principles expressed in the Declaration of Helsinki and approved by the Institutional Review Board of Kyoto University (844 and E-921). All patients provided written informed consent for the collection of samples and subsequent analysis.

### Antibodies

The following antibodies were purchased from BD PharMingen; purified monoclonal antibody (mAb) for human CD3 (UCHT1), CD4 (RPA-T4), CD8a (RPA-T8), CD45RA (NI100) and CTLA-4 (BNI3). Purified mAbs for human CD25 (BC96), GITR (eBio AITR) and FoxP3 (236A/E7) were purchased from eBioscience.

### Flow cytometric analysis

PBMCs were isolated with Ficoll-Isopaque (GE Healthcare) gradient centrifugation. Flow cytometric analyses were carried out using a FACS CantoII with Diva Software (BD Pharmingen), and the data were analyzed by FlowJo software (Treestar). To discriminate dead cells, we used LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen). For cell surface staining, $10^6$ cells were incubated with mAbs for 30 minutes at 4°C, and then analyzed. For intracellular staining, we used a human FoxP3 staining kit according to the manufacture's protocol (eBioscience). To distinguish $FoxP3^+$ and $FoxP3^-$ cell population clearly, we used isotype control according to the manufacture's recommendation. To detect the viral antigen Tax, we cultured PBMCs from ACs or HAM/TSP patients for 12–18 hours and stained with monoclonal antibodies against FoxP3 or Tax (MI-73) [54], and then analyzed by flow cytometry.

### Statistical analysis

To compare 2 groups when data were determined to have a Gaussian distribution, the Student *t* test was used. If data did not have a Gaussian distribution, the Mann–Whitney *U* test was used for unpaired data, and the Wilcox signed-ranks test was used for paired data. The AC group and HD did not differ significantly in sex or age, using chi-squared test and Mann–Whitney *U* test. Differences with $P < 0.05$ were considered to be statistically significant. Correlations were evaluated using Spearman's rank correlation.

## Additional files

**Additional file 1: Figure S1.** Absolute cell numbers of each $CD4^+$T-cell subset in HTLV-1 infected individuals. (A) Absolute cell numbers of $CD4^+$ T cells in 4 distinct subjects. Data shown are gated on lymphocyte fraction based on the dot plot pattern of SSC and FSC. (B and C) Absolute cell numbers of $FoxP3^-CD45RA^+$ naïve $CD4^+$ T cells (B) or $FoxP3^-CD45RA^-$ effector/memory $CD4^+$ T cells (C). (D) Absolute cell numbers of $FoxP3^+$ cells in $CD4^+$ T cells.

**Additional file 2: Figure S2.** Effect of *ex vivo* cultivation on FoxP3 and CD45RA expression. The percentages of FoxP3 and CD45RA expression in $CD4^+$ T cells both before and after *ex vivo* culture are shown from 5 distinct ACs.

**Additional file 3: Figure S3.** Frequency of each CD4 T-cell subset in Tax-expressing cell population in AC. Cumulative results from 23AC individuals are shown in the graph.

### Competing interests

The authors declare that they have no competing interests.

### Acknowledgments

This study was supported by Grant-in-Aid for Scientific Research from Japanese Society for the Promotion of Science and Ministry of Health Labor and Welfare, a grant from Takeda Science Foundation, a grant from Naito Foundation. We thank Prof. Charles R.M. Bangham for critical reading of the manuscript and Ms. M. Nakashima for preparation of peripheral blood of patients. We are most grateful to the patients and healthy donors who participated in this study.

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### Authors' contributions

This study was designed by YS and MM. Laboratory analysis was performed by YS and JT. Data analysis was performed by YS, AU, JT and MM. Clinical samples and data were provided by AU, MN and KN. YS and MM wrote the paper. All authors read and approved the final manuscript.

Received: 24 December 2011 Accepted: 30 May 2012
Published: 30 May 2012

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doi:10.1186/1742-4690-9-46
**Cite this article as:** Satou *et al.*: HTLV-1 modulates the frequency and phenotype of FoxP3$^+$CD4$^+$ T cells in virus-infected individuals. *Retrovirology* 2012 **9**:46.

Tropical Medicine and Health Vol. 40 No. 2, 2012, pp. 55-58
doi:10.2149/tmh.2012-02



**Original article**

# Distribution of Two Subgroups of Human T-Lymphotropic Virus Type 1 (HTLV-1) in Endemic Japan

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Received 20 January, 2012 Accepted 5 June 2012 Published online 4 August, 2012

**Abstract:** Endemic areas of human T-lymphotropic virus type 1 (HTLV-1) have been reported in Japan as well as tropical Africa, Central and South America and Melanesia. The existence of two subgroups, i.e., the transcontinental and Japanese subgroups, was reported in Japan. In the present study, we provide data on the ratio of the two subgroups in each endemic area and infection foci and examine the distribution of HTLV-1 in Japan and neighboring areas. A 657 bp fragment of env region of HTLV-1 proviral genome was successfully amplified for 183 HTLV-1 positive DNA samples. The subgroup determination was done by RFLP reactions using endonucleases *HpaI* and *HinfI*. The northern part of mainland Kyushu, represented by Hirado and Kumamoto, was monopolized by the Japanese subgroup, while the transcontinental subgroup ranged from 20 to 35% in the Pacific coast areas of Shikoku (Kochi), the Ryukyu Archipelago (Kakeroma and Okinawa) and Taiwan. An interesting finding in the present study is the presence of the transcontinental subgroup in Kochi, suggesting the endemicity of the transcontinental subgroup along the Kuroshio Current.

**Key words:** Japanese subgroup, transcontinental subgroup, human migration, Kuroshio Current

## INTRODUCTION

Human T-lymphotropic virus type 1 (HTLV-1) was first isolated in 1980 [1] and has been identified as a causative agent of adult T cell leukemia (ATL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 has three major transmission routes: from mother to infant through breast milk, from male to female through semen, and to blood recipients through the lymphocytes of HTLV-1 carriers. These transmission routes, especially mother-to-child transmission, allow HTLV-1 to pass from generation to generation and localize within family, community and ethnic groups. Thus, the elucidation of the geographical distribution of HTLV-1 has important ethnoepidemiological implications [2].

In view of this unique fact, a large number of phylogeographycal and epidemiological studies have been conducted within and beyond the borders of Japan, and valuable results have been obtained. Firstly, endemic areas were reported in tropical Africa, the Caribbean basin, Central and South America, Papua New Guinea and other islands of Melanesia, as well as Japan [3, 4]. Secondly, there are three major lineages existing worldwide: the Melanesian subtype, the Central African subtype, and the cosmopolitan subtype, ubiquitous in endemic areas around the world [5, 6]. Thirdly, the cosmopolitan subtype is further divided into three major subgroups: A, B, and C, which correspond to the transcontinental subgroup, the Japanese subgroup, and the West African subgroup, respectively [7, 8]. Fourthly, within Japan, endemicity is found in Kyushu and Okinawa, and small infection foci are seen in coastal islands of the Japan Sea and the Pacific side of Shikoku, Kii and Tohoku, while most of Honshu is HTLV-1-free [3]. Furthermore, a few endemic areas have been found in areas neighboring Japan: Nogliki of Sakhalin, Kinmen, Fujian and Taiwan [7, 9–11]. Fifthly, the existence of two different subgroups of

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HTLV-1, i.e., the transcontinental and Japanese subgroups, in Japan and clusters of the former subgroup in Kyushu and the Ryukyu islands were reported [12].

In the present study, we provide data on the ratio of the two subgroups in each endemic area and infection foci within Japan and use that data to elucidate the distribution of HTLV-1 in Japan and neighboring area.

## MATERIALS AND METHODS

DNA samples from a total of 197 anonymous HTLV-1 positive donors were obtained from the Joint Study on Predisposing Factors of ATL Development (JSPFAD) and used in the present study. Of the 197 samples, 40 were gathered in Hokkaido (Hokkaido University Hospital), four in Iwate (Iwate Medical University), 30 in Kochi (Kochi Medical School Hospital), 50 in Hirado (Nagasaki University Hospital), 23 in Kumamoto (Kumamoto University Hospital) and 50 in Okinawa (Okinawa Kyodo Hospital).

Furthermore, DNA was extracted from peripheral blood donated by five anonymous HTLV-1 carriers on Ishigaki Island, Japan (Yaeyama County, Ishigaki City, Okinawa Prefecture). The analysis of samples donated by the Yaeyama residents was approved by the ethics committee of the Institute of Tropical Medicine, Nagasaki University, Japan (Approval No. 10012147).

