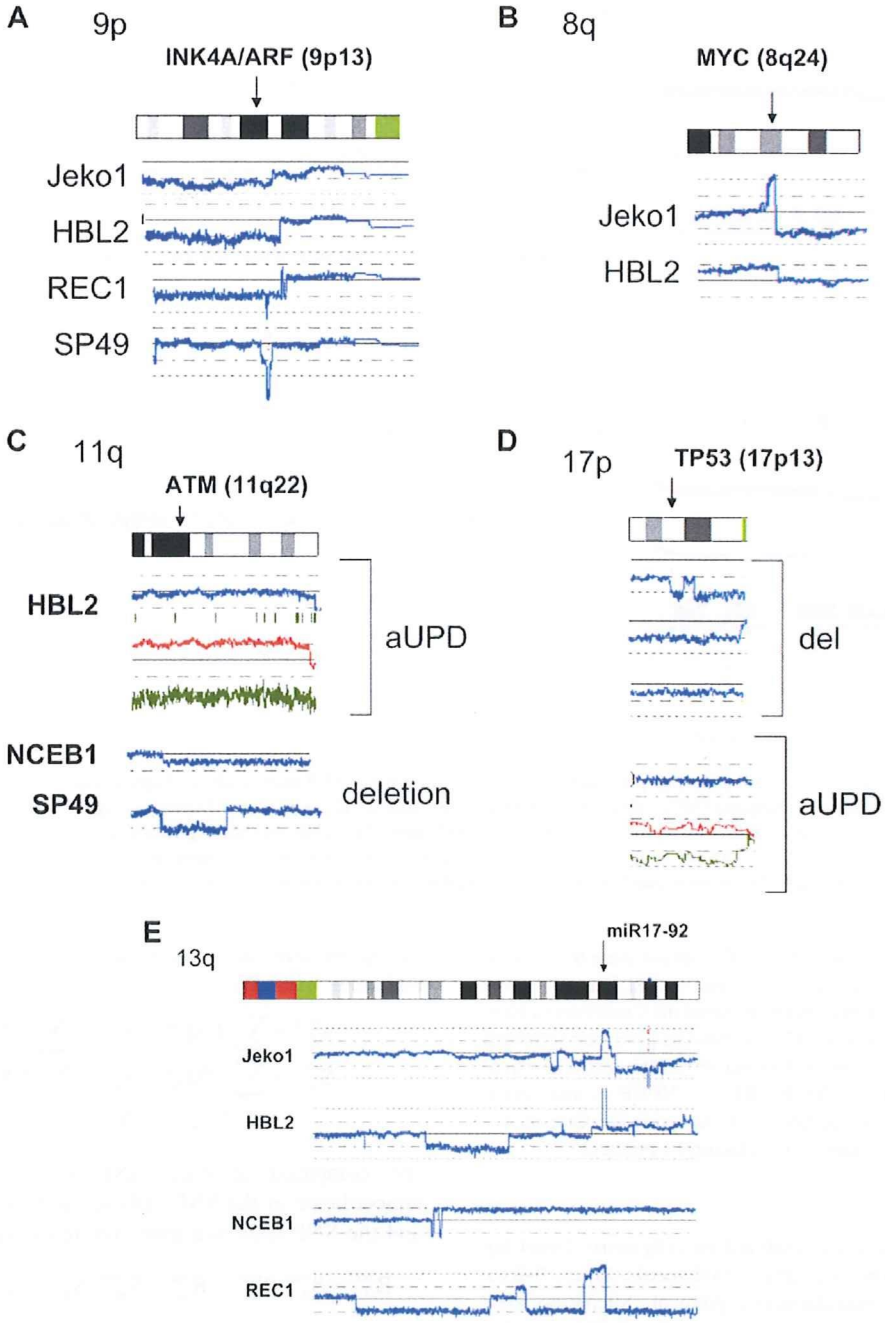

Figure 2. Common genomic alterations in mantle cell lymphoma (MCL). Representative single nucleotide polymorphism genomic microarray (SNP-chip) results of common genomic alterations found in MCL are shown. Blue lines indicate gene dosage of each case. (**A**) Deletion of INK4A/ARF (9p 21.3). (**B**) Amplification and duplication of c-MYC (8q24). (**C**) Acquired uniparental disomy (aUPD) and deletion of 11q involving ATM. Gene dosages are indicated by blue lines. NCEB1 and SP49 have deletion of 11q22. HBL2 shows normal gene dosage (blue line), but allele-specific gene dosage levels (green and red lines) indicate that one of the parental alleles (green line) is deleted and the other parental allele (red line) is duplicated, suggesting aUPD. (**D**) Deletion and aUPD of 17p involving TP53. Top three cases show deletion of 17p13. The bottom case demonstrates normal gene dosage at 17p13 (blue line), but allele-specific gene dosage levels (green and red lines) indicate that one of the parental alleles (green line) is deleted and the other parental allele (red line) is duplicated, suggesting aUPD. (**E**) Amplification and duplication of 13q involving miR17-92. Mantle cell lines: Jeko1, HBL2, REC1, SP49, NCEB1. This figure is available in color online at www.exphem.org.

Table 1. Common genomic alterations found in mantle cell lymphoma by single nucleotide polymorphism genomic microarray

Chromosome locus	Type of alteration	Frequency	Candidate gene
1p	Deletion/aUPD	9/33	
1q	Deletion	7/33	
3q	Duplication	13/33	PIK3CA, BCL6
6q	Deletion	13/33	
8q	Duplication/amplification	2/33	c-MYC
9p	Deletion	4/33	INK4A/ARF
9q	aUPD	6/33	
11q	Deletion/aUPD	8/33	ATM
11q	Duplication/amplification	4/33	CCND1
17p	Deletion	6/33	TP53
18q	Duplication/amplification	4/33	MALT1, BCL2

aUPD = acquired uniparental disomy.

Then these values were averaged over the references that have a concordant genotype for each SNP in a given set of references (K), and $R^K_{AB,\,i}$, $R^K_{A,\,i}$ and $R^K_{B,\,i}$ were obtained. To minimize noise in allele-specific gene-dosage analysis, we selected the optimized references using stepwise reference selection as reported previously [20].

Results

Five common well-known abnormalities detected by SNP-chip in MCL

Representative results of SNP-chip analysis in 33 MCL, including homozygous and hemizygous deletions, duplications, amplifications, and aUPDs are shown in Figure 1A–F. An overview of all of the genomic gains and/or losses detected by SNP-chip analysis is shown in Figure 1G.

We have found four common genomic abnormalities in MCL, including deletion of 9p, duplication/amplification of 8q, deletion/aUPD of 11q, and deletion/aUPD of 17p (Fig. 2 and Table 1). A commonly deleted gene at 9p13 was the INK4A/ARF locus (Fig. 2A); deletion of these genes was found in 4 of 5 cell lines and 6 of 28 primary MCL samples. SNP-chip was able to identify a homozygous region as small as 200kb in the REC1 cell line (one allele had deletion of 9p21.3-pter; the other allele had deletion of nucleotide 21,884,299–22,093,813; http://genome.ucsc.edu/). A commonly duplicated/amplified region in 8q was 8q24 involving the c-MYC gene (Fig. 2B), occurring in 2 of 5 cell lines and 3 of 28 primary MCL samples. Although deletion/aUPD of 11q involved relatively large regions, ATM (11q22) is one of the genes always involved in this abnormal region (Fig. 2C), and deletion/aUPD of this gene was detected in 3 of 5 cell lines (two deletions and one aUPD) and 4 of 28 primary MCL samples (three deletions and one aUPD). Deletion/aUPD of 17p encompassed a large region including TP53 (17p13) (Fig. 2D). Deletion/aUPD of 17p13 was detected in 3 of 5 cell lines (all deletions) and 9 of 28 primary MCL (6 deletions and 3 aUPDs). We found duplication/amplification of 13q in four of five cell lines, and the commonly amplified/duplicated region involved miR17-92 (Fig. 2E).

Recurrent deleted regions with unidentified target genes

In addition to the five well-known abnormal regions in MCL described here, we have found a number of abnormal regions (Fig. 1G and Table 1). Deletions involving 1p, 1q, 2q, 6q, 7q, 8p, 10p, 13q, and X chromosome were common in MCL (Fig. 1G and Table 1). Deletion of 1p was found in 3 of 5 cell lines and 6 of 28 primary MCL, and the commonly deleted region was 1p22.1–21.2 (nt. 93,123,689–nt. 103,840,794) involving 34 genes (Fig. 3A). Deletions involving 1q were detected in 2 of 5 cell lines and 5 of 28 primary MCL (Fig. 3B). However, we could not identify a commonly deleted region in this abnormality. This region may contain multiple target genes deleted in MCL.

We found deletions of 6q in 5 of 5 cell lines and 8 of 28 primary MCL samples. Data from these 13 cases allowed us to identify two commonly deleted regions, 6q21 (nt. 108,581,628–nt. 113,358,672), which involve 31 genes, including FYN, TRAF2IP2, and WISP3 (CCN6); and 6q25.1-25.3 (nt. 151,908,208–nt. 157,547,535), which encompass 17 genes including FBX05 (Fig. 3C).

Recurrent duplicated/amplified regions

We found duplication of 3q (8 of 28 primary MCL), trisomy 3 (4 of 28 primary MCL), and tetrasomy 3 (1 of 28 primary MCL), leading to gain of genomic material in 3q in 45% of the primary MCL (Fig. 4A). This region was relatively large and involved PIK3CA (3q26) and BCL6 (3q27).

We also found duplication/amplification of 11q in 2 of 28 primary MCL and 2 of 5 MCL cell lines. This commonly amplified/duplicated region involved cyclin D1. All of the cases with duplication/amplification of 11q also had duplication/amplification of the IgH gene (Fig. 4B), suggesting that rearranged IgH-CCND1 region was duplicated/amplified.

We identified duplication/amplification of 18q (one cell line and three primary MCL samples), and this region is relatively large. This DNA segment contains MALT1 (18q21) and BCL2 (18q21), which are frequently overexpressed in low-grade lymphomas and associated with antiapoptosis.

