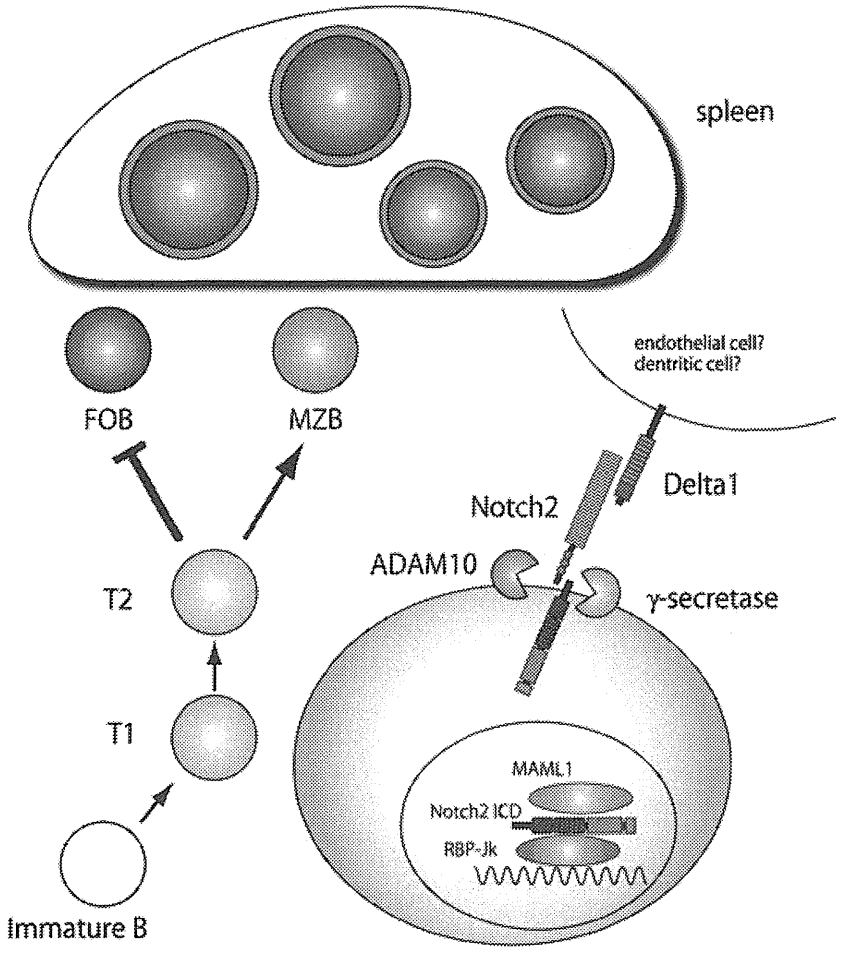

Fig. 1 Notch2 regulates marginal zone B cell development. Dll1 engagement of the Notch2 receptor expressed on splenic immature B cells initiates the Notch2 signaling cascade through ADAM10-mediated cleavage. This results in the formation of Notch-RBP-J-MAML1 proteins, which regulates gene expression and skews the differentiation program toward MZB cells rather than FOB cells

genes of the Notch signaling cascade were inactivated. Mind bomb (Mib), an E3 ubiquitin ligase, activates Notch signaling through endocytosis of Notch ligands. Deletion of *Mib1* in nonhematopoietic cells recapitulates defects in MZBs, whereas deletion in hematopoietic cells resulted in MZB levels that were comparable to control mice (Song et al. 2008).

Deletion of *ADAM10*, which encodes a matrix metalloprotease that processes the extracellular domain of the Notch receptor after ligand binding, also results in defects in MZB cells (Gibb et al. 2010). In contrast, deletion of *Msx2-interacting nuclear target protein* (*MINT*), which is a repressor of RBP-J mediated transcriptional activity and thus considered to be a negative regulator for Notch signaling, showed decreased numbers of FOB cells and increased numbers of MZB cells (Kuroda et al. 2003). Notch receptors can be modified by fringe glycosyl transferases. Lunatic and manic fringe were shown to cooperatively enhance Dll1-Notch2 interaction, and thereby induce MZB development (Tan et al. 2009).

Taken together, these data suggest that Dll1 engagement of the Notch2 receptor expressed on splenic immature B cells initiates the Notch2 signaling cascade through ADAM10-mediated cleavage. This results in the formation of a multi-protein complex including Notch-RBP-J-MAML1 proteins, which regulates gene expression and thereby skews the differentiation program toward MZB cells rather than FOB cells (Fig. 1).

3 Notch2 Signaling in Peripheral T Cell Differentiation and Activation

Signaling through Notch1 has been proven to be among the most important systems for immature T cell differentiation in the thymus (Radtke et al. 1999). During T cell differentiation, Notch signaling is also an essential component for functional maturation and activation of peripheral T cells, in which Notch2 appears to be among the main players (Maekawa et al. 2008). Notch2 expression is increased along with activation of CD8+ cytotoxic T lymphocytes (CTL) (Maekawa et al. 2008). CTLs of both *Notch2* and *RBP-J* conditional knockout mice show an impaired activation potential in vitro as well as in vivo (Maekawa et al. 2008). Cleaved Notch2 (N2IC) directly interacts with CREB and p300 and binds to the promoter of the granzyme B gene, an effector molecule of CTL (Maekawa et al. 2008). Conditional inactivation of *Notch2* in CD8+ T cells results in a decreased antitumor response (Sugimoto et al. 2010). Notch1 appears to be dispensable for an efficient CTL response given the fact that deletion of *Notch1* in CD8+ T cells shows an antitumor response comparable to control mice (Sugimoto et al. 2010). However, this view was recently challenged by a report showing that Notch1 also directly controls main players of CTL, including Eomes, perforin, and granzyme B (Cho et al. 2009).

Notch2/Notch1 double deficient animals reveal impaired differentiation of na CD4+ T cells toward helper T2 (Th2) cells. Notch was shown to directly regulate the transcription of the transcription factor GATA3, and the cytokine interleukin-4 (IL-4) (Amsen et al. 2004, 2007; Fang et al. 2007), both of which are important mediators of Th2 differentiation. *RBP-J* deficient animals recapitulate the phenotype observed in *Notch2/Notch1* deficient animals indicating that this process is mediated through canonical Notch signaling (Amsen et al. 2004). The role of Notch signaling in Th1 differentiation is less clear. Several reports demonstrated that Th1 differentiation is augmented by Notch signaling (Maekawa et al. 2003; Sun et al. 2008); however, a Th1 response is maintained in *Notch2/Notch1* double deficient, and *RBP-J* deficient animals (Amsen et al. 2004), as well as in mice expressing a dominant negative *MAML1* (Tu et al. 2005), questioning the importance of Notch signaling in Th1 differentiation.

With certainty, it can thus be summarized that Notch2 signaling induces cytotoxic T cell differentiation and activation, and that Notch1 and Notch2 concordantly induce Th2 cell differentiation.

