Hepatitis C virus genotype 6 prevalence, spontaneous clearance and diversity amongst members of the Li ethnic minority in Baisha County, China

Running title: HCV genotype 6 prevalence in Li minority

Ru Xu[1#], Yongjuan Yu[2#], E. Carol McWilliam Leitch[3#], Min Wang[1], Ke Huang[1], Jieting Huang[1], Xi Tang[4], Qiao Liao[1], Dandan Song[4], Zhengang Shan[1], Chengyao Li[4], John Mclauchlan[3], Xia Rong[1]

1 Institute of Clinical Blood Transfusion, Guangzhou Blood Center, Guangzhou, Guangdong, China, 510095

2 Department of Clinical Laboratory, People's Hospital of Baisha Li Autonomous County, Hainan, China, 572800

3 MRC-University of Glasgow Centre for Virus Research, Glasgow, United Kingdom, G61 1QH

4 Department of Transfusion Medicine, School of Biotechnology, Southern Medical University, Guangzhou, Guangdong, China, 510515

[#] Ru Xu, Yongjuan Yu and E. Carol McWilliam Leitch should be considered joint first authors.

Correspondence: Xia Rong PhD., Institute of Clinical Blood Transfusion, Guangzhou Blood Center, Guangzhou, Guangdong, China, 510095. E-mail:joyjoy@126.com; Tel/Fax: +86-20-83595903.

Acknowledgements

We are grateful to Professor Ming Zhang (Department of Epidemiology and Biostatistics, Faculty of Infectious Diseases, University of Georgia, Athens, GA, USA) for assistance in modifying the manuscript.

Abstract

The epidemiology of hepatitis C virus varies widely across geographic regions and ethnic groups. Our previous study showed that 6 strains isolated from Baisha County, Hainan Island, China were all new genotype 6 (gt6) subtypes which differed significantly from subtypes of other regions. In the current study, we conducted a comprehensive epidemiological survey of HCV in the Li ethnic group, native to Baisha County. Anti-HCV antibodies were detected by 2 independent ELISAs in all participants, positive results confirmed by the recombinant immunoblot assay (RIBA) and HCV RNA viral loads were measured. Univariate chi-square test and multivariable logistic regression analyses were used to determine the risk factors for HCV infection and spontaneous clearance rates. Indeterminate RIBA results were excluded or included in analyses, consequently findings were expressed as a range. Direct sequencing of partial regions within NS5B and E1 were employed for genotyping. Among 1682 participants, 117 to 153 were anti-HCV positive (7.0-9.1%), with 42.7-52.6% confirmed to have cleared infection. Anti-HCV positivity was associated with older age (≥60years) (OR=0.02, 95%CI 0.01-0.05, P<0.01) and surgery (OR=2.75, 95%CI 1.36-5.57, P<0.01), with no significant difference found between the HCV infection group and the HCV spontaneous clearance group. The gt6 subtype distribution characteristics of Baisha County were unique, complex and diverse. The sequences did not cluster with known gt6 subtypes but formed 4 Baisha community-specific groups. HCV infection in members of the Li minority ethnic group is characterized by high prevalence rates in the elderly, high spontaneous clearance rates and broad gt6 diversity.

Key words: Hepatitis C; Seroprevalence; Risk factors; Li minority; Genotype

Introduction

Hepatitis C virus (HCV) is a blood-borne virus that is a major cause of end-stage liver diseases, including decompensated cirrhosis and hepatocellular carcinoma. Approximately 6-40 million Chinese people are HCV antibody positive (anti-HCV+), with a general prevalence of 0.43% to 3.2%[1-3]. Recent reports from the Chinese Ministry of Health showed that the number of new HCV infections increased from 39,380 cases in 2004 to 214,023 cases in 2017 (http://en.nhfpc.gov.cn/). Currently, there is no vaccine against HCV. Even though new direct acting antivirals (DAAs) can achieve high sustained virology response (SVR) rates, they are extremely costly and emerging resistance-associated variants (RAVs) have been linked to DAA treatment[4-7]. Prevention of new infections is therefore considered the most cost-effective policy to reduce overall HCV prevalence in China[8].

The prevalence of HCV is distributed unevenly in different regions of China and among different populations. Very high rates of HCV infection were often found in men who have sex with men (MSM) and in human immunodeficiency virus (HIV)-infected people in recent years[9,10]. Also, high HCV infection prevalence is often found in some remote regions of the country. For example, HCV prevalence was as much as 9.6% in Linxian, a rural community in Henan Province of China[11]. As reported, HCV is most frequently transmitted by certain medical and non-medical procedures[12-15]. Understanding the prevalence and risk factors for HCV infection in regions with high prevalence could effectively prevent virus transmission to other geographical areas. Following initial exposure to HCV, two outcomes can occur, either chronic infection or spontaneous clearance of the virus, which occurs in 15%-40%

of cases[16-18]. Due to the fact that symptoms occur in only ~15% of HCV-infected patients[19], the clearance rate may be underestimated.

HCV has been classified into 7 genotypes according to genetic variation. Generally, gt1, gt2, and gt3 are distributed globally, gt4 is prevalent in Middle Eastern countries, gt5 is restricted to the northern part of South Africa[20], and gt6 is endemic in Southeast Asia and south China[21-24]. Previously, we conducted a comprehensive study [25] and found that HCV gt6a has become the second most prevalent subtype on Hainan Island. Other than HCV gt6a, we also identified 6 novel unassigned gt6 variants, all of which were discovered in the Li ethnic within Baisha County, Hainan Island[26]. Baisha County is located in a mountainous area on Hainan Island and most native residents belong to the Li ethnic minority that is derived from a sub-group of Austronesians who are descended from the aborigines of the Neolithic Age. In order to understand whether the novel gt6 unassigned variants were an unusual phenomenon, we designed this study to understand the epidemiology of HCV infection in the Hainan Li ethnic community. Such information could help improve strategies for HCV prevention and control in this county and potentially avoid transmission to other regions of Hainan Island. Moreover, it could give insight into the evolution of gt6 in the Li ethnic group.

