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Hepcidin exerts a negative immunological effect in pulmonary tuberculosis without HIV co-infection, prolonging the time to culture-negative

Ken Tashiro[a], Masaki Yamamoto[b,*], Ryota Ushio[b], Nobuaki Kobayashi[a], Takashi Sato[a], Makoto Kudo[b], Takeshi Kaneko[a]

[a] Department of Pulmonology, Yokohama City University Graduate School of Medicine, Yokohama, Japan
[b] Respiratory Disease Centre, Yokohama City University Medical Centre, Yokohama, Japan

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ABSTRACT

Objectives: A major regulatory peptide in iron metabolism, hepcidin, has been shown to predict mortality in HIV-infected tuberculosis patients. The aim of this study was to evaluate whether plasma hepcidin levels on admission can be used to predict the treatment outcome of patients with smear-positive pulmonary tuberculosis (PTB) without HIV co-infection.
Methods: In this prospective observational study, a total of 35 PTB patients with *Mycobacterium tuberculosis*-positive sputum smears were enrolled. The relationship between plasma hepcidin levels on admission and the time period to sputum culture-negative was explored.
Results: Plasma hepcidin levels of PTB patients were significantly higher than those of healthy subjects ($p < 0.001$). A positive correlation between hepcidin level on admission and the period until culture-negative was also observed ($r = 0.46$, $p = 0.006$). Furthermore, the hepcidin level showed a negative correlation with spot numbers in the positive control wells of the T-SPOT.TB assay; thus the effect of the peptide on interferon-gamma production in T cells was explored. Hepcidin reduced interferon-gamma gene transcription and interferon-gamma production in a dose-dependent manner in Jurkat cells stimulated with phytohaemagglutinin, an antigen non-specific stimulation.
Conclusions: These findings indicate that hepcidin alters immunological reactions against *M. tuberculosis* infection and has an influence on the outcomes of PTB patients without HIV co-infection.

Introduction

Tuberculosis (TB), a contagious disease, is usually transmitted by an aerosol of *Mycobacterium tuberculosis*, such as that caused by coughing. Although the global incidence of TB is gradually declining, there were still 9 million cases and 1.5 million deaths in 2013. In some parts of the world, especially in Africa, the incidence and drug resistance of TB remain a concern (Zumla et al., 2015).

Iron is an essential factor for both humans and pathogenic microbes, including *M. tuberculosis* (Ganz, 2018; Posey and Gherardini, 2000; Ratledge, 2004). Hepcidin is considered a major regulatory peptide in iron metabolism. The major role of this peptide is controlling the expression of ferroportin, a transmembrane protein responsible for iron transportation from the inside to the outside of a cell. Iron transportation by ferroportin is especially important in iron absorption from enterocytes, macrophages, and hepatocytes (Donovan et al., 2005). Internalization and degradation of ferroportin are promoted by binding to hepcidin (Delaby et al., 2005; Donovan et al., 2005; Ganz, 2006; Nemeth et al., 2004; Rivera et al., 2005).

Along with plasma concentrations and body stores of iron, hepcidin production is regulated by other factors, such as inflammation and hypoxia. For example, interleukin 6 (IL-6) is an important upstream mediator of hepcidin induction by inflammation (Sangkhae and Nemeth, 2017). Hypoxia has been reported to influence hepcidin expression (Silva et al., 2018; Sonnweber et al., 2014).

Hepcidin also has potency to modulate immunological reactions. Ferroportin (a target of hepcidin) overexpression on macrophages has been found to significantly impair intracellular *M. tuberculosis* growth during the early stages of infection in vivo

* Corresponding author at: Respiratory Disease Centre, Yokohama City University Medical Centre, 4-57 Urafune, Minami-ku, Yokohama 232-0024, Japan.
E-mail address: masakiy@yokohama-cu.ac.jp (M. Yamamoto).

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(Johnson et al., 2010). Hepcidin suppresses lipopolysaccharide-induced IL-6 and tumour necrosis factor alpha transcription and production in macrophages (De Domenico et al., 2010). Human hemochromatosis protein, another iron metabolism peptide, also has various immunological functions (Reuben et al., 2017).

These findings raise the question of whether iron-related factors might have some influence on immunological reactions and the clinical course in *M. tuberculosis* infection. Hepcidin levels have already shown predictive ability for the prospective risk of *M. tuberculosis* infection (Minchella et al., 2014) and mortality by active *M. tuberculosis* infection (Kerkhoff et al., 2016) in individuals with HIV infection. Furthermore, hepcidin levels are constant over age in healthy individuals except premenopausal women. On close investigation, a diurnal variation of hepcidin levels has been recognized (Schaap et al., 2013); however, the range of fluctuation of this diurnal variation is difficult to detect by enzyme-linked immunosorbent assay (ELISA) (Galesloot et al., 2011).

These features of the peptide appear favourable to adapt in clinical practice. However, reports on the predictive utility of the peptide in TB patients without HIV co-infection are limited. In a study only including a small proportion of HIV co-infected subjects, hepcidin levels of active TB patients at diagnosis were significantly higher than those of both tuberculin skin test (TST)-negative and positive contacts without active disease (Minchella et al., 2015). Another study conducted in Tanzania addressed household TB contacts and found that those who developed active disease possessed higher hepcidin levels than those who did not develop active disease, even in subjects without HIV co-infection (Hella et al., 2018).

