studies to report on the feasibility of systematic BIMA use. Deep sternal wound infection (DSWI) is one of the significant complications of coronary artery bypass grafting using BIMA grafting particularly in diabetic patients. Skeletonized harvesting of BIMA grafts is reported to be associated with reduction in DSWI following BIMA grafting.

In their study, authors used skeletonized technique to harvest BIMAs. Did they use the harvest technique of sparing the bifurcation of IMA to the chest wall and pericardiacophrenic artery at least on 1 side while harvesting the mammary arteries? It was reported that adoption of this technique in BIMA grafting strategy reduced the incidence of DSWI to less than 1%, even in diabetic patients [2, 3].

What was the HbA1c percentage in these patients prior to surgical revascularization? Was it higher than 8% in all patients who had sternal wound infection? A higher percentage of HbA1c >8% is associated with higher incidence of DSWI following BIMA harvest [4].

What was the mean body surface area (BSA) in their patient population who had graft failure? It was reported that lower BSA appeared to have worse long-term survival when BIMA grafting strategy was used [5].

In the control group, no patient had been subjected to reangiography, and authors concluded that there was no graft failure. However, the majority of grafts that fail do so with little immediate clinical consequence to the patient which was also evident from our study in which we observed asymptomatic patients when subjected to graft angiography at 6–72 months postoperatively to evaluate graft patency, and found 2% of the left IMA grafts to be occluded [6]. How did the authors assess graft patency in the control group?

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Reply to Sajja

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Keywords: Coronary artery bypass grafting • Bilateral internal thoracic arteries

We thank Dr Sajja for his interest in our recent article published in the *European Journal of Cardiothoracic Surgery* [1, 2].

The incidence of sternal wound complications reported in CATHEXIS (5.5%) is lower than that reported in the bilateral internal thoracic artery arm of the Arterial Revascularization Trial (9.6%) [3]. This is notable as only 16.4% of the

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研究テーマ	肺移植に関する実験的・臨床的研究 Experimental or clinical research on lung transplantation				
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 取得単位数 26 ／取得すべき単位総数 34
学生本人が行った研究の概要	（1）　ラット肺移植モデルを用いた肺移植後拒絶反応の新しい診断法の研究：急性拒絶反応を 18fluorodeoxygloucose-positron emission tomography (FDG-PET)によって評価する実験モデルを作成した。さらに急性拒絶時に移植肺への FDG 取り込みの増加があることを見出した。 （2）　胸腺腫の悪性度と発生部位の関係についての臨床研究：当院の胸腺腫手術症例を対象とした後方視的観察研究を行い、胸腺腫発生部位が前縦隔上部の場合、前縦隔下部発生に比べて術後生存率が有意に低いことを明らかにした。（下記論文２） （3）　Peer review のある英文論文２編を出版または出版予定とした。（以下） 1. Tian D, Nakajima J, et al.J Thorac Cardiovasc Surg 2019 (ahead of print) PMID31548078 2. Tian D, Nakajima J, et al. Thoracic Cancer 2019; 10: 2096-2105　PMID31499597	
総合評価	【良かった点】 熱心に、また当科教員や研究生と協力的に研究に取り組み、英文論文をまとめられたこと 【改善すべき点】 特になし 【今後の展望】 上記（1）の研究成果を論文化する。内容がよろしければ博士論文として認められる可能性がある。３年目からは貴財団からの助成を受けられないため、東京大学大学院の「学外研究指導」という形式にて中国の病院での臨床研究を行ってもらう予定。	
学位取得見込	通常はあと２年必要。(来年度第３学年だが)特例で大学院３年次に取得できる可能性もある（が稀）。	
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研究テーマ	肺移植に関する実験的・臨床的研究 Experimental or clinical research on lung transplantation				
専攻種別	論文博士	☐	課程博士	☑	

1．研究概要(1)

1)目的(Goal)Although the cuff technique in rat lung transplantation (LTx) has a long history, it remains technically challenging(1-3). We have developed key tricks and modifications in the devices and the cuff technique that optimize the rat LTx model to achieve successful operations during a short learning period.

2)戦略(Approach)Altogether, 180 consecutive rats underwent orthotopic left LTx performed by a single surgeon using our modified devices and procedures. Allogeneic and syngeneic transplantation were performed using Lewis rats as recipients and Brown Norway and Lewis rats as donors. Allogeneic recipients were treated with cyclosporine during the first week. Recipients were sacrificed at various time points after ≥2 weeks.

3)材料と方法(Materials and methods)Small, handheld surgical instruments and larger equipment for rat LTx were used as in previous studies of Kyoto University(4). We have modified the device for cuff preparation. We used a petri dish attached to two foam blocks. A groove and hole were carved on the foam blocks to match the ends of a bulldog clamp (Natsume Seisakusho Co., Ltd, Tokyo, Japan). To prevent lung tissue injury, we modified the ends of the cuff tail from rectangular to round.Male Brown Norway and Lewis rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Models of syngeneic and allogeneic transplantation were performed as described previously. Animals were sacrificed at ≥2 weeks postoperatively. All rats were maintained under specific pathogen-free conditions at The University of Tokyo. Animals were fed a standard diet and provided with water adlibitum.

4)実験結果(Results)A special cuff-preparation plate was created using a petri dish and two foam blocks. This modified plate stabilizes the preparation and prevents donor lung compression. A “⊥”-shaped incision was carved into the front wall of the pulmonary artery (PA) using micro-scissors. “V”-shaped incisions were made from the inferior-to-superior branches of the pulmonary vein (PV) and bronchus. A “pendulum model” was applied at implantation to make the hilar anastomosis tension-free and technically easier to perform. There were no intraoperative complications. Ten rats (5.6%) experienced partial or full pulmonary atelectasis. Five deaths (2.8%) due to pleural effusion occurred during the follow-up period. The operative times for heart-lung block retrieval, cuff preparation, cold ischemia, warm ischemia, and total procedure time were 8.4±0.8, 11.6±1.5, 25.1±2.2, 8.1±1.2, and 46.7±2.8 min, respectively.

5)考察(Discussion)The key tricks and improvements we made in the cuff technique for rat LTx provided the advantages of expeditiousness, a low complication rate, and a high success rate.

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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 be

論文名 1 Title	Should All Donors Be Treated by ex vivo Lung Perfusion?					
掲載誌名 Published journal	JAMA Surgery (IF: 10.688)					
	2020 年 3 月	25 巻（号 ）	頁 ～ 頁	言語 Language	English	
第1著者名 First author	Dong Tian	第2著者名 Second author	Yu Wang	第3著者名 Third author	Shi-Yang Deng	
その他著者名 Other authors						
論文名 2 Title	Rat lung transplantation model: modifications of the cuff technique					
掲載誌名 Published journal	Annals of Translational Medicine (IF: 3.689)					
	2020 年 3 月	20 巻（号 In press 頁 ～1	頁	言語 Language	English	
第1著者名 First author	Dong Tian	第2著者名 Second author	Haruhiko Shiiya	第3著者名 Third author	Masaaki Sato	
その他著者名 Other authors	Jun Nakajima					
論文名 3 Title	Tumor location impact in thymic epithelial tumors: clinicopathological features and prognosis evaluation					
掲載誌名 Published journal	Thoracic Cancer(IF: 2.6)					
	2019 年 11 月	10 巻（号）	2096 頁 ～ 2105 頁	言語 Language	English	
第1著者名 First author	Dong Tian	第2著者名 Second author	Haruhiko Shiiya	第3著者名 Third author	Masaaki Sato	
その他著者名 Other authors	Chang - Bo Sun, Masaki Anraku, and Jun Nakajima					
論文名 4 Title	Clinical Outcomes in Lung Transplantation of Marginal Donor after Ex vivo Lung Per-fusion: A Systematic Review and Meta-analysis.					
掲載誌名 Published journal	The Journal of Thoracic and Cardiovascular Surgery (IF: 5.3)					
	2020 年 2 月	15 9(2) 巻（号）	720 頁 ～ 730 頁	言語 Language	English	
第1著者名 First author	Dong Tian	第2著者名 Second author	Yu Wang	第3著者名 Third author	Haruhiko Shiiya	
その他著者名 Other authors	Chang-Bo Sun, Yukari Uemura, Masaaki Sato, Jun Nakajima					
論文名 5 Title	Differences Between Patients With Idiopathic Pleuroparenchymal Fibroelastosis and Those With Other Types of I diopathic Interstitial Pneumonia in Candidates for Lung Transplants					
掲載誌名 Published journal	Transplant Proc (I F:0.95 9)					
	2019 年 7 月	51(6) 巻（号）	2014 頁 ～ 2021 頁	言語 Language	English	
第1著者名 First author	Haruhiko Shiiya	第2著者名 Second author	Dong Tian	第3著者名 Third author	Masaaki Sato	
その他著者名 Other authors	Karasaki T, Kitano K, Nagayama K, Anraku M, Kaga K, Matsui Y, Nakajima J					

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 be

論文名 6 Title	T2期食管癌环形肌与纵形肌细分的临床意义研究进展				
掲載誌名 Published journal	中华胸心血管外科杂志				
	2019 年 5 月	35 (1) 巻 (号)	55 頁 ～ 58 頁	言語 Language	中文
第1著者名 First author	黄桁	第2著者名 Second author	王喻	第3著者名 Third author	田东
その他著者名 Other authors					
論文名 7 Title	Don' t go too far when choosing intentional segmentectomy for small sized lung cancer				
掲載誌名 Published journal	J Thorac Cardiovasc Surg. **(IF: 5.3)**				
	2020 年 3 月	Accepted 巻 (号)	頁 ～ 頁	言語 Language	English
第1著者名 First author	Cheng-Wu Liu	第2著者名 Second author	**Dong Tian (Co-first)**	第3著者名 Third author	Qiang Pu
その他著者名 Other authors					
論文名 8 Title	Clinical Nomogram for Lymph Node Metastasis in Pathological T1 Esophageal Squa-mous Cell Carcinoma: A Multicenter Retrospective Study				
掲載誌名 Published journal	Annals of Translational Medicine. **(IF: 3.689)**				
	2020 年 3 月	20 巻 (号)	Inpress 頁 ～ 頁	言語 Language	English
第1著者名 First author	**Dong Tian**	第2著者名 Second author	Kai-Yuan Jiang	第3著者名 Third author	Heng Huang
その他著者名 Other authors	Hong-Ying Wen				
論文名 9 Title	Depth of Circular and Longitudinal Muscle Invasion in T2 Esophageal Squamous Cell Carcinoma Does Not Affect Prognosis or Lymph Node Metastasis: A Multicenter Ret-rospective Study				
掲載誌名 Published journal	World J Surg. **(IF: 2.768)**				
	2020 年 1 月	44(1) 巻 (号)	171 頁 ～ 178 頁	言語 Language	English
第1著者名 First author	**Dong Tian**	第2著者名 Second author	Heng Huang	第3著者名 Third author	Yu-Shang Yang
その他著者名 Other authors	Kai-Yuan Jiang, Xi He, Xiao-Guang Guo & Long-Qi Chen				
論文名 10 Title	Lung volume reduction surgery: Only short-term evaluation is enough?				
掲載誌名 Published journal	Ann Thorac Surg **(IF: 3.919)**				
	2019 年 7 月	1975(19) 巻 (号)	Inpress 頁 ～ 頁	言語 Language	English
第1著者名 First author	**Dong Tian**	第2著者名 Second author	Kai-Yuan Jiang	第3著者名 Third author	Long-Qi Chen
その他著者名 Other authors					

