Table 1. The development of Novel vaccines for *M. tuberculosis* using animal model

Vaccine	Mouse	Guinea pig	Monkey	SCID-PBL/hu	Human
	Prophylactic Effect 10,000 fold than BCG	effective	effective		plan (phase I, II)
HVJ-Envelope/HSP65 DNA+IL-12 DNA	Therapeutic Effect	plan	effective	effective	
	Therapeutic Effect against MDR-TB XDR-TB	plan	plan		
HVJ-liposome/HSP65 DNA+IL-12 DNA	prophylactic Effect 100 fold effective than BCG	effective	effective (100% survival)		
recombinant 15 K granulysin	Therapeutic Effect		plan		
15 K granulysin DNA	Therapeutic Effect		plan		

HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine was evaluated by using mouse, guinea pig, monkey and SCID-PBL/hu model. Therapeutic efficacy as well as prophylactic efficacy was shown in this vaccine. HVJ-liposome/HSP65 DNA+IL-12 DNA vaccine and granulysin vaccine were also evaluated by using these models.

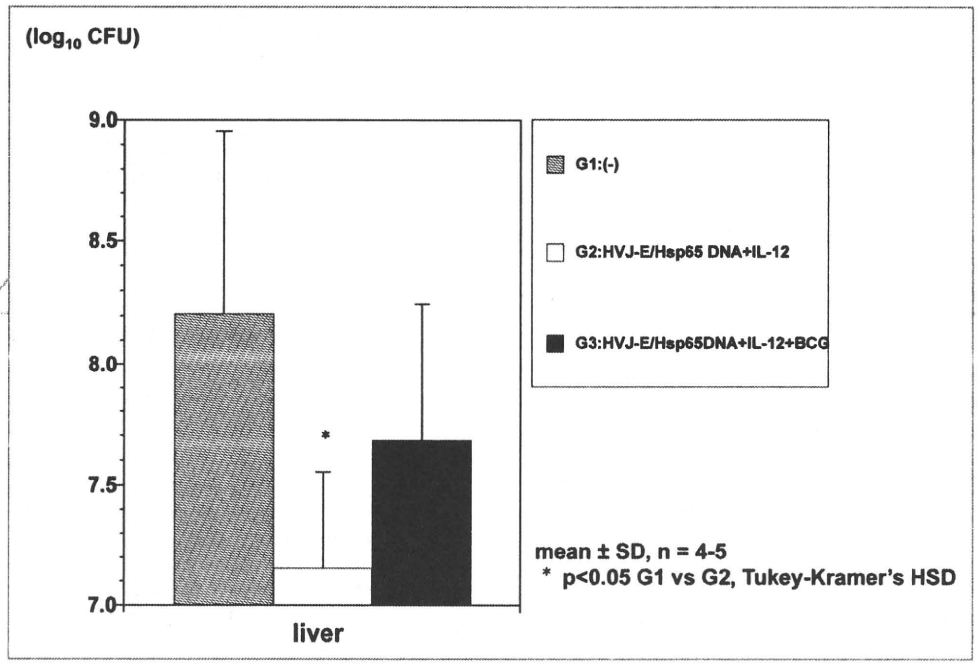

Figure 4. ~~(A) Therapeutic efficacy of TB vaccines using chronic TB infection model by aerosol challenge of TB. Therapeutic efficacy was evaluated by chronic TB infection model of mice using aerosol challenge of TB. Mice were challenged intratracheally by aerosol of *M. tuberculosis* H37Rv in saline (15 CFU/mouse) using Madison aerosol exposure chamber. Five weeks after the challenge of TB, mice were treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA six times, every 3 days i.m. Eight weeks after the challenge of TB, therapeutic efficacy was measured by enumerating bacterial loads (CFU) in the lungs, liver and spleen from vaccinated mice. (B) Therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine on chronic TB infection model of mice using aerosol chamber. DBA/1 mice were challenged intratracheally by aerosol of *M. tuberculosis* H37Rv (15 CFU/mouse). After the treatment of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine 6 times i.m., therapeutic efficacy was measured by enumerating bacterial loads (CFU) in the lungs from vaccinated mice.~~

significantly therapeutic efficacy even in SCID-PBL/hu mice which exerted human T cell immune responses (Table 1).

Therapeutic efficacy of granulysin vaccine on TB infected mice. Two major protein products, 15 kDa (15 K) granulysin and 9 kDa (9 K) granulysin, are detected in CTL and NK cells. Granulysin exhibits potent cytotoxic activity against a broad panel of microbial targets, including bacteria, fungi and parasites. Granulysin is present in human $CD8^+$ (and some $CD4^+$) CTLs, NK cells, NK T Cells and γ/δ T cells. We found that 15 K granulysin was secreted from CD8 positive CTL, could enter into human macrophages and killed *M. tuberculosis* in the cytoplasm of macrophages (Fig. 5). Recombinant 15 K granulysin protein enhanced the in vitro induction of human cytotoxic T cells in the 5 day MLC culture (Fig. 6). Synergistic effect of recombinant 15 K granulysin in the presence of IL-6-related DNA vaccine (IL-6 DNA+IL-6 receptor DNA+gp130 DNA vaccine) was shown by in vivo induction of CTL specific for HSP65 TB antigen in the mice stimulated with killed TB antigens (Fig. 7). Granulysin vaccines (recombinant 15 K granulysin and 15 K granulysin DNA vaccine) exerted strong

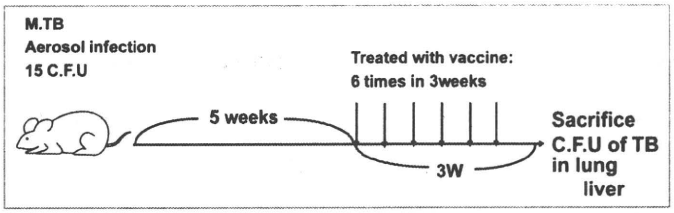

Figure 5. The hypothesis models of anti-tuberculosis immunity by granulysin produced from human cytotoxic T cell.

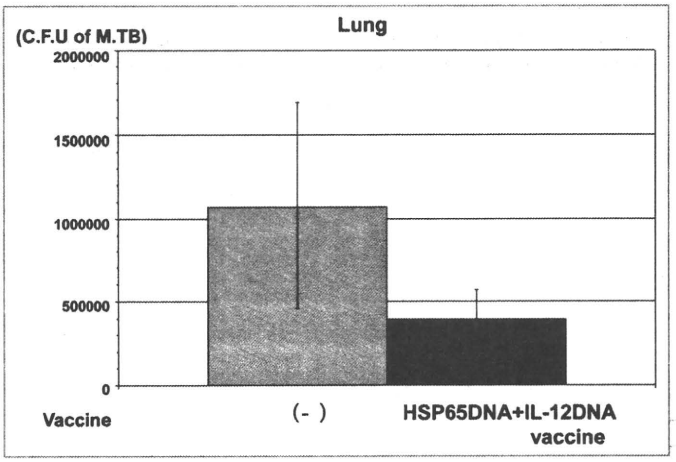

Figure 6. In vitro induction of human cytotoxic T cell by the stimulation with recombinant 15 K granulysin protein. T cells from human PBL were obtained by nylon wool column method. 1×10^6 T cells were cultured with human $CESS_{MMC}$ cells (Mitomycic C treated CESS tumor cells) in the presence of 15 K granulysin for 5 days. CTL activity of effector cells was assayed using ^{51}Cr labbelled CESS cells. Results are expressed as % Specific cytotoxicity ± SD. % Specific cytotoxicity was calculated as;

$$\frac{\text{Experimental } ^{51}\text{Cr release-Spontaneous } ^{51}\text{Cr release}}{\text{Maximum } ^{51}\text{Cr release-Spontaneous } ^{51}\text{Cr release}} \times 100;$$

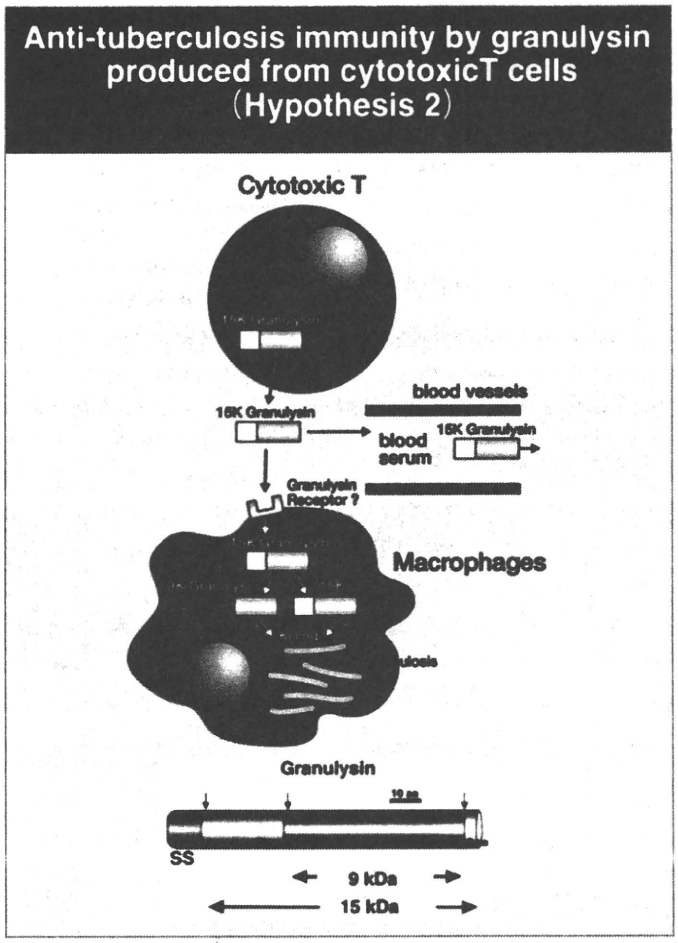

Figure 7. Synergistic effect of recombinant 15 K granulysin + IL-6 related DNA on the in vivo induction of CTL specific for HSP65 antigen. C57BL/6 mice were injected with killed TB H37Ra and then treated with recombinant 15 K granulysin protein i.p 6 times and/or IL-6 DNA + IL-6 Receptor DNA + gp130 DNA using adenovirus vector i.m. 3 weeks after killed TB challenge, CTL activity against HSP65 antigens of TB in the spleen cells was assessed by ^{51}Cr release assay. HSP65 DNA (derived from TB) was transfected into EL-4 tumor cells syngenic to C57BL/6 mice, and used for target cells. Results are expressed as % specific cytotoxicity ± SD.

therapeutic efficacy (decrease in the number of TB in the lungs, liver and spleen) in the mice infected with TB by aerosol challenge (**Table 1**).

Monkey models. Furthermore, the therapeutic activity of this vaccine was evaluated in a nonhuman primate model infected with *M. tuberculosis*. We studied therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine on TB-infected monkeys using GMP-level-vaccine which contains two kinds of DNA in one plasmid vector for clinical therapeutic trial (Fig. 8A).

Therapeutic efficacy was evaluated by survival, ESR, body weight, immune responses, chest X-ray findings and PPD skin test (Fig. 8B).

Immune responses of cynomolgus monkey were augmented at 11 weeks after the challenge of *M. tuberculosis* Erdman strain by intratracheal instillation. The proliferation of PBL was also augmented by therapeutic vaccination of monkeys with HVJ-Envelope/HSP65 DNA+IL-12 DNA (data not shown). This vaccine also improved the survival of monkeys, compared to the saline (control) group, after TB challenge (Fig. 9). All 5 monkeys were alive in the group of HVJ-Envelope/HSP65DNA+IL-12DNA vaccine (100% survival) at 16 weeks after challenge. In contrast, only 3 monkeys out of 5 were alive in the saline control group (60% survival) (Fig. 9 and **Table 2**). The number of monkeys which showed an increase in body weight was larger in the group treated with this DNA vaccine than in control group (**Table 2**). This vaccine improved ESR (Erythrocyte Sedimentation Rate) of TB-infected monkeys as shown in Figure 10. The proliferation of PBL by the stimulation with HSP65 antigens, H37Ra-killed TB antigens and PPD antigens was examined, and it was more augmented by the treatment with this DNA vaccine than the treatment with saline (data not shown). Furthermore, IL-2 production from PBL by the stimulation with killed TB H37Ra antigens was also examined and it was more augmented by the treatment with this vaccine than that with saline (Fig. 11). The induction of IL-2

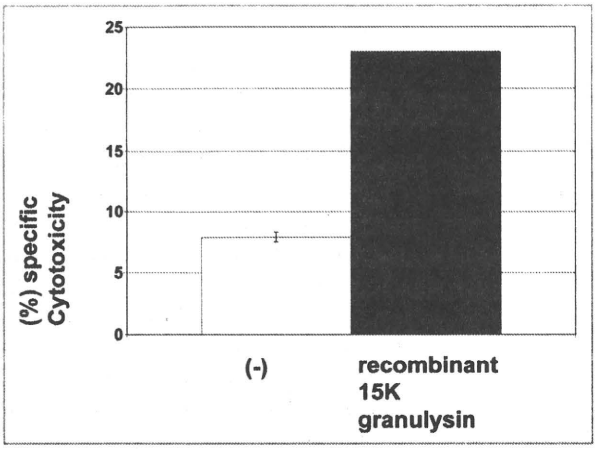

Figure 8. (A) Construction of DNA vaccine for clinical trial. HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine was constructed for GMP-level vaccine which contains two kinds of DNA in one plasmid vector (pVAX1) for clinical therapeutic trial. (B) Evaluation of therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine on TB-infected cynomolgus monkeys. Cynomolgus monkeys were vaccinated nine times with HVJ-Envelope with expression plasmid of both HSP65 and human IL-12 (HSP65+hIL-12/HVJ: 400 ug i.m.), one week after the challenge with the *M. tuberculosis* Erdman strain (5×10^2) by intratracheal instillation. Therapeutic efficacy was evaluated by survival, chest X-P findings, immune responses, body weight and erythrocyte sedimentation rate (ESR) for one year.

