陽性を示し感染例として化学予防の対象となった(画像的検索において発症は確認されず).また、管轄保健所により患児の通学する小学校児童を対象とした接触者検診が実施されたが感染例と判断された児童はなかった.

【考察】

今回報告した症例は、①小学校低学年児童に発症した塗沫陽性重症肺結核症例、さらに②喉頭結核病変を合併した、という二つの点において極めて稀な症例である.

近年、本邦における小児結核症例は年間 120 例前後へと減少し、新登録塗沫陽性小児肺結核患者数は 2000 年以降年間 10 例未満の少数例で推移している.特に 5～9 歳の年齢層での塗沫陽性肺結核患者数は毎年 0～1 例と稀である.この年齢層において本例のように両側全肺野に拡がる広汎な病巣を形成した重症肺結核を経験することは極めて稀であり、過去 20 年間の本邦の文献に同様な症例報告は確認されなかった.また、小児における喉頭結核症例の報告も世界的にも極めて少なく[1)、2)、3)、4)]、本邦では 1986 年以降現在まで江口らの粟粒結核に伴う学童例の報告[5)]が見られるのみである.喉頭結核はその進展経路により 1)進行した活動性肺結核病巣から経気管的に喉頭粘膜へ直接侵入し二次的に病巣を形成するもの、及び 2)血行性、リンパ行性に進展し粟粒結核の部分症状として現れるもの、の二つのタイプに分類される[6)].本例はその胸部CT所見より経気道性及び血行性の両機序により肺野病変が進展拡大したものと判断したが[7)]、喉頭病変についてはその喉頭ファイバー所見、即ち喉頭のみならず声門下気管粘膜にも連続した病変を認めたことより前者の進展様式が強く疑われた.

一般に小児結核症例では塗沫陽性例が少なく、また咳嗽も無いか軽度であることがほとんどであり、周囲への感染源となることは極めて稀である.しかしながら、Curtisら[8)]は空洞を有する 9 歳の塗沫陽性肺結核症例において患児を中心に多数の感染例が確認された事例を報告し、小児であっても空洞を有する肺結核や喉頭結核症例は周囲への感染源として極めて重要であり、症例を中心とした慎重な接触者検診の実施が必要である、と述べている.本症例も長期に亘って湿性咳嗽が持続した空洞を伴う塗沫陽性肺結核例、喉頭結核合併例であり、集団感染事例への進展が懸念された.管轄保健所の指導のもと、患児を中心とした接触者検診が実施され、患児の姉、兄、従妹 3 人が感染例として化学予防対象とされたが、通学していた小学校では幸いにも感染・発症例は 1 例も検出されなかった.

では、なぜ本症例では両側全肺野にびまん性に拡がる広汎な病巣が形成され、高度の嗄声や吸気性喘鳴を伴う喉頭結核病巣を呈するまで重症化してしまったのであろうか?

本症例では診断時ツ反陰性、CD4 陽性 T 細胞数減少など細胞性免疫能減弱を示唆する所見を認めたが、過去に BCG 感染を含む易感染性を示唆するエピソードはなく、抗結核剤治療開始後は順調に治癒傾向が見られ、治療終了時にはツ反は再度陽転、リンパ球サブセットも正常化した.これらより診断時には重症結核に伴う続発性細胞性免疫不全状態にあったが、T 細胞の欠陥や INF-γ及び IL-12 レセプター変異を伴う原発性免疫不全の存在は否定的と判断した.

本症例の経過を振り返り、①同居祖父結核発症時の接触者検診の不備、②有症状受診例に対する診断の遅れ＝“Doctor's delay”、③問診を中心とした学校結核健診の限界、などの要因が重なり合った結果、本症例の重症化に繋がったものと考察した.

①同居祖父結核発症時の接触者検診の不備

患児が 4 歳時、連日濃厚な接触を有する同居祖父が咳嗽、血痰などの症状を伴って肺結核を発症し管轄保健所において接触者検診が実施された.二重発赤を伴うツ反強陽性（発赤径 32mm）が明らかとなったが、旧厚生省化学予防適応基準に沿った判断により化学予防の対象とはされなかった(BCG既接種で塗沫陽性患者との接触があった場合、ツ反発赤径 30mm 以上で化学予防対象となるが本例では感染源が塗沫陰性であった).また、初回検診時に胸部単純写真のチェックを受けた後は定期的な検診実施を指示されることなく放置されてい

た．喀痰塗沫検鏡の結果を問わず、呼吸器症状を有する結核患者と頻回かつ濃厚な接触を有する幼児において二重発赤を伴うツ反強陽性が判明した場合には、感染成立の可能性を強く疑って化学予防を開始する、或いは発症の可能性も考慮に入れ定期的な経過観察を行う、などの慎重な対応が必要であったのではなかろうか．雉本ら[9]や高松ら[10]はそれぞれの小児結核症例に関する検討のなかで、結核発症例のうち約 3 割が旧厚生省基準の化学予防適応外に相当し、同基準を機械的に適用することにより化学予防が必要な症例の相当数が見逃される危険性を指摘している．BCG接種歴やツ反結果のみを機械的に当てはめるのでなく、それぞれの症例において感染源の状況（排菌状況や症状、有症状期間）、感染源との接触頻度、検診対象児の年齢などを含む感染成立のリスクを丁寧に評価した上で感染（＝化学予防適応）の有無を判断する態度が必要であろう．また、感染の可能性が疑われるケースについては常に発症の可能性を念頭においた慎重なフォロー・アップ（概ね接触判明後 2 年間に亘る画像的検索）を継続することも重要である．本ケースでは初回検診結果の機械的解釈、さらにその後の慎重な経過観察体制の欠如が重症化へ繋がった可能性は否定できない．
また、さらに接触者検診の精度を向上させる為、結核菌特異抗原刺激によるINF-γ測定検査（QuantiFERON® -TB2G）の導入に関して検討することも必要であろう．小児結核症例診断における本検査の有効性は既に報告されている[11]が、今後小児潜在感染例診断における有用性の検討、さらに小児を対象とした接触者検診のおける本検査適応及び結果解釈に関するガイドラインの作成も強く望まれる．
②有症状受診例に対する診断の遅れ＝“Doctor's delay”
本症例では診断される半年以上前より嗄声や咳嗽などの呼吸器症状が遷延し、頻回に医療機関を受診していた．しかしながら、適切な診断、治療開始が遅れ、結果として多量の排菌を伴う重症肺結核、喉頭結核へと進展し、高度の貧血や低蛋白血症、成長障害を伴う状態に至った．即ち、患者側の医療機関受診の遅れ（”Patient's delay”）ではなく、医療機関における診断の遅れ（”Doctor's delay”）が重症化に繋がった．遷延する呼吸器症状を主訴とする症例で、その原因が明らかでない、或いは初期治療に対して抵抗性で改善傾向が乏しい場合には胸部聴診所見や発熱の有無にかかわらず早期に画像的評価を実施する姿勢が必要である．また、診察に際しては予防接種歴や家族歴を含む問診を徹底、再確認することも重要である．
③問診を中心とする学校結核健診の限界
小児結核症例の減少を受けて2003年春より学校における結核健診の方法が大きく変更され、それまでの結核予防法に基づくツベルクリン反応による定期健診は中止され、学校保健法による定期健診の中に結核に関する問診が導入された．即ち、結核に関する治療歴・予防内服歴・家族歴、結核高蔓延地域での最近の居住歴、長く続く咳や痰などの呼吸器症状の有無、BCG 接種歴などに関する問診結果を教育委員会に設置された結核対策委員会において検討し、精検の要否を判断する方法へと変更された．
本症例において学校結核健診は有効に機能したのであろうか？本症例では 2004 年春（小学校入学時）の問診票に家族の結核既往歴が記載されていたにもかかわらず、レントゲン検査を含む精検対象とされなかった．結核に関する治療歴、予防内服歴、家族歴に関する問診結果が要検討例に該当していても保健所からの情報により適切に事後管理がされている、或いは発病のおそれなし、等と判断されれば精検対象から外されてしまう．同様に有症状例であっても医療機関を受診し検査・治療中であれば要精検例から除外されることとなる．
学校結核健診方法の変更により、これまでツ反結果のみから適応ありと判断されていた過剰な化学予防対象例が著減した一方で、学校健診により発見される患者数も減少しており[12]新たに導入された健診方法の有効性（特にその感度）について再度検討する必要があろう．

以上、本症例が重症化するに至った 3 つの要因は、小児結核の予防及び早期診断に向けて特に重点を置いて取り組むべき重要な方策である．即ち、小児結核症例は身近な親族から強い感染を受けて発症するケースが多く、周囲に感染源が判明した場合には迅速かつ徹底した

