

Original article

Identification of tryptase- and chymase-related gene clusters in human mast cells using microarrays

Tryptase and chymase are the two major granular proteases present in human mast cell (MC)s. We used oligonucleotide microarray to measure the levels of approximately 22 000 transcripts in cord blood-derived MCs at 4 weeks, 8 weeks, 12 weeks and 18 weeks in culture. Tryptase (*TPSB2*) was expressed at the highest level among all transcripts and its expression level reached a plateau at 8 weeks. On the other hand, the expression level of chymase (*CMAI*) doubled every 4–6 weeks. A similar tendency was found at the protein levels with FACS analysis. After filtering the transcripts with MC-specificity, hierarchical clustering analysis identified 494 and 81 transcripts in the same clusters with tryptase and chymase, respectively. MC-specific genes, *KIT* and *HDC* were found in the tryptase cluster. In the chymase cluster, a critical suppressor for cell senescence, *BM1* and the several related genes were found, suggesting that chymase expression may be closely related to cell senescence/quiescence events.

C. Dahl¹, H. Saito², M. Kruhøffer³, P. O. Schiøtz⁴

¹A Research, Department of Pediatrics, Aarhus University Hospital, Aarhus, Denmark; ²Research Unit for Allergy Transcriptome, RIKEN Research Center for Allergy and Immunology, Tokyo, Japan; ³Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo, Japan; ⁴Molecular Diagnostic Laboratory, Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark

Key words: chymase; cord blood-derived cultured human mast cells; mast cells; microarray; tryptase.

Christine Dahl
A Research
Department of Pediatrics
Aarhus University Hospital
Brendstrupgaardsvej 100
8200 Aarhus N
Denmark

Accepted for publication 24 June 2005

Rodent mast cell (MC) were classified into two types, i.e. connective tissue MCs preferentially located in tissues such as skin and mucosal MCs dominantly found in mucosa such as intestine (1–4). Although they are distinct in staining characteristics (1, 2), T cell dependency (3), functions (4) and cytokine requirement for development (5, 6), both cell types are considered to be closely related (7) and even interchangeable by some authors (8).

For human MCs, two subtypes have been recognized by the distribution of granular neutral proteases. TC-type MCs (MC_{TC}) contain tryptase together with chymase, cathepsin-G and carboxypeptidase, while T-type MCs (MC_T) contain tryptase only. These two MC types differ in tissue distribution (9). However, Toru et al. observed that interleukin (IL)-4 promoted cell maturation with the expression of both tryptase and a high amount of chymase from tryptase single-positive MCs and raised a possibility that MC_T give rise to MC_{TC} accompanied by maturation (10). In addition, we have previously reported that all human MCs in culture are capable of producing chymase, and the production is regulated in part by a cytokine-independent programme in their progenitors as well as by cytokine-dependent mechanisms (11). We used

oligonucleotide microarray (GENECHIP; Affymetrix, Santa Clara, CA, USA) to examine maturation-related MC-specific genes in this study. We report that chymase gene expression doubled every 4–6 weeks in culture, whereas tryptase and other major MC-specific gene expression reached a plateau at 8 weeks in culture.

Materials and methods

Cytokines and antibodies

Ficoll–Paque cell separation solution was purchased from Amersham Pharmacia Biotech (Hilleroed, Denmark). AC133 cell isolation kit and LS+ cell separation columns were purchased from Miltenyi Biotec (Slangerup, Denmark). Fetal calf serum (FCS) and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Human recombinant stem cell factor (rh-SCF), human rh-IL-6 and human rh-IL-3 were purchased from R&D systems (Abingdon, UK). Serum-free medium StemSpan was purchased from Stem Cell technologies (Vancouver, Canada). IgG₁ antibodies against chymase and tryptase were purchased from Chemicon (Aarhus, Denmark). IgG₁ phycoerythrin (PE)-conjugated monoclonal antibody (mAb) against CD117 and its isotype control was purchased from Serotec (Hamar, Norway).

Anti-Fc ϵ RI α mAb, CRA-1 was purchased from Kyokuto Pharmaceutical (Tokyo, Japan).