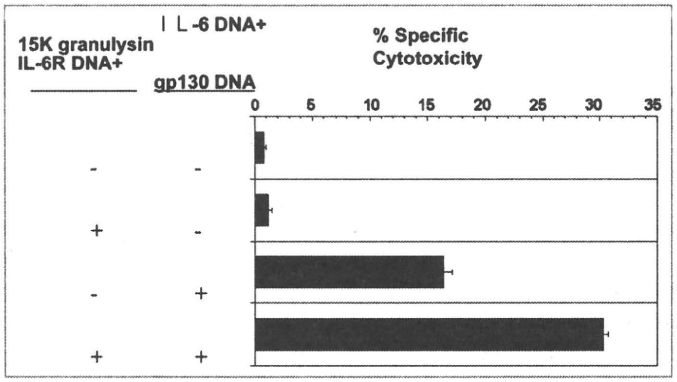

Figure 9. Survival of monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine after the infection of TB. Therapeutic efficacy was evaluated by survival of monkeys. Survival of monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12DNA at 19 weeks after the challenge of TB by intratracheal instillation was shown.

from PBL by the stimulated with PPD was significantly lower in control monkeys died of TB within 19 weeks after TB challenge than that in survived monkeys in the same group, (data not shown). IL-2 production by the stimulation with HSP65 protein was also extremely low in the control monkeys died of TB (data not shown). Thus, this GMP-level of DNA vaccine which contains two kinds of genes in one plasmid vector exerted therapeutic efficacy in TB-infected monkeys. These results demonstrate that HVJ-Envelope/HSP65DNA+IL-12DNA vaccine could provide strong therapeutic efficacy against TB in the cynomolgus monkey models as well as murine models.

Discussion

In the present study, the HSP65+hIL-12/HVJ vaccine exerted a significant therapeutic effect against TB, as indicated by: (1) extension of survival of mice infected with XDR-TB, (2) decrease in the CFU of TB in lungs, liver and spleen of mice infected with MDR-TB as well as drug-sensitive TB(H37RV), (3) decrease in the CFU of TB in these organs of mice challenged with TB in the in vivo humanized immune model of SCID-PBL/hu and (4) prolongation of survival and augmentation of immune responses, in a cynomolgus monkey model which closely mimics human TB disease. It is important to evaluate the survival of monkey.[2,5,13] Increases in the survival rate of the monkeys treated with this vaccine were observed, compared to the control monkeys treated with saline. In the recent study, it is demonstrated that granulysin vaccine shows therapeutic efficacy against TB in mice (**Table 1**). Therefore, the combination of these therapeutic vaccines might be useful in the future.

MDR-TB and XDR-TB are becoming big problems around the world. About 500,000 new patients with MDR-TB are shown every year. However, the effective drugs against MDR-TB are few.

The HVJ-Envelope/HSP65DNA+IL-12DNA vaccine exerted the therapeutic activity even against XDR-TB, which is resistant to RFP, INH, SM, EB, KM, EVM, TH, PAS, LVFX, PZA and only sensitive to CS. Thus, our results with the HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine in the murine therapeutic model and cynomolgus monkey therapeutic model should provide a significant rationale for moving this vaccine into clinical trial. Furthermore, we have established chronic TB disease model using a mouse infected with TB in the aerosol chamber. Therapeutic efficacy of this vaccine was also observed in this model.

DNA vaccine is a relatively new approach to immunization for infectious diseases.[1,2,5,16-19]

Prophylactic and therapeutic DNA vaccines were established by using several kinds of vectors such as (1) HVJ-liposome, (2) HVJ-envelope, (3) adenovirus vector, (4) adeno-associated virus vector (AAV) and (5) lenti-virus vector.[1,2]

We have developed a hemagglutinating virus of Japan envelope (HVJ-Envelope) using inactivated Sendai virus, as a non-viral vector for drug delivery.[7-9] It can efficiently deliver DNAs, siRNAs, proteins and anti-cancer drugs into cells both in vitro and in vivo.[9-11] Therefore, HVJ-Envelope was used as an efficient and safe vector for DNA vaccine against TB in the present study.

It will be a high priority for the clinical development programs to evaluate the current vaccines for post-exposure vaccine which prevents reactivation of TB in the large proportion of the global population latently infected with TB.

It is very important to evaluate the long survival period in a monkey model, as human TB is a chronic infection disease. Furthermore, the decrease in the body weight of TB patients is usually accompanied by a progression of the disease.[20]

Most importantly, this is the leading report of novel therapeutic vaccine using monkey models as well as murine models.

According to our knowledge, only a few therapeutic vaccine against TB has been reported in reference 21 and 22.

Table 2. Body weight and survival of cynomolgus monkeys treated with HSP65 DNA+IL-12 DNA vaccine

	Increase in body weight at 16 weeks		Survival
G_1 (DNA 9 times)	+	2/5 (40%)	5/5
	+		
	-		
	-		
	0		
	-		
G_2 (control saline)	+	1/5 (20%)	3/5
	0		
	-		
	-		

Increase in body weight and survival of monkeys treated this DNA vaccine at 16 weeks after TB challenge.

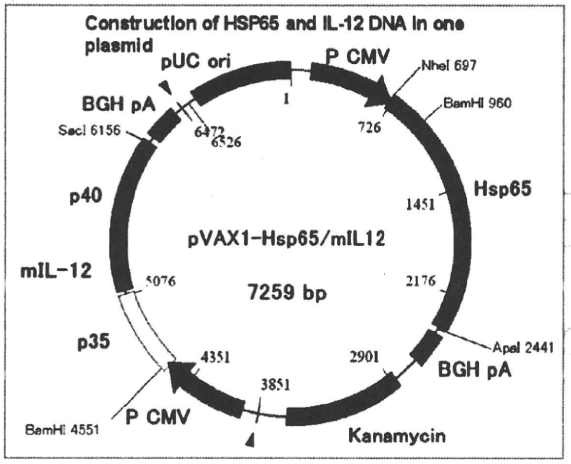

Figure 10. ~~Improvement of ESR by the treatment of HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine. Therapeutic efficacy was evaluated by ESR of the monkeys at 11 weeks after *M. tuberculosis* infection.~~

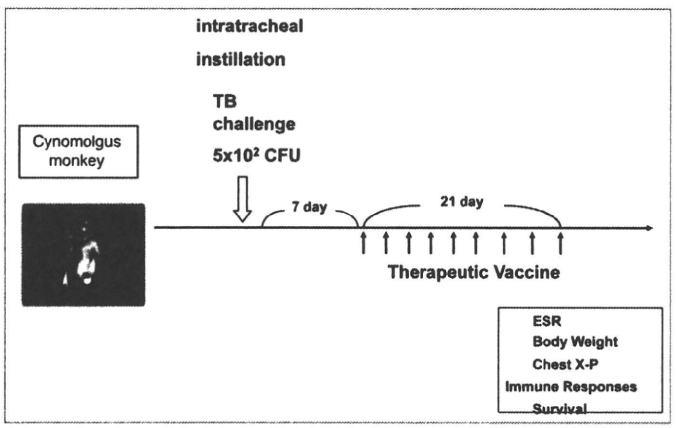

Figure 11. Augmentation ~~of IL-2 production from PBL in the monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine. Peripheral blood lymphocytes (PBL) were cultured with killed TB, H37Ra for 3 days. Supernatants were harvested after 3 day culture. IL-2 activity in the culture supernatants was assessed by ELISA. IL-2 activity (U/ml) was shown.~~

Hsp65DNA+IL-12 DNA vaccine exerted strong therapeutic efficacy (100% survival at 16 weeks after challenge and augmentation of immune responses) in the TB-infected monkeys. In contrast, the survival rate of the saline control group was 60%. HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine increased the body weight of TB-infected monkeys, improved the erythrocyte sedimentation rate and augmentated the immune responses (proliferation of PBL, IL-2 production). The enhancement of IL-2 production from monkeys treated with this vaccine was correlated with the therapeutic efficacy of the vaccine.

Thus, we are taking advantage of the availability of multiple animal models to accumulate essential data on the HVJ-envelope DNA vaccine in anticipation of a phase I clinical trial.

In conclusion, these data indicate that HVJ-Envelope/HSP65DNA+IL-12DNA vaccine and granulysin vaccine might be useful against TB including XDR-TB and MDR-TB for human therapeutic clinical trials.

Acknowledgements

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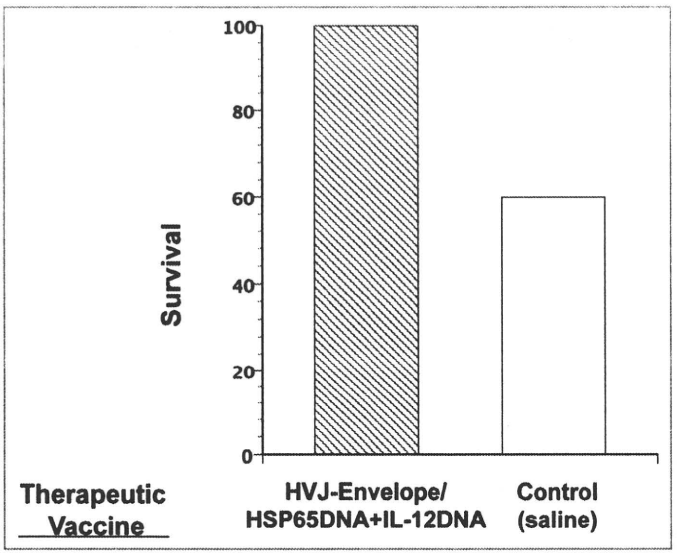

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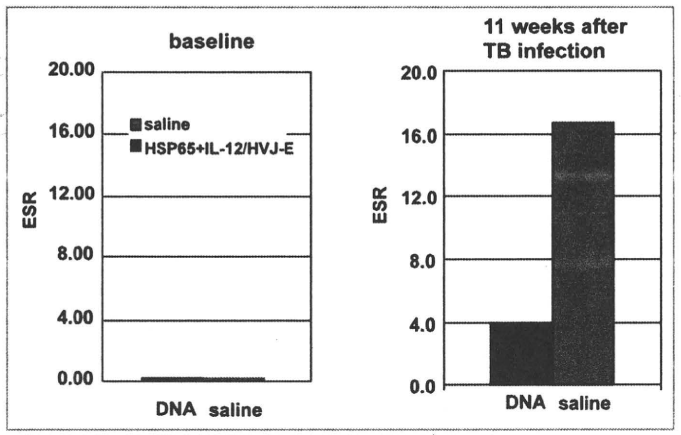

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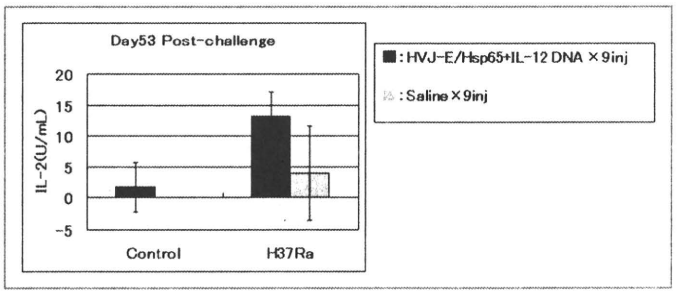

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Development of therapeutic and prophylactic vaccine against tuberculosis using monkey and transgenic mice models

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Key words: monkey, prime-boost method, HVJ-envelope/HSP65DNA + IL-12DNA, *Mycobacterium tuberculosis*, vaccine, 15 KDa granulyin, granulysin transgenic mouse, patients with tuberculosis, prophylactic efficacy, SCID-PBL/hu

Abbreviations: HVJ, hemagglutinating virus of Japan; Tg, transgenic; MDR-TB, multi-drug resistant tuberculosis; 15 K granulysin, 15 kilodalton granulysin; 9 K granulysin, 9 kilodalton granulysin; PBL, peripheral blood lymphocyte; ESR, erythrocyte sedimentation rate

Purpose: BCG is not efficacious against *M. tuberculosis* (TB) in adult. Therefore, novel TB vaccines were established by using three kinds of animal models (cynomolgus monkey model which is the best animal model of human TB, IL-2R knock out SCID mice as a human immune model and granulysin transgenic mouse).