This prospective observational study investigated hepcidin levels on admission and the time required to achieve sputum culture-negative status, as a clinical indicator for anti-TB treatment. Hepcidin levels and the time to culture-negative showed a positive correlation. Due to the negative correlation found between hepcidin levels and spot numbers in the positive control wells of enzyme-linked immunospot assays (T-SPOT.TB; Oxford Immunotec, Oxford, UK) observed in the pulmonary TB (PTB) patients in this study, the influence of the peptide on the immunological function of T cells was investigated, as the influence of hepcidin on macrophages has been evaluated previously. In the present study it was found that hepcidin has the potency to reduce interferon-gamma gene (*IFNG*) transcription and interferon-gamma (IFN-γ) production in CD4+T cells against non-specific stimulations.

Materials and methods

Ethics statement

The study protocol was approved by the Institutional Review Board of Yokohama City University School of Medicine and was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN000016454). Informed consent was obtained appropriately for the study subjects.

Subjects

From February 2015 to February 2016, sputum smear-positive PTB patients without HIV infection, admitted for isolation and treatment at Yokohama City University Hospital, were enrolled in this prospective observational study. All PTB patients were confirmed to have *M. tuberculosis* infection with conventional commercially available nucleic acid amplification tests, using acid-fast test-positive specimens. HIV infection status was evaluated by HIV Ag/Ab Combo test (Abbott Laboratories, Abbott Park, IL, USA) at the time of admission. Subjects with negative sputum culture, multidrug-resistant *M. tuberculosis*, those who had already started anti-TB therapy before hospitalization, those who were younger than 18 years of age, and those who had been admitted repeatedly due to TB were excluded.

The patients underwent clinical examinations and investigations of the levels of iron-related factors at admission for isolation and initiation of anti-TB chemotherapy. Laboratory tests except those for hepcidin were conducted in the hospital laboratory using peripheral blood specimens collected in vacutainer tubes containing clot activator. Serum iron was measured with a colorimetric method (Nitroso-PSAP method), ferritin by latex agglutination method, and transferrin and C-reactive protein (CRP) with an immunoturbidimetric method, using a LABOSPECT 008 analyzer (Hitachi High-Technologies Co., Tokyo, Japan). Blood samples for T-SPOT.TB assays were obtained at the time of hospitalization before treatment initiation using vacutainer tubes containing heparin. Samples for the T-SPOT.TB were transferred to an outside laboratory (SRL Inc., Tokyo, Japan) for the assays. Spot numbers of wells (NIL, ESAT6, CFP10, and mitogen) for each sample were reported. The results of the assays were interpreted by subtracting the spot count in the negative control (NIL) from the spot count in panels A and B; the results were reported as positive >8 spots, negative <4 spots, borderline 5, 6, or 7 spots, or invalid. All healthy control subjects enrolled were healthcare workers from the facility in the hospital and were confirmed not to have had a previous *M. tuberculosis* infection by T-SPOT.TB.

Bacteriological examination

Patient sputum samples were collected weekly. Samples were decontaminated, pelleted by centrifugation, examined for acid-fast bacilli (AFB) by fluorescence microscopy, and tested for *M. tuberculosis* by liquid culture with mycobacterial growth indicator tubes (Becton Dickinson, Sparks, MD, USA). The period from hospitalization to the day on which the third weekly consecutive culture-negative sputum sample was obtained was defined as the time to sputum culture-negative. The smear grade showing bacterial load was classified into four classes based on the Japanese guidelines using Ziehl–Neelsen stain (×1000): (−), 0 AFB/300 fields; (±), 1–2 AFB/300 fields; (+), 1–9 AFB/100 fields; (2+), 10–999 AFB/100 fields; and (3+), ≥10 AFB/field.

Anti-TB chemotherapy

Treatment regimens used in this study were the standard regimens recommended by the Japanese Society for Tuberculosis. Basically, subjects in this study received 2 months of daily oral isoniazid (5 mg/kg of body weight; maximum 300 mg/day), rifampicin (10 mg/kg; maximum 600 mg/day), pyrazinamide (25 mg/kg; maximum 1.5 g/day), and ethambutol (15 mg/kg; maximum 750 mg/day), followed by 4 months of isoniazid and rifampicin at the same daily doses. In cases with drug-induced liver dysfunction, treatment with 2 months of daily oral isoniazid, rifampicin, and ethambutol, followed by 7 months of isoniazid and rifampicin at the same daily doses given above was employed.

Measurement of plasma hepcidin levels

Peripheral blood specimens were collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA)-Na (Terumo, Tokyo, Japan). Whole blood samples were centrifuged for 10 min at 200 *g* (1500 rpm). Then, supernatant plasma samples were dispensed and preserved at −80 °C until measurement. Measurement of plasma hepcidin levels was conducted using an ELISA kit (Hepcidin 25 Bioactive; DRG Instruments GmbH, Marburg, Germany) according to the manufacturer's instructions.

Cells and media

Jurkat cells (ATCC TIB-152) were cultured in RPMI 1640 (ThermoFisher Scientific, Pittsburgh, PA, USA) with 10% foetal bovine serum (FBS) at 37 °C in 5% CO_2. Recombinant hepcidin (Wako, Osaka, Japan) was employed to evaluate the effect on *IFNG* transcription and IFN-γ production. According to the manufacturer, the recombinant hepcidin used in the study contains the active form hepcidin-25 with purity of 98% or more. Hepcidin was dissolved in phosphate-buffered saline (PBS) and the concentration was adjusted.