Purification of cord blood CD133⁺ cells

Human cultured MCs were obtained from cord blood according to the method described previously (12, 13). Briefly, heparinized, sterile cord blood was obtained within 30 min after delivery. The mothers gave informed consent based on guidance from the regional ethics committee. The blood was diluted three times with phosphate-buffered saline (PBS) and overlaid on a Ficoll-Paque gradient and centrifuged at room temperature for 30 min (400 g). The mononuclear cell layer was collected and washed in PBS at 4°C, for 7 min (450 g).

CD133⁺ cells were separated using AC133 cell isolation kit, and a magnetic LS⁺ separation column according to the manufacturer's instructions. Briefly the mononuclear cells were resuspended in 300 μ l MACS buffer [PBS containing bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), pH 7.2] at 10⁸ total cells. The cells were pretreated with FcR-blocking reagent at 100 μ l/10⁸ cells, reacted with AC133 MicroBeads, and incubated for 30 min at 4°C. After incubation the cells are washed in MACS buffer, the pellet was resuspended in 500 μ l MACS buffer and applied to the separation column. The column was washed four times with 3 ml MACS buffer in the magnetic field. The column was removed from the magnet and the cells eluted with 5 ml of MACS buffer. Cells were counted in alcian blue.

Generation of MCs

Purified CD133⁺ cells were cultured for the first 2 weeks in StemSpan serum-free medium supplemented with 100 ng/ml human rh-SCF, 50 ng/ml rh-IL-6, 1 ng/ml human rh-IL-3 (IL-3 was added only for the first 2 weeks) and 100 μ g/ml penicillin/streptomycin. After the week 8, the medium was supplemented with 10% FCS. The cells were passed on to new medium every week.

Flow cytometric analyses

Intracytoplasmic staining for tryptase and chymase using FACS was performed as reported previously (11). Briefly, 6–10 $\times$ 10⁴ cells were fixed with 4% paraformaldehyde, washed and incubated with PBS-saponin containing 0.1% BSA for 1 h at 37°C. These cells were then incubated with 50 mg/ml of human immunoglobulin G (IgG; ICN Biomedicals, Aurora, OH, USA). For tryptase staining, cells were incubated with 3 μ g/ml of mouse antihuman tryptase (Chemicon) for 1 h at 4°C, washed, and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Becton-Dickinson, San Jose, CA, USA) for 30 min at 4°C. For chymase staining, cells were stained with 3 μ g/ml of mouse antihuman chymase (Chemicon) by the same way as was done for tryptase staining. After staining, all cells were washed and resuspended in PBS containing 0.1% BSA. Control cells were stained with an irrelevant mouse IgG₁ (Becton-Dickinson). Cell analysis was performed using a FACScan and CELLQUEST software.

GENECHIP expression analysis

GENECHIP expression analysis was done by the methods previously reported (14, 15). Total cellular RNA was isolated from MCs with RNeasy Mini Kits (Qiagen, Valencia, CA, USA), according to the manufacturer's specifications. The purity of RNA was assessed on the basis of the A260/A280 ratio, and the integrity of RNA was

verified by agarose gel electrophoresis. The yield of RNA per 10⁶ MCs was 2.4 (2.0–2.8) μ g (n = 6). Human genome-wide gene expression was examined by using the Human Genome U133A probe array (GENECHIP; Affymetrix), which contains the oligonucleotide probe set for approximately 22 000 full-length genes and expressed sequence tags (ESTs), according to the manufacturer's protocol (Expression Analysis Technical Manual) and the previous reports (16). Total RNA (3–10 μ g) was extracted from approximately 10⁷ cells. Double-stranded cDNA was synthesized and the cDNA was subjected to *in vitro* transcription in the presence of biotinylated nucleoside triphosphates. The biotinylated cRNA was hybridized with a probe array for 16 h at 45°C, and the hybridized biotinylated cRNA was stained with streptavidin-PE and then scanned with a Hewlett-Packard Gene Array Scanner (Hewlett-Packard, Palo Alto, CA, USA). The fluorescence intensity of each probe was quantified using a computer program, GENECHIP ANALYSIS SUITE 5.0 (Affymetrix). The expression level of a single mRNA was determined as the average fluorescence intensity among the intensities obtained by 11-paired (perfect-matched and single nucleotide-mismatched) probes consisting of 25-mer oligonucleotides. If the intensities of mismatched probes were very high, gene expression was judged to be absent even if a high average fluorescence was obtained with the GENECHIP ANALYSIS SUITE 5.0 program. The level of gene expression was determined as the average difference (AD) using the GENECHIP software.

