1. Introduction

Recent basic and epidemiologic data reveal that non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome that is closely associated with multiple factors such as obesity, hyperlipidemia, type 2 diabetes mellitus and hypertension as well as a constellation of clinical problems that arise from insulin resistance [1]. Consequently, no effective monotherapy for the prevention or treatment of NAFLD has been developed to date. The complicated pathogenesis of NAFLD suggests that combination therapy may be more effective for the treatment of NAFLD in humans not complying to a healthy lifestyle. Actually, multiple combination therapies are known to exert greater beneficial effects on type 2 diabetes mellitus and hypertension than monotherapy [2,3]. Similarly, several studies have reported that multifactorial treatment was also effective against NAFLD [4]. However, an effective combination therapy for NAFLD has yet to be established. We hypothesized that a combination therapy comprising ezetimibe (EZ) and acarbose (AC), which have different medicinal actions, might improve the conditions of NAFLD patients with hyperlipidemia and diabetes mellitus. In dyslipidemic subjects, EZ is a useful cholesterol absorption inhibitor that prevents the absorption of dietary and biliary cholesterol by selectively binding to the intestinal cholesterol transporter Niemann–Pick C1-Like1 [5,6]. Several reports have concluded that EZ monotherapy not only protects against diet-induced hyperlipidemia, but also attenuates liver steatosis in an experimental NAFLD model [7–9]. In diabetic subjects, on the other hand, AC (an α-glucosidase inhibitor) delays the digestion of complex carbohydrates and disaccharides to absorbable monosaccharides by reversibly inhibiting α-glucosidases within the intestinal brush border. This drug has also been reported to exert protective effects against liver steatosis in mice [10]. In this study, we investigated the efficacy of long-term combination therapy with EZ and AC for the prevention of NAFLD in a mouse model.

2. Materials and methods

2.1. Reagents and special diet

EZ and AC were kindly provided by Schering-Plough Co., Ltd. (Osaka, Japan) and Bayer Co., Ltd. (Osaka, Japan), respectively. High-fat diet 32 was obtained in powdered form from Japan CLEA (Tokyo, Japan); this animal feed contains 506.8 kcal/100 g (57.5% from fat; fatty acid, 32.7 g/100 g; cholesterol, 12.9 mg/100 g; 19.7% from protein and 22.8% from carbohydrate). Basal diet (BD) was obtained from ORIENTAL YEAST Co., Ltd. (Tokyo, Japan); this feed contains 360 kcal/100 g (13.3% from fat, 26.2% from protein, and 60.5% from carbohydrate).

2.2. Animal treatment and experimental procedures

All animals were treated humanely according to the guidelines of the National Institutes of Health and the AERI-BBRI Animal Care and Use Committee. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yokohama City University School of Medicine.

We used male C57BL/6J mice (6 weeks old; Charles River, Japan) in this study. The animals were allowed free access to food and tap water throughout the acclimatization and experimental periods.

After acclimatization for one week, the mice were randomly divided into five experimental treatment groups (n = 8 mice each) as follows: Group 1 was maintained on BD only; Group 2 was maintained on HFD only; Group 3 received HFD with EZ (5 mg/kg/day); Group 4 received HFD with AC (100 mg/kg/day); and Group 5 received HFD with both EZ (5 mg/kg/day) and AC (100 mg/kg/day). All five groups received their respective treatments for 24 weeks. Each drug was admixed in the HFD. The average doses of the consumed drugs were calculated based on the food intake and the body weight of each mouse, which were monitored weekly.

2.3. Measurement of plasma and serum biochemical markers

Serum alanine aminotransferase (ALT) was measured using Spotchem SP-4410 (Arklay Co, Kyoto, Japan). Total serum cholesterol (Chol), total serum triglyceride (TG) levels and serum lipoproteins were analyzed using an online dual-enzymatic method for the simultaneous quantification of Chol and TG using high-performance liquid chromatography (HPLC) at Skylight Biotech Inc. (Akita, Japan), according to a procedure described by Usui et al. [11]. Blood insulin resistance was estimated using the homeostasis model assessment for insulin resistance (HOMA-IR) derived from the following equation: IR = fasting plasma glucose level in mg/dL × fasting serum insulin level in ng/mL/22.5.

2.4. Liver histopathological evaluations

Liver samples were excised and embedded in Tissue-Tek OCT compound and paraffin for histological analysis. The liver sections were processed routinely for hematoxylin–eosin (H & E) staining. The presence of collagen, an index of fibrosis in the lesions, was examined in Masson's trichrome-stained preparations. To evaluate fat deposition, the liver sections were stained with oil red O. All histopathological findings were scored by the same two pathologists, who were unaware of the treatments that the animals had received. The histological features were grouped into three broad categories: steatosis, inflammation, and fibrosis.

2.5. Measurement of liver triglyceride and cholesterol contents

Liver samples were homogenized in 50 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA and 1 mM PMSF. Liver TG and Chol contents were analyzed using HPLC at Skylight Biotech Inc. (Akita, Japan).

2.6. RNA isolation and reverse transplantation

Total RNA was isolated from the samples using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription to produce cDNA was performed using a TaqMan Gold RT-PCR Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

2.7. Quantification of gene expressions using real-time RT-PCR

The hepatic mRNA levels of several markers associated with lipid metabolism as well as that of a housekeeping gene, β-actin, were determined using fluorescence-based real-time RT-PCR on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed using the Power SYBR® Green PCR Master Mix reagent, according to the manufacturer's instructions. The values in all the samples were normalized to the expression level of β-actin. The gene expression ratio was determined using data from the BD-only group.

2.8. Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 12. All results are expressed as the means ± SEM. Statistical comparisons were made using the Student *t*-test or Scheffe's method after an analysis of variance (ANOVA). The results were considered significantly different at <0.05.

3. Results

3.1. Food consumption, body weight and liver weight

After 24 weeks of treatment, no significant differences in caloric intake were seen among the four groups fed HFD with or without additional treatment. However, significant differences in the body weights, liver weights and body weight per liver weight ratios were seen among the groups at the end of the study. The mice fed HFD only had significantly higher body weights, liver weights, HOMA-IR values than the control mice that were fed BD. Regarding the therapeutic effects, the effects of the combination therapy with EZ and AC on the liver weight and the liver weight per body weight ratio were the most remarkable (Table 1). During this study period, no apparent adverse effects of the drugs, such as diarrhea, were seen.

3.2. Effects of monotherapy with ezetimibe or acarbose and combination therapy on NAFLD

3.2.1. Liver steatosis

Oil-red O-stained liver samples from the four groups fed HFD revealed fatty liver. While the HFD only group showed macrovesicular steatosis, the AC monotherapy group showed a slightly reduced size of lipid deposits and the EZ monotherapy group showed only microvesicular steatosis. Furthermore, the combination therapy group showed even smaller lipid deposits, almost to the extent observed in the BD group (Fig. 1A). Quantification of the liver TG and liver Chol contents showed that the combination therapy was the most effective at decreasing the liver TG and liver Chol contents among the four groups fed HFD (Fig. 1B and C).

3.2.2. Liver inflammation

H & E-stained liver samples obtained from the four groups fed HFD showed signs of liver inflammation. While the HFD only group showed diffuse lobular inflammation and hepatocyte ballooning, the AC monotherapy group showed slightly reduced levels of inflammatory cell infiltrates and hepatocyte degeneration, and the EZ monotherapy group showed an even greater reduction. Furthermore, the combination therapy group showed minimal inflammatory cell infiltrates and only a small amount of hepatocyte degeneration (Fig. 2A). The serum ALT level in the combination therapy group was significantly lower than the levels in the HFD only group and the monotherapy groups (Fig. 2B).

3.2.3. Liver fibrosis

Masson-stained liver samples from the three groups revealed liver fibrosis. While the HFD only group

Table 1
Comparison of characteristics between mice fed BD and mice fed HFD with or without EZ and/or AC.

	BD ($n = 8$)	HFD ($n = 8$)	HFD + EZ ($n = 8$)	HFD + AC ($n = 8$)	HFD + EZ + AC ($n = 8$)
Calorie intake (kcal/day)	9.9 ± 0.1*	18.1 ± 0.0	19.0 ± 0.0	19.2 ± 1.4	19.0 ± 1.7
Ezetimibe intake (mg/day)	–	–	0.19 ± 0.00	–	0.19 ± 0.17
Acarbose intake (mg/day)	–	–	–	3.78 ± 0.27	3.74 ± 0.34
Starting body weight (g)	24.0 ± 1.3	24.6 ± 0.3	24.5 ± 0.6	24.2 ± 0.5	24.8 ± 0.3
Final body weight (g)	30.1 ± 0.8*	54.6 ± 1.1	47.9 ± 2.1*,†	49.7 ± 1.3*,†	40.9 ± 1.5*
% vs. BD-only	100	181	159	165	136
Liver weight (g)	1.4 ± 0.5*	5.0 ± 0.2	2.8 ± 0.3*,†	3.8 ± 0.2*,†	1.6 ± 0.1*
% vs. BD-only	100	357	200	271	114
Liver weight/body weight ratio	0.048 ± 0.001*	0.091 ± 0.002	0.058 ± 0.004*,†	0.075 ± 0.002*,†	0.039 ± 0.001*
% vs. BD-only	100	182	116	150	78
Fasting glucose (mg/dl)	93.7 ± 10.7*	128.9 ± 21.3	86.3 ± 5.1*	101.1 ± 8.8	87.9 ± 6.2*
Fasting insulin (ng/ml)	0.23 ± 0.03*	4.84 ± 0.87	3.20 ± 0.88	3.89 ± 0.96	1.91 ± 0.36*
HOMA-IR	1.0 ± 0.1*	36.2 ± 10.1	12.2 ± 3.4*	13.0 ± 2.9*	7.7 ± 1.7*

BD, basal diet; HFD, high-fat diet; EZ, ezetimibe; AC, acarbose; HOMA-IR, homeostasis model assessment for insulin resistance. Data are expressed as the mean ± SEM.

* $P < 0.05$, compared with the HFD group.

† $P < 0.05$, compared with the HFD + EZ + AC group.