Measurement of IFNG *transcription levels*

Jurkat cells (5×10^5 cells/well) were cultured in 48-well plates with or without hepcidin (Wako, Osaka, Japan) for 2 h, then stimulated with a final concentration of 0.0005% phytohaemagglutinin (PHA) for 2 h. To measure *IFNG* mRNA transcription levels, total RNA was isolated from Jurkat cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Synthesis of cDNA from 100 ng total RNA was conducted using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR was performed on the StepOne Plus Real-Time PCR system with TaqMan Fast Advanced Master Mix and Gene Expression Assays for *IFNG* and the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) as a standard (Applied Biosystems, Waltham, MA, USA). All experiments were conducted according to the manufacturer's instructions.

Measurement of IFN-γ production levels

Jurkat cells (2.5×10^5 cells/well) were cultured in 48-well plates with or without hepcidin (Wako, Osaka, Japan) for 2 h, then stimulated with a final concentration of 0.001% PHA for 24 h. The cell culture supernatants of Jurkat cells were measured with human IFN-γ Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) as per the manufacturer's procedures.

Statistical analysis

The Mann–Whitney *U*-test was used to compare plasma hepcidin levels of PTB patients and healthy subjects. To evaluate correlations between plasma hepcidin levels and other clinical characteristics, Spearman's correlation test was used. To evaluate time-dependent event outcomes, the Kaplan–Meier curve and log-rank test for trend were applied. The Cox proportional hazards model was used to assess the impact of laboratory data and time to culture-negative. The relationship between hepcidin levels and smear results on admission was evaluated by Mann–Whitney *U*-test. Sputum smear on admission was converted to a binary outcome 0, 0.5, 1, 2 or 3. The paired *t*-test was used to identify significant differences between the two groups in quantitative real-time RT-PCR analysis for *IFNG* and ELISA for IFN-γ. *p*-Values of less than 0.05 were considered significant. In the in vitro study, all experiments were conducted more than three times independently. Data are expressed as the mean value ± standard error of the mean (SEM). All analyses were conducted using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA) and JMP Pro 12 (SAS Institute Inc., Cary, NC, USA).

Results

Patient and subject characteristics

From February 2015 to February 2016, 35 PTB patients and 15 control subjects were enrolled. Characteristics of the subjects are shown in Table 1. The mean age of the control group subjects was significantly lower than that of the PTB group patients. The type (with or without cavities) and distribution of pulmonary lesions were evaluated by computed tomography (CT). For subjects with symptom (s) unlikely to be from respiratory lesions, CT and/or magnetic resonance imaging examinations were conducted to evaluate extrapulmonary lesion(s) (Table 2). No strains isolated from PTB patients in this study showed resistance to the four standard treatment drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol).

Table 1
Characteristics of the study subjects.

	PTB patients ($n = 35$)	Controls ($n = 15$)	*p*-Value
Age (years)	66.2 ± 19.2	30.5 ± 3.9	<0.001
Sex, male	20 (57.1%)	10 (66.7%)	0.54
Hepcidin (ng/ml)	45.1 ± 20	26.4 ± 9.7	<0.001

PTB, pulmonary tuberculosis. Age data and hepcidin levels are expressed as the mean value ± standard deviation. All *p*-values were derived by Mann–Whitney *U*-test.

Plasma hepcidin levels in PTB patients

Plasma hepcidin levels of PTB patients were significantly higher than those of healthy subjects ($p < 0.001$) (Table 1). Serum iron levels of PTB patients were lower than the lower limit of normal (Table 2). As hepcidin has a major role in the regulation of serum iron concentration, it was expected that hepcidin levels would show a significant correlation with serum iron levels, but they did not (Figure 1A). There was a negative correlation between hepcidin levels and serum transferrin levels (Figure 1B). The levels of the peptide showed a positive correlation with levels of serum ferritin, a peptide complex related to iron storage (Figure 1C). Since hepcidin production is regulated by IL-6, the correlation between hepcidin and CRP, another IL-6-induced protein, was examined and showed a positive correlation (Figure 1D). To identify any influence of infection burden on hepcidin levels, AFB smear status, the types of lung lesions (with or without cavities), and the distribution of lung lesions (unilateral or bilateral) were evaluated with regard to hepcidin levels; no significant differences were identified (Table 3A–C). These results indicate that hepcidin production is controlled by factors other than iron levels in PTB patients.

Hepcidin levels associated with time to culture-negative

The correlation between hepcidin levels on admission and time to achieve sputum culture-negative status was examined, and a positive correlation was found ($r = 0.46$, $p = 0.006$) (Figure 2A). CRP

Table 2
Clinical features of pulmonary tuberculosis patients.