Material and methods

Survey cohort

From July 2014 to October 2015, 1682 volunteers from all 4 Baisha communes

and 7 townships aged 11-95 years were invited to participate in this study. The participants were selected by a random sampling method, including adults who received health examinations and children in high school who received a thalassemia test. The inclusion criteria were: 1) residing in the study locale, 2) having the cognitive ability required to respond to the questionnaire survey and 3) having no prior HCV treatment. The physicians ensured that individuals were personally interviewed to assure their complete understanding of the study and the participants provided written informed consent prior to enrollment. People who refused to provide the detailed information contained in questionnaire survey forms were excluded from the study. Participants' socio-demographic features and the presence of risk factors were recorded and blood samples were collected for an assessment of HCV markers. This study was approved by the Medical Ethics Committee of Guangzhou Blood Center and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All study subjects signed an informed consent.

Anti-HCV assays and HCV RNA detection

The 1682 plasma specimens were tested for HCV antibody using two independent HCV enzyme-linked immunosorbent assays (ELISA) (Shanghai Kehua, China and Ortho HCV 3.0 ELISA, Ortho-Clinical Diagnostics, Inc, USA). HCV RNA levels were tested by quantitative real-time polymerase chain reaction (qPCR) (Diagnostic Kit for Quantification of Hepatitis C virus RNA, Da An Gene Co, Ltd. of Sun Yat-Sen University, China) and the limit of detection (LOD) was 50 IU/ml. The presence of HCV antibody was further confirmed by RIBA-HCV BLOT 3.0 (MP Biomedicals Asia Pacific Pte. Ltd., Singapore). The RIBA-HCV BLOT 3.0 assay utilizes well-defined

antigens derived from HCV immunodominant proteins from core, NS3 helicase, NS4 and NS5 regions. Band reactivity was graded by visual calibration against the human immunoglobulin (IgG) control band (1+) and anti-IgG control band (3+). A sample was considered positive when at least two HCV bands had a reactivity of ≥1 or a single core band had reactivity of ≥2. Indeterminate results were defined when only a single band had a reactivity (except core reactivity of ≥2), and a result was considered negative when no single HCV antigen line had a reactivity of ≥1.

Sequence determination

HCV sequences from the E1 region [nucleotide (nt) 729 to 1322 according to the numbering of the H77 genome] and NS5B region (nt 8256 to 8641) were amplified following previously described protocols[22]. Briefly, viral RNA was extracted using a QIAamp Viral RNA Mini Kit (QIAGEN Inc, Valencia, CA, USA). Reverse transcription PCR (RT-PCR) was performed by RevertAid First Strand cDNA Synthesis Kit (Fermentas, CA, USA). Amplification was completed by a nested PCR with E1 or NS5B-specific primers[22]. The amplified products were bi-directionally sequenced using an Applied Biosystems (ABI) PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Version 3.1 (Applied Biosystems, Foster City, CA, USA). The bi-directional sequences were inspected and assembled using SeqMan to obtain the consensus sequence. Sequences were aligned using MegAlign. The above software used is contained in the Lasergene 8.1 Package (DNASTAR Inc, Madison, WI).

Phylogenetic analysis

Phylogenetic analysis was performed using MEGA 7.0. The maximum likelihood approach (using the General Time Reversible model and Gamma distributed with Invariant site (G+I) rate) with 500 iterations of bootstrap sampling was performed. HCV genotype reference sequences were retrieved from the HCV database (http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html). Reference sequences in the phylogenetic analysis were chosen according to consistent criteria[27] and HCV gt6 unassigned sequences were included (detailed information is shown in Supplementary Table 1). The significance of the tree topology was evaluated using the distance program (method: p-distance), implemented in Mega 7.0. Bootstrap values of >70% were considered significant.

Statistical analysis

The Chi-square test and multivariate logistic regression analysei~~s~~ were applied to determine the predictors of anti-HCV positivity. Measurement data were expressed as the mean ± standard deviation (SD) and compared using the two-sample t-test, where appropriate. $P<0.05$ was considered statistically significant. All statistical analyses were performed with SPSS Statistics for Windows, version 19.0 (IBM Corp., Armonk, New York).

Nucleotide sequence accession numbers

The nucleotide sequences reported in this study were deposited in Genbank with the following accession numbers: MH298078-MH298132 and MH298133-MH298190.

Results

HCV infection in the Baisha Li minority

Samples from the 1682 Baisha Li subjects were tested for HCV antibodies, and 324 were reactive (19.3%) based on the Ortho ELISA assay. Reactive samples were retested with Kehua ELISA assay, and 249 (14.8%) were reactive with both independent assays. HCV RNA was detected in 67 of these samples with a mean viral load of 6.76×10^4 IU/ml ± 9.55 (Table 1). No samples were found that were both anti-HCV negative and HCV RNA positive (HCV RNA [+]). When compared with the viral RNA titer (3.90×10^5 IU/ml ± 6.92) in chronically HCV-infected subjects in China in our previous study[28], the HCV RNA titer in the Li minority of Baisha County was significantly lower ($t=20.79$, d.f.=102, $P<0.01$, t test).

We determined that positivity in both the HCV RNA test and 2 independent ELISAs was sufficient to confirm HCV infection and no further serological testing was performed for these 67 samples. For the remaining 182 seropositive samples that were HCV RNA negative [HCV RNA (-)], an anti-HCV confirmatory assay (RIBA) was used to further assess the presence of HCV antibodies. Using RIBA, 96 of the 182 HCV ELISA (+) samples were determined to be negative and subsequently excluded from the anti-HCV positive group (Table 1). Patients testing HCV RNA (+) (n=67) or RIBA positive (n=50) were defined as the anti-HCV (+) group. HCV RNA (+) individuals were considered as having a persistent infection whereas RIBA positive individuals but who were HCV RNA (-) were defined as spontaneous clearers. There were 36 RIBA indeterminate samples which were either included or excluded from

subsequent analysis to give both liberal and conservative estimates. Using these criteria, the prevalence of HCV infection in the Baisha Li minority was 7.0-9.1% (n= [67+50]/1682 or [67+50+36]/1682). Surprisingly, HCV prevalence varied greatly between subjects ⩾60 years old and < 60 years old (Table 2). Only 5 of the HCV (+) samples were from individuals < 60 years old. The prevalence of virus infection for participants aged 60 or more was 16.4-21.6% (n=112/685, or 148/685 including RIBA indeterminate results), but only 0.5% (n=5/997) for those <60 years of age. Furthermore, we found that HCV spontaneous clearance was high (42.7-52.6%, n=50/117 or 86/153 minus or plus RIBA indeterminate results respectively) across the entire infected group. In terms of the antigen reactivity of the 86 HCV RNA (-), RIBA (+) or indeterminate samples, a wide reactivity was observed with core antigen. 69 samples (80.2%) reacted with core antigen, 65 (75.6%) reacted with NS3 antigen (NS3-1 and NS3-2), 50(58.1%) with NS4 antigen and 12(14.0%) with NS5 antigen (Supplementary Table 2).