A 657 bp fragment of env region was amplified by nested PCR. The first reactions were performed in 20 μl volumes containing 1 μl (ca. 50 ng) of the extracted DNA, 200 μM (final conc.) of dNTP mixture, 0.25 μM (final conc.) of the primer sets, 2 μl of 10 × Ex Taq Buffer and 0.5U TaKaRa Ex Taq HS (TAKARA BIO Inc., Shiga, Japan). The external primers were TAATAGCCGCCAGTGGAA AG (nucleotide positions according to the J02029 sequence: 5027–5046) and AGTCCTTGGAGGCTGAACG (6786–6768). The thermal conditions were as follows: 5-min denature at 94°C, 40 cycles of 40 sec at 94°C, 30 sec at 61°C and 40 sec at 72°C, and 10-min final extention at 72°C. The second reactions were performed in 40 μl volumes containing 2 μl of the first PCR product, 200 μM (final conc.) of dNTP mixture, 0.25 μM (final conc.) of the primer sets, 4 μl of 10 × Ex Taq Buffer and 1U TaKaRa Ex Taq HS. The internal primers were CTCCCTTCTAGTCGACGCTCCAGG (5685–5708) and CGTCTGTTCTGGGCAGCATA (6341–6322). The thermal conditions were as follows: 2-min denature at 95°C, 35 cycles of 20 sec at 95°C, 20 sec at 58°C and 30 sec at 72°C, and 2-min final extention at 72°C.

All of the 35 samples from Hokkaido, all of the four from Iwate, 28 of 30 from Kochi, 44 of 50 from Hirado, 21 of 23 from Kumamoto, 46 of 50 from Okinawa and all of the five from Yaeyama were well amplified. RFLP reactions were performed using endonucleases *HpaI* and *HinfI* as designed by Yang et al. [7]. The digested DNA fragments were electrophoresed on 2% agarose gel pre-stained with ethidium bromide and visualized.

## RESULTS AND DISCUSSION

All except one of the HTLV-1 isolates from Iwate, Hirado and Kumamoto were determined as the Japanese subgroup, while 20–35% of the isolates from Hokkaido, Kochi, Okinawa and Yaeyama were determined as the transcontinental subgroup (Fig. 1). The electrophoresis profile of two isolates (Hokkaido and Kochi) was consistent with neither the Japanese nor the transcontinental subgroup but similar to the West African/Caribbean subgroup shown by Yang et al. [7]. Thus, these were tentatively treated as "undetermined" in the present paper.

The uneven distribution of the transcontinental and Japanese subgroups in the endemic areas of Japan was clarified in the present study, whereas only the transcontinental subgroup was reported from neighboring areas such as Nogliki of Sakhalin, Kinmen, and Fujian [9–11].

The northern part of mainland Kyushu, represented by Hirado and Kumamoto, seems to be monopolized by the Japanese subgroup. On the other hand, the presence of the transcontinental subgroup ranges from 20 to 35% in the Pacific coast areas of Shikoku (Kochi), the Ryukyu Archipelago (Kakeroma [13] and Okinawa) and Taiwan [7]. An interesting finding in the present study is the presence of the transcontinental subgroup in Kochi, suggesting the endemicity of the transcontinental subgroup along the Kuroshio Current.

A north-flowing ocean current on the west side of the Pacific Ocean, the Kuroshio Current has played the role of an aorta for migration and transportation along the Pacific coast of southwestern Japan since prehistoric times. The endemicity of the transcontinental subgroup along the Kuroshio Current might reflect this human movement. If so, we need to pay more attention to the date and mode of local human movements which may have implications in the epidemiology of HTLV-1 and other infectious agents such as hepatitis B virus [14].

## ROLE OF FUNDING SOURCE

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (20390186, 221S0001, 23659354, 23590800), Cooperative Research Grant (2009-E-1) of the Institute of Tropical Medicine, Nagasaki University and by the Global Center of Excellence Program at Nagasaki Uni-

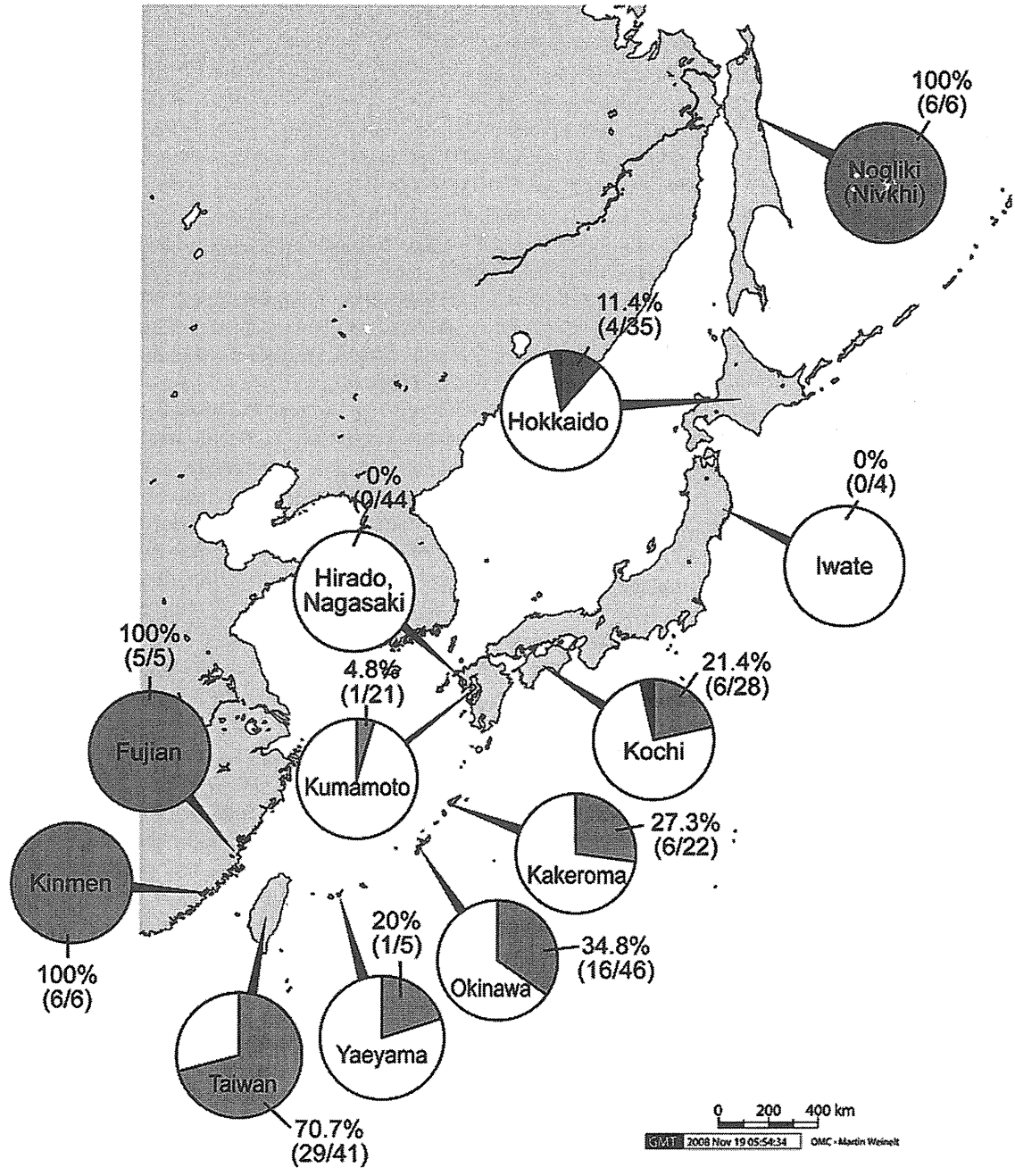


Fig. 1. Ratio of the transcontinental subgroup (grey) to the Japanese subgroup (white) of HTLV-1 cosmopolitan subtype in various localities of East Asia. The data of Nogliki, Kakeroma, Taiwan, Kinmen and Fujian were cited from Syrtsev et al. [10], Eguchi et al. [13], Yang et al. [7], Chen et al. [9] and Wang et al. [11], respectively.

versity. No sponsor, however, participated in the study design, in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

## AUTHOR DISCLOSURE STATEMENT

Drs. Otani, Yamamoto and Eguchi have full access to all the data in the study and hold final responsibility for the decision to submit for publication. All authors declare that they have no conflict of interest.