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 be

論文名 11 Title	Tumour size: an unneglectable prognostic factor for patients with thymoma.					
掲載誌名 Published journal	Eur J Cardiothorac Surg **(IF: 3.847)**					
	2020 年 2 月	57(2) 巻(号)	411 頁 ～ 412 頁	言語 Language	English	
第1著者名 First author	**Dong Tian**	第2著者名 Second author	Heng Huang	第3著者名 Third author	Kai-Yuan Jiang	
その他著者名 Other authors	Long-Qi Chen					
論文名 12 Title	Neoadjuvant Chemotherapy with Irinotecan and Nedaplatin in Single Cycle Followed by Esophagectomy Versus Surgery Alone on cT1 Potential Resectable Esophageal Squamous Cell Carcinoma: A Prospective Nonrandomized Trial for Short-term Out-comes					
掲載誌名 Published journal	Dis Esophagus **(IF: 2.702)**					
	2019 年 3 月	32(3) 巻(号)	頁 ～ 頁	言語 Language	English	
第1著者名 First author	**Dong Tian**	第2著者名 Second author	Lin Zhang	第3著者名 Third author	Yu Wang	
その他著者名 Other authors	L Chen, K-P Zhang, Y Zhou, H-Y Wen, M-Y Fu					
論文名 13 Title	Experience from AATS Foundation for Thoracic Surgery Training Fellowship: Lung Transplantation in Toronto General Hospital					
掲載誌名 Published journal	J Thorac Cardiovasc Surg **(IF:5.3)**					
	2018 年 8 月	156(2) 巻(号)	929 頁 ～ 930 頁	言語 Language	English	
第1著者名 First author	**Dong Tian**	第2著者名 Second author	Shaf Keshavjee	第3著者名 Third author		
その他著者名 Other authors						
論文名 14 Title	Strategies to achieve long-term success of lung transplantation.					
掲載誌名 Published journal	Annals of Translational Medicine. **(IF: 3.689)**					
	2020 年 3 月	20 巻(号)	Inpress 頁 ～ 頁	言語 Language	English	
第1著者名 First author	Masaaki Sato	第2著者名 Second author	**Dong Tian**	第3著者名 Third author		
その他著者名 Other authors						
論文名 15 Title	Length of Intensive Care Unit stay: an unneglectable risk factor for postoperative delirium in patients undergoing esophagectomy					
掲載誌名 Published journal	Ann Thorac Surg. **(IF: 3.919)**					
	2019 年 12 月	108 (6) 巻(号)	1921 頁 ～ 1925 頁	言語 Language	English	
第1著者名 First author	**Dong Tian**	第2著者名 Second author	Yu Wang	第3著者名 Third author	**Long-Qi Chen**	
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

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

学会名 Conference	The 72th Annual Scientific Meeting of the Japanese Association for Thoracic Surgery				
演 題 Topic	A cadaveric anatomical study of recurrent laryngeal nerve in esophagectomy				
開催日 date	2019 年 10 月 31 日	開催地 venue	Kyoto, Japan		
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	Jing-Ya Deng, Heng Huang, Kai-Yuan Jiang, Huan-Yu Tang, Xi He				
学会名 Conference	The Annual Meeting of 39th Japanese Association for Research on the Thymus				
演 題 Topic	Tumor location impact in thymic epithelial tumors: clinicopathological features and prognosis evaluation				
開催日 date	2020 年 2 月 15 日	開催地 venue	Sapporo, Japan		
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	Haruhiko Shiiya, Masaaki Sato, Jun Nakajima				
学会名 Conference	The 36th Annual Meeting of the Japanese Association for Chest Surgery				
演 題 Topic	Tumor location impact in thymoma: clinicopathological features and prognosis evaluation				
開催日 date	2019 年 5 月 17 日	開催地 venue	Osaka, Japan		
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	Haruhiko Shiiya, Masaaki Sato, Jun Nakajima				
学会名 Conference	2019无锡国际肺移植大会				
演 題 Topic	Rat lung transplantation model				
開催日 date	2019 年 11 月 1 日	開催地 venue	无锡，中国		
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	Masaaki Sato				

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

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	東京大学
助成金名称 Grant name	2019年度博士課程研究遂行協力制度
受給期間 Supported period	2019 年 8 月 ～ 2020 年 1 月
受給額 Amount received	300,000 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	Chinese Government
奨学金名称 Scholarship name	China Scholarship Council
受給期間 Supported period	2018 年 9 月 ～ 2020 年 4 月
受給額 Amount received	170,000 円 /月

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) 中島 淳

Letters

COMMENT & RESPONSE

Should All Donors Be Treated by Ex Vivo Lung Perfusion?

To the Editor We read with great interest the article by Divithotawela et al,[1] which demonstrated the posttransplant long-term outcomes were similar between ex vivo lung perfusion (EVLP)-treated lung transplant (LTx) and standard LTx, although the quality of donor lungs was worse with EVLP-treated LTx. To our knowledge, this study is the largest study of EVLP and includes 230 EVLP cases. We congratulate Divithotawela et al[1] for this excellent study.

In our 2019 meta-analysis[2] of 8 studies including 186 EVLP-treated LTx, we concluded the outcomes after LTx of EVLP on marginal donor lungs were comparable with those of standard LTx. However, we only analyzed the short-term outcomes because of limited studies with long-term outcomes met the inclusion criteria. One subsequent study about EVLP-treated LTx with 10-year follow-up published by Ghaidan et al[3] has reported no differences were found between conventional LTx and EVLP-treated LTx regarding long-term outcomes, with a limited case number of EVLP-treated LTx (n = 6). Divithotawela et al[1] showed a large volume series that provided a higher evidence level. However, the mean preprocurement arterial oxygen tension/inspired oxygen fraction (Pao_2/FiO_2) ratio of EVLP-treated donor lungs was up to 348 mm Hg, which was the highest value comparing with other previous studies. Most previous studies performed EVLP on initially rejected donor lungs with Pao_2/FiO_2 ratio less than 300 mm Hg.[2] Whitford et al[4] proposed even the threshold of 300 mm Hg was excessively conservative and resulted in the waste of donor lungs and the application of unnecessary EVLP. We wonder whether EVLP-treated donor lungs in the Divithotawela et al study[1] are the real high-risk donor lungs. In this view, it would be extremely interesting if Divithotawela et al[1] could provide data regarding Pao_2/FiO_2 ratio less than 300 mm Hg in EVLP-treated and compare with conventional LTx group in their series.

Divithotawela et al[1] also showed the long-term outcomes are similar between the 2 groups. However, the trends of most subanalysis after 9 years were better in EVLP-treated group. In this point, should all donors be treated by EVLP? The biggest obstacle may be the cost of EVLP, which was not analyzed in this study.[1] McMeekin et al[5] reported the EVLP currently may not be cost-effective using the National Institute for Health and Clinical Excellence threshold, but several plausible scenario analyses results indicate that a service incorporating EVLP would be considered cost-effective.

Dong Tian, MD, PhD
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Conflict of Interest Disclosures: None reported.

1. Divithotawela C, Cypel M, Martinu T, et al. Long-term outcomes of lung transplant with ex vivo lung perfusion. *JAMA Surg*. 2019;154(12):1-9. doi:10.1001/jamasurg.2019.4079

2. Tian D, Wang Y, Shiiya H, et al. Outcomes of marginal donors for lung transplantation after ex vivo lung perfusion: a systematic review and meta-analysis. *J Thorac Cardiovasc Surg*. 2019;S0022-5223(19)31641-1.

3. Ghaidan H, Fakhro M, Andreasson J, Pierre L, Ingemansson R, Lindstedt S. Ten year follow-up of lung transplantations using initially rejected donor lungs after reconditioning using ex vivo lung perfusion. *J Cardiothorac Surg*. 2019;14(1):125. doi:10.1186/s13019-019-0948-1

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5. McMeekin N, Chrysos AE, Vale L, Fisher AJ. Incorporating ex-vivo lung perfusion into the UK adult lung transplant service: an economic evaluation and decision analytic model. *BMC Health Serv Res*. 2019;19(1):326. doi:10.1186/s12913-019-4154-6

Original Article

Rat lung transplantation model: modifications of the cuff technique

Dong Tian[1,2,3#], Haruhiko Shiiya[1,4#], Masaaki Sato[1], Jun Nakajima[1]

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Contributions: (I) Conception and design: D Tian, H Shiiya, M Sato; (II) Administrative support: None; (III) Provision of study materials or patients: D Tian, H Shiiya; (IV) Collection and assembly of data: D Tian, H Shiiya; (V) Data analysis and interpretation: D Tian, H Shiiya; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Background: Although the cuff technique in rat lung transplantation (LTx) has a long history, it remains technically challenging. We have developed key tricks and modifications in the devices and the cuff technique that optimize the rat LTx model to achieve successful operations during a short learning period.

Methods: Altogether, 180 consecutive rats underwent orthotopic left LTx performed by a single surgeon using our modified devices and procedures. Allogeneic and syngeneic transplantation were performed using Lewis rats as recipients and Brown Norway and Lewis rats as donors. Allogeneic recipients were treated with cyclosporine during the first week. Recipients were sacrificed at various time points after ≥2 weeks.