Risk factors for hepatitis C

The prevalence of HCV infection did not differ significantly by univariate analysis based on gender, transfusions, alcohol consumption, piercings, acupuncture, familial HCV infections or dental procedures, but did vary significantly for tattooing, surgery and age ⩾60 years, both including and excluding the RIBA indeterminate samples (Tables 3 and 4). The results obtained by including or excluding RIBA indeterminate samples from the analysis were similar; only the inclusion group is shown below and details for the exclusion group are given in table 4. In the anti-HCV (+) group, there were more persons who had been tattooed and undergone surgery than in the anti-HCV

(-) group (OR=2.82, 95%CI 1.78-4.46, P<0.01; OR=4.05, 95% CI 2.18-7.51, P<0.01, overall chi-square test). We also found that individuals aged ≥60 years had a significantly higher prevalence of HCV infection than people aged <60 years (OR=0.02, 95% CI 0.01-0.05, P<0.01, chi-square test) (Table 3). Multivariate logistic regression analysis revealed that an age≥60 years (OR=0.02, 95% CI 0.01-0.05, P<0.01) and surgery (OR=2.75, 95%CI 1.36-5.57, P<0.01) were the significant predictors of anti-HCV positivity (Table 3) with the confounders adjusted. In addition, we found that there was no significant difference between HCV spontaneous clearance and HCV persistent infection for risk factors including gender and age, as well as history of transfusion, alcohol consumption, tattooing, surgery, piercings, acupuncture, a family history of HCV and dental operations (Supplementary Table 3). There was also no significant difference in the relative prevalence of persistent and recovered HCV infection according to gender, either excluding or including the RIBA indeterminate results (supplementary table 4).

Molecular characterization of Baisha HCV strains

In order to determine the HCV genotype/subtype distribution in the study subjects, we amplified and sequenced the 67 HCV RNA (+) samples. 55 sequences from the E1 region and 58 from the NS5B region were obtained, while neither E1 nor NS5B sequences were amplified from 8 samples because of their very low viral loads and limited plasma volume. For these 8 samples, we tried to amplify the 5'UTR and Core regions, but only 1 sequence from the 5'UTR region was amplified which was determined as gt6new by phylogenetic analysis (data not shown). No amplified products were obtained from sample 114 in the NS5B region and samples 257, 292,

429 and 555 in the E1 region despite several attempts, suggesting a lack of primer specificity for these HCV strains which potentially represent new subtypes. Indeed all the amplified products in our analysis did not cluster clearly with any of the previously assigned gt6a-6xd or other unassigned gt6 variants which were collected from some south Asian countries such as Thailand, Vietnam, Cambodia, Laos and Myanmar[23,29] (Fig 1). Interestingly, the NS5B Baisha sequences formed four specific groups (designated Ⅰ, Ⅱ, Ⅲ and Ⅳ) which included 6 database sequences collected from Baisha County of Hainan Island and 1 database sequence obtained from the Guangdong province of China. 24.1% (14/58) of the Baisha strains clustered within group Ⅰ, 19.0% (11/58) clustered with group Ⅱ, 36% (21/58) clustered with group Ⅲ and 15.5% (9/58) clustered with group Ⅳ, all with significant bootstrap values. Isolates BSM397, 371 and 396 were distinctly divergent from the above four groups and the gt6 reference, but their bootstrap values were lower than 70%. It should be noted that sequence homology between these three isolates and gt6 reference strains ranged between 61-83%. Full length viral genome sequencing is required to confirm their diversity in a future study. Although the subtype 6w (EU643834, DQ278892) database sequences were the closest strains to group Ⅰ, these sequences cannot be considered as belonging to 6w because of high variability with the database strains (ranging between 19.4%-22.9%), which is greater than the recognized within-subtype distance of 15%. Six isolates (KJ470620, KJ470621, KJ470622, KJ470623, KJ470624, KJ470625; designated gt6new) that were previously collected from Baisha County clustered with group Ⅱ and Ⅲ. The distance between KJ470623 and samples 9, 41, 46, 22, 239 and 525 was 9.3%-12.4%, which is within the acceptable subtype range. However at least one full-length (or at least complete open reading frame) is needed to designate a new subtype according to the HCV nomenclature criteria of the International Committee on

Taxonomy of Viruses (ICTV). The distances between other database sequences and the Baisha sequences were greater than 15%. Even KC844040, the gt6new sequence which was collected from Guangdong province of China that clustered with group Ⅳ, was 15%-19.3% distant from the group Ⅳ Baisha isolates, indicating that the Baisha sequences are divergent from all other current gt6 sequences. To illustrate the diversity within the Baisha sequences, strains 282 and 305 were identified from a couple but clustered with different groups and the sequence divergence was 29%. No other participants within the study were related according to our questionnaire survey.

We also analyzed E1 sequences using the same methodology as that for NS5B. The phylogenetic tree inferred by E1 sequences (Fig 2) had a similar topology to the NS5B phylogenetic tree. All the E1 sequences in the current study also did not clearly cluster with any of the currently assigned gt6a-6xd subtypes or with unassigned gt6 variants which were collected from some south Asian countries[23,29]. Similar to the results with NS5B, the E1 sequences demonstrated a trend and formed four specific groups (groups Ⅰ, Ⅱ, Ⅲ and Ⅳ) although the bootstrap values of groups Ⅰ and Ⅱ were lower than 70%. Full length viral genome sequencing is required to confirm the diversity of these two groups in a future study. The sequences from the couple (strains 282 and 305) that were divergent in the NS5B region also clustered with different groups in the E1 region.