接触者検診を実施し、慎重な事後処置を行うことが非常に大切である．また、小児結核の発見機会としては家族検診（約 50%）に次いで医療機関受診（約 25%）が多く[13]、有症状受診に対する適切な対応も非常に重要である．長期に遷延する呼吸器症状を主訴とする症例に対しては結核感染症の可能性も念頭に置き早期に適切な画像的評価を行うことが必要である．さらに、10 歳代の新登録患者数は近年横這いで推移しており、学校結核健診の必要性は決して低下していない．健診問診結果を慎重に検討し、要精検例に対して精度の高い精密検査を実施することも重要である．また、効率的、かつ精度の高い学校健診を行う為に、現在の学校結核健診制度に関する批判的な検討を行うことも必要であろう．

【結語】

7 歳女児に発症した塗沫陽性重症肺結核症の一例を経験した．両側全肺野に広汎に分布する多彩な病巣を認め、さらに喉頭結核病変の合併も確認された．肺野病変はその CT 所見より経気道性及び血行性の両機序により全肺野に散布したものと判断した。診断に至るまで長期間に亘って咳嗽や嗄声、発熱等の症状が持続し、頻回に医療機関を受診していた．患児を中心とする接触者検診では家族内に 3 例の潜伏感染例を認めたが、幸いにも通学する小学校における感染・発症例は認めなかった．同居祖父結核発症時の接触者検診の不備、有症状受診例に対する診断の遅れ（“Doctor’s delay”）、問診を中心とした学校結核健診の限界、などの要因が重なりあって、本症例の重症化に繋がったものと考えた．未だ年間 120 例前後の小児結核新規発症例が報告され、小児の親の世代に当たる 20 歳代、30 歳代の結核登録患者数も横這いで推移しており、小児結核を取り巻く環境は未だ油断できる状況にはない．小児結核の予防及び早期診断の為、乳児期早期からの BCG 接種徹底に加え、小児の特殊性を考慮に入れた精度の高い接触者検診の実施、有症状受診例に対する早期の画像検査を含む適切な対応、学校結核健診要精検例に対する慎重な対応、などの対策徹底が強く望まれる．

謝辞：本症例の画像所見につき御教授頂きました当院放射線科游逸明先生、古市健治先生、呼吸器内科佐藤敦夫先生に深謝致します。

Key Words
小児結核・肺結核・喉頭結核・接触者検診・Doctor’s delay

表1．入院時検査所見

WBC	12300	/μl
Stab	8.5	%
Seg	70.0	%
Mono	7.5	%
Baso	0.5	%
Lym	13.5	%
RBC	454X10⁴	/μl
Hb	8.4	g/dl
MCV	61.5	fl
MCH	18.6	pg
Plt	69.2X10⁴	/μl
ESR(1hr)	97	mm
TP	6.1	g/dl
Alb	2.8	g/dl
AST	22	IU/l
ALT	11	IU/l
LDH	299	IU/l
BUN	3.0	mg/l
Cr	0.25	mg/l

Fe	3	μg/d
Ferritin	76.0	ng/l
CRP	20.13	mg/d
IgG	1360.0	mg/l
IgA	234.0	mg/l
IgM	96.7	mg/l
CH50	50.7	U/ml
Mycoplasma Ab	X320	
β-D-glucan	<4.76	pg/l
CD3+	30.3	%
CD3+CD4+	14.4	%
CD3+CD8+	13.6	%
CD4/CD8 ratio	1.06	

髄液一般検査

細胞数	0/3	
Protein	11	mg/l
Glucose	52	mg/l
ADA	4	IU/l

細菌検査

喀痰）

抗酸菌塗沫	(2+)
PCR (*M..tuberculosis* complex)	(+)
培養（小川）	(+);30コロニー
薬剤感受性（各種抗結核剤）	良好

髄液）

抗酸菌塗沫	(-)
PCR (*M..tuberculosis* complex)	(-)
培養	(-)

ツベルクリン反応　0X0/0X0mm;(-)