Methods and Results: DNA vaccine expressing TB Hsp65 and IL-12 was delivered by the hemagglutinating virus of Japan (HVJ)-envelope. The BCG prime followed by Hsp65 + IL-12/HVJ vaccine boost showed a synergistic effect in the TB-infected cynomolgus monkey (100% survival). In contrast, 33% of monkeys were alive in BCG alone group. Furthermore, the prolongation of survival period of the monkey was observed by the combination of BCG and DNA vaccine even when the boost was performed after long-term period (4 month) from prime. This combination also improved the erythrocyte sedimentation rate (ESR), increased the body weight and augmented the proliferation of PBL and IL-12 production at higher levels than BCG alone or saline. Furthermore, this vaccine exerted therapeutic efficacy in IL-2R knock out SCID-PBL/hu mice, which were transplanted with human T cells. Granulysin is an important defensive molecule expressed by human T cells and NK cells and has a cytolytic activity against microbes including *Mycobacterium tuberculosis* (TB) and tumors. Expression of 15 kD (15 K) granulysin protein and mRNA in CD8 positive T cells in the patients infected with drug sensitive (TB) or multi-drug resistant M. tuberc*ulosis (MDR-TB)* were lower than that in the healthy volunteers, suggesting that granulysin treatment might improve the tuberculous disease in human. Therefore, we established two kinds of granulysin transgenic mice (15 K granulysin transgenic mice and 9 K granulysin transgenic mice). It was demonstrated that 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB effect, including the decrease of the number of TB and augmentation of the CTL activity. These are the first findings which demonstrate in vivo effects of 15 K granulysin and 9 K granulysin against TB infection. Moreover, DNA vaccine expressing 15 K granulysin showed a therapeutic activity against TB in mice.

Conclusion: These data indicate that monkey, IL-2R gene-knock out SCID-PBL/hu and granulysin transgenic mice models provide useful tools for the development of novel vaccines (HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine and granulysin vaccine) against TB.

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Introduction

Tuberculosis is a major global threat to human health, with about 2 million people dying every year from *Mycobacterium tuberculosis* (TB) infection. The only tuberculosis vaccine currently available is an attenuated strain of *Mycobacterium bovis* BCG (BCG), although its efficacy against adult TB disease remains controversial. Furthermore, multi-drug resistant tuberculosis (MDR-TB) and extremely drug resistant TB (XDR-TB) are becoming big problems in the world. In such circumstances, the development of therapeutic as well as prophylactic vaccines against TB is required.

Cynomolgus monkey model is the best animal TB model as reported by Walsh and Tan.[1] TB infection in the cynomolgus monkey is very similar to human TB disease.[1-3] In the present study, the long term prime-boost period prophylactic efficacy of

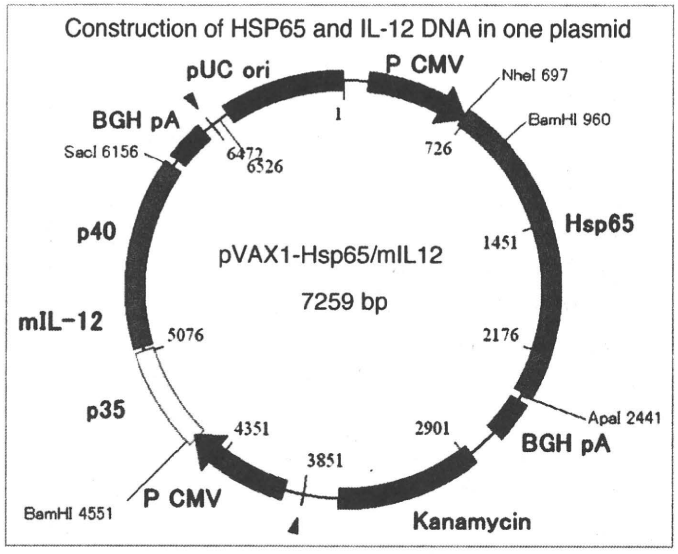

Figure 1. Construction of DNA vaccine for clinical trial. HVJ-Envelope/HSP65DNA + IL-12DNA vaccine was constructed for GMP-level-vaccine which contains two kinds of DNA in one plasmid vector (pVAX1) for clinical prophylactic trial.

vaccine was investigated using monkey models. In vivo humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice constructed with human PBL (SCID-PBL/hu) provide a useful tool for investigating human immune responses activated by vaccine.[4,5] Transgenic mice which contain the components of vaccine also provide a lot of information about novel TB vaccines. Therefore, using cynomolgus monkey model, SCID-PBL/hu mice model and transgenic mice model, we have developed several kinds of novel vaccines against TB.

Granulysin, a member of the saposin-like protein family, colocalizes with perforin and granzymes in the cytolytic granules of human CTL and NK cells, has cytotoxic activity against intracellular pathogens in infected cells in the presence of perforin and has a cytotoxic effect against tumor cells.[6-8] The granulysin is expressed in human CD8 positive cytotoxic T cells and NK cells. It has been suggested that the granulysin has the function of in vitro cytotoxic activity against *M. tuberculosis* outside the macrophage cells, and contributes the in vitro reduction of *M. tuberculosis* in the macrophage in the presence of perforin.[7] However, the precise role of granulysin in the in vivo defense for the tuberculosis infection has not been elucidated yet. Therefore, we have established 15 K and 9 K granulysin transgenic mice to elucidate in vivo role of granulysin and to develop novel vaccines against the infection of *M. tuberculosis*. These 15 K granulysin transgenic mice and 9 K granulysin transgenic mice showed in vivo anti TB effect. This is the first demonstration of an in vivo action of granulysin for TB using granulysin transgenic mice. We have also developed novel TB vaccine of HVJ-Envelope/HSP65 DNA + IL-12 DNA.[3,9,10] Therefore, these findings suggest that granulysin or granulysin DNA may be useful as a TB vaccine, in the combination of other DNA vaccine.

Results

Cynomolgus monkey model. The prophylactic efficacy of HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine against TB was

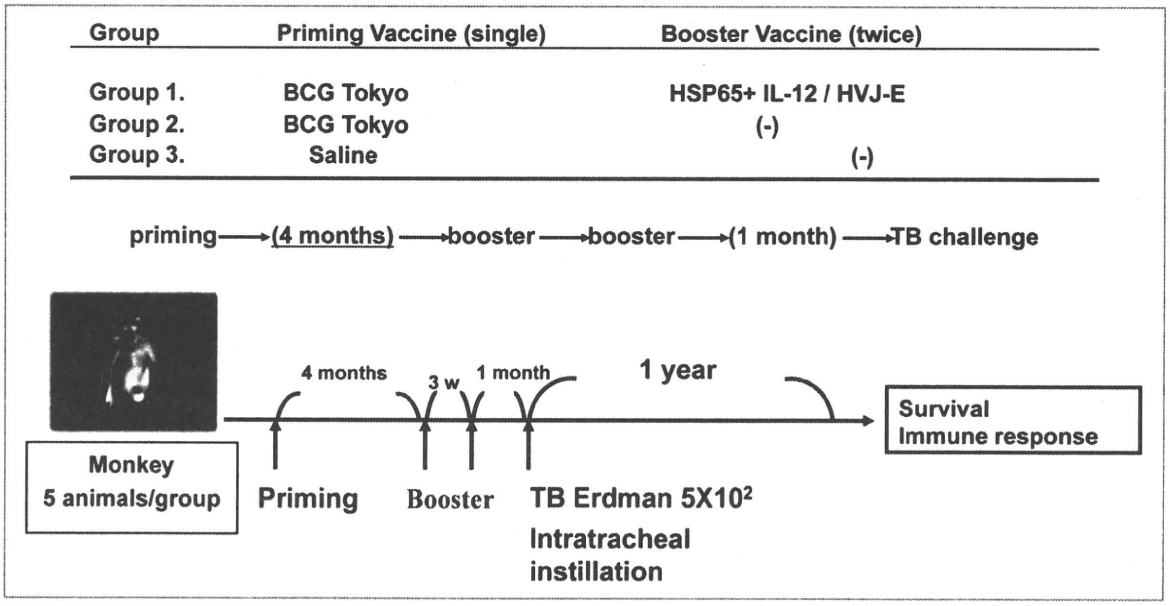

Figure 2. Evaluation of prophylactic efficacy of HVJ-Envelop/HSP65DNA + IL-12DNA vaccine on the infection of cynomolgus monkeys. Protective efficacy of HSP65 + IL-12/HVJ and BCG using prime-boost method against TB challenged cynomolgus monkeys. Group of animals were vaccinated three times (every 3 weeks) with (1°) BCG Tokyo, (2°) HSP65 + IL-12/HVJ, (3°) HSP65 + IL-12/HVJ = G_1. BCG prime-HVJ/DNA boost group; (1°) BCG, (2°) saline, (3°) saline = G_2. G_2 group animals were vaccinated with BCG once; (1°) saline, (2°) saline, (3°) saline = G_3. 4 month after the prime BCG vaccine, 2° vaccine was immunized. 3 weeks after the 2° vaccine, 3° vaccine was treated. One month after the third vaccination, monkeys were challenged with the *M. tuberculosis* (5 x 10^2 CFU) by intratracheal instillation. Prophylactic efficacy was evaluated by survival periods, erythrocyte sedimentation rate (ESR), body weight, chest X-rays, immune responses and DTH reaction against PPD for 16 months.

investigated using GMP-level-vaccine which contains two kinds of DNA in one plasmid vector for clinical trial (**Fig. 1**).

Long-term interval model (4 months) between prime and boost vaccinations was used in this study prior to intratracheal instillation of the challenge dose (**Fig. 2**).

In Group1 (BCG prime—DNA vaccine boost) monkeys, the regimen of vaccines improved ESR, compared to the regimen of Group3 (saline control group) or that of Group2 (BCG alone control group) (**Fig. 3**).

This vaccination method (BCG prime—DNA vaccine boost) also increased the body weight of 4 TB-infected monkeys out of 5 in Group1 as shown in **Figure 4**. In contrast, 2 monkeys in Group3 (saline) or 2 monkeys in Group2 (BCG alone) showed the decrease in body weight after the infection of TB.

The proliferation of PBL from monkeys in the base-line period was almost same among these G_1, G_2 and G_3 groups. However, proliferation of PBL from monkeys in G_1 group, (BCG prime-DNA vaccine boost group), was higher than those in G_2 (BCG alone group) and G_3 (saline control group) at 4 weeks after third vaccinations (G_1–G_3: $p < 0.05$) (**Fig. 5**).

Furthermore, IFNγ production from PBL in G_1 group (BCG prime-DNA vaccine boost group) was higher than those in G_2 (BCG alone group) and G_3 (saline control group) (data not shown).

By using long-term prime-boost method and vector containing two kinds of genes in one plasmid, the most reproducible and prophylactic efficacy based on the prolongation of survival was observed in Group1 monkeys (BCG prime-DNA boost, **Fig. 6**). The combination of BCG prime and DNA vaccine boost improved the survival (100% at 230 days and 80% at 360 days after TB challenge, respectively). In contrast, BCG vaccine alone in G_2 group monkeys showed 60% survival at 355 days and 40% survival at 360 days. The treatment of saline (G_3) showed 50% survival at 360 days.

Thus, even using the experimental model of long-term interval (4 months interval) between prime period and boost period, we could observe the prophylactic efficacy of this BCG prime-HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine in monkeys.

Transgenic mice model. The granulysin expression in the $CD3^+CD8^+CD4^-$ PBL-T cells of the patients with drug sensitive TB and MDR-TB was significantly lower than that of normal volunteer (data not shown).

We also analyzed the 15 K granulysin in the culture supernatants of PBL from patients with MDR-TB and healthy volunteer. The amounts of 15 K granulysin were measured after the stimulation with PPD, Hsp65 protein and killed TB H37Ra antigen. The production of 15 K granulysin was suppressed in the culture supernatants of PBL from patients with MDR-TB, compared to that from healthy volunteer (data not shown). Thus, it was suggested that granulysin treatment might improve the tuberculosis disease in human.