## ACKNOWLEDGEMENTS

We gratefully thank Dr. Osamu Ikehara (Okinawa Prefectural Yaeyama Hospital, Japan) who gathered samples on Ishigaki I., and Drs. Junko Okumura, Masahiro Hashizume, Toshihiko Sunahara and Hidefumi Fujii for their important suggestions. The authors thank the staff members in all the collaborating institutions and Mr. Makoto Nakashima, Ms. Takako Akashi, and other staff members in the central office of the JSPFAD for their efforts in sample processing and biologic assays.

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VIROLOGY JOURNAL

RESEARCH Open Access

# Paradoxical expression of *IL-28B* mRNA in peripheral blood in human T-cell leukemia virus Type-1 mono-infection and co-infection with hepatitis C Virus

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**Abstract**

**Background:** Human T-cell leukemia virus type-1 (HTLV-1) carriers co-infected with and hepatitis C virus (HCV) have been known to be at higher risk of their related diseases than mono-infected individuals. The recent studies clarified that IL-28B polymorphism rs8099917 is associated with not only the HCV therapeutic response by IFN, but also innate immunity and antiviral activity. The aim of our research was to clarify study whether IL-28B gene polymorphism (rs8099917) is associated with HTLV-1/HCV co-infection.

**Results:** The genotyping and viral-serological analysis for 340 individuals showed that IL-28B genotype distribution of rs8099917 SNP did not differ significantly by respective viral infection status. However, the IL-28B mRNA expression level was 3.8 fold higher in HTLV-1 mono-infection than HTLV-1/HCV co-infection. The high expression level was associated with TT (OR, 6.25), whiles the low expression was associated with co-infection of the two viruses (OR, 9.5). However, there was no association between down-regulation and ATL development (OR, 0.8).

**Conclusion:** HTLV-1 mono-infection up-regulates the expression of IL-28B transcripts in genotype-dependent manner, whiles HTLV-1/HCV co-infection down-regulates regardless of ATL development.

**Keywords:** IL-28B, IL-λ3, HTLV-1, HCV, SNP

## Introduction

A retrovirus, human T-cell leukemia virus type-1 (HTLV-1), and a positive-strand RNA virus, hepatitis C virus (HCV), are completely different in terms of virologic characteristics. Nevertheless, they play a similar role in the pathogenesis of viral-induced malignant neoplasms, such as adult T-cell leukemia (ATL) in HTLV-1- infected individuals, and hepatocellular carcinoma (HCC) and B-cell lymphoma in HCV- infected individuals, during long-term chronic infections.

Furthermore, it is known that co-infection with HCV and HTLV-1 is frequently observed in an area endemic for HTLV-1. HCV/HTLV-1 co-infected individuals have been reported to be at higher risk for developing HCC than those infected with HCV alone [1-3]. Although the pathologic mechanism of the co-infection remains to be elucidated, it is thought that the impaired immunity due to HTLV-1 infection may contribute to HCV infection and HCV-related disorders, which is suggested by previous reports. Kohno et al. reported that the severe immunodeficiency and anergic state in patients with ATL may be associated with a functional property of leukemic cells originating from regulatory T-cells expressing CD4, CD25, CCR4, GITR and Foxp3 [4]. Kishihara et al. also reported that impairment of the immune response by HTLV-1 could explain the reduced effectiveness of interferon (IFN) treatment in patients co-infected with HTLV-1 and HCV [5].

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Recently, genome-wide association studies of patients with HCV have made great advances in viral clearance associated with *IL-28B* single nucleotide polymorphisms (SNP) [6,7]. IL-28B is a type III Lambda interferon (IFN-λ) and a cytokine similar to IL-10 with IFN-like activities [8]. This new IFN-λ family includes IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) [9]. Although the IFN-λ genomic structure resembles that of the IL-10 family [10], the amino acid and functional level of IFN-λs are more closely related to type I IFNs than IL-10. The IFN-λs are induced by stimulation with several single-strand RNAs (ssRNA) and several kinds of viruses. The IL-28B SNPs, such as rs8099917, rs12979860, and 12980275, have been reported to be associated with spontaneous clearance [10], innate HCV immunity [9], HCV-related disease chronicity, and therapeutic response to pegIFN-α and ribavirin (RBV) [6,7].

From these observations, we hypothesized that IFN-λ3 encoded from the *IL-28B* gene would be associated with HTLV-1 infection. The aim of the present study was to examine the mutual association between IL-28B polymorphism (rs8099917 SNP) and mono-infected-HTLV-1 and co-infected HTLV-1 with HCV subjects.

## Materials and methods

### Clinical subjects

All subjects were of Japanese origin living in Nagasaki City, an endemic area for HTLV-1 in Japan. For genomic specimens, 340 blood samples were randomly collected from patients who visited a liver clinic and liver transplantation center from April 2009 to March 2011 from the departments of Hepatology and a Hematology Clinic. One hundred and twenty-four of the 340 samples were also available for total RNA tests. Accordingly, most patients had either chronic liver disease (CLD) or adult T-cell leukemia (ATL). This study was done under informed consent after the approval of the Nagasaki University hospital IRB (IRB Approval No.10050). Since the samples used here were un-linked materials, patient information was restricted.

### Cell lines

Eight HTLV-1-infected T-cell lines, Hut 102, MT-1, MT-2, ST1, KK1, KOB, OMT, and LMY-1, were used for IL-28B mRNA quantification. The first three were purchased and latter five were established in our laboratory [11].

### Serological and genetic tests for HCV and HTLV-1

HCV and HTLV-1 infections were mainly serologically detected using commercially available kits, CLEIA-anti-HTLV-1, Lumipulse-II Ortho HCV (Fujirebio-INC, Tokyo, Japan). The confirming examination was genetically performed by the Cobas TaqMan HCV test (TaqMan HCV; Roche Tokyo INC, Tokyo, Japan) for HCV and in-house HTLV-1 proviral real-time RT quantifiable PCR [12]. Genomic DNA and total RNA were extracted from peripheral blood mononuclear cells (PBMC) using commercially available QuickGene DNA Whole blood kits (FUJIFILM Corp., Tokyo, Japan) and PureLink RNA Micro Kits (Invitrogen Corp., Carlsbad, Ca, USA). The extraction protocol was performed according to the manufacturer's instructions.

### Genotyping for SNPs

SNP genotyping was performed using multiplex PCR amplification and Pyrosequencing technology. To amplify target regions, newly designed biotinylated-primers were employed: sense and anti-sense for rs8099917, 5'-TCCTCCTTTTGTTTTCCTTTCTG-3' and 5'-AAAAAGCCAGCTACCAAACTGT-3'. Then, the amplicon was sequenced according to the manufacturer's instructions based on Pyrosequence technology (Qiagen, Hilden, Germany). Biotin-labeled amplicons from the 1st PCR were captured by binding to streptavidin-coated Sepharose beads, and DNA was denatured to produce an ssDNA template for the Pyrosequencing assay. The ssDNA was released and combined with the sequencing primer, which was extended during the Pyrosequencing reaction to provide the sequence of the template DNA. Pyrosequencing data were produced in the form of Pyrograms, and genotypes were assigned by the peak pattern presented in the Pyrogram.

### Real-time reverse transcription (RT) quantifiable PCR for IL-28B mRNA

mRNA for IL-28B transcribed into cDNA by a GoScript™ RT System (Promega, Madison, WI, USA) was quantified by a LightCycler System (Rosche, Mannheim, Germany) using newly designed sense and anti-sense primers, 5'-AAGGACTGCAAGTGCCGCT-3' and 5'-GCTGGTCCAAGACATCCC-3' (AY129149). A standard curve was generated using a tenfold dilution method with a reference material derived from pTAC-1.2735 inserted with 166 base fragments including the target. The amplicon was assayed by the Cyber green method. The raw data were normalized by *abl* mRNA density and evaluated as the relative value for *abl* gene expression calculated by *IL-28B* data/*abl* data × $10^4$, modified from our previous mRNA real time RT qPCR method [12].

### Statistical analysis

The minor-allele frequency (MiAF) was set as the less frequent allele in a population for SNPs analyzed. Viral infectious status was divided into 4 groups of HTLV-1 mono-infection, HCV mono-infection, HTLV-1/HCV-co-infection, and non-infection (double negative; DN).

Differences in the genotype distribution of IL-28B SNPs among groups were compared using the Chi-square or Fisher exact test. The level of mRNA expression was compared using the Mann Whitney U test. Correlation analysis was performed by the Nonparametric Spearman's rank correlation method. The relationship between a factor and an outcome was estimated the magnitude of the association by the odds ratio with 95% confidence intervals (95%CI). Statistical analysis was performed using SAS 9.1. The statistical significance level was set at 0.05.