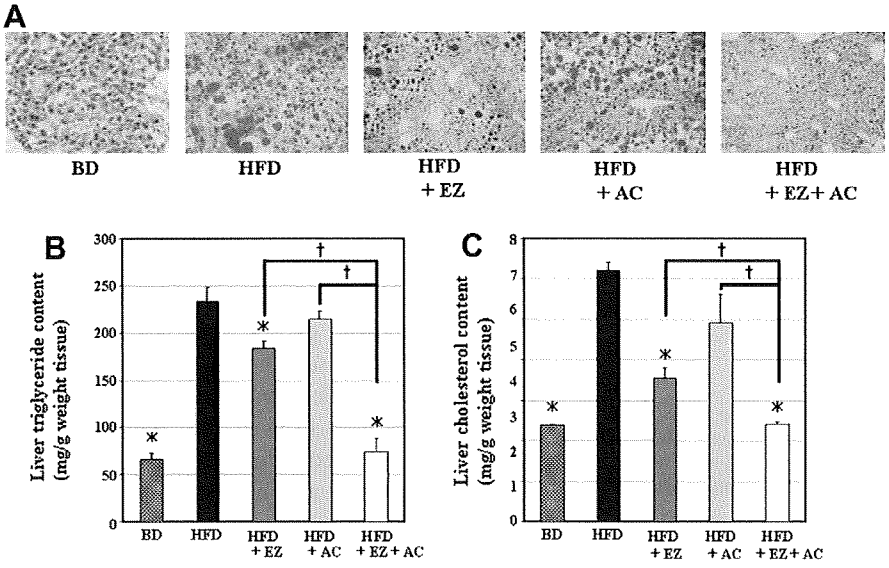

Fig. 1. Analysis of liver steatosis. (A) Oil red O staining (red color) shows lipid deposits in liver samples (magnification: 100×). Minimal fatty deposits are visible in the HFD + EZ + AC group, almost to the extent seen in the BD group. (B and C) The liver TG and liver Chol contents were significantly lower in the HFD + EZ + AC group than in the monotherapy groups (data are expressed as the mean ± SEM. $^{*}P < 0.05$, compared with the HFD group; $^{\dagger}P < 0.05$, compared with the HFD + EZ + AC group).

showed combined pericellular portal fibrosis, the EZ and AC monotherapy groups both showed minimal perivenular and perisinusoidal fibrosis. Furthermore, the combination therapy group showed little fibrosis (Fig. 3A). The liver collagen I mRNA expression level in the combination therapy group was significantly lower than the levels in the HFD only group and the monotherapy groups (Fig. 3B).

3.3. Insulin resistance

The fasting glucose levels in the combination therapy group and the EZ monotherapy group, and the fasting insulin level in the combination therapy group were significantly lower than that in the HFD only group, but no significant difference was seen among the three therapeutic groups. The HOMA-IR levels were significantly

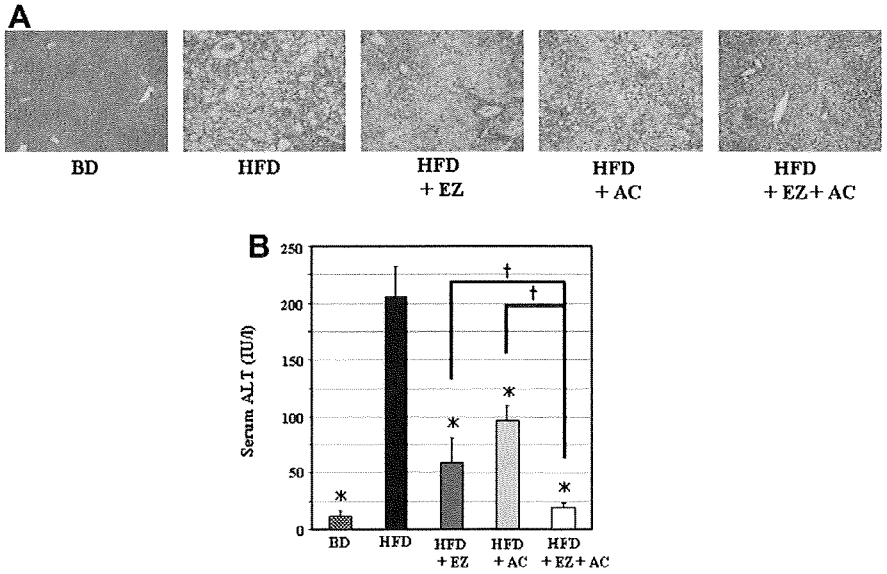

Fig. 2. Analysis of liver inflammation. (A) Liver samples were stained with H & E (magnification: 100×). Minimal inflammatory cell infiltrates and only a small amount of hepatocyte degeneration are visible in the HFD + EZ + AC group. (B) The serum ALT level was significantly lower in the HFD + EZ + AC group than in the monotherapy groups (data are expressed as the mean ± SEM. $^{*}P < 0.05$, compared with the HFD group; $^{\dagger}P < 0.05$, compared with the HFD + EZ + AC group).

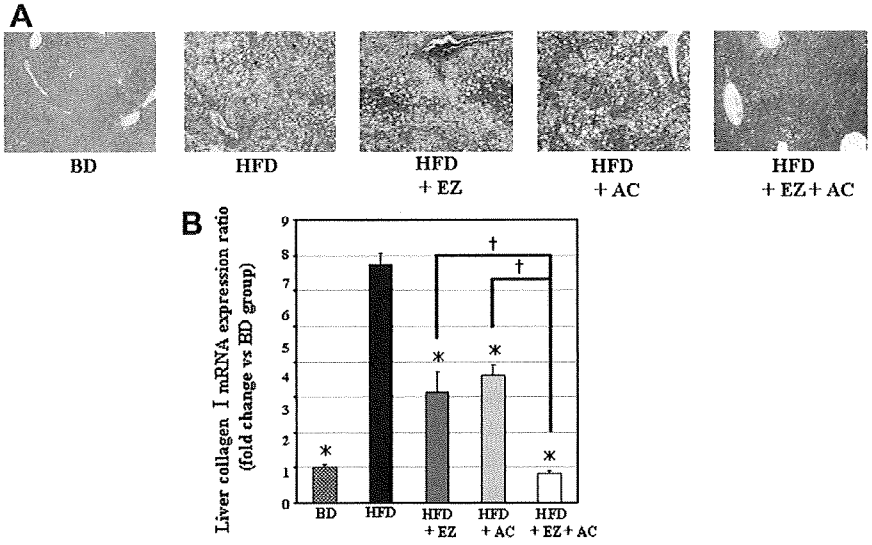

Fig. 3. Analysis of liver fibrosis. (A) Liver samples were stained with Masson's trichrome (magnification: 100×). No obvious fibrosis is visible in the liver specimens from the HFD + EZ + AC group or the BD group. (B) The liver collagen I mRNA expression level was significantly lower in the HFD + EZ + AC group than in the monotherapy groups. The gene expression ratio was determined by comparison with the BD group (data are expressed as the mean ± SEM. $^{*}P < 0.05$, compared with the HFD group; $^{\dagger}P < 0.05$, compared with the HFD + EZ + AC group).

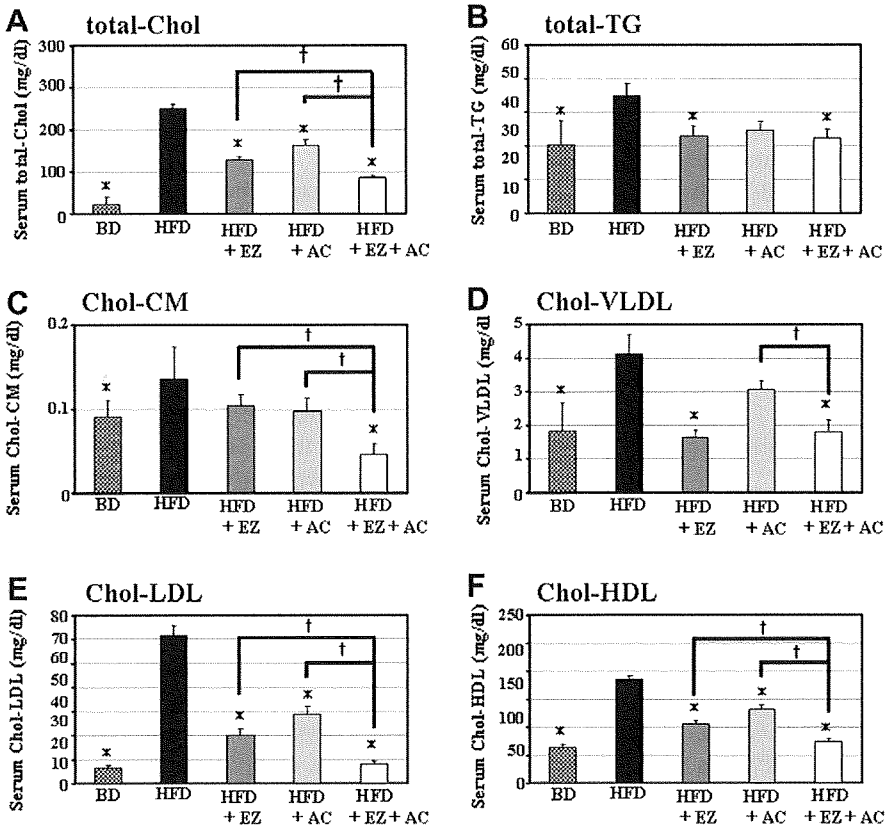

Fig. 4. Analysis of serum biochemical markers of lipid metabolism and lipoproteins. (A, C, E and F) The serum total-Chol, the serum Chol-CM, the serum Chol-LDL and the serum Chol-HDL levels were significantly lower in the HFD + EZ + AC group than in the monotherapy groups. (B) The serum total-TG level was not significantly different among the three therapeutic groups. (D) The serum Chol-VLDL level was significantly lower in the HFD + EZ + AC group than in the HFD + AC group (data are expressed as the mean ± SEM. $^{*}P < 0.05$, compared with the HFD group; $^{\dagger}P < 0.05$, compared with the HFD + EZ + AC group).

lower in the combination therapy group and the AC and EZ monotherapy groups than in the HFD only group. However, no significant difference was seen among the three therapeutic groups (Table 1).