Therefore, to elucidate the in vivo mechanism of granulysin, we have established granulysin transgenic mice. We established eleven distinct transgenic mice including 15 K granulysin transgenic mice and 9 K granulysin transgenic mice. We confirmed the expression of mRNAs and proteins of 15 K granulysin and

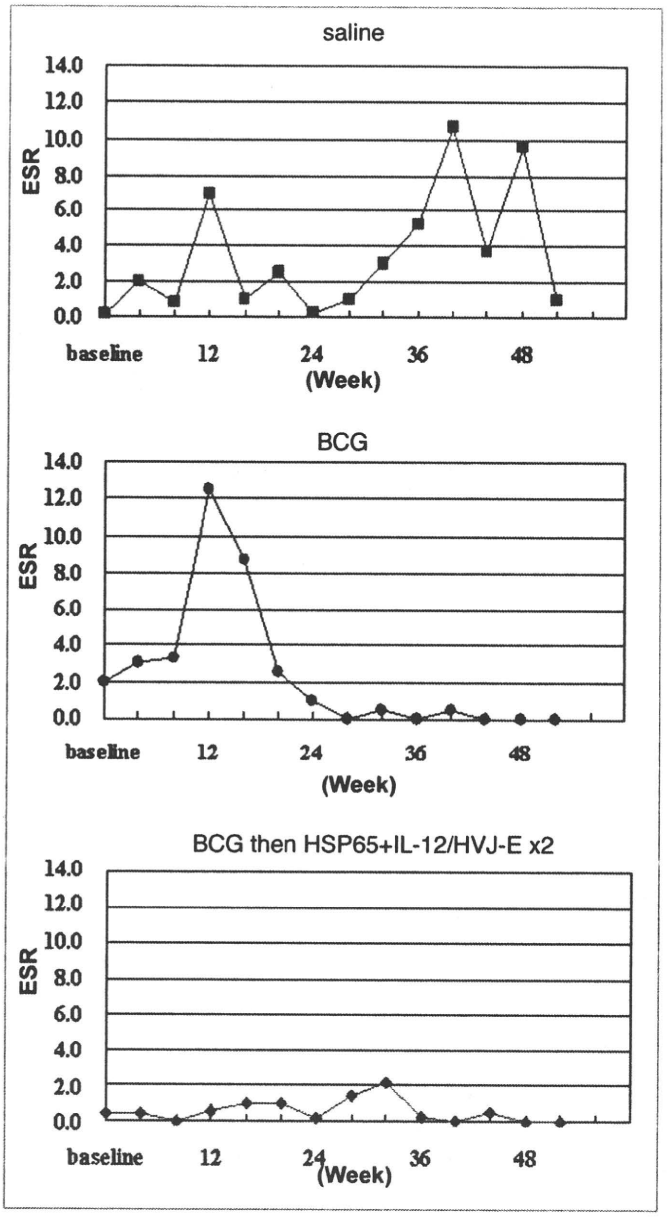

Figure 3. Improvement of erythrocyte sedimentation rate (ESR) in the cynomolgus monkeys immunized with BCG prime-HVJ-envelope/HSP65DNA + IL-12DNA boost vaccine. Cynomolgus monkeys were immunized and challenged as described in Figure 2. ESR of all monkeys was evaluated every month and mean values of ESR of 5 monkeys were shown.

9 K granulysin in established transgenic mice, respectively (data not shown). 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB effects, in vivo induction of cytotoxic T cells specific for TB, proliferation of T cells after the stimulation with TB antigens and augmentation of cytokine production.

As shown in **Figure 7**, in vivo anti-TB efficacy of 15 K granulysin transgenic mouse was observed.

CFU of *M. tuberculosis* was decreased at 4 weeks after the intravenous injection of 5×10^5 TB in the lungs of 15 K granulysin transgenic mice compared to those of wild type mice and

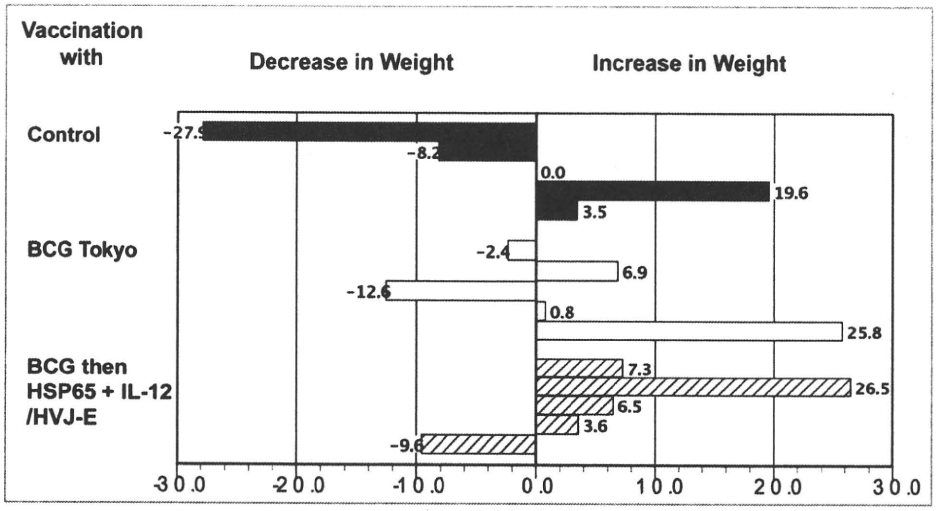

Figure 4. The increase in the body weight of monkeys vaccinated with HVJ-Envelope/Hsp65 DNA + IL-12 DNA. Monkeys vaccinated with BCG prime-HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine boost were challenged as described in Figure 2. Body weight of all monkeys was evaluated every month and values of body weight of monkeys at 16 weeks after TB challenge were shown.

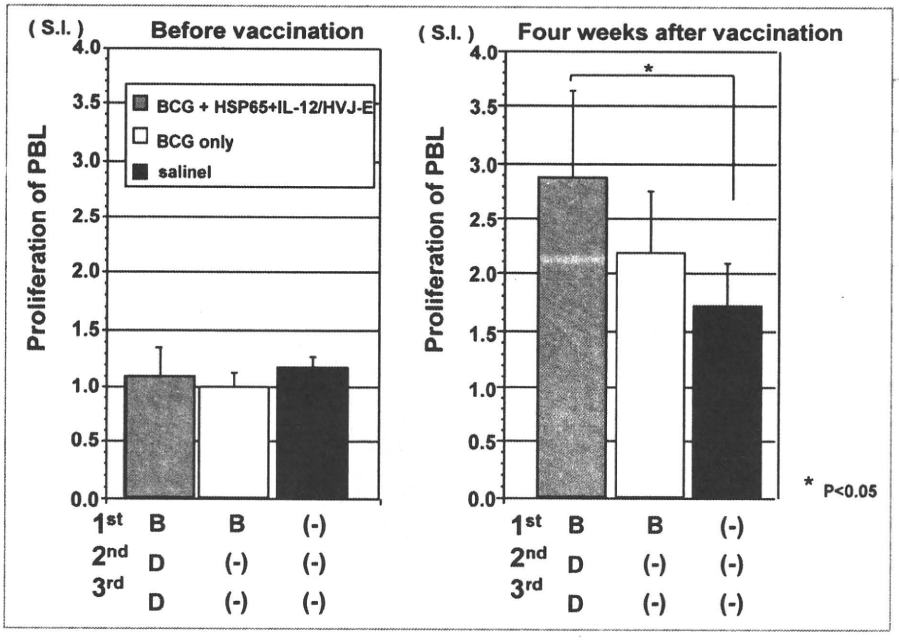

Figure 5. Augmentation of the proliferation of PBL in the monkeys immunized with BCG prime—HVJ-Envelope/Hsp65 DNA + IL-12 DNA boost vaccine. The proliferation of PBL (base line: before vaccination) from monkeys and PBL from monkeys vaccinated and challenged as described in Figure 2 were shown. Stimulation Index (S.I) of the ^{3}H-TdR uptake of monkey PBL at 11 weeks after TB challenge were shown. Student's t test were used to compare T cell proliferation between groups (p-value: G_1–G_3 $p < 0.05$).

normal C57BL/6 mice ($p < 0.05$). Furthermore, CFU of TB in the lungs of 9 K granulysin transgenic #1 mice and 9 K granulysin transgenic #17 mice were also decreased at 4 weeks after TB injection compared to that of wild type mice ($p < 0.05$) (**Fig. 8**). Thus, 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB efficacy and decreased the number of TB in the lungs.

SCID-PBL/hu model. We have very important and interesting SCID-PBL/hu models capable of analyzing in vivo human T cell immune responses and evaluating the efficacy of novel vaccines against TB, as reported first in Cancer Research 1997.

We used IL-2 receptor γ-chain gene knock out SCID-PBL/hu mice to analyze human immune responses.

Now, the therapeutic effects of HSP65 + IL-12 DNA vaccine in G_3 group (50 μg i.m.) on TB infection is observed in this IL-2 receptor γ-chain gene disrupted SCID-PBL/hu-model ($p < 0.05$) (**Table 1**). Human CTL activity against TB was associated with the efficacy of TB vaccine (data not shown).

Taken together, using cynomolgus monkey model, SCID-PBL/hu mice model and granulysin transgenic mice model, we have developed two kinds of novel vaccines, HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine and granulysin vaccine, against TB.

Discussion

In the present study, using cynomolgus monkey model, SCID-PBL/hu mice model and granulysin transgenic mice model, we have developed two kinds of novel vaccines, HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine and granulysin vaccine, against TB.

Most importantly, we demonstrated that even when the boost was performed after long-term period (4 month) from prime, the prolongation of monkey survival was observed by the combination of BCG and this HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine in the present study. This combination improved the erythrocyte sedimentation rate (ESR), increased the body weight and augmented the proliferation of PBL and IFNγ production, more than BCG alone or saline.

In the mouse system, by using BCG prime-HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine boost method, the number of *M. Tuberculosis* in the lungs of DNA-vaccinated mice were 10,000 (ten thousand) times lower compared to BCG alone vaccinated mice in our study (data not shown).

In parallel with the protective effect of HVJ-Envelope/DNA vaccines + BCG vaccine using prime-boost method on bacterial loads, histopathological analysis shows that mice vaccinated with

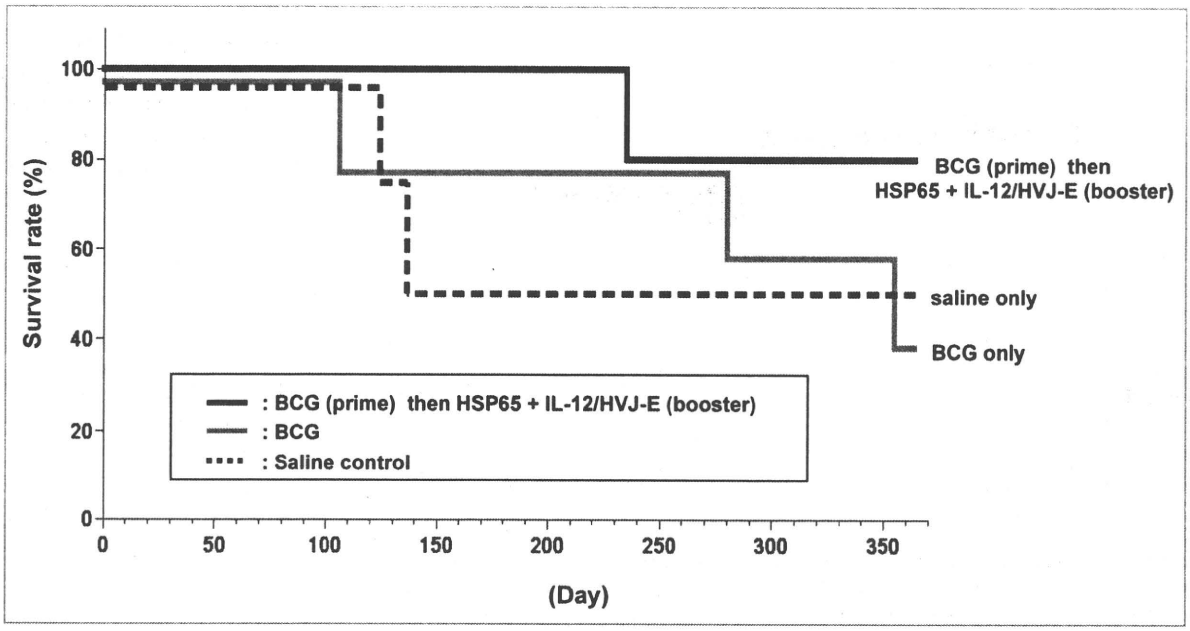

Figure 6. Protective efficacy of Hsp65 + IL-12/HVJ and BCG using prime—boost method against TB challenged cynomolgus monkeys. Group of animals were vaccinated three times as described in Figure 2. Group of animals were vaccinated with (1°) BCG Tokyo, (2°) Hsp65 + IL-12/HVJ, (3°) Hsp65 + IL-12/HVJ = G_1(—). BCG prime-HVJ/DNA boost group; (1°) BCG, (2°) saline, (3°) = G_2(—). G_2 group animals were vaccinated with BCG once; (1°) saline, (2°) saline, (3°) saline = G_3(—). One month after the third vaccination, monkeys were challenged with the *M. tuberculosis*.

this BCG prime-HVJ-Envelope/HSP65 DNA + IL-12 DNA boost had fewer and smaller lesions in the lungs and significantly less lung granuloma than the naïve mice and mice vaccinated with BCG alone (data not shown).