## Results

### IL-28B genotypes and the sero-status

Three hundred and forty samples were genotyped on IL-28B rs8099917 SNP and were serologically examined for viral infection of HTLV-1 and HCV. As summarized in Table 1. They consisted of 263 (77.4%) major TT homozygotes, 171 (20.9%) minor TG heterozygotes, and 6 (1.8%) minor GG homozygotes. The virus tests revealed that 59 were negative for both HTLV-1 and HCV, 73 were positive for HTLV-1 alone, 179 were positive for HCV alone and 29 were positive for both viruses. The genotypic distributions, as well as minor allele frequency (MAF) of the IL-28B gene, did not significantly differ among each viral infection status as a control of no-infection.

Since the HTLV-1 mono-infection group consisted of 47 ATL patients and 26 HTLV-1 carriers, we stratified the two groups of ATL patients and carriers and the minor allele frequencies of the two groups were compared; the difference between that of ATL and carriers was not statistically significant ($p$ = 0.21). The prevalence of TT was not different statistically either ($p$ = .495).

Next, the expression levels of IL-28B were quantified using 124 samples randomly collected during this study period.

**Table 1 IL-28B genetic distribution and allele frequency in stratification based on the combination of HTLV-1 and HCV infection**

| | | Genotype r(rs8099917) | | | Allele fequency | | |
|---|---|---|---|---|---|---|---|
| | No | TT | TG | GG | T | G | |
| All cases | 340 | 263 (77.4%) | 71 (20.9%) | 6 (1.8%) | 0.86 | 0.14 | |
| 1) non-Infection | 59 | 45(76.3) | 10 (16.9) | 4(6.8) | 0.84 | 0.15 | |
| 2) HTLV-1 mono | 73 | 55(75.3) | 17 (23.3) | 1(1.9) | 0.87 | 0.13 | $P$ = 0.90 |
| ATL patients | 47 | 37(78.7) | 10 (21.3) | 0(0.0) | 0.89 | 0.11 | $P$ = 0.11 |
| carriers | 26 | 18(69.2) | 7(26.9) | 1(3.8) | 0.82 | 0.18 | $P$ = 0.46 |
| 3) HCV-mmono | 179 | 141 (78.7) | 37 (20.7) | 1(1.0) | 0.89 | 0.11 | $P$ = 0.68 |
| 4) co-infection | 29 | 22(75.9) | 7(24.1) | 0(0.0) | 0.88 | 0.14 | $P$ = 0.9 |

There was no significant difference in the genetic distribution and allele frequency among respective infectious states

$P$ values were compared with non infection

### IL-23B mRNA expression level and HCV/HTLV-1 co-infection

The expression levels of IL-28B mRNA were generally low in most cases with a median value of 5.2 in no-infection, 10.6 in HTLV-1 mono-infection, 3.9 in HCV mono-infection, and 2.8 in HTLV-1/HCV co-infection (Figure 1a). Notably, a small number of measurement values shown as open circles was high, and they were distributed only within the HTLV-1 mono-infected and HCV mono-infected groups. Moreover, all of those who had high values were exclusively TT homozygous, as shown in Figure 1b (samples marked by $^{A\text{-}E)}$ were the same in Figure 1(a) and Figure 1(b)). Surprisingly, the median value was the highest in HTLV-1 mono-infection and the lowest in the co-infection group (10.6 versus 2.8; $p$ = 0.013). Therefore, to clarify whether ATL cells directly affect the expression of IL-28B mRNA, we compared the mRNA expression levels in mainly HTLV-1 carriers, ATL patients with ATL cells, and ATL cell lines. As shown in Figure 2, the median values were significantly higher in mono HTLV-1 carriers with TT (17.9 vs 5.6, $P < 0.05$) and ATL patients with TT having ATL cells than those of non-infected individuals (13.4 vs 5.6, $p < 0.05$). No high expression level was observed in two ATL or 16 carriers with HTLV-1/HCV co-infection. Surprisingly, these data were lower rather those from TG/GG. On the other hand, IL-28B mRNA expression in 8 HTLV-1-infected T-cell lines was undetectable in all but one (Hut 102). The genotype was TT in all cell lines.

In addition, there was no correlation between the IL-28B mRNA levels and HCV-RNA levels (non-parametric Spearman's rank correlation, $R^2$ = 0.0543, Figure 3).

### Assessment by odds ratio analysis for an outcome if a risk factor is present

As shown in Figure 2, HTLV-1 was revealed to have an association similar to HCV and IL-28B mRNA. However, the up-regulated-action of HTLV-1 was nullified if the virus was co-infected with HCV. The prevalence of a major TT and minor TG/GG was similar among individuals infected with either HTLV-1 or HCV, as well as the allele frequency, indicating that there is no specific correlation between IL-28B and HTLV-1. Thus, to approach a causative clue, assessment by odds ratio (OR) analysis was performed (Table 2). Only the high mRNA level besides 3 states of HTLV-1 mono-infection,

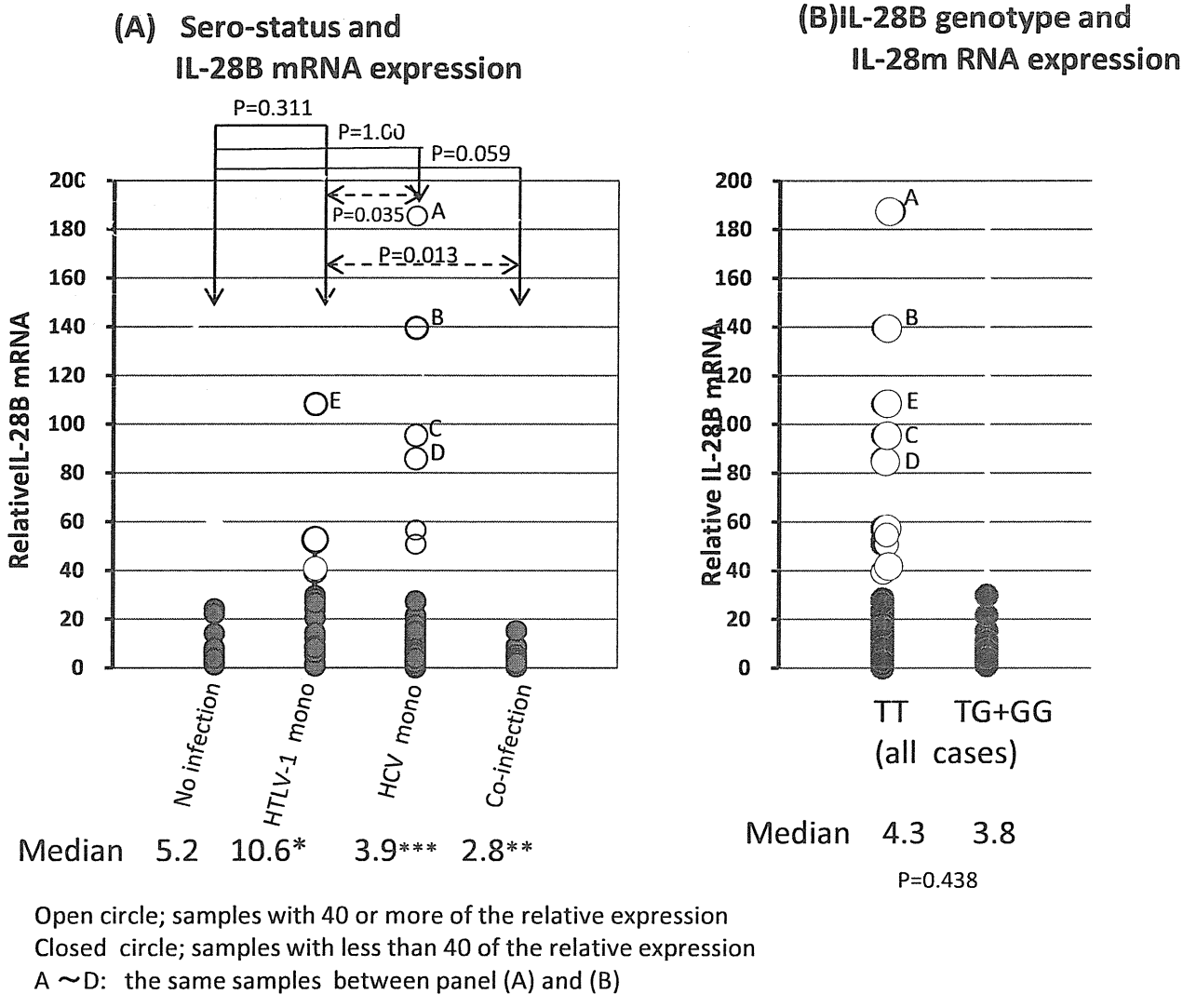


**Figure 1 Relative individual IL-28B mRNA levels in peripheral blood mononuclear cells among viral infectious groups (a) and among the TT and TG/GG genotypes (b)**. The median values were significantly different between * vs ** and *** vs **. Open circles; those with high IL28BmRNA with TT homozygotes.

co-infection with HCV and ATL was associated with TT genotype (OR = 6.25). On the other hand, down-regulation of the mRNA density was defined as HTLV-1/HCV co-infection (OR = 9.5 $p$ = 0.004), but low expression was not associated with ATL development (OR = 0.8, $p$ = 0.81).