4 Notch2 Signaling in Mast Cells

Mast cells arise from HSCs in the bone marrow, migrate to peripheral tissues as immature progenitors, where they subsequently differentiate into mature mast cells (Galli et al. 2005). However, the detailed process of their development is still disputed. Mast cells can be generated in vitro by culturing mouse bone marrow cells with a defined cocktail of cytokines. This in vitro system allows to partially mimic the physiologic development of mast cells. Notch2 signaling instructs myeloid progenitors to adopt a mast cell fate as opposed to differentiate into neutrophils or macrophages, through the coordinated regulation of *Hes1* and *GATA3* (Sakata-Yanagimoto et al. 2008). Mast cells are divided into two subtypes; mucosal and connective tissue type mast cells (Gurish and Boyce 2006; Miller and Pemberton 2002). Each subtype features specific mast-cell proteases (mMCP) (Miller and Pemberton 2002). Notch2 signaling skews cultured mast cells toward the mucosal type rather than connective tissue type (M.S.-Y. and S.C., unpublished data). The *Strongyloides venezuelensis* (*SV*) infection model is useful for analyzing mast cell-mediated mucosal immunity (Maruyama et al. 2000). This nematode evokes intraepithelial mast cell hyperplasia in the small intestine (Maruyama et al. 2000). *Notch2*-null mice show impaired expulsion of *SV*, possibly because of a delayed mast cell progenitor production in the bone marrow, impaired migration of mast cells from the lamina propria to the intraepithelium of the intestine, and impaired activation of intestinal mast cells (Sakata-Yanagimoto et al. 2011). The number and distribution of connective tissue-type mast cells are normal in *Notch2*-null mice (Sakata-Yanagimoto et al. 2011), suggesting that Notch2 signaling is specifically required for proper migration and activation of intestinal mast cells.

5 Notch2 Signaling in Dendritic Cells

Dentritic cells (DCs) initiate immune responses by presenting antigen to na T cells (Steinman and Idoyaga 2010). DCs arise from common bone marrow progenitors that can give rise to both DCs and macrophages (Steinman and Idoyaga 2010). DCs comprise two subclasses, i.e., the so-called plasmacytoid DCs and classical DCs. Classical DCs residing in the spleen are further classified into two main subsets; CD8+CD11b− DCs which mediate cross-presentation to cytotoxic T cells via MHC class I pathway (Dudziak et al. 2007; den Haan et al. 2000) and CD8−CD11b+ DCs which preferentially present MHC class II restricted antigens to CD4+ helper T cells (Dudziak et al. 2007). CD8−CD11b+ DCs are mainly localized in the marginal zone, adjacent to the Dll1-expressing cells (Caton et al. 2007). CD11b+ DCs in the lamina propria of the intestine contain two distinct subsets; CD11b+CD103+ DCs and CD11b+CD103− DCs. CD11b+CD103+ DCs migrate to mesenteric lymph nodes and are presumed to have antigen presenting potential to helper T cells (Denning et al. 2011; Bogunovic et al. 2009).

DC-specific deletion of either *Notch2* or *RBP-J* impairs the development of CD8−CD11b+ DCs in the spleen (Caton et al. 2007; Lewis et al. 2011). CD8+ DCs are also decreased by deletion of *Notch2* but are not affected by the deletion of *RBP-J* (Caton et al. 2007; Lewis et al. 2011). Splenic CD11b+ DCs are divided into two subsets according to the expression levels of Esam and Cx3cr1 (Lewis et al. 2011). CD11b+Esam^{high}Cx3cr1^{low} but not CD11b+Esam^{low}Cx3cr1^{high} DCs are almost abrogated by deletion of either *Notch2* or *RBP-J* (Lewis et al. 2011). CD11b+Esam^{high}Cx3cr1^{low} cells are required for proper priming of T cells in the spleen, which are reduced in *RBP-J*-null mice (Lewis et al. 2011).

Notch2 selectively controls CD11b+CD103+ DCs in the lamina propria of the intestine as well as those that migrate toward mesenteric lymph nodes, which in turn are important for supporting IL-17 producing CD4+ T cells. CD11b+CD103+ DCs are not affected by the inactivation of *RBP-J* (Lewis et al. 2011).

Taken together, Notch2 regulates tissue-specific subsets of DCs in the spleen and in the intestine. Notch2 function might be partly mediated by a RBP-J independent/noncannonical pathway.

6 Notch2 Signaling in Hematopoietic Stem Cells

Notch signaling plays an essential role in self-renewal of stem cells as well as in the growth and differentiation of diverse progenitors within various organs. In contrast, the role of Notch signaling in self-renewal of HSC has been disputed over the years. Early in vitro gain-of-function experiments, such as introduction of a constitutive active form of Notch (Varnum-Finney et al. 2000; Stier et al. 2002) or the transcription factor Hes1 (Kunisato et al. 2003), and stimulation of HSCs with cell-surface expressed ligands or ligand-immunoglobulin chimeric proteins (Karanu et al. JEM 2000; Ohisi et al. JCI 2002; Suluki et al. Stem cells 2006), indicated that Notch signaling supports self-renewal of HSCs and has a role in HSC expansion. On the contrary, several loss-of-function experiments suggest that Notch signaling is dispensable for maintenance of HSCs. HSCs lacking *RBP-J* and those expressing dominant negative *MAML1*, a potent inhibitor of the Notch transcriptional complex, achieve long-term reconstitution comparable to wild-type HSCs, when transplanted into irradiated mice (Maillard et al. 2008). The reconstitution potential of HSCs null for both *Notch1* and *Jagged1* was shown to be comparable to that of wild-type HSCs (Mancini et al. 2005).

However, recently, such negative findings were partially challenged. At a very early time point after treatment with 5-fluorouracil, the number of multipotent progenitors (MPPs) was decreased in *Notch2*-null mice, compared to that in control mice (Varnum-Finney et al. 2011). Similarly, shortly after transplantation, both MPPs and long-term HSCs were decreased in *Notch2*-null BM transplanted mice (Varnum-Finney et al. 2011).

In summary, these data suggest that although Notch signaling is dispensable for homeostasis of HSCs, in challenge and stress situations signaling through Notch2 seems to play a role in the process of HSCs expansion.

7 Notch2 Signaling in Transformation of Blood Cells

7.1 Notch2 Mutations in B Cell Lymphomas

Notch1 is among the most important molecules for physiologic development of T cells (Radtke et al. 1999), and Notch2 is indispensable for MZB cell development (Saito et al. 2003) as described above. Discovery of hyperactivation of Notch1 and Notch2 through gain-of-function mutations in immature T cell neoplasms (Weng et al. 2004) (T cell acute lymphoblastic leukemia or T-ALL in humans) and in subtypes of mature B cell neoplasms (Lee et al. 2009; Troen et al. 2008), respectively, appears to echo the physiologic roles of these molecules in specific lineages and differentiation stages. Those mutations are concentrated in the extracellular heterodimerization (HD) domain and the intracellular proline-, glutamic acid-, serine-, and threonine-rich (PEST) domain of *Notch1* in T-ALL (Weng et al. 2004), and only in the PEST domain of *Notch2* in mature B cell lymphomas (Lee et al. 2009; Troen et al. 2008). The distribution of mutations suggests that hyperactivation of Notch2 signaling in B cell lymphomas still requires binding of the ligand, whereas mutations within the HD domain of *Notch1* in T-ALL results in ligand independent activation of Notch1 signaling. In contrast to the fact that *Notch1* mutations are found in approximately 50 % of T-ALL cases (Weng et al. 2004), *Notch2* mutations were identified in only five out of 63 cases (8 %) of diffuse B-cell lymphoma (Lee et al. 2009) and in two out of 41 cases (5 %) of MZB cell lymphoma (Troen et al. 2008).

The relationship between B cell development and gain-of-function mutations in Notch2 is not as clear as in the context of T cell development and Notch1 mutations. Genetic evidence described above strongly suggests an oncogenic role of deregulated Notch2 in B lineage transformations. On the contrary, there has been a series of reports describing the tumor suppressive function of Notch signaling in B lineage cells, particularly in B-cell lymphoblastic leukemia (B-ALL) (Zweidler-McKay et al. 2005; Kannan et al. 2011), although loss-of-function mutations have not been found in the Notch2 signaling pathway. Integrating these pieces of information, it seems likely that Notch2 signaling can context dependently promote or suppress growth of B lineage cells. Another complexity was recently added by the identification of Notch1 mutations through the genome-wide screening of patient samples suffering from chronic lymphocytic leukemia (Puente et al. 2011), a type of intermediately mature B cell neoplasm and mantle cell lymphoma (Kridel et al. 2012), another type of mature B neoplasms.