We extended our studies to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis, to evaluate the HSP65 + IL-12/HVJ.[1,2] Vaccination with BCG prime-HSP65 + IL-12/HVJ boost provided better protective efficacy as assessed by the Erythrocyte Sedimentation Rate, chest X-ray findings and immune responses than BCG alone. Importantly, HSP65 + IL-12/HVJ resulted in an increased survival for over a year. This was the first report of successful DNA vaccination against *M. Tuberculosis* in the monkey model which closely mimics human TB disease.[3]

Furthermore, the protective efficacy of the HSP65 + IL-12/HVJ and BCG using the prime-boost method in the TB-infected cynomolgus monkeys was very strong. All four monkeys from the group of BCG-prime and the DNA vaccine (HVJ-liposome/HSP65 + IL-12 DNA vaccine) boost were alive more than 12 months post-infection.[3] In contrast, only 2 monkeys out of 6 from the BCG Tokyo alone group were alive (33% survival).

Prime-boost method was reported in the study of MVA85A vaccine, which is a modified vaccine virus Ankara (MVA) strain expressing antigen 85A. In phase I studies in humans, this vaccine has induced high immune responses in previously BCG-vaccinated individuals.[14] Boosting of BCG vaccination with MVA85A downregulates the immunoregulatory cytokine TGFβ.[15] Aeras-402 DNA (DNA that expressed 85A, 85B and TB10.4) vaccine using adenovirus vector is intended for use as a boosting vaccine in BCG-primed individuals.[16] Several vaccines use a prime-boost strategy to enhance the immune responses.[17]

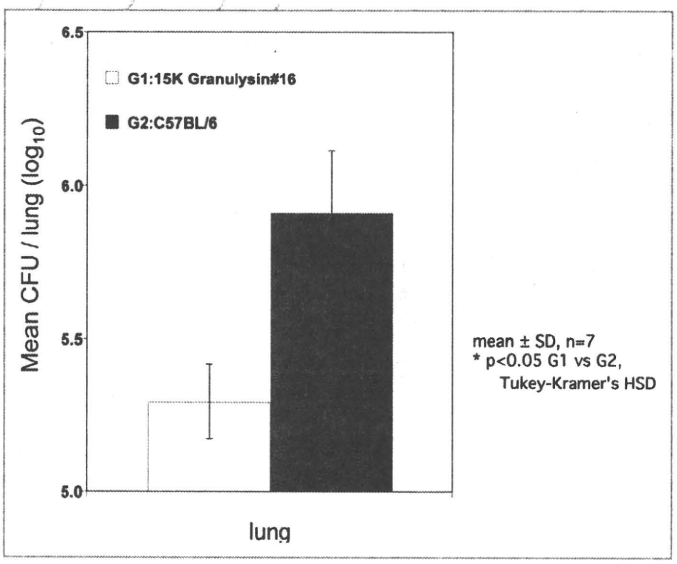

Figure 7. In vivo inhibition of the growth of *M. tuberculosis* in the 15 K granulysin transgenic mice. (In vivo anti-TB effect of 15 K granulysin transgenic mouse). Seven 15 K granulysin #16 transgenic mice and seven wild type C57BL/6 mice were injected with 5 x 10^5 H37RV *M. tuberculosis* i.v. 4 weeks after the challenge of *M. tuberculosis*, mice were sacrificed. CFU of *M. tuberculosis* in the lungs of these mice were assessed described in Material and Methods. CFU of 15 K granulysin #16 transgenic mice (□). CFU of control wild C57BL/6 mice (■). Student's t-test was used ($p < 0.05$).

In Japan and other countries, the BCG vaccine is inoculated into human infants (0–6 months after birth). Therefore, BCG prime in infants and HSP65 + hIL-12/HVJ boosts for adults

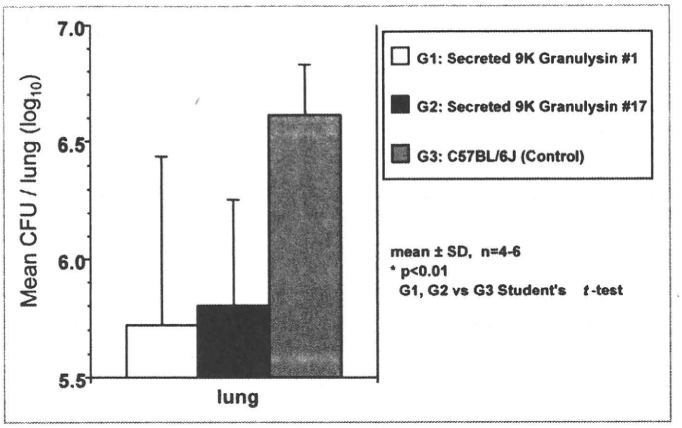

Figure 8. In vivo inhibition of the growth of *M. tuberculosis* in the 9 K granulysin transgenic mice. (In vivo anti-TB effect of 9 K granulysin transgenic mouse). Five 9 K granulysin #1 transgenic mice, five 9 K granulysin #7 mice and five wild type C57BL/6 mice were injected with 5 x 10^5 H37RV *M. tuberculosis* i.v. and 4 weeks after the challenge of *M. tuberculosis*, mice were sacrificed. CFU of *M. tuberculosis* in the lungs of these mice were assessed described in Material and Methods. CFU of 9 K granulysin #1 transgenic mice G_1 (□). CFU of 9 K granulysin #17 transgenic mice G_2 (■). CFU of control wild type C57BL/6 mice G_3 (■). Student's t-test was used to compare the CFU of each group(G_1–G_3: $p < 0.01$ G_2–G_3: $p < 0.01$).

Table 1. Efficacy of HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine against tuberculosis infection using IL-2 Receptor (-/-) NOD-SCID mice (SCID-PBL/hu)

Group	Treated	CFU of TB (log)
G1	(-)	6.03 ± 0.06
G2	HSP65DNA + IL-12 DNA vaccine (10 μg)	5.96 ± 0.15
G3	HSP65DNA + IL-12 DNA Vaccine (50 μg)	5.40 ± 0.97

Therapeutic efficacy of HVJ-envelope/HSP65DNA + IL-12DNA, using in vivo humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice (SCID-PBL/hu). Groups of animals were treated with 3 times with HVJ-envelope/HSP65DNA + IL-12DNA (50 ug i.m. or 10 ug i.m.). 10 days after the third vaccination, mice were sacrificed and CFU of TB in the liver of mice were assessed as described in Materials and Methods. 1 x 10^7 PBL from a healthy human volunteer were injected i.p. into IL-2 receptor γ-chain disrupted NOD-SCID mice. 21 days after injection of PBL, mice were challenged with 5 x 10^5 H37Rv i.v. and then treated with vaccine. G1, (-) control; G2, treated with HVJ-envelope/HSP-65DNA + IL-12DNA 10 μg; G3, treated with HVJ-envelope/HSP65DNA + IL-12DNA 50 μg. Student's t-test was used to compare the CFU of TB of each group (G1–G3: $p < 0.05$).

(including junior high school students, high school students and old persons) may be required for the significant improvement of clinical protective efficacy against TB.

In the present study, using very long-term period (4 month interval between prime and boost), protective efficacy of the combination of vaccines was evaluated. In human, long-term interval (5~15 years) between prime vaccine and boost vaccine might be used in the clinical application of a novel TB prophylactic vaccine.

Thus, our results with the HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine in the murine prophylactic model and cynomolgus monkey prophylactic model should provide a significant rationale for moving this vaccine into clinical trial.

On the other hand, we established transgenic mice and a vaccine expressing granulysin. The granulysin expression in the $CD3^+CD8^+$ PBL-T cells of the patients with drug sensitive TB was significantly lower than that of normal volunteer (data not shown). The granulysin expression in $CD3^+CD8^+$ T cells from MDR-TB patients was lower than that in $CD8^+$ T cells from drug sensitive TB patients.

The production of 15 K granulysin was also suppressed in the culture supernatants of PBL from 10 patients with MDR-TB, compared to that from healthy volunteer (data not shown). Thus, these data suggest that granulysin vaccine treatment provides a useful tool to regulate the human TB infection disease.

Two major protein products, 15 K granulysin and 9 K granulysin, are detected in CTL and NK cells. Granulysin exhibits potent cytotoxic activity against a broad panel of microbial targets, including bacteria, fungi and parasites.

We found that 15 K granulysin was secreted from CD8 positive CTL, and it could enter human macrophages and kill *M. tuberculosis* in the cytoplasm (data not shown). Therefore, we established 15 K granulysin transgenic mice and 9 K granulysin transgenic mice.

It was demonstrated that 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB effect and decrease in the number of TB. Thus, granulysin DNA vaccine therapy and recombinant granulysin therapy might provide a weapon against MDR-TB and XDR-TB.

In conclusion, we have the advantage of the availability of multiple animal models to accumulate essential data on the HVJ-Envelope DNA vaccine and granulysin vaccine in anticipation of a phase I clinical trial.

Materials and Methods

Methods for the evaluation of the prophylactic efficacy of the vaccine on the TB infection of the monkeys. Cynomolgus monkeys were housed in a BSL 3 animal facility of the Leonard Wood Memorial Research Center. All animal experiments were approved by the Leonard Wood Memorial Animal Care and Use Committee and the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. The animals were vaccinated three times with the HVJ-envelope with expression plasmid of both HSP65 and human IL-12 (HSP65 + hIL-12/HVJ: 400 ug i.m.), and then challenged with the *M. tuberculosis* Erdman strain (5 x 10^2) by intratracheal instillation. Survival, immune responses (proliferation of PBL and cytokines production), body weight, ESR, PPD skin test and chest X-P findings were examined as described in our previous studies.[3,9,10]

Methods for the establishment of granulysin transgenic mouse. 15 K granulysin gene, 9 K granulysin gene or secreted 9 K granulysin DNA (15 K granulysin secretory signal DNA was fused into N terminal of 9 K granulysin DNA) were transferred to expressing plasmid DNA (pCAGGS) having CAG promoter. DNA fragment was injected to pronuclei embryo and grafted to 200 foster parents. 2 types of 15 K granulysin Tg mice (#3, #16), 3 types of 9 K granulysin Tg mice (#15,

#17, #18) and 6 types of secreted 9 K granulysin Tg mice(#1, #3, #11, #14, #17, #25) were made. Granulysin activity was assessed by monoclonal antibody targeting 15 K granulysin and 9 K granulysin. *Mycobacterium tuberculosis* H37Rv 5 x 10^5 CFU was intravenously injected to 15 K granulysin Tg mice, 9 K granulysin Tg mice, wild type (control) mice and normal C57BL/6 mice (8-12 weeks).[3,10] From 2 to 12 weeks after injection, these mice were sacrificed. The lungs, the liver and the spleen of these mice were removed, homogenized and cultivated for 14 days on 7H11 agar medium. Then, the number of colony of *Mycobacterium tuberculosis* was measured.[3,10] Mice were maintained in isolator cages, manipulated in laminar flow hoods and used between 8–10 weeks of age. All animal experiments were approved by the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. All vaccinations and experiments on isolate tissue of animal were done under anaesthetic state with sevoflurane. Infected animals were housed in individual micro-isolator cages in a Biosafety Level (BSL) 3 animal facility of the NHO Kinki-chuo Chest Medical Center.

CTL activity in the spleen cells of mice were assessed using ^{51}Cr release assay.[11-13]

Methods for the establishment of SCID-PBL/hu model. IL-2 receptor γ-chain disrupted NOD-SCID-PBL/hu was constructed as described in our previous study.[4,5] CTL activity was assessed using the method as described previously in reference 12 and 13.

Statistical analysis. Student's t tests were used to compare log 10 value of CFU between groups following challenge of TB. Student's t test were also performed to compare immune responses between groups in T cell proliferation assay. A p-value of <0.05 was considered significant.

Acknowledgements

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Genotypes and Characteristics of Clustering and Drug Susceptibility of *Mycobacterium tuberculosis* Isolates Collected in Heilongjiang Province, China[▽]

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For the last decade China has occupied second place, after India, among the top five countries with high burdens of tuberculosis (TB). Heilongjiang Province is located in northeastern China. The prevalence of drug-resistant TB in Heilongjiang Province is higher than the average level in China. To determine the transmission characteristics of *Mycobacterium tuberculosis* strains isolated in this area and their genetic relationships, especially among the Beijing family strains, we investigated their genotypes. From May 2007 to October 2008, 200 *M. tuberculosis* isolates from patients presenting pulmonary TB were analyzed by molecular typing using PCR-based methods: spacer-oligonucleotide typing (spoligotyping), Beijing family-specific PCR (detection of the deletion of region of difference 105 [RD105]), and mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) analysis. Different combinations of MIRU-VNTR loci were evaluated to define the genotypes and clustering characteristics of the local strains. We found that Beijing family strains represented 89.5% of the isolates studied. However, the rates of multidrug-resistant (MDR) *M. tuberculosis* among Beijing and non-Beijing family strains were not statistically different. The 15-locus set is considered the optimal MIRU-VNTR locus combination for analyzing the *M. tuberculosis* strains epidemic in this area, while the 10-locus set is an ideal set for first-line molecular typing. We found that the clustering rate of all the *M. tuberculosis* isolates analyzed was 10.0% using the 15-locus set typing. We conclude that the Beijing family genotype is predominant and that highly epidemic TB and MDR TB are less likely associated with the active transmission of *M. tuberculosis* in the study area.