Besides the previously mentioned common regions of duplications/amplifications, other less frequent duplications and amplifications were also detected in a number of chromosomal loci (Fig. 1G).

aUPD in MCL

We found a number of novel aUPD regions in MCL (Fig. 5), including 1p, 9p, 9q, 11q, 17p, and 19p. As described here, aUPD of 17p was detected in three primary MCL, and aUPD of 11q was found in two MCL (one cell line and one primary tumor). aUPD involving chromosome 9 was the most common aUPD in our study; whole chromosome 9 aUPD was found in three MCL, and aUPD of the whole short arm of chromosome 9 was found in one MCL (Fig. 5). Two cases had aUPD of

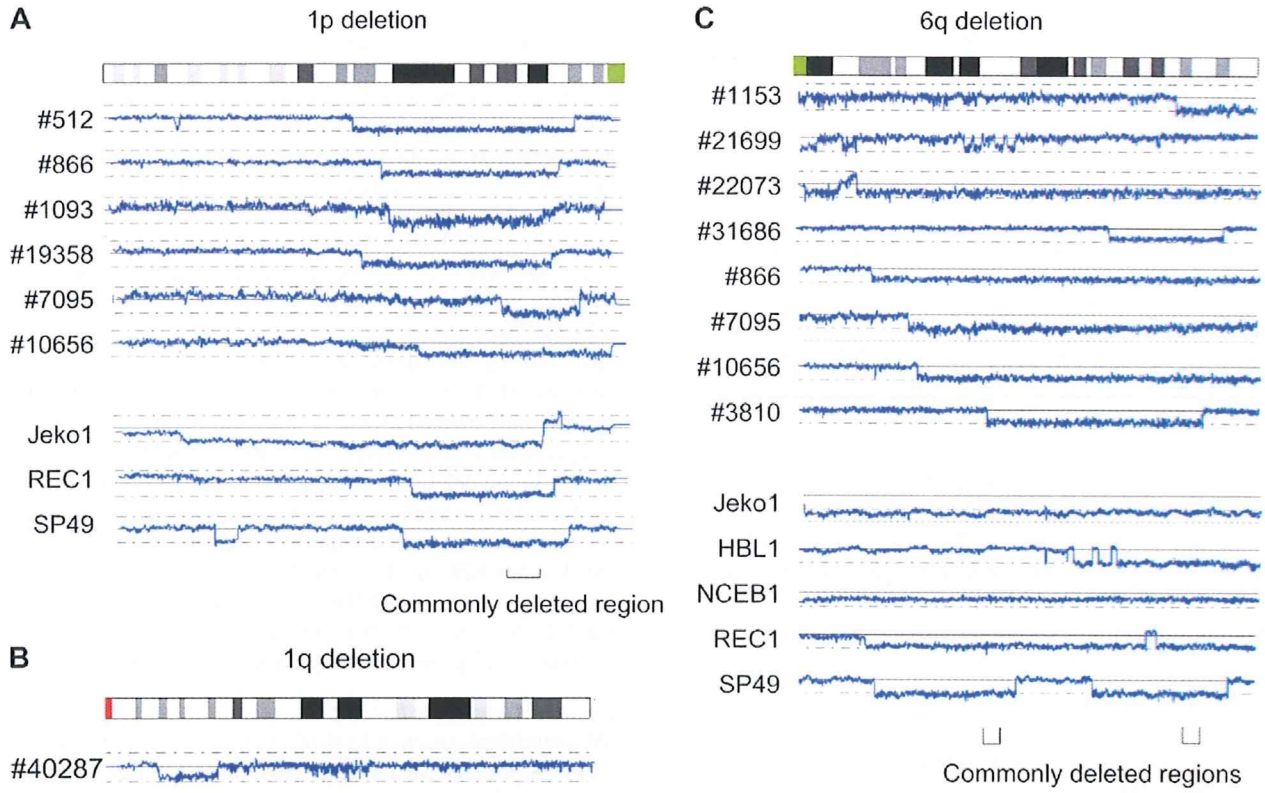

Figure 3. Recurrent deletions in mantle cell lymphoma (MCL). Recurrent deletions of 1p, 1q, and 6q are shown. (**A**) Deletion of 1p. (**B**) Deletion of 1q. (**C**) Deletion of 6q. Commonly deleted regions are indicated by brackets. Cell line names and sample numbers are indicated. Blue lines indicate gene dosage levels of each case. This figure is available in color online at www.exphem.org.

whole long arm of chromosome 9. None of the cases with aUPD involving whole chromosome 9 or 9p had homozygous deletion of INK4A/ARF (9p21.3) (data not shown).

In primary MCL #21699, we found a homozygous deletion of 19p13.1 caused by aUPD. This homozygously deleted region contained eight genes, including three TNF superfamily genes (TNFSF9, TNFSF7, and TNFSF14) (Fig. 6).

Discussion

Most MCL samples have genomic abnormalities in addition to t(11;14) [8,9], and a transgenic murine model carrying overexpression of cyclin D1 suggests that additional genetic events are needed for development of MCL [6,7]. Nevertheless, genomic alterations of MCL have not been fully examined as yet. In this study, we employed high-resolution SNP-chip analysis on 33 MCL samples to explore genomic abnormalities in this disease. This technique can detect alterations as small as 200kb.

Previously, we have validated the reliability of SNP-chip analysis by fluorescence in situ hybridization and genomic quantitative polymerase chain reaction, which showed that this method has low background and the data are reliable [17,18]. In this study, we have found well-known genomic abnormalities, including deletion of INK4A/ARF [23,24], validating that the SNP-chip analysis can sensitively detect genomic abnormalities in MCL.

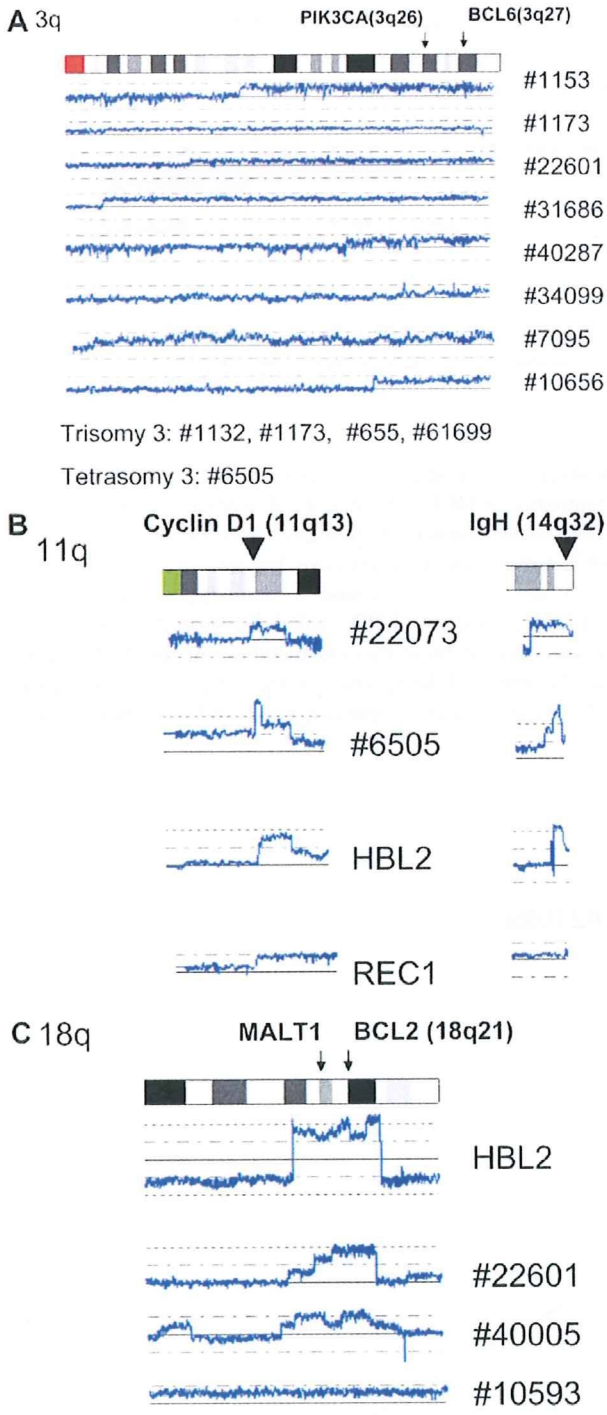

Figure 4. Recurrent duplication/amplification in mantle cell lymphoma (MCL). (**A**) Duplications of 3q are shown. Positions of PIK3CA (3q26) and BCL6 (3q27) are indicated by arrows. Sample numbers with trisomy 3 and tetrasomy 3 are also shown. (**B**) Amplifications/duplications of cyclin D1 and IgH. The positions of cyclin D1 and IgH are indicated by arrowheads. (**C**) Amplifications/duplications of 18q. Positions of MALT1 and BCL2 are indicated by arrows. Sample numbers and cell line names are shown. This figure is available in color online at www.exphem.org.

Copy number variants (CNV) are polymorphic difference observed in normal individuals [25–27]. This change may be misinterpreted as a deletion and/or a duplication/amplification. CNV have been reported by a number of investigators; and the sites and sizes of CNV have been collected in a database (http://genome.ucsc.edu/ and http://projects.tcag.ca/variation/). We compared all altered genomic sites that we found in this study with CNV sites in the databases and excluded CNV from our results. Therefore, all genomic altered sites in this study are most likely pathogenic and acquired during development of MCL. However, rare CNV sites that have not been reported may be included in our results. Accumulation of additional data of CNV sites will further exclude the false-positive sites of deletions/duplications.

We found gain of the 3q region in 45% of primary MCL. This 3q region is relatively large and contains a number of genes, including PIK3CA and BCL6. PIK3CA is one of the important molecules in signal transduction for cellular proliferation [28]. Slight increase of protein levels of PIK3CA by gain of genomic material in this region may cause stimulation of this signal-transduction pathway in MCL [29]. Another interesting duplicated/amplified site found in MCL is 18q involving MALT1 and BCL2. Overexpression of these genes by duplication/amplification may cooperate with cyclin D1 in development of MCL. We also found duplication/amplification of cyclin D1; and these same samples had duplication/amplification of IgH. Therefore, these MCL probably have increased expression of cyclin D1, similar to duplication of der(22)t(9;22) carrying the BCR-ABL fusion gene in chronic myelogenous leukemia transforming to blastic crisis [30]. A duplication of IgH-cyclin D1 may also play a role in transforming MCL to an even more aggressive form of the disease [31].

We also found a number of aUPD sites in MCL. These aUPDs may contain an activation mutation of an oncogene; the wild-type allele becomes deleted and the mutated (activated) allele is duplicated as is often observed with JAK2 mutations [20,32]. Also, these aUPDs are detected in the regions of tumor suppressor genes. Duplication of a chromosome that has a point mutation or segmental deletion involving a tumor suppressor gene leads to homozygous deletions of the tumor suppressor gene. This is often observed with homozygous deletion of INK4A/ARF (9p21.3) by aUPD in a variety of cancers [17]. Interestingly, although we found four cases with aUPD involving whole chromosome 9 or 9p, none of these four MCL samples had homozygous deletion of INK4A/ARF. In our previous study analyzing 399 samples of pediatric acute lymphoblastic leukemia by SNP-chip, 9p aUPD usually was associated with homozygous deletion of INK4A/ARF [17]. However, cases with whole chromosome 9 aUPD rarely had homozygous deletion of this gene [17]. In MCL, an unidentified altered tumor suppressor gene or oncogene may be duplicated by aUPD involving chromosome 9.

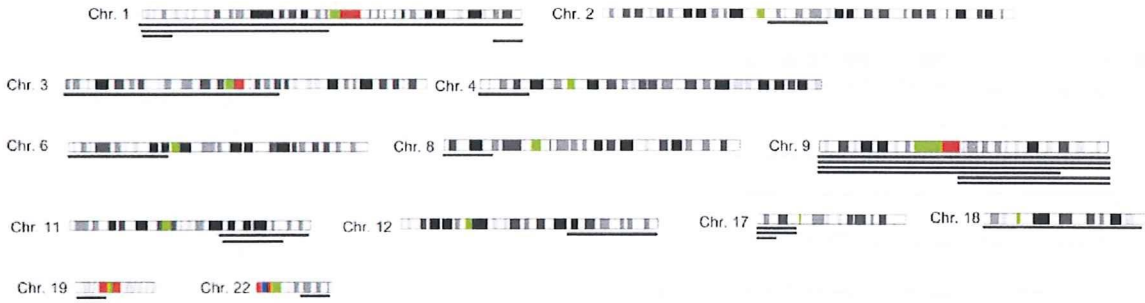

Figure 5. Acquired uniparental disomy in mantle cell lymphoma. Regions of acquired uniparental disomy (aUPD) are shown by lines under each chromosome. Each line under a chromosome reflects a mantle cell lymphoma (MCL) sample. Only the chromosomes having aUPD are shown. This figure is available in color online at www.exphem.org.