7.2 Notch2 Signaling in Myeloid Neoplasms

Recently, Notch signaling was proven to function as a tumor-suppressor in chronic myelomonocytic leukemia (CMML) (Klinakis et al. 2011); several components of the Notch pathway, including *Nicastrin* (*NCSTN*), *APH1A*, *MAML1*, and *Notch2*

Table 1 Role of Notch signaling as tumor activator or tumor suppressor in hematopoietic leukemia/lymphoma

	Gain-of-function			Loss-of-function	
	T	B	M	B	M
Notch1	T-ALL ATL	CLL MCL DLBCL	AML	(B-ALL)[a]	
Notch2		MZB lymphoma DLBCL			CMML

T-ALL, T-cell acute lymphocytic leukemia; ATL, adult T-cell leukemia/lymphoma; CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; DLBCL, diffuse large B-cell lymphoma; MZB lymphoma, marginal zone B-cell lymphoma; AML, acute myeloid leukemia; B-ALL, B-cell acute lymphocytic leukemia; CMML, chronic myelomonocytic leukemia

[a] Loss-of-function mutations in Notch signal components have not been found in B-ALL

itself were found to be mutated and defective in CMML patients. This conclusion is also supported by the phenotype of mice lacking *NCSTN*, a component of γ-secretase, as well as that of *Notch1*-, *Notch2*-, and *Notch3*- triple null mice (Klinakis et al. 2011). These animals show enhanced granulocyte-monocyte progenitor potential and develop a fatal CMML-like disease (Klinakis et al. 2011). On the contrary, activating mutations of Notch1 were found in acute myeloid leukemia, a precursor myeloid neoplasms, though the frequency is less than 1 % (Wouters et al. 2007; Fu et al. 2006). Thus, as is the case of B cell malignancies, Notch signaling can function as either tumor promoter or suppressor within myeloid neoplasms.

These oncogenic and tumor suppressive functions of Notch1 and Notch2 signaling in T cell, B cell, and myeloid lineages have been summarized in Table 1. Knowledge about this area will expand rapidly in the very near future using current sequencing technology.

8 Conclusion

Signaling through Notch2 has an essential role in two major cell types present in the marginal zone of the spleen, splenic MZB cells, and splenic DCs. Notch2 signaling also mediates intestinal immunity by regulating development and localization of intestinal DCs and mast cells, and development of helper T cells and CTLs. Genetic and biologic evidence indicates that abnormal Notch2 signaling is involved in transformation of immune cells, although its functions appear to be bivalent; oncogenic signaling for mature B neoplasms and tumor suppressive signaling for mature myeloid neoplasms. The reason of specificity and non-redundant functions of Notch2 in the immune system may be partly attributed to the differences in expression patterns among Notch family genes, although this issue needs to be elucidated in more detail in future studies.

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ORIGINAL ARTICLE

Identification of unbalanced genome copy number abnormalities in patients with multiple myeloma by single-nucleotide polymorphism genotyping microarray analysis

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Abstract Single-nucleotide polymorphism genotyping microarray (SNP array) analysis provides detailed information on chromosomal copy number aberrations. To obtain detailed information on genomic abnormalities related to pathogenesis or prognosis of multiple myeloma (MM), we performed 250K SNP array analysis in 39 MM patients and 11 cell lines. We identified an accumulation of deletions and uniparental disomies at 22q12.1. Among the hyperdiploid MM cases, chromosomal imbalance at this locus was associated with poor prognosis. On sequencing, we also found a mutation in the seizure-related 6 homolog (mouse)-like (*SEZ6L*) gene located at ch.22q12.1 in an MM cell line, NOP1. We further found isolated deletions in 17 genes, five of which are known tumor suppressor genes. Of these, deletion of protein tyrosine phosphatase, receptor type D (*PTPRD*) was found in three samples, including two patients. Consistent with previous reports, non-hyperdiploid MM, deletion of 13q (del13q) and gain of 1q in non-hyperdiploid MMs were predictive of poor prognosis ($p = 0.039$, $p = 0.049$, and $p = 0.013$, respectively). However, our analysis revealed that unless accompanied by gain of 1q, the prognosis of non-hyperdiploid MM was as good as that of hyperdiploid MM. Thus, SNP array analysis provides significant information useful to understanding the pathogenesis and prognosis of MM.

Keywords Multiple myeloma · Snp array · Chromosomal copy number imbalances

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Introduction

Multiple myeloma (MM) is a hematological neoplasm characterized by the clonal expansion of malignant plasma cells within the bone marrow. In conventional chromosomal analysis, 60–70 % of MM patients reveal a normal karyotype, at least in part because the bone marrow is often only partially infiltrated by MM cells and metaphases are more readily available in normal myeloid cells than in MM cells [1]. Interphase fluorescence in situ hybridization (FISH), a technique recently used more commonly in clinical practice, has demonstrated frequencies of many genetic abnormalities that are potentially involved in the pathogenesis of MM in a more precise manner [2–4]. Owing to FISH analysis, we now understand that >80 % of MM patients have genetic abnormalities visible at the chromosomal level [1, 4, 5]. About half of all MM cases are a hyperdiploid type, wherein the odd-numbered chromosomes such as 3, 5, 7, 9, 11, 15, 19, and 21 tend to be increased in number [6–8]. The remaining cases are the non-hyperdiploid type, and chromosomes 8, 13, 14, and 16 are frequently lost [1]. In non-hyperdiploid MM, the immunoglobulin heavy chain *(IgH)* gene recurrently forms a fusion with fibroblast growth factor receptor 3 (*FGFR3*;

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at 4p16), multiple myeloma SET domain/Wolf-Hirschhorn syndrome candidate1 (*MMSET*; at 4p16.3), cyclin D3 (*CCND3*; at 6p21), cyclin D1 (*CCND1*; at 11q13), and musculoaponeurotic fibrosarcoma oncogene homolog (*MAF*; at 16q23). In MM, deletion of tumor protein 53 (*TP53*; at 17p13) or retinoblastoma 1 (*RB1*; at 13q14) is considered to be predictive of poor prognosis [9, 10]. Monosomy 13q (del13q) is observed in 30–35 % of hyperdiploid MM cases and in as many as 85 % of non-hyperdiploid MM cases [1].

Single-nucleotide polymorphism genotyping microarray (SNP array) analysis provides more detailed information on the genomic copy number changes in cancers. We and others previously demonstrated that genes localized to such genomic regions with copy number changes are often a target of mutations and involved in tumorigenesis of hematologic malignancies [11–14]. In this paper, we report the findings from an MM cohort analyzed by a SNP array that has an improved algorithm over those previously used for other MM cohorts. We discovered several small deletions and uniparental disomies (UPDs), which might include candidate genes responsible for the pathogenesis of MM. We further compared these SNP array data with clinical information to clarify the impact of genomic abnormalities on the prognosis of MM. We found several new pieces of information, including the impact of the presence or absence of gain of 1q gain in non-hyperdiploid MM cases.

Materials and methods

Patient samples

Thirty-nine bone marrow samples from patients who were diagnosed as having multiple myeloma from May 1992 through December 2010 were collected at Tsukuba University Hospital. Thirty-two of the samples were obtained from patients at the time of diagnosis and the remaining 7, from patients in the later course of the disease. The first-line treatment was MP (melphalan and prednisolone) in 12 (30.7 %), MCP (melphalan, cyclophosphamide, and prednisolone) in 9 (23.0 %), VAD (vincristine, doxorubicin, and dexamethasone) in 10 (25.6 %), and prednisolone alone in 1 (2.6 %). Seven patients (18.0 %) received high-dose chemotherapy with autologous peripheral stem cell transplantation after VAD treatment. Allogeneic stem cell transplantation was performed for 2 patients after relapse following bortezomib or thalidomide treatment. Plasma cells constituted 4–83 % (average 33 %) of the bone marrow nucleated differential cell count at the time of sample preparation. The clinical characteristics of the 39 patients are summarized in Table 1 and supplemental table 3. This study was approved by the institutional ethics committee of Tsukuba University Hospital.