GENECHIP data analysis

The AD levels were normalized to compare the data obtained in different laboratories with the median value of approximately 22 000 transcripts in each GENECHIP. The normalized data were further filtered using the information about MC specificity as previously reported (17) and published online (<http://www.nch.go.jp/imal/GeneChip/JACI.htm>). For duplicate MC sample at 8 and 12 weeks, the average values were used to identify MC specificity (see the raw data in our web site at <http://www.nch.go.jp/imal/GeneChip/Dahl.htm>). Data were considered MC-specific when (i) either one of expression levels of a certain transcript in the four MC samples was increased at least twofold (activation programme) compared with the maximal value expressed by eosinophils, neutrophils, monocytes, CD4 cells, CD8 T cells, B cells, platelets and fibroblasts (<http://www.nch.go.jp/imal/GeneChip/JACI.htm>), and (ii) increased gene expression included at least one presence call (Affymetrix algorithm). Then, these data about 1600 MC-specific transcripts were analysed for hierarchical clustering using Eisen's GENE CLUSTER and TREE VIEW (<http://rana.lbl.gov/EisenSoftware.htm>) to identify gene clusters. These data were logarithmically transformed, centred and the normalized with the mean of the four data in a transcript.

Results and discussion

Microarray analysis of MCs on 4, 8, 12 and 18 weeks in culture

We used GENECHIP U133A in this study to examine approximately 22 000 kinds of transcripts expressed by MCs on 4, 8, 12 and 18 weeks in culture, and obtained the expression level data normalized with the median values. We have previously used the same GENECHIP version to examine genome-wide gene expression of seven different types of leucocytes, cord blood-derived cultured MCs and lung MCs (17). Using the online repository data

(<http://www.nch.go.jp/imal/GeneChip/JACI.htm>), we calculated the maximum value of the normalized (with the median value) AD levels expressed by eosinophils, neutrophils, CD4⁺ cells, CD8⁺ cells, CD14⁺ cells, CD19⁺ cells, platelets and fibroblasts. The data obtained in the present study were compared with the maximum values. We then identified 16 000 MC-specific transcripts as shown in the Supplementary Table 1 (<http://www.nch.go.jp/imal/GeneChip/Dahl.htm>) based on the criteria written in the Materials and methods.

As shown in Supplementary Table 1, the data obtained from MCs cultured for 4 weeks selectively expressed genes unlikely to be MC-specific, such as eosinophil peroxidase (*EPO*) and neutrophil elastase 2 (*ELA2*). Although these genes were selected as MC-specific in the present criteria in comparison with other cell types including mature eosinophils and neutrophils, these granulocytes are known to decrease mRNA transcription of some granular proteins when they are fully mature (18, 19). Therefore, it is speculated that some immature granulocytes may be contaminated in the MC population at 4 weeks in culture.

Among the well known MC-specific molecules, only α -chain of the high affinity receptor for IgE (*Fc ϵ RI α*) was not selected in the present study, because cord blood-derived MCs express lower levels of *Fc ϵ RI α* compared with adult type MCs, and other cell types such as eosinophils express its mRNA at substantial levels as has been reported (14, 17). The expression levels of other major MC-specific molecules were mostly found to reached plateaus at 8 weeks such as several transcript variants of β -tryptase (*TPSB2*) and *KIT* (kit receptor for SCF). The expression levels of some *TPSB2* transcripts

were highest among all transcripts present in MCs after 8 weeks (Supplementary Table 1). On the contrary, the expression of *CMA1* (chymase) doubled (from 2.4 at 4 weeks to 28 at 18 weeks, the normalized values) every 4–6 weeks until 18 weeks as shown in Supplementary Table 1.

Protein expression of tryptase and chymase

To confirm the delayed expression of chymase compared with tryptase, we performed flow cytometric analysis using the intracytoplasmic staining of these granular proteins. As illustrated in Fig. 1 as we expected, chymase levels were very low until 6 weeks, and gradually increased thereafter. On the contrary, MCs expressed tryptase at considerable levels even at 6 weeks in culture. Thus, the results obtained using GENECHIP seem to be reliable.