Results: A special cuff-preparation plate was created using a petri dish and two foam blocks. This modified plate stabilizes the preparation and prevents donor lung compression. A "⊥"-shaped incision was carved into the front wall of the pulmonary artery (PA) using micro-scissors. "V"-shaped incisions were made from the inferior-to-superior branches of the pulmonary vein (PV) and bronchus. A "pendulum model" was applied at implantation to make the hilar anastomosis tension-free and technically easier to perform. There were no intraoperative complications. Ten rats (5.6%) experienced partial or full pulmonary atelectasis. Five deaths (2.8%) due to pleural effusion occurred during the follow-up period. The operative times for heart-lung block retrieval, cuff preparation, cold ischemia, warm ischemia, and total procedure time were 8.4±0.8, 11.6±1.5, 25.1±2.2, 8.1±1.2, and 46.7±2.8 min, respectively.

Conclusions: The key tricks and improvements we made in the cuff technique for rat LTx provided the advantages of expeditiousness, a low complication rate, and a high success rate.

Keywords: Cuff technique; key trick; lung transplantation (LTx); modification; rat

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Introduction

The cuff technique of the rat lung transplantation (LTx) model was first reported by Mizuta *et al.* in 1989 (1). Various modifications and improvements have been reported in recent years (2-5). Although it has been thought that any trained surgeon could perform this technique successfully, the difficulties and disadvantages of this model still exist and often require a long learning curve (6). In addition, complications such as twisted blood vessels, pulmonary atelectasis, bleeding, and thoracic effusion still occur, although some recipient rats survive without any symptoms (4).

Figure 1 Graft preparation plate is modified by two foam blocks attached to a petri dish. A groove and hole were carved on the foam blocks to match the ends of a bulldog clamp.

Because improper devices and procedures for this technique could potentially lead to frustration and failure to complete one's training, disclosing the improvements of the devices and procedures may shorten and simplify complex steps, thereby encouraging the trainee to continue. This article describes the key tricks and modifications of devices used in this improved cuff technique that optimize the rat LTx model, helping to achieve successful operations in a shorter time with a lower complication rate.

Methods

Devices and instruments

Small, handheld surgical instruments and larger equipment for rat LTx were used as in previous studies of Kyoto University (7,8) and as described in *Figures S1-S3*. We have modified the device for cuff preparation. We used a petri dish attached to two foam blocks. A groove and hole were carved on the foam blocks to match the ends of a bulldog clamp (Natsume Seisakusho Co., Ltd, Tokyo, Japan) (*Figure 1*). To prevent lung tissue injury, we modified the ends of the cuff tail from rectangular to round (*Figure S4*).

Animals

Male Brown Norway and Lewis rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Models of syngeneic and allogeneic transplantation were performed as described previously (7) (further details are described in supplementary material). Animals were sacrificed at ≥2 weeks postoperatively. All rats were maintained under specific pathogen-free conditions at The University of Tokyo. Animals were fed a standard diet and provided with water ad libitum.

This study was approved by the Experimental Animal Ethics Committee of The University of Tokyo (No. H19-027). All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at The University of Tokyo.

Time-interval definitions

The donor operation time was the interval from incision of the donor rat to excision of the heart-lung block. The cuff preparation time was the interval from excision of the heart-lung block to completion of cuff placement. The cold ischemia time was defined at the time from flushing the donor lungs *in situ* with ET-Kyoto solution (Otsuka, Tokushima, Japan) to graft removal from hypothermic storage. The warm ischemia time was set as the interval from donor lung removal from the ice until restoration of the reperfusion. The total procedure time was the interval from the donor's skin incision until closure of the recipient's incision.

Donor procedures

Donor heart-lung block retrieval was performed as in previous studies (7,8). After the hilar structures of the left main bronchus, pulmonary artery (PA), and pulmonary vein (PV) with part of the left atrium were dissected, the hilar structures were consecutively everted around the cuff and secured by a preparatory 6-0 slipknot. Thereafter, the graft was rewrapped by organ perfusate-soaked paper and stored on ice for implantation (*Videos 1,2*).

Recipient procedure

A thoracotomy incision was made from the area of a palpable cardiac impulse on the chest wall and extended dorsally for about 3–4 cm. A blepharostat was used as a chest retractor (Speculum BANGERTER Large Right; Inami, Tokyo, Japan) to expose the thoracic cavity. The hilar structures were dissected and then clamped distally and proximally using two Satinsky clamps. The Satinsky clamps were then fixed in plastic clay to place mild tension on the bronchovascular structures. A ⊥-shaped incision was made

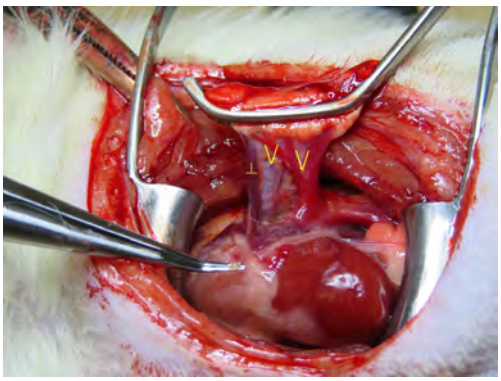

Figure 2 Hilar bronchovascular structure incisions are "V"-shaped on both bifurcations of the pulmonary vein and bronchus and "⊥"-shaped for the pulmonary artery.

on the front wall of the PA using micro-scissors. V-shaped incisions were made from the inferior-to-superior branches of the PV and bronchus (*Figure 2*).

The "pendulum model" method was used for implantation. The bronchus was implanted first. The native bronchus and cuff body were secured using a preparatory 6-0 nylon knot positioned loosely around the recipient bronchus. The superior bifurcation of the bronchus was excised to eliminate any overlaps between the bronchus and the PA. The donor lung was then repositioned to allow a PA anastomosis without tension. The donor lung was repositioned again to allow creation of the PV anastomosis without tension. The PA and PV were implanted in analogous fashion as with the bronchus. After excising the left native lung parenchyma, the end rim of the native structures was cut for debonding before reperfusion (*Figure S5*) (*Video 3*).

Assessment of chest-radiography and histology

Post-transplantation rats underwent chest plain radiography on postoperative day 7 to screen for complications such as atelectasis or to have recorded complications at the time of sacrifice. For routine histological evaluation, lung grafts were fixed in 10% formalin, embedded in paraffin, sectioned into 4-μm thickness, and stained with hematoxylin-eosin.

Statistical analysis

Results were expressed as means ± SD. The results were analyzed by SPSS 24.0 software (IBM, Armonk, NY, USA).

Results

Key tricks and modifications on techniques and devices

The following devices and techniques were improved and modified in our series (*Table 1*).

- Regarding the devices, we modified the cuff preparation plate, which now contains a petri dish attached to two foam blocks. A groove and hole were carved on the foam blocks to match the ends of a bulldog clamp. This modified plate stabilized the operation, was less injurious, and made the procedure easier to master.
- When locating the incision site on the recipient, the region of apical impulse, or palpable cardiac impulse on the chest wall, was first confirmed and then extended dorsally for about 3–4 cm. This position was located more easily and was suitable for the following implantation. Before implantation, a ⊥-shaped incision was made on the PA, and V-shaped incisions were made on the PV and bronchus. These improvements made implantation easier and lacerations on the structures less likely to occur.
- The "pendulum model" method was used for implantation. First, the bronchus was implanted. Thereafter, the donor lung was repositioned to close the PA and PV, thereby allowing a tension-free anastomosis. In addition, we modified the cuff so it had a round tail, used the slip-knots for cuff preparation, and debonded the end rim of the native structures after implantation.

Outcomes of rat LTx

In a consecutive series, 180 (syngeneic 33, allogeneic 147) anastomoses were completed by one performer (D Tian) without lacerations, twisting, or angulation of intraoperative bronchovascular structures and without bleeding at the vascular anastomosis. There were no intraoperation deaths, pneumothorax, or vessel thromboses. Partial or full pulmonary atelectasis was observed in 10 cases (5.6%). Five deaths (2.8%) were found at a follow-up time of 3–7 days, most likely due to pleural effusion.

Operation time of rat LTx of all procedures

The mean weights of the rats at the time of transplant of Lewis and Brown Norway rats were 239.7±42.8 and

Table 1 Key tricks and modifications on techniques and devices

Procedures and devices	Previous status	Modifications	Advantages
Cuff	Rectangular tail	Round tail (*Figure S4*)	Round tail can prevent accidental injury
Cuff preparation plate	Petri dish only	Two foam blocks with a petri dish (*Figure 1*)	Modified plate can keep operation stable, cause less injury, is easy to master
Recipient thoracotomy	3rd/4th Intercostal space; inferior margin of the scapula	Region of apical impulse (*Figure 5,* 3"–4")	This anatomical landmark can easily confirm the correct skin site for incision
Hilum structure incisions	Transverse or ├-shaped incision on the front wall	(I) ⊥-shaped incision for PA; (II) V-shaped incisions on the bifurcations of the PV and bronchus (*Figure 2*)	(I) ⊥-Shaped incision can prevent laceration of pulmonary artery; (II) V-shaped incisions form a wide rim of structure ostium that can be easily implanted and free of laceration
Implantation	Single direction with PV or PA first	"Pendulum model" for implantation (*Figure 5*: 3'40"–7'03")	"Pendulum model" method is easy for implantation
Reperfusion	No record	Cut the end rim of native structures (*Figure S5*)	Debonding can prevent compression after reflation and reperfusion

PA, pulmonary artery; PV, pulmonary vein.

Table 2 Overall operative time for rat lung transplantation, including all procedures

Single procedure*	Operative time (min)
Heart-lung block retrieval	8.4±0.8
Cuff preparation	8.1±1.4
Cold ischemia	26.4±2.2
Warm ischemia	8.1±1.2
Total procedure	48.0±2.8

*The procedures are defined as follows: heart-lung block retrieval: from incision on donor rat to excision of heart-lung block; cuff preparation: from excision of left donor lung to completion of cuff placement; cold ischemia: from flushing the donor lungs *in situ* with ET-Kyoto solution to graft removal from hypothermia storage; warm ischemia: from donor lung removal from ice until restoration of reperfusion; total procedure: from donor skin incision until closure of the recipient incision.

291.2±23.0 g, respectively. The mean operative times of heart-lung block retrieval, cuff preparation, cold ischemia, warm ischemia and total procedure time are seen in *Table 2*.