Cell lines

Eleven human myeloma cell lines, AMO1, FR4, oda, JJN3, KMS-11, KMS-12PE, NCU-MM1, NOP1, SK-MM-1, U266, and XG7, were kindly provided by Dr. S. Iida (Nagoya City University Graduate School of Medical Sciences). All cell lines were cultured in Roswell Park Memorial Institute (RMPI) 1640 medium (Sigma-Aldrich Japan, Tokyo, Japan). 1×10^6 cells were collected for DNA extraction.

DNA extraction

Mononuclear cells (MNCs) were separated from bone marrow samples and stored at −80 °C until DNA extraction. CD138-positive cells were further purified from MNCs from 7 patients using CD138 microbeads and a MACS separation column (Miltenyi Biotec, Tokyo, Japan). DNA extraction was performed with the column purification protocol attached to the PureGene Kit (Qiagen, Tokyo, Japan).

Table 1 Clinical characteristics of patients with multiple myeloma ($n = 39$)

Parameter at diagnosis	No. of patients (%)
Median	
Age (years)	61.9 (34–85)
Sex	
Male	20 (51.2)
Female	19 (48.7)
Subtype	
IgG λ/κ	16 (41.0)/12 (30.7)
IgA λ/κ	4 (10.2)/0
IgD λ/κ	0/2 (5.1)
BJP λ/κ	3 (7.6)/1 (2.5)
Others (IgM-κ)	1 (2.5)
Clinical stage (ISS)	
1	10 (25.6)
2	10 (25.6)
3	13 (33.3)
ND	6 (15.3)
FISH analysis	
Del(13q) ($n = 12$)	7 (58.3)
Del(17p13)/TP53 ($n = 12$)	4 (33.3)
Del(13q) and Del(17p13) ($n = 12$)	2 (16.6)
Karyotype (G-band)	
Normal	29 (74.3)
Hyperdiploid	4 (10.3)
Non-hyperdiploid	4 (10.3)
ND	2 (5.1)

BJP Bence-Jones protein, *ISS* International Staging System, *ND* not determined

SNP-based mapping array

The genomic DNA obtained from the bone marrow samples and cell lines was subjected to a 250K GeneChip SNP genotyping microarray (Affymetrix, Tokyo, Japan) [11]. The array data were analyzed with Copy Number Analyzer for GeneChips (CNAG) software for allele-specific copy number analysis (http://www.genome.umin.jp) [15]. On the basis of the SNP array results and in accordance with the International System for Human Cytogenetic Nomenclature, MM patients who had 47–57 chromosomes were defined as having hyperdiploid MM and the remaining patients, as having non-hyperdiploid MM (2009) [16].

Clinical data and statistical analysis

Patients' clinical information such as routine blood analyses, bone marrow chromosomal studies (G-banding and interphase FISH for 13q14.3 [D13S319] and 17p13), treatments and responses, and overall survival (OS) was retrospectively obtained from the medical records. All G-banding and interphase FISH analyses were outsourced to SRL (Tokyo, Japan). The cut-off values for deletion of 13q14.3 and 17p, respectively, were adopted from the false-positive ratios announced by the SRL. Based on the results of the SNP array analysis, Kaplan–Meier survival curves were calculated using Statistical Product for Service and Solution (SPSS) version 18.0 software (IBM Corporation, Tokyo, Japan), and significance was defined as a probability value of <0.05. The median follow-up time was 52.3 months (range 3.0–201.3 months).

Mutation analysis of the seizure-related 6 homolog (mouse)-like (*SEZ6L*) gene

All exons of *SEZ6L* were sequenced in the 11 cell lines and in 8 bone marrow samples in which the plasma cells constituted more than 80 % of the bone marrow cells, with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The primer sequences for the *SEZ6L* exons were obtained from a previous report [17]. All sequence data were compared with the *SEZ6L* sequence provided in the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) database.

Results

Inclusion of patients, karyotyping, and FISH analysis

Among 39 MM patients, male-to-female ratio was 20:19 and the most common subtype was IgG λ (41.0 %). When the patients were classified according to the stage of International Staging System (ISS), 10 patients (25.6 %) were ISS 1 or 2, 13 patients (33.3 %) were ISS 3 (median OS: not reached, 87.7 and 70.2 months, respectively). G-banded karyotypes were obtained from the medical records of 37 patients. An abnormal karyotype was reported in 8 of those patients (20.6 %), and a normal karyotype in the remaining 29 (74.3 %). Records of interphase FISH to detect deletions of 13q14.3 and 17p13 were available for 12 patients. Of those 12 samples, del(13q) was found in 7 patients (58.3 %) and del(17p13), in 4 patients (33.3 %). Two patients (16.6 %) had both del(13q) and del(17p13) (Table 1). All abnormalities that could be detected by G-banding and FISH analysis were also detected by SNP array.

Diploidy abnormalities

Genome-wide copy number changes detected by SNP array analysis are listed in Table 2 and Supplemental Figure 1. In the current cohort of 39 patients, 23 (59.0 %) had

Table 2 Summary of chromosomal copy number changes in 39 MM patients

Deletions (%)		Gains (%)		UPD (%)	
Ch	HD/NHD/All	Ch	HD/NHD/All	Ch	HD/NHD/All
1p	13.0/12.5/12.8	1q	52.1/50.0/51.2	1p	4.3/12.5/7.6
6q	8.6/18.7/12.8	3q	43.4/12.5/30.7	1q	0.0/18.7/7.6
8p	26.0/37.5/30.7	5[a]	43.7/0.0/25.6	2p	17.3/18.7/18.2
9p	0.0/12.5/5.1	6p	34.7/0.0/20.5	2q	8.6/12.5/10.2
11q	0.0/25.0/10.2	7q	43.4/12.5/30.7	6q	8.6/6.2/7.6
13q	26.0/56.2/38.4	9q	60.3/12.5/41.0	8q	8.6/12.5/10.2
16q	8.6/25.0/15.3	11q	65.2/0.0/38.4	9p	13.0/6.2/10.2
22q	13.0/18.7/15.3	15[a]	54.5/6.2/41.4	13q	4.3/12.5/7.6
		18[a]	21.7/0.0/12.8	16q	4.3/12.5/7.6
		19p	69.5/12.5/46.1	22q	8.6/6.2/7.6
		19q	47.8/0.0/25.6		
		21q	17.3/0.0/10.2		

Ch chromosome, *HD* hyperdiploid, *NHD* non-hyperdiploid

[a] Gain of both chromosomal arms

hyperdiploid-type MM and 16 had non-hyperdiploid-type MM (41.0 %).

Numeric chromosomal changes

Gains of a single arm or of entire chromosomes resulted in hyperdiploid MM in most cases, and in non-hyperdiploid MM in only a small number of cases. These chromosomal gains were frequent at odd-numbered chromosomes, as previously described (Table 2) [8]. Gains of 1q, 9q, 11q, 15, and 19p were found in more than 50 % of the hyperdiploid MM cases. Gain of 1q was also found in 50 % of the non-hyperdiploid MM cases. Loss of a single chromosomal arm was frequent at 1p, 8p, 13q, and 22q in more than 10 % of both the hyperdiploid and the non-hyperdiploid MM cases, and preferentially in the non-hyperdiploid MM cases. Loss of 6q, 11q, and 16q was found in more than 10 % of the non-hyperdiploid MM cases. Among these, 13q loss was found in more than 50 % of the non-hyperdiploid MM cases (Table 2).