In development of cancer, a number of functional cellular pathways are dysregulated, including those involved in proliferation, apoptosis, and differentiation. Dysregulation of multiple pathways by multiple genomic alterations may be needed for development of MCL. Cyclin D1 plays a key role in the cell cycle and overexpression of this molecule inappropriately drives the cell cycle. Dysregulation of cyclin D1 is necessary but not sufficient to cause MCL. Additionally, deletion of genes associated with detection of DNA damage, including p53 and ATM, have also been detected frequently in MCL. These latter abnormalities might accelerate accumulation of genomic alterations transforming MCL cells to a more aggressive disease.

In this study, we found a homozygous deletion of 19p13.3 caused by aUPD, and this region contains seven genes; three of these include members of the TNF superfamily genes. Likely, one of the genes in this region behaves as a tumor suppressor gene, and is homozygously

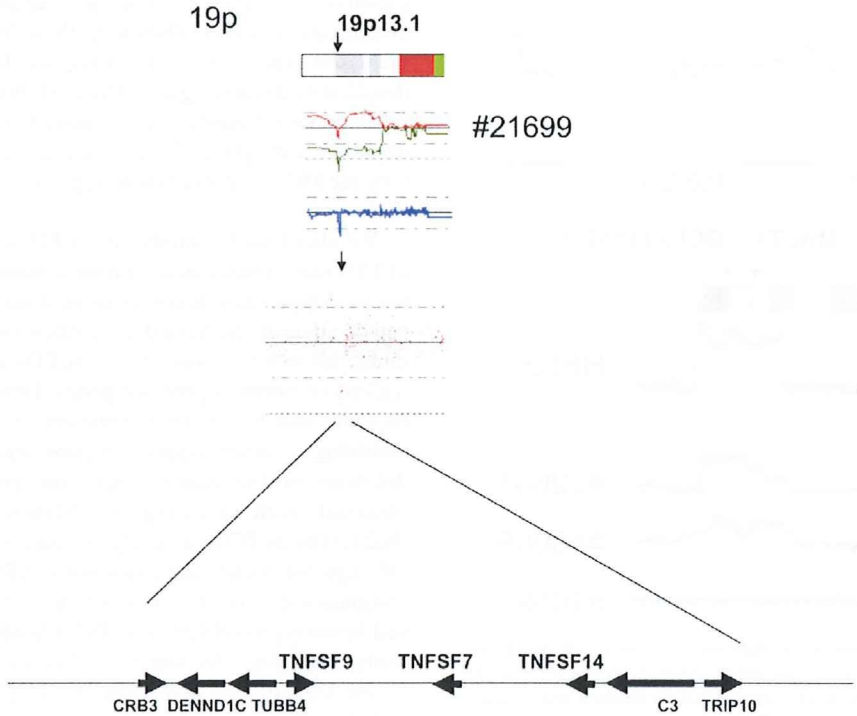

Figure 6. Homozygous deletion of 19p13.3 by acquired uniparental disomy in mantle cell lymphoma (MCL). Top panel: acquired uniparental disomy (aUPD) was found on 19p leading to homozygous deletion of 19p13.1 in MCL case no. 21699. Middle panel: Single nucleotide polymorphism genomic microarray (SNP-chip) probe signals on 19p. Bottom panel: Eight genes involved in homozygous deletion caused by aUPD. TNF superfamily genes (TNFSF 7, 9, 14) are homozygously deleted in this case. Arrows indicate the directions and approximate relative sizes of the genes located in this region. This figure is available in color online at www.exphem.org.

deleted by aUPD. Accumulation of additional MCL SNP-chip data should lead to identification of the tumor suppressor gene in this region.

In this study, we found candidate genomic events that could contribute to development of MCL in concert with overexpression of cyclin D1. These genes include tumor suppressor genes, the function of which is impaired by deletions and/or mutations, including INK4A/ARF, TP53, ATM1, and TNF superfamily genes on 19p13.3. We also identified commonly deleted regions on chromosome 1p, 1q, and 6q, where unidentified tumor suppressor genes may be localized. The other collaborating abnormalities are overexpression of oncogenes, which may occur by their duplication or amplification. Examples from our study include c-MYC, miR17-92, PIK3CA, BCL6, MALT1, and BCL2.

Accumulation of additional SNP-chip data will complement our study, leading to identification of novel target signal transduction pathways that can become targets for treatment of this frequently fatal disease.

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Conflict of Interest

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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The Journal of Immunology

Notch Activation Induces the Generation of Functional NK Cells from Human Cord Blood CD34-Positive Cells Devoid of IL-15

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Notch Activation Induces the Generation of Functional NK Cells from Human Cord Blood CD34-Positive Cells Devoid of IL-15[1]

Kyoko Haraguchi,*† Takahiro Suzuki,[2]*‡ Noriko Koyama,‖ Keiki Kumano,*‡ Fumio Nakahara,[3]*‡ Akihiko Matsumoto,[4]* Yasuhisa Yokoyama,[5]*‡ Mamiko Sakata-Yanagimoto,[5]*‡ Shigeo Masuda,[6]*‡ Tsuyoshi Takahashi,‡ Aki Kamijo,[7]§ Koki Takahashi,§ Minoko Takanashi,# Yoshiki Okuyama,† Koji Yasutomo,** Seiji Sakano,†† Hideo Yagita,‖ Mineo Kurokawa,‡ Seishi Ogawa,‡¶ and Shigeru Chiba[8]*‡‡

The development of NK cells from hematopoietic stem cells is thought to be dependent on IL-15. In this study, we demonstrate that stimulation of human cord blood CD34$^+$ cells by a Notch ligand, Delta4, along with IL-7, stem cell factor, and Fms-like tyrosine kinase 3 ligand, but no IL-15, in a stroma-free culture induced the generation of cells with characteristics of functional NK cells, including CD56 and CD161 Ag expression, IFN-γ secretion, and cytotoxic activity against K562 and Jurkat cells. Addition of γ-secretase inhibitor and anti-human Notch1 Ab to the culture medium almost completely blocked NK cell emergence. Addition of anti-human IL-15-neutralizing Ab did not affect NK cell development in these culture conditions. The presence of IL-15, however, augmented cytotoxicity and was required for a more mature NK cell phenotype. CD56$^+$ cells generated by culture with IL-15, but without Notch stimulation, were negative for CD7 and cytoplasmic CD3, whereas CD56$^+$ cells generated by culture with both Delta4 and IL-15 were CD7$^+$ and cytoplasmic CD3$^+$ from the beginning and therefore more similar to in vivo human NK cell progenitors. Together, these results suggest that Notch signaling is important for the physiologic development of NK cells at differentiation stages beyond those previously postulated. *The Journal of Immunology*, **2009, 182: 6168–6178.**

Natural killer cells are critical for host immunity because they rapidly mediate cellular cytotoxicity against pathogen-infected or malignantly transformed cells and produce a wide variety of cytokines and chemokines that influence other components of the immune system. Unlike other lymphocytic lineages, however, the continuous staging scheme of human NK cell development in vivo has yet to be elucidated (1). One reason for this may be the difficulty in closely correlating our knowledge of mouse NK cell biology with human NK cell biology (2), because mouse NK cells do not express a homolog of CD56, which is the marker most representative of human NK cells; instead, the most widely used markers of NK cells in various mouse strains are NK1.1 and DX5, mouse-specific Ags. Among the molecules involved in NK cell development, IL-15 has a particularly important role. For example, IL-15-deficient mice lack NK1.1$^+$ cells (3), indicating that IL-15 is essential for NK cell development in mice. The requirement of IL-15 for mouse NK cell development has also been demonstrated by other studies (4, 5). In humans, IL-15 is considered to be required for in vitro NK cell development and virtually most current protocols for human NK cell differentiation culture depend on IL-15. IL-15-independent NK cell differentiation has been reported in which human cord blood (CB)[9] cells are cocultured with murine stromal cell lines (6). Signaling, however, substituting IL-15 signaling that is responsible for the NK cell differentiation in this culture system was not described.

NK cells are thought to be derived from hematopoietic stem cells through a T/NK precursor stage. The Notch signaling pathway influences cell fate decisions in numerous cellular systems,

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[9] Abbreviations used in this paper: CB, cord blood; cy, cytoplasmic; FL, Fms-like kinase 3 ligand; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *tert*-butyl ester; CMA, concanamycin A.

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including various hematopoietic and immune cells (7–9). To date, four Notch receptors (Notch1– Notch4) and at least four Notch ligands (Delta1, Delta4, Jagged1, and Jagged2) have been identified in mammals. Signaling through Notch1 is crucial in the early stages of T cell development (10–12). In culture, ligand-induced Notch signaling drives human CB $CD34^+$ cells to differentiate into T/NK cell precursors (13). Furthermore, Notch signaling drives the T/NK precursors toward differentiation into T and NK cells, although the results for the NK cells are controversial. For example, inhibition of Notch signaling suppresses T cell development and stimulates NK cell development (14–16), whereas activation of Notch signaling contributes to the efficient development of NK cells in mice (17, 18) and humans (19). It is not concluded, however, whether Notch signaling is involved in the function of NK cells or whether IL-15 is necessary for NK cell development in culture.

In this report, to gain further insight into the physiologic significance of Notch signaling in NK cell development, we examined whether IL-15 is dispensable for the generation of functional NK cells and whether Notch signaling has a role in the later stages of NK cell development. Our results indicated that Notch signaling, but not IL-15 stimulation, was essential for inducing $CD34^+$ cells to give rise to $CD7^+$ and cytoplasmic (cy) $CD3^+$ cells that express CD56 in stroma-free culture. Surprisingly, cells cultured with Delta4-coated plates, but lacking IL-15 in the medium, were functional NK cells with cytotoxic activity. IL-15, along with Delta4, further augmented NK cell activity and phenotypic maturation. The addition of IL-15 without exogenous Notch ligand, however, did not allow $CD34^+$ cells to take a NK cell developmental pathway resembling physiologic NK cell precursors. Notch signaling might have a significant role in the development of NK cells in vivo.

Materials and Methods

Reagents and Abs

Recombinant human Delta4-Fc chimeric protein was generated as described previously (20). Recombinant human IL-7 and IL-15 were purchased from R&D Systems. Human stem cell factor and human Fms-like kinase 3 ligand (FL) were a gift from Amgen. Human IL-6/IL-6 receptor fusion protein (FP6) and human thrombopoietin were provided by Kirin Pharma. Anti-IL-15 Ab (MAB2471) and isotype control mouse IgG1 were purchased from R&D Systems. Anti-CD3 (UCHT1), CD8 (SK1), CD14 (M5E2), CD44 (G44-26), CD45 (HI30), CD45RA (HI100), CD56 (B159), CD94 (HP-3D9), CD161 (DX12), NKG2D (1D11), CCR7 (3D12), granzyme B (GB11), and IFN-γ (25723.1) Abs were purchased from BD Biosciences. Anti-CD2 (T11), CD4 (13B8.2), CD7 (8H8.1), CD11a (25.3), CD11b (Bear1), CD25 (B1.49.9), CD27 (1A4CD27), CD33 (D3HL60.251), CD57 (NC1), CD62L (DREG56), CD117 (YB5.B8), CD122 (CF1), CD158a (EB6), and CD158b (GL183) Abs were purchased from Beckman Coulter. Anti-CD34 and CD133 Abs were purchased form Miltenyi Biotec. RIK-2, anti-TRAIL mAb, was prepared as described previously (21).