TB lesions	
Bilateral pulmonary lesions	21 (60.0%)
With cavity lesion(s)	17 (48.6%)
With extrapulmonary lesion(s)	3 (8.6%)
Comorbidities	
Immunosuppressive state	3 (8.6%)
Chronic liver disease	1 (2.9%)
Chronic renal disease	3 (8.6%)
Active malignancy	2 (5.7%)
Laboratory data	
Haemoglobin (g/dl)	11.5 ± 2.2
Fe (μg/dl)	41.9 ± 31.7
Ferritin (ng/ml)	364.8 ± 427.5
Transferrin (mg/dl)	159.2 ± 55.7
CRP (mg/dl)	5.82 ± 5.5
Sputum smear AFB ≥2+	16 (45.7%)[a]

TB, tuberculosis; CRP, C-reactive protein; AFB, acid-fast bacilli. Laboratory data are expressed as mean values ± standard deviation.
[a] Number and ratio of patients with more than 10 AFB/100 fields.

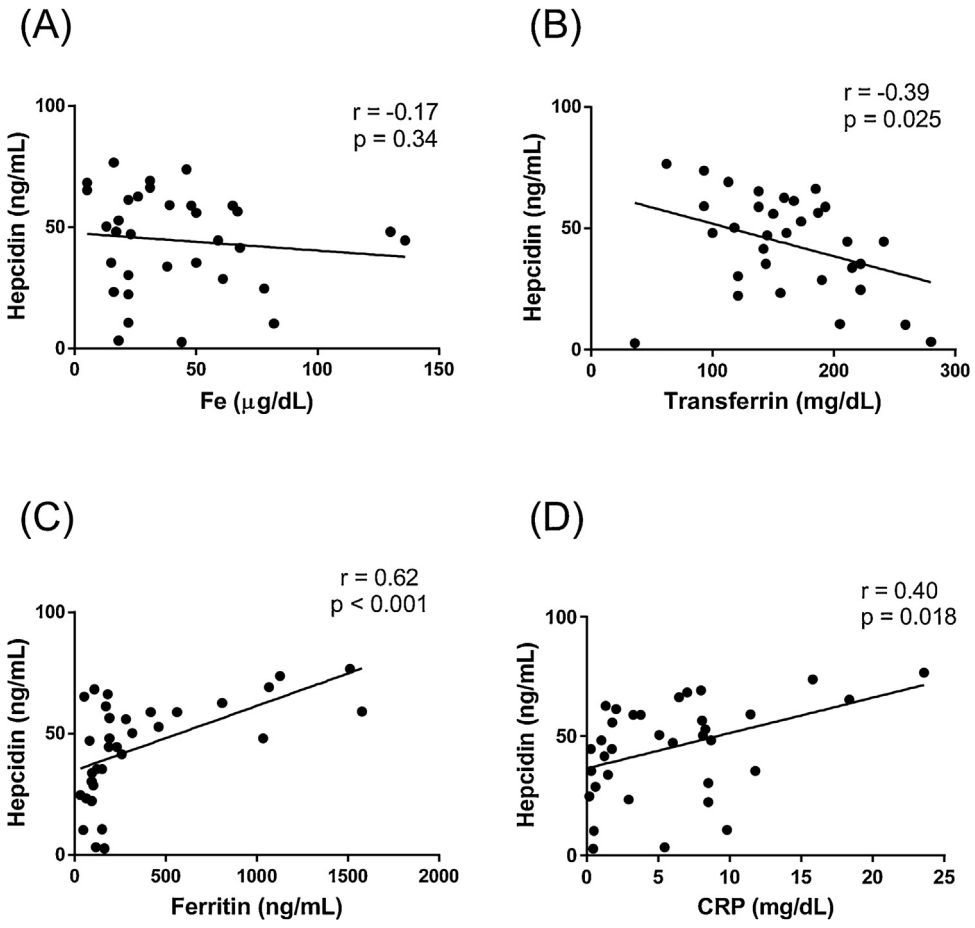

Figure 1. Scatter plot of hepcidin levels on admission versus laboratory data. The vertical position of each dot indicates the hepcidin level. The horizontal position of each dot shows the level of the indicated factor. (A) Serum iron; (B) serum transferrin; (C) serum ferritin; (D) serum CRP. Correlation between two variables was evaluated using Spearman's correlation test. *r*: Spearman's rank correlation coefficient.

levels also showed a positive correlation with time to culture-negative ($r = 0.45$, $p = 0.007$) (Figure 2B), consistent with the correlation between them. Other factors did not exhibit better correlations than hepcidin levels (Figure 2C–E). As the overall median value of hepcidin in the PTB group was 48.2 ng/ml, the PTB patients were categorized into two groups in order to assess the influence of hepcidin: high hepcidin (≥50 ng/ml) and low hepcidin (<50 ng/ml). The time to achieve culture-negative status was compared between the two groups using the Kaplan–Meier curve and log-rank test for trend. The high hepcidin group required significantly longer to achieve negative culture ($p = 0.005$) (Figure 3). Next, the burden of infection, in addition to the distribution of lung lesions (unilateral or bilateral) and smear grade were compared between the high hepcidin and low hepcidin groups. There were no statistically significant differences between them (Table 3). Multivariate analysis was performed on laboratory data (hepcidin, CRP, and iron), with significant differences found in the univariate analysis. In the Cox proportional hazards analysis, serum iron and hepcidin were found to independently affect the time to culture-negative (Table 4).

Table 3
Relationship between hepcidin levels and infection burden.