Discussion

Although several studies have introduced technical modifications for rodent LTx after Mizuta and colleagues first reported the cuff technique (1), some important and difficult key procedures have not been described. Rat LTx remains a difficult procedure, necessitating a long learning curve. In this study, we modified the operative devices and the cuff techniques to make it easier to master the procedure with a low complication rate and a high success rate. Using this modified technique, one author (H Shiiya) completed the first successful case at the 10th trying. Because it is a more expeditious operation, the ischemic times were shorter than those in a previous study (1) with fewer complication and no intraoperative deaths.

Only a few studies have reported on the instruments used for cuff preparation, although instruments can determine the success of this technique in the rat LTx model. Sugimoto *et al.* (9) used a petri dish for cuff preparation. With their technique, the donor lung was compressed and unstable when the cuff was created. A previous study reported the cuff preparation time at 18.7 min, which more than twice that of our technique (5). We introduced a modified cuff preparation plate that could stabilize the cuff preparation, making it easier to master and more expeditious. With this improved device, the cuff preparation time was only 8.1 min, with no structural lacerations in our series.

The skin incision of the recipient is important for transplantation. It is impossible to measure the intercostal space on a rat before surgery. Most previous reports that described the incision location, however, relied on different intercostal spaces (3-6,10,11). Habertheuer *et al.* (12) started

the skin incision 1 cm below the inferior margin of the scapula, which is unreliable because of the scapula is easily mobilized. In our series, we located the region of palpable cardiac impulse on the chest wall. This apical impulse region is at the level of the third or fourth intercostal space, as previously described (4,6,13,14). It seems more useful, however, to follow this apical impulse when performing rat LTx. Our series showed no failures in placing a suitable incision.

Another key trick is to locate the incisions on the recipient's PV, bronchus, and PA. To shorten the warm ischemia period and minimize pulmonary injury, an expeditious technique is important. Most of the previous reports made a transverse incision on the front wall of the recipient's hilar structures (3,9,12,15,16). Guo *et al.* (5) made the incision in a ├shape toward all the structures. Jungraithmayr *et al.* (17) made the incision on the lower branch of the PV. We first introduced the V-shaped incisions from the inferior-to-superior branches on the PV and bronchus cuffs. This V-shaped incision follows a wide rim of structure ostium, which allows the donor graft to be easily implanted without laceration. To match the lengths of the Br and PV, the ┴-shaped incision is made on the front wall of the PA, which can well prevent proximal laceration at implantation. Guo *et al.* (5) reported a 5% intraoperative failure rate of their technique due to bleeding and PV injury during the operation. Our study showed no intraoperative failures with hilar structure lacerations.

The "pendulum model" method for implantation was proposed in our study. Previous investigators often chose vessels as the first step. Rajab *et al.* (11) addressed PV first, followed sequentially with the bronchus and PA. Sugimoto *et al.* (12) went in the opposite direction, with the PA, bronchus, and PV, respectively. In our study, we anastomosed the bronchus first because, compared with vessels, it is structurally solid and easy to anastomose without laceration. In addition, we cut the superior bifurcation of the bronchus before the PA anastomosis because the bronchus and PA are always located adjacent to each other. Previous studies have not been concerned about the rim of the native hilum structures before reperfusion. Because the rim of the structures may contract and compress the graft structures, we cut the rim of the native structures to release them. With this technique, no vessel thrombosis, inadequate blood supply, or airway compression occurred after reperfusion and reinflation. Regarding the implantation time, previous studies reported an implantation time (warm ischemia time) of 15.2–20.0 min (5,6,12). With our modified implantation technique, the warm ischemia time was only 8.1 min.

Previous studies also showed follow-up survival rates of <90%. The intraoperative deaths were mainly due to pleural effusion, venous cuff failure, or pyothorax (1,5,12). Habertheuer *et al.* (12) reported about 18.9% cases were lost because of severe pleural effusions during the postoperative period. With our approach, the total follow-up survival rate was 97.2% (175/180), with no intraoperative deaths. A high success rate not only saves cost and time, it can strengthen the operator's confidence during surgery. All five deaths in our study were most likely due to pleural effusion. Minor bleeding from intercostal muscles is difficult to detect and may have caused the pleural effusion. As effusion accumulates, it may compress the grafted lung, causing a life-threatening complication. Thus, careful inspection is necessary before chest closure.

Pulmonary atelectasis is a common, severe complication of rat LTx. Although a rat can survive without obvious symptoms, the animal may not be used for further evaluation. In our study, only 5.6% of rats experienced graft atelectasis during the follow-up period, which appears to be an acceptable result for the rat LTx model. In our series, the post-LTx complications and follow-up deaths were rare. Reperfusion injury, a major cause of graft injury, is related to the ischemia time. A decrease in reperfusion pressure is thought to minimize this complication (17-19).

Conclusions

We developed modifications of devices and procedures of the cuff technique for the rat LTx model. The advantages of this modified cuff technique include its expeditiousness, low complication rate, and high rate of success. Our improvements could further facilitate rat LTx in a feasible, reproducible manner.

Acknowledgments

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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. The institutional Animal Care and Use Committee of The University of Tokyo (Tokyo, Japan) authorized this study (approval No. H19-027).

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For allogeneic transplant, nine- to ten-week-old Brown Norway weighing about 250 g are used as donors and eleven- to twelve-old Lewis weighing 300 g are used as recipients.

For syngeneic transplant, Lewis rats are used as both donors and recipients. Allogeneic LTx recipients were subcutaneously administered 25 mg/kg CsA (Novartis pharma, Tokyo, Japan) on 4 days in the first week.

All recipients were executed 0.5 g/kg cefazolin sodium (Nichi-Iko Pharmaceutical Co, Toyama, Japan), 30 mg/kg Methylprednisolone Sodium Succinate (Pfizer, Tokyo, Japan) and 10 mg/kg Furosemide (Teva Takeda Pharma Ltd, Aichi, Japan) at postoperative day 1 and day 2.

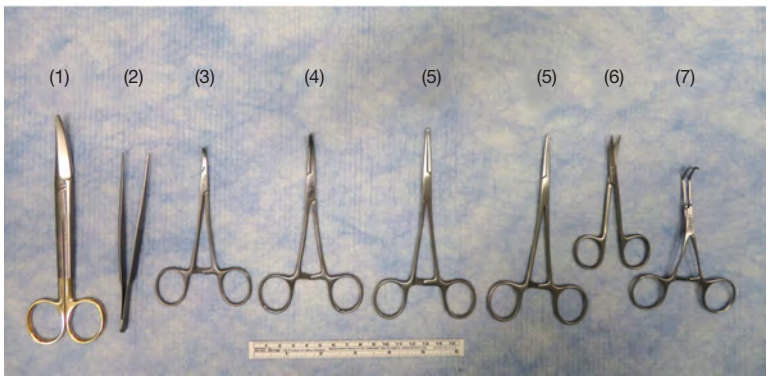

Figure S1 These instruments are used for donor graft retrieval. (1) Mayo scissors curved with carbide tips L140 mm, Natsume Seisakusho Co., Ltd, Tokyo, Japan. (2) Tweezers with fine tip without hook straight type L130 mm, Natsume Seisakusho Co., Ltd, Tokyo, Japan. (3) Micro-Mosquito Angled 90, Fine Science Tools, , Inc British Columbia, Canada. (4) Kelly hemostatic forceps 14 cm curved, Bioresearch Center Co., Ltd., Aichi, Japan. (5) Kelly hemostatic forceps 14 cm straight, Bioresearch Center Co., Ltd., Aichi, Japan. (6) Extra Fine Bonn Curved sharp/sharp 8.5 cm, Fine Science Tools, Inc., British Columbia, Canada. (7) Blood Vessel Clamp Satinsky 12 cm, Dr.Frigz International(Pvt)Ltd, Sialkot, Pakistan.

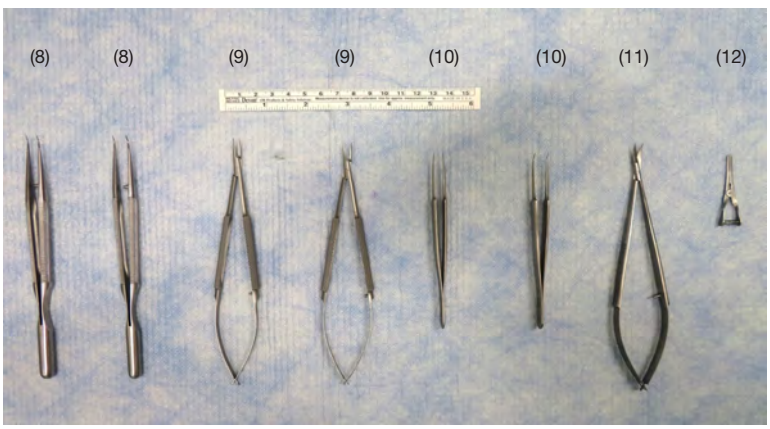

Figure S2 These instruments are used for graft cuff preparation. (8) Vessel Dilator (Angled), Fine Science Tools, British Columbia, Canada. (9) Micro Needle holder curved L155 mm, Natsume Seisakusho Co., Ltd, Tokyo, Japan. (10) Dumont #5/45, Fine Science Tools, Inc., British Columbia, Canada. (11) Microspring scissors 15 cm curved, Bioresearch Center Co., Ltd., Aichi, Japan. (12) Bulldog clamp straight W 0.9 mm × L 17 mm, Natsume Seisakusho Co., Ltd, Tokyo, Japan.