## Discussion

Although co-infection with HTLV-1 and HCV has been shown to result in higher rates of cirrhosis and increased death from liver diseases [1,2], the caustic mechanism by which the co-infection affects HCV pathogenesis remains to be elucidated. Some clue to the mechanism may be found by studying the relation between IL-28B genotypes and co-infection, because IL-28B encoding IFN-λs are categorized as type 3 IFNs and are potent endogenous anti-viral cytokines. They signal via JAK/STAT intracellular pathways and up-regulated transcription of IFN-stimulated genes (ISGs) that are required to control viral infection [13]. Here, we investigated whether IL-28B polymorphism rs8099917 is associated with co-infection status.

The present study is the first to reveal that the IL-28B genotype is not associated with stratification based on the combination of HTLV-1 and HCV infection; no infection for both (double negative; DN), HTLV-1 mono-infection, HCV mono-infection and HTLV-1/HCV co-infection. Similarly, the frequency of the major TT homozygotes was not associated among ATL patients and HTLV-1 carriers (Table 2). These two

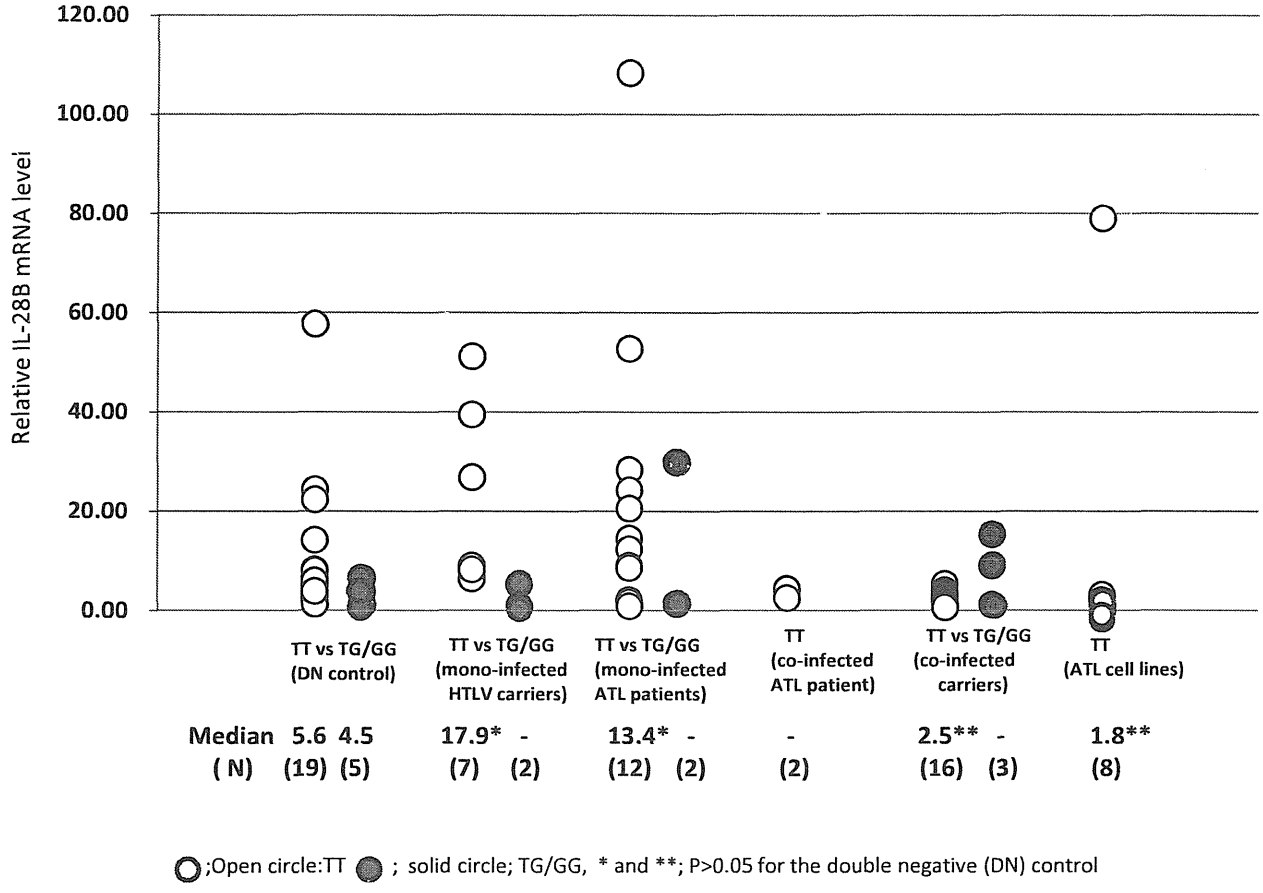


**Figure 2 Individual relative expression density of IL-28B mRNA in TT vs TG/GG derived from 24 double-negative (DN) controls consisting of 19TT and 5TG/GG, 9 mono- infected HTLV-1 carriers, 14 mono-infected and 2 co-infected ATL patients, 19 co-infected carriers and 8 ATL cell lines samples.**

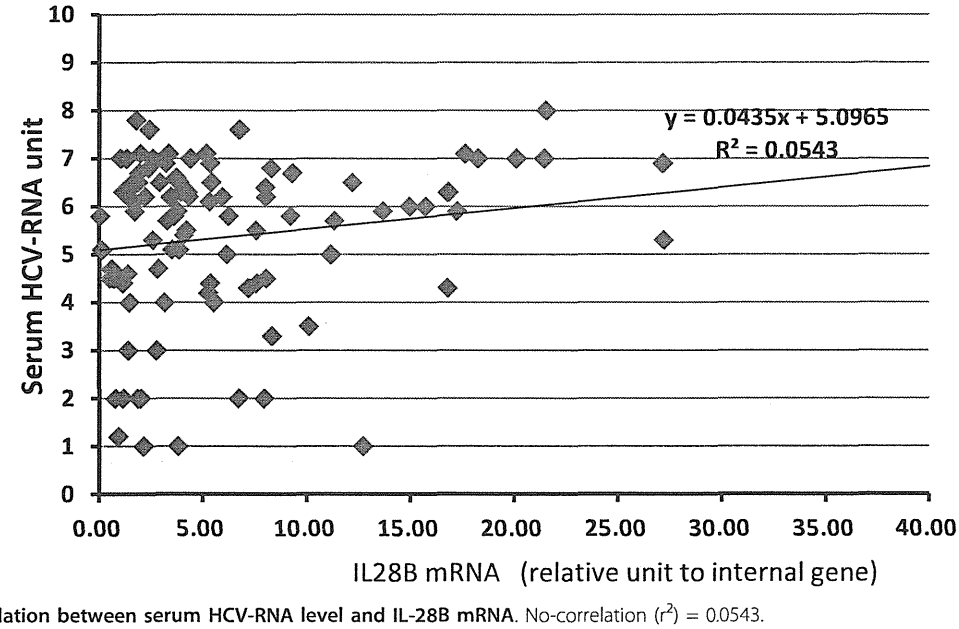


**Figure 3 Correlation between serum HCV-RNA level and IL-28B mRNA.** No-correlation ($r^2$) = 0.0543.

**Table 2 Assessment by odds ratio analysis for an outcome if a risk factor is present**

| | factor | | | |
|---|---|---|---|---|
| **(A) Outcome** | **dependent independent** | **Odds ratio** | **95%CI** | **P** |
| 1) HTLV-1 mono-infection | TT vs TG/GG | 1.11 | 0.62-1.99 | 0.72 |
| 2) Co-infection | TT vs TG/GG | 0.54 | 0.04-6.88 | 1.00 |
| 3) High mRNA Expression* | TT vs TG/GG | 6.25 | 1.16-33.75 | 0.04 |
| 4) ATL | TT vs TG/GG | 1.50 | 0.60-3.75 | 0.39 |
| (B) | | | | |
| 5) Low mRNA Expression | HTLV-1 mono vs DN | 0.34 | 0.06-2.04 | 0.24 |
| 6) Low mRNA Expression | HCV mono vs DN | 0.29 | 0.07-2.23 | 0.15 |
| 7)) Low mRNA Expression | Co-Inf** vs HTLV-1-mono** | 9.5 | 2.06-43.76 | 0.004 |
| 8) ATL | low expression or not | 0.8 | 0.14-4.74 | 0.81 |