3.4. Serum biochemical markers of lipid metabolism and lipoproteins

The combination therapy group and the EZ and AC monotherapy groups all exhibited significant reductions in the serum total-Chol level, compared with the HFD only group, and the serum total-Chol level in the combination therapy group was also significantly lower than the levels in the monotherapy groups. The serum total-TG levels in the combination therapy group and the EZ monotherapy group were significantly lower than that in the HFD only group, but no significant difference was seen among the three therapeutic groups. The serum cholesterol-chylomicron (Chol-CM) level was significantly lower only in the combination therapy group, compared with the HFD only group. Furthermore, the serum Chol-CM level was significantly lower in the combination therapy group than in the monotherapy groups. The serum cholesterol-very low-density lipoprotein (Chol-VLDL) levels in the combination therapy and EZ monotherapy groups were significantly lower than that in the HFD only group. The combination therapy significantly decreased the Chol-VLDL level, compared with AC monotherapy. The serum cholesterol-low-density lipoprotein (Chol-LDL) and the serum cholesterol-high-density lipoprotein (Chol-HDL) levels in the combination therapy group and the EZ and AC monotherapy groups were significantly lower than that in the HFD only group. Furthermore, the combination therapy significantly decreased the serum Chol-LDL and the serum Chol-HDL levels, compared with those in the monotherapy groups (Fig. 4).

3.5. Liver mRNA expression levels of markers associated with lipid metabolism

No significant differences in the liver sterol regulatory element-binding protein-1c (SREBP-1c) mRNA expression levels were seen among the groups. The liver SREBP-2 mRNA expression levels were significantly upregulated in the combination therapy group and the EZ monotherapy group, compared with the levels in the HFD only group. The liver peroxisome proliferators-activated receptor-α1 (PPAR-α1), the liver microsomal triglyceride transfer protein (MTP) and the liver low-density lipoprotein receptor (LDLR) mRNA expression levels were significantly upregulated only in the combination therapy group, compared with the levels in the HFD only group. Furthermore, the combination therapy significantly upregulated the liver SREBP-2, liver PPAR-α1, liver MTP and liver LDLR mRNA expression levels, compared with those in the monotherapy groups (Fig. 5).

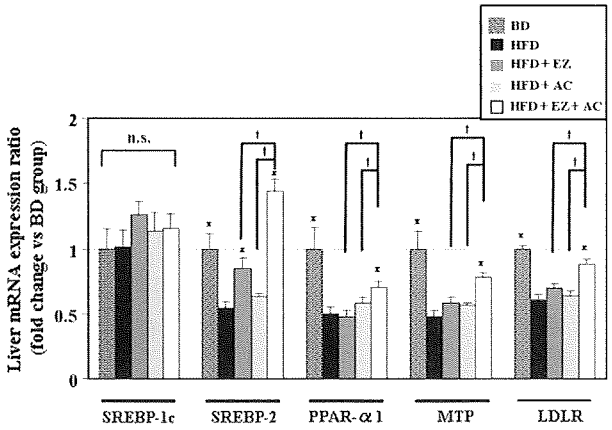

Fig. 5. Analysis of liver mRNA expression levels of markers associated with lipid metabolism. No significant differences in the liver SREBP-1c mRNA expression levels were apparent among the five groups. The liver SREBP-2, liver PPAR-α1, liver MTP and liver LDLR mRNA expression levels were significantly higher in the HFD + EZ + AC group than in the monotherapy groups. The gene expression ratio was determined by comparison with the BD group (data are expressed as the mean ± SEM. $^{*}P < 0.05$, compared with the HFD group; $^{\dagger}P < 0.05$, compared with the HFD + EZ + AC group; ns, not statistically significant).

4. Discussion

Our experimental NAFLD model of mice fed HFD for 24 weeks was confirmed by histopathological findings for the liver, which showed fatty liver and hepatitis; meanwhile, mice fed BD for the same period showed almost normal findings. Mice fed HFD were shown to have obesity, insulin resistance and hyperlipidemia. When this NAFLD model was initially treated using either EZ or AC monotherapy or combination therapy for 24 weeks, the EZ monotherapy prevented NAFLD and the AC monotherapy showed a tendency to prevent NAFLD, but the combination therapy achieved the most dramatic effects in preventing the progression of NAFLD. Two possible mechanisms responsible for the attenuation of the NAFLD pathology findings can be considered: first, EZ and AC may have additive or multiplier effects on the reduction in the serum Chol level and the improvement of insulin resistance by inhibiting the intestinal absorption of both Chol and glucose, which are the major elements of lipid synthesis in the liver; and second, the lipid metabolic disorder in the liver may be improved through the promotion of lipid discharge from the liver and the β-oxidation of lipids in the liver.

Regarding the first pathway, the combination therapy may have efficiently reduced caloric absorption, mainly of Chol, despite the absence of any significant alterations in the daily caloric consumption. This pathway is

supported by the observations that the combination therapy significantly decreased the body weight and the liver weight, the serum total-Chol and the serum Chol-CM levels, compared with the results in the monotherapy groups. While only the combination therapy significantly decreased the fasting insulin level, compared with that in the HFD only group, no significant difference in the improvement of insulin resistance was seen among the three therapeutic groups. Thus, reducing the serum Chol level in the presence of improved insulin resistance may be the key to the dramatic prevention of NAFLD achieved by the combination therapy. This potential pathway is easy to understand in view of a previous study, which reported that dietary Chol leads to hepatic inflammation in an NASH model [12]. Previous studies have also reported that either EZ or AC monotherapy can decrease the serum Chol level [8,13,14] and improve insulin resistance [8,13,15]. So, in this study, the combination therapy of both drugs might decrease the serum Chol more efficiently and improve insulin resistance equally, compared with either EZ or AC monotherapy. Our data showed that the serum TG levels were significantly decreased in the EZ monotherapy and combination therapy groups, compared with the HFD only group, but that it was not significantly different among the three therapeutic groups. Thus, although serum TG is known to be an important factor in NAFLD, the present findings suggest that the mechanism responsible for the dramatic prevention of NAFLD by the combination therapy mainly involves a decrease in the serum Chol level relative to the serum TG level in our model. Although some clinical and experimental studies have documented that EZ monotherapy did not alter body weight but did decrease liver weight, the treatment periods of these studies were not as long as that used in the present study. On the other hand, some clinical and experimental studies have shown that monotherapy with an α-glucosidase inhibitor significantly decreased body weight, especially when administered at a high dose and for a long treatment period [13–15]. We treated mice with a Chol absorption inhibitor and/or an α-glucosidase inhibitor for a long treatment period, which may explain the reduction in body weight and liver weight in our model. Thus, long-term therapy might also be essential for preventing NAFLD. These results suggest that long-term combination therapy comprising EZ and AC may prevent NAFLD more effectively than either the EZ or AC monotherapy by efficiently decreasing the serum Chol level while attenuating insulin resistance.

Regarding the second pathway, we examined the MTP and PPAR-α1 gene expression levels to evaluate the outflow of lipids from the liver and the level of lipid β-oxidation in the liver. MTP is a heterodimeric lipid transfer protein that is essential for VLDL synthesis and secretion, and the activity of hepatic MTP is suppressed by insulin [16]. PPARs are expressed in the liver and other metabolically active tissues and play a key role in modulating hepatic TG accumulation. PPAR-α1, an isoform of PPAR, regulates fatty acid β-oxidation [17]. Activating liver MTP and PPAR-α1 gene expression might promote the discharge of TG and Chol from the liver and accelerate the mitochondrial and peroxisomal fatty acid β-oxidation pathways [18]. In our experiment, the liver MTP and PPAR-α1 mRNA expression levels in the combination therapy group were significantly upregulated, compared with those in the HFD only group and the monotherapy groups. Consequently, the release of VLDL from the liver and the β-oxidation of lipids in the liver were thought to be accelerated in the livers of mice treated with the combination therapy, compared with in the livers of mice treated with EZ or AC monotherapy, possibly leading to an improvement in lipid metabolic disorders in the livers of mice treated with the combination therapy. The transcription factors in the SREBP family are key regulators of the lipogenic genes in the liver [19]. SREBP-2 is known as a key protein regulating Chol synthesis and uptake and is responsible for the increased transcription that follows sterol depletion [20]. Among the SERBP-1 family members, SREBP-1c retains some ability to stimulate fatty acid synthesis, but it has very little ability to stimulate Chol synthesis [21]. So, in response to sterol-epletion, the liver produces more SREBP-2 and processes it more efficiently, thereby activating Chol synthesis [22]. On the other hand, LDLR expression is primarily regulated by SREBP-2, and diminished hepatic Chol probably enhances the expression of hepatic LDLR, increasing Chol synthesis. This compensatory increase in Chol synthesis would decrease the plasma LDL level [23]. In our study, this compensative reaction might upregulate SREBP-2 and LDLR mRNA expression in an attempt to store Chol in the liver. Recent studies have reported the selective compensatory induction of hepatic HMG-CoA reductase and LDLR in response to the inhibition of Chol absorption [23], so the presently reported combination therapy plus statin therapy may be more effective on poorly controlled NAFLD.

In summary, both the EZ and the AC monotherapies decreased the serum Chol level to a certain extent and improved insulin resistance but did not change the liver MTP and PPAR-α1 mRNA expression levels. On the other hand, the combination of EZ and AC decreased the serum Chol level more efficiently in the presence of improved insulin sensitivity by inhibiting both intestinal Chol and glucose absorption, thus suppressing the inflow of lipids to the liver. Furthermore, only the combination therapy among the three therapeutic groups promoted the discharge of lipids from the liver and the β-oxidation of lipids through the activation of liver MTP and PPAR-α1, thereby reducing the hepatic TG and Chol stores and dramatically attenuating the pathology of NAFLD (Fig. 6).

	serum total-Chol	serum Chol-CM	fasting insulin	insulin resistance	liver MTP	liver PPAR-α1	liver TG content	liver Chol content
EZ	↓	→	→	↓	→	→	↓	↓
AC	↓	→	→	↓	→	→	→	→
EZ+AC	↓↓	↓	↓	↓	↑	↑	↓↓	↓↓

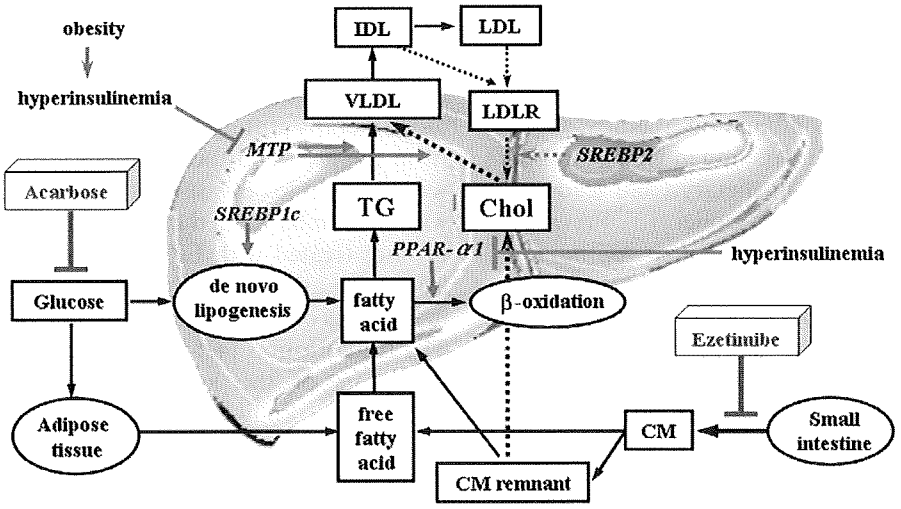

Fig. 6. Schema showing lipid metabolism in the liver and summarizing the results of this study. The combination therapy of EZ and AC decreased the serum total-Chol and Chol-CM levels more efficiently in the presence of improved of insulin sensitivity by inhibiting both the intestinal Chol and glucose absorption, compared with either EZ or AC monotherapy, thus suppressing the inflow of lipids into the liver. Furthermore, only the combination therapy among the three therapeutic groups promoted the discharge of lipids from the liver and the β-oxidation of lipids through the activation of liver MTP and PPAR-α1, thereby reducing the hepatic TG and Chol stores and dramatically attenuating the pathology of NAFLD.