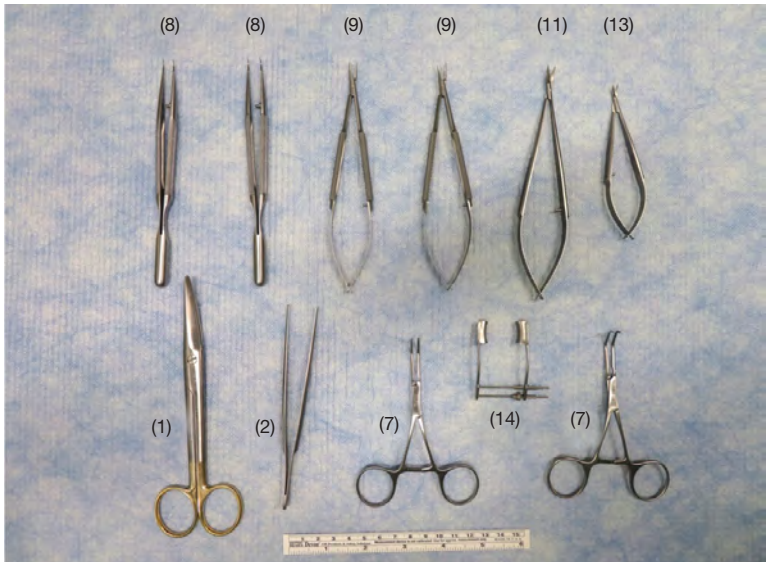

Figure S3 These instruments are used for implantation/anastomosis. (1) Mayo scissors curved with carbide tips L140 mm, Natsume Seisakusho Co., Ltd, Tokyo, Japan. (2) Tweezers with fine tip without hook straight type L130 mm, Natsume Seisakusho Co., Ltd, Tokyo, Japan. (7) Blood Vessel Clamp Satinsky 12 cm, Dr.Frigz International(Pvt)Ltd, Sialkot, Pakistan. (8) Vessel Dilator (Angled), Fine Science Tools, Inc., British Columbia, Canada. (9) Micro Needle holder curved L155 mm, Natsume Seisakusho Co., Ltd, Tokyo, Japan. (11) Microspring scissors 15 cm curved, Bioresearch Center Co., Ltd., Aichi, Japan. (13) Micro scissors curved L105 mm Cutting Edge 7 mm, Natsume Seisakusho Co., Ltd, Tokyo, Japan. (14) Speculum BANGERTER Large Right, Inami & Co., Ltd., Tokyo, Japan.

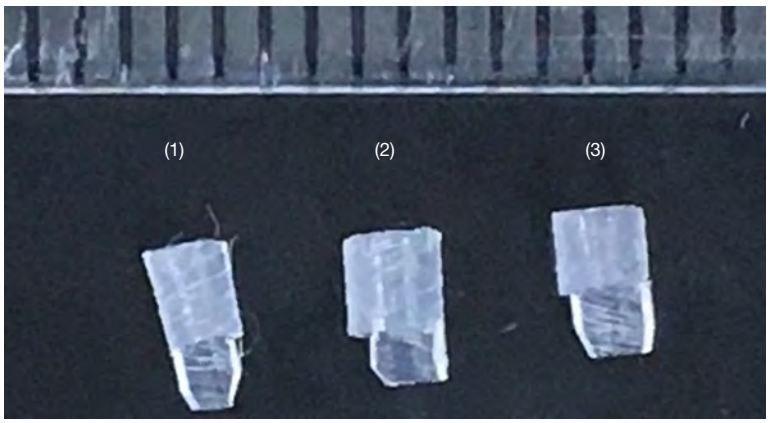

Figure S4 Cuffs with round tail.

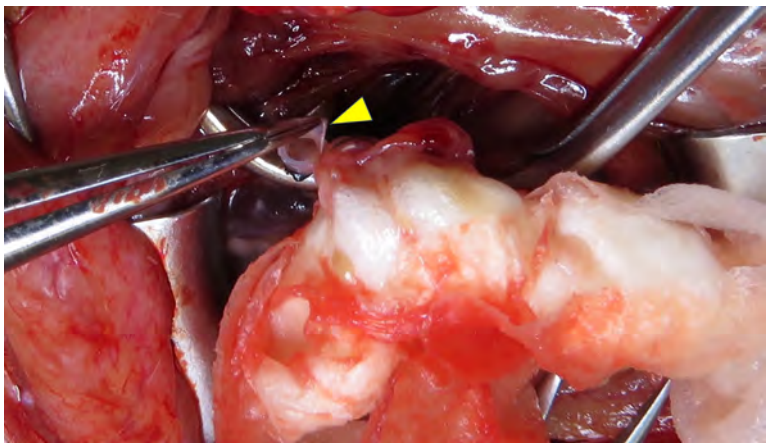

Figure S5 Cut the end rim of native structures (arrow).

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ORIGINAL ARTICLE

Tumor location may affect the clinicopathological features and prognosis of thymomas

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Keywords
Clinicopathological features; prognosis; recurrence; thymoma; tumor location.

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Abstract

Background: The current staging systems do not consider the tumor location of thymomas, and its clinical relevance is poorly understood. This study aimed to evaluate the impact of tumor location on the clinicopathological features and prognosis of thymomas.

Methods: We performed a retrospective review of patients at our institution who underwent an extended thymectomy for a thymoma from 1976 to 2015. The tumor location was classified as either the superior or inferior mediastinum based on the maximum tumor diameter. The clinicopathological characteristics of the thymoma were also evaluated. Kaplan-Meier estimates and Cox proportional hazards models were used to analyze the survival outcomes and risk factors for recurrence.

Results: A total of 194 patients with thymoma were eligible for this study. Compared with the inferior mediastinum group (n = 167), the superior mediastinum group (n = 27) had a higher frequency of myasthenia gravis (MG), advanced Masaoka-Koga staging, disease progression and recurrence ($P < 0.05$). The Kaplan-Meier analysis demonstrated thymomas in the superior mediastinum had worse survival outcomes that included overall survival, progression-free survival and disease-free survival ($P < 0.05$). The multivariate analysis showed tumor location was an independent prognostic factor for all the survival outcomes ($P < 0.05$). Furthermore, the tumor location ($P = 0.004$) and Masaoka-Koga stage ($P < 0.001$) were the only two independent risk factors for recurrence in the multivariate analysis.

Conclusions: The clinicopathological features of thymomas on MG, Masaoka-Koga staging, disease progression, and recurrence were different between locations of superior and inferior mediastinum locations. Thymomas in the superior mediastinum tended to be associated with worse survival and increased recurrence.

Introduction

Thymomas are regarded as rare, low-grade malignant tumors of the thymic epithelium that do not have a consensus treatment.[1,2] Approximately 1.3–2.2/10^6 people are affected by thymomas, according to worldwide cancer statistics.[3–5] However, up to half of the anterior mediastinal tumors are diagnosed as thymomas, which are widely considered to be the most common tumors in the anterior mediastinum.[6]

The typical clinical manifestations of thymomas are a mass that is identified on chest images with or without symptoms such as myasthenia gravis (MG), intermittent coughing, different degrees of chest pain, and vena cava syndrome.[7] Although thymomas are considered to be malignant, their 10-year survival rates are more than 90%.[8,9]

The clinicopathological and prognostic risk factors for thymomas are difficult to confirm because of the heterogeneous nature of the disease.[10] In fact, many retrospective

case series have described its characteristics and assessed different prognostic factors for long-term survival as well as risk factors for tumor recurrence. While the Masaoka-Koga staging system, which is based on the extent of tumor invasion, implantation, lymph node and/or hematogenous metastases, is widely accepted as the most important independent prognostic and risk factor for recurrence for thymomas, there is no consensus on the roles of other factors.[9–15] The International Association for the Study of Lung Cancer (IASLC) Staging Prognostic Factors Committee and International Thymic Malignancy Interest Group (ITMIG) recently proposed a staging system for thymomas and thymic carcinomas (TCs) that was in accordance with their survival outcomes.[16] This staging system was approved for the eighth edition of the tumor, nodes, and metastasis (TNM) staging classification by the American Joint Committee on Cancer (AJCC) / the Union for International Cancer Control (UICC) consortium.[16–18] Despite their extensive use, both the Masaoka-Koga and the TNM staging systems have ambiguities that have not been confirmed. Although thymomas can occur in any location of the anterior mediastinum, differences based on their anatomical tumor location have not been described in either of the current staging systems. As a result, it is currently unknown if tumor location can impact the clinicopathological features and survival outcomes of this disease. This retrospective study aimed to describe the clinicopathological features of thymomas that were located in different tumor locations. We also analyzed patient clinical outcomes to determine if tumor location is an independent prognostic factor and a risk factor for recurrence.

Methods

Patients

The Ethics Committees and Review Board of the University of Tokyo Hospital approved this study (No. 2406), and the need for patient consent was waived. This retrospective review included all the patients at our institute with a thymoma who underwent an extended thymectomy from 1 January 1976 to 31 December 2015. The clinical information was retrieved from electronic (2000–2015) and archived paper charts (1976–1999). The inclusion criteria for patients in this study were as follows: (i) a pathologically confirmed thymoma; (ii) complete clinical and pathological data accessible for restaging based on the Masaoka-Koga stage[19]; and (iii) the patient had undergone an extended thymectomy via a median sternotomy, as described in our previous studies.[20] We excluded thymic carcinomas or neuroendocrine tumors because they are histopathologically and clinically distinct from thymomas.[21,22] During the 40-year study period, 201 consecutive patients with thymomas were reviewed. Three patients with ambiguous WHO classifications[23] and four patients that lacked detailed imaging of the tumor location were excluded from the cohort. The clinicopathological features and survival outcomes of 194 patients were included in this retrospective review. The evaluated variables included tumor location, age, tumor diameter, sex, MG, Myasthenia Gravis Foundation of America (MGFA) classification, anti-acetylcholine receptor (anti-AchR), WHO classification, Masaoka-Koga stage, multiple primary malignant tumors (MPMT), surgical radicality, lymph node dissection, lymph node metastasis, preoperative induction therapy, postoperative adjuvant therapy, disease progression, recurrence, overall survival (OS), progression-free survival (PFS) and disease-free survival (DFS).

Definitions

The boundaries of the superior mediastinum were defined by previous publications as the level between the sternal angle to the inferior margin of the fourth thoracic vertebrae.[24,25] The tumor location was determined from preoperative thoracic imaging examinations and were based on the maximum diameter of the tumor from electronic images (year 2000–2015) or from the descriptions on paper images (year 1976–1999). The tumor diameter of each thymoma was measured by pathologists who recorded its value in the clinical charts. The presence of MG or MPMT was determined using preoperative information or data from the postoperative follow-up period. Both the pre- and postoperative incidence of other cancers were included in this study. The MGFA classification[26] was only performed for patients who experienced comorbid MG. The anti-AchR was determined by preoperative radioimmunoassays. A classification of positive (more than 0.3 nmol/L) or negative (0.3 nmol/L or less) of anti-AchR was used in accordance with the terms of the previous study.[27] The staging and histological classification of thymomas were performed according to the Masaoka-Koga staging system[19] and the WHO histologic classification criteria.[23] Surgical radicality was defined as either an R0 resection (with no residual tumor on microscopy) or a non-R0 resection (with microscopic or macroscopic residual tumor). The preoperative induction therapy and postoperative adjuvant therapy consisted of either chemotherapy or radiotherapy. The definitions of disease progression, recurrence, OS, PFS and DFS were compliant with the standard outcome measures for thymic malignancies of the ITMIG.[28] The disease progression, OS and PFS were analyzed for the whole population, while the recurrence and DFS were only calculated for patients who underwent an R0 resection.[28]

Follow-up

The postoperative follow-up interval was every 3–6 months, and chest X-ray or computed tomography (CT) were performed annually. Any other necessary examinations were determined by the patient's clinical needs. The final follow-up visit was in October 2018 to evaluate survival and recurrence outcomes.