(A) Upper 4 lines; assessing the risk of 1) HTLV-1 persistent infection, 2) super-imposed HTLV-1 infection with HCV (co-infection), 3) high IL-28B mRNA expression, and 4) ATL development when the genotype is a risk factor (B) Lower 4 lines; assessing the risk factors described in the outcome, the IL-28B mRNA expression level in peripheral blood (5, 6, and 7), and ATL development (8). Consequently, similarly to HCV, HTLV-1 is associated with up-regulation of IL-28B mRNA along with the TT homozygote, and co-infection with HTLV-1 and HCV paradoxically down-regulates the mRNA level

*; IL-28B Expression level, Co-inf = co-infection with HTLV-1 and HCV, mono = mono-infection

findings suggest that the SNP rs8099917 is not associated with susceptibility to HTLV-1 infection or the development of ATL. On the other hand, all of ATL cell- or HTLV-1-infected T-cell- lines examined were exclusively TT homozygous, implying that HTLV-1-infected cells carrying TT homozygotes may immortalize easily in vitro.

Next, we found a strange phenomenon that the IL-28B mRNA expression levels in peripheral blood were lower in samples with HTLV-1/HCV co-infection than in samples with either HTLV-1 or HCV alone, especially significantly for HTLV-1 mono-infection. In particular, samples carrying TT homozygotes were strongly down-regulated, more than the minor TG hetero- and GG-homozygotes. Why are the mRNA expression levels different in mono- and dual-infection? Although it is not known how rs8099917 affects the action of IL-28B, presumably it alters the immune function to viruses. In addition to a common anti-viral IFN-stimulating signal pathway, HTLV-1 may use an alternative anti-viral pathway like HBV [14], because the HTLV-1 provirus is integrated into host genomic DNA and replicates in distinctive life cycle kinetics. Moreover, ATL originates from Treg cells, which play a central role in suppressing immunity [15]. However, this cannot fully explain the impairment in the HTLV-1 carrier's immunity because no ATL cells are present during the carrier period. Thus, we noted IFN-λ(IL-29, IL-28), which was recently discovered as a type III IFNs with anti-virus ability, anti-tumor and immune responses [16-18].

From our results, the IL-28B expression level was higher in HTLV-1 mono-infected individuals including ATL patients. IFN-λis usually up-regulated through activation of the NF-kappaB pathway after viral infection. Actually, the Sendai virus, an influenza A virus, and others have been demonstrated to activate the NF-kappa B pathway, resulting in up-regulated IL-28B expression [19,20]. Accordingly, the highest up-regulation of IFN-λ3 in HTLV-1 mono-infection may be explained by virtue of a viral protein of HTLV-1 having strong NF-kB activating ability. Moreover, it is instructive that IFN-λhas a potent function to expand Treg cells [21], which are mainly infected with HTLV-1, predisposing development of ATL. However, there has not yet been evidence that co-infection with HCV damages Tax action.

Of IL-28B producing cells in the literature, most cells in the blood are described as having a weak or absent expression under the steady state conditions. Li et al. [9] reported that IL-28B mRNA is not always expressed in virally infected cells. Actually, our findings in HTLV-1-infected cases also showed that at least the main producing cells are likely to be cells other than ATL cells because most cell lines from ATL and some blood samples containing ATL cells were expressed faintly. At present, plasmacytoid dendritic cells are indicated to be the most potent producers of IFN-λs [19]. On the other hand, IFN-λ3 reportedly has the functions of proliferating Treg cells which are the origin of ATL cells, suggesting that HTLV-1 is associated with up-regulation via Treg cells infected with HTLV-1.

In conclusion, we found an unusual phenomenon in that the expression of IL-28B mRNA was affected by not only the IL-28B rs8099917 genotype, but also co-infected HTLV-1 with HCV. This will contribute to a better understanding the enigmatic impairment of immunity in the HTLV-1 carrier state, including co-infection with HTLV-1 and HCV.

**Abbreviations**

HTLV-1: Human T-cell leukemia virus type -1; HCV: Hepatitis C virus; SNP: Single nucleotide polymorphism; IFN: Interferon; PBMC: Peripheral blood

mononuclear cell; PCR: Polymerase chain reaction; MAF: Minor-allele frequency.

**Acknowledgements**
This work was supported by the Japanese Government, Kakken-B No. 213901832.

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**Authors' contributions**
SK designed the study and wrote the manuscript, and SM, TU, KN, DS HH, KY, NU, YM analyzed the genotype, TK collected samples, and TK, KN, MI and SK organized and assessed the data. All authors interpreted the data and were financially supported. All authors read and approved the final manuscript.

**Competing interests**
The authors declare that they have no competing interests.

Received: 5 July 2011 Accepted: 15 February 2012
Published: 15 February 2012

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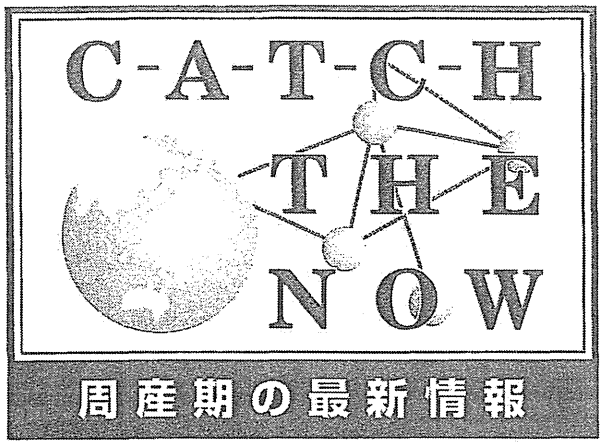
**Cite this article as:** Kamihira *et al.*: Paradoxical expression of *IL-28B* mRNA in peripheral blood in human T-cell leukemia virus Type-1 mono-infection and co-infection with hepatitis C Virus. *Virology Journal* 2012 **9**:40.




*2011年から全妊婦に対して妊婦健診でHTLV-1抗体検査が行われるようになった．今後，キャリア減少を目指して取っていくべき方策と，キャリア妊婦へのかかわり方など，母子感染対策のために産科スタッフが知っておきたい知識を解説する．*

# HTLV-I母子感染対策のために助産師が知っておきたい知識

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## はじめに

これまで九州・沖縄など，ごく一部の地域で行われてきたHTLV-I母子感染対策が，2011年から全国で行われるようになった．2011年度からは全国の都道府県でHTLV-I母子感染対策協議会が設置される予定で，各地域での保健指導およびカウンセリング体制の検討，市町村職員などへの研修，HTLV-I母子感染対策に関する普及啓発が行われることになっている．この中で保健所，女性健康支援センターでは授乳に関する指導，助言や不安や悩みのカウンセリングを担当し，産婦人科医療機関では，妊婦のHTLV-I抗体スクリーニング，確認検査の実施，結果の説明の後に授乳に関する指導，助言を行うことになっている[1]．HTLV-Iは後述するように成人T細胞白血病（ATL）やHTLV-I関連脊髄症（HAM）などの難治性の疾患を，キャリアのごく一部に引き起こすが，母乳を介して母子感染することが知られている[2, 3]．母子感染を防ぐために母乳を制限することを指導するが，従来のように断乳の上，人工乳で育てる方法以外に，母乳を搾乳していったん凍らせてから，解凍して哺乳瓶で与える凍結母乳法[4]，3カ月までの短期母乳も母子感染を減少させることが分かっている[5]．臨床現場でHTLV-Iキャリア妊婦に母乳哺育法を直接指導する立場にある助産師や看護師にも，HTLV-Iの知識は必須事項となっており，また不安や悩みのカウンセリングにも対応することが今後増してくると思われる．本稿ではHTLV-I母子感染について概説する．

## HTLV-Iについて

1981年に京都大学の日沼頼夫らにより，成人T細胞白血病（ATL）の原因ウイルスとしてHTLV-Iが発見された[6]．このウイルスはCD4陽性のT細胞に感染し，いったん感染すると，生涯にわたりウイルスを持ち続ける（キャリアという）．HTLV-Iキャリアは1990年ごろは120万人と推定され，九州・沖縄にキャリアの大半が居住していた[7]．その後，2006年，2007年の全国調査（厚生労働研究：山口班）で，キャリア数は108万人と少し減少したが，キャリアの居住地が九州・沖縄から，全国に拡散していることが明らかとなった（表1）[8]　重要なことと