(A) Relationship between hepcidin levels and AFB			
	Hepcidin ≥50 ng/ml ($n = 17$)	Hepcidin <50 ng/ml ($n = 18$)	*p*-Value
AFB (0.5, 1, 2, or 3)	1 (1–2.5)	1 (1–2)	0.61
(B) Plasma hepcidin levels and types of lung lesions (with or without cavities)			
	With cavity lesion(s) ($n = 17$)	No cavity lesion ($n = 18$)	*p*-Value
Hepcidin (ng/ml)	48.2 ± 20.9	42.0 ± 19.2	0.26
(C) Plasma hepcidin levels and distribution of lung lesions (unilateral or bilateral)			
	Bilateral pulmonary lesions ($n = 21$)	Unilateral pulmonary lesion(s) ($n = 14$)	*p*-Value
Hepcidin (ng/ml)	45.3 ± 21.0	44.6 ± 19.3	0.91

AFB, acid-fast bacilli. The AFB value is expressed as the median value with interquartile range. The *p*-value was derived by Mann–Whitney *U*-test.
Hepcidin levels are expressed as the mean value ± standard deviation. The *p*-value was derived by Mann–Whitney *U*-test.

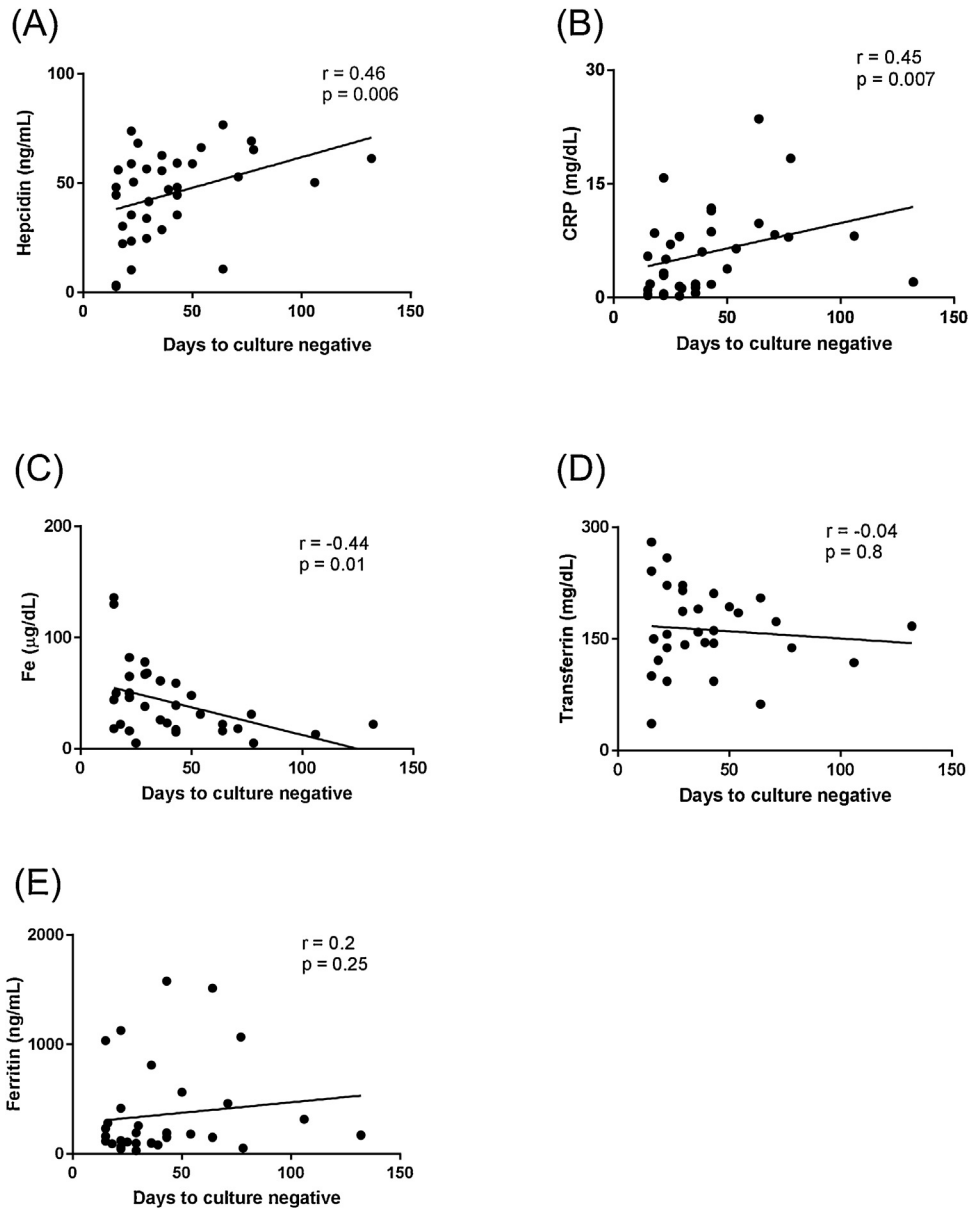

Figure 2. Scatter plot of laboratory data on admission versus days to culture-negative. The vertical position of each dot shows the level of the indicated factor. (A) Hepcidin, (B) serum CRP, (C) serum iron, (D) serum transferrin, (E) serum ferritin. The horizontal position of each dot indicates the days to culture-negative. Correlation between these two variables was evaluated using Spearman's correlation test. *r*: Spearman's rank correlation coefficient.