Characterization of highly divergent Baisha HCV viral strains

In order to determine whether or not the high sequence divergence observed for samples 282 and 305 was the consequence of a complex mixture of multiple infections,

the E1 amplified product was cloned and 50 clones from each sample were sequenced and phylogenetically analyzed (Supplementary Fig 1). Strain 409 was cloned and similarly sequenced as a control. Within each sample tested, the cloned sequences were closely related, with a sequence homology of 99-100% for sample 282, 95-100% for 305 and 98-99% for 409. Phylogenetic analysis showed that the cloned sequences for each isolate clustered together and with the corresponding PCR-derived sequence (Supplementary Fig 1). When the cloned sequences were included in the E1 phylogenetic analysis, the topology and bootstrap values were similar to those obtained with the PCR-derived consensus sequences (data not shown). These data confirmed the genomic divergence of the strains from samples 282 and 305, indicating that this couple had been independently infected and sexual transmission can be excluded.

Discussion

In the current study, high HCV prevalence (7.0-9.1%), high spontaneous clearance rates (42.7-52.6%) and a broad gt6 diversity were observed in Baisha County, which is remarkably different from that observed in other regions. The specific epidemiology resulting in such high divergence is unknown but may be indicative of transmission trends or specific HCV evolution.

The prevalence of HCV in the Li minority of Baisha County in our study was between 7.0% and 9.1%, which is higher than previous reports in China (3.2% in 1992[30], 2.2% in 1997[2], 0.43% in 2006[3] and 3.0% reported by a more recent study whose subjects were recruited in Jilin province from 2010 to 2013[1]). One reason for the varied HCV prevalence between studies was the testing procedures used, i.e. utilizing one or two ELISA screening assays, and whether a confirmatory assay (RIBA) was

334 applied. For example, the 0.43% positive rate was based on the utilization of two
335 ELISA screening assays plus RIBA[1] whereas the 3.0% prevalence in Jilin province was
336 based on two ELISA screening assays only[3]. In our study, after performing two
337 independent ELISA screening assays, we further employed a highly specific RIBA
338 assay to exclude false positive reactions from the ELISA screening assays which have
339 been reported to have up to 35% false-positive results in immunocompetent individuals,
340 blood donors, the military and the general population[31]. RIBA indeterminate results
341 indicate that the clinical status of such individuals cannot be determined. Previous
342 studies showed that a RIBA indeterminate test may be observed in recently infected
343 individuals who are in the seroconversion phase or in elderly individuals who have
344 recovered from previous or distant HCV infection[32-34]. Makuria et al[33] reported that
345 RIBA-indeterminate blood donors were older than chronic HCV carriers. All of the
346 RIBA indeterminate individuals in our study were ⩾60 years old and there is no other
347 available RIBA assay to perform a second test. Therefore, we considered these RIBA
348 indeterminate samples as both recovered and not recovered from infection in our
349 calculations. Thus, the 7.0 to 9.1% prevalence of HCV infection in the Li minority
350 group in this remote, rural county is relatively reliable. Another likely reason for
351 discrepancies between studies is the socio-demographics of the participants within each
352 cohort, including geography and age. Interestingly, HCV infection in the Baisha Li
353 minority demonstrates a birth cohort effect, with 95.7-96.7% (n=[112/117]-[148/157])
354 of HCV infections occurring in individuals ⩾60 years old and the prevalence rate in
355 this age group was 16.4 to 21.6%. Of the five infected individuals whose age was <60
356 years old, four sequences (BSY159, BSY517, BSM217 and BSM694) had a scattered
357 distribution among the elder age sequences (Fig 1 and Fig 2); the fifth was HCV RNA
358 (-) and no viral sequences were obtained from PCR amplification. Both univariate

Chi-square and multiple logistic regression analysis showed that age ⩾60 years old was strongly associated with anti-HCV positivity. We speculate that perhaps specific lifestyle behaviors or poor medical practices in the past led to HCV transmission in the Li ethic group from Baisha County. Furthermore, surgery was an important risk factor for HCV infection both by univariate Chi-square and multiple logistic regression analysis, probably due to the use of unsterile devices, as demonstrated in previous studies[35,36]. Another important risk factor was tattooing based on the results from the univariate Chi-square between HCV infection prevalence in tattooed verses non-tattooed persons (Table 3 and 4). Unsafe practices could have exposed individuals to contaminated tattooing tools because of a lack of awareness of infection control practices, lack of sterilization equipment, not purchasing disposable tattoo tools and so forth. There is evidence that tattooing is a route for HCV transmission[37,38]. The Li minority of Baisha County has reportedly had less contact with individuals from other ethnic groups, and tattooing as a custom persists throughout their life, especially in elderly individuals, as demonstrated by the average age of the persons who were tattooed (73.33±14.54 years old) being greater than non-tattooed individuals (47.77±22.41 years old) ($t=98.98$, $P<0.01$, t test, data not shown). However, in terms of the multivariate analysis, HCV prevalence was not significantly different between the tattooed and non-tattooed participants. These inconsistent results may be attributable to the strong association between age and tattooing ($\chi2=1.37E2$, OR=12.89; 95%CI = 7.57-21.95, P=1.37E-31, chi-square test, data not shown). Furthermore, some reports indicate that the male sex is associated with HCV infection[39-41]. Our results lack an association between males and HCV infection, which differs from the literature on this topic. One reason for this discrepancy could be that the prevalence of tattooing in females was significantly higher than in males (female (11.0%) vs male

(3.4%): $\chi 2=31.50$, $P<0.01$, data not shown), and that the custom of tattooing leads to higher HCV prevalence (tattooed (20.0%) vs non-tattooed (8.1%): ($\chi 2=21.10$, $P<0.01$, data not shown)) in females.