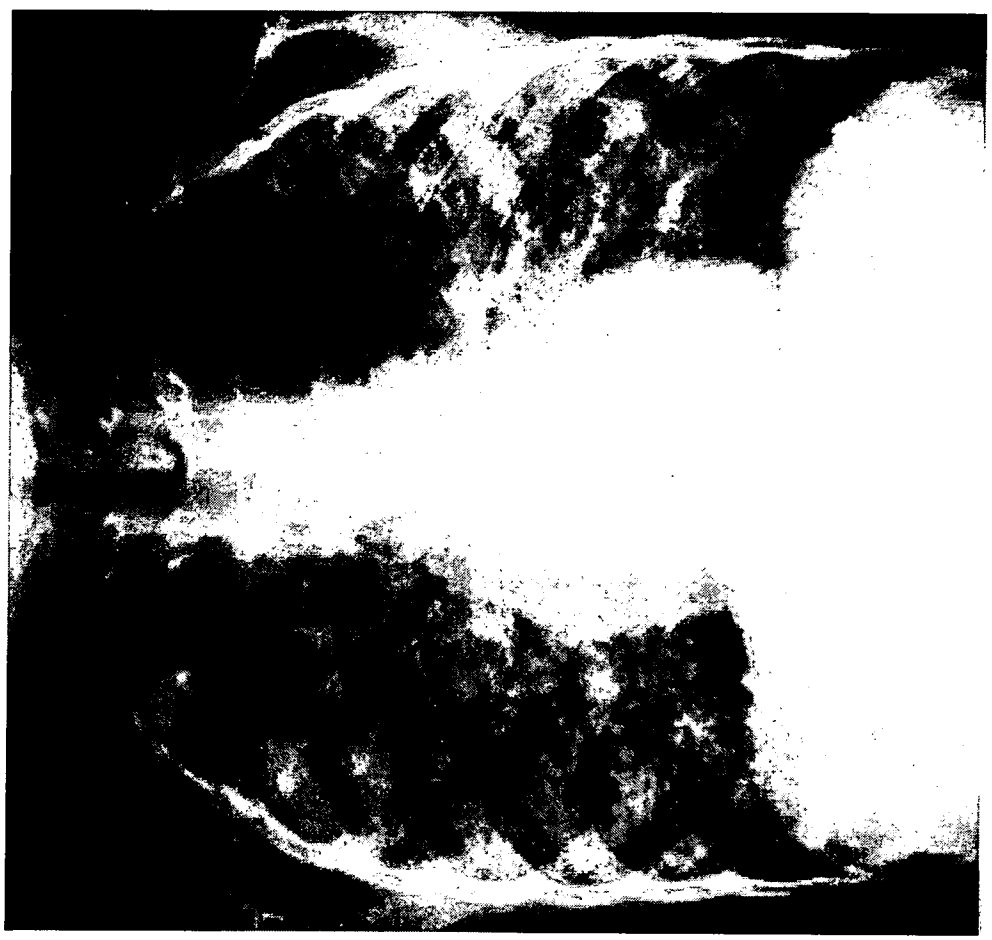

図1

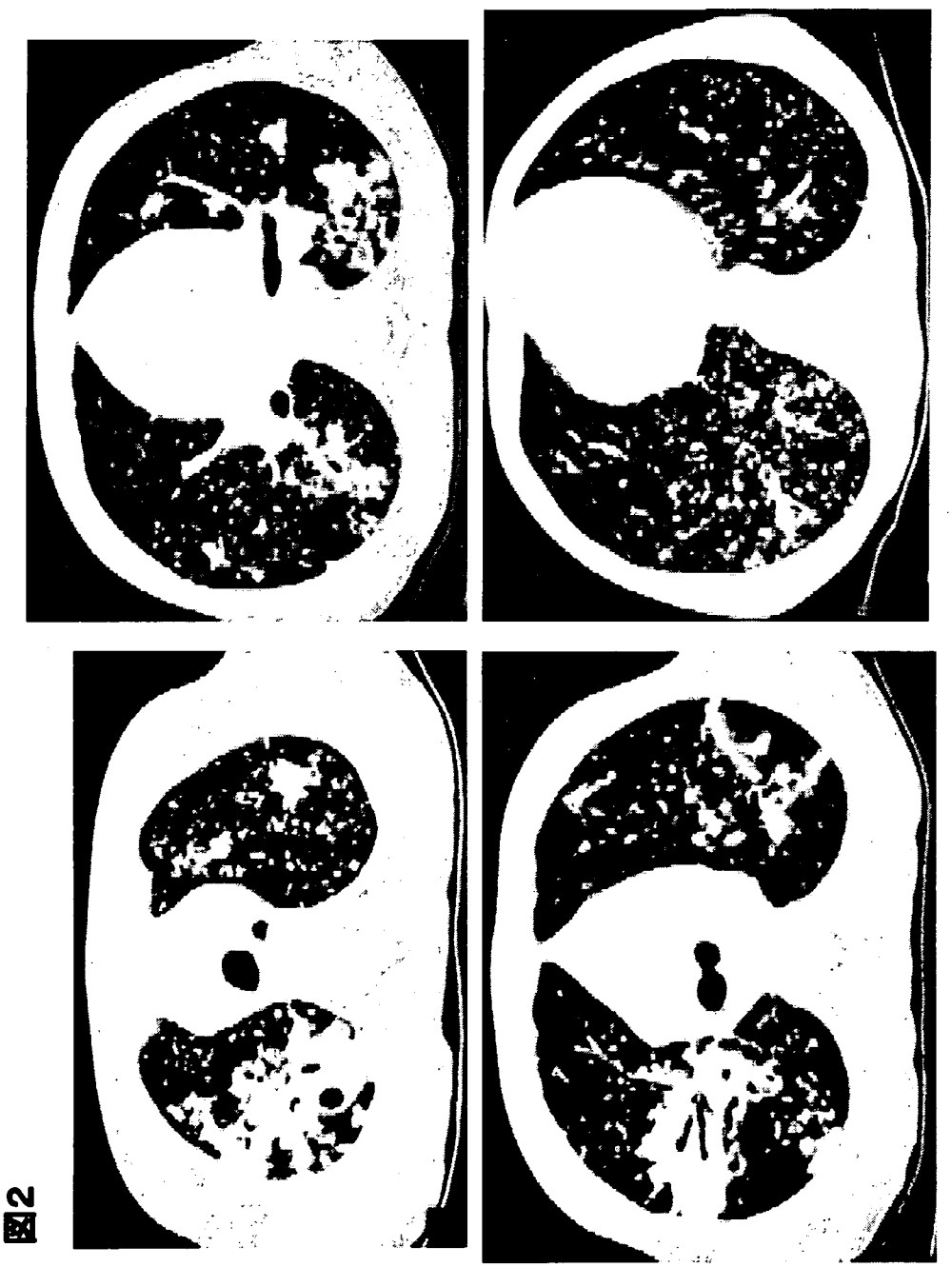

図2

図3

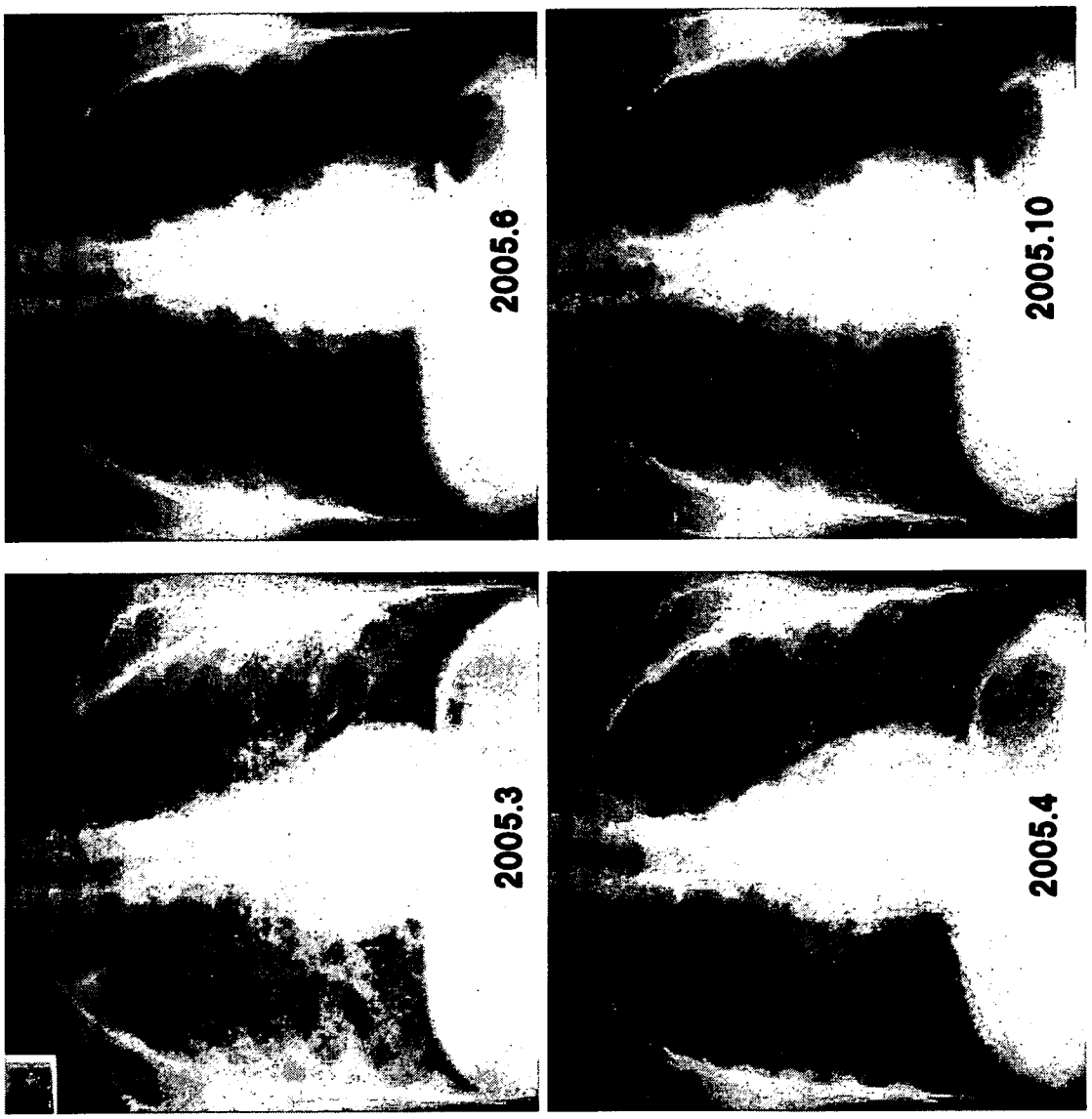

図4

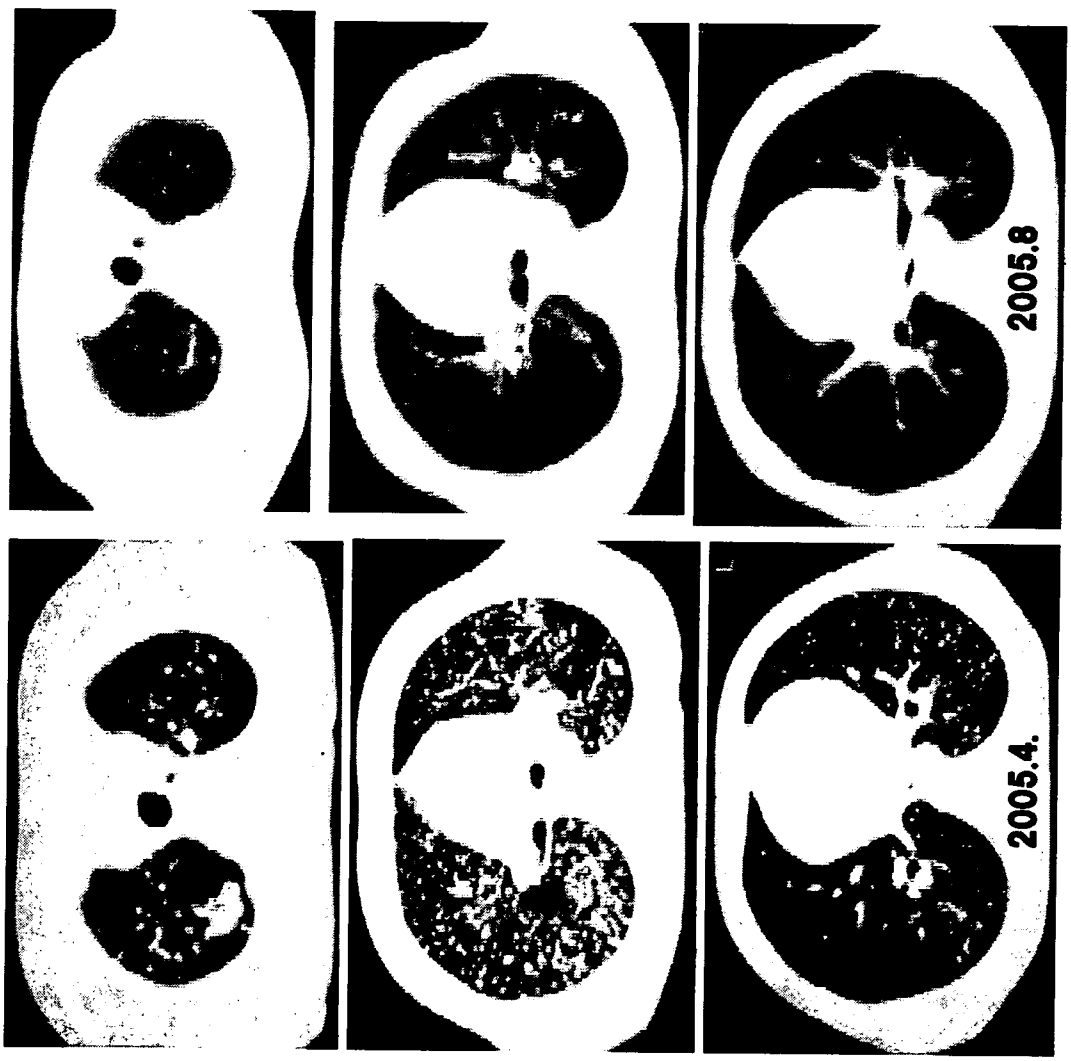

図5

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2005.02985.x

Novel roles of osteopontin and CXC chemokine ligand 7 in the defence against mycobacterial infection