Hierarchical clustering analysis of MC-specific transcripts

We next used hierarchical clustering analysis to investigate what other molecules are similarly regulated in developing MCs with *TPSB2* and *CMA1*. We identified 494 and 81 transcripts in the same clusters with tryptase and chymase, respectively. Among 494 transcripts present in the tryptase-related gene cluster, many well known MC-specific molecules such as *KIT* and histidine decarboxylase (*HDC*) were found (Supplementary Table 2, Fig. 2). On the contrary, among 81 transcripts present in the chymase-related gene cluster, several transcriptional regulators were found (Supplementary Table 3, Fig. 2).

The polycomb gene, *BM11* has recently been found to be required for the maintenance, quiescence or self-renewal

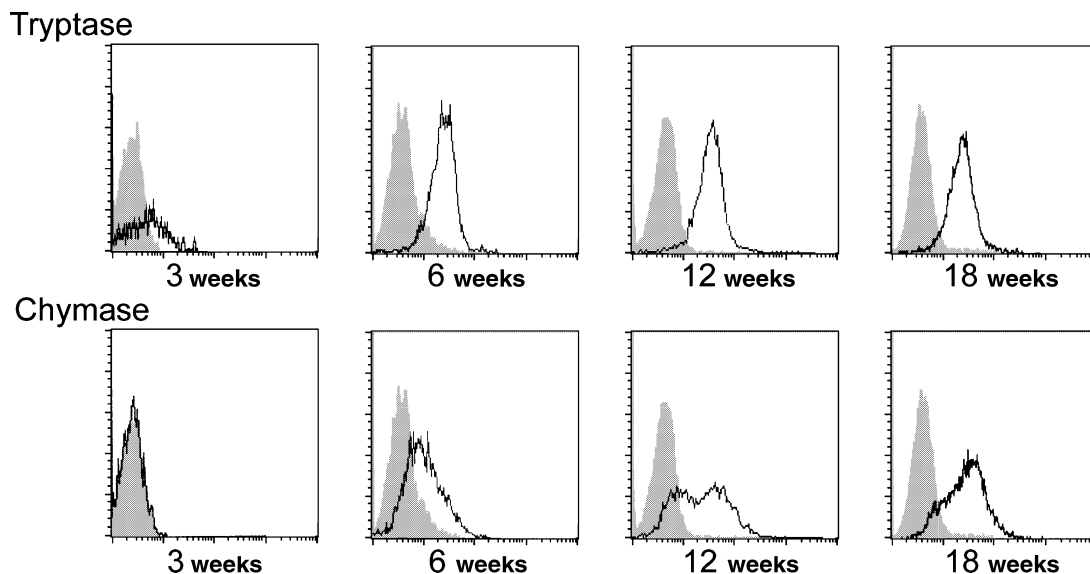


Figure 1. FACS analysis for intracytoplasmic tryptase (upper panels) and chymase (lower panels) in cord blood-derived cultured mast cells (MCs). CD34⁺ cells were cultured in the presence of stem cell factor (SCF) and interleukin (IL)-6 for 3, 6, 12 and 18 weeks. After being permeabilized, the cells were reacted with antitryptase, antichymase or an irrelevant mouse IgG₁. Each figure illustrates the results of three independent experiments. Control (shaded area); sample (unshaded area).