Isolation of $CD34^+$ and $CD133^+$ cells

Human CB samples were collected from normal full-term deliveries. The parents of all donors provided written informed consent to participate in the study. The procedures were approved by the institutional review board. Mononuclear cells were separated from blood samples by density gradient centrifugation (Lymphoprep; AXIS-SHIELD PoC). CD34- and CD133-enriched cells were separated from mononuclear cells using a MACS Direct CD34 Progenitor Cell Isolation Kit and MACS CD133 MicroBead Kit (Miltenyi Biotec), respectively, according to the manufacturer's protocol. The purity of the $CD34^+$ and $CD133^+$ cells was 97.3 ± 2.3% (n = 15) and 95.4 ± 3.2% (n = 4), respectively. Residual $CD3^+$ and $CD56^+$ cells were 0.73 ± 0.42% and 0.41 ± 0.32%, respectively, in either purification strategy.

Cell culture

Nontissue culture-type 24-well plates were precoated by applying 10 μg/ml Delta4-Fc or control Fc fragments of human Ig G (Fc) (Athens Research & Technology) to the plates at 37°C for 1 h. Cells were cultured in MEM Eagle, α modification (Sigma-Aldrich) supplemented with 20% FBS (Thermo Trace) and penicillin-streptomycin at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. The number of CD34 or CD133 magnetic bead-sorted cells seeded in each well was 0.25–1.2 × 10^5. Cytokines were added at concentrations of 10 ng/ml for IL-7, 100 ng/ml for stem cell factor and 100 ng/ml for FL. one-half of the culture medium was changed every 3 or 4 days. Ten nanograms of thrombopoietin per ml and 100 ng/ml FP6 were added only into the starting culture medium for effective proliferation, although they were not essential (data not shown). IL-15 was added at 5 ng/ml when indicated. Anti-IL-15 or isotype IgG was added at 10 μg/ml when indicated. To inhibit Notch signaling, 10 μmol/L γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *tert*-butyl ester (DAPT; Calbiochem) was added to the culture medium. $CD161^+$ and $CD161^-$ cells from the culture were isolated using FACSAria (BD Biosciences) after staining with anti-CD161-PE Ab.

Phenotyping assay

Immunofluorescence staining for flow cytometry was performed according to standard procedures. To exclude dead cells from the analysis, 7-aminoactinomycin D (Beckman Coulter) was used. Cytoplasmic staining was performed as follows: after staining the cells with anti-CD56-allophycocyanin and fixing with FACS lysing solution (BD Biosciences), the cells were permeabilized using FACS permeabilizing solution (BD Biosciences) and stained with anti-CD3-PE Ab. For staining for granzyme B, the same fixing and permeabilizing procedure was performed after cell surface staining with anti-CD56-PE and anti-CD3-allophycocyanin. For staining for TRAIL, the cells were incubated with 1 μg of RIK-2 for 30 min at 4°C followed by anti-mouse IgG1-PE (A85-1). Cells were analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences).

Cytotoxicity assays

A ^{51}Cr release assay to determine cytotoxicity was performed using standard procedures. In brief, 5 × 10^3 K562 or Jurkat cells were labeled with $Na_2{}^{51}CrO_3$ (Amersham Biosciences) and cocultured with effector cells at various ratios in 96-well round-bottom microtiter plates in 200 μl of culture medium. The cocultured cells were incubated for 4 h, and 100 μl of supernatant was collected from each well and counted with a Packard COBRA gamma counter (Packard Instruments). The percentage of specific ^{51}Cr release was calculated as follows: [cpm experimental release − cpm spontaneous release]/(cpm maximal release − cpm spontaneous release) × 100. The ratio of spontaneous release to maximal release was <20% in all experiments. In experiments to test the mode of cytotoxicity, we used concanamycin A (CMA; Sigma-Aldrich) as a selective inhibitor of the perforin-mediated cytotoxicity, and anti-TRAIL Ab RIK-2. Effectors were pretreated with 100 nmol/L CMA for 2 h before the cytotoxicity assays (22). RIK-2 was added at a final concentration of 10 μg/ml at the start of the cytotoxicity assay.

Intracellular cytokines

The cells were stimulated by PMA (25 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of monensin (2 μmol/L; Sigma-Aldrich) for 4 h. After staining the cells with anti-CD56-PE, they were fixed and permeabilized as described above and stained with anti-IFN-γ-FITC Ab. The cells were analyzed on a FACSCalibur using CellQuest software.

Anti-Notch Abs

For cell surface staining, we used biotinylated Abs and streptavidin-PE (BD Biosciences). To block Notch1, we added 10 (μg/ml) MHN1-519 to the medium. Mouse IgG1 (R&D Systems) was used as the control. The anti-human Notch1 (MHN1-519, mouse IgG1), Notch2 (MHN2-25, mouse IgG2a), and Notch3 (MHN3–21, mouse IgG1) mAbs were generated by immunizing BALB/c mice with human Notch1-Fc (R&D Systems), Notch2-Fc (the Fc portion of human IgG1 was fused to the 22nd epidermal growth factor repeat of the extracellular region of human Notch2), or Notch3-Fc (R&D Systems) and screening hybridomas producing mAbs specific for Notch1-Fc, Notch2-Fc, or Notch3-Fc by ELISA. MHN1-519, MHN2-25, and MHN3-21 reacted with CHO(r) cells (23) expressing human Notch1, Notch2, and Notch3, respectively, as demonstrated by flow cytometry (supplemental Fig. S4*A*[10]). MHN1-519 and MHN3h21 blocked Notch1-Fc and Notch3-Fc binding to CHO(r) cells expressing human Delta4, respectively, but MHN2-25 did not block Notch2-Fc binding (supplemental Fig. S4*B*).

[10] The online version of this article contains supplemental material.

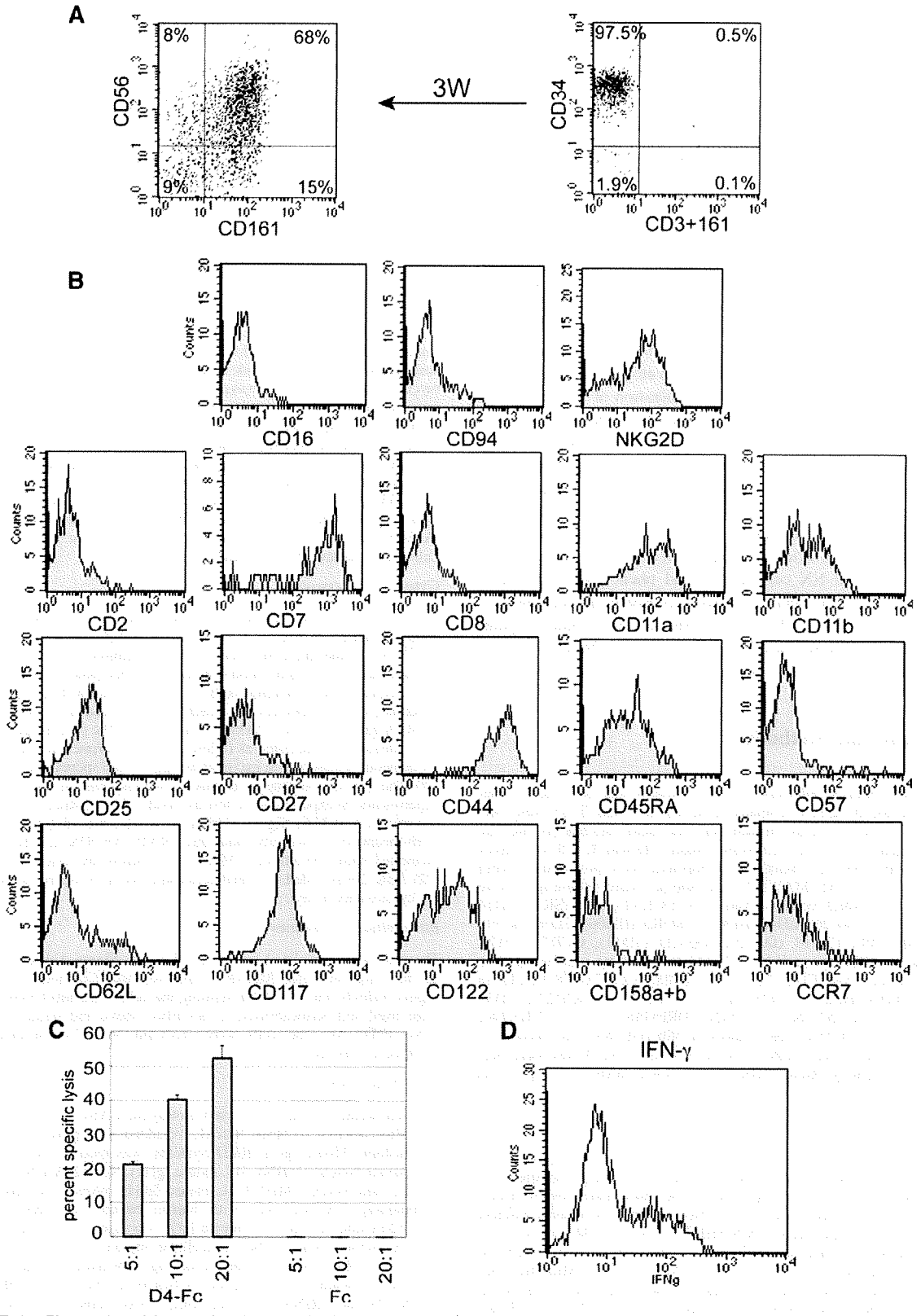

FIGURE 1. Phenotypic and functional analysis of cells derived from $CD34^+$ cells on Delta4-Fc-coated plates. *A*, Representative dot plot illustrating CD161 vs CD56 expression in the cells generated on Delta4-Fc-coated plates from $CD34^+$ CB cells after culture for 3 wk, and dot plot illustrating CD161/CD3 vs CD34 of the sorted CB population before culture. *B*, Various phenotypic analyses of the 3-wk cultured cells that were gated on $CD161^+$ events. Results are representative of at least four experiments. *C*, The 2.5-wk cultured cells were cytotoxic against K562 target cells at the indicated E:T ratios. The ratio of $CD161^+$ cells cultured on Delta4-Fc-coated plates and those Fc-coated plates in this experiment was 40 and 0%, respectively. Results are representative of four experiments. *D*, IFN-γ production by the 3-wk culture cells, as analyzed by intracellular expression. The histogram plots were gated on $CD56^+$ events. Results are representative of five experiments.

