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Molecular Analysis of Tumor Suppressor Genes, *Rb*, *p53*, *p16INK4A*, *p15INK4B* and *p14ARF* in Natural Killer Cell Neoplasms

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Natural killer (NK) cell neoplasms, which are derived from mature or precursor NK cells, are rare diseases and are observed predominantly in Asian countries. We analyzed the status of the *Rb*, *p53*, *p15INK4B*, *p16INK4A* and *p14ARF* genes in these diseases by Southern blot, polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) and western blot analysis. We used 31 NK cell neoplasms, including four cell lines derived from NK cell neoplasms, 3 myeloid/NK cell precursor acute leukemias, 4 blastic NK cell lymphoma/leukemias, 4 aggressive NK cell leukemia/lymphomas, 4 nasal NK cell lymphomas, and 12 chronic NK lymphocytosis. We found gene amplification of the *p53* gene in one nasal NK cell lymphoma, and point mutations of the *p53* gene in one blastic NK cell lymphoma/leukemia and one chronic NK lymphocytosis. In addition, homozygous deletions of *p15*, *p16* and *p14* genes in 5 out of 31 samples were detected; 3 were from nasal NK cell lymphoma and 2 from blastic NK cell lymphoma/leukemia. Also hemizygous deletion of the *Rb* gene in one blastic NK cell lymphoma was detected. Rb proteins were highly expressed in one cell line as well as two myeloid/NK cell precursor acute leukemias. In other cell lines, complete loss and an aberrant migration pattern of Rb protein expression were observed. Comparative genomic hybridization suggested that the homozygous deletions of the *p15*, *p16* and *p14* were subtle chromosomal deletions and could not be identified by standard karyotyping in some cases. Although the number of cases we analyzed was not large, alterations identified in the *Rb*, *p53*, *p16*, *p15* and *p14* genes are of significance and might be associated with tumorigenesis in NK cell neoplasms.

Key words: Homozygous deletion — Point mutation — PCR-SSCP — Comparative genomic hybridization

Natural killer (NK) cell neoplasms are rare malignancies derived from natural killer cells.[1)] This disease entity is subclassified into six subtypes, including acute lymphoblastic leukemia with NK phenotype,[1)] myeloid/NK cell precursor acute leukemia,[2)] blastic NK cell lymphoma/leukemia,[3)] nasal and nasal type NK cell lymphoma,[4)] aggressive NK cell leukemia/lymphoma[5)] and chronic NK lymphocytosis.[4)] Neoplastic cells in these diseases of all six subclasses typically have CD2, CD56 and cytoplasmic CD3 antigens, and lack surface CD3 antigen and rearrangements of the T cell receptor (*TCR*) genes.[1)] Chronic NK lymphocytosis is usually an indolent disease and rarely shows chromosomal abnormalities.[1)] Thus, it is not clear as to whether chronic NK lymphocytosis is a monoclonal proliferation of a transformed NK cell. Besides chronic NK lymphocytosis, NK cell neoplasms are fatal diseases when the malignant cells systemically disseminate.[1, 6, 7)]

In nasal and nasal type NK cell lymphomas as well as aggressive NK cell leukemia/lymphoma, EB virus is frequently detected in the neoplastic cells.[8–11)] The etiology of NK cell neoplasms remains to be disclosed. However, some recurrent chromosomal abnormalities have been reported.[12–14)] Recently, comparative genetic hybridization (CGH) analysis was performed, indicating the presence of numerous genetic alterations in NK cell neoplasms.[15)]

The *p53* gene is one of the most important tumor suppressor genes, and is frequently mutated in human cancers.[16, 17)] In response to DNA damage, *p53* induces transcription of *p21*, one of the cyclin-dependent kinase inhibitor (*CDKI*) genes, leading to cell cycle arrest.[18)] The mutation of this gene has been reported in nasal NK/T lymphoma.[19)]

The *p15INK4B* and *p16INK4A* genes are localized on chromosome 9p21, and are frequently deleted in human cancers.[20)] These genes encode CDKIs, which bind to CDK and block the cell cycle.[21)] The *p16* gene has an alternative exon 1 (exon 1 β) and encodes another protein, so-called p14ARF, via a different reading frame.[22)] p14ARF binds mdm2 protein and induces degradation of mdm2.[22)] Mdm2 protein is an oncoprotein, which binds p53 and impairs p53 function.[22)] Gene amplification of the *mdm2* gene is frequently detected in sarcomas.[23)] *p14ARF* prevents p53 from binding mdm2 and induces cell cycle arrest.[22)] Homozygous deletions of these genes are a domi-