The published data reveals that the HCV clearance rate varies in different populations[16]. HCV spontaneous clearance of 18% occurred in children in a contemporary US cohort after 6 months of infection[42]. Two recent studies from north and southwest China involving a limited number of patients with acute HCV infection reported recovery rates of 28.2%[43] and 30.2%[44]. The Irish Hepatology Research Group revealed that there was up to 50% recovery among a population of Irish women who were exposed to HCV-contaminated anti-D immune globulin[45,46]. Therefore, the HCV spontaneous clearance rate in Baisha Li ethnic group is relatively high (42.7 to 56.2%). There is a possibility that a lower spontaneous clearance rate is masked by fast progression to disease and HCV-related mortality thus increasing the observed clearance rate among elderly individuals. Compared to a population of Chinese chronically HCV-infected subjects, the significantly lower viral load observed in the Li population provides indirect evidence to support a higher spontaneous clearance rate which would correlate with a previous study[39]. No significant association was found with gender in our study, although it has been well-established that women are more likely to clear HCV than males[41,43,44,47]. However, three other studies, including U.S. injection drug users, U.S. blood donors and the Italian general population, also found no significant association of HCV clearance with gender[48]. One possible interpretation for these differing results is that the association with gender may be confounded by tattooing, the probable route of HCV transmission in Baisha County, which is practiced significantly more in females than males as discussed above. In addition the genotype 6 strains from our study may differ in this respect compared to the genotypes that

predominate in other studies that have evidence linking gender and clearance rates. According to reports, the different HCV spontaneous clearance rates might be related to host and/or viral factors. Host factors include humoral and cellular immune responses. In this study, only the humoral response was examined, particularly in the reactivity to individual viral proteins. From the data collected here, the anti-core reactivity seemed to persist, while the anti-NS5 reactivity appeared to be short-lived. It has been reported that reactivity to core protein may represent either non-specific reactivity or resolved infection with antibodies to other HCV proteins that are no longer reactive. In addition, NS3 and core antibodies are the last two remaining reactive proteins in cases of seroconversion[49].

There is a geographical distribution of the HCV genotypes in China. For example, subtypes 1b and 2a were predominant in north China[50], subtype 6a was first found in Guangdong province and transmitted to other regions[40], and subtypes 3a and 3b were found in south China[51]. Subtype 6a was initially detected in Guangdong province and has become the second most prevalence subtype since 2008[22]. Importantly, subtype 6a has rapidly spread from Guangdong to other regions in China over the past 10 years[22]. The distribution of HCV genotypes on Hainan Island which seceded from Guangdong province in 1988 (http://www.npc.gov.cn/npc/dbdhhy/content_5602.htm), is still largely unknown especially in the Li ethnic minority. Fig 3 shows the HCV genotype distribution of previous studies on Hainan Island from 2009 to 2016; subtype 1b has gradually decreased, while subtype 6a significantly increased from no detected cases to 35% of cases in the Han ethnic population[24,26,52,53]. Only 6 isolates were from the Li minority, and interestingly, they were novel gt6 variants[20]. According to the above results, we speculate that there is a different HCV genotype distribution between the Li minority

and Han ethnic groups on Hainan Island. We found that novel HCV gt6 predominate in the Li minority group of Baisha County according to phylogenetic trees constructed from NS5B and E1 sequences. The predominant gt6 strains were diverse and did not cluster into any assigned subtypes from other regions in China, including the rest of Hainan island[24,52,53]. The reason for this discrepancy could be the distinct geographical location of Li and Han populations or a lack of interaction between the groups. In previous studies, samples were collected from the Han population of Haikou city, a modern city. Samples in our study were collected from Baisha County, a remote community, most of whom are of the Li minority. We concluded that the broad diversity of gt6 is a likely characteristic of HCV infection in the Li minority group of Baisha County. One possible biological interpretation for this tree topology (Fig 1 and Fig 2) is that most of the variants have evolved over time rather than a recent introduction into Baisha County. Further support for this hypothesis is that most of the HCV variants were isolated from elderly (≥60 years) individuals with limited exposure to HCV transmission routes outside their own community. A similar endemic spread seems to have occurred in Ghana, west Africa with gt2[54]. However, there is no evolutionary data to support this hypothesis in our current analysis. Another possible reason is that some isolates were transmitted from Guangdong province, as KC844040 was the closet sequence from group Ⅳ. To test these two hypotheses, comprehensive evolutionary and diversity analyses will be required in a future study. Moreover, full-length genome sequencing will be required to provide more detailed sequence information and meet the requirement of new subtype designation according to the HCV nomenclature criteria of ICTV[27].

In summary, HCV is common in a rural Li ethnic minority community among its

older residents (≥60years), demonstrating a birth cohort effect. Moreover, the anti-HCV confirmatory assay revealed a high rate of spontaneous HCV clearance and infection in the Baisha Li minority group. HCV infection within the community is characterized by the predominance of gt6, with broad genetic diversity that potentially contains new subtypes for this genotype.

Conflict of interest

All authors declare that they have no competing interest.

Funding:

This research was supported by the grants from National Natural Science Foundation of China (No.81772208), ECML and JM were supported by funding from the UK Medical Research Council (MC_UU_12014/1).

Author Contributions: Ru Xu drafted the main manuscript. Yongjuan Yu, Min Wang, Jieting Huang, Xi Tang, Qiao Liao, Dandan Song and Zhengang Shan contributed to donor recruitment and data collection. Ru Xu and Ke Huang performed statistical analysis and the interpretation of the data. E. Carol McWilliam Leitch, Chengyao Li and John Mclauchlan revised the manuscript. Xia Rong conceived the study and approved the manuscript. All authors reviewed and approved the final version of the manuscript.

References

1. Fu-qiang CY-sLLC. A sero-epidemiological study on hepatitis C in China. Chin J Epidemioil 2011;32:888-91.

2. Kang LY SY, Hao LJ, Cao XY, Pan QC. Studies on epidemiology of the population with HCV and HEV infections and their epidemic factors in China. Chinese Journal of Infectious Diseases 1997;15(2):71-5.

3. Zhang Q, Qi W, Wang X, et al. Epidemiology of Hepatitis B and Hepatitis C Infections and Benefits of Programs for Hepatitis Prevention in Northeastern China: A Cross-Sectional Study. Clin Infect Dis 2015.

4. Feld JJ, Kowdley KV, Coakley E, et al. Treatment of HCV with ABT-450/r-ombitasvir and dasabuvir with ribavirin. The New England journal of medicine 2014;370:1594-603.

5. Fried MW, Buti M, Dore GJ, et al. Once-daily simeprevir (TMC435) with pegylated interferon and ribavirin in treatment-naive genotype 1 hepatitis C: the randomized PILLAR study. Hepatology 2013;58:1918-29.

6. Jacobson IM, Gordon SC, Kowdley KV, et al. Sofosbuvir for hepatitis C genotype 2 or 3 in patients without treatment options. The New England journal of medicine 2013;368:1867-77.

7. Jacobson IM, McHutchison JG, Dusheiko G, et al. Telaprevir for previously untreated chronic hepatitis C virus infection. The New England journal of medicine 2011;364:2405-16.