Statistical analysis

Demographic and clinicopathologic variables were collected and included in the statistical analysis. The descriptive statistics are reported as the mean ± standard deviation. The categorical variables are reported as frequencies and proportions. The differences between the groups of superior and inferior mediastinum were assessed using a Student's *t*-test, chi-square test, linear-by-linear association, likelihood ratio detection or Fisher's exact test. The OS and PFS were evaluated using the Kaplan-Meier method. The differences between survival curves were analyzed by a log-rank test. Furthermore, patients who only underwent an R0 surgery were selected to evaluate DFS with the same methods described above. To assess the significance of tumor location as an independent prognostic risk factor for OS, PFS and recurrence, univariate and multivariate analysis were performed using the Cox's proportional hazards model. The hazard ratio (HR) was estimated with a 95% confidence interval (CI) for both the univariate and multivariate analysis. Since only a subset of the patients were comorbid for MG and underwent a lymph node dissection, we did not include the MGFA classification or lymph node metastasis as covariates in the univariate or multivariate analysis. The following variables were finally included as the starting set of covariates for the univariate analysis: tumor location (Superior/Inferior), age (as continuous), tumor diameter (as continuous), sex (male/female), MG (Yes/No), anti-AchR (positive/ negative), WHO histologic classification (A/AB/B1/B2/B3), Masaoka-Koga stage (I/II/III/IV), MPMT (Yes/No), surgical radically (R0 resection/non-R0 resection), lymph node dissection (Yes/No), preoperative induction therapy (Yes/No), and postoperative adjuvant therapy (Yes/No). Only variables with a $P < 0.1$ in the univariate analysis were included as covariates for the multivariate analysis. Two-sided tests were applied and a $P < 0.05$ was considered to be a statistically significant difference. The statistical analysis was performed using SPSS 24.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinicopathological features

A total of 194 patients with thymomas were eligible to be included in this study, including 98 males and 96 females with a mean age of 53.2 ± 13.5 years versus 53.9 ± 14.7 years ($P = 0.811$), respectively. Patients with thymomas in the superior mediastinum group ($n = 27$) accounted for 13.9% of the total cases while those with thymomas in the inferior mediastinum group ($n = 167$) accounted for 86.1%. The clinicopathological characteristics of patients with superior and inferior mediastinum thymomas are shown in Table 1. There were no significant differences in age, tumor diameter, sex, MGFA classification, anti-AchR, WHO histological classification, MPMT, surgical radicality, lymph node dissection, lymph node metastasis, preoperative induction therapy or postoperative adjuvant therapy between the two groups ($P = 0.811$, 0.448, 0.881, 0.492, 0.134, 0.069, 0.382, 0.721, 0.881, 0.268, 0.114, and 0.998, respectively). However, MG, Masaoka-Koga stage, disease progression and recurrence were significantly different between the two groups ($P = 0.007$, 0.005, <0.001 and < 0.001, respectively). More patients with thymomas in the superior mediastinum had MG (55.6% vs. 29.3%), Masaoka-Koga stage III/IV disease (40.7% vs.18.6%), and disease progression (44.4% vs. 8.4%) than those with thymomas in the inferior mediastinum. Only patients who underwent an R0 resection were included to evaluate recurrence. Patients with thymomas in the superior mediastinum tended to have a higher frequency of recurrence (37.5% *versus* 7.2%) than those with thymomas in the inferior mediastinum ($P < 0.001$).

Survival outcomes

The median follow-up period was 115.0 months (range: 0–399 months). The OS and PFS were 188.5 ± 21.4 months and 133.7 ± 23.2 months in the superior mediastinum group versus 349.3 ± 11.7 months and 316.2 ± 15.4 months in the inferior mediastinum group, respectively. The differences in OS and PFS between the superior and inferior mediastinum groups were significant ($P = 0.003$ and $P < 0.001$, respectively). Patients with thymomas in the superior mediastinum experienced a significantly worse OS and PFS than those with thymomas in the inferior mediastinum. The Kaplan-Meier curves are shown in Figures 1 and 2.

DFS was also assessed in this study, but only with patients who underwent an R0 resection. The DFS for patients with superior and inferior mediastinum were 149.3 ± 24.5 days and 330.4 ± 13.5 days, respectively. There was a significant difference in DFS between the two groups ($P < 0.001$). Thymomas in the superior mediastinum were associated with significantly worse DFS. The Kaplan-Meier curves are shown in Figure 3.

Prognostic factors for survival outcomes

To verify that the tumor location itself was associated with survival outcomes in patients with thymomas, both

Table 1 Summary of patient clinicopathologic characteristics in thymoma

Parameters	All patients (*n* = 194)	Superior (*n* = 27)	Inferior (*n* = 167)	*P*-value
Age (years) (mean ± SD) (range)	53.8 ± 14.5 (15–83)	53.2 ± 13.5 (28–78)	53.9 ± 14.7 (15–83)	0.811a
Tumor diameter (cm) (mean ± SD) (rang)	5.2 ± 2.5 (1.0–19.4)	5.6 ± 3.1 (2.5–16.0)	5.2 ± 2.4 (1.0–19.4)	0.448a
Sex				0.881b
Male	98 (50.5%)	14 (51.9%)	84 (50.3%)	
Female	96 (49.5%)	13 (48.1%)	83 (49.7%)	
MG				0.007*,b
Yes	64 (33.0%)	15 (55.6%)	49 (29.3%)	
No	130 (67.0%)	12 (44.4%)	118 (70.7%)	
MGFA classification†,‡‡				0.492c
I	28 (14.4%)	8 (29.6%)	20 (12.0%)	
II	22 (11.3%)	4 (14.8%)	18 (10.8%)	
III	5 (2.6%)	1 (3.7%)	4 (2.4%)	
IV	5 (2.6%)	2 (7.4%)	3 (1.8%)	
V	4 (2.1%)	0 (0)	4 (2.4%)	
Anti-AchR¶				0.134b
Positive	62 (32.0%)	12 (44.4%)	50 (29.9%)	
Negative	132 (68.0%)	15 (55.6%)	117 (70.1%)	
WHO classification‡				0.069d
A	17 (8.8%)	2 (7.4%)	15 (9.0%)	
AB	45 (23.2%)	4 (14.8%)	41 (24.6%)	
B1	64 (33.0%)	9 (33.3%)	55 (32.9%)	
B2	50 (25.8%)	5 (18.5%)	45 (26.9%)	
B3	18 (9.3%)	7 (25.9%)	11 (6.6%)	
Masaoka-Koga stage§				0.005*,c
I	98 (50.5%)	9 (33.3%)	89 (53.3%)	
II	54 (27.8%)	7 (25.9%)	47 (28.1%)	
III	24 (12.4%)	5 (18.5%)	19 (11.4%)	
IV	18 (9.3%)	6 (22.2%)	12 (7.2%)	
MPMT				0.382e
Yes	27 (13.9%)	2 (7.4%)	25 (15.0%)	
No	167 (86.1%)	25 (92.6%)	142 (85.0%)	
Surgical radicality††				0.721e
R0 resection	176 (90.7%)	24 (88.9%)	152 (91.0%)	
Non-R0 resection	18 (9.3%)	3 (11.1%)	15 (9.0%)	
Lymph node dissection				0.881b
Yes	41 (21.1%)	6 (22.2%)	35 (21.0%)	
No	153 (78.9%)	21 (77.8%)	132 (79.0%)	
Lymph node metastasis§§				0.268e
Yes	7 (3.6%)	2 (33.3%)	5 (14.3%)	
No	34 (96.4%)	4 (66.7%)	30 (85.7%)	
Preoperative induction therapy				0.114e
Yes	9 (4.6%)	3 (11.1%)	6 (3.6%)	
No	185 (95.4%)	24 (88.9%)	161 (96.4%)	
Postoperative adjuvant therapy				0.998b
Yes	79 (14.3%)	11 (40.7%)	68 (40.7%)	
No	115 (85.7%)	16 (59.3%)	99 (59.3%)	
Disease progression				<0.001*,b
Yes	26 (13.4%)	12 (44.4%)	14 (8.4%)	
No	168 (86.6%)	15 (55.6%)	153 (91.6%)	
Recurrence¶¶				<0.001*,b
Yes	20 (11.4%)	9 (37.5%)	11 (7.2%)	
No	156 (88.6%)	15 (62.5%)	141 (92.8%)	

*$P < 0.05$. †Alfred Jaretzki *et al*. 2000. ‡Muller-Hemelink *et al*. 1999. §Koga *et al*. 1994. ¶Positive: The serum titer of antiAchR ≥ 0.3 nmol/L. Negative: The serum titer of antiAchR<0.3 nmol/L (Nakajima *et al*. 2008). ††R0 resection: no residual tumor on microscopy; Non-R0 resection: microscopic or macroscopic residual tumor. ‡‡Only patients with MG were included (*N* = 64). §§Only patients with lymph node dissection were included (*N* = 41). ¶¶Only patients with complete resection were included (*N* = 176). aStudent's *t*-test was used. bChi-square test. cLinear-by-linear association. dLikelihood ratio detection. eFisher's exact test was used. MG, myasthenia gravis; MGFA, Myasthenia Gravis Foundation of America; anti-AchR, anti-acetylcholine receptor; WHO, World Health Organization; MPMT, multiple primary malignant tumors.