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nant type of mutation in human malignancies, and point mutations of these genes are a minor type of mutation, although on rare occasions nucleotide substitutions of these gene occur.[20, 24]

The *Rb* gene is a causative gene of retinoblastoma as well as a tumor suppressor gene.[25] Rb protein is important for the cell cycle, binding E2F, one of the transcriptional factors, in early S phase. A complex of D-type cyclin and CDK4/6, and cyclin E/CDK2 complex phosphorylate Rb at late G1 phase. After phosphorylation, Rb protein releases E2F, and induces transcription of genes which are necessary for DNA synthesis, and the cell enters the S phase.[26] Abnormalities of the *Rb* gene have been reported in cancers other than retinoblastoma.[27, 28]

Since the genetic changes in NK cell neoplasms are not fully elucidated, here we analyzed the genes, *Rb*, *p53*, *p15INK4B*, *p16INK4A* and *p14ARF*, in NK cell neoplasms.

MATERIALS AND METHODS

Cell lines, clinical samples and DNA extraction Four cell lines derived from NK cell neoplasms were kindly provided by Dr. M. J. Robertson (NKL), Dr. Y. Kagami (HANK1), Dr. J.-H. Gong (NK-92) and Dr. J. Tsuchiyama (NK-YS).[29] Peripheral blood, lymph nodes or tumor biopsy samples were obtained from 27 cases with NK cell neoplasms, including 12 chronic NK lymphocytosis, 4 aggressive NK cell leukemia/lymphomas, 4 nasal NK cell lymphomas, 4 blastic NK cell lymphoma/leukemias, and 3 myeloid/NK cell precursor acute leukemias after informed consent had been obtained. Normal human DNA and cDNA were obtained from bone marrow cells of normal volunteers.

In all cases, neoplastic cells accounted for over 70% of the samples, as confirmed by flow-cytometric analysis. High-molecular DNAs were extracted from the samples as previously reported.[30] The DNAs were subject to Southern blot analysis and polymerase chain reaction (PCR).

Probes and Southern blot analysis Probes for Southern blot analysis were generated from normal human DNA or cDNA by PCR. *p16* exon 1 β probe was generated using primers, β-S: 5′-CGC GCC TGC GGG GCG GAG AT-3′, β-AS: 5′-CTG GTC TTC TAG GAA GCG GC-3′. *p15* cDNA probe was generated from cDNA of normal human bone marrow cells using primers *p15*-S: 5′-GCT GCG GAA TGC GCG AGG-3′ and *p15*-AS: 5′-CCT GGC GTC AGT CCC CCG-3′. Using this *p15* cDNA probe, exons 1 and 2 of the *p15* gene, and exon 2 of the *p16* gene were simultaneously identified by Southern blotting, since the sequence of exon 2 of the *p15* and that of the *p16* gene are homologous.[31, 32] *p53* probe was a PCR product of exon 5 of the *p53* gene generated from normal human DNA by PCR using primers *p53*E5-S: 5′-TCT GTT CAC TTG TGC CCT GAC TTT C-3′, *p53*E5-AS: 5′-ACC CTG GGC AAC CAG CCC TGT CGT C-3′. *Rb* probe was generated from an *Rb* cDNA plasmid provided by Health Science Research Resources (Tokyo) by PCR using primers *Rb*-S: 5′-CAT ATG TCT TTA TTG GCG-3′ and *Rb*-AS: 5′-AAG AGG ACA AGC AGA TTC-3′. All probes used were ligated into pGEM-T vectors (Promega, Madison, WI) and the sequences were confirmed. The probes were radio-labelled by random priming methods using a Ready-to-go DNA labelling kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

High-molecular DNAs (10 μg) were digested with restriction enzyme *Eco*RI (TaKaRa, Kyoto) and the fragments were electrophoresed on 0.8% agarose gels. The fractionated DNAs were transferred onto nylon membranes (Hybond N+, Amersham Pharmacia Biotech) according to the manufacturer's recommendations. Prehybridization, hybridization and washing were performed as previously described.[30] The membranes were exposed on an imaging plate and the signals were detected with the BAS 2500 imaging analyzer (Fuji, Tokyo).