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Accepted for publication 19 October 2005
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Summary

Granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced human monocyte-derived macrophage (GM-Mϕ) or macrophage CSF (M-CSF)-induced human monocyte-derived Mϕ (M-Mϕ) are distinct in terms of the resistance to *Mycobacterium tuberculosis*. To elucidate the role of molecules involved in the functional differences between these Mϕs, we investigated the gene expression profiles using microarray. After culture of $CD14^+$ monocytes with CSFs, Mϕs were cultured with or without bacillus Calmette–Guérin (BCG) (GM-Mϕ-BCG and M-Mϕ-BCG). The gene expression profiles from these cells were compared. Chemokines highly expressed in M-Mϕs were selected and evaluated for anti-mycobacterial activity and superoxide production. *FN1* and *FCGR2B* were the most up-regulated genes in GM-Mϕ and M-Mϕ, respectively. After stimulation with BCG, three chemokine genes (*Osteopontin* (*SPP1*), *CXC chemokine ligand 7* (*CXCL7*) and *CC chemokine ligand 11* (*CCL11*)) were highly expressed in M-Mϕ-BCG when compared to those in GM-Mϕ-BCG. A significantly increased resistance to *M. tuberculosis* H37Ra was observed after the stimulation of GM-Mϕ with SPP1 or CXCL7. Superoxide production levels of SPP1- or CXCL7-stimulated GM-Mϕs were higher than those of GM-Mϕs without stimulation. These results indicate that both SPP1 and CXCL7 might have a role in the resistance against mycobacteria, at least in part, through augmenting reactive oxygen intermediate production in Mϕs.

Keywords: GM-CSF, M-CSF, macrophage, microarray

Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, is one of the most important burdens on human health [1]. Both environmental and genetic factors contribute to the development of the disease, which approximates 10% of the infected subjects [2]. Twin studies provided the evidence that human genetic factors could influence the development of tuberculosis [3]. The genetic basis of susceptibility to mycobacteria has been clarified partly by the recent identification of defects in the molecules of the interferon (IFN)-γ-mediated immune pathway, such as IFN-γ receptors 1 and 2 [4,5], interleukin (IL)-12 receptor-β1 [6], IL-12p40 [7] and STAT1 [8]. In addition, linkage and/or association studies have demonstrated many susceptibility genes, such as *HLA* [9], *NRAMP1* [10], *IFN-G* [11], *TNF-A* [12], *IL-10* [12], *IL-1RA* [13], *MBL* [14], *VDR* [15] and *TLR2* [16].

The immune response against mycobacteria is mounted in a complex process. In the host, mycobacteria dwell chiefly within macrophages (Mϕs). Following activation, Mϕs produce a wide range of cytokines/chemokines and activate T cells. IFN-γ secreted by activated T cells and natural killer (NK) cells is one of the principal Mϕ activating factors, and acts as the central cytokine in the control of mycobacterial infection. Activated T cells stimulate anti-mycobacterial machinery in Mϕs, which includes reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) [17].

Mϕs that play a pivotal role in the mycobacterial infections are heterogeneous in nature, with different phenotypes and functions. They are derived predominantly from peripheral blood monocytes, and differentiate to specific cells in target tissues. Peripheral blood monocytes need colony-stimulating factors (CSFs) such as granulocyte-macrophage (GM)-CSF or macrophage (M)-CSF for their survival and

differentiation *in vitro*. GM-CSF-induced monocyte-derived macrophage (GM-Mϕ) and M-CSF-induced monocyte-derived macrophage (M-Mϕ) are distinct in their morphology, cell surface antigen expression and function. GM-Mϕ and M-Mϕ show susceptibility and resistance to mycobacteria, respectively [18,19].

To determine novel host resistance or susceptibility genes in mycobacteria infection, we investigated the differences in the gene expression profiles between GM-Mϕ and M-Mϕ with a high-density oligonucleotide microarray containing approximately 30 000 human genes. The expression profiles of each Mϕ subset were analysed with and without the stimulation of bacillus Calmette–Guérin (BCG) (GM- and M-Mϕ-BCG). Our results enlarged the views in the immunological mechanisms against mycobacteria, especially in the roles of several chemokines.

Materials and methods

Mϕ culture

Peripheral blood mononuclear cells (PBMC) were prepared from blood buffy coats of eight different healthy donors separately by density gradient centrifugation using lymphocyte separation medium (Cappel, Aurora, OH, USA). $CD14^+$ monocytes were purified (> 95%) from PBMC using a magnetic cell separation system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), with anti-CD14 monoclonal antibody (mAb)-coated microbeads and an FcR blocking reagent (Miltenyi Biotec). $CD14^+$ monocytes were cultured at a concentration of 5×10^4 cells/100 µl in 96-well tissue culture plates or at a concentration of 5×10^5 cells/2 ml in 6-well tissue culture plates with RPMI-1640 (Invitrogen Japan KK, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL, Gaithersburg, MD, USA), and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml; Sigma-Aldrich, St Louis, MO, USA) in an incubator containing 5% CO_2 at 37°C. Cells were stimulated with GM-CSF (100 ng/ml, PeproTech, London, UK) (GM-Mϕ) or M-CSF (75 ng/ml, PeproTech) (M-Mϕ). Optimal conditions were maintained by refreshing the medium and cytokines every 3 days. After 7 days of culture, a fraction of the cells were stimulated with BCG (10 mg/ml, BCG Tokyo 172; Japan BCG Laboratory, Japan) for 3 h (GM- and M-Mϕ-BCG). During BCG stimulation, a culture medium without antibiotics was used. For the analysis of anti-mycobacterial function and superoxide production, GM-Mϕs were stimulated with or without different concentrations of a chemokine: osteopontin (SPP1) (Biogenesis, Poole, UK: 0·02, 0·25 and 2·5 µg/ml), CXCL7 (Sigma-Aldrich: 0·05, 0·15 and 0·5 µg/ml) or CCL11 (Wako, Osaka, Japan: 0·5, 5 and 50 ng/ml) for another 6 days.

Bacterial preparation and infection to Mϕs

M. tuberculosis H37Ra was grown for 1 week in Middlebrook 7H9 liquid medium (Difco, Detroit, USA) at 37°C and aliquots were frozen at −80°C. In each experiment, an aliquot was thawed and grown in 7H9 medium to mid-exponential growth phase. The culture was sonicated (time: 10 s, output: 1, duty: 80%) (Branson Sonifier 250, CT, USA) to disperse bacilli before the infection. Both types of Mϕ layers were exposed to H37Ra for 3 h in a multiplicity of infection ratio of 1 : 1 in triplicate, washed three times and reincubated in the culture medium (RPMI-1640 plus 10% FBS) with antibiotics. After culture, the medium was removed and sterile phosphate-buffered saline was added to each well. The cells in the bottoms of the wells were scraped with a sterile scraper (Techno Plastic Products AG, Transadingen, Switzerland) and then sonicated as mentioned previously. Serial dilutions of the bacterial suspensions were plated on Middlebrook 7H10 agar plates (Difco). Colonies on the agar plates were counted 3 weeks after inoculation.

RNA isolation

Mϕs were harvested at 7 days after culture with CSF, and after further 24 h with BCG stimulation. Total RNA was extracted using RNA Extraction Kit, Isogen (Nippon Gene, Osaka, Japan), according to the manufacturer's instructions. All experiments were performed according to the guidelines of the ethics committee of Kyushu University.

Microarray processing

mRNA was amplified linearly using an Amino Allyl MessageAmp aRNA Kit (Ambion, Austin, TX, USA). In brief, mRNA (1·5 µg) was reverse transcribed to synthesize complementary DNA (cDNA) using an oligo(dT) primer bearing a T7 RNA polymerase promoter. Second-strand synthesis was carried out to make a transcription template. *In vitro* transcription of the cDNA with incorporation of amino allyl UTP was performed to produce multiple copies of amino allyl-labelled anti-sense RNA (aRNA). After purification, amino allyl-labelled aRNA was reacted with N-hydroxy succinimide esters of Cy3 and Cy5 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for the Mϕ samples and a standard control, respectively. Uncoupled dye molecules were removed using Micro Bio-Spin P-30 Tris chromatography columns (Bio-rad, Hercales, CA, USA). Cy3- and Cy5-labelled products were mixed together in the same amounts. After the aRNA was fragmented in a buffer containing 40 mM Tris-acetate, 100 mM CH_3COOK and 30 mM $(CH_3COO)_2Mg.4H_2O$ at 94°C for 15 min, the hybridization buffer (5 × SSC, 0·5% SDS, 4 × Denhardt's solution, 100 µg/ml salmon sperm DNA, 10% formamide) was added. The hybridization was performed by incubating 60 µl of the product into three Acegene Human oligo chip 30K slides (Hitachi Software Engineering, Yokohama, Japan). Each slide was rinsed with a solution provided by the manufacturer (Hitachi Software Engineering). Two microarray experiments for each Mϕ subset were conducted, using two

RNA mixtures, each one equally combined from four independent cell cultures.