Tuberculosis (TB) remains a major public health threat worldwide. China has occupied second place, after India, among the top five high-burden countries for the last decade (http://www.who.int/tb/en). Although both the incidence and prevalence of TB in China have shown a steady decline in recent years, it remains a leading notifiable infectious disease (http://www.moh.gov.cn). Currently, the spread of drug-resistant TB, especially multidrug-resistant (MDR) TB, in China, presents a major challenge. The most up-to-date data from the World Health Organization (WHO) indicates that the rate of MDR TB in China was 8.3% (the rates of primary and acquired MDR TB were 5.7% and 25.6%, respectively) in 2007 (50), significantly higher than the global average rate (3.6%). The higher levels of TB prevalence and drug resistance have become the main public health concern of the Chinese government.

Heilongjiang Province, located in northeastern China, is one of the regions where the prevalence of both TB and drug-resistant TB is higher than the average level in China. The most recently updated epidemiological data, from 2007 to 2008, show that the rates of primary and acquired MDR TB were 18.3% and 37.8%, respectively, in Heilongjiang Province (our unpublished data). The reasons for the high prevalence and drug resistance of TB in Heilongjiang Province are still unknown and should be investigated to facilitate control of the TB epidemic in this area and throughout China.

In several Asian countries with high TB rates, a unique genotype of *Mycobacterium tuberculosis*, known as the Beijing family genotype, has been found to be the dominant genotype (3, 17, 34). During the last decade, Beijing family strains have been spreading in various geographic locations worldwide and now account for more than a quarter of all TB cases worldwide (12). Possible associations of the epidemic caused by this genotype with its drug resistance (1, 41) and its high adaptability to the host intracellular environment (8) have been reported. In China, Beijing family strains have spread widely; however, the proportion of Beijing family strains in Heilongjiang Province remains unknown. It is thus unclear if the high prevalence and high drug resistance of epidemic TB are directly related to the spread of Beijing family strains. The answers to these

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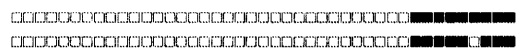
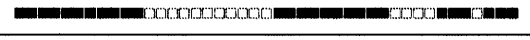
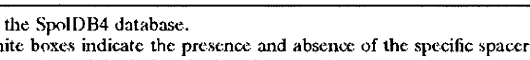
TABLE 1. The profile of spoligotypes and RD105 deletion of the *M. tuberculosis* isolates (n = 44)

SIT no.[a]	Spoligotype[b]	Label	No. of isolates[c]	RD105 deletion
1		Beijing	39	+
190		Beijing	1	+
1793		Undesignated	1	−
—		New	1 (2460)	+
—		New	1 (2488)	−
—		New	1 (2495)	−

[a] —, unassigned in the SpolDB4 database.
[b] The black and white boxes indicate the presence and absence of the specific spacer, respectively.
[c] The identification number of the isolate is given in parentheses.

questions would be very helpful in achieving effective control of TB in Heilongjiang Province as well as throughout China.

To determine the spreading characteristics and the genetic relationships among the *M. tuberculosis* isolates in a given geographic region, a suitable genotyping method is the most important means, i.e., one that is discriminatory, economical, and reproducible. Spacer-oligonucleotide typing (spoligotyping) is a PCR-based method which is simple, highly reproducible, and applicable to a digital format (22). Spoligotyping possesses the most features of an ideal typing method and is the gold standard for identifying strains as belonging to the Beijing family. However, due to the genetic homogeneity of this family, this method is uninformative in the areas where the Beijing family is prevalent (47) and is therefore limited to use as a first-line typing method.

A genotyping method based on mycobacterial interspersed repetitive unit-variable-number tandem repeats (MIRU-VNTR), which is technically flexible to facilitate interlaboratory comparison and database management, is well accepted and has recently been adapted to high-throughput techniques (7, 39). The digital format of MIRU-VNTR patterns makes this method suitable for the global study of the molecular epidemiology of *M. tuberculosis*. A number of studies have investigated the usefulness of different VNTR loci for typing *M. tuberculosis* and have shown that the discriminatory power of this method may be comparable to that of IS*6110* typing, which is the most reliable method for the genetic differentiation of *M. tuberculosis* isolates (33, 38). Because Beijing family strains are prevalent in many developing areas of Asia, where resources are definitely limited, MIRU-VNTR typing would be the best choice here to study the molecular epidemiology of *M. tuberculosis* strains.

Because TB, especially drug-resistant TB, constitutes a heavy burden in Heilongjiang Province, it is important to understand the possible spreading mechanisms of *M. tuberculosis* and particularly drug-resistant TB. In the present study, we conducted the genotyping of *M. tuberculosis* isolates to determine the prevalence of Beijing family strains and the genotypes of *M. tuberculosis* isolates in Heilongjiang Province using MIRU-VNTR analysis. The appropriate VNTR locus combination for genotyping the *M. tuberculosis* isolates in our area was also evaluated and optimized.

MATERIALS AND METHODS

Mycobacterial specimens. The study included 200 *M. tuberculosis* samples isolated between May 2007 and October 2008 from 200 patients from various regions of Heilongjiang Province who had been diagnosed with pulmonary TB in the Harbin Chest Hospital. All the patients were HIV-1 negative. The average age of the patients was 49.1 years, and 71.5% (143/200) were male. *M. tuberculosis* H37Rv was used as the reference strain.

DNA extraction and molecular identification of *M. tuberculosis* isolates and RD105 deletion detection. Mycobacterial cultures were obtained from clinical specimens after incubation in a BACTEC Mycobacterium Growth Indicator Tube (MGIT) 960 Automated System (Becton Dickinson). The cultures were inactivated by 70% ethanol for 2 h (9). DNA was extracted using lysozyme and the phenol-chloroform method (46). Molecular identification of the mycobacterial isolates was performed based on PCR amplification of the 16S rRNA gene and the Rv0577, Rv2073c, and Rv3120 genes (15). Rv2073c amplification was modified as follows: PrimeSTAR HS DNA polymerase (TakaRa. China) was used instead of *Taq* polymerase, and 2 μl of dimethyl sulfoxide was added to ensure good amplification. The amplification cycle was 5 min at 98°C followed by 25 cycles of 1 min at 98°C, 5 s at 65°C, and 40 s at 72°C, with a final 10 min at 72°C. The identification of genomic deletions in region of difference 105 (RD105) was performed by PCR as previously described (14, 26). Briefly, each PCR mixture was prepared in a volume of 20 μl containing 50 ng of DNA, 0.25 U of *Taq* polymerase (TakaRa. China), a 0.2 mM concentration of each deoxynucleoside triphosphate (dNTP), and 0.2 μM (each) primer. The amplification cycle was 10 min at 94°C followed by 25 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, with a final step for 10 min at 72°C.

Spoligotyping. Spoligotyping of the isolates was performed as described by Kamerbeek et al. (22). The direct repeat (DR) region was amplified with the primer pair, and the PCR products were hybridized to a set of 43 oligonucleotide probes corresponding to each spacer, which were covalently bound to a mem-

TABLE 2. Allelic diversity of different MIRU-VNTR loci[a]

MIRU-VNTR locus	No. of alleles (range)		*h*	
	Isolate set 1	Isolate set 2	Isolate set 1	Isolate set 2
QUB26	7 (4–10)	8 (3–10)	0.706	0.581
QUB11b	6 (3–8)	9 (0–8)	0.644	0.730
MIRU31	6 (2–7)	7 (2–8)	0.599	0.500
MIRU16	4 (2–6)	4 (2–6)	0.520	0.230
MIRU4	5 (1–5)	6 (0–5)	0.517	0.260
MIRU26	5 (4–9)	8 (3–10)	0.467	0.649
Mtub4	4 (2–5)	6 (0–5)	0.413	0.463
MIRU40	5 (1–5)	6 (1–6)	0.373	0.358
Mtub21	6 (1–8)	9 (1–9)	0.307	0.493
Mtub39	4 (1–6)	6 (1–6)	0.229	0.243
MIRU10	4 (1–4)	4 (1–4)	0.229	0.300
Mtub30	4 (2–5)	4 (2–5)	0.191	0.267
Mtub29	3 (3–5)	5 (2–6)	0.189	0.138
MIRU39	3 (1–3)	4 (1–4)	0.149	0.388
ETR A	2 (3–4)	7 (0–6)	0.146	0.329
QUB4156	2 (2–4)	5 (0–4)	0.107	0.182
ETR C	3 (2–5)		0.068	
ETR B	2 (1–2)		0.066	
MIRU23	1 (5)		0	

[a] Set 1 consists of 44 isolates obtained from May 2007 to November 2007. Set 2 consists of 200 isolates obtained from May 2007 to October 2008.

TABLE 3. The cumulative HGDI with successive addition of each MIRU-VNTR locus

Locus combination[a]	VNTR locus (*h*)[b]	No. of patterns	No. of clusters	No. of clustered isolates	No. of isolates in each cluster	Clustering rate (%)	HGDI (cumulative)
1	QUB11b (0.730)						
2	MIRU26 (0.649)	35	19	184	2–41	82.5	0.9042
3	QUB26 (0.581)	84	33	149	2–23	58.0	0.9686
4	MIRU31 (0.500)	112	33	121	2–18	44.0	0.9808
5	Mtub21 (0.493)	126	33	107	2–16	37.0	0.9867
6	Mtub4 (0.463)	135	30	95	2–15	32.5	0.9888
7	MIRU39 (0.388)	150	23	73	2–14	25.0	0.9913
8	MIRU40 (0.358)	159	18	59	2–12	20.5	0.9935
9	ETR A (0.329)	164	16	52	2–12	18.0	0.9943
10	MIRU10 (0.300)	169	14	45	2–12	15.5	0.9950
11	Mtub30 (0.267)	169	14	45	2–12	15.5	0.9950
12	MIRU4 (0.260)	176	12	36	2–10	12.0	0.9967
13	Mtub39 (0.243)	178	10	32	2–10	11.0	0.9968
14	MIRU16 (0.230)	179	11	32	2–8	10.5	0.9976
15	QUB4156 (0.182)	180	10	30	2–8	10.0	0.9977
16	Mtub29 (0.138)	180	10	30	2–8	10.0	0.9977

[a] The successive addition of each VNTR locus.
[b] The *h* value represents the diversity determined from the 200 isolates.

brane (22). Spoligotypes in binary format were compared with the SpolDB4 database, and the spoligotype international type (SIT) numbers and the clades were also determined (4).

MIRU-VNTR typing. To identify a suitable MIRU-VNTR locus set for genotyping *M. tuberculosis* isolates in this area, 19 loci were selected for analyzing the first set of 44 *M. tuberculosis* isolates (38). The PCR mixture and conditions were the same those for the RD105 deletion identification described above. Genomic DNA of the H37Rv strain and sterile distilled water were used as the positive and negative controls, respectively. PCR products were analyzed on a 1.5% agarose gel against a 100-bp DNA ladder (TakaRa, China), and the copy number at each locus was calculated using a Quantity 1 gel imaging system (Tanon, China). The MIRU-VNTR allelic diversity (*h*) at a given locus was calculated as follows: $h = 1 - \Sigma x_i^2[n/(n-1)]$, where x_i is the frequency of the *i*th allele at the locus, and n is the number of isolates (35). The discrimination of the locus combination was calculated using the Hunter-Gaston discriminatory index (HDGI) (16):

$$\mathrm{HGDI} = 1 - \frac{1}{N(N-1)}\sum_{j=1}^{s} n_j(n_j - 1)$$

where N is the total number of isolates in the typing method, s is the number of distinct patterns discriminated by MIRU-VNTR, and n_j is the number of isolates belonging to the *j*th pattern.

Phylogenetic and cluster analysis. We used the R software, version 2.11.1 (http://cran.r-project.org), for phylogenetic and cluster analysis. A dendrogram was produced from the MIRU-VNTR genotypes of the 200 *M. tuberculosis* isolates. First, the repeat numbers of MIRU-VNTR genotypes were standardized based on a *z*-score normalization. Then, a similarity coefficient matrix of the *M. tuberculosis* isolates was obtained by calculating the Euclidean distances between isolates from the standardized data. Finally, clustering was performed, and a phylogenetic tree was constructed using Ward's parameter with the matrix. The *M. tuberculosis* isolates analyzed in this study were classified into two groups, characterized by clustered and nonclustered *M. tuberculosis* isolates. A molecular cluster was defined as two or more *M. tuberculosis* isolates having identical genetic patterns as determined by MIRU-VNTR genotyping. The isolates with unmatched genetic profiles were considered nonclustered strains. Assuming that one patient from each cluster corresponded to the index case at the origin of infection, the clustering rate was calculated using the following formula: clustering rate = $(n_c - c)/n$, where n_c is the total number of clustered isolates, c is the number of isolate clusters, and n is the total number of isolates in the sample (37).