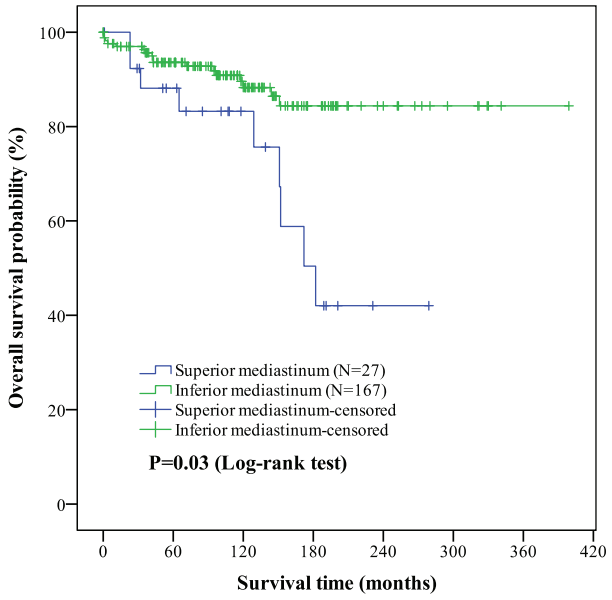

Figure 1 Kaplan-Meier curves of tumor location (superior/inferior mediastinum) on overall survival (OS) outcomes in thymoma. The mean overall survival in superior and inferior mediastinum of thymoma were (188.5 ± 21.4) months and (349.3 ± 11.7) months. The difference of OS between two groups was significant (P = 0.003). Of the 194 patients, the OS was (327.3 ± 13.3) months.

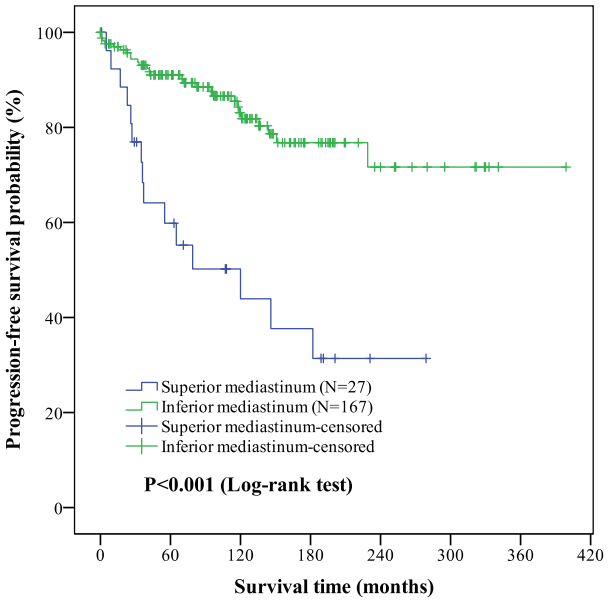

Figure 2 Kaplan-Meier curves of tumor location (superior/Inferior mediastinum) on progression-free survival (PFS) outcomes in thymoma. The mean PFS in superior and inferior mediastinum of thymoma were (133.7 ± 23.2) months and (316.2 ± 15.4) months. The differences of PFS between two groups were significant (P < 0.001). Of the 194 patients, the PFS was (294.7 ± 14.8) months.

univariate and multivariate analysis using a Cox proportional hazard model were performed for OS and PFS.

In the univariate analysis, both OS and PFS were associated with tumor location, age, Masaoka-Koga stage and preoperative induction therapy (P < 0.05). In addition, the tumor diameter, WHO classification and surgical radicality were shown to be predictors of PFS in the univariate analysis (P < 0.05). In the multivariate analysis, only tumor location and age were found to be independent prognostic factors of OS (P = 0.048 and < 0.001, respectively) and PFS (P < 0.001 and 0.002, respectively). Thymomas in the superior mediastinum were associated with a worse OS (HR, 0.371; 95% CI, 0.139–0.989; P = 0.048) and PFS (HR, 0.250; 95% CI, 0.117–0.533; P < 0.001). Additionally, preoperative induction therapy was shown to be an independent prognostic factor for OS (P = 0.030), but not for PFS (P = 0.466). The Masaoka-Koga stage was an independent prognostic factor for PFS (P = 0.004) but not for OS (P = 0.272). In contrast, sex, MG, anti-AchR, MPMT, lymph node dissection, and postoperative adjuvant therapy were not prognostic factors in either the univariate or multivariate analysis (P > 0.05). The results are presented in Tables 2 and 3.

Risk factors for recurrence

Only the patients with an R0 resection were included in the present analysis of recurrence. For the 176 patients,

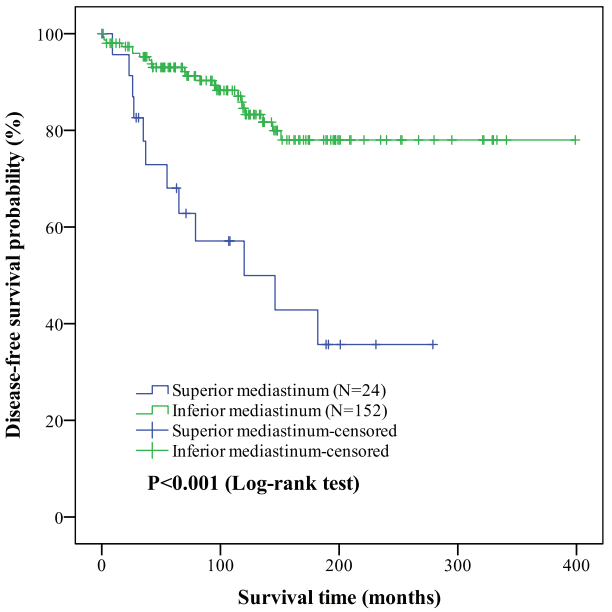

Figure 3 Kaplan-Meier curves of tumor location (superior/inferior mediastinum) on disease-free survival (DFS) outcomes in thymoma. The mean DFS in superior and inferior mediastinum of thymoma were (149.3 ± 24.5) months and (330.4 ± 13.5) months. The differences of DFS between two groups were significant (P < 0.001). Of the 176 patients, the DFS was (308.1 ± 13.9) months.

Table 2 Univariate and multivariate analysis of prognostic factors according to OS in thymoma

	OS	Univariate analysis		Multivariate analysis	
Prognostic factors	*n* (%)	HR (95% CI)	*P*-value	HR (95%CI)	*P*-value
Tumor location (Superior/Inferior)	18 (66.7)/150 (89.8)	0.318 (0.142–0.715)	0.006*	0.371 (0.139–0.989)	0.048*
Age (continue)	-	1.070 (1.034–1.107)	<0.001*	1.070 (1.034–1.107)	<0.001*
Tumor diameter (continue)	-	1.087 (0.953–1.239)	0.215	-	-
Sex (Male/Female)	85 (86.7)/83 (86.5)	1.079 (0.499–2.333)	0.847	-	-
MG (Yes/No)	54 (84.4)/114 (87.7)	1.136 (0.514–2.510)	0.752	-	-
Anti-AchR (positive/negative)§	53 (85.5)/115 (87.1)	1.008 (0.449–2.263)	0.985	-	-
WHO classification(A/AB/B1/B2/B3)†	14 (82.4)/43 (95.6)/57 (89.1)/40 (80.0)/14 (77.8)	1.403 (0.973–2.023)	0.070	1.093 (0.752–1.587)	0.641
Masaoka stage (I/II/III/IV)‡	87 (88.8)/49 (90.7)/18 (75.0)/14 (77.8)	1.541 (1.072–2.214)	0.019 *	1.277 (0.826–1.974)	0.272
MPMT (Yes/No)	21 (77.8)/147 (88.0)	2.218 (0.885–5.556)	0.089	1.894 (0.677–5.297)	0.224
Surgical radicality (R0/non-R0)¶	153 (86.9)/15 (83.3)	2.179 (0.646–7.353)	0.209	-	-
Lymph node dissection (Yes/No)	34 (82.9)/134 (87.6)	1.454 (0.611–3.463)	0.397	-	-
Preoperative therapy (Yes/No)	5 (55.6)/163 (88.1)	5.387 (1.823–15.917)	0.002*	3.813 (1.143–12.724)	0.030*
Postoperative therapy (Yes/No)	65 (82.3)/103 (89.6)	1.395 (0.642–3.031)	0.400	-	-

*$P < 0.05$. †Muller-Hemelink *et al.*, 1999. ‡Koga *et al.*, 1994. §Positive: The serum titer of Anti-AchR ≥0.3nmol/L. Negative: The serum titer of Anti-AchR<0.3nmol/L (Nakajima *et al.* 2008). ¶R0 resection: no residual tumor on microscopy; Non-R0 resection: microscopic or macroscopic residual tumor. OS, overall survival; HR, harzard ratio; CI, confidence interval; MG, myasthenia gravis; anti-AchR, anti-acetylcholine receptor; WHO, World Health Organization; MPMT, multiple primary malignant tumors.

tumor location, tumor diameter, WHO classification, Masaoka-Koga stage and preoperative induction therapy were identified as significant variables by the univariate analysis ($P < 0.05$), while tumor location (OR, 0.294; 95% CI, 0.107–0.803; $P = 0.017$) and Masaoka-Koga stage (OR, 3.355; 95% CI, 1.756–6.409; $P < 0.001$) were the only independent risk factors identified in the multivariate analysis. Recurrence was more likely for thymomas in the superior mediastinum and advanced Masaoka-Koga stages than for thymomas in the inferior mediastinum (Table 4).

Discussion

It is particularly important to determine the specific characteristics of thymomas. However, no previous studies have discussed the importance of tumor location in determining clinicopathological features or its relationship to prognosis. While many authors have attempted to identify prognostic factors and risk factors for the recurrence of thymomas, none have identified tumor location as a risk factor.[10,13,29,30]

Clinicopathological characteristics of thymomas

In the study by Padda *et al.* 33.0% of patients with thymomas had MG, which was similar to the incidence observed in the ITMIG database.[31] However, thymomas located in the superior mediastinum were more frequently associated with MG than those located in the inferior mediastinum. There is no consensus on the pathophysiological link between thymomas and MG.[32] However, previous studies have demonstrated a correlation between MG and anti-AChR antibodies.[33,34] In our study, there were more patients with positive anti-AchR in the superior mediastinum group but the difference between the two groups was not statistically significant.

Masaoka-Koga stage III/IV accounts for approximately 30% of all patients with thymomas.[15,35–38] In this study, we found that thymomas located in the superior mediastinum were more likely to be an advanced Masaoka-Koga stage than those located in the inferior mediastinum. The observation that there were more advanced stages of thymomas in the superior mediastinum can be explained from an anatomical standpoint as follows. Thymomas in the superior mediastinum are closely adjacent to the great vessels, such as the innominate vein, superior vena cava and others, which facilitates the invasion of the thymoma to neighboring organs. Furthermore, the superior mediastinum thymomas surround the great vessels, which makes them rich in blood supply and enables them to become metastatic even early in their disease history.