表1　HTLV-Iキャリア推定数（献血者からの陽性率から推定）

| 地　域 | 1990年 | | 2006年，2007年 | |
|---|---|---|---|---|
| | キャリア数 | キャリア地域別（%） | キャリア数 | キャリア地域別（%） |
| 北海道・東北 | 108,000 | 9.1 | 74,753 | 6.9 |
| 関東（東京） | 128,300 | 10.8 | 190,609 | 17.7 |
| 北陸・東海 | 82,100 | 6.9 | 81,802 | 7.6 |
| 近　畿 | 202,300 | 17.0 | 171,843 | 15.9 |
| 中国・四国 | 65,000 | 5.4 | 67,133 | 6.2 |
| 九州・沖縄 | 607,300 | 50.9 | 492,582 | 45.7 |
| 全　国 | 1,193,000 | 100.0 | 1,078,722 | 100.0 |

平成2年度厚生省成人T細胞白血病（ATL）の母子感染防止に関する研究（園祖班），平成20年度厚労省研究本邦におけるHTLV-I感染及び関連疾患の実態調査と総合対策（山口班）のデータを一部改変

して，HTLV-Iキャリアのすべてが ATL や HAM を発症するのではなく，ATL だと 40 歳を過ぎたころから毎年キャリア 1,000 人に 1 人くらいの発病（生涯発病率は約 5%），HAM では生涯発病率は 0.25% と低率であることを認識する必要がある．HTLV-I ウイルスの感染を家系調査したところ，家族内集積が認められ，母親がキャリアであれば高率に子どもに感染することが知られていた[9]．その後の詳細な疫学調査，基礎研究により，HTLV-I は母乳を介して子どもに感染することが判明した．そのほかの感染ルートとして，輸血感染，性行為を介した夫婦間感染があるが，輸血感染に関しては，1986 年より献血時に感染症スクリーニングを行っているため，現在は皆無である．ATL の発病には，感染してから数十年を要することから（平均発症年数は 58 歳である），ATL 患者のすべては母子感染例である．従って ATL を撲滅するためには母子感染対策が極めて重要になる．

国はこれらのことを受け，HTLV-I を地方の風土病ではなく，全国に広がるウイルスとして認め，母子感染を防ぐために，妊婦に対する HTLV-I 抗体検査を 2010 年度より公費負担とし，各都道府県に HTLV-I 母子感染対策協議会を設置し，スムーズに母子感染対策が行われるように，各都道府県に働き掛けた．

## 母乳を介した母子感染

HTLV-I は CD4 陽性 T リンパ球（免疫を司るリンパ球）の中に潜んでおり，血漿中には存在しない．感染したリンパ球が，非感染リンパ球と接触することにより感染が生じる（図 1）．感染は生きたリンパ球同士でないと起こらないので，いったん母乳を凍らせると母乳中のリンパ球は死んでしまうため，感染が起こらなくなる．また母体血中には感染を中和する抗体が存在する．これらの抗体（IgG，IgM，IgA，IgE）の中の IgG 抗体は，胎盤を通過して胎児に移行するため，胎児は生まれた際，母体と同量の感染中和抗体を持っている．母体由来の感染中和抗体の半減期は 1 カ月であるため，出生後 3 カ月ま

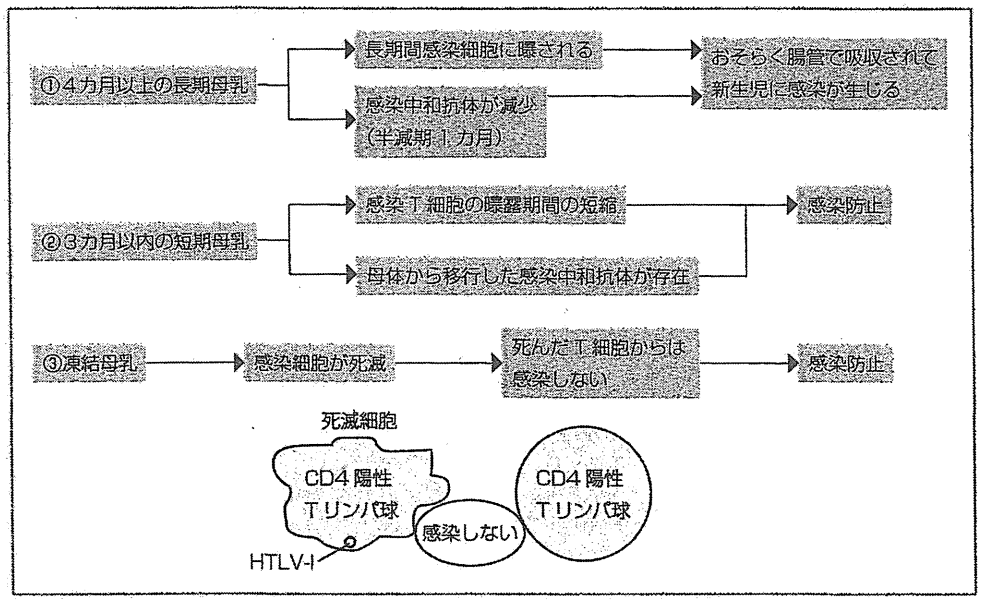


図1　HTLV-I感染様式

では感染中和抗体は，少なくとも8分の1以上存在する．3カ月までの短期授乳だと，感染曝露期間も短くなり，中和抗体も存在するため母子感染が生じにくい（図1）．

表2に1990年までのHTLV-I母子感染率のデータと1990年以降のデータを示す[5]．1990年ごろまでは，HTLV-I母子感染防止のためには，人工哺育の選択肢しかなかったが，人工乳では母乳哺育に比べて母子感染率が明らかに減少している．1990年以降は，凍結母乳，3カ月までの短期母乳でも十分に人工乳と同程度の母子感染防止効果があることが判明した[5]．このため2011年発刊の『産婦人科診療ガイドライン　産科編2011』では人工哺育，凍結母乳，3カ月までの短期母乳のいずれかを勧めている[10]．しかし，一方的に勧めるのではなく，妊婦の意思を尊重する．母子感染のリスクを承知で強く母乳哺育を希望する際は，妊婦の希望に添うように対応する．

## 完全人工栄養，3カ月までの短期母乳，凍結解凍母乳法のメリットとデメリット

HTLV-Iキャリア妊婦ならびに夫に，上記3つの栄養法を提示した際，必ず各栄養法のメリットとデメリットを質問される．

人工栄養は，感染したリンパ球を子どもが飲むことがなく，これまでに1,500例以上のデータがあり，最も確実に母子感染を防ぐことが証明されている方法である．しかし，約3%に母子感染以外の感染ルートが見られたことから，完全には母子感染を予防できない．また，母子間の母乳哺育を介したスキンシップ，愛情形成が不十分となるデメリットがある．そのほか，母乳中に含まれるIgA抗体が補給され

表2　HTLV-I母子感染率

〈1990年まで〉
- 母乳哺育：103/788（13.1%）
- 人工哺育：36/953（3.8%）

〈1990年以降〉
- 母乳哺育
  4カ月以上：93/525（17.7%）
  3カ月以下：3/162（1.9%）
- 人工哺育：51/1,553（3.3%）
- 凍結母乳：2/64（3.1%）

ないデメリットもある．人工乳の際は，しっかりと子どもを抱きしめ，目を見つめるようにして哺乳するように指導してほしい．また，母乳をあげたいのに母乳をやめたお母さんのその気持ちは，子どもに対する強い愛情であることをカウンセリングしていただきたい．筆者の経験では，人工乳哺育を行ったために母子関係がうまく形成されなかったということはない．

凍結解凍母乳哺育では，まず搾乳を行い，母乳パックに母乳を移してから，1晩から1日間，家庭用冷凍庫（－18度）で母乳を凍らせる．凍結することで感染リンパ球は死滅し，感染性が消失するので，しっかりと凍っていることを確認する．その後，ぬるま湯（37度前後）で解凍し，母乳が人肌の温度になるまで温めて哺乳瓶で哺乳させる．栄養学的には母乳と全く同じなので，IgA抗体も補給される上に，3カ月以上の長期母乳も可能である．ただし手間がかかることと，直接母乳を与えることができないという不満感はある．しかし，この方法は理論的にも妥当性があり，母乳の持つ栄養成分を損なわずに栄養できるので，もう少し普及してもよい方法と思われる．