Signal detection and data analysis

Fluorescence signals for approximately 30 000 spots in slides were detected separately by fluorescent image analyser FLA-8000 (Fuji Film, Tokyo, Japan) for Cy3 and Cy5. Hybridization intensities were processed using Arrayvision software version 6·0 (Imaging Research, Ontario, Canada). Signal and background intensities were determined by the median pixel values. Local background values were determined as the average of four background spots around each gene spot. All spots in the image (for both Cy3 and Cy5 signals) were evaluated for a possibility of dusts, to lower the probability of false data in all experiments. GeneSpring version 6·2. (Silicon genetics, Redwood City, CA, USA) was used for data analysis. According to the GeneSpring instruction, normalization of the data was performed using the 'Lowess method' [20]. Spots with dust, or with signal values of which the Cy5 or Cy3 channels were less than three times of background, were excluded.

*Taq*Man real-time quantitative reverse transcriptase-PCR (qRT-PCR)

The same RNA used in the microarray analysis was employed for qRT-PCR. The cDNA was synthesized from the RNA using a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA), as described previously [21]. PCR primers and the target probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from ABI (Applied Biosystems, Foster City, CA, USA) as a *Taq*Man GAPDH control reagent kit. PCR primers and *Taq*Man probes for *FN1* and *FCGR2B* genes were purchased from ABI as assay reagents (Assays-on-Demand™, Gene Expression Products) with the following numbers: Hs00415006_m1 for *FN1* and Hs00414000_m1 for *FCGR2B*, and used according to the instructions of the manufacturer. The qRT-PCR was performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems) [22]. To calculate the relative amount of gene expression, the value of each gene expression was divided by that of the internal control, GAPDH. The analysis was carried out in duplicate samples.

Flow cytometry

Flow cytometric analysis was performed using an EPICS XL (Beckman Coulter, Miami, FL, USA). Multi-colour staining was performed by fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or PE-cyanin 5·1 (PC5)-conjugated mAbs against the following markers: HLA-DR, CD14, CD71, CD44, CXCR2 and appropriate controls (Immunotech, Marseille, France).

Superoxide production assay

Superoxide production by Mϕs was determined as described previously [23]. GM-Mϕs were cultured with or without a chemokine for 7 days. After treatment with trypsin (Invitrogen), cells were harvested, washed and resuspended in Hanks's balance salt solution (HBSS) (Invitrogen) (5×10^4/0·5 ml). They were stimulated with antibody-opsonized zymosan (1 mg/ml, Sigma-Aldrich) at 37°C, and the reaction was terminated by the addition of SOD (50 μg/ml, Sigma-Aldrich). The chemiluminescence was counted for 35 min with an enhancer-containing luminol-based detection system (DIOGENES; National Diagnostics) using a luminometer (Auto Lumat LB953; EG & G Berthold).

Statistical analysis

Data in qRT-PCR, colony forming unit (CFU) counting and superoxide production assays were assessed by Student's *t*-test using SPSS software version 11.

Online supplemental material

The microarray data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series Accession number GSE3408.

Results

Characterization of GM- and M-Mϕ

After culture with GM- and M-CSF for 1 week, peripheral blood monocytes differentiated into GM-Mϕ and M-Mϕ, respectively. These two types of Mϕs showed distinct features in their phenotypes and functions. Although both Mϕs expressed HLA-DR, GM-Mϕs strongly expressed CD71 and M-Mϕs were strongly positive for CD14 (Fig. 1a). M-Mϕs showed a higher resistance to *M. tuberculosis* H37Ra and a higher superoxide production than GM-Mϕs (Figs 2 and 3B), as reported by Akagawa [18].

Comparison of the constitutive gene expression levels between GM- and M-Mϕ, by microarray and quantitative PCR

To identify the molecules involved in the functional differences between GM- and M-Mϕ, we compared the constitutive gene expression profiles in each Mϕ using microarray (Fig. 1b). The 10 most up-regulated genes, which are a result of comparison between these Mϕ, are listed in Table 1. *FN1* and *FCGR2B* were the most up-regulated genes in GM-Mϕs and M-Mϕs, respectively, both of which encode proteins that potentially interact with *M. tuberculosis*. These microarray data were confirmed by qRT-PCR. As shown in Fig. 1c, the

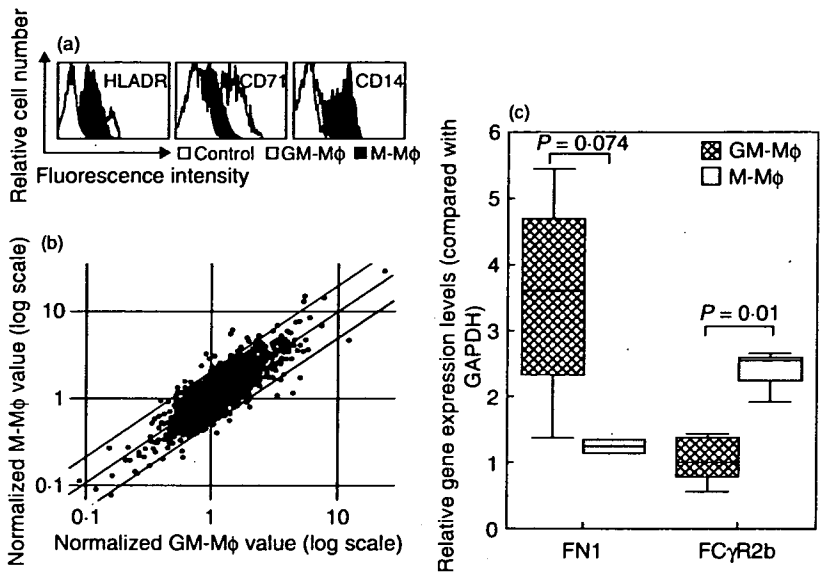

Fig. 1. (a) Phenotypic characteristics of GM-Mϕ and M-Mϕ, generated from human CD14$^+$ monocyte. (b) The scatter-plot between two types of Mϕs in their constitutive states. Each spot is the representative of normalized data in logarithmal scale from the average of two values from each cell type. (c) The qRT-PCR analysis for *FN1* and *FCGR2b* gene expression levels, which were the most up-regulated genes in each Mϕ (Table 1).

expression levels of *FN1* and *FCGR2B* genes were increased in GM-Mϕs and M-Mϕs, respectively, although the difference of *FN1* expression levels did not reach the statistical significance.

Comparison of the gene expression levels between GM- and M-Mϕ with and without BCG exposure by microarray

When we compared the gene expression profiles between GM- and M-Mϕ with and without BCG, *IL-1B* showed the highest expression among BCG-stimulated genes in both Mϕs (Table 2). Also, *SOD2* gene was listed among highly expressed genes in both Mϕs after BCG stimulation (Table 2). Then, we compared the gene expression profiles between GM-Mϕ-BCG and M-Mϕ-BCG (Table 3). *Osteopontin* (*SPP1*) was the most up-regulated gene in M-Mϕ-BCG compared with GM-Mϕ-BCG, suggesting the protective role of *SPP1* in M-Mϕ against mycobacteria. Analysis of genes according to the gene ontology (GeneSpring software) revealed that four HLA-related genes were included in the 10 most up-regulated genes in GM-Mϕ-BCG compared with M-Mϕ-BCG, while three chemokine genes (*SPP1*, *CXC chemokine ligand 7* (*CXCL7*) and *CC chemokine ligand 11* (*CCL11*)) were included in the 10 most up-regulated genes in M-Mϕ-BCG compared with GM-Mϕ-BCG.

Effects of 3 chemokines on the growth of *M. tuberculosis* H37Ra in GM-Mϕ

We selected three chemokine genes (*SPP1*, *CXCL7* and *CCL11*) as the candidate genes that potentially contribute to the protective function of M-Mϕs. To clarify the possible effects of these chemokines on the resistance of M-Mϕs against *M. tuberculosis*, GM-Mϕs were cultured in the presence of different concentrations of one of these chemokines for 6 days, and their protective abilities against *M. tuberculosis* H37Ra were evaluated (Fig. 2). SPP1 or CXCL7-stimulated GM-Mϕs significantly inhibited the growth of H37Ra 6 days after the infection with the organism, while CCL11 stimulation had no effects on it (Fig. 2).