In this study, we used mice with a high level of NPC1L1 mRNA expression in the small intestine but a low expression level in the liver; thus, we were able to examine the effect of EZ on the inhibition of Chol absorption in the small intestine. In humans, both the small intestine and the liver show high levels of NPC1L1 mRNA expression, so EZ inhibits not only dietary and biliary Chol absorption in the small intestine, but also biliary Chol absorption in the liver [5].

One limitation of this study is that the drugs were administered to the mice at the beginning of treatment; thus, the present data show that combination therapy is effective for preventing NAFLD, but not necessarily for treating the late phase of the disease. Further study is needed to confirm that the effect is maintained when the drugs are administered later during the disease course.

In conclusion, our study suggests that combination therapy involving a sterol absorption inhibitor and an antidiabetic agent administered for 24 weeks might improve the histopathology results in a mouse model of NAFLD. The extrapolation of our data to humans, however, remains complicated, and further investigations are needed to match the results in humans.

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Prevalence and Risk Factors for Tuberculosis Infection among Hospital Workers in Hanoi, Viet Nam

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Abstract

Background: Transmission of tuberculosis (TB) to health care workers (HCWs) is a global issue. Although effective infection control measures are expected to reduce nosocomial TB, HCWs' infection has not been assessed enough in TB high burden countries. We conducted a cross-sectional study to determine the prevalence of TB infection and its risk factors among HCWs in Hanoi, Viet Nam.

Methodology/Principal Findings: A total of 300 HCWs including all staff members in a municipal TB referral hospital received an interferon-gamma release assay (IGRA), QuantiFERON-TB Gold In-Tube™, followed by one- and two-step tuberculin skin test (TST) and a questionnaire-based interview. Agreement between the tests was evaluated by kappa statistics. Risk factors for TB infection were analyzed using a logistic regression model. Among the participants aged from 20 to 58 years (median = 40), prevalence of TB infection estimated by IGRA, one- and two-step TST was 47.3%, 61.1% and 66.3% respectively. Although the levels of overall agreement between IGRA and TST were moderate, the degree of agreement was low in the group with BCG history (kappa = 0.29). Working in TB hospital was associated with twofold increase in odds of TB infection estimated by IGRA. Increased age, low educational level and the high body mass index also demonstrated high odds ratios of IGRA positivity.

Conclusions/Significance: Prevalence of TB infection estimated by either IGRA or TST is high among HCWs in the hospital environment for TB care in Viet Nam and an infection control program should be reinforced. In communities with heterogeneous history of BCG vaccination, IGRA seems to estimate TB infection more accurately than any other criteria using TST.

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Introduction

Transmission of *Mycobacterium tuberculosis* (MTB) in health care facilities is a problem worldwide [1–3]. Occupational tuberculosis (TB) can lead to the loss of skilled workers and impact health care service adversely, which has serious consequences in association with recent spread of multi-drug resistant (MDR) MTB strains [1]. Effective infection control measures are expected to reduce nosocomial TB [3–6]. In this sense, estimation of prevalence and risk for TB infection among health care workers (HCWs) involved in TB care is one of the essential steps to review and reinforce TB control measures.

In TB high burden countries, however, occupational risk for TB has often been neglected and concealed by the high prevalence in the general population. Furthermore, in those countries, widespread use of BCG vaccination has interfered with interpretation of tuberculin skin testing (TST) [1,7], which was the only measure to detect TB infection until recently.

A newly developed diagnostic test designated as the interferon-gamma release assay (IGRA) uses a principle that MTB-specific antigens provoke immune reaction in the whole blood after TB infection [8]. With the advent of IGRA, many investigators have reported that latent TB infection could be detected more specifically than using TST [9–11]. QuantiFERON-TB Gold test, an ELISA-based IGRA, is also recommended by the US Centers for Disease Control and Prevention (CDC) for initial and sequential-testing of latent TB infection among HCWs [12].

Viet Nam is one of the 22 TB high burden countries defined by WHO, with prevalence of TB being 227/100,000 population and drug resistance TB is ever-increasing [13]. In Hanoi, the capital of Viet Nam, prevalence of smear positive pulmonary TB is 146/100,000 population [14] and the annual risk of TB infection reported from the suburban area is 0.8% [15]. Despite the high burden, little is known about TB infection among HCWs. We conducted this study to estimate the prevalence and risk factors for TB infection among HCWs in a crowded TB referral hospital together with an adjacent general hospital in Hanoi, Viet Nam, by comparing IGRA with conventional TST one- and two-step methods.

Methods

Ethics statement

A written informed consent was obtained from each participant. The study was approved by ethical committees of the Ministry of Health, Viet Nam and International Medical Center of Japan respectively.

Study design and setting

We conducted a cross-sectional study in November 2007 in two hospitals adjacently located in the same block of Hai Ba Trung District in Hanoi city (Figure 1). A 110-bed "TB hospital", which receives 2,000 TB in-patients and 46,000 turns of examination per year, is mainly assigned for taking care of TB patients in the entire city. The other is a 460-bed "non-TB hospital", which is a general hospital but transfers all TB-suspected patients to the aforementioned TB hospital.

Participants and data collection

Sample size was determined by the number of all staff members in the TB hospital, since our goal was to clarify the situation of all available HCWs working in the environment. The same categories of departments, such as outpatient clinic, intensive care unit, departments of internal medicine, laboratory and administration were selected from the non-TB hospital and the equivalent number was randomly extracted from each category. Demographic information, history of BCG vaccination and factors potentially associated with TB exposure were collected by an interview using a structured questionnaire. Those factors included job category, duration of working, practice of wearing mask, and professional or household contact with TB patients. For all participants, the blood was collected for IGRA, then TST was administered but not for those with pregnancy, breast-feeding or allergy to tuberculin. To rule out active TB, chest X-ray was taken for all participants with positive IGRA results. Sputum test was performed for participants with productive cough. They also had the chance of receiving INH for treatment of latent TB infection if they wished, after consultation with TB doctors there.

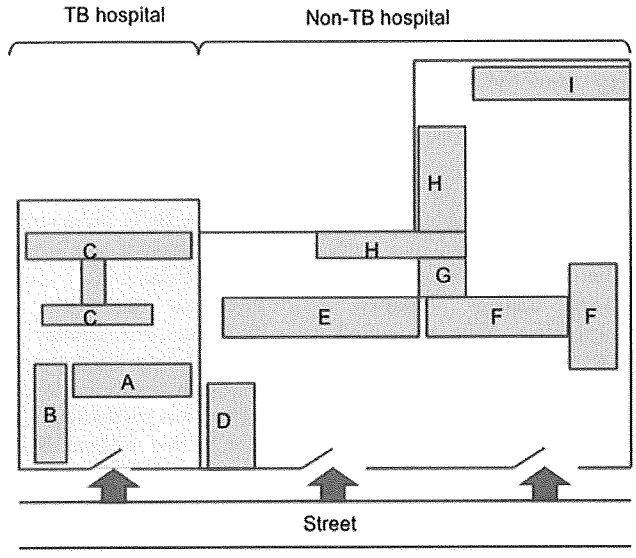

Figure 1. Allocation of two hospitals. TB: Tuberculosis. TB hospital buildings: A = Administration; B = Outpatient clinic and Laboratory; C = Wards and Imaging diagnosis. Non-TB hospital buildings: D = Emergency; E = Wards; F = Laboratory; G = Administration and Imaging diagnosis; H = Out-patient clinic and Wards; I = Imaging diagnosis.
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TST and IGRA

As the first TST, a 5-tuberculin unit dose of Purified Protein Derivatives (Pasteur institute, Nha Trang, Viet Nam), authorized by the Ministry of Health of Viet Nam, was administered by well-trained technicians. Diameter of the induration size was measured after 48 to 72 hours, using a standardized ruler. If the size was less than 10 mm, the second administration with the same dosage was given after 14 days and the results were interpreted similarly (the second TST). From experience in Viet Nam [15], a cut-off value of 10 mm was used in this study, unless otherwise specified. Possible effects of changing cut-off values from 11 mm to 15 mm were also evaluated.

IGRA for TB is a method to measure interferon-gamma induced by MTB-specific antigens (TB antigen) to detect infection. In this study, the newest version of ELISA-based IGRA, QuantiFERON-TB Gold In-Tube^{TM} (Cellestis, Victoria, Australia), was used. One milliliter of the whole blood was collected separately in each heparin-containing tube pre-coated with nil for negative control, mitogen for positive control, and TB antigen. After 18-hour incubation in 37°C, each tube was centrifuged and plasma was harvested. Concentration of interferon-gamma in the plasma was measured using the ELISA method and calculated using analytical software recommended by the manufacturer. The cut-off value of interferon-gamma concentration was 0.35 IU/ml calculated from TB antigen minus negative control. Based on the algorithm of the software, the result was considered to be indeterminate in one of the following two conditions: the nil value itself was higher than 8.0 IU/ml, or mitogen minus nil value was less than 0.5 IU/ml in addition to TB antigen minus nil was less than 0.35 IU/ml. The testing procedure was carefully monitored [16] and quality control of the test was done in each run, following the manufacturer's instruction.