8. Martin NK, Vickerman P, Grebely J, et al. Hepatitis C virus treatment for prevention among people who inject drugs: Modeling treatment scale-up in the age of direct-acting antivirals. Hepatology 2013;58:1598-609.

9. Dong Y1 QC, Xia X, Wang J, Zhang H, Zhang X, Xu J. Hepatitis B virus and hepatitis C virus infection among HIV-1-infected injection drug users in Dali, China: prevalence and infection status in a cross-sectional study. Arch Virol 2015;160:929-36.

10. van de Laar TJ MG, Prins M, Danta M. Acute hepatitis C in HIVinfected men who

510 have sex with men: an emerging sexually transmitted infection. AIDS 2010;24:1799–
511 812.
512 11. Zhang M, Sun XD, Mark SD, et al. Hepatitis C virus infection, Linxian, China.
513 Emerg Infect Dis 2005;11:17-21.
514 12. Delarocque-Astagneau E, Pillonel J, De Valk H, Perra A, Laperche S, Desenclos
515 JC. An incident case-control study of modes of hepatitis C virus transmission in France.
516 Ann Epidemiol 2007;17:755-62.
517 13. Esteban JI, Sauleda S, Quer J. The changing epidemiology of hepatitis C virus
518 infection in Europe. J Hepatol 2008;48:148-62.
519 14. Santantonio T ME, Ferrari C. Risk factors and outcome among a large patient
520 cohort with community-
521 acquired acute hepatitis C in Italy. Clin Infect Dis 2006;43:1154-9.
522 15. Sohn HS, Kim JR, Ryu SY, et al. Risk Factors for Hepatitis C Virus (HCV)
523 Infection in Areas with a High Prevalence of HCV in the Republic of Korea in 2013.
524 Gut Liver 2016;10:126-32.
525 16. Micallef JM, Kaldor JM, Dore GJ. Spontaneous viral clearance following acute
526 hepatitis C infection: a systematic review of longitudinal studies. J Viral Hepat
527 2006;13:34-41.
528 17. Strasak AM, Kim AY, Lauer GM, et al. Antibody dynamics and spontaneous viral
529 clearance in patients with acute hepatitis C infection in Rio de Janeiro, Brazil. BMC
530 Infect Dis 2011;11:15.
531 18. Tobler LH, Bahrami SH, Kaidarova Z, et al. A case-control study of factors
532 associated with resolution of hepatitis C viremia in former blood donors (CME).
533 Transfusion 2010;50:1513-23.
534 19. Maheshwari A, Ray S, Thuluvath PJ. Acute hepatitis C. Lancet 2008;372:321-32.

20. Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. J Hepatol 2014;61:S45-57.

21. Fu Y, Qin W, Cao H, et al. HCV 6a prevalence in Guangdong province had the origin from Vietnam and recent dissemination to other regions of China: phylogeographic analyses. PLoS One 2012;7:e28006.

22. Rong X, Xu R, Xiong H, et al. Increased prevalence of hepatitis C virus subtype 6a in China: a comparison between 2004-2007 and 2008-2011. Arch Virol 2014;159:3231-7.

23. Wan Z, Chen Q, Chen X, et al. HCV Diversity among Chinese and Burmese IDUs in Dehong, Yunnan, China. PLoS One 2016;11:e0163062.

24. Wu T, Xiong L, Wang F, et al. A Unique Pattern of HCV Genotype Distribution on Hainan Island in China Revealed by Evolutionary Analysis. Cell Physiol Biochem 2016;39:316-30.

25. Huang K, Chen J, Xu R, et al. Molecular evolution of hepatitis C virus in China: A nationwide study. Virology 2018;516:210-8.

26. An Y, Wu T, Wang M, et al. Conservation in China of a novel group of HCV variants dating to six centuries ago. Virology 2014;464-465:21-5.

27. Smith DB, Bukh J, Kuiken C, et al. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology 2014;59:318-27.

28. Rong X, Lu L, Wang J, et al. Correlation of viral loads with HCV genotypes: higher levels of virus were revealed among blood donors infected with 6a strains. PLoS One 2012;7:e52467.

29. Lu L, Wu T, Xiong L, Li C, Nguyen MH, Murphy DG. Analysis of HCV-6 isolates among Asian-born immigrants in North America reveals their high genetic diversity

and a new subtype. Virology 2016;492:25-31.

30. Xia GL, Liu, C. B., Cao, H. L., et al. Prevalence of hepatitis B and C virus infections in the general Chinese population. Results from a nationwide cross-sectional seroepidemiologic study of hepatitis A, B, C, D, and E virus infections in China,1992. Int Hepatol Commun 1996;5:62-37.

31. Alter MJ, Kuhnert WL, Finelli L, Centers for Disease C, Prevention. Guidelines for laboratory testing and result reporting of antibody to hepatitis C virus. Centers for Disease Control and Prevention. MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports 2003;52:1-13, 5; quiz CE1-4.

32. Bes M, Esteban JI, Casamitjana N, et al. Hepatitis C virus (HCV)-specific T-cell responses among recombinant immunoblot assay-3-indeterminate blood donors: a confirmatory evidence of HCV exposure. Transfusion 2009;49:1296-305.

33. Makuria AT, Raghuraman S, Burbelo PD, et al. The clinical relevance of persistent recombinant immunoblot assay-indeterminate reactions: insights into the natural history of hepatitis C virus infection and implications for donor counseling. Transfusion 2012;52:1940-8.

34. Ponde RA. Enzyme-linked immunosorbent/chemiluminescence assays, recombinant immunoblot assays and nucleic acid tests in the diagnosis of HCV infection. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology 2013;32:985-8.

35. Moosavy SH, Davoodian P, Nazarnezhad MA, Nejatizaheh A, Eftekhar E, Mahboobi H. Epidemiology, transmission, diagnosis, and outcome of Hepatitis C virus infection. Electron Physician 2017;9:5646-56.