Frequency and distribution of uniparental disomies

Uniparental disomy (UPD), characterized by copy number-neutral LOH, results in LOH without allelic loss in SNP array analysis [8, 18]. UPD often implies that genes localized in such abnormal genome regions are involved in tumorigenesis if they are repeated in multiple cases. Here, the frequency of UPD was almost the same in the hyperdiploid and non-hyperdiploid MM cases. In total, 17 of the 39 cases (43.5 %) had UPDs. Two or more UPDs were found in 10 cases (25.6 %); in particular, 2 cases (MM45 and MM74) demonstrated more than 10 UPDs (15 and 12 lesions, respectively). Among those 10 cases with 2 or more UPDs, UPD at a single region of 2p was present in 7 cases (18.2 %; Table 2).

Analysis of repetitive copy number-abnormal regions and identification of a missense mutation in *SEZ6L*

We found that repetitive copy number-abnormal regions that had overlapping gains, deletions, or UPDs of chromosomes in more than 10 samples are confined to a very short length at 4 sites: at 16q21 including cadherin 11 (*CDH11*), at 19q12 including HERV-K_19q12 provirus Rec protein, at 22q11.22 including immunoglobulin lambda constant 1 (*IGLC1*), and at 22q12.1. Among these, the region at 22q12.1 (chromosome 22: 24,650,773-25,107,435, with a size of 456 kbp) was found to include both deletions and UPDs (Supplemental Figure 2a). When analyzed in all cases, abnormalities of this chromosomal lesion had no impact on OS; however, when analyzed in hyperdiploid MM cases only, patients with these abnormalities had poorer prognosis than did those without them (median, 60.1 vs not reached; $p = 0.040$; Supplemental Figure 2d). The minimal commonly affected region at 22q12.1 contained 2 genes, myosin XVIIIB *(MYO18B)* and *SEZ6L*. Both these genes are considered to be tumor suppressors; their deletions, mutations, and DNA methylation at promoter regions have been reported in lung and gastric cancers and in melanoma [17, 19–21]. We performed direct sequencing for *SEZ6L* and found a missense mutation, G563A, in exon 2 in an MM cell line, NOP1 (Supplemental Figure 2b) [17].

Isolated gene gains and deletions

The SNP array analysis identified isolated gains of 10 genes and deletions of 17 genes. Except for *c-myc*, these genes have not been described as amplified or deleted in MM (Supplemental Table 1) [22]. All cases having isolated gene deletions were cases of non-hyperdiploid MM. Among the 17 deleted genes, protein phosphatase 2, regulatory subunit B, beta (*PPP2R2B*) (1 case), protein tyrosine phosphatase, receptor type, D (*PTPRD*) (2 cases), discs, large homolog 2 (*DLG2*) (1 case), breast cancer cell protein 2 (*BRCC2*) (1 case), and protein tyrosine phosphatase, receptor type, T (*PTPRT*) (1 case) are considered to be tumor suppressors (Fig. 1 and Supplemental Figure 3) [23–28]. Notably, isolated deletion of *PTPRD* was found in 3 samples, including 2 from patients, and 1 MM cell line had deletion from 9p23 to 9q34.3, a region that includes *PTPRD* (Fig 1).

Significance of copy number abnormalities on prognosis

We evaluated the effects of genome copy number abnormalities on OS (Table 3). Non-hyperdiploid MM cases had poorer prognosis than did hyperdiploid MM cases (median, 52.2 vs 87.7 months; $p = 0.039$; Fig 2a). Patients with del13q also had shorter survival (median, 54.4 vs 87.7 months; $p = 0.049$; Fig 2b). Among the non-hyperdiploid MM cases, those with del13q had shorter survival than did those without it, although the difference between them was not significant (median, 28.9 vs 84.3; $p = 0.099$; Table 3 and Fig 2d). The presence of del13q had no impact on prognosis in the hyperdiploid MM cases (Table 3). Furthermore, Gain of 1q showed a substantially negative impact on OS (median, 24.3 vs 84.3 months; $p = 0.013$) when compared only within the non-hyperdiploid cases. In the whole population, gain of 1q did not show an impact on OS, although there was a tendency of poorer prognosis in those having gain of 1q in the current cohort. Patients with gain of 15q or 19q tended to have a better prognosis than did those without, although the differences were not

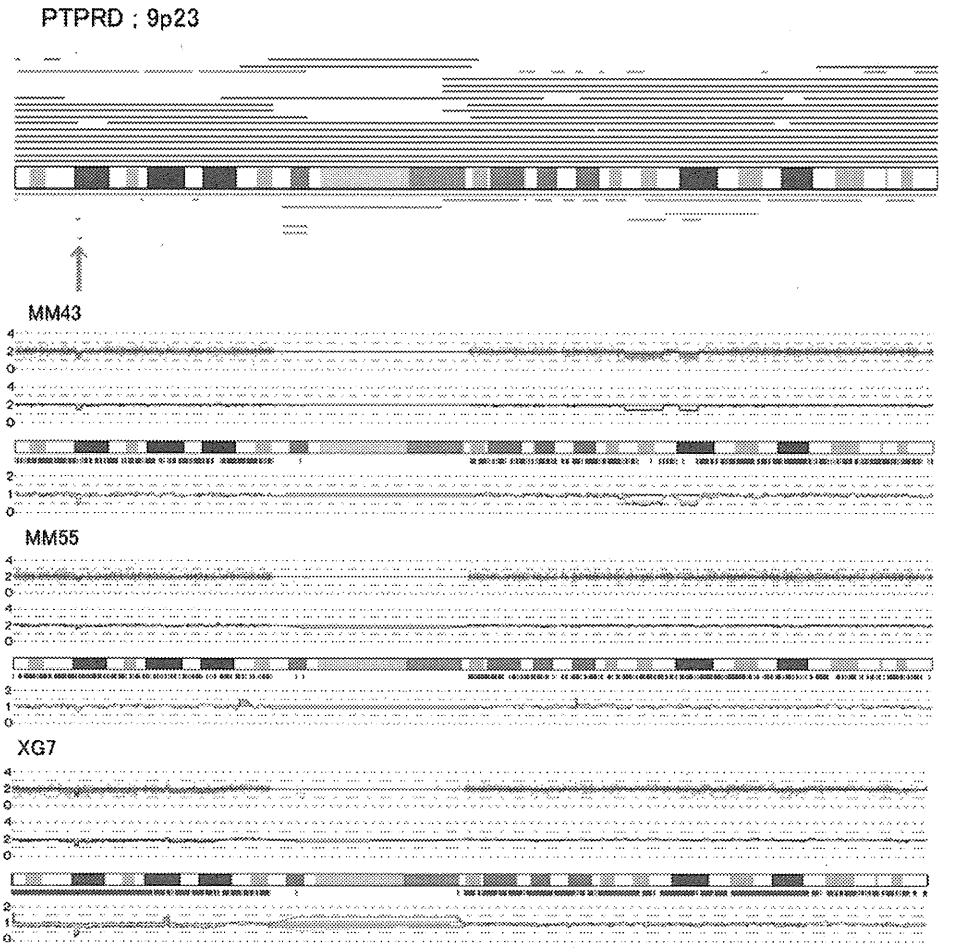

Fig. 1 Deletion of *PTPRD* located at 9p23 Isolated deletion of *PTPRD*, a tumor suppressor gene, was observed in 2 MM patients and 1 MM cell line (*red arrow*). In the top chromosomal view, amplified regions are shown as *red lines* and deleted regions, as *green lines*. In each sample's view, the upper *small red dots lines* are the signals of the SNP array probes, the *middle blue lines* show the allele copy number calculated by CNAG, and the *bottom red* and *green lines* show individual alleles

statistically significant ($p = 0.058$ and $p = 0.065$, respectively; Supplemental Figure 4). Gains of 6p (20.5 %) or ch.18 (12.8 %) were observed only in the hyperdiploid MM cases, but they had no impact on OS ($p = 0.538$ and $p = 0.550$, respectively; Table 3). OS for patients with UPDs and for those without UPDs was not significantly different ($p = 0.193$), and each UPD also had no impact on OS.