Influence of hepcidin on T cell IFN-γ production under non-specific stimulation

During exploration for clinical features associated with hepcidin levels, a negative correlation was unexpectedly identified between hepcidin levels and the spot numbers in positive control wells of the T-SPOT.TB assay ($r = -0.38$, $p = 0.028$) (Figure 4A). Although the cause was not identified, it has been reported that the spot numbers in the positive control wells of active PTB patients are lower than those of patients with latent *M. tuberculosis* infections (LTBI) (Wang et al., 2016). Hepcidin levels have been shown to be higher in active PTB patients than in those without active TB lesions (Minchella et al., 2015), as shown in the present study. These results led to the hypothesis that hepcidin directly affects IFN-γ production in T cells responding to PHA, a non-specific stimulation.

To clarify the possible negative effect of hepcidin on IFN-γ production in T cells, *IFNG* mRNA transcription levels in Jurkat cells, immortalized T lymphocytes, were quantified. Jurkat cells were cultured in several concentrations of hepcidin and then stimulated with PHA. The results showed that hepcidin decreased *IFNG* transcription ($p = 0.015$) and IFN-γ production ($p = 0.001$) levels in a dose-dependent manner in PHA-stimulated Jurkat cells (Figure 5A and B, respectively).

Next, the effect of hepcidin on the CD4+T cell response to antigen-specific stimulation was investigated in T-SPOT.TB assays. There was no significant difference in mean spot numbers or ratio against spots in the positive control well (**Supplementary Material**, Table S1); however, a negative correlation between hepcidin levels and spot numbers was identified in ESAT6 ($r = -0.45$, $p = 0.007$), but not in CFP10 ($r = -0.076$, $p = 0.7$) (Figure 4B and C).

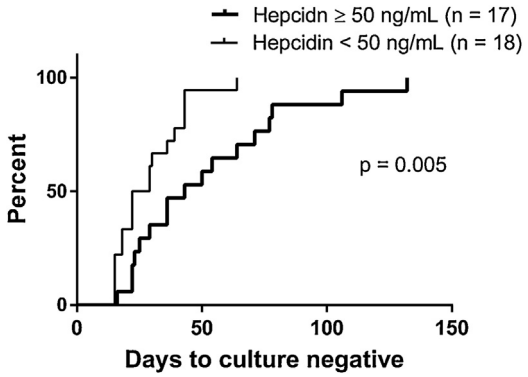

Figure 3. Kaplan–Meier analysis for time to culture-negative. Pulmonary tuberculosis subjects were categorized into two groups: high hepcidin (≥50 ng/ml hepcidin) and low hepcidin (<50 ng/ml). The time taken to achieve culture-negative status was compared between the two groups using the Kaplan–Meier curve and log-rank test for trend. *p*: log-rank test for trend.

Table 4
Cox proportional hazards analysis.

	HR (95% CI)	*p*-Value
Hepcidin	0.977 (0.959–0.997)	0.024
CRP	0.992 (0.911–1.075)	0.856
Fe	1.013 (1.002–1.023)	0.023

HR, hazard ratio; CI, confidence interval; CRP, C-reactive protein.

Discussion

This study suggests that the response to anti-TB chemotherapy in PTB patients varies with hepcidin concentration.

Hepcidin is a peptide hormone secreted mainly by hepatocytes, but also by a variety of other cells. Hepcidin was initially identified as a member of the defensin family. As an antimicrobial peptide, this peptide has been reported to have the ability to cause structural damage to *M. tuberculosis* residing in phagosomes of macrophages, subsequently inhibiting *M. tuberculosis* growth (Sow et al., 2007). Along with this direct antimycobacterial activity, hepcidin plays an important role in iron homeostasis. In short, hepcidin binds to the cell surface iron transporter ferroportin, leading to its internalization and degradation (Zhao et al., 2013). Hepcidin synthesis is not only influenced by iron levels (Ganz and Nemeth, 2015), and hepcidin secretion is increased in response to infection or inflammation, leading to hypoferraemia caused by inflammation (Goodnough et al., 2010; Nicolas et al., 2002).

Iron redistribution caused by increased hepcidin can be protective against malarial and extracellular bacterial infections (Arezes et al., 2015; Ganz and Nemeth, 2015). In contrast, as reported by studies in mouse models, hepcidin-induced iron sequestration may be beneficial for intracellular microorganisms (Nairz et al., 2009), including *M. tuberculosis* (Olakanmi et al., 2007).

With regard to clinical findings, higher hepcidin levels were found to indicate a significantly greater risk of developing *M. tuberculosis* infection in subjects with newly diagnosed HIV infection (Minchella et al., 2014). High hepcidin concentrations have also been strongly associated with disseminated disease and a poor prognosis in patients with HIV-associated TB (Kerkhoff et al., 2016). However, information about the influence of hepcidin on the clinical course of *M. tuberculosis* without HIV co-infection is limited. Therefore, the present study was performed to explore the possible effect of hepcidin on the clinical course of *M. tuberculosis* without HIV infection.