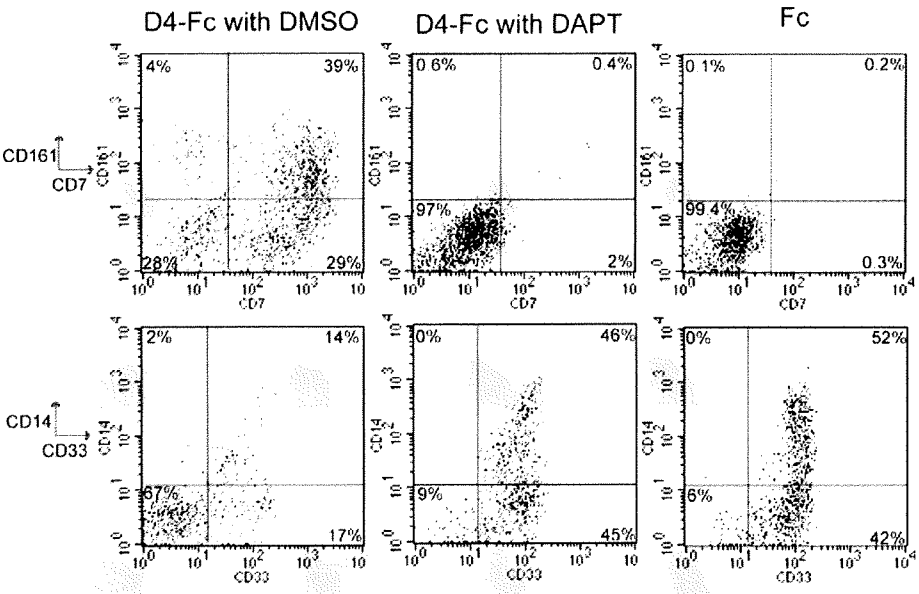

FIGURE 2. Phenotypic analysis of cells cultured in the presence of γ-secretase inhibitors. Representative dot plots of CB $CD34^+$ cells that were cultured for 2.5 wk on Delta4-Fc-coated plates with DMSO (the solvent for the γ-secretase inhibitors: D4-Fc with DMSO), Delta4-Fc-coated plates with DAPT (D4-Fc with DAPT), and Fc-coated plates (Fc). Results are representative of three experiments.

Results

Human CB $CD34^+$ and $CD133^+$ cells gave rise to functional NK cells by Notch signaling in a stroma-free culture without exogenous IL-15

$CD34^+$ or $CD133^+$ cells were cultured on Delta4-Fc-coated plates. The cells became almost immunophenotypically homogeneous after culture for ~3 wk (Fig. 1*A*). The proliferation efficiency depended on CB batches; fold increases in the cell number after the 3-wk culture were 10.3 ± 7.74-fold (n = 11). These cells expressed CD56 and CD161, but did not express surface CD3 or TCRα/β (data not shown). CD56/CD161 double-positive cells also expressed NKG2D and CD117, but were essentially negative for CD16 and killer Ig-like receptors (CD158a and CD158b). The cells had cytotoxic activity against K562 (Fig. 1*C*) and Jurkat cells (see Fig. 5*Bii*), and secreted IFN-γ (Fig. 1*D*). These results indicate that the culture products meet the general criteria for functional NK cells. The products generated from CB $CD34^+$ and $CD133^+$ had the same characteristics (data not shown).

Virtually no NK cells developed in culture on control Fc-coated plates; the vast majority of the cells were $CD33^+$ myeloid cells, a significant part of which expressed CD14 (Fig. 2). The absolute cell numbers with control Fc are ~5-fold higher than that with Delta4-Fc, and the fold increases in the cell number after the 3-wk culture were 45.7 ± 31.6-fold (n = 11). To confirm that the NK cell differentiation was Notch dependent, we added a γ-secretase inhibitor, DAPT, which strongly inhibits its ligand-dependent Notch activation (24, 25). The cells cultured on Delta4-Fc-coated plates in the presence of DAPT had the same immunophenotype as those cultured on the control Fc-coated plates and did not give rise to NK cells (Fig. 2), indicating that the observed NK cell development was Notch activation dependent. The number of cells generated increased to the level of that in the control Fc protein-coated plates (data not shown).

We cultured $CD34^+$ cells and $CD133^+$ cells purified from G-CSF-mobilized peripheral blood cells. Both cell types gave rise to $CD56^+CD161^+$ NK cells that were similar to those derived from CB $CD34^+$ or $CD133^+$ cells. The amount of time required for mobilized peripheral blood $CD34^+$ or $CD133^+$ cells (~5 wk) to develop to a major population of $CD56^+CD161^+$ NK cells was greater than that required for CB $CD34^+$ or $CD133^+$ cells (supplemental Figs. S1*A* and S2 and Fig. 3), although the time courses varied to some degree from batch to batch (supplemental Fig. S2 and data not shown).

We next examined the effects of other soluble Notch ligands, human Delta1-Fc and Jagged1-Fc, on NK cell development from CB $CD34^+$ cells. Delta1-Fc had an effect similar to that of Delta4-Fc, although with lower efficiency (supplemental Fig. S1*B*), and Jagged1-Fc showed no potential to induce NK cell development (data not shown). Therefore, we used Delta4-Fc as the soluble Notch ligand and CB $CD34^+$ cells as the starting material for the remaining experiments.

IL-15 is dispensable for in vitro NK cell development from CB $CD34^+$ cells in the presence of Delta4 stimulation, whereas Notch stimulation appears to be essential for physiologic NK cell development

When IL-15 was added to the culture medium on control Fc-coated plates, $CD56^+CD161^+$ NK cells emerged (Fig. 3 and supplemental Fig.S2, Fc plus IL-15; cf with Fig. 3 and supplemental Fig.S2, Fc); this effect was blocked by anti-IL-15- neutralizing Ab (Fig. 3 and supplemental Fig.S2, Fc plus IL-15 plus anti-IL-15). IL-15 does not affect the absolute cell number; fold increases in the cell number after the 3-wk culture were 46.8 ± 36.3-fold, 43.1 ± 35.7-fold, and 48.4 ± 9.48-fold with IL-15 (n = 7), without IL-15 (n = 7), and with IL-15 and anti-IL-15 (n = 3) in the control Fc-coated plate condition. The rate of NK cell development by IL-15 stimulation, however, was much slower than that by Delta4-Fc stimulation. In the absence of Notch stimulation, but with IL-15, the percentage of total NK-lineage cells represented by positive CD161 was only 2.6 ± 2.9%, 6.3 ± 4.6%, and 9.0 ± 4.5% at 2, 3, and 4 wk, respectively (Fig. 3 and supplemental Fig.S2, Fc plus IL-15); whereas in Delta4-Fc with IL-15 (Fig. 3 and supplemental Fig.S2, D4-Fc plus IL-15) or without IL-15 (Fig. 3 and supplemental Fig.S2, D4-Fc), the percentage of total NK-lineage cells was 56 ± 17%, 77 ± 11%, and 81 ± 5.8% (with IL-15) or 52 ± 18%, 74 ±

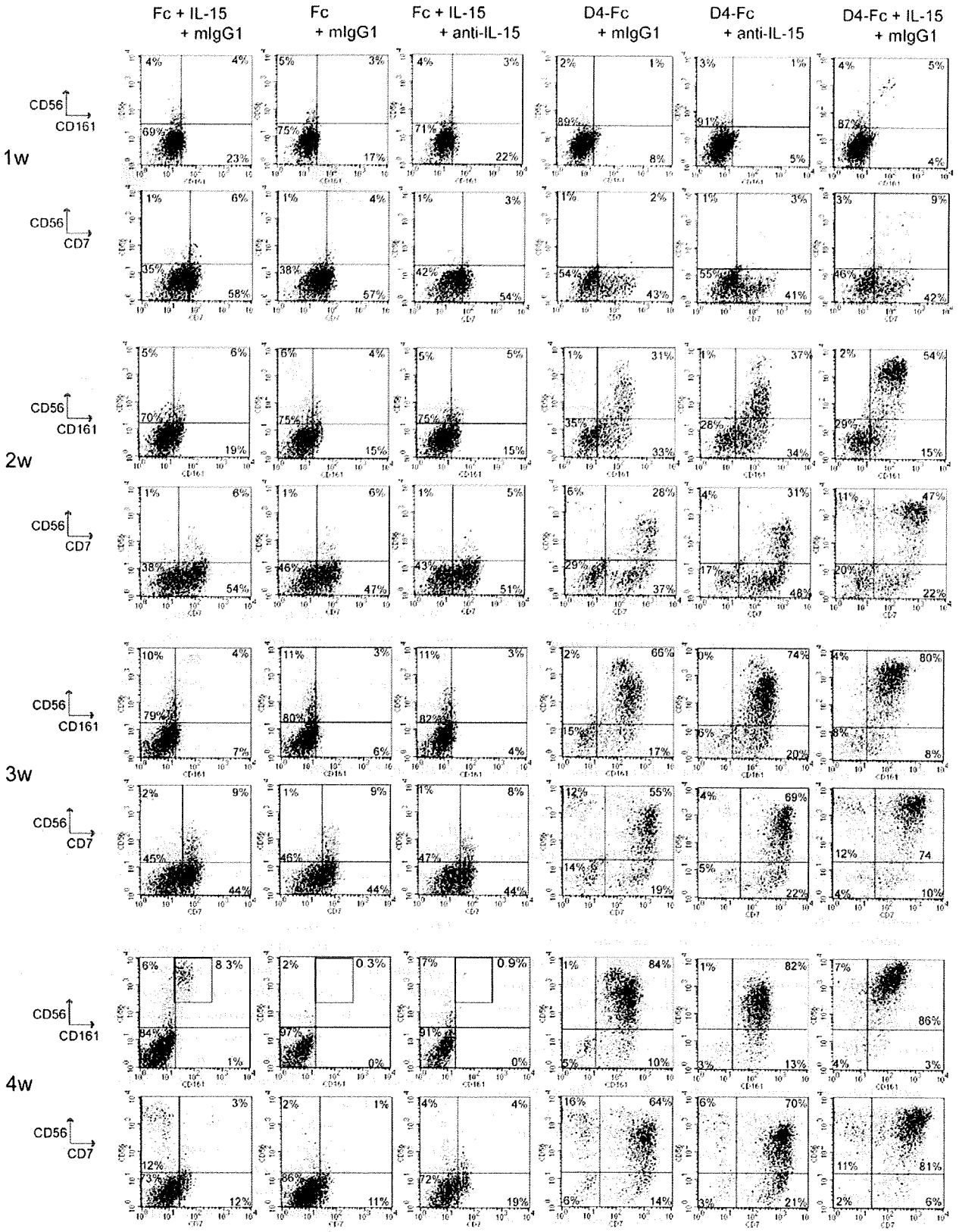

FIGURE 3. Phenotypic analysis during culture under several culture conditions. Representative dot plots illustrating CD161 vs CD56 and CD7 vs CD56 of cells that were cultured from CB CD34$^+$ cells for the indicated number of weeks on Fc-coated plates with IL-15 and mouse (m) IgG1-containing medium (Fc + IL-15 + mIgG1), Fc-coated plates with mouse IgG1-containing medium (Fc + mIgG1), Fc-coated plates with anti-IL-15 Ab-containing medium (Fc + anti-IL-15), Delta4-Fc-coated plates with mouse IgG1-containing medium (D4-Fc + mIgG1), Delta4-Fc-coated plates with anti-IL-15 Ab-containing medium (D4-Fc + anti-IL-15), and Delta4-Fc-coated plates with IL-15 and mouse IgG1-containing medium (D4-Fc + IL-15 + mIgG1). Results are representative of at least three experiments. The means and SD of each CD161 vs CD56 quadrant in replicate experiments are shown in supplemental Fig. S2.