36. Zamani F, Sohrabi M, Poustchi H, et al. Prevalence and risk factors of hepatitis C

585 virus infection in amol city, north of iran: a population-based study (2008-2011). Hepat
586 Mon 2013;13:e13313.
587 37. Hellard ME, Aitken CK, Hocking JS. Tattooing in prisons--not such a pretty picture.
588 Am J Infect Control 2007;35:477-80.
589 38. Jafari S CR, Baharlou S, Etminan M, Buxton J. Tattooing and the risk of
590 transmission of hepatitis C: a systematic review and meta-analysis. Int J Infect Dis
591 2010;14:e928±40.
592 39. Fabricio-Silva GM, Poschetzky BS, de Mello Perez R, Dos Santos RC, Cavalini
593 LT, Porto LC. Association of cytokine gene polymorphisms with hepatitis C virus
594 infection in a population from Rio de Janeiro, Brazil. Hepat Med 2015;7:71-9.
595 40. Fu Y, Wang Y, Xia W, et al. New trends of HCV infection in China revealed by
596 genetic analysis of viral sequences determined from first-time volunteer blood donors.
597 J Viral Hepat 2011;18:42-52.
598 41. Inoue G, Horiike N, Michitaka K, Onji M. Hepatitis C virus clearance is prominent
599 in women in an endemic area. J Gastroenterol Hepatol 2000;15:1054-8.
600 42. Wang CC, Krantz E, Klarquist J, et al. Acute hepatitis C in a contemporary US
601 cohort: modes of acquisition and factors influencing viral clearance. The Journal of
602 infectious diseases 2007;196:1474-82.
603 43. Xu CJ, Zhang CP, Luo BF, et al. Prevalence and characterization of hepatitis B and
604 C virus infections in a needle-sharing population in Northern China. BMC Public
605 Health 2015;15:460.
606 44. Liu S, Cheng M, Mu M, Yang Q. [Natural clearance of hepatitis C virus in 96
607 patients with infection acquired by blood transfusion from a single donor in Guizhou].
608 Zhonghua Gan Zang Bing Za Zhi 2014;22:251-4.
609 45. Kuniholm MH, Kovacs A, Gao X, et al. Specific human leukocyte antigen class I

610 and II alleles associated with hepatitis C virus viremia. Hepatology 2010;51:1514-22.
611 46. Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated
612 anti-D immune globulin. Irish Hepatology Research Group. The New England journal
613 of medicine 1999;340:1228-33.
614 47. Bakr I, Rekacewicz C, El Hosseiny M, et al. Higher clearance of hepatitis C virus
615 infection in females compared with males. Gut 2006;55:1183-7.
616 48. Murphy EL, Fang J, Tu Y, et al. Hepatitis C virus prevalence and clearance among
617 US blood donors, 2006-2007: associations with birth cohort, multiple pregnancies, and
618 body mass index. The Journal of infectious diseases 2010;202:576-84.
619 49. Rafik M, Bakr S, Soliman D, et al. Characterization of differential antibody
620 production against hepatitis C virus in different HCV infection status. Virol J
621 2016;13:116.
622 50. Lu L, Nakano T, He Y, Fu Y, Hagedorn CH, Robertson BH. Hepatitis C virus
623 genotype distribution in China: predominance of closely related subtype 1b isolates and
624 existence of new genotype 6 variants. J Med Virol 2005;75:538-49.
625 51. Wu Z, Cui L, Zhao W, et al. Molecular epidemiology of hepatitis C infections in
626 Ningxia, China: genotype, phylogeny and mutation analysis. Virol J 2016;13:172.
627 52. Junxia Zhou JX. The research of Hepetitis C Virus genotyping in Haikou region.
628 Modern preventive medicine 2012;39:4507-10.
629 53. Tao wu JW, Xiaozhen Xu, Lu Xiong,Jiao Wang, Fuli Wang, Feng Lin. Genotype
630 distribution of Hepetitis C Virus among 163 isolates from ethic Han in Hainan Province.
631 China tropical medicine 2016;16:873-6.
632 54. Candotti D, Temple J, Sarkodie F, Allain JP. Frequent recovery and broad genotype
633 2 diversity characterize hepatitis C virus infection in Ghana, West Africa. J Virol
634 2003;77:7914-23.
635

Supplementary material

Supplementary Fig 1 Phylogenetic tree of isolates 282, 305 and 409 E1 sequences. Solid yellow circles, solid green circles and solid red circles represent clones of the 282 isolate, 305 isolate and 409 isolate respectively. A maximum Likelihood tree was constructed using Mega 7.

Supplementary table 1 Supplementary table 1 Detailed information of reference sequences in the phylogenetic analysis

Supplementary table 2 Details of RIBA reactivity to core, NS3, NS4 andNS5 antigens

Supplementary table 3 Socio-demographics of subjects according to HCV Persistent infection and HCV Spontaneous clearance

Figure legends:

Fig 1 The estimated maximum likelihood phylogeny for 58 Baisha Li minority NS5B sequences. Sequences determined in this study are shown in solid green circle. Solid yellow circle represents unassigned HCV gt6 references which were collected from China and unassigned reference sequences from other south Asian countries except China are indicated by solid red circle. Other reference sequences which were named by subtype and accession number represent assigned HCV gt1-gt7.

Fig 2 The estimated maximum likelihood phylogeny for 55 Baisha Li minority E1 sequences. Sequences determined in this study are shown in solid green circle. Solid yellow circles represent unassigned HCV gt6 references which were collected from China and unassigned reference sequences from other south Asian countries except China are indicated by solid red circle. Other reference sequences which were named by subtype and accession number represent assigned HCV gt1-7.

Fig 3 The location of Hainan island in China and the HCV genotype distribution in previous studies. The Li minority population reside in the central southern mountainous region (colors) whereas the Han ethic population occupy the northern coastal region (blank).

671 Table 1 Confirmatory results of HCV antibody by RIBA

	Both Ortho and Kehua ELISA positive (n=249)				ELISA negative,
	RNA positive	RNA negative, ELISA positive (n=182)			RNA negative
		RIBA positive	RIBA indeterminate	RIBA negative	
Total Samples = 1682	67	50	36	96	1433
HCV RNA titer (IU/ml)	$6.76x10^4$ (± 9.55)	-	-	-	-
Anti-HCV positive		117§	153§		
Prevalence of HCV infection		7.0 (117/1682)§	9.1% (153/1682) §		
Prevalence of HCV spontaneous clearance		42.7 (50/117) §	56.2% (86/153) §		

672 § Lower value refers to the exclusion of RIBA indeterminate data and higher value to the
673 inclusion of RIBA indeterminate data.