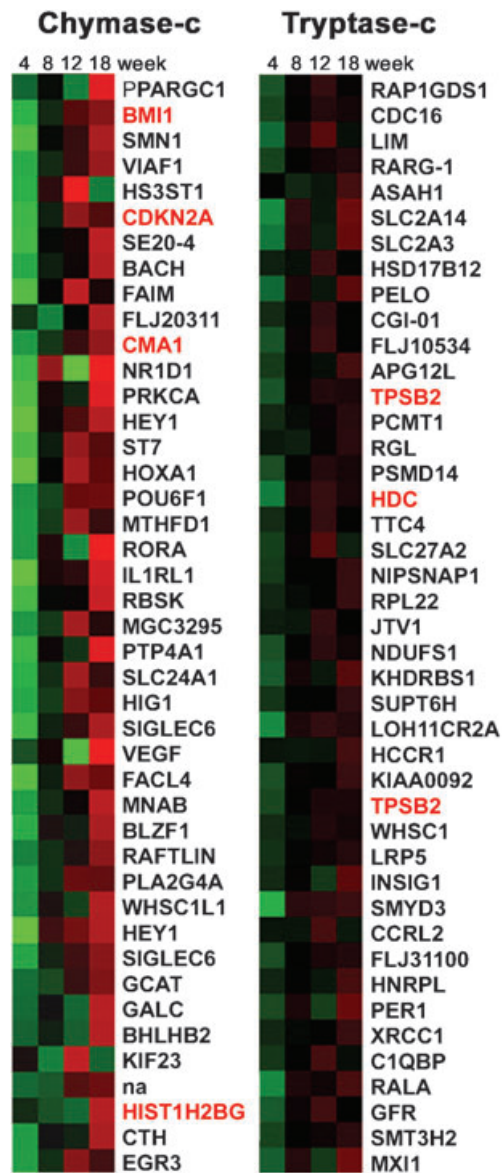


Figure 2. Representative gene lists identified in the chymase-related cluster (left column) and in the tryptase-related cluster (right column). Red, green and black (or dark) blocks represent high, low and intermediate expression levels after normalization, respectively. It should be noted that the expression pattern of transcripts present in the chymase cluster tended to be highly variable between 4 and 18 weeks, whereas that in the tryptase cluster tended to be less variable.

References

1. Enerback L. Mast cells in rat gastrointestinal mucosa: dye-binding and metachromatic properties. *Acta Pathol Microbiol Scand* 1966;**66**:303–312.

2. Mayrhofer G. Fixation and staining of granules in mucosal mast cells and intraepithelial lymphocytes in the rat jejunum, with special reference to the relationship between the acid glycosaminoglycans in the two cell types. *Histochem J* 1980;**12**:513–526.

3. Miller HRP, Walshaw R. Immune reactions in mucous membranes. *Am J Pathol* 1972;**69**:195–208.

of adult stem cells in some tissues partly because it represses genes that induce cellular senescence and cell death. (20) Thus, *BMII* may support maintenance of MCs by suppressing the apoptosis and senescence. *BMII* can suppress the differentiation/senescence-inducing gene expression by modifying histone/DNA complex. As shown in (red letters) Fig. 2 and (bold letters) in Supplementary Table 3, three different transcripts of histone octamer protein complex were found in this gene cluster. *CDKN2A* was also found in the chymase cluster. Loss of *BMII* leads to haematological defects in which de-repression of the *CDKN2A* is critically implicated (21). Taken together, in the later culture period, *BMII* and the several related genes, which may support maintenance, quiescence or self-renewal, were upregulated in MCs accompanied by chymase expression. Although the mechanisms and the roles of chymase expression are still largely unclear, the present observation is expected to provide a new insight into human MC development.

Acknowledgments

Authors thank Bente Devantié for excellent technical assistance. Technician Ellen Margrethe Raaby is thanked for her interest, skilfulness and persistent work in performing cell culturing. This work was supported in part by grants from the RIKEN Yokohama Institute and the Organization for Pharmaceutical Safety and Research of the Ministry of Health, Labour and Welfare (Millennium Genome Project, MPJ-5). Also, this work was supported by the Lundbeck Foundation, Denmark, the Velux Foundation, Denmark, by Glaxo Smith and Cline, England, by the Aarhus University Foundation, Denmark, the AP Møller Foundation for the Advancement of Medical Science, the Danish Asthma and Allergy Association and the Danish Lung Association.

Supplementary material

The following supplementary material is available for this article from <http://www.Blackwell-Synergy.com>

Table 1. Mast cell-specific gene list in comparison with six leucocyte types, platelets and fibroblasts. The AD (signal) values normalized with the median value of the whole transcripts in a array. P, M and A at flag column indicate that the signal was detected with presence, marginal and absence call, respectively.

Table 2. Gene symbol list for the tryptase-related gene cluster.

Table 3. Gene symbol list for the chymase-related gene cluster.