Statistical analysis. Associations among multiple categorical variables were assessed using R, version 2.11.1, by a chi-square test or Fisher's exact test when the theoretical frequency was less than five. Two-by-two tables were assessed by a chi-square test (here, Yates' continuity correction was needed when the value was less than five), and results were expressed as odds ratios (OR) with 95% confidence intervals (95% CI). The agreement between spoligotyping and RD105 deletion typing was assessed using kappa statistics; the agreement was considered good for values of kappa above 0.75. *P* values of <0.05 were considered statistically significant.

RESULTS

Epidemic of Beijing family strains in Heilongjiang Province. During the study period, 200 *M. tuberculosis* isolates identified using both the BACTEC 960 automated system and molecular methods were collected. First, we analyzed the correlation between spoligotyping and RD105 deletion for the identification of the Beijing genotype using 44 isolates collected from May 2007 to November 2007. Among the 44 *M. tuberculosis* isolates, spoligotypes of 41 isolates were classified into three designated SITs according to the SpolDB4 database (Table 1). Among these, 40 isolates were Beijing family strains. The most frequent genotype (39/41) was the typical Beijing spoligotype SIT1, which has only spacers 35 to 43; the only other Beijing genotype belonged to spoligotype SIT190. One isolate, with an SIT number of 1793, was not designated in the database. The remaining three isolates showed new spoligotypes, which were not registered in SpolDB4 database. Interestingly, one isolate (2460) showed a unique genotype with only two spacers, 35 and 36. We found that 40 isolates lacking RD105 exhibited Beijing family spoligotypes (Table 1). The *M. tuberculosis* isolate 2460 also lacked RD105. The results of the kappa statistics analysis showed that the agreement of spoligotyping and RD105 deletion detection in identifying the Beijing family genotype was high ($\kappa = 0.8451$). Subsequently, instead of using spoligotyping, RD105 deletions in the other 156 *M. tuberculosis* strains were examined, and we found that 179 of the 200 isolates (89.5%) had the Beijing family genotype, while 21 (10.5%) were non-Beijing family strains.

Optimal combination of MIRU-VNTR loci for genotyping *M. tuberculosis* isolates in Heilongjiang Province. First, to evaluate and determine the most suitable loci for genotyping the *M. tuberculosis* isolates epidemic in Heilongjiang Province, we analyzed 19 MIRU-VNTR loci, which had been previously identified as a suitable locus combination for genotyping *M. tuberculosis* isolates in the regions where the Beijing family is

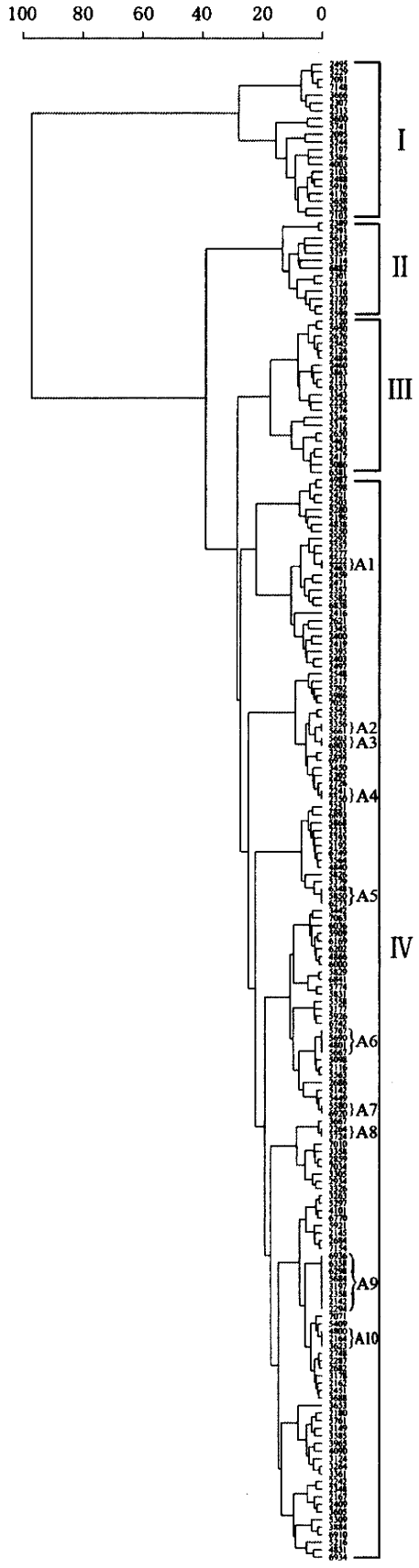


FIG. 1. Dendrogram of 200 *M. tuberculosis* isolates from Heilongjiang Province. The phylogenetic tree was produced from the MIRU-VNTR genotypes which were derived from 16 of the 19 loci by excluding ETR B, ETR C, and MIRU23. A1 to A10, cluster names.

dominant (19, 27) (Table 2). The allelic diversity (h) of the first set of 44 *M. tuberculosis* isolates at each MIRU-VNTR locus varied significantly. Among the 19 loci, the allelic diversity for 2 loci (QUB11b and QUB26) exceeded 0.6, suggesting that they are highly discriminating (30). Seven loci (MIRU4, MIRU16, MIRU26, MIRU31, MIRU40, Mtub21, and Mtub4) showed moderate discrimination ($0.3 \leq h \leq 0.6$), but ETR C ($h = 0.068$) and ETR B ($h = 0.066$) were less polymorphic. Diversity was not observed for the MIRU23 locus ($h = 0$). Thus, the loci ETR C, ETR B, and MIRU23, having discriminatory powers of less than 0.1, were excluded from the subsequent MIRU-VNTR analysis.

Next, we analyzed the 200 *M. tuberculosis* isolates collected from May 2007 to October 2008 using the remaining 16 MIRU-VNTR loci. All 16 loci displayed an allelic diversity similar to the original 19 loci (Table 2). The highest diversity among the 200 isolates was observed at QUB11b ($h = 0.730$), and the lowest diversity was observed at Mtub29 ($h = 0.138$). The HGDI of the 16-locus set was as high as 0.9977. However, because the 16-locus procedure still did not meet the requirements of cost and labor expenditure for high-throughput genotyping, we then tried to optimize the locus combination while minimizing the number of loci. Based on the allelic diversity of each MIRU-VNTR locus, the cumulative HGDI of the locus combination by successive addition of a locus was compared (Table 3). The cumulative HGDI and clustering rate of the 10-locus set were equal to that of the 11-locus set (HGDI, 0.9950; clustering rate, 15.5%); they were also the same for the 15- and the 16-locus sets (HGDI, 0.9977; clustering rate, 10.0%). The set of the first seven loci with the highest allelic diversity gave an HGDI of 0.9913 and a clustering rate of 25.0%.

VNTR profiles and genotypes of the *M. tuberculosis* isolates in Heilongjiang Province. The MIRU-VNTR genotyping results showed that the 200 isolates were classified into 180 genotypes. A total of 170 isolates had unique patterns, while the remaining 30 isolates were in 10 clusters. A dendrogram was constructed based on the genotypes of 200 isolates using 16 loci (Fig. 1). The isolates were divided into four groups based on phylogenetic clustering and genotypic characteristics. Groups I to IV contained 21, 13, 21, and 145 isolates, respectively. Among the 179 Beijing family isolates, 144 (99.3%) were in group IV; the remaining 35 isolates were in groups I, II, and III ($P < 0.0001$), and all the clustered isolates were in group IV ($P = 0.0018$), suggesting that the distributions of the Beijing family isolates and clustered isolates were distinctive among the four groups (Table 4).

Characteristics of the clustered isolates. Thirty Beijing family isolates (30/179, or 16.8% of the Beijing family strains) were determined to be in 10 clusters (A1 to A10), with the clustering rate of 10.0% based on 15-locus MIRU-VNTR patterns. In contrast, none of 21 non-Beijing family isolates were clustered (OR, 0; 95% CI, 0 to 1.024; $P = 0.087$) (Table 5). Most of the

TABLE 4. Differences of *M. tuberculosis* characteristics among the four subgroups

Isolate characteristic	Total no. of isolates	No. (%) of isolates by subgroup[a]				*P* value[b]
		I (n = 21)	II (n = 13)	III (n = 21)	IV (n = 145)	
Resistance						
Streptomycin	85	9 (42.9)	6 (46.2)	8 (38.1)	62 (42.8)	0.9704*
Isoniazid	92	9 (42.9)	8 (61.5)	7 (33.3)	68 (46.9)	0.4317*
Rifampin	55	4 (19.0)	7 (53.8)	3 (14.3)	41 (28.3)	0.0815
Ethambutol	48	4 (19.0)	6 (46.2)	3 (14.3)	35 (24.1)	0.2070
MDR	51	4 (19.0)	6 (46.2)	2 (9.5)	39 (26.9)	0.1032
Four-drug susceptibility	77	8 (38.1)	3 (23.1)	9 (42.9)	57 (39.3)	0.6786*
Four-drug resistance	23	2 (9.5)	3 (23.1)	1 (4.8)	17 (11.7)	0.4402
Obtained from a patient with acquired TB	126	17 (81.0)	9 (69.2)	11 (52.4)	89 (61.4)	0.2294
Obtained from a patient with hemoptysis	169	15 (71.4)	9 (69.2)	20 (95.2)	125 (86.2)	0.0562
Beijing strain	179	3 (14.3)	12 (92.3)	20 (95.2)	144 (99.3)	<0.0001
Clustered	30	0 (0)	0 (0)	0 (0)	30 (20.7)	0.0018

[a] n, number of isolates in the subgroup.
[b] Values marked with an asterisk were determined by a chi-square test; other values were determined by a Fisher's exact test.

clusters were small: six (A1 to A4, A7, and A8) contained only two members; two (A5 and A10) contained three members; cluster A6 contained four members. The largest cluster, A9, contained eight members. In addition, the clustering rates of the two periods, May 2007 to May 2008 (106 isolates) and June 2008 to October 2008 (94 isolates), were 6.4% and 12.8%, respectively (OR, 0.3240; 95% CI, 0.1161 to 0.8265; P = 0.0088).

To determine if there was any correlation between the clustering characteristics and the geographical origins of the isolates, we investigated the home addresses of the patients in the clusters from the available medical records. We found that the isolates belonging to clusters A2 and A7 were scattered throughout the Heilongjiang Province while clusters A3 to A6, A9, and A10 were registered in Harbin City.

Drug susceptibility patterns of the *M. tuberculosis* isolates in Heilongjiang Province. To determine the association between drug resistance patterns and genotypic characteristics, drug susceptibility to the four first-line antituberculosis drugs, i.e., streptomycin, isoniazid, rifampin, and ethambutol, was examined using an automated BACTEC MGIT 960 SIRE system (Becton Dickinson). A total of 77 isolates (38.5%) were susceptible to all four drugs; 123 (61.5%) were resistant to at least one drug, and 51 (41.5%) were MDR *M. tuberculosis* (Tables 4 and 5). The drug susceptibility patterns of the isolates among the four genotype groups were not significantly different (Table 4). Of the 51 MDR *M. tuberculosis* isolates, 48 isolates were found to be the Beijing family strains, and 3 were non-Beijing family strains. Of the Beijing family strains, 26.8% (48/179) were MDR, and 14.3% (3/21) of the non-Beijing family strains were MDR. The rates of MDR *M. tuberculosis* among Beijing and non-Beijing family strains were not statistically different (OR, 0.4564; 95% CI, 0.0824 to 1.6670; P = 0.2127). Resistance to at least one drug was observed more frequently among Beijing family strains (63.1%, or 113/179) than among non-Beijing family strains (47.6%, or 10/21), but the difference was not statistically significant (OR, 1.8771; 95% CI, 0.6828 to 5.2244; P = 0.1670) (Table 5).

DISCUSSION

The Beijing family strains currently prevail throughout China. RD105 deletion has recently been reported to serve as a genetic marker for Beijing family strains (44), and several

TABLE 5. Differences of *M. tuberculosis* characteristics between Beijing and non-Beijing family

Isolate characteristic	Total no. of isolates	No. (%) of isolates[a]		OR	95% CI	*P* value[b]
		Beijing (n = 179)	Non-Beijing (n = 21)			
Resistance						
Streptomycin	85	78 (43.6)	7 (33.3)	0.6488	0.2110–1.8176	0.3691
Isoniazid	92	84 (46.9)	8 (38.1)	0.6972	0.2381–1.9189	0.4423
Rifampin	55	52 (29.1)	3 (14.3)	0.4086	0.0739–1.4876	0.1517
Ethambutol	48	45 (25.1)	3 (14.3)	0.4978	0.0898–1.8235	0.4056*
MDR	51	48 (26.8)	3 (14.3)	0.4564	0.0824–1.6670	0.2127
Four-drug susceptibility	77	66 (36.8)	11 (52.4)	1.8771	0.6828–5.2244	0.1670
Four-drug resistance	23	21 (11.7)	2 (9.5)	0.7928	0.0837–3.6902	0.9510*
Obtained from a patient with acquired TB	126	109 (60.9)	17 (90.0)	2.7174	0.8390–11.5638	0.0717
Obtained from a patient with hemoptysis	169	153 (85.5)	16 (76.2)	1.8323	0.4830–5.8459	0.4275*
Clustered	30	30 (16.8)	0 (0)	0	0–1.0241	0.0869*

[a] n, number of isolates in the group.
[b] Values marked with an asterisk were determined by a continuity-adjusted chi-square test; other values were determined by a chi-square test.

studies have used this method to identify them (6, 26, 45). It is financially economical, labor saving, and especially suitable for high-throughput analysis. In this study, we found good agreement between RD105 deletion detection and spoligotyping. One strain (2460) showed a novel spoligotype containing only spacers 35 and 36 and an RD105 deletion. According to the definition of the Beijing family spoligotype, these strains contain at least three spacers among direct repeats 35 to 43; however, strain 2460 can be included in the Beijing family because it lacks RD105.