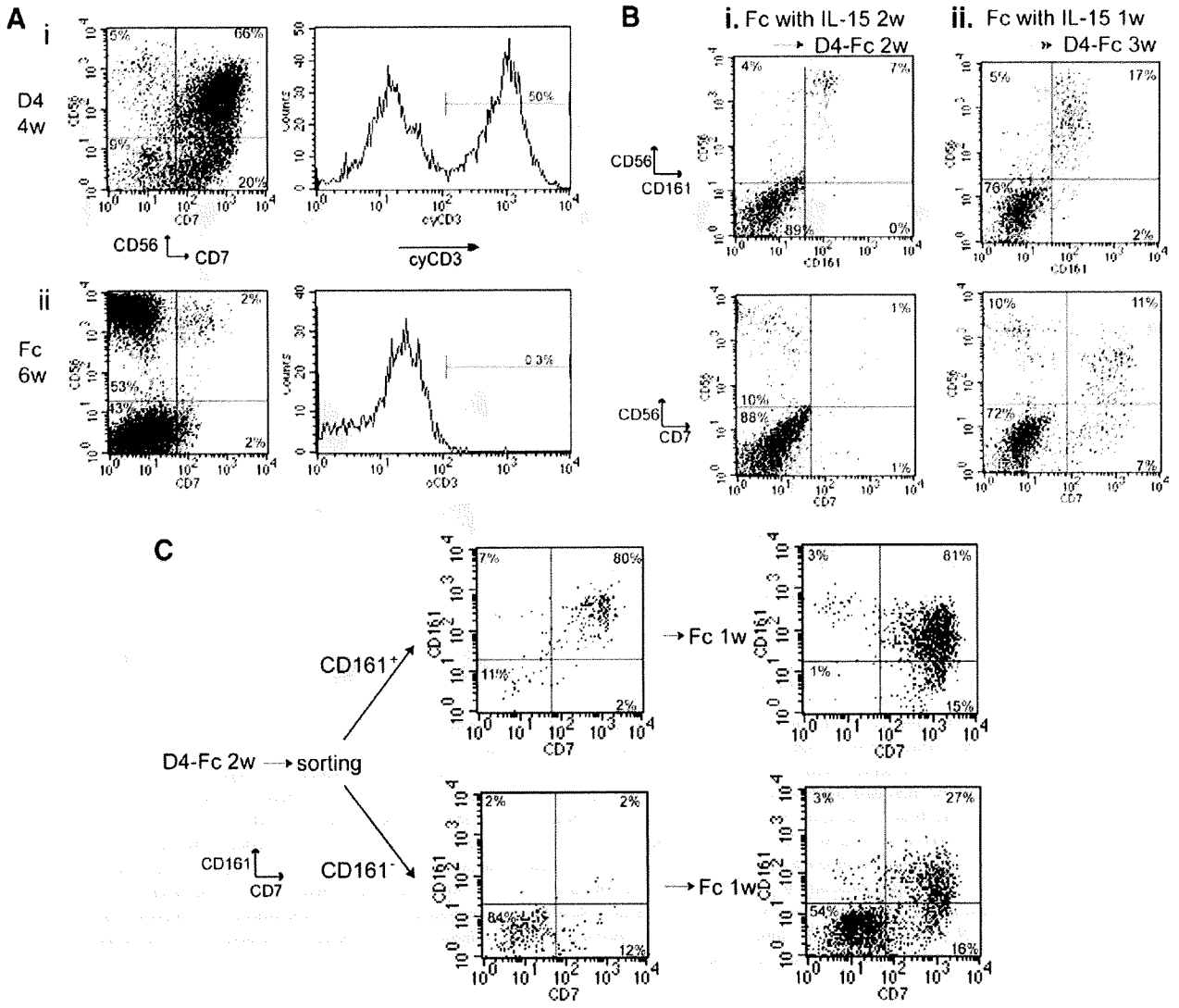

FIGURE 4. Phenotypic analysis of cells after various culture conditions. *A*, Representative dot plots illustrating CD7 vs CD56 cells that were cultured from CB CD34$^+$ cells for 4 wk on Delta4-Fc-coated plates (D4, *Ai*) and for 6 wk on Fc-coated plates in the presence of IL-15 (Fc, *Aii*). Histogram plots illustrating cyCD3 of the same cells that were gated on CD56$^+$ events. Results are representative of six and five experiments, respectively. *B*, Representative dot plots of cells that were cultured from CB CD34$^+$ cells for 2 or 1 wk on Fc-coated plates with IL-15-containing medium and were then transferred to Delta4-Fc-coated plates and cultured for 2 or 3 wk, respectively, with IL-15-free medium (*Bi* and *Bii*). Results are representative of three experiments. *C*, Representative dot plots illustrating CD7 vs CD161 expression in the cells that were sorted into CD161$^+$ or CD161$^-$ after 2-wk culture from CB CD34$^+$ cells on Delta4-Fc-coated plates, and dot plots of cells that were cultured another week on Fc-coated plates with IL-15-free medium. Results are representative of three experiments.

11%, and 88 ± 6.7% (without IL-15) at 2, 3, and 4 wk, respectively. (supplemental Fig. S2*Bi*) The differences were statistically significant between the D4-Fc group and the Fc group ($p < 0.001$). The adjusted absolute numbers of NK-lineage cells cultured on Delta4-Fc tended to be greater than those cultured on Fc with IL-15, although the differences were not always statistically significant (supplemental Fig. S3*C*). CD56$^+$ CD161$^+$ NK cells eventually comprised a major population after 6 wk of culture with IL-15 but without Notch stimulation (Fig. 4*Ai*). No CD56$^+$CD7$^+$ (Fig. 3, Fc plus IL-15) or CD56$^+$ cyCD3$^+$ (Fig. 4*Aii*) cells were detected during culture with IL-15 but without Delta4-Fc, whereas Delta4-Fc stimulation induced the generation of CD7$^+$cyCD3$^+$ cells, which could represent naturally arising T/NK cell progenitors (26, 27), at the early phase of the culture. Although CD7^{low} cells appeared in culture with IL-15 alone, they might represent monocytes, because a substantial amount of CD14$^+$ cells emerged regardless of the presence of IL-15 when Delta4-Fc was absent and peripheral blood monocytes express CD7 at low levels.

Delta4-Fc stimulation without IL-15 efficiently induced NK cell development (Figs. 1 and 3 and supplemental Fig. S2, D4-Fc). Most of the cells became CD7^{high} in the first 2 wk. A few CD161$^+$ cells were detected at the first week, the number of which increased at the next week. Only a part of the CD161$^+$ cells was positive for CD56 during the early phase of the culture, but at the later time points, most CD161$^+$ cells were CD56$^+$. This observation may indicate that CD161$^+$CD56$^-$ cells emerge at first and they gradually become CD161$^+$ CD56$^+$, although there is another interpretation such as simultaneous generation of double-positive and CD161 single-positive cells, expansion of double-positive cells, and apoptotic disappearance of the single-positive cells. Given the previous

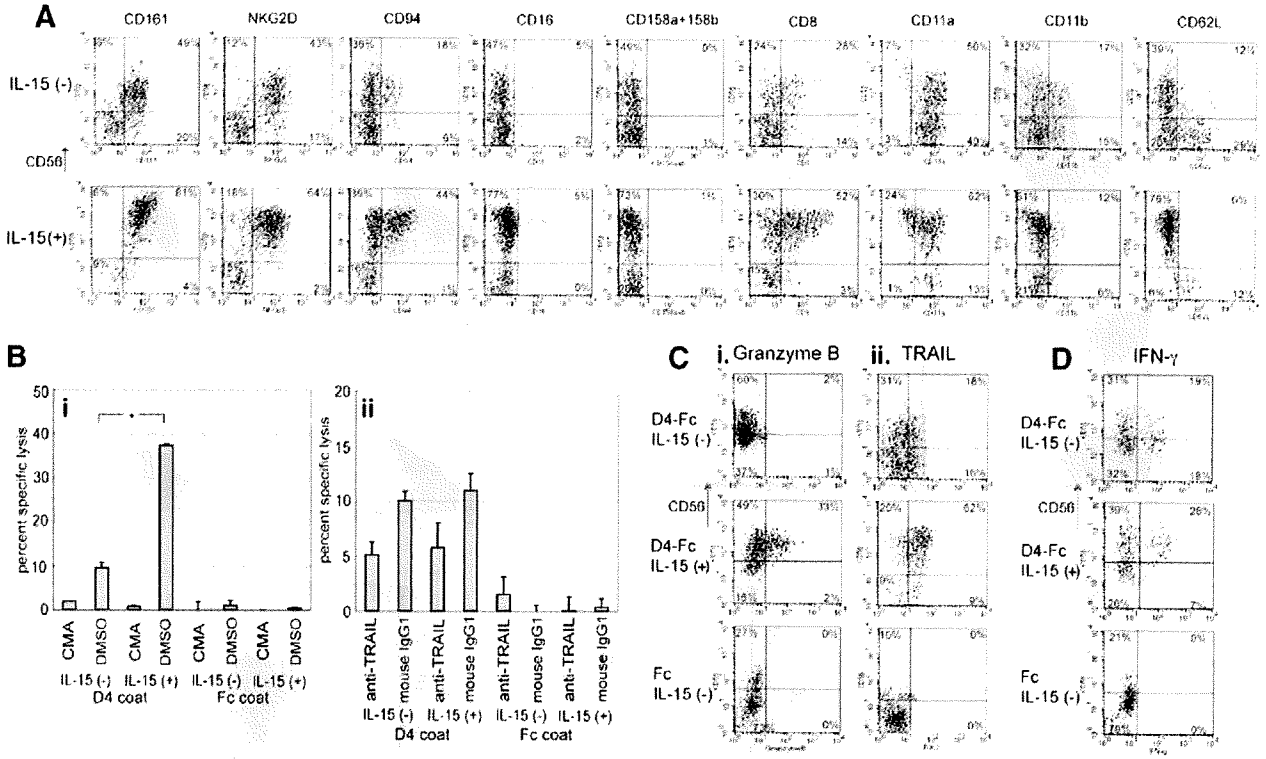

FIGURE 5. Phenotypic and functional differences between cells cultured in IL-15-containing and IL-15-free medium on Delta4-Fc-coated plates. *A*, Representative dot plots illustrating CD56 vs indicated Ags of cells cultured for 3 wk from CB $CD34^+$ cells in IL-15-containing or IL-15-free medium on Delta4-Fc-coated plates. Results are representative of six experiments. *B*, Cytotoxicity against K562 (*Bi*) or Jurkat (*Bii*) target cells at an E:T ratio of 5:1. Effectors were developed in the indicated conditions for 2.5 wk. In this experiment, the ratio of $CD161^+$ cells cultured on Delta4-Fc-coated plates with or without IL-15 condition and those cultured on Fc-coated plates with or without IL-15 condition were 53, 46, 0.6, and 0%, respectively. Effectors were pretreated with CMA or DMSO (the solvent for CMA) (*Bi*). Anti-TRAIL RIK-2 or its isotype control mouse IgG1 was added at the start of the cytotoxicity assay (*Bii*). Results are representative of three (*Bi*) and six (*Bii*) experiments. Batch to batch variation can be seen by comparing this figure with Fig. 1. *C*, Representative dot plots illustrating intracellular granzyme B (*Ci*) or TRAIL (*Cii*) vs CD56 of the cells cultured for 3 wk in medium with or without IL-15 on Delta4-Fc-coated plates and without IL-15 on Fc-coated plates. Results are representative of four experiments. *D*, Representative dot plots illustrating intracellular IFN-γ vs CD56 of cells cultured for 3 wk in medium with or without IL-15 on Delta4-Fc-coated plates and without IL-15 on Fc-coated plates. Results are representative of four experiments.

demonstration that CD161 is expressed on the cell surface earlier than CD56 (28), the former possibility appears more likely. To explore the possibility that IL-15 is secreted by a certain population of cells during culture and contributes to NK cell development, we added anti-IL-15-neutralizing Ab to the culture. The addition of anti-IL-15-neutralizing Ab to the culture medium blocked NK cell development in the presence of IL-15 (Fig. 3, IL-15 plus anti-IL-15), but did not affect either the rate or efficiency of Delta4-Fc-dependent NK cell emergence (Fig. 3, D4-Fc plus anti-IL-15, fold increase in the cell number after 3-wk culture on Delta4-coated plate with anti-IL-15 was 8.75 ± 4.18-fold (n = 5), which was not statistically different from those cultured on Delta4-coated plates without anti-IL-15 or with IL-15), further supporting the possibility that IL-15 is dispensable for NK cell development from human CB $CD34^+$ cells.