4. Befus AD, Pearce FL, Gauldie J, Horsewood P, Bienenstock J. Mucosal mast cells: I. Isolation and functional characteristics of rat intestinal mast cells. *J Immunol* 1982;**128**:2475–2480.
5. Ihle JN, Keller J, Oroszlan S, Henderson LE, Copeland TD, Fitch F. Biologic properties of homogeneous interleukin 3: 1. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, P cell-stimulating factor activity, colony-stimulating factor activity and histamine-producing cell-stimulating factor activity. *J Immunol* 1983;**131**:282–287.
6. Nocka K, Buck J, Levi E, Besmer P. Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. *EMBO J* 1990;**9**:3287–3294.
7. Kanakura Y, Thompson H, Nakano T, Yamamura T, Asai H, Kitamura Y et al. Multiple bidirectional alterations of phenotype and changes in proliferative potential during the in vitro and in vivo passage of clonal mast cell populations derived from mouse peritoneal mast cells. *Blood* 1988;**72**:877–885.
8. Xia Z, Ghildyal N, Austen KF, Stevens RL. Post-transcriptional regulation of chymase expression in mast cells. A cytokine-dependent mechanism for controlling the expression of granule neutral proteases of hematopoietic cells. *J Biol Chem* 1996;**271**:8747–8753.
9. Irani AM, Craig SS, DeBlois G, Elson CO, Schechter NM. Deficiency of the tryptase-positive, chymase-negative mast cell type in gastrointestinal mucosa of patients with defective T lymphocyte function. *J Immunol* 1987;**138**:4381–4386.
10. Toru H, Eguchi M, Matsumoto R, Yanagida M, Yata J, Nakahata T. Interleukin-4 promotes the development of tryptase and chymase double-positive human mast cells accompanied by cell maturation. *Blood* 1998;**91**:187–195.
11. Ahn K, Takai S, Pawankar R, Kuramasu A, Ohtsu H, Kempuraj D et al. Regulation of chymase production in human mast cell progenitors. *J Allergy Clin Immunol* 2000;**106**:321–328.
12. Saito H, Ebisawa M, Tachimoto H, Shichijo M, Fukagawa K, Matsumoto K et al. Selective growth of human mast cells induced by Steel factor, IL-6, and prostaglandin E2 from cord blood mononuclear cells. *J Immunol* 1996;**157**:343–350.
13. Dahl C, Saito H, Nielsen HV, Schiotz PO. The establishment of a combined serum-free and serum-supplemented culture method of obtaining functional cord blood-derived human mast cells. *J Immunol Methods* 2002;**262**:137–143.
14. Iida M, Matsumoto K, Tomita H, Nakajima T, Akasawa A, Ohtani NY et al. Selective down-regulation of high-affinity IgE receptor (Fc ϵ RI) α -chain messenger RNA among transcriptome in cord blood-derived versus adult peripheral blood-derived cultured human mast cells. *Blood* 2001;**97**:1016–1022.
15. Nakajima T, Matsumoto K, Suto H, Tanaka K, Ebisawa M, Tomita H et al. Gene expression screening of human mast cells and eosinophils using high-density oligonucleotide probe arrays: abundant expression of major basic protein in mast cells. *Blood* 2001;**98**:1127–1134.
16. Kato A, Ogasawara T, Homma T, Saito H, Matsumoto K. Lipopolysaccharide-binding protein critically regulates lipopolysaccharide-induced IFN- β signaling pathway in human monocytes. *J Immunol* 2004;**172**:6185–6194.
17. Nakajima T, Iikura M, Okayama Y, Matsumoto K, Uchiyama C, Shirakawa T et al. Identification of granulocyte subtype-selective receptors and ion channels by using a high-density oligonucleotide probe array. *J Allergy Clin Immunol* 2004;**113**:528–535.
18. Gruart V, Truong MJ, Plumas J, Zandecki M, Kusnierz JP, Prin L et al. Decreased expression of eosinophil peroxidase and major basic protein messenger RNAs during eosinophil maturation. *Blood* 1992;**79**:2592–2597.
19. Date Y, Nakazato M, Shiomi K, Toshimori H, Kangawa K, Matsuo H et al. Localization of human neutrophil peptide (HNP) and its messenger RNA in neutrophil series. *Ann Hematol* 1994;**69**:73–77.
20. Park I-K, Morrison SJ, Clarke MF. *Bmi1*, stem cells, and senescence regulation. *J Clin Invest* 2004;**113**:175–179.
21. Leung C, Lingbeek M, Shakhova O, Liu J, Tanger E, Saremaslani P et al. *Bmi1* is essential for cerebellar development and is overexpressed in human medulloblastomas. *Nature* 2004;**428**:337–341.