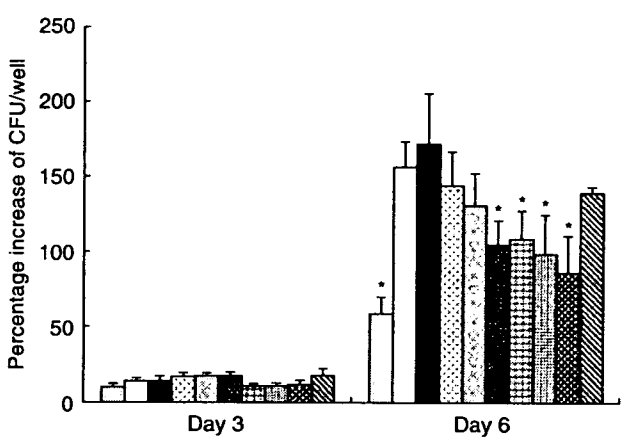

Fig. 2. Inhibition of *M. tuberculosis* H37Ra growth in Mϕs by SPP1 and CXCL7. Mtb colony-forming unit (CFU) assay was performed on days 3 and 6 after H37Ra-Mϕ exposure. M-Mϕ, GM-Mϕ with granulocyte macrophage-colony stimulating factor (GM-CSF) only □ and GM-Mϕ without any cytokines ■ were cultured as controls. GM-Mϕs were stimulated with three different chemokines: for SPP1, 0.02, 0.25 and 2.5 μg/ml; for CXCL7, 0.05, 0.15 and 0.5 μg/ml. Data for CCL11 are shown only for the results using the highest concentration in the experiments (see Methods for details). Mean values and standard deviations of triplicates are shown. *Indicates that P value was <0.05 in comparison with GM-Mϕ.

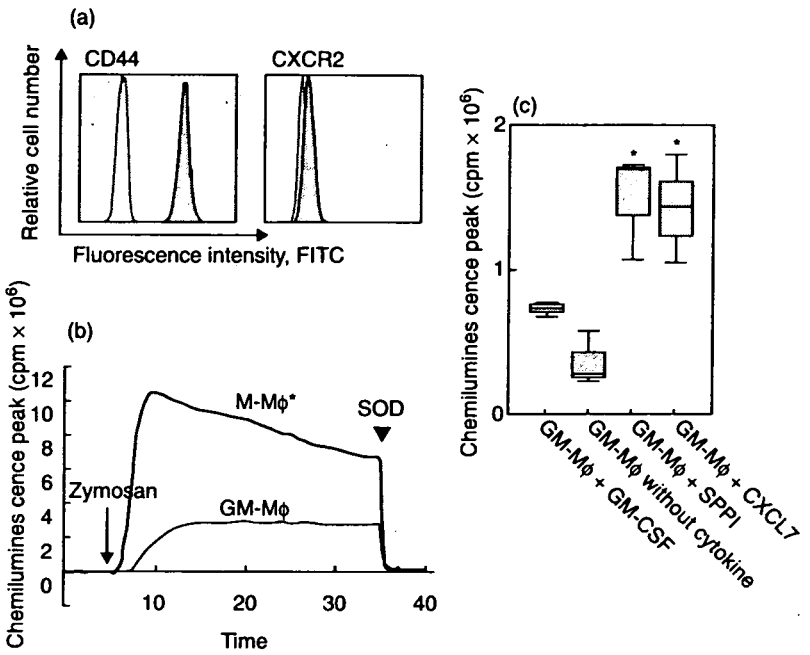

Fig. 3. Increased superoxide production from GM-Mϕs after the stimulation with SPP1 and CXCL7. (a) Receptor expression for SPP1 (CD44) and CXCL7 (CXCR2) on the surface of GM-Mϕ, determined by flow cytometry. (b) Superoxide production by the Mϕs measured by a change of chemiluminescence. The Mϕs (5.0×10^4 cells) were stimulated with antibody-opsonized zymosan (arrow), and the chemiluminescence change was monitored continuously for 40 min with DIOGENES. SOD was added to terminate the reaction (arrowhead). (c) Superoxide production from GM-Mϕs with or without chemokine stimulation. Representatives of three independent experiments are shown. *Indicates that P value was <0.05 in comparison with GM-Mϕ.

Table 1. Gene expression profiles of GM-Mϕ and M-Mϕ. (a) Genes which up-regulated in GM-Mϕ (top 10) compared to those in M-Mϕ; (b) genes which up-regulated in M-Mϕ (top 10) compared to those in GM-Mϕ.

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	FN1	ENSG00000115414	Fibronectin 1	3·81
2	Unknown	ENSG00000079310	ensembl prediction	3·12
3	Unknown	ENSG00000085063	ensembl prediction	2·93
4	CCL7	NM_006273–1	Monocyte chemotactic protein 3 precursor; scya7	2·86
5	AD 158	AL136919–1	Hypothetical protein; dkfzp586j1119	2·83
6	ARPC2	AF116702–1	pro2446	2·56
7	KIAA1838	XM_035688–1	Hypothetical protein xp_035688; loc94580	2·53
8	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	2·53
9	HBD	NM_000519–1	Haemoglobin, delta	2·35
10	ABCC3	AF085692–1	Multidrug resistance-associated protein 3b; mrp3	2·17
(b)				
1	FCGR2B	NM_004001–1	fc fragment of igg, low affinity iib, receptor for (cd32)	4·14
2	Unknown	ENSG00000024862	ensembl prediction	3·90
3	MHC Ag	L34093–1	MHC class ii hla-dq-alpha chain	3·64
4	Unknown	ENSG00000126461	ensembl prediction	3·01
5	C15orf12	AK001830–1	cDNA flj10968 fis clone place1000863 moderately similar to putative mitochondrial 40 s ribosomal protein yhr148w	2·90
6	Unknown	AC069384·3.87217·105230·1	ensembl genscan prediction	2·85
7	Unknown	AP002767·1.52387·73825·2	ensembl genscan prediction	2·84
8	MMP9	NM_004994–1	Matrix metalloproteinase 9 preproprotein	2·82
9	TM7SF1	NM_003272–1	Transmembrane 7 superfamily member 1 (up-regulated in kidney)	2·70
10	Unknown	AC003958·1.1·127834·1	ensembl genscan prediction	2·50

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Table 2. Genes (top 10) whose expression was up-regulated in GM-Mϕ–BCG compared to those in GM-Mϕ (a), and in M-Mϕ-BCG compared to those in M-Mϕ (b).

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	IL1B	NM_000576–1	Interleukin 1, beta	46·66
2	SOD2	NM_000636–1	Superoxide dismutase 2, mitochondrial	13·62
3	MT1G	XM_048213–1	Metallothionein 1 g	10·71
4	CLECSF9	AB024718–1	Macrophage c-type lectin mincle; mincle	6·28
5	CCL1	M57502–1	Secreted protein i-309; scya1	5·96
6	BCL2A1	NM_004049–1	bcl2-related protein a1	5·85
7	AKR1C3	L43839–1	3-alpha-hydroxysteroid dehydrogenase; 3alpha-hsd	5·72
8	Unknown	AC005027·2.1·157073·2	ensembl genscan prediction	5·62
9	GRO1	NM_001511–1	Gro1 oncogene (melanoma growth stimulating activity, alpha)	5·61
10	MT1H	NM_005951–1	Metallothionein 1 h	5·38
(b)				
1	IL1B	NM_000576–1	Interleukin 1, beta	74·62
2	CCL20	NM_004591–1	Small inducible cytokine subfamily a (cys-cys), member 20	59·71
3	ARHGEF1	NM_004706–1	Rho guanine nucleotide exchange factor 1	56·29
4	CCL7	NM_006273–1	Monocyte chemotactic protein 3 precursor	27·49
5	SOD2	NM_000636–1	Superoxide dismutase 2, mitochondrial	25·94
6	IL8	NM_000584–1	Interleukin 8	17·98
7	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	16·33
8	SERPINB2	NM_002575–1	Serine (or cysteine) proteinase inhibitor, clade b (ovalbumin), member 2	15·81
9	TNFAIP6	NM_007115–1	Tumour necrosis factor, alpha-induced protein 6	15·29
10	H1F2	NM_005319–1	h1 histone family, member 2	14·06

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Table 3. Genes (top 10) whose expression was up-regulated in GM-Mϕ–BCG compared to those in M-Mϕ-BCG (a) and in M-Mϕ-BCG compared to those in GM-Mϕ-BCG (b).