Statistical analysis

To compare proportions in two groups, chi-squared test was used. Mantel-Haenszel method for stratified data was also attempted. Agreement between TST and IGRA was quantified using kappa statistic. Symmetry test equivalent to McNemar test was used to evaluate the symmetry of discordant results, TST+/IGRA- and TST-/IGRA+. To determine whether history of BCG vaccination or other factors interprets discordant results, unadjusted and adjusted odds ratios were calculated using a logistic regression model. The associations between potential risk factors and TB infection estimated by IGRA positivity were also evaluated by multivariate analysis using a logistic regression model, with IGRA result as outcome and factors possibly related to tuberculosis infection as independent variables. Biologically significant variables such as sex and other variables showing p

values <0.20 in the univariate analysis were included in the multivariate model. All statistical analyses were performed using Stata version 10 (StataCorp, College Station, TX) and $p<0.05$ was considered to be statistically significant.

Results

Characteristics of study population

As shown in Table S1, a total of 300 HCWs of the two hospitals participated in our study and the majority of these were female. The median age was 40 years old, ranging from 20 to 58. Educational levels depended on job categories, but two thirds were at pre-university level or lower. More than one third of the participants had a history of BCG vaccination, of which more than 95% had actual BCG scar (data not shown).

Participants to the study included all of the 150 HCWs working in the TB hospital and 150 of 803 HCWs from the non-TB hospital (Table S1). Two thirds of HCWs in the TB hospital were less than 40 years old and this proportion was larger than in the non-TB hospital ($p<0.0001$, table not shown).

Study flow

As shown in Figure 2, all 300 participants provided blood for IGRA, while 288 of the 300 received TST. Out of them, 112 (38.9%) HCWs whose induration size of the first TST was less than 10 mm took the second TST. IGRA results were indeterminate in 35 (11.7%) individuals, in which 33 received TST and 2 did not. Since IGRA-TST data sets were analyzable when positive or negative results were obtained from both tests, these 33 IGRA indeterminate results were subtracted from 288 TST results, making 255 valid data sets. For check-up of active pulmonary TB, 131 of 142 IGRA-positive HCWs took chest radiography. Spontaneously cured tuberculosis was not completely excluded in 11 individuals (data not shown). Although active TB could not be ruled out from the chest radiography in one individual, all of the IGRA-positive individuals did not report any signs or symptoms in the follow-up period and were regarded as having latent TB infection. TST results were not emphasized in making this decision, because we expected that false-positive TST results due to previous BCG vaccination were not clinically negligible. None of them agreed to take INH treatment.

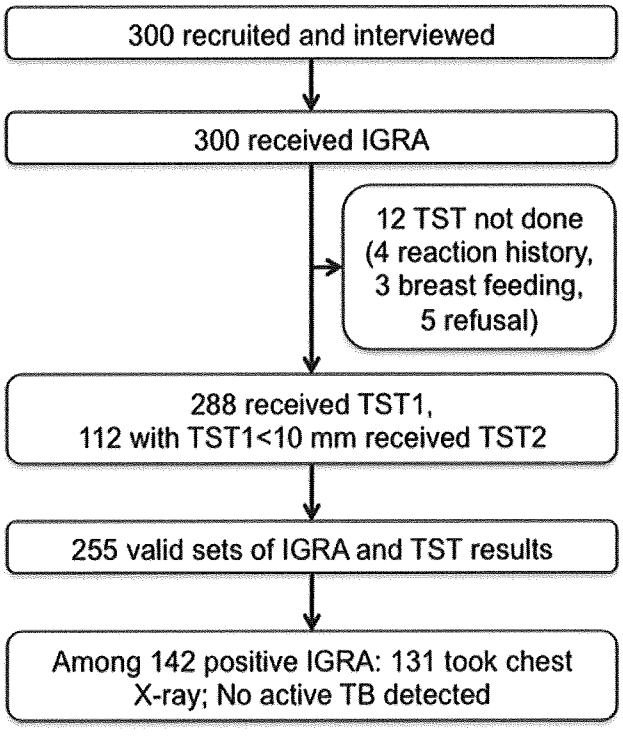

Figure 2. Study flow diagram. IGRA: Interferon-gamma release assay; TST1: The first Tuberculin skin test; TST2: The second Tuberculin skin test; TB: Tuberculosis.
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TST and IGRA positivity

TST measurements were obtained in 288 individuals. With a cut-off value of 10 mm, 176 of 288 (61.1%) were positive after the initial injection as a result of conventional "one-step" TST (Table not shown). Of 112 participants with negative TST initially, 15 turned into positive after the second injection, increasing the overall positivity up to 66.3% as a result of "two-step" TST. When 15 mm of induration size was used as the cut-off value, positive results were decreased to half (29.5%). Positive or negative IGRA results were obtained in 265 out of 300 individuals (88.3%). With the cut-off point of IGRA described in the method section, 142 were positive out of the total 300 tested (47.3%).

Since age distribution was rather different between the two hospitals, data stratified by age of each hospital was shown in Table 1. In the TB hospital, positive TST results with one-step TST and 10-mm cut-off value accounted for 54.9%, 72.7%, 86.5% and 85.7% in groups of 20–29, 30–39, 40–49 and ≥50 years old respectively. IGRA results in the same hospital revealed positive in 38.2%, 47.9%, 51.3% and 87.5% for the corresponding age groups. The proportion of TST ≥10 mm was higher in the TB hospital than in the non-TB hospital by the Mantel-Haenszel test. IGRA positivity in the TB hospital had a similar tendency as compared with that in the non-TB hospital when stratified by age, although the difference did not reach statistical significance (Table 1).

Agreement between TST and IGRA

TST using different cut-off values were compared with IGRA in 255 sets of data (Table 2). Overall kappa values showed moderate agreement (kappa = 0.4 to 0.6) between TST and IGRA, whereas high cut-off values such as 13 mm and 15 mm of TST did not further increase the degree of agreement. As compared with one-step TST with the cut-off value of 10 mm (agreement rate = 72.5%, kappa = 0.44), two-step TST did not have any favorable effect on the degree of agreement (agreement rate = 71.0%, kappa = 0.41). In the group with BCG history, the degree of agreement was rather low, in contrast to the group without BCG history (kappa = 0.29 vs. 0.55) (Table 2).

These findings prompted us to investigate the source of disagreement. With the cut-off value of 10 mm, the number of TST+/IGRA- individuals was disproportionately larger than that of TST-/IGRA+ individuals, which was statistically significant by the symmetry test ($p = 0.0008$). This disproportion was predominant in the subgroup with BCG history, but not in that without BCG history ($p = 0.0013$ vs. 0.20 respectively), when the same cut-off value was applied. Conversely, TST-/IGRA+, the other type of discordance, was strikingly increased when cut-off values of 13 or 15-mm were used ($p = 0.0008$ and $p<0.0001$ respectively) (Table 2).

Consequently, we investigated more deeply into factors associated with discordant results. In univariate analysis, BCG vaccination showed a significant association with TST+/IGRA- discordant results, with OR = 2.34 (95%CI, 1.14–4.81), when the other combinations were set as controls [10,11]. In multivariate analysis, when age, working hospital and working

Table 1. Proportion of IGRA and TST positivity, stratified by age.

		Non-TB hospital		TB hospital		*p* value
		No. positive/No. tested	**(%)**	**No. positive/No. tested**	**(%)**	
IGRA						
	20–29*	6/26	(23.1)	21/55	(38.2)	
	30–39	4/13	(30.8)	23/48	(47.9)	
	40–49	43/87	(49.4)	20/39	(51.3)	
	≥50	18/24	(75.0)	7/8	(87.5)	
	Combined**					0.13
One-step TST, ≥10 mm						
	20–29*	7/26	(26.9)	28/51	(54.9)	
	30–39	5/13	(38.5)	32/44	(72.7)	
	40–49	47/86	(54.7)	32/37	(86.5)	
	≥50	19/24	(79.2)	6/7	(85.7)	
	Combined**					<0.0001
One-step TST, ≥15 mm						
	20–29*	2/26	(7.7)	5/51	(9.8)	
	30–39	5/13	(38.5)	15/44	(34.1)	
	40–49	27/86	(31.4)	17/37	(45.9)	
	≥50	12/24	(50.0)	2/7	(28.6)	
	Combined**					0.45
Two-step TST, ≥10 mm						
	20–29*	8/26	(30.8)	28/51	(54.9)	
	30–39	5/13	(38.5)	33/44	(75.0)	
	40–49	60/86	(69.8)	32/37	(86.5)	
	≥50	19/24	(79.2)	6/7	(85.7)	
	Combined**					0.0004

TB: Tuberculosis; IGRA: Interferon-gamma release assay; TST: Tuberculin skin test.
*Years old.
**Mantel-Haenszel test for stratified data.
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Table 2. Agreement between IGRA and TST using different cut-off values of TST.

		One-step TST						Two-step TST
		≥10 (mm)*			**≥11 (mm)***	**≥13 (mm)***	**≥15 (mm)***	**≥10 (mm)***
			BCG (−)**	**BCG (+)****				
TST+/IGRA+ (n)		114	44	39	102	86	69	119
TST+/IGRA- (n)		49	14	27	30	21	13	58
TST-/IGRA+ (n)		21	8	8	33	49	66	16
TST-/IGRA- (n)		71	33	23	90	99	107	62
Agreement, %		72.5	77.8	63.9	75.3	72.5	69.0	71.0
Kappa (SE)		0.44 (0.06)	0.55 (0.10)	0.29 (0.09)	0.50 (0.06)	0.46 (0.06)	0.39 (0.06)	0.41 (0.06)
Symmetry test***	Chi-squared value	11.2	1.64	10.3	0.14	11.2	35.6	23.8
	p value	0.0008	0.20	0.0013	0.71	0.0008	<0.0001	<0.0001

IGRA: Interferon-gamma release assay; TST: Tuberculin skin test; SE: Standard error; BCG (−): Without history of BCG vaccination; BCG (+): With history of BCG vaccination;
*N = 255; all subjects with valid data sets.
**N = 99 and 97 for BCG (−) and BCG (+) groups, respectively.
***Equivalent to McNemar test for evaluation of the symmetry of TST+/IGRA- and TST-/IGRA+.
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duration were included in the model, BCG was the only parameter showing significant association with this discordance (OR = 2.26 [95%CI, 1.09–4.71]) (Table not shown). No factors analyzed in this study showed association with TST-/IGRA+ discordance.