Discussion

In this study, we analyzed genome copy numbers at high resolution using 250k SNP array analysis in 39 MM patients and 11 MM cell lines. The patterns of genome copy number abnormalities, the frequencies of gains of 3q, 11q, chromosomes 15 and 21, and deletions of 8p and chromosome 22 in our cohort were similar to those found in previous cohorts analyzed by SNP array [7, 8], whereas the frequency of gain of 1q was higher and those of deletions of 1p, 6p, 13q and 16q were fewer in our cohort.

In our cohort, hyperdiploid MM had a better prognosis than did non-hyperdiploid MM. Some previous reports that used FISH or CGH array analysis also showed that non-hyperdiploid MM had poor prognosis [29]. However, we found that the frequency of gain of 1q was unexpectedly high in non-hyperdiploid MM. Although the poorer prognosis of non-hyperdiploid MM with gain of 1q than of non-hyperdiploid MM without it is known, comparison of the prognosis of non-hyperdiploid MM without gain of 1q with that of hyperdiploid MM with or without gain of 1q has not been described before. The current analysis suggests that the poorer prognosis of non-hyperdiploid MM is true only when it is accompanied by gain of 1q and that without the gain of 1q, the prognosis of non-hyperdiploid MM may be as good as that of hyperdiploid MM (Fig 2c).

Many copy number changes of large genomic regions were frequently identified, among which del13q was the most common (38.4 %), followed by 8p loss (30.7 %; Table 3). Nevertheless, of all these abnormalities, only 13q deletion and 1q gain had a statistically significant impact on survival: both had a negative impact on survival when analyzed in all MM cases as well as in non-hyperdiploid cases only. The impact of del13q on MM survival is a matter of controversy [30]. It was first reported to be a poor

Table 3 Frequencies and prognostic impact of chromosomal copy number abnormalities

Copy number abnormality	Cases, *n* (%)	Prognosis	*p* value
1q+	20 (51.2)	–	0.082
3q+	12 (30.7)	–	0.893
5q+	10 (25.6)	–	0.716
6p+	8 (20.5)	–	0.538
7q+	12 (30.7)	–	0.618
9q+	16 (41.0)	–	0.223
11q+	15 (38.4)	–	0.365
15q+	15 (38.4)	–	0.058
ch 18+	5 (12.8)	–	0.550
19q+	17 (43.5)	–	0.065
del(8p)	11 (30.7)	–	0.155
del(13q)	15 (38.4)	poor	0.049*
del(16q)	6 (15.3)	–	0.190
del(17p)	5 (12.8)	–	0.118
del(22q)	6 (15.3)	–	0.217
All UPDs	17 (43.5)	–	0.193
NHD vs HD	16 (41.0)	poor	0.039*
NHD with 1q+ vs NHD without 1q+	8 (50.0)	poor	0.013*
HD with 1q+ vs HD without 1q+	12 (52.1)	–	0.965
NHD with del(13q) vs NHD without del(13q)	9 (56.2)	–	0.099
HD with del(13q) vs HD without del(13q)	6 (26.0)	–	0.542
Deletion and UPD of *SEZ6L* (22q12.1)	11 (30.7)	–	0.254
HD with deletion or UPD of *SEZ6L*[a]	5 (27.7)	poor	0.040*
NHD with deletion or UPD of *SEZ6L*[b]	6 (37.5)	–	0.497

ch chromosome, *del* deletion of chromosome, *UPD* uniparental disomy, *NHD* non-hyperdiploid, *HD* hyperdiploid, *SEZ6L* seizure-related 6 homolog (mouse)-like

* Significant *p* value ($p < 0.05$)

\+ Gain of chromosome

[a] Compared with HD without deletion or UPD of *SEZ6L*

[b] Compared with NHD without deletion or UPD of *SEZ6L*

prognostic factor when found by conventional cytogenetic studies [10, 31]. However, some subsequent reports showed that del13q detected either by cytogenetic or interphase FISH analysis failed to prove an impact on poor prognosis [29, 32, 33]. In our cohort, the frequency of del13q was higher in the non-hyperdiploid MM cases (non-hyperdiploid MM, 56.2 % vs hyperdiploid MM, 26.0 %).

To make a shortlist of candidate genes, we surveyed genes localized to regions that were amplified or deleted, shorter than 3 Mbp, and contained less than 10 genes (Supplemental Table 2). Among those genes, *CDKN2C* and Fas-associated factor 1 (*FAF1*) at 1p32.3 might be interesting, given that deletion of 1p was observed in 20–30 % of MM patients and is predictive of poor prognosis by SNP array analysis [7, 34]. In the current cohort, 2 hyperdiploid MM cases and 1 non-hyperdiploid case had the 1p32 deletion. One of the 2 hyperdiploid MM cases had a 1.2 Mb small deletion at 1p32.3, which contained only *CDKN2C* and *FAF1*. Other potentially interesting genes are *CDKN2A* and *CDKN2B* in 9p21.3, which were deleted in 2 cases, with one having a 0.2 Mb small deletion.

Most 1q gains were those at the whole long arm level, but 1q21.1-21.2 could be a candidate region relevant to the pathophysiology of MM [35]. CDC28 protein kinase regulatory subunit 1B (*CKS1B*), located at 1q21.2, functions in ubiquitination and degradation of a cyclin-dependent kinase inhibitor, *p27Kip1* [36] and thus was ascribed as one of the genes responsible for the progression of MM [35, 37]. In the current analysis, 1 patient had a 1.2 Mbp gain of 1q21.1. This region contains 13 genes, including B cell CLL/lymphoma 9 (*BCL9*), which is involved in B cell malignancies [38].

A small region in 9p23 containing *PTPRD* was deleted in 2 non-hyperdiploid cases and 2 cell lines, XG7 and AMO1 (Fig 1). Importantly, *PTPRD* was identified as a unique gene in 3 out of the 4 samples. Whereas deletions, mutations, and promoter methylations of *PTPRD* have been observed in various kinds of cancers such as melanoma, lung cancer, colon cancer, gastric cancer, and glioblastoma [25, 39–41], the relevance of this gene to the pathogenesis of MM has not been previously described. Given that *PTPRD* suppresses cell proliferation by dephosphorylation of signal transducer and activator of transcription 3 (*STAT3*), which acts as a signal transducer of interleukin-6 [42] and is an important factor for survival and proliferation of MM cells [43, 44], the deletion of *PTPRD* might play a role in MM, as it does in other cancers. Other than *PTPRD*, 16 genes were identified as