Progression and recurrence after the initial thymoma is resected is observed in 6.9%–18.0% of patients and can

Table 3 Univariate and multivariate analysis of prognostic factors according to PFS in thymoma

	PFS	Univariate analysis		Multivariate analysis	
Prognostic factors	*n* (%)	HR (95% CI)	*P* value	HR (95%CI)	*P* value
Tumor location (Superior/Inferior)	12 (44.4)/140 (83.8)	0.238 (0.126–0.449)	<0.001*	0.250 (0.117–0.533)	<0.001*
Age (continue)	-	1.033 (1.009–1.058)	0.007 *	1.043 (1.015–1.071)	0.002*
Tumor diameter (continue)	-	1.155 (1.061–1.258)	0.001*	1.098 (0.981–1.229)	0.105
Sex (Male/Female)	78 (79.6)/74 (77.1)	1.126 (0.614–2.065)	0.701	-	-
MG (Yes/No)	46 (71.9)/106 (81.5)	1.487 (0.806–2.744)	0.204	-	-
Anti-AchR (Positive/Negative)§	45 (72.6)/107 (81.1)	1.395 (0.753–2.585)	0.290	-	-
WHO classification (A/AB/B1/B2/B3)†	13 (76.5)/41(91.1)/51(74.0)/37(74.0)/10(55.6)	1.468 (1.097–1.963)	0.010 *	0.968 (0.710–1.322)	0.840
Masaoka stage (I/II/III/IV)‡	87 (88.8)/46(85.2)/14 (58.3)/5(27.8)	2.452 (1.863–3.227)	<0.001*	1.890 (1.223–2.922)	0.004*
MPMT (Yes/No)	18 (66.7)/134(80.2)	1.953 (0.928–4.111)	0.078	2.105 (0.918–4.829)	0.079
Surgical radicality (R0/non-R0)¶	142 (80.7)/10(55.6)	4.421 (2.025–9.651)	<0.001*	2.303 (0.791–6.705)	0.126
Lymph node dissection (Yes/No)	28 (68.3)/124(81.0)	1.857 (0.965–3.574)	0.064	0.802 (0.389–1.655)	0.551
Preoperative therapy (Yes/No)	4 (44.4)/148(80.0)	4.122 (1.597–10.639)	0.003*	1.559 (0.473–5.136)	0.466
Postoperative therapy (Yes/No)	59 (74.7)/93(80.9)	1.083 (0.588–1.992)	0.798	-	-

*$P < 0.05$. †Muller-Hemelink *et al*., 1999. ‡Koga *et al*., 1994. §Positive: The serum titer of anti-AchR ≥0.3 nmol/L. Negative: The serum titer of anti-AchR<0.3 nmol/L (Nakajima *et al*. 2008). ¶R0 resection: no residual tumor on microscopy; Non-R0 resection: microscopic or macroscopic residual tumor. PFS, progression-free survival; HR, harzard ratio; CI, confidence interval; MG, myasthenia gravis; anti-AchR, anti-acetylcholine receptor; WHO, World Health Organization; MPMT, multiple primary malignant tumors.

Table 4 Univariate and multivariate analysis of risk factors according to recurrence in thymoma

	Recurrence	Univariate analysis		Multivariate analysis	
Prognostic factors	*n* (%)	HR (95% CI)	*P*-value	HR (95%CI)	*P*-value
Tumor location (superior/inferior)	9 (37.5)/11(7.2)	0.158 (0.065–0.381)	<0.001*	0.294 (0.107–0.803)	0.017*
Age (continue)	-	1.002 (0.969–1.035)	0.926	-	-
Tumor diameter (continue)	-	1.236 (1.122–1.361)	<0.001*	1.027 (0.885–1.193)	0.724
Sex (Male/Female)	10 (10.8)/10(12.0)	1.067 (0.444–2.565)	0.884	-	-
MG (Yes/No)	10 (18.2)/10(8.3)	2.166 (0.900–5.212)	0.084	1.431(0.521–3.929)	0.486
Anti-AchR (positive/negative)§	8 (15.4)/12(9.7)	1.536 (0.627–3.760)	0.348	-	-
WHO classification (A/AB/B1/B2/B3)†	1 (6.3)/2(4.4)/8(13.8)/4(8.9)/5(41.7)	1.704 (1.110–2.615)	0.015*	0.967 (0.597–1.566)	0.891
Masaoka stage (I/II/III/IV)‡	2 (2.0)/6(11.1)/5(29.4)/7(100.0)	4.131(2.672–6.385)	<0.001*	3.355 (1.756–6.409)	<0.001*
MPMT (Yes/No)	4 (15.4)/16(10.7)	1.655 (0.551–4.971)	0.369	-	-
Lymph node dissection (Yes/No)	7 (20.0)/13(9.2)	2.259 (0.901–5.665)	0.082	0.899 (0.334–2.421)	0.833
Preoperative therapy (Yes/No)	5 (55.6)/15(9.0)	10.037 (3.545–28.413)	<0.001*	1.949 (0.510–7.442)	0.329
Postoperative therapy (Yes/No)	11 (15.7)/9(8.5)	1.505 (0.621–3.651)	0.366	-	-

*$P < 0.05$. †Muller-Hemelink *et al*., 1999. ‡Koga *et al*., 1994. §Positive: The serum titer of anti-AchR ≥0.3 nmol/L. Negative: The serum titer of anti-AchR<0.3 nmol/L (Nakajima *et al*. 2008). HR, harzard ratio; CI, confidence interval; MG, myasthenia gravis; anti-AchR, anti-acetylcholine receptor; WHO, World Health Organization; MPMT, multiple primary malignant tumors.

occur even decades after the primary resection.[22,29,39–42] In this study, thymomas located in the superior mediastinum were more likely to be associated with disease progression and tumor recurrence than those in the inferior mediastinum. The differences between the two anatomic locations of thymomas can be attributed to the following reasons. The superior mediastinum thymomas are linked to the great vessels which increases the likelihood of hematogenous metastasis when compared to thymomas of the inferior mediastinum. Regarding surgical treatment, the R0 resection is difficult to perform for thymomas in the superior mediastinum, which may also increase the disease progression rate.[38] Even with an R0 resection of the superior mediastinum thymoma, the probability of an intraoperative tumor implantation is increased, and the progression and recurrence of the patients will be affected accordingly. The clinical stage is often correlated with progression and recurrence rates.[38,43] Our results from the Masaoka-Koga stage of the thymomas demonstrated that thymomas located in the superior mediastinum were more likely to be an advanced Masaoka-Koga stage, which may result in higher progression and recurrence rates for the tumors in the superior mediastinum.

Survival outcomes of and prognostic factors for thymomas

When compared to tumors located in the inferior mediastinum, the thymomas of the superior mediastinum constituted a small proportion of the thymomas in this case series. However, the patients of the superior mediastinum group had an overall worse prognosis according to our survival analysis, and thus, their thymomas may have been more aggressive in nature. The OS, PFS and DFS in the inferior mediastinum group were largely consistent with previous reports, but those in the superior mediastinum group were worse than what has been previously reported.[14,20,34] To our knowledge, this is the first study to characterize the relationship between survival outcomes and the tumor location of thymomas.

It is difficult to determine a clear set of prognostic factors for thymomas due to the excellent patient survival. A review of prognostic risk factors for thymomas summarized 29 studies that used multivariate analysis to determine risk factors for survival and showed that the Masaoka-Koga stage was the most important factor associated with survival outcomes, followed by the WHO classification, surgical radicality and tumor size.[12]In our study, the Masaoka-Koga stage was regarded as a prognostic factor for PFS and the age for both OS and PFS, which was consistent with previous results.[4,44–46] Additionally, this study is the first demonstration that tumor location can also serve as a prognostic factor for OS and PFS. Thymomas in the superior mediastinum may result in poor survival. This is likely due to thymomas in the superior mediastinum reaching a more advanced Masaoka-Koga stage, which is associated with an increased disease progression and recurrence, as our results demonstrated. We also found that preoperative induction therapy was associated with a worse prognosis for OS, which may be due to the low number of patients who received this therapy (4.6%) in our study. However, the Japanese Association for Research on the Thymus (JART) reported the same results for preoperative induction therapy. The investigators concluded that this is likely because patients with more advanced staging are more likely to receive preoperative induction treatment.[46] There were no beneficial effects observed for postoperative adjuvant therapy in OS nor PFS, which is also consistent with previous reports by JART.[46]

Risk factors for the recurrence of thymomas

The ITMIG established that the tumor recurrence rates are the best measure of treatment efficacy in thymomas from at any site.[47] Recurrence is highly relevant for thymoma patients and more eligible patients are needed to improve the statistical analysis.

The results from a prospective multicenter database identified associations between higher Masaoka stage and increasing tumor size with increased recurrence rates. Sex, WHO classification, and adjuvant therapy were not risk factors for recurrence.[38] A review showed that an advanced tumor stage predicted a higher recurrence rate in all the included studies.[12] However, none of these studies discussed the tumor location as a potential predictive factor for recurrence or disease-free survival. In this study, the tumor location was categorized as either the superior or inferior mediastinum and the Masaoka-Koga stage was shown to be an independent risk factor for recurrence in both the univariate and the multivariate analysis. We showed that thymomas in the superior mediastinum were more likely to promote tumor recurrence. The neighboring great vessels and the more advanced Masaoka-Koga stages of the tumors in the superior mediastinum may contribute to these findings.

Collectively, these results demonstrate that tumor location should be included in the future staging systems for thymomas. Furthermore, there might be differences in the optimal treatment protocols for superior and inferior mediastinum thymomas, such as induction treatments, adjuvant therapies and follow-up intervals. Future multicenter and prospective studies are necessary to confirm this new proposal.

Limitations

There were several limitations of this study. First, this was a retrospective study with data that was collected over a period of approximately 40 years, which may have led to different diagnoses from the pathologists and surgeons. Second, the preoperative imaging data generated prior to 1999 could only be collected from chart records, which may be less accurate for distinguishing tumor locations than the information obtained directly from electronic records. Third, we could not include every potential confounding variables in our multivariate model. Additional multicenter prospective studies of the primary tumor location are required to confirm our findings and improve insights into the staging and treatment of thymomas.

In conclusion, this is the first report to demonstrate the significant impacts of tumor location on a range of clinicopathological characteristics and prognosis in patients with thymomas. The clinicopathological features of MG, Masaoka-Koga stage, disease progression and recurrence were different between the superior and inferior mediastinum thymomas. Importantly, we showed that thymomas in the superior mediastinum were associated with worse survival and more recurrence.

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Disclosure

The authors have no conflicts of interest to disclose.

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