3カ月までの短期母乳は人工乳と同等の感染予防効果を有し，かつ直接自分の乳房から哺乳できるため，母親の満足感も高く，母子間愛情形成にも有利に働く．ただし，症例数が少ないことと，途中で母乳哺育をやめられずズルズルと長期母乳になるケースが散見されることがある．2カ月の終わりごろ，もしくは3カ月に入ったころより徐々に粉ミルクと母乳の混合栄養にしていき，3カ月末には母乳から人工乳に切り替えることが重要である．また3カ月まで母乳を与えた場合，薬剤で母乳分泌を止めることは難しい．そのため，しばらくは乳房緊満は続くが，乳房を冷やしたりして対応する．4カ月目から凍結母乳に切り替えることは可能かと質問されることがあるが，この点にはいまだエビデンスはない．おそらく感染のリスクは増加しないと思うが，実行される場合は，いまだエビデンスにはなっていないと説明した上で，行っていただきたい．

## HTLV-Iキャリアと説明した後の対応

図2に示すように，妊娠30週ごろまでにHTLV-I抗体スクリーニング法を公費で行う．陽性となった場合，偽陽性である可能性があるので，必ず確認検査が必要であると説明した上で，ウエスタンブロット（WB）法を保険診療で行う[5]．九州・沖縄などの一部の地域を除いては，一次検査で陽性となった症例の20～30%のみが，真の陽性（キャリア）である．そのため一次検査の結果のみでキャリアと判断せずに，必ずWB法を行い，陽性であれば，その時点でキャリアと説明し，十分な時間をとって対応，説明する[5, 10]．WB法陰性の場合は，母乳哺育を勧める．ただし，WB法で10～20%に

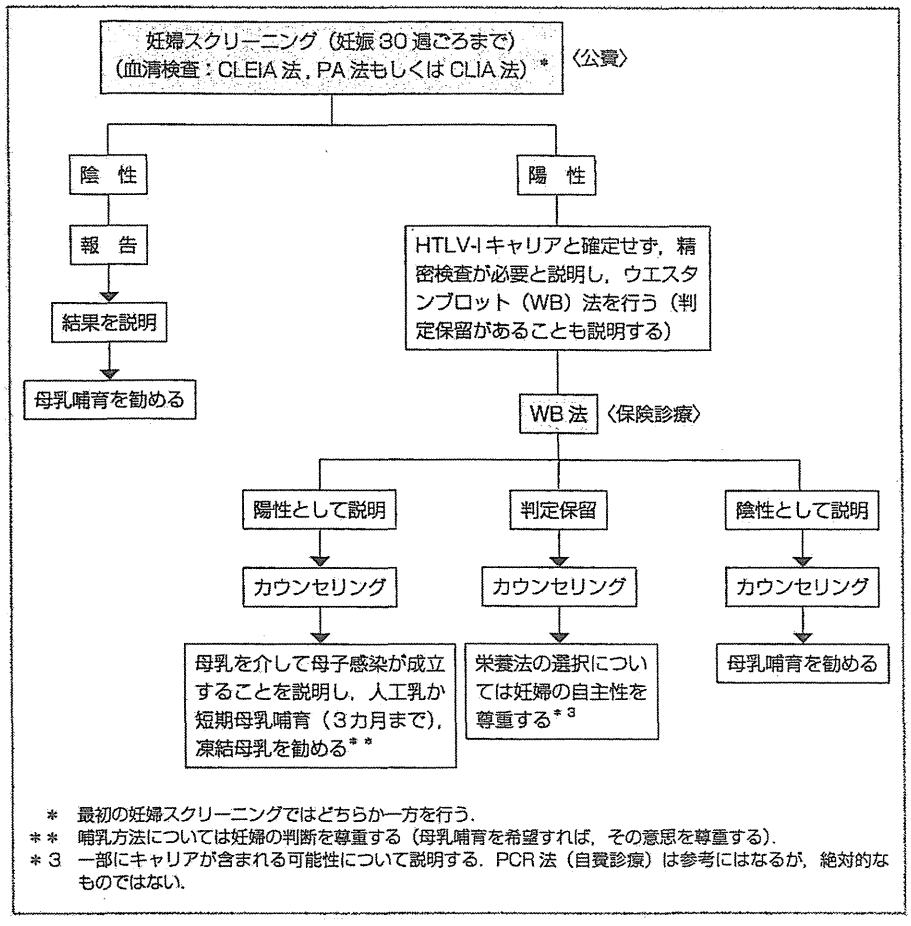


図2　HTLV-Iスクリーニングの進め方

判定保留となるケースがある．このような場合，自費診療となるがPCR法で精査することも可能である[5]．しかし，高価な検査であるため，厚生労働研究：板橋班，浜口班が検査会社と協力して無償で判定保留例にPCR法を行い，その結果を伝える事業を始めたので，これを利用いただきたい．余ったサンプルで，PCR法の評価を7カ所の施設で行い，将来HTLV-I PCR法が保険適用される際の試料とさせていただく．また血漿は判定保留の少ないWB法の確立に役立てる計画である．

## 典型的なHTLV-Iキャリア

表3に示すように，まず自分がHTLV-Iキャリアであることに大きなショックを受ける．HTLV-Iキャリアのすべてが ATL，HAM になるのではなく，40歳を過ぎてからキャリア1,000人に1人の割合で発症する（喫煙による肺癌の発癌の半分程度）と話し，無用の心配をしないよう説明するが，この際，カウンセリングが必要なこともある．

表3　典型的なキャリア例

1. HTLV-Iキャリアであることを知り，大きなショックを受ける．
2. 母子感染予防法があることを知り，子どもには感染させたくないと訴える．
3. 夫，家族に結果を知らせるべきか悩む．
4. 夫と相談し，母乳栄養法について決定する．
5. 自分自身のATL，HAMのことで不安になる．

※1～5の間，カウンセリングが必要なことがある．

次に，子どもにHTLV-Iを感染させることを減らす方法があると説明すると，ほぼすべてのキャリア妊婦は，自分の子どもには感染させたくないと訴える．このときは，分かりやすく人工乳，凍結母乳，3カ月までの短期母乳について説明する．

次に必ず，自分がHTLV-Iキャリアであることを夫や家族に伝えるべきか悩む．本人の悩みは深刻なので，医療者側からアドバイスをするとよい．夫婦の状況によって変わると思うが，可能であれば夫には相談したほうがよいと考える．その理由は，HTLV-Iは「親の意思」によって防ぐことが可能な感染症であり，子どもの将来を決定するためには，2人で相談したほうがよいからである．またキャリアである妊婦を支えてくれるのは夫であるからである．しかし，そのほかの家族にキャリアであることを伝える必要は原則的にはなく，またメリットも特別の場合を除いてない．

その後，夫と本人とに対して3つの授乳方法を説明し，いずれかの授乳方法を選択した際は，助産師・看護師は母乳外来などで対応することになる．人工乳を選択した場合は，目の前でほかの患者に乳房管理の指導を行うことは避けるなど，配慮していただきたい．場合によっては相談にも乗ってほしい．凍結母乳もしくは短期母乳を選択した場合は，積極的に母乳外来などで出産前の乳房管理に努めてほしい．凍結母乳を選択した場合は，搾乳の方法や母乳パックなどの情報を提供していただきたい．3カ月までの短期授乳を選択された場合，2カ月末から3カ月に入った時点で，徐々に粉ミルクに切り替えていく方法を提示していただきたい．

またいったん，授乳法を決めたが，その後に気持ちが揺らぐこともあるので，その際も時間を取って，本人が納得するまで十分に相談に乗ってあげてほしい．

最後にキャリア妊婦は「自分がこの先どうなるのか？」ということで必ず悩む．分娩後もしくは40歳以降になってから，2011年度に全国で設置される予定となっている「HTLV-Iキャリア外来」を紹介して，専門的な話をしてもらうことを勧めていただきたい．種々の不安を解消した上で，お産に臨み，その後の育児もスムーズにいくよう支援していただきたい．また出産後の赤ちゃんは小児科でフォローしてもらい，3歳時の採血で感染の有無が分かることも説明してほしい．小児科外来でも看護師は育児に関する種々の悩みや質問を受けると思うので，その際も丁寧な説明をお願いしたい．

## おわりに

国が本格的にHTLV-I撲滅に向けて動き出した．この中で母子感染予防対策は，最重要課題である．全国で毎年約3,000人の妊婦が突然，HTLV-Iキャリアであると告げられることになるが，十分な説明の上で，医師，助産師，看護師が協力し合って，HTLV-I母子感染が減少し，かつキャリアの健康が維持されることを望む．