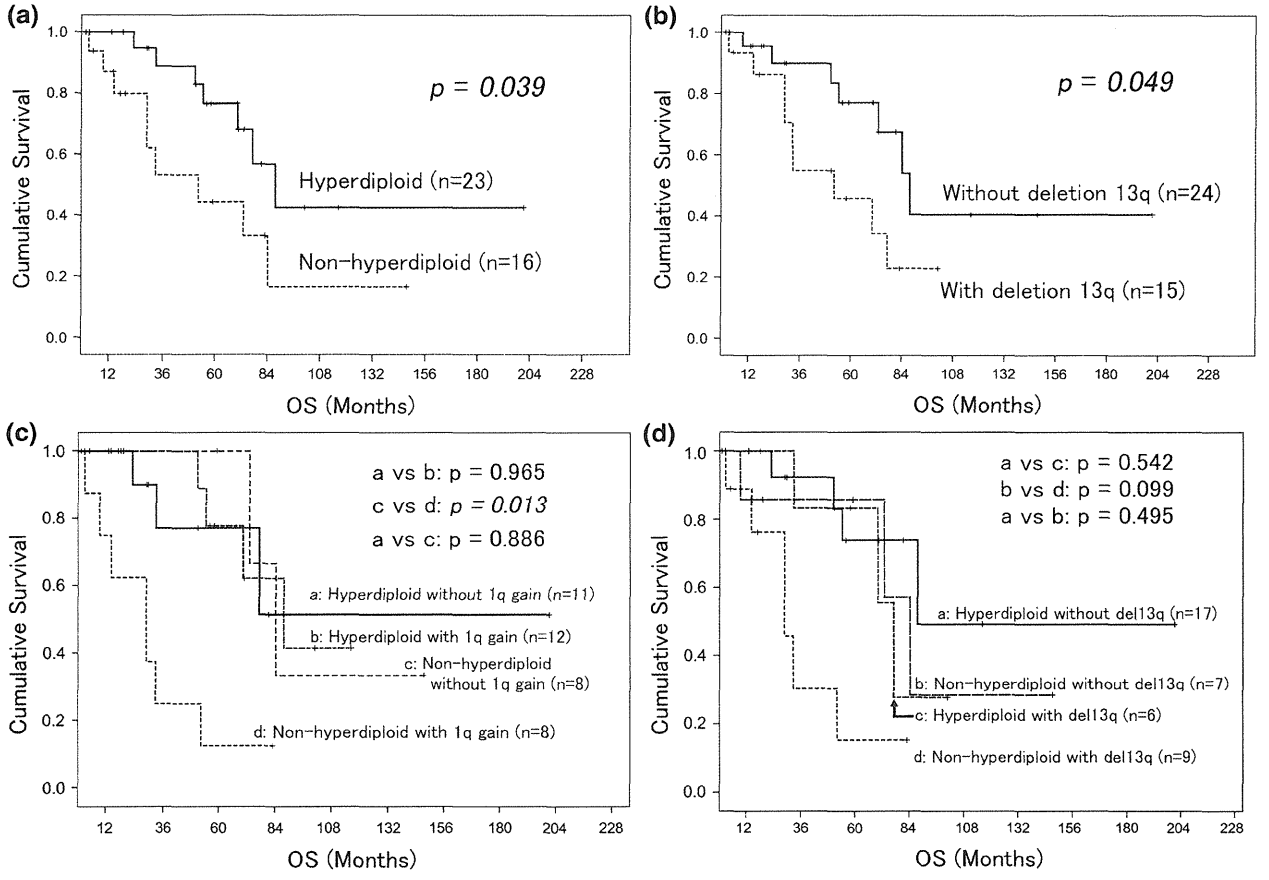

Fig. 2 Effect of chromosomal abnormalities on overall survival in MM patients Overall survival (OS) was calculated by the Kaplan–Meier method. **a** Effect of diploidy status on OS ($p = 0.039$). **b** OS in cases with deletion of 13q versus OS in cases without it ($p = 0.049$). **c** Effect of status of diploidy and gain of 1q on OS. Non-hyperdiploid MM with gain of 1q had a significantly poor prognosis ($p = 0.013$). **d** Effect of status of diploidy and deletion of 13q on OS. *vs* versus

unique in the deleted regions, and all of these isolated gene deletions were found only in the non-hyperdiploid cases. Among the MM cell lines, deletions of the fragile histidine triad *(FHIT)*, WW domain-containing oxidoreductase *(WWOX)*, and deleted in colorectal carcinoma *(DCC)* genes might be meaningful because promoter methylation of *FHIT* and *DCC* as well as deletion of *WWOX* were reported as factors of poorer prognosis in MM patients [45–47].

On the other hand, isolated gene gains were observed in 10 regions (Supplemental Table 1b). Among these genes, *c-myc* and *PVT-1* might be relevant to MM development [48, 49]. These genes are targets of translocation to immunoglobulin loci in B cell malignancies including MM and are located at 8q24. In the current study, 1 MM case (MM57) had a small amplification at 8q24, and 7 MM cell lines (JJN3, KMS11, KMS12-PE, Oda, NCU-MM1, SK-MM1, and XG 7) had more than 4 copies at the same locus.

Recently, whole genome and exome sequencing analysis of 38 MM patients was reported [50]. Most of the genes found to be mutated or deleted in that report were included in the amplified or deleted regions in our SNP array analysis. Deletion of *PTPRD* (1 case) and mutations of Eph receptor A7 *(EPHA7)* (2 cases) and *DLG2* (1 case) were found in their analysis, and isolated deletions of these genes were also found in our SNP array analysis. However, deletion of *PPP2R2B*, *BRCC2*, and *PTPRT* was detected in our SNP array analysis, but not by whole genome/exome sequencing.

SNP array is a powerful tool for detecting detail genomic abnormalities. The abnormalities found in this study might be involved in the etiology of MM. Combination of SNP array analysis with other genetic analysis such as whole genome sequencing will also be helpful in thoroughly understanding the genetic basis of MM.

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Conflict of interest All authors have no conflict of interest to declare.

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Rapid T-cell chimerism switch and memory T-cell expansion are associated with pre-engraftment immune reaction early after cord blood transplantation

Cord blood (CB) contains immature immune cells and is thought to be less active in inducing allogeneic immune reaction than other sources of stem cells. However, a high incidence of immune-mediated complications has been reported, such as pre-engraftment immune reaction (PIR) and haemophagocytic syndrome (HPS) early after cord blood transplantation (CBT) (Kishi *et al*, 2005; Narimatsu *et al*, 2007; Frangoul *et al*, 2009; Takagi *et al*, 2009; Patel *et al*, 2010). In addition, we reported that human leucocyte antigen (HLA) disparity in the graft-*versus*-host (GVH) direction adversely affected engraftment kinetics when single calcineurin inhibitors were used for GVH disease (GVHD) prophylaxis (Matsuno *et al*, 2009). These observations suggested that the GVH reaction plays a critical role in engraftment. Here, we report the engraftment kinetics of donor-derived T cells using a multicolour flow cytometry-based method (HLA-Flow method) (Watanabe *et al*, 2008) and also describe the results of naïve/memory T-cell phenotype analyses early after CBT.

Between November 2009 and September 2010, 73 adult patients underwent single-unit CBT at Toranomon hospital. This study reports 41 patients who were eligible for chimerism analysis using the HLA-Flow method and survived more than 14 d after CBT. Characteristics of the patients and CB are summarized in Table SI. All patients provided written informed consent, and the study was conducted in accordance with institutional review board requirements. Peripheral blood was collected at 1, 2, 3, 4, and 8 weeks after CBT. Anti-HLA monoclonal antibodies in combination with lineage-specific antibodies were used to analyse the lineage-specific chimerism as previously reported (Watanabe *et al*, 2008). Anti-HLA antibodies specific for donor and recipient HLA in all patients are summarized in Table SII. At 2, 4, and 8 weeks after CBT, T-cell subsets were analysed using the following monoclonal antibodies: peridinin-chlorophyll-protein – cyanin 5·5 (PerCP-Cy5·5)-CD8, phycoerythrin – cyanin 7 (PE-Cy7)-CCR7, allophycocyanin (APC)-CD4, APC-Cy7-CD3 (BD Pharmingen, San Jose, CA, USA), and Pacific Blue-CD45RA (CALTAG, Carlsbad, CA, USA). Absolute numbers of $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), and naïve ($CD45RA^+CCR7^+$) and memory ($CD45RA^-CCR7^{+/-}$) T cells were calculated by multiplying the peripheral lymphocyte counts by the percentage of positive cells. PIR was characterized by non-infectious high-grade fever (>38·5°C) coexisting with skin eruption, diarrhoea, jaundice and/or body weight gain greater than 5% of baseline, developing 6 or more days before engraftment (Kishi *et al*, 2005; Uchida *et al*, 2011). Cumulative incidence of neutrophil engraftment, PIR, and GVHD were calculated using Gray's method. Intergroup comparisons were performed using the Mann–Whitney *U*-test.