We found that 89.5% of the *M. tuberculosis* isolates in Heilongjiang Province were Beijing family strains. This genotype accounts for 80 to 90% of the *M. tuberculosis* strains currently epidemic in the Beijing area (19); it is also prevalent in Ningxia (67%), Shanghai (89%), Zhejiang (70%), Tianjin (91.7%), and Guangxi (55.3%) but less prevalent in Guangdong (25%) (5, 24, 25, 36, 48). Hence, Heilongjiang Province is one of the regions where the proportion of the Beijing genotype is the highest. This genotype is thought to be associated with drug resistance (1, 10, 23, 41). However, less association has been reported in other geographic settings (2, 3, 20, 43). In the present study, the statistical analysis showed that there was no difference between the Beijing and non-Beijing genotype strains in drug resistance patterns, indicating that the Beijing genotype is less likely to be associated with the high prevalence of drug resistance and *M. tuberculosis* TB in our area.

Molecular typing by MIRU-VNTR has been used in epidemiology studies, and its stability is adequate for tracking recent transmission and distinguishing relapses and reinfections (39). Currently, the system based on 12 loci (29) is most widely used among the different sets of MIRU-VNTR loci. However, it is not effective for the analysis of clustered isolates (7). Other sets of MIRU-VNTR loci, such as the 14-locus set and the 15-locus set, have improved the discrimination of unrelated isolates (23, 38). An optimized set of 24 loci has also been defined; however, not all 24 loci are required for genotyping *M. tuberculosis* strains in any given situation (38) as the number of loci required depends on the lineage known to be prevalent in the investigated area.

In the present study, we found that the 16 of the 19 loci had high discriminatory diversity. This 16-locus set showed strong discriminatory power in analyzing the *M. tuberculosis* strains in our area (HGDI of 0.9977). Because the ability of the different locus combinations to differentiate the *M. tuberculosis* strains varied, we evaluated various sets of MIRU-VNTR loci to identify a minimal subset that provided discrimination comparable to that of the 16 loci. We found that the locus Mtub29 could be excluded from the set because the HGDI and clustering rate of the remaining 15 loci were the same as those of the 16 loci. The HGDI and the clustering rate of a 10-locus set were comparable to those of the 16-locus set. Therefore, we suggest that this 10-locus set be used as a first-line set for genotyping *M. tuberculosis* isolates in Heilongjiang Province, especially for routine epidemiological investigation and large-scale genotyping. Comparing the HGDI and the clustering rate of this locus set with those of various locus sets reported in other areas of China, we found that the discriminatory power of the 15-locus set used in the present study was the highest and that the clustering rate was the lowest (Table 6).

However, MIRU-VNTR loci showed variation in the ability to differentiate Beijing genotype strains from different geographical areas. Trying to explore the loci showing high discriminatory power among Beijing genotype strains in various areas of the world (Table 7), we found that at least 11 and 14 loci showed high enough diversity among the locally circulating Beijing genotype strains in China and Japan, respectively. Therefore, we recommend them as the predominant candidates (Table 7, underlined median *h* value for China and Japan). Since the Beijing genotype is dominant in China and Japan, we also suggest taking the 14 loci that show high diversity among the strains epidemic in the two countries as the predominant candidates for Asia (Table 7, underlined Asian median values). Meanwhile, the loci showing very low diversity (Table 7, boldface), 11 from China and 9 from Japan, may not need to be included for future studies. However, there are still some loci that showed high variation in differentiating Beijing genotype strains. For example, the loci MIRU10 and MIRU16 showed moderate diversity in Hong Kong and Gansu but low diversity in the other areas of China. The locus VNTR4120 was highly discriminatory in Japan (*h* of 0.902) but less discriminatory in China (*h* of 0.092).

In Japan, most of the loci reported showed comparatively high discriminatory power; therefore, considering labor and cost, some loci with moderate *h* values (>0.3) may not need to be included. Russia is much different from Japan and China in allelic diversity of the MIRU-VNTR loci, and the *h* values of most loci are much lower than those in China and Japan. This difference may imply that the loci which are suitable for genotyping the isolates epidemic in Asia may not be suitable for genotyping isolates in Russia.

Active transmission of drug-resistant *M. tuberculosis* strains in a community is an emerging problem. It is generally assumed that the proportion of clustered strains in a population reflects the level of active transmission (11, 28). The present study using 15 loci showed that the clustering rate in Heilongjiang Province is 10.0%, which is lower than the rates reported in other areas (Table 6). Though some loci that show moderate or high discriminatory power in other areas were not included in the present study, omitting them will not increase the clustering rate in this area because the loci may decrease the clustering trend of the strains by decreasing the diversity. The

TABLE 6. Discriminatory index of different locus sets used in various regions of China and the clustering rates

Area	Locus set	Clustering rate (%)	HGDI	Reference
Hong Kong	17 loci	17.4	0.9900	23
Shanghai	16 loci	16.1	0.9982	52
	7 loci	25.0	0.9957	
Beijing	24 loci	15.3	0.9920	19
	15 loci	18.1	0.9900	
	12 loci	59.7	0.7880	
Fujian	12 loci	17.1	0.9808	18
Gansu	15 loci	42.1		42
Zhejiang	15 loci	30.0	0.9905	49
Eight regions	12 loci	33.5	0.9780	13
Five regions	19 loci	15.7	0.9949	27
Heilongjiang	15 loci	10.0	0.9977	This study
	10 loci	15.5	0.9950	
	7 loci	25.0	0.9913	

TABLE 7. Allelic diversity of different MIRU-VNTR loci for differentiating *M. tuberculosis* Beijing family strains in different areas

Locus	Allelic diversity (h) by region[a]														
	Russia[b]			Japan[c]				China[d]							Median for Asia
	St. Petersburg (n = 48)	West Siberia (n = 51)	Median	Kobe (n = 181)	Japan (n = 240)	Chiba (n = 185)	Median	Beijing (n = 72)	Shanghai (n = 189)[e]	Hong Kong group 1 (n = 51)	Hong Kong group 2 (n = 243)	Gansu (n = 202)	Heilongjiang (n = 179)	Median	
VNTR4120	0.370		0.370	0.902	0.902	0.882	0.902		0.092*					0.092	0.892
QUB3232	0.729		0.729	0.880	0.909	0.813	0.880				0.804			0.804	0.847
VNTR3820	0.542		0.542	0.800	0.871	0.817	0.817		0.821					0.821	0.819
QUB11b	0.205	0.210	0.208	0.772	0.815	0.763	0.772	0.651	0.689	0.618	0.669		0.704	0.669	0.697
QUB18		0.740	0.740			0.629	0.629		0.607	0.740	0.488			0.607	0.618
Mtub24					0.591	0.614	0.603		0.223					0.223	0.591
QUB26	0.636	0.780	0.708	0.741	0.764	0.215	0.741	0.518	0.630	0.299	0.314		0.607	0.518	0.563
Mtub21	0.330	0.110	0.220	0.393	0.598	0.537	0.537	0.556	0.544			0.690	0.396	0.550	0.544
QUB11a				0.685	0.752	0.535	0.685		0.538	0.384	0.514			0.514	0.537
QUB3336				0.487	0.642	0.482	0.487				0.214			0.214	0.485
QUB4156	0.082		0.082	0.611	0.623	0.603	0.611	0.395	0.469		0.167		0.182	0.289	0.469
Mtub4	0.000		0.000	0.459	0.468	0.581	0.468	0.306	0.266				0.391	0.306	0.425
MIRU26	0.520		0.520	0.383	0.314	0.283	0.314	0.353	0.614	0.200		0.560	0.596	0.560	0.368
QUB1895				0.364	0.337	0.468	0.364		0.365	0.229	0.206			0.229	0.351
VNTR2372					0.595	0.345	0.470		0.177*					0.177	0.345
QUB15					0.537	0.629	0.583		0.032*		0.132			0.082	0.335
MIRU31	0.160	0.000	0.080	0.322	0.270	0.379	0.322	0.169	0.328		0.156	0.370	0.395	0.328	0.325
ETR F					0.237	0.499	0.368		0.290					0.290	0.290
MIRU10	0.082		0.082	0.419	0.431	0.291	0.419	0.144	0.239	0.377		0.160	0.154	0.160	0.265
MIRU40	0.122	0.390	0.256	0.327	0.229	0.473	0.327	0.194	0.147*	0.196		0.350	0.292	0.196	0.261
MIRU16	0.082		0.082	0.310	0.258	0.421	0.310	0.068	0.131	0.058		0.580	0.200	0.131	0.229
ETR A	0.158	0.000	0.079	0.147	0.223	0.165	0.165	0.232	0.031*	0.201	0.188	0.280	0.238	0.217	0.201
Mtub39	0.000	0.000	**0.000**	0.186	0.215	0.271	0.215	0.171	0.061*			0.120	0.174	0.146	0.174
MIRU39	0.000		**0.000**	0.221	0.156	0.160	0.160	0.119	0.141	0.320	0.040	0.100	0.290	0.141	0.158
Mtub30	0.042		0.042	0.403	0.379	0.210	0.379	0.068	0.091*			0.090	0.133	**0.090**	0.133
Mtub29	0.087	0.180	0.134	0.043	0.095	0.103	**0.095**	0.119	0.061*				0.123	**0.119**	**0.103**
MIRU23	0.000	0.000	**0.000**	0.176	0.158	0.124	0.158	0.014	0.061*			0.030		**0.030**	**0.093**
QUB5(MIRU27)	0.000		**0.000**	0.115	0.081	0.074	**0.081**	0.014	0.031*			0.100		**0.031**	**0.078**
MIRU4	0.000		**0.000**	0.086	0.049	0.000	**0.049**	0.120	0.061	0.019	0.072		0.212	**0.061**	**0.067**
MIRU20	0.120		**0.120**	0.022	0.065	0.063	**0.063**	0.014	0.061*					**0.038**	**0.061**
ETR C	0.042	0.000	**0.021**	0.022	0.057	0.063	**0.057**	0.094		0.165	0.057	0.000		**0.076**	**0.057**
Mtub34	0.000		**0.000**	0.066	0.033	0.000	**0.033**	0.014	0.089*					**0.052**	**0.033**
QUB23					0.025		0.025				0.016			0.016	0.021
ETR B	0.000		**0.000**	0.033	0.017	0.032	**0.032**	0.014	0.000*	0.000	0.064	0.020		**0.014**	**0.019**
VNTR0569						0.011	0.011		0.000*					0.000	0.006
QUB1451				0.033			0.033		0.000*		0.008			0.004	0.008
MIRU2	0.000		**0.000**	0.000	0.008	0.000	**0.000**	0.000	0.000*					**0.000**	**0.000**
MIRU24	0.000		**0.000**	0.000	0.000	0.042	**0.000**	0.000	0.000*					**0.000**	**0.000**
ETR E		0.000	**0.000**												0.000

[a] n, number of isolates; underlining, corresponding locus was recommended; boldface, corresponding locus was not recommended.
[b] See the following references: for St. Petersburg, 31; for West Siberia, 40.
[c] See the following references: for Kobe, 17; for Japan, 32; for Chiba, 51. The Japan strains were from a drug resistance survey in Japan in 2002.
[d] See the following references: for Beijing, 19; for Shanghai, 52; for Hong Kong group 1, 23; for Hong Kong group 2, 21; and for Gansu, 42. Hong Kong strains were collected in 2001 (group 1) or 2001 to 2003 (group 2).
[e] For values marked with an asterisk, the number of samples was 65.

comparatively low rate and the small size of the clusters suggest that the high resistance of *M. tuberculosis* in Heilongjiang Province is not related to recent transmission but, rather, may be related to reactivation or inappropriate therapy. However, the clustering rate is still increasing and was much higher in late 2008 (12.8%) than in 2007 (6.4%), suggesting that more effective control strategies are needed.

This is the first report describing the molecular epidemiology of *M. tuberculosis* isolated from patients with pulmonary TB in Heilongjiang Province, China. The low clustering rate in our area indicates that only mild active transmission occurred in the time period studied. We defined the most suitable MIRU-VNTR locus set for analyzing the *M. tuberculosis* isolates in Heilongjiang Province, where Beijing family strains are prevalent. In our hands, the 15-locus set provided a high degree of discrimination; the 10-locus set was shown to be ideal for use in first-line molecular typing in future research although we still need to examine the discriminatory power of the rest of the recommended loci.

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