Factors associated with IGRA positivity

We tried to identify factors associated with IGRA positivity. In univariate analysis, non-occupational factors such as age and the high body mass index (BMI) were significantly associated with having a positive IGRA result (OR = 1.05 [95%CI, 1.02–1.07] per one year and OR = 5.10 [95%CI, 1.45–17.99], respectively) (Table 3), whereas occupational factors including job category, working duration, and mask use did not show significant associations with IGRA positivity (Table 3).

In multivariate analysis, significantly increased odds of IGRA positivity were observed with non-occupational factors such as increase in age (OR = 1.06 [95%CI, 1.00–1.11]), high BMI (OR = 4.18 [95%CI, 1.14–15.36]), education lower or equal to high school level (OR = 4.28 [95%CI, 1.28–14.27]) and pre-university level (OR = 3.54 [95%CI, 1.18–10.59]). Among occupational factors tested, working in TB hospital was the only parameter showing the significant association (OR = 1.94 [95%CI, 1.04–3.64]) (Table 3).

Discussion

Our study demonstrated the high prevalence of latent TB infection estimated by either TST or IGRA positivity among hospital workers and higher risk of infection adjusted for age and other factors in the TB hospital than in a general hospital in Hanoi, Viet Nam. Disagreement between TST and IGRA positivity was largely affected by BCG vaccination history and it was not improved by changing cut-off values of TST. As far as we know, this is the first report on TB infection among HCWs evaluated by IGRA in Southeast Asia.

The overall prevalence of IGRA positivity among HCWs in our study (47.3%) is high and comparable to previous estimates from India, Russia and Georgia (40.0%, 40.8%, and 60.0% respectively) [17–19]. Direct comparison is difficult among the studies, because in the previous studies particularly the Russian one, detailed information about age strata has not been shown, which strongly affects the prevalence of TB infection.

The prevalence of TST positivity in our study population was higher than that of IGRA. High false-positive TST reaction due to BCG vaccination given after infancy has been reported [7,20], especially in individuals less than 40 years old [21]. In fact, the degree of TST/IGRA agreement was low in the group with BCG vaccination in our study, with a significant disproportional increase in TST+/IGRA- over TST-/IGRA+. Furthermore,

Table 3. Logistic regression analysis results for the associations between potential risk factors and IGRA positivity (n = 265).

		Proportion of positive results		Uni-variate		Multi-variate	
		n	(%)	Odds Ratio	(95%CI)	Odds Ratio	(95%CI)
Non-occupational factors:							
Age	/year	NA*	NA*	1.05	(1.02–1.07)	1.06	(1.00–1.11)
Sex	Female	102/197	(51.8)	1.00	(reference)	1.00	(reference)
	Male	40/68	(58.8)	1.33	(0.76–2.32)	1.10	(0.56–2.16)
BMI	18.5≤<25.0	114/223	(51.1)	1.00	(reference)	1.00	(reference)
	<18.5	12/23	(52.2)	1.04	(0.44–2.46)	1.50	(0.57–3.94)
	25.0≤	16/19	(84.2)	5.10	(1.45–17.99)	4.18	(1.14–15.36)
Education	University and higher	47/93	(50.5)	1.00	(reference)	1.00	(reference)
	High school and lower	25/36	(69.4)	2.22	(0.98–5.04)	4.28	(1.28–14.27)
	Pre-university	70/136	(51.5)	1.04	(0.61–1.76)	3.54	(1.18–10.59)
Occupational factors:							
Hospital	Non-TB	71/136	(52.2)	1.00	(reference)	1.00	(reference)
	TB	71/129	(55.0)	1.12	(0.69–1.82)	1.94	(1.04–3.64)
Job	Others	45/74	(60.8)	1.00	(reference)	1.00	(reference)
	Doctor	38/66	(57.6)	0.88	(0.45–1.72)	2.60	(0.82–8.29)
	Nurse	45/98	(45.9)	0.55	(0.30–1.01)	0.78	(0.28–2.19)
	Technician	14/27	(51.9)	0.69	(0.29–1.69)	1.02	(0.31–3.35)
Working years	<2	12/29	(41.4)	1.00	(reference)	1.00	(reference)
	2≤<5	20/47	(42.6)	1.05	(0.41–2.68)	0.94	(0.34–2.58)
	5≤<10	22/44	(50.0)	1.42	(0.55–3.65)	0.85	(0.28–2.56)
	10≤	88/145	(60.7)	2.19	(0.97–4.92)	0.91	(0.27–3.13)
Mask use	Frequently	65/124	(52.4)	1.00	(reference)	1.00	(reference)
	Occasionally	40/82	(48.8)	0.86	(0.50–1.51)	1.02	(0.55–1.88)
	Rarely/never	37/59	(62.7)	1.53	(0.81–2.88)	1.78	(0.81–3.94)

*NA = Not applicable.

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among parameters tested in our study, BCG history was the only factor to be associated with TST+/IGRA-discordance in univariate and multivariate analysis. Our finding is consistent with the previous reports [10,11], but different from that of another recent study, where BCG did not account for this discordance [22]. This may be simply due to difference in age of BCG vaccination. Involvement of other unknown factors for the discordance cannot be excluded. Exposure to nontuberculous mycobacterium might be another factor for TST+/IGRA- discordance [11], although nontuberculous mycobacterium is rarely found among smear-positive patients in Viet Nam (unpublished data).

These findings indicate that IGRA is more advantageous than TST with different cut-off values [23]. In Viet Nam, BCG vaccination has been included in the Extended Program of Immunization since 1986 and given within one month after birth. Before this point of time, there were no national guidelines and BCG vaccination was sporadically implemented in several areas and mostly given during childhood. In the heterogeneous background of BCG vaccination, it seems difficult to interpret TST result of the present Vietnamese HCWs even with a higher cut-off value as recommended elsewhere [21,24]. High agreement level between TST and IGRA in a study from India [17] is probably attributed to the fact that most of their participants were vaccinated at birth.

On the other hand, our study did not find significant associations between BCG history and TST-/IGRA+ discordant results and this finding is consistent with the previous reports [10,11,22]. Age was associated with TST-/IGRA+ in one study [10] but this was not confirmed in our study.

The CDC [3] and others [25] recommend performing a two-step TST on all newly employed HCWs to identify HCWs who have had MTB infection. Two-step TST is known to evoke remote infection, weak response by nontuberculous mycobacteria, past BCG or other factors, while IGRA appears to reflect recent rather than remote MTB infection [23]. In our study, the influence of two-step TST on TST/IGRA discordance was not much different from that of one-step TST.

In the TB hospital, the proportion of young HCWs who should lower the overall IGRA positivity was larger than in non-TB hospital. Despite this fact, the IGRA positivity in TB hospital was not low. Occupational factors as well as non-occupational factors have been expected to be associated with latent TB infection. In multivariate analysis using a logistic regression model, working in the TB hospital was significantly associated with twofold increase in odds of TB infection estimated by IGRA. Although previous studies have shown that occupational factors, such as working duration and job category, confer a risk on IGRA positivity [17–19], our results did not support their data. Working duration is closely related to age and it was difficult to assess its independent effect on TB infection in our study. On the other hand, our data imply that many staff members pursuing a variety of job in the TB hospital might have a considerable chance of exposure to infectious droplet nuclei. While non-TB hospital is a large hospital including an eleven-story building and located in a site with a large yard, the TB hospital is smaller and more enclosed, where all TB patients and HCWs share the same ambulatory route from the entrance (Figure 1). Personal protective equipment used is mostly surgical mask, which cannot prevent the transmission effectively. This finding suggests that the overall working environment and currently used administrative measures should be reconsidered. The cores of infection control programs should be understood deeply to avoid health-care associated infection of TB or MDR-TB at the worst, when a number of MDR-TB patients are hospitalized for treatment.

Non-occupational risk factors for IGRA positivity have been shown in several studies [17,19]. Age reflects cumulative exposure to MTB and it was significantly associated with IGRA positivity in our study. Education levels may indicate potential risk of TB infection in non-working environment as well as high risk of nosocomial infection. Although these two risks were not separately assessed in our study design, training may be necessary to increase awareness of prevention of nosocomial infection towards workers with low educational levels. Possible risk of high BMI for TB infection was unexpected, but the effect was highest among all covariates. In fact, high BMI was associated with both TST and IGRA positivity. TB development associated with diabetes accompanied by overweight is known [26], but the relationship between overweight and TB infection itself has not been reported. The results may have been produced by chance. Another independent investigation is necessary to determine whether it can be reproducible.

Among the HCWs with positive results of IGRA, no one took INH for treatment of latent TB infection. INH treatment is a safe and low-cost intervention and recommended by WHO [27] and others [28]. However, in typical health care facilities of TB high burden countries where the risk of TB exposure is high and continuous, HCWs still doubt significance of one-time INH treatment.

Our study has several limitations. Firstly, we were not able to evaluate the risk of infection from non-working environment as mentioned above, although the prevalence of TB infection could be estimated roughly from the annual risk of TB infection based on the TST surveys using a formula recommended elsewhere [29]. In Viet Nam, the infection rate was too different between areas to estimate it (data not shown). Secondly, we did not measure HIV infection in our study population. According to the data from a household survey in Ho Chi Minh city, estimated prevalence of HIV infection there is 0.7% in 2005 [30]. We assume that the prevalence is lower in Hanoi. Thirdly, it was not possible to identify the cause of indeterminate cases of IGRA. We re-performed ELISA for all preserved plasma samples with indeterminate results and obtained completely the same results. In addition, we have paid careful attention to maintain the high quality of this test [16]. All of the indeterminate cases showed low response to both TB antigen and mitogen and the pattern of TST measurements in IGRA indeterminate cases was similar to that of IGRA negative cases. For this reason, while calculating the IGRA positivity we did not include indeterminate cases in the numerator but did include them in the denominator, although our results might have underestimated the true proportion.

In conclusion, there is a potential high risk of TB infection among HCWs, particularly those working in TB health facilities in a TB high burden country. Prompt attention is necessary to prevent TB infection among HCWs, preparing for recent spread of MDR-TB in resource-limited settings. For this purpose, IGRA seems appropriate to estimate latent TB infection accurately, contributing to improve infection control strategy especially for young vulnerable HCWs who have heterogeneous history of BCG vaccination after birth.