We analysed lineage-specific chimerism for 32, 40, 40, 34, and 34 patients at a median of 8 (range, 7–11; week 1), 15 (14–20; week 2), 22 (21–25; week 3), 29 (28–36; week 4), and 57 (56–62; week 8) days post-transplant, respectively. Fig 1A shows representative results for $CD4^+$ T-cell chimerism. $CD4^+$ and $CD8^+$ T-cell chimerism results in all patients are shown in Fig 1B. Of 41 enrolled patients, 37 achieved neutrophil engraftment at a median of 19 d (range, 13–38 d). Thirty-nine patients achieved donor-dominant T-cell chimerism (>90%) by 3 weeks after CBT, whereas the remaining two patients, with recipient-dominant T-cell chimerism (>90%) at every point tested, developed graft failure because of early relapse (day 14 post-transplant) and rejection, respectively. Among the 39 patients who achieved donor-dominant T-cell chimerism, two died before engraftment due to non-relapse causes on day 28 (infection) and day 25 (diffuse alveolar haemorrhage), respectively. Among those with donor-dominant chimerism, 24 (63%) of 38 evaluable patients developed PIR at a median of 8 (6–11) days after CBT. Patients who achieved donor-dominant T-cell chimerism (>90%) at 1 week had a higher incidence of PIR compared to those who did not ($P = 0{\cdot}017$, Fig 1C). In a representative patient at 2 weeks after CBT, rapid conversion from naïve to memory phenotype was observed in both $CD4^+$ and $CD8^+$ T cells (Fig 2A). Fig 2B shows the relative proportion of naïve $CD4^+$ and $CD8^+$ T cells at 2, 4, and 8 weeks after CBT in 37 evaluable patients who achieved donor-dominant T-cell chimerism. Patients who developed PIR had significantly more lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+$ memory T cells, and $CD8^+$ memory T cells at 2 weeks after CBT compared with those without PIR (Fig 2C and data not shown).

Our data confirmed that a majority of patients achieved donor-dominant T-cell chimerism around 2 weeks after CBT. We also found that early recipient-type T-cell chimerism was closely associated with graft rejection. A remarkable finding was that a rapid recipient-to donor-dominant switch

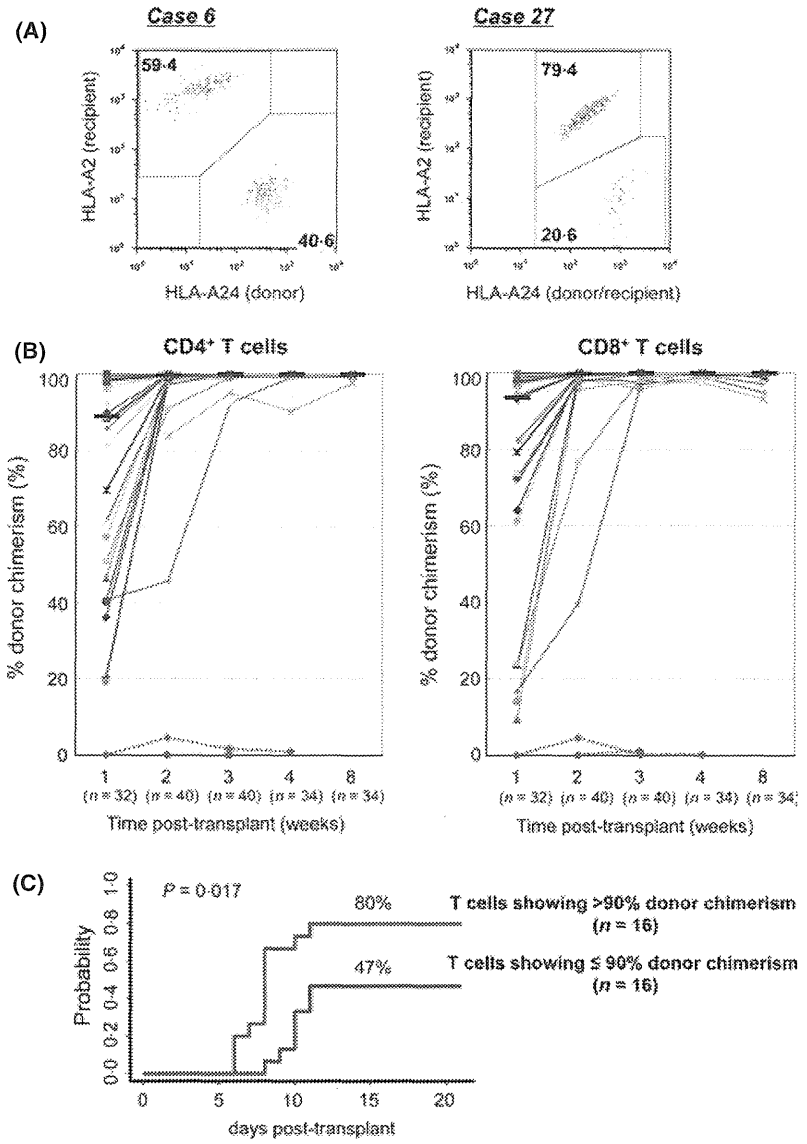

Fig 1. T-cell chimerism analysed by HLA-Flow method. (A) Chimerism analysis by the HLA-Flow method separated donor- vs. recipient-derived cells among $CD4^+$ T cells at 1 week after cord blood transplant (CBT). In Case 6, human leucocyte antigen (HLA)-A2 was recipient-specific and HLA-A24 was donor-specific. In Case 27, HLA-A2 was recipient-specific, whereas HLA-A24 was shared by both donor and recipient, indicating that HLA-A2-negative and HLA-A24-positive cells were donor-derived. (B) The median percentages of donor-derived $CD4^+$ T cells and $CD8^+$ T cells at 1 week after CBT were 88·9%, and 93·5%, respectively. Red dotted lines indicate recipient-dominant chimerism in two patients who developed graft failure. (C) Cumulative incidence of pre-engraftment immune reaction (PIR) according to chimerism status of T cells at 1 week after CBT

of T-cell chimerism at 1 week post-transplant was associated with a higher incidence of PIR, supporting a hypothesis that PIR could be an early variant form of GVH reaction caused by donor-derived T cells. CB T cells are naïve and do not include pathogen-specific effector T cells. Grindebacke *et al* (2009) demonstrated that about 80% of $CD4^+$ T cells kept the naïve phenotype during the first 18 months after birth. In contrast, we found a rapid conversion from naïve to memory phenotype at 2 weeks after CBT. In addition, PIR could be associated with peripheral expansion of donor-derived memory T cells. Recently, Gutman *et al* (2010) reported that $CD8^+$ T cells predominately expressed effector memory or effector phenotype early after double-unit CBT, reflecting an immune response of the dominant unit against the non-engrafting unit. These findings suggest that donor-derived naïve T cells will be activated by alloantigens and differentiate into mature cells early after CBT. Most of the present patients with PIR responded promptly after a