IL-2 is also suggested to be involved in the NK cell development. To examine whether IL-2, which might be secreted by a certain population of the cells, was present in the culture, the IL-2 concentration in the supernatant was measured by ELISA. No IL-2 was detected (cutoff level, 7 pg/ml; data not shown), indicating that IL-2 was not involved in the NK cell development induced by Delta4-Fc.

To examine the NK cell developmental stages that are critically dependent on Notch signaling, we cultured CB $CD34^+$ cells on control Fc-coated plates with IL-15 for 1 or 2 wk and then transferred them onto Delta4-Fc-coated plates and cultured them further for 3 or 2 wk without IL-15, respectively (culturing for a total of 4 wk). Approximately 50% of the $CD56^+$ $CD161^+$ population expressed $CD7^+$ at 4 wk in the 1-wk IL-15 condition (Fig. 4*Bii*). In contrast, very few $CD56^+$ cells that emerged in the 2-wk IL-15 condition expressed CD7 (Fig. 4*Bi*). These observations indicated that CB $CD34^+$ cells cultured with IL-15, but without Notch stimulation, for 1 wk retained the capacity to generate $CD56^+CD7^+$ cells, but that they lost this capacity when cultured without Notch stimulation for 2 wk. We also examined whether the Notch stimulation at early phases of the culture irreversibly determines NK cell developmental fate. To examine the early phase of NK cell development, we cultured CB $CD34^+$ cells for 2 wk on Delta4-Fc-coated plates and sorted the product into $CD161^+$ and $CD161^-$ cells, because CD161 is known to be expressed earlier than CD56 on the cell surface (28). We then transferred each population onto control Fc-coated plates and cultured them for another week without IL-15. More than 80% of the population derived from the $CD161^+$ cells expressed $CD7^+$. Interestingly, the $CD161^-$ cells also gave rise to $CD161^+CD7^+$ cells among one of the two major populations (Fig. 4*C*). These observations indicate that Notch activation irreversibly drives a subset of $CD34^+$ cell

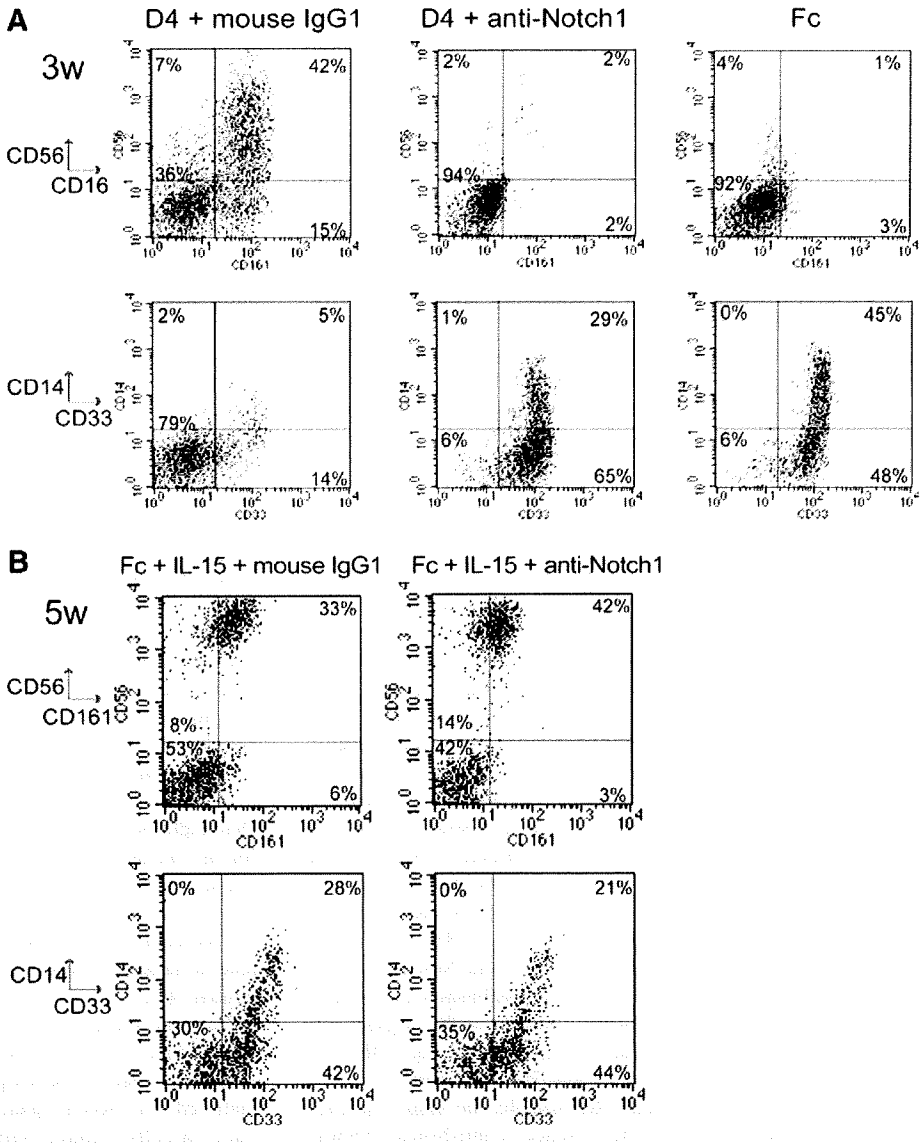

FIGURE 6. Phenotypic analysis of cells cultured in the presence of human Notch1-blocking Ab. *A*, Representative dot plots of cells that were cultured for 3 wk from CB $CD34^+$ cells on Delta4-Fc-coated plates with mouse IgG1-containing medium, Delta4-Fc-coated plates with anti-human Notch1-containing medium, and Fc-coated plates. Results are representative of six experiments. *B*, Representative dot plots of cells that were cultured for 5 wk from CB $CD34^+$ cells on Fc-coated plates with IL-15 and mouse IgG1-containing medium and Fc-coated plates with IL-15 and anti-human Notch1-containing medium. Results are representative of three experiments.

progenies to the $CD161^+CD7^+$ NK cell fate within 2 wk, presumably before $CD161^+$ is expressed.

IL-15, along with Delta4 stimulation, induces phenotypic maturation and functional augmentation of CB $CD34^+$ cell-derived NK cells

We compared the immunophenotype of the CB $CD34^+$ cell-derived NK cells generated in the culture with Delta4-Fc but lacking IL-15 (D4-Fc) and in culture with Delta4-Fc and IL-15 (D4-Fc plus IL-15). IL-15 does not affect the absolute cell number; fold increases in the cell number after the 3-wk culture were 10.6 ± 6.16-fold and 10.2 ± 6.71-fold with and without IL-15 in the D4-Fc-coated plate condition (n = 8). The cells grew slightly faster with D4-Fc plus IL-15 than with D4-Fc alone, but there were no significant differences in the frequency of $CD56^+CD161^+$ population in both conditions after 3 wk (cf Fig. 3 and supplemental Fig. S2*A*, D4-Fc and D4-Fc plus IL-15; supplemental Fig. S2*Bii*; and Fig. 5). The expression levels of CD7 and NKG2D were similar. CD94 was expressed at a higher level in the D4-Fc plus IL-15 condition. CD16 and CD158 were not expressed in the D4-Fc condition, but were expressed at low levels in the D4-Fc plus IL-15 condition. The expression levels of adhesion molecules, i.e., CD11a, CD11b, and CD62L, were higher in the D4-Fc condition (Fig. 5*A*). The other markers shown in Fig. 1 (CD2, CD7, CD25, CD27, CD44, CD45RA, CD57, CD117, CD122, and CCR7; data not shown), as well as IFN-γ (Fig. 5*D*), were expressed at similar levels under both conditions. There was a remarkable difference in the expression level of CD56, which was markedly higher in the D4-Fc plus IL-15 condition.

Cytotoxic activity against K562 cells was significantly higher in NK cells generated in the D4-Fc plus IL-15 condition than that in the D4-Fc condition. CMA, an inhibitor of perforin-mediated cytotoxicity, had a stronger suppressive effect on the cytotoxic activities of NK cells generated in the D4-Fc plus IL-15 condition (Fig. 5*Bi*). Interestingly, granzyme B, which enhances the perforin-mediated cytotoxicity and whose expression was not detected in the D4-Fc condition, was up-regulated in the D4-Fc plus IL-15 condition (Fig. 5*Ci*). This might explain the stronger suppression of NK cell cytotoxic activity by CMA when generated in the D4-Fc plus IL-15 condition compared with the D4-Fc condition. In contrast, there was no significant difference in the killing activities against Jurkat cells of the NK cells generated under

either condition (Fig. 5*Bii*), and CMA did not affect the cytotoxic activities against Jurkat cells, irrespective of the culture conditions (data not shown). This finding suggests that perforin or granzyme B does not have a major role in killing Jurkat cells. We evaluated whether TRAIL had a role by adding anti-TRAIL-blocking Ab RIK2 to the medium. RIK2 partially but clearly suppressed the cytotoxic activities against Jurkat cells generated in both conditions without significant differences (Fig. 5*Bii*), although TRAIL expression was slightly higher in the NK cells generated in the D4-Fc plus IL-15 condition (Fig. 5*Cii*). From these observations, we concluded that IL-15 does not influence the killing activity through TRAIL but does enhance the killing activity through perforin/granzyme B. The cytotoxic activity of immature NK cells is TRAIL dependent, while that of mature NK cells is mainly dependent on perforin (29). Therefore, IL-15 might contribute to the maturation of NK cells and confer on them the capacity to exact perforin/granzyme B-medicated cytotoxicity.