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	HLA-DRA	NM_019111–1	Major histocompatibility complex, class ii	6·86
2	HLA-DMA	NM_006120	Major histocompatibility complex	6·79
3	ID2	NM_002166–1	Inhibitor of dna binding 2, dominant negativ ehelix-loop-helix protein	6·06
4	HLA-DP	S66883–1	Major histocompatibility complex class ii antigen beta chain	5·81
5	HLA-DQA	L34093–1	MHC class II hla-dq-alpha chain	5·30
6	PRG1	NM_002727–1	Proteoglycan 1, secretory granule	4·88
7	RGC32	NM_014059–1	rgc32 protein	4·72
8	TNFSF13B	NM_006573–1	Tumour necrosis factor (ligand) superfamily, member 13b	4·44
9	Unknown	AC026785·3.13728·33112·2	ensembl genscan prediction	4·31
10	Unknown	XM_016170–1	Hypothetical protein xp_016170; loc88021	4·17
(b)				
1	SPP1	NM_000582–1	Secreted phosphoprotein 1 (osteopontin)	22·37
2	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	19·20
3	CXCL7	NM_002704–1	Pro-platelet basic protein (NAP2, SCYB7, CTAP3, PPBP)	15·08
4	FLJ20033	NM_017629–1	Hypothetical protein flj20033	9·43
5	LOC64182	NM_022359–1	Similar to rat myomegalin	8·82
6	Unknown	BC000845–1	Unknown (protein for image:3457769)	7·01
7	C8B	NM_000066–1	Complement component 8, beta polypeptide	6·78
8	Unknown	BC006174–1	Unknown (protein for image:4053618)	6·73
9	STK4	NM_006282–1	Serine/threonine kinase 4	6·62
10	CCL11	NM_002986–1	Small inducible cytokine subfamily a (cys-cys), member 11 (eotaxin)	6·60

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Effects of SPP1 and CXCL7 on GM-Mϕs

The expression of cell surface receptors for SPP1 (CD44) and CXCL7 (CXCR2) on GM-Mϕ were confirmed (Fig. 3a). To investigate further the mechanism of increased resistance of SPP1- or CXCL7-stimulated GM-Mϕs against *M. tuberculosis*, superoxide production by Mϕs was investigated. After the stimulation with antibody-opsonized zymosan, M-Mϕs produced a higher amount of superoxide than GM-Mϕ (Fig. 3b). Superoxide production by GM-Mϕs was significantly enhanced after the stimulation with SPP1 or CXCL7 (Fig. 3c). The reaction was terminated by SOD, which inhibits cytochrome *c* reduction (Fig. 3b). These results suggested that increased superoxide production was one of the mechanisms of increased resistance of SPP1- or CXCL7-stimulated Mϕs against *M. tuberculosis*.

Discussion

GM-Mϕs and M-Mϕs show distinct features, although both Mϕs come from the same origin ($CD14^+$ PMNC). It has been reported that GM-Mϕs show a susceptibility to *M. tuberculosis*, while M-Mϕs have a resistance to *M. tuberculosis* with a greater FcγR-mediated phagocytic capacity and a higher capability of ROI production [18].

In our experiment, *FN1* that encode fibronectin (FN) was expressed predominantly in GM-Mϕs compared with M-Mϕs (Table 1a). FN is expressed constitutively in the lung [24]. *M. tuberculosis* binds to the FN by FN attachment proteins on the surface of *M. tuberculosis*. After fibronectin opsonization, *M. tuberculosis* can be phagocytosed easily via complement receptors and integrin receptors [25,26]. Therefore, it is possible that increased FN production led to the enhanced *M. tuberculosis* load into the cells. On the other hand, *FCGR2B* was highly expressed in M-Mϕs compared with GM-Mϕs (Table 1b). It was reported that *FCGR2B* expression levels were increased in peripheral blood monocytes in patients with tuberculosis compared with healthy controls by microarray analysis [27]. In contrast to FcγR1, FcγR2a and FcγR3, FcγR2b is an inhibitory receptor that does not contain immunoreceptor tyrosine-based activation motifs (ITAM) [28]. Therefore, FcγR2b seems to modulate inflammatory responses and inhibits phagocytosis of Mϕs [25]. Further analysis for the role of FcγR2b in *M. tuberculosis* infection would be necessary.

IL-1B and *SOD2* expression levels were up-regulated in both types of Mϕs after the stimulation with BCG (Table 2), which was consistent with the previous report [29]. IL-1β is produced by activated Mϕs following *M. tuberculosis* infections, and is an important mediator of cellular anti-mycobacterial activities [30]. The importance of IL-1 for the generation of protective immunity against mycobacterial infection was clarified using *IL-1*-knock-out mice [31]. Cell wall components of *M. tuberculosis* are known to induce *IL-1B* expression in human monocytes and macrophages [32]. In addition, increased *IL-1B* gene expression was observed in bronchoalveolar lavage cells from tuberculosis patients compared with those from healthy individuals [33]. Mϕ stimulation triggers an oxidative burst and the generation of superoxide anions (O_2^-) and other ROI in Mϕs [34]. SOD2 may play a role in protection of Mϕs against ROI in *M. tuberculosis*-infected Mϕs. None the less, the protective function of M-Mϕs against *M. tuberculosis* in contrast to GM-Mϕs do not seem to be obtained solely by the increased expression of these molecules, because these molecules were also highly expressed in GM-Mϕs.

Three chemokines were included in the 10 most up-regulated genes in M-Mϕ-BCG compared with GM-Mϕ-BCG (Table 3b). In *M. tuberculosis* infections, chemokines contribute to the recruitment of other immune cells, especially of T cells, and the formation and maintenance of granuloma [35]. We also found that GM-Mϕs, which were stimulated with SPP1 and CXCL7, were more bacteriostatic to *M. tuberculosis* than unstimulated GM-Mϕs (Fig. 2). This is the first description that these two chemokines played protective roles against *M. tuberculosis* in humans. After BCG stimulation, the ratio of *SPP1* expression was highest in M-Mϕ compared with GM-Mϕ (Table 3b). SPP1 is a multi-functional protein that is expressed in both alveolar and peritoneal Mϕs [36]. *SPP1* knock-out mice were more susceptible to *M. tuberculosis* with small and immature granuloma formation in their lungs [37]. *M. tuberculosis* infection of primary human alveolar macrophages causes a substantial increase in *SPP1* gene expression [38]. Many investigators recognize SPP1 as a proinflammatory cytokine, which causes cellular adhesion of inflammatory leucocytes. Furthermore, SPP1 promotes chemotaxis and adhesion of human peripheral blood T cells [39] and enhances their IFN-γ production [40]. It is worthy of notice that its expression can be used as a prognostic marker in patients with *M. tuberculosis* infection [41].

CXCL7 is a cleavage product of platelet basic protein with a length of 70 amino acids [42]. In neutrophils, CXCL7 induces an increase of cytosolic calcium concentration, chemotaxis, exocytosis, production of ROI, degranulation and elastase release [42,43]. Although there is a recent report showing that CXCL7 can modulate the synthesis of IL-12 in Mϕs [44], the role of CXCL7 in Mϕs has not been well determined.

In addition, we demonstrated that SPP1 and CXCL7 facilitated the production of superoxide in GM-Mϕs after the stimulation with antibody-opsonized zymosan particles. The high production of ROI in Mϕs following *M. tuberculosis* infections may be compatible with the high expression of SOD2 in microarray results from BCG-stimulated Mϕs, which may play an important role in preventing Mϕ damages induced by ROI. On the other hand, RNI production in GM-Mϕs was not increased after the stimulation with SPP1 or CXCL7 (data not shown). Immunologically activated Mϕs can generate superoxide anion and other ROI [34]. Mice

deficient in the NADPH oxidase complex have a susceptibility to *M. tuberculosis* infection [45]. In humans, patients with chronic granulomatous disease are more susceptible to *M. tuberculosis* [46]. Although our data showed that SPP1 and CXCL7 may play an important role against *M. tuberculosis*, possibly through the up-regulation of superoxide production in Mϕs, it is possible that their anti-mycobacterial effect could be based on other mechanisms.

In summary, our data showed that Mϕs can secret a large array of molecules that induce host defence after the exposure to BCG. Among them, we found new roles of SPP1 and CXCL7 against *M. tuberculosis* in Mϕs. Further analysis of these molecules using iRNA will confirm the role of these chemokines in *M. tuberculosis* infection more clearly.

Acknowledgements

This work was supported by the Ministry of Education, Culture, Sport, Science and Technology of Japan.

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doi: 10.1111/j.1744-313X.2007.00653.x

Association of *IL12RB1* polymorphisms with susceptibility to and severity of tuberculosis in Japanese: a gene-based association analysis of 21 candidate genes

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Summary

Tuberculosis (TB) is the second commonest cause of death from infectious disease after HIV/AIDS worldwide. Association studies have revealed that host genetic factors, such as human leukocyte antigen and solute carrier family 11 member A1 (NRAMP1), play roles in susceptibility to TB. To identify host genetic factors involved in the susceptibility to TB in Japanese, we performed a gene-based association analysis of 21 candidate genes on 87 TB patients and 265 controls using marker single nucleotide polymorphisms (SNPs). For the genes with two or more marker SNPs exhibiting significant allele association, we subsequently analysed the association between adjacent coding SNPs (cSNPs) and TB. Among a total of 118 marker SNPs, 3 of *IL1B* and 2 of *IL12RB1* showed association with TB. Non-synomymous cSNPs were not identified in *IL1B*. Association studies on four non-synomymous cSNPs of *IL12RB1* (641A/G, 1094T/C, 1132C/G, 1573G/A) in linkage disequilibrium showed that three of them (641A/G, 1094T/C, 1132C/G) were significantly associated with the development of TB. Haplotype analysis on the four cSNPs demonstrated that frequency of ATGG haplotype was significantly lower in TB patients than in controls. When TB patients were divided into two subgroups according to the severity of lung disease, advanced subgroup showed a prominent association with 641A/G, 1094T/C and 1132C/G SNPs. These data suggested that genetic variants of *IL12RB1*, at least in part, confer genetic susceptibility to TB, and are associated with the progression of the disease, in Japanese.