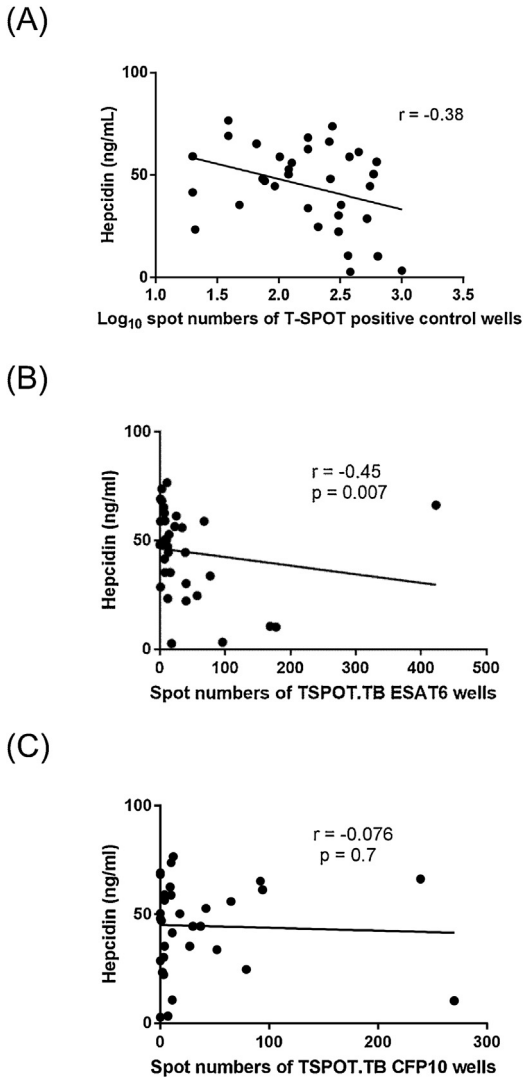

Figure 4. Scatter plot of hepcidin levels on admission versus spot numbers of positive control, ESAT6, and CFP10 wells in the T-SPOT.TB assays. The vertical position of each dot indicates the hepcidin level. The horizontal position of each dot indicates the spot numbers in the T-SPOT.TB assays. (A) Log_{10} positive control; (B) ESAT6; (C) CFP10. Correlation between these two variables was evaluated using Spearman's correlation test. *r*: Spearman's rank correlation coefficient.

To assess the effect of hepcidin on the clinical course of *M. tuberculosis* infection, the time to culture-negative status in PTB patients was investigated. In Japan, loss of infectivity is defined as three consecutive negative sputum cultures; thus, microbiological examinations of respiratory specimens are regularly conducted. Moreover, microbiological testing directly reflects the efficacy of anti-TB chemotherapy. Other elements, for example, hospitalization days or the period until hospital death, could be strongly influenced by comorbidities and medical or social conditions. The results of this study showed that higher hepcidin concentrations at treatment initiation were associated with a longer time to culture-negative status (Figure 2A and 3).

As reported previously (Kerkhoff et al., 2016; Wisaksana et al., 2013), plasma hepcidin levels were found to be significantly higher in PTB patients than in healthy control subjects (Table 1). A significant difference in age between the PTB and healthy groups was observed; however, plasma hepcidin levels are reportedly not influenced by age (Galesloot et al., 2011). In general, hepcidin production is regulated by iron levels. In the present study, levels of hepcidin and iron did not show a stronger association than

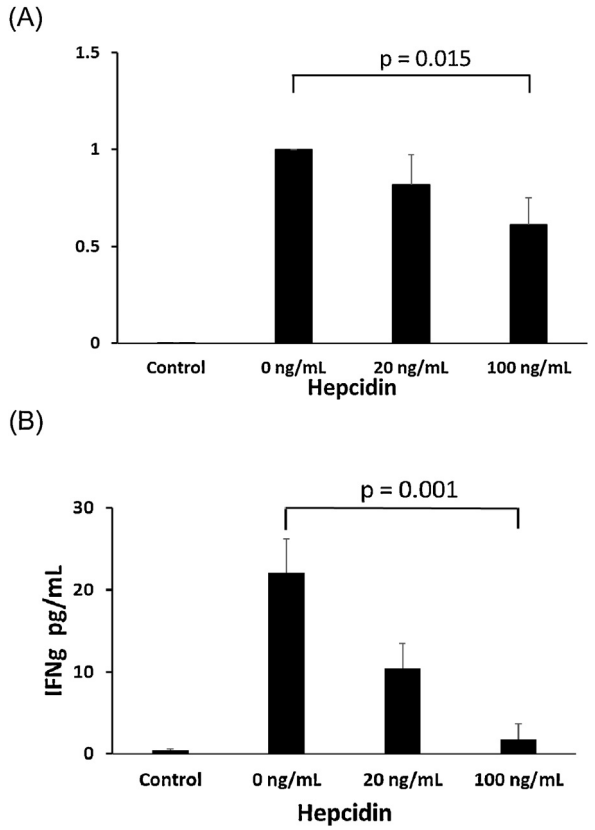

Figure 5. *IFNG* transcription and IFN-γ production levels in Jurkat cells stimulated with antigen non-specific stimulation. (A) Jurkat cells (5×10^5 cells/well) were cultured in 48-well plates with indicated hepcidin concentrations for 2 h, and then stimulated with a final concentration of 0.0005% phytohaemagglutinin for 2 h. Total RNA was isolated and reverse-transcribed. Quantitative real-time PCR for *IFNG* was subsequently performed. Values are presented as the mean ± SEM ($n = 4$ independent experiments). All *p*-values were derived by two-tailed paired *t*-test. (B) Jurkat cells (2.5×10^5 cells/well) were cultured in 48-well plates with indicated hepcidin concentrations for 2 h, and then stimulated with a final concentration of 0.001% phytohaemagglutinin for 24 h. Cell culture supernatants of Jurkat cells were analysed with human IFN-γ Quantikine enzyme-linked immunosorbent assay kits. Values are presented as the mean ± SEM ($n = 5$ independent experiments). All *p*-values were derived by two-tailed paired *t*-test.