Supporting Information

Table S1 Characteristics of population studied. TB: Tuberculosis. *Others mainly consist of administrative staff and pharmacists.

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Author Contributions

Conceived and designed the experiments: LTL NTLH NK HY ET SS PHT VCC NH KH LAT NK. Performed the experiments: NTLH VCC IM. Analyzed the data: NTLH AN TM NK. Contributed reagents/materials/analysis tools: LTL NK HY ET SS PHT IM LAT NK. Wrote the paper: LTL NTLH NK.

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原　著

我が国における鈍的外傷患者の生存予測ロジスティック回帰式の検討 －日本外傷データバンクの解析から－

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木村　昭夫

本研究は，1．日本の鈍的外傷患者の予測生存確率（Ps）を，最適に行うロジスティック回帰式を作成すること，2．呼吸数データ欠損していても生存予測可能な式を作成することを目的とした．2004～2007年の間，日本外傷データバンク（JTDB）に登録されたPs計算可能なデータ17,564のうち，鈍的外傷12,975登録データを無作為に2分割し，一方をTraining data（6,487）とし，他方をValidation data（6,488）とした．また，同期間にJTDBに登録されたPs計算不可能なデータ4,574で，呼吸数を必要としない式による生存予測を試みた．独立変数として，ISSと連続変数としての年齢，コード化された収縮期血圧・呼吸数・GCSスコアを用いることにより，日本の鈍的外傷患者により適した式を作成し得た．また，独立変数に呼吸数を必要としない式でも，ほぼ同等のaccuracyを持つことが示された．係数を単純化して使用しやすい式としても，accuracyは高い精度で保たれた．

索引用語：JTDB，TRISS，非穿通性的外傷，呼吸数

背景と目的

我が国において，2004年より日本外傷データバンク：Japan Trauma Data Bank（JTDB）への登録が開始され，2007年までには20,257の症例登録がなされている．これを基に米国のTRISS法[1]と同等以上の精度をもつ生存予測回帰式の作成をするべきとの声[2)-4)]が高まっている．一方でJTDB登録データのうち呼吸数データが欠損しているものは多数あり，呼吸数データを必要としない生存予測式も期待されている．

本研究では，日本の鈍的外傷患者の予測生存確率（Ps）を，最適に行うロジスティック回帰式を作成すると同時に，呼吸数データがなくても，生存予測可能にするロジスティック回帰式を作成することを目的とした．

対象と方法

2004～2007年の間，JTDBに登録された日本外傷学会トラウマレジストリー委員会にて洗浄された20,257登録データを対象とした．そのうち呼吸数が欠損しているのは，3,814（18.8%）登録であった．Ps計算可能なデータは17,564であり，そのうち鈍的外傷12,975登録データを無作為に2分割し，一方をTraining（Derivation）dataとし，他方をValidation dataとした．一方，同期間にJTDBに登録された鈍的外傷でかつPs計算不可能なデータは4,574であった．

Training dataを用いてロジスティック回帰分析を行った．独立変数には，Injury Severity Score（以下ISS），年齢（AGE）もしくはコード化された年齢（cAGE），Glasgow Coma Scale（以下GCS）スコア，収縮期血圧（以下BP），呼吸数（以下RR）をそれぞれコード化したcGCS，cBP，cRR（Table 1）並びにそれらを用いたRevised Trauma Score（以下RTS）を用い，従属変数は生死の2カテゴリーとした．推定法として，最尤推定法を用いた．最小および飽和モデルの尤度比検定にて，各々のロジスティック回帰式の当てはまり度合を評価した．

検証にはValidation dataを用い，receiver operating characteristic（以下ROC）曲線の曲線下面積（以

下AUC）と予測生存のaccuracyについて，Training dataのそれぞれの値と比較した．

統計処理コンピュータソフトウエアーには，JMP8.0（SAS社）を用いた．

結　果

Training dataは6,487，Validation dataは6,488となり，Table 2に各データ群の独立因子候補の分布を示した．

Training Dataより算出された各回帰モデル式の係数をTable 3に示した．各モデルとも最少モデルとの検定では$p<0.0000$，飽和モデルとの検定では$p=0.6304\sim1.000$となり，当てはまりは非常によいと判断された．元来のTRISS法でも，AUCは0.9627と高値あったが，cAGEのかわりに年齢を連続変数としてそのまま用いたもの，さらにはRTSを算出せずにcBP，cGCS，cRRをそのまま独立変数として用いたもののほうが，AUCは0.9674とより大きかった．また，cRRを省いてもAUCは0.9670とほとんど変わらなかった（Figure 1）．

Training dataとValidation dataから算出されたAUCとaccuracyの比較をTable 4に示す．両者間にそれぞれの値において，大きな変化はなく，再現性を検証することができた．

最も当てはまりのよかった回帰式とその独立変

Table 1　Coded values

Coded value	GCS score	Systolic blood pressure	Respiratory rate	Age
4	13-15	>89mmHg	10-29/min	
3	9-12	76-89mmHg	>29/min	
2	6-8	50-75mmHg	6-9/min	
1	4-5	1-49mmHg	1-5/min	>55
0	<4	No pulse	0	0-55

GCS：Glasgow Coma Scale

Table 2　Comparison of factors between Training Data & Validation Data

	Training Data Set	Validation Data Set
Number	6487	6488
AGE	47.1±23.1	46.6±23.3
ISS	17.6±14.2	17.4±14.0
RTS	7.8[6.9, 7.8]	7.8[6.9, 7.8]
cBP	4[4, 4]	4[4, 4]
cGCS	4[3, 4]	4[3, 4]
cRR	4[4, 4]	4[4, 4]

Mean±SD, Median［25%, 75%］range
ISS：Injury Severity Score, RTS：Revised Trauma Score
cBP：coded value（0～4）of systolic blood pressure
cGCS：coded value（0～4）of Glasgow Coma Scale score
cRR：coded value（0～4）of respiratory rate

Table 3　Coefficients of Logistic Regression Models

Regression Model	Intercept	βISS	βRTS	βAGE	βcAGE	βcBP	βcGCS	βcRR
TRISS	−0.4499	−0.0835	0.8085	×	−1.743	×	×	×
ISS, RTS, cAGE	−1.7163* (0.2791) [2.98]	−0.0675* (0.005) [181]	0.9301* (0.0368) [639]	×	−1.439* (0.137) [111]	×	×	×
ISS, RTS, AGE	−0.5373 (0.3114) [2.98]	−0.0703* (0.0051) [189]	0.9526* (0.0379) [634]	−0.0381* (0.0032) [141]	×	×	×	×
ISS, AGE, cBP, cGCS, cRR	−0.6982 (0.3681) [3.60]	−0.0707* (0.0051) [190]	×	−0.0385* (0.0032) [142]	×	0.662* (0.077) [73.0]	0.867* (0.092) [336]	0.386* (0.041) [17.7]
ISS, AGE, cBP, cGCS	−0.1997 (0.3107) [0.41]	−0.0698* (0.0051) [188]	×	−0.0369* (0.0032) [135]	×	0.832* (0.063) [172]	0.927* (0.046) [415]	×

βx：regression coefficients
*：$p<0.0001$
(=standard error)，［$=\chi 2$］
ISS：Injury Severity Score, RTS：Revised Trauma Score
cAGE：0－55 years = 0，>55years = 1
cBP：coded value（0～4）of systolic blood pressure
cGCS：coded value（0～4）of Glasgow Coma Scale score
cRR：coded value（0～4）of respiratory rate

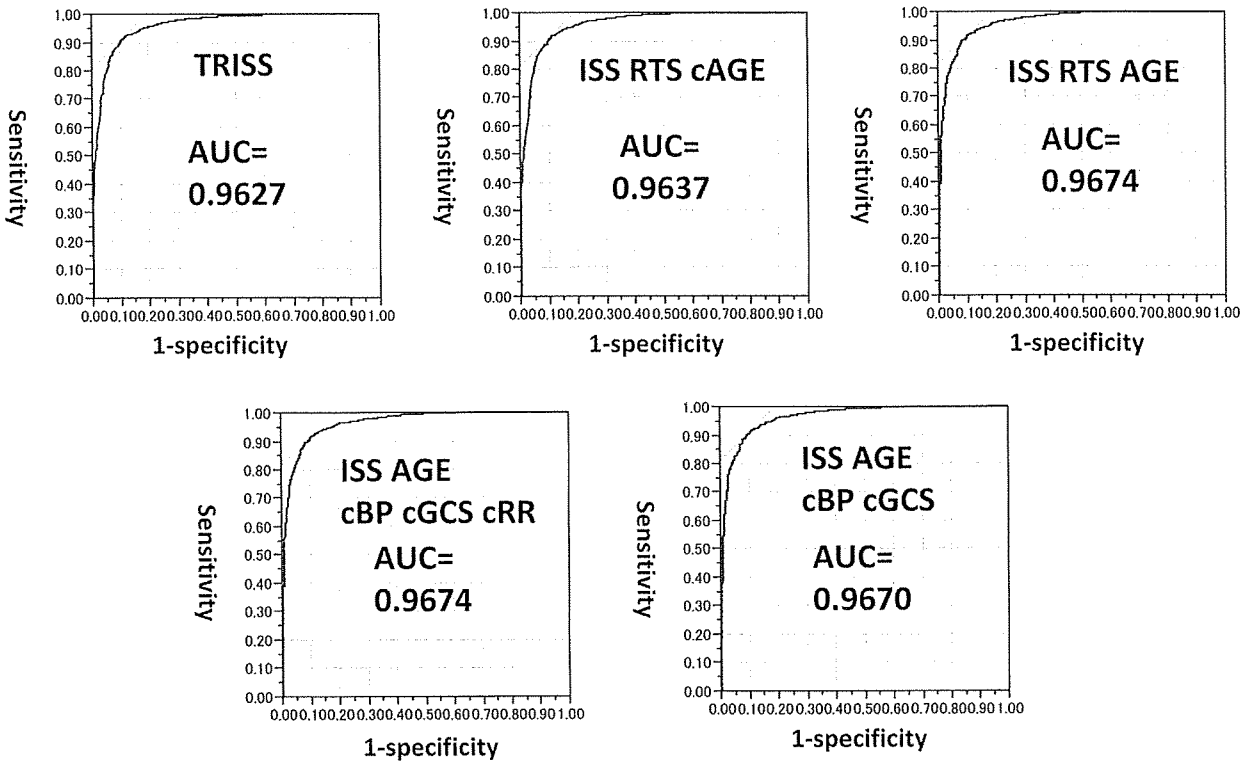

Figure 1　Receiver operating curves (ROC) of each regression model derived from the training data sets.
ISS：Injury Severity Score, RTS：Revised Trauma Score, cAGE：0-55 years =0, >55 years =1,
cBP：coded value (0～4) of systolic blood pressure, cGCS：coded value (0～4) of Glasgow Coma Scale score,
cRR：coded value (0～4) of respiratory rate, AUC：area under the curve of ROC