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Received 26 February 2006; revised 8 June 2006 and 5 October 2006; accepted 26 November 2006

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Abbreviations

TB, tuberculosis; IL, interleukin; NRAMP1, natural resistance associated macrophage protein 1; SLC11A1, solute carrier family 11 member A1; VDR, vitamin D receptor; NTM, non-tuberculous environmental mycobacteria; IFN, interferon; MSMD, Mendelian susceptibility to mycobacterial disease; SNP, single, nucleotide polymorphism; cSNP, coding SNPs; IFN-γR, IFN-γ receptor; IL-12R, IL-12, receptor; STAT, signal transducer and activator of transcription; IL-18R, IL-18 receptor; IL-23R, IL-23 receptor; TNF, tumor necrosis factor; TNFRSF, TNF receptor superfamily; UBE3A, ubiquitin protein ligase E3A; LD, linkage disequilibrium; UTR, untranslated region.

Introduction

Tuberculosis (TB) is the second commonest cause of death from infectious disease after HIV/AIDS worldwide. The World Health Organization estimated 8–9 million new cases of clinical TB and 2 million deaths resulting from the disease every year (WHO, 2005). Only about 10% of the individuals infected with *Mycobacterium tuberculosis* develop TB, whereas the remaining 90% stay free from the disease throughout their life (Murray *et al.*, 1990). Almost half of the patients show rapid progression and develop clinical disease within 2 years after infection (Frieden *et al.*, 2003). In addition to these clinical observations, epidemiological, twin and adoption studies support the role of host genetic factors in the susceptibility to TB (Comstock, 1978; Sorensen *et al.*, 1988). Previous association studies demonstrated the association of several genes, such as human leucocyte antigen (HLA), natural resistance associated macrophage protein 1 (NRAMP1 or solute carrier family 11 member A1 [SLC11A1]) and vitamin D receptor (VDR) genes and interleukin (IL)-1 locus, with the susceptibility to TB (Singh *et al.*, 1983; Bellamy *et al.*, 1998, 1999; Goldfeld *et al.*, 1998; Wilkinson *et al.*, 1999; Greenwood *et al.*, 2000). A linkage analysis on sib-pairs conducted in Africa (Bellamy *et al.*, 2000) has mapped TB susceptibility loci to chromosomes 15q11–13 and Xq26, although another genome-wide scan for a Brazilian TB patient did not replicate it (Miller *et al.*, 2004).

On the other hand, genetic analysis of severe or recurrent cases with clinical diseases caused by weakly virulent mycobacterial species, such as BCG and non-tuberculous environmental mycobacteria (NTM) revealed the congenital deficiencies of the molecules involved in IL-12/interferon

(IFN)-γ axis named 'Mendelian susceptibility to mycobacterial disease (MSMD, MIM 209950)' (Dupuis *et al.*, 2000). Increased susceptibility to TB is also observed in this type of genetic disorders. Therefore, it is possible that mutations causing MSMD are responsible for the development of TB and/or that any functional polymorphisms of the genes encoding molecules of IL-12/IFN-γ axis may affect the genetic control of *M. tuberculosis*.

In the present study, we screened 21 candidate genes for TB susceptibility in Japanese by a gene-based association analysis using marker single nucleotide polymorphisms (SNPs) and subsequently analysed the association between TB and coding SNPs (cSNPs) adjacent to the positive marker SNPs in terms of susceptibility and disease severity.

Materials and methods

Subjects

The study population comprised 87 unrelated Japanese patients with TB (mean age: 52.7 ± 21.1 years; 18 women and 69 men) and 265 unrelated healthy Japanese individuals (mean age: 56.5 ± 12.7 years; 112 women and 153 men), who resided in Kyushu Island in the southern part of Japan. All the TB patients had been given a diagnosis of pulmonary TB on the basis of clinical symptoms and chest radiographic findings with bacteriological confirmation (culture, 82 patients; smear and/or polymerase chain reaction [PCR], 5 patients). Eleven patients were having TB relapses. Common clinical symptoms were cough (77%), sputum (53%) and fever (30%). Patients with known immunodeficient states, such as HIV infection and are undergoing immunosuppressive therapy were excluded. Lung disease on standard posterior–anterior chest radiograph of each patient was graded according the International Classification of Tuberculosis (Falk *et al.*, 1969; Van Lettow *et al.*, 2004):

(1) minimal lung disease was defined as infiltrates of slight to moderate density; disease present in a small portion of both lungs; the total volume of infiltrate(s) being the volume of one lung present above the second chondrosternal junction and the spine of the fourth junction or the body of the fifth thoracic vertebra and no cavitations present.
(2) moderately advanced disease was defined as disease present in one or both lungs; the total extending not more than as follows:
    (i) scattered lesions of slight to moderate density do not involve more than the total volume of one lung or the equivalent volume of both lungs
    (ii) dense, confluent lesions do not involve more than one-third of the volume of one lung, and
    (iii) the total diameter of the cavities are less than 4 cm; and
(3) far advanced lung disease was defined as: lesions more extensive than moderately advanced disease. Thirty-four, 38 and 15 patients had minimal, moderately advanced and far advanced lung disease, respectively. Twenty-nine patients had cavitary lesion(s). Subjects with diabetes were not included in the control group. After full explanation of the study by research personnel, written informed consent was obtained from the subjects or guardian(s). This study was approved by the ethical committees of Kyushu University and by the other participating institutions.

Screening of the candidate genes

Genomic DNAs were extracted from whole blood by using QIAamp DNA Blood Kit (Qiagen, Germantown, MD). Twenty-one candidate genes selected for analysis consisted of three genes whose association with TB has been observed in Japanese and/or other ethnic population (SLC11A1, VDR and IL-1β genes), 14 genes associated with IL-12/IFN-γ axis (IFN-γ, IFN-γR [IFN-γ receptor] P, IFN-γR2, IL-12 p40, IL-12p35, IL-12R [IL-12 receptor] β1, IL-12Rβ2, signal transducer and activator of transcription [STAT]-1, IL-18, IL-18R [IL-18 receptor], IL-23p19, IL-23R [IL-23 receptor], IL-27p28 and IL-27R [IL-27 receptor, WSX-1] genes), three genes associated with tumor necrosis factor (TNF)-α signaling (TNF-α, TNFRSF [TNF receptor superfamily]1 A and TNFRSF1B genes), and ubiquitin protein ligase E3A (UBE3A) gene, a putative TB susceptibility gene in chromosome 15q11–13 based on the sib-pair linkage analysis (Cervino *et al.*, 2002). All of them are located on autosomal chromosomes. HLA genes were not analysed in this study because of their complexity. These candidate genes were screened by association analysis of marker SNPs, which were validated by the TaqMan™ Validated SNP Genotyping Assays (Applied Biosystems, Foster City, CA). A total of 118 marker SNPs with 62–23 572 base pair (bp) interval within each gene (median 5633 bp interval) were genotyped by Assays-On-Demand™ primer and probe sets (Applied Biosystems) using ABI PRISM 7900HT (Applied Biosystems) according to the manufacturer's protocol.

SNPs detection and genotyping by PCR sequencing

For genes with two or more marker SNPs exhibiting significant allele association with TB (cut-off at $P < 0.05$), we subsequently searched for adjacent cSNPs by PCR and direct sequencing. Genomic DNAs extracted from whole blood of 24 TB patients randomly selected from the total TB population were used. Twenty-four samples are sufficient to detect SNPs with minor allele frequencies over 5%. To analyse exons 1–7 and 3′ UTR of *IL1B* adjacent to three marker SNPs with positive association (rs1143629, rs1143643 and rs3917368), we constructed eight pairs of oligonucleotide primer pairs according to the human *IL1B* gene sequence (GenBank Accession No. AY137079), as follows: 5′-AAACAGCGAGGGAGAAACTG-3′ and 5′-GCATACACACAAAGAGGCAGAG-3′ for exon 1, 5′-ACACATGAACGTAGCCGTCA-3′ and 5′-AGGGGAA-AAATCTGGTCTCC-3′ for exon 2, 5′-GCAGGCT-GTTTGCAGTTTCT-3′ and 5′-TCCTTGGGTTGGGAG-TTAAA-3′ for exon 3, 5′-CTCCCTCCCTCGCTCTCT-3′ and 5′-CTGCCTGCTCTTGGCTAACT-3′ for exon 4,