hepcidin levels and other factors, suggesting that hepcidin production is strongly controlled by factors other than iron status under the condition of *M. tuberculosis* infection. Levels of hepcidin showed a positive relationship with CRP, a biomarker of inflammation, and ferritin (Figure 2C, D). Ferritin, as an acute phase protein, is also strongly influenced by inflammation (Namaste et al., 2017). The effects of inflammation on nutritional biomarkers, especially iron-related factors, have been assessed by a collaborative research group called Biomarkers Reflecting Inflammation and Nutrition Determinants of Anemia (BRINDA) (Suchdev et al., 2016). While this project was not conducted with TB subjects, reports from the research group can be applied to interpret iron status and iron-related factors under inflammation caused by TB. It remains uncertain whether hepcidin has direct effects on ferritin production or not.

In addition to its influence on macrophages (De Domenico et al., 2010; Ganz and Nemeth, 2015), hepcidin was found to reduce *IFNG* transcription and IFN-γ production against non-specific stimulation in T cells (Figure 5A, B). These findings indicate that hepcidin could directly affect IFN-γ production in T cells. IFN-γ is essential in the host defence against mycobacterial infection, including infection by *M. tuberculosis* (Flynn et al., 1993; Kampmann et al., 2005). In HIV-infected individuals, the risk of TB incidence is increased with declining CD4+ cells count, a major source of IFN-γ (Ellis et al., 2017). Decreased IFN-γ production in T cells caused by hepcidin may impair the protective capability against *M. tuberculosis*, resulting in a prolonged time to culture-negative. This suppressive effect of hepcidin on IFN-γ production in peripheral blood T cells from PTB patients was only observed under stimulation with ESAT6 and not with CFP10 (Figure 4).

Several limitations of this study should be addressed. First, this was a single-centre study in a developed country with moderate TB incidence. Therefore, the range and effect of hepcidin might differ in other settings, such as developing countries with a high TB incidence. In developing countries with high TB incidence, inadequate nutritional conditions, especially for iron, may have an influence on the clinical course of PTB. Second, all of the PTB patients in this study required isolation due to respiratory lesions with transmission capacity. In patients with less or more severe conditions, for instance patients with no transmission capacity or with disseminated and/or central nervous system lesions, the influence of hepcidin could be different from that observed in the present study cohort. Third, the evaluation of hepcidin levels was conducted only at treatment initiation in this study. Fluctuations in hepcidin levels after starting anti-TB chemotherapy should be evaluated in a further study. Furthermore, the evaluation of IFN-γ levels in clinical specimens was not conducted in this study. An effect of hepcidin on IFN-γ transcription and production was observed in ex vivo cells with artificial stimulation. Unfortunately, the direct relationship between hepcidin and IFN-γ levels in clinical specimens could not be evaluated in this study. Fourth, an effect of hepcidin on *M. tuberculosis*-specific antigen stimulation could be identified only in the ESAT6 wells in this study (Figure 4B and C). In general, a low frequency of CD4 + T cells specific for an antigen makes it difficult to conduct direct ex vivo analysis from clinical subjects, because of sample volume requirements (Uchtenhagen et al., 2016). To evaluate an effect on antigen-specific stimulation, more efficient direct ex vivo analysis or an alternative method must be considered. While these limitations do not allow definitive conclusions to be drawn on the direct impact of hepcidin on the clinical course of PTB patients under treatment, the study findings may help in the development of an alternative therapeutic intervention strategy for TB elimination.

As hepcidin pathways are linked with haematological, metabolic, and infectious diseases, therapeutic modulations of hepcidin pathways have already been under evaluation (Katsarou and Pantopoulos, 2018). These trials for hepcidin modulations have mainly been conducted in patients with iron metabolism disorders; however, studies on immunological functions of hepcidin could help to expand the clinical utility of hepcidin modulators in other fields, especially TB infection control.

In conclusion, the plasma hepcidin level on admission for PTB treatment in the absence of HIV co-infection has the ability to predict the therapeutic response. The negative influence of the peptide in reducing IFN-γ production from T cells with a non-specific stimulation partly help to support this finding. It is necessary to elucidate relevant mechanisms, especially the response to antigen-specific stimulation and intracellular alterations in T cells by this peptide.

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Ethical approval

The study protocol was approved by the Institutional Review Board of Yokohama City University School of Medicine and was registered in the University Hospital Medical Information Network

Clinical Trials Registry (UMIN000016454). Informed consent was obtained appropriately for subjects.

Conflict of interest

The authors declare no conflicts of interest associated with this article.

Author contributions

KT and MY designed the study. KT conducted the in vitro and ex vivo experiments and analysed the data. KT and MY wrote a draft of the manuscript. MY, a principal investigator, and TK were responsible for critical revision of the manuscript. RU, NK, TS, and MK contributed to the data collection.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.06.023.

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