674

675 Table 2 Age-related HCV infection in the Baisha Li minority community

ages	Anti-HCV (-) (%)	Anti-HCV (+) (%)	total
10-20y	293(99.3)	2(0.7)	295
20-30y	148(100)	0(0)	148
30-40y	128(100)	0(0)	128
40-50y	225(99.1)	2(0.9)	227
50-60y	198(99.5)	1 (0.5)	199
60-70y	189-190 (89.6–90.0)	21-22 (10.0-10.4)	211
70-80y	262–284 (76.8–83.3)	57-79(16.7-23.2)	341
80-95y	86–99 (64.7–74.4)	34-47(25.6-35.3)	133
total	1529–1565 (90.9–93.0)	117-153(7.0-9.09)	1682

676

677 Table 3 Socio-demographics of subjects according to anti-HCV positivity including

678 RIBA indeterminate results

	Univariate chi-square analysis				Multivariate analysis	
	Anti-HCV(+)§ n=153	Anti–HCV(-) n=1529	OR（95%CI）	P-value	OR（95%CI）	P-value
Gender						
Male	57(37.3)	597(39.0)	0.93（0.28-1.28）	0.78	0.73（0.47-1.13）	0.16
Female	96(62.7)	932(61.0)				
Age						
<60y	5(3.3)	982(64.2)	0.02（0.01-0.05）	4.11E-54	0.02（0.01-0.05）	6.66E-18
>60y	148(96.7)	547(35.8)				
Transfusion						
Yes	4(2.6)	25(1.6)	1.62（0.56-4.73）	0.51	1.17（0.34-3.98）	0.81
No	149(97.4)	1504(98.4)				
Alcohol Consumption						
Yes	61(39.9)	614(40.2)	0.99（0.7-1.39）	0.95	0.97（0.67-1.43）	0.89
No	92(60.1)	915(59.8)				
Tattooing						
Yes	27(17.6)	108(7.1)	2.82（1.78-4.46）	1.35E-06	1.01（0.57-1.81）	0.96
No	126(82.4)	1421(92.9)				
Surgery						
Yes	15(9.8)	40（2.6）	4.05（2.18-7.51）	1.88E-06	2.75（1.36-5.57）	0.005
No	138（90.2）	1489（97.4）				
Piercings						
Yes	44(28.8)	382(25)	1.21（0.84-1.75）	0.31	1.46（0.91-2.35）	0.12
No	109(71.2)	1147(75)				
Acupuncture						
Yes	4(1.6)	24(2.6)	1.68（0.58-4.92）	0.34	0.54（0.17-1.72）	0.30
No	149(98.4)	1505(97.4)				
Family history of HCV						
Yes	2(1.3)	7（0.5）	2.88（0.59-13.99）	0.17	1.28(0.23-7.23)	0.78
No	151(98.7)	1522（99.5）				
Dental procedures						
Yes	4(2.6)	39(2.6)	0.98（0.36-2.9）	0.96	0.70(0.22-2.24)	0.55
No	149(97.4)	1490(97.4)				

679

680 **§：HCV RNA positive, RIBA positive and RIBA indeterminate samples**
681 **constituted the anti-HCV (+) group**

682

683 Table 4 Socio-demographics of subjects according to anti-HCV positivity excluding

684 RIBA indeterminate results

	Univariate Chi-square analysis				Multivariate analysis	
	Anti–HCV(+)[#] n=117	Anti–HCV(-) n=1565	OR（95%CI）	P-value	OR（95%CI）	P-value
Gender						
Male	44(37.6)	610(39.0)	0.94（0.64-1.39）	0.77	0.69（0.42-1.12）	0.14
Female	73(62.4)	955(61.0)				
Age						
<60y	5(4.3)	992(62.4)	0.03（0.01-0.06）	**3.82E-36**	0.03（0.01-0.06）	**2.39E-15**
≥60y	112(95.7)	573(37.6)				
Transfusion						
Yes	3(2.6)	26(1.7)	1.56（0.46-5.22）	0.47	1.24（0.32-4.82）	0.76
No	114(97.4)	1539(98.3)				
Alcohol consumption						
Yes	46(39.3)	629(40.2)	0.96（0.66-1.42）	0.85	0.96（0.63-1.47）	0.86
No	71(60.7)	936(59.8)				
Tattooing						
Yes	20(17.1)	115(7.3)	**2.60（1.55-4.36）**	**1.82E-04**	1.01（0.57-1.81）	0.96
No	97(82.9)	1450(92.7)				
Surgery						
Yes	11(9.4)	44 (2.8)	**3.59（1.80-7.15）**	**1.11E-04**	**2.36（1.09-5.10）**	**0.03**
No	106 (90.6)	1521 (97.2)				
Piercings						
Yes	35(29.9)	391(25)	1.28（0.85-1.94）	0.24	1.63（0.96-2.75）	0.07
No	82(70.1)	1174(75)				
Acupuncture						
Yes	4(3.4)	24(1.5)	2.27（0.78-6.67）	0.12	1.08（0.31-3.70）	0.91
No	113(96.6)	1541(98.5)				
Family history of HCV						
Yes	2(1.7)	7(0.4)	3.87（0.80-18.85）	0.07	1.82(0.32-10.27)	0.49
No	115(98.3)	1558(99.6）				
Dental procedures						
Yes	2(1.7)	41(2.6)	0.65（0.15-2.71）	0.55	0.37(0.08-1.76)	0.21
No	115(98.3)	1524(97.4)				

685 **§：HCV RNA positive and RIBA positive samples only constituted the anti-HCV**

686 **(+) group**

687

688 **Supplementary Table 4**

	Male n (%)	Female n (%)	P Value*	RIBA indeterminate results
HCV(+)	57 (8.7)	96 (9.3)	0.78	Included
HCV(-)	597 (91.3)	932 (90.7)		
Total	654	1028		
HCV(+)	44 (6.7)	73 (7.1)	0.77	Excluded
HCV(-)	610 (93.3)	955 (92.9)		
Total	654	1028		
Spontaneous clearance	29 (50.9)	57 (59.4)	0.31	Included
Persistent infection	28 (49.1)	39 (40.6)		
Total	57	96		
Spontaneous clearance	16 (36.4)	34 (46.6)	0.28	Excluded
Persistent infection	28 (63.6)	39 (53.4)		
Total	44	73		

689 * Statistical analysis was performed with a Chi-square test.
690
691