Inhibitory effect of anti-Notch1 Ab on Delta4-dependent NK cell development

We prepared mAbs specific for the extracellular domain of Notch1, Notch2, and Notch3 (supplemental Fig. S4*A*). The expression patterns of Notch1, Notch2, and Notch3 in fresh CB mononuclear cells, $CD34^+$ cells, and products during the culture of $CD34^+$ cells are shown in supplemental Fig. S3, *A* and *B*. Notch1 was expressed at higher levels on NK and T cells than on B cells and monocytes. Notch2 was expressed at higher levels on monocytes than on lymphocytes. Notch3 expression was virtually negative on all types of lymphocytes and positive on monocytes. Notch1 and Notch2, but not Notch3, were expressed on $CD34^+$ cells. The $CD34^+$ cell-derived $CD56^+$ NK cells also expressed Notch1 and Notch2, but not Notch3. All three Notch receptors were expressed on cells grown on the control Fc-coated plates (supplemental Fig. S3*B*).

Because $CD34^+$ cells expressed Notch1 and Notch2, but not Notch3 (supplemental Fig. S3*B*), and the established anti-Notch1 Ab, but not anti-Notch2 Ab, blocked binding of the cognate soluble Notch receptor to the ligands (supplemental Fig. S4*B*), we cultured CB $CD34^+$ cells on Delta4-Fc-coated plates in anti-Notch1 Ab-containing medium. Remarkably, the immunophenotype of the cells grown under the presence of anti-Notch1 Ab was almost the same as that of cells grown on control Fc-coated plates, indicating that the effect of Delta4 was completely blocked and NK cell development was shut down by the anti-Notch1 Ab (Fig. 6*A*). Anti-Notch2 Ab did not have such an effect, consistent with the fact that it did not block ligand binding to the cognate receptors (data not shown). CB $CD34^+$ cells cultured with IL-15 on Fc-coated plates in the presence of the anti-Notch1 Ab gave rise to NK cells in a manner indistinguishable from that of cells grown without the Ab (Fig. 6*B*). These results suggest that Notch1 might be a physiologic Notch receptor that mediates Delta4 signaling for NK cell development from CB $CD34^+$ cells and further support the notion that Notch signaling has a role distinct from that of IL-15.

Discussion

In the present study, we demonstrated that functional NK cells developed from CB $CD34^+$ cells when stimulated with the Notch ligand Delta4. Previous reports indicated that NK cells can be derived from in vitro culture of human $CD34^+$ cells prepared from fetal liver, bone marrow, or CB with either IL-2 or IL-15 (30–33), which signal through the shared IL-2/IL-15 receptor β-chain and the common γ-chain. IL-15 has been considered to have a more physiologic role than IL-2 in NK development (30). Notably, IL-15-independent NK cell differentiation has recently been published (6). This culture system, however, has been reported to be stromal cell dependent while the potential molecules and signaling pathways are unknown and, thus, the conclusion whether IL-15 is indispensable is yet to be determined. Notch signaling has been examined in the context of NK cell development as well and appears to affect the very early phase of progenitor development (17–19). In studies of human NK cell development, however, culture systems containing IL-15 and/or a coculture system with the fetal thymus organ or stromal cells are used exclusively. A novel and unexpected finding in the present study was the fact that stimulation of CB $CD34^+$ cells with a soluble Notch ligand, Delta4-Fc, coated onto the plate in the presence of stem cell factor, FL, and IL-7 was sufficient to induce the development of functional NK cells.

Our data do not officially exclude the possibility that endogenous IL-15 is involved in NK cell development in a manner, e.g., that cell-autonomously produced IL-15 activated the signaling by binding to the receptor intracellularly. Given the fact, however, that the exogenous addition of IL-15 resulted in the qualitative rather than quantitative difference in the NK cells developed in the presence of Delta4-Fc, in addition to inefficient blockade by anti-IL-15-neutralizing Ab, IL-15 is likely to be dispensable for human NK cell development in the presence of Delta4-Fc.

The finding that IL-15 is not necessary for human NK cell development in culture contrasts with the absolute necessity of IL-15 signaling for NK development in some mouse phenotypes; mice lacking a gene for IL-15 (3) (34, 35), IL-15 receptor α-chain (36), common β-chain (37), or common γ-chain (38, 39) lack NK cells. This might be due to differences between the in vitro culture conditions and the in vivo environment in which NK cells develop. Another explanation might be a difference between mice and humans, as in the case of IL-7 requirement for T cell development; IL-7 is required for the V-D-J rearrangement of the TCR β-chain gene in humans, whereas it is dispensable in mouse T cell development (40).

Previous studies reported that the effect of Notch signaling in the presence of IL-15 on NK cell development is confined to the very early stages of development. In the present study, we demonstrated that Notch signaling confers CD7 expression competence on cells cultured with or without IL-15 for 1 wk or less, but not for 2 wk, unless also stimulated by Notch. This finding is similar to that in a previous report demonstrating that Notch signaling confers cyCD3 expression competence only on prethymic but not thymic NK cell progenitors or peripheral blood $cyCD3^-$ NK cells (19). We confirmed the Notch signal dependency of cyCD3 expression during NK cell development. Coexpression of CD7 and CD45RA on $CD34^+$ cells might be associated with a restriction toward NK cell development (26, 33). Our data strongly suggest that the vast majority, if not all, of the NK cells derived from $CD34^+$ cells without Notch signaling were generated through $CD7^-$ cells. Therefore, although it is yet to be elucidated whether all of the NK cell progenitors are $CD7^+$ (41), NK cells established in vitro without Notch stimulation might not develop from a physiologic NK progenitor or might skip the physiologic NK/T progenitor stage. Furthermore, our data suggest that the effect of Notch stimulation on CD7 expression is imprinted on cells only if it is administered at the initial stage of the $CD34^+$ cell culture. We, however, failed to prospectively identify the subpopulations in the $CD34^+$ cells that are targets of Delta4 to develop NK-lineage cells. Delta4 stimulation induced NK cell development from both the most immature $CD34^+CD38^-$ and more mature $CD34^+CD38^+$ progenitor populations and both $CD34^+$

$CD45RA^+$ lymphoid progenitors and $CD34^+CD45RA^-$ populations (data not shown).

The findings of the present study extend our understanding to more mature stages of NK cell differentiation: the presence of Notch signaling induces generation of functional NK cells in culture conditions that do not generate $CD56^+$ cells without Notch stimulation per se. The precise stages of NK cell development during which Notch signaling determines the progression toward functional NK cells is not known.

In our experiments, even cells cultured with a Notch ligand alone had cytotoxic activity. The level of this activity, however, was weaker than that in NK cells generated by Notch stimulation with IL-15. Indeed, the perforin-mediated cytotoxicity of NK cells generated in the absence of IL-15 was significantly weaker, despite the fact that this is the major pathway of mature NK cells to kill target cells (42). In contrast, the TRAIL-mediated cytotoxicity was almost the same regardless of presence or absence of IL-15. This finding, along with the change in the expression level of CD56, might indicate that IL-15 induces the maturation of $CD56^{low}$ $CD161^+$ immature NK cells generated by Notch stimulation without IL-15. Another difference between the cells cultured with or without IL-15 was the down-regulation of adhesion molecules (CD11a, CD11b, CD62L) on the cell surface. These molecules might be important for homing of the NK cells to the sites at which they function.

To our surprise, cytotoxic activities were not detected in the cell populations generated in the control Fc plus IL-15 condition at either 3 or 6 wk (Fig. 5*B* and data not shown), although these results might be affected by the facts that the frequency of $CD56^+CD161^+$ cells was very low at 3 wk and that culture for 6 wk might be too long to evaluate cytotoxic activities while the frequency of $CD56^+CD161^+$ cells was much greater. In any case, when clinical application of progenitor-derived NK cells is considered, a Delta4-Fc-coating system would give a significant advantage.

In conclusion, Notch stimulation by Delta4 (or Delta1) was required for initial NK cell differentiation and the development of $CD161^+CD56^{low}$ immature NK cells. Among Notch receptors, Notch1 might be essential for physiologic NK cell development, although the involvement of other Notch receptors is yet to be elucidated. IL-15 was not essential for differentiation, but was necessary for maturation. IL-15 might have an indispensable role only in the later part of the NK development. This knowledge might be useful for future approaches toward the ex vivo generation and manipulation of NK cells and their therapeutic application.

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Disclosures

The authors have no financial conflict of interest.

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High-Resolution Genomic Copy Number Profiling of Glioblastoma Multiforme by Single Nucleotide Polymorphism DNA Microarray

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Abstract

Glioblastoma multiforme (GBM) is an extremely malignant brain tumor. To identify new genomic alterations in GBM, genomic DNA of tumor tissue/explants from 55 individuals and 6 GBM cell lines were examined using single nucleotide polymorphism DNA microarray (SNP-Chip). Further gene expression analysis relied on an additional 56 GBM samples. SNP-Chip results were validated using several techniques, including quantitative PCR (Q-PCR), nucleotide sequencing, and a combination of Q-PCR and detection of microsatellite markers for loss of heterozygosity with normal copy number [acquired uniparental disomy (AUPD)]. Whole genomic DNA copy number in each GBM sample was profiled by SNP-Chip. Several signaling pathways were frequently abnormal. Either the p16(INK4A)/p15(INK4B)-CDK4/6-pRb or p14(ARF)-MDM2/4-p53 pathways were abnormal in 89% (49 of 55) of cases. Simultaneous abnormalities of both pathways occurred in 84% (46 of 55) samples. The phosphoinositide 3-kinase pathway was altered in 71% (39 of 55) GBMs either by deletion of *PTEN* or amplification of epidermal growth factor receptor and/or vascular endothelial growth factor receptor/platelet-derived growth factor receptor α. Deletion of chromosome 6q26-27 often occurred (16 of 55 samples). The minimum common deleted region included *PARK2, PACRG, QKI,* and *PDE10A* genes. Further reverse transcription Q-PCR studies showed that *PARK2* expression was decreased in another collection of GBMs at a frequency of 61% (34 of 56) of samples. The 1p36.23 region was deleted in 35% (19 of 55) of samples. Notably, three samples had homozygous deletion encompassing this site. Also, a novel internal deletion of a putative tumor suppressor gene, *LRP1B,* was discovered causing an aberrant protein. AUPDs occurred in 58% (32 of 55) of the GBM samples and five of six GBM cell lines. A common AUPD was found at chromosome 17p13.3-12 (included *p53* gene) in 13 of 61 samples and cell lines. Single-strand conformational polymorphism and nucleotide sequencing showed that 9 of 13 of these samples had homozygous *p53* mutations, suggesting that mitotic recombination duplicated the abnormal *p53* gene, probably providing a growth advantage to these cells. A significantly shortened survival time was found in patients with 13q14 (RB) deletion or 17p13.1 (*p53*) deletion/AUPD. Taken together, these results suggest that this technique is a rapid, robust, and inexpensive method to profile genome-wide abnormalities in GBM. (Mol Cancer Res 2009;7(5):665–77)

Introduction

Glioblastoma multiforme (GBM) is an extremely malignant subtype of astrocytoma, with survival times being <12 to 15 months. These tumors typically have a very high proliferative rate with widespread microvascular proliferation and areas of focal necrosis. Genetic abnormalities have been identified in GBM using cytogenetics, fluorescence *in situ* hybridization, and comparative genomic hybridization. These studies have shown several notable abnormalities. The p16(INK4A)/p15 (INK4B)-CDK4/6-pRb pathway was found to be aberrant in the vast majority of GBMs either as a result of inactivation of either p16(INK4A) or Rb or overexpression of either CDK4 or CDK6 (1, 2). Homozygous deletion of *p16(INK4A)* occurs in approximately 31% to 50% of GBMs (3, 4). The *CDK4* gene is amplified on chromosome 12q13-14 in ~15%

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