Table 4　Area under Receiver Operating Curves (AUC) & Accuracy of Models

Regression Model	AUC Derivation	Accuracy Derivation	AUC Validation	Accuracy Validation
TRISS	0.9627	93.17%	0.9621	93.33%
ISS, RTS, cAGE	0.9637	93.14%	0.9643	93.49%
ISS, RTS, AGE	0.9674	93.10%	0.9667	93.61%
ISS, AGE, cBP, cGCS, cRR	0.9674	93.16%	0.9670	93.69%
ISS, AGE, cBP, cGCS	0.9670	93.16%	0.9654	93.48%

AUC：area under curve
ISS：Injury Severity Score, RTS：Revised Trauma Score
cAGE：0-55 years＝0，>55 years＝1
cBP：coded value（0～4）of systolic blood pressure
cGCS：coded value（0～4）of Glasgow Coma Scale score
cRR：coded value（0～4）of respiratory rate

数からcRRを省いた回帰式の係数を，小数点2以下で四捨五入して単純化したところ，cRR以外では，切片とcBPの係数のみ異なり，残りの係数は同じにすることができた（Table 5）．それぞれの回帰モデル式のAUCとaccuracyは，Training（Derivation）dataとValidation dataとの間に大きな違いは認められなかった（Table 5）．

TRISSではPsを算出できない鈍的外傷4,574登録データに対して，呼吸数を含まない回帰モデル式を適用したところ，1,744登録についてPsが算出可能であり，AUCは0.9023，accuracyは77.12%であった（Figure 2）．

考　察

小関ら[3)4)]は，1994年から1998年までに13施設か

Table 5　Proposed Regressions Models with and without Respiratory Rate

	Intercept	βISS	βAGE	βcBP	βcGCS	βcRR
AGE, ISS, cBP, cGCS, cRR	−0.7	−0.07	−0.04	0.7	0.9	0.4
AGE, ISS, cBP, cGCS	−0.2	−0.07	−0.04	0.8	0.9	×

	AUC Derivation	Accuracy Derivation	AUC Validation	Accuracy Validation
ISS, AGE, cBP, cGCS, cRR	0.9674	93.21%	0.9669	93.62%
ISS, AGE, cBP, cGCS	0.9670	93.39%	0.9654	93.60%

ISS：Injury Severity Score, RTS：Revised Trauma Score
βx：Regression coefficients
cAGE：0-55 years＝0，>55 years＝1
cBP：coded value（0～4）of systolic blood pressure
cGCS：coded value（0～4）of Glasgow Coma Scale score
cRR：coded value（0～4）of respiratory rate
AUC：area under receiver operating curve

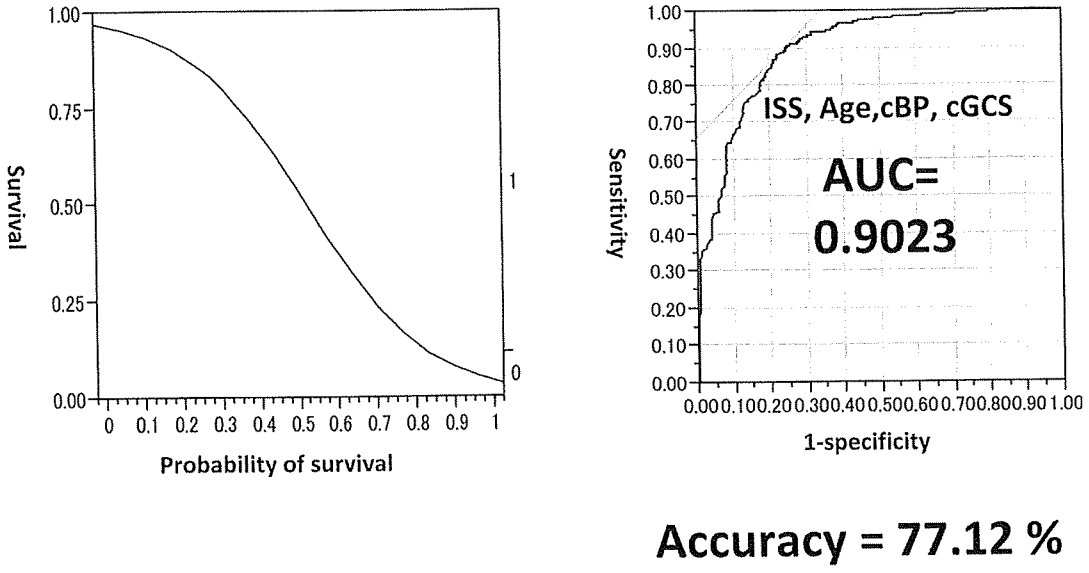

Figure 2　Application of the regression model proposed for data without respiratory rate.
ISS：Injury Severity Score, cBP：coded value（0～4）of systolic blood pressure, cGCS：coded value（0～4）of Glasgow Coma Scale score, cRR：coded value（0～4）of respiratory rate, AUC：area under the curve of receiver operating curve

ら登録された4,747例の鈍的外傷のデータについて，cross validationによるロジスティック回帰分析を行い，TRISS法を構成する変数を決定した．その結果，本研究と変数は若干異なるものの，AUCは0.971，accuracyは93.3%とほぼ同様の結果であり，再現性が確認された．

また同解析で，TRISS原法を用いてもAUCは0.968，accuracyは93.0%とほとんど同等の値であった．本研究結果においても，我が国の鈍的外傷例に米国のTRISS原法を用いた場合，ROC曲線のAUCが0.96，accuracyは93%程度あることが示された．このことは，日本の鈍的外傷の治療成績が，米国とほとんど変わらないことを物語っていると考えられる．我が国の鈍的外傷診療体制は，一般外科中心の米国とは異なっている．しかしながら，救急医の積極的関与や放射線科による塞栓術，脳神経外科の高い機動力などに支えられており，一般外科以外の多くの診療科を必要とする鈍的外傷の診療レベルは，現時点では米国とほぼ同等にまで達しているのではないかと思われる．

多変量ロジスティック回帰式の独立変数として何を用いるかは興味深い問題であるが，精度を高

めるためにその数をTRISS法より大幅に多くすることは，Psを計算できないデータを増加させる危険性がある．また，ただでさえ多いJTDBの入力項目を，さらに増やす必要性を伴う独立変数を設けることも現実的ではない．よって筆者は，TRISS法の独立変数の構成要素を最小限変化させることのみで，よりよい生存予測回帰式を作成することとした．

小関ら[3]は，日本版TRISSを作成する場合には，年齢は実変数として用いること推奨していたが，JTDB　2004～2007年の報告[5]からみても，年齢と死亡割合の関係において，米国のように55歳から急に上昇することはなく，年齢の上昇とともに比較的緩やかに死亡割合が上昇しているため，年齢をカテゴリー化しないほうが，より精度の高い生存予測式となることが，若干ではあるが本研究においても示された．

小関ら[3][4]が日本版TRISSを作成する場合に提唱していた，AIS≧5頭部外傷のカテゴリー変数の追加は，情報がISSと重複することもあり，本研究では，あえて行わなかった．というのも，この問題について，生存予測回帰式の精度を上げるためには，独立変数の追加で解決するより，AISスコアが適正に改定されることで解決されるべきと考えられるからであり，その方向性も実現に向かっていると思われる[6]．

呼吸数については，かねてから欠損値が多くPsが計算できないことが，問題となっていた．RTSの代わりにGCSスコアのみを用いて精度のよい生存予測式が導けることは，Bouamraら[7]が示していたが，その代わりに性別や年齢要素を考慮する必要があった．本研究では，RTSに用いるコード化された因子のうち，収縮期血圧とGCSスコアのみを用いることでも，ほとんど精度が下がらない生存予測式を導くことができた．さらに係数を単純化することにより，cRRを独立因子として含む回帰式から含まない回帰式への変更を最小限にすることができた．この生存予測式により，Psが計算できなかったデータの38.1％で，Psを計算することができるようになった．精度の高い生存予測が呼吸数抜きでも可能なことを，我が国の外傷診療に携わる多くの臨床家は，経験的に知っており，それゆえ診療録に記載することをおろそかにしてきた可能性がある．

先に示したBouamraら[7]の回帰式では，性別を独立変数として加えていた．しかし，解析の準備段階で性別を検討したものの，独立変数としてのp値は有意水準に達しなかったため，本研究ではあえて変数として加えることはしなかった．ただ，より多くのデータを解析する場合や，他の独立変数の選択の仕方によっては，予後を左右する因子となる可能性は十分にある．

きちんと証明した文献はほとんどないものの，これまで様々な場面で，日本の外傷患者の救命割合が米国のそれより低いと，強調されてきた感がある．前述してきた鈍的外傷の治療成績では両国間に大きな違いがないことからすると，我が国の穿通性外傷の治療成績が悪いことが，救命割合の低さの主たる原因と推測される．筆者は米国での留学経験から，日本の平均的な穿通性外傷診療は，TRISSが作られた20年前の米国の診療システムや手術治療レベルに，残念ながらいまだ達していないと判断している．日本の現状を肯定するような穿通性外傷の生存予測式を作成してpreventable trauma deathの評価に用いるよりは，先進している米国の生存予測式をそのまま用いて評価したほうが，日本の外傷診療の質の向上につながる可能性が高い．よって本研究では，非穿通性・鈍的外傷についての生存予測式のみを提唱することとした．

結　語

独立変数としてのISSと連続変数としての年齢，コード化された収縮期血圧・GCSスコア・呼吸数を用いることにより，日本の鈍的外傷患者により適した生存予測ロジスティック回帰式を作成した．また，独立変数から呼吸数を省いた回帰式でもほぼ同等の正診精度を持ち，係数を単純化して使用しやすい回帰式にしても精度は保たれることが示された．

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文　献

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