PCR-single strand conformational polymorphism (PCR-SSCP) and sequencing Exons 5 to 8 were amplified by PCR using primers *p53*E5-S and *p53*E5-AS for exon 5, *p53*E6-S: 5′-CAG GGC TGG TTG CCC AGG GTC CCC A-3′ and *p53*E6-AS: 5′-ACT GAC AAC CAC CCT TAA CCC CTC C-3′ for exon 6, *p53*E7-S: 5′-CTC CTA GGT TGG CTC TGA CTG T-3′ and *p53*E7-AS: 5′-GAG GCT GGG GCA CAG CAG GCC AGT G-3′ for exon 7, *p53*E8-S: 5′-TAG GAC CTG ATT TCC TTA CTG CCT C-3′ and *p53*E8-AS: 5′-AAC TGC ACC CTT GGT CTC CTC CAC C-3′ for exon 8. The PCR was performed as previously described.[33]

The PCR products were then electrophoresed in 0.5× Supershift detection gel (Toyobo, Tokyo) at 300 V for 18 h according to the manufacturer's recommendation. The gels were dried and exposed on X-ray films for 48 h at room temperature. Shifted bands were cut out and PCR products were eluted into ddH_2O, after which PCR amplification was performed again using the eluted PCR products as templates. Nucleotide sequences of the PCR products from shifted bands were directly determined using a Prism sequence kit on a genetic analyzer 310 (Applied Biosystems, Foster City, CA)

Western blot analysis Neoplastic cells were lysed with RIPA buffer and the protein concentration was measured with a Protein Concentration assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The protein solutions were fractionated by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described.[30] The proteins were transferred onto nitrocellulose filters (Amersham Pharmacia Biotech) and Rb proteins were detected using anti-Rb antibody (Cosmo Biotec, Tokyo). The same membranes were incubated with anti-β-actin antibody (Sigma, St. Louis, MO) to determine

the quantity of proteins used. Blocking and washing were performed as previously reported.[30] Rb and β-actin proteins were detected with secondary horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulin antibody (New England Biolabs, Beverly, MA) and the signals were detected on X-ray films using a West Pico Western Detection Kit (Pierce, Rockford, IL).

CGH CGH was performed as previously reported.[15] Briefly, DNAs extracted from samples were labeled by nick translation with SpectrumGreen dUTP (Vysis, Naperville, IL) and normal reference DNAs with SpectrumRed dUTP. The sample and normal labeled DNAs were mixed and ethanol-precipitated in the presence of Cot-1 DNA (Gibco BRL, Gaithersburg, MD). The DNA mixture was

Table I. Characterization of Samples

Sample	Age/Sex	Diagnosis	TCR	CD3	CD56	EBV	Outcome
Cell lines							
HANK1	46/F	nasal	germ	–	+	ISH	dead[a]
NK-YS	19/F	nasal	germ	–	+	TR	dead[a]
NK-92	50/M	aggressive[b]	germ	–	+	–	dead[a]
NKL	62/M	aggressive	germ	–	+	NE	dead[a]
Clinical samples							
Patient No.							
1	15/F	aggressive	germ	–	+	TR	dead
2	13/M	aggressive	NE	–	+	NE	dead
3	19/F	aggressive	NE	–	+	NE	dead
4	88/M	aggressive	NE	–	+	NE	dead
5	65/M	nasal	germ	–	+	NE	dead
6	67/F	nasal	germ	–	+	TR	dead
7	27/M	nasal	germ	–	+	NE	dead
8	57/M	nasal	NE	–	+	NE	unknown
9	61/M	blastic	germ	–	+	NE	dead
10	37/M	blastic	germ	–	+	NE	alive
11	50/F	blastic	NE	–	+	NE	unknown
12	69/M	blastic	NE	–	+	NE	dead
13	66/M	chronic	germ	–	–[c]	NE	AWD
14	84/M	chronic	NE	–	+	NE	dead
15	62/F	chronic	germ	–	+	NE	AWD
16	51/F	chronic	germ	–	–[c]	NE	AWD
17	51/F	chronic	germ	–	+	NE	AWD
18	55/F	chronic	germ	–	+	NE	AWD
19	34/M	chronic	germ	–	+	NE	AWD
20	58/M	chronic	germ	–	+	NE	AWD
21	18/F	chronic	NE	–	+	ISH/TR	AWD
22	62/M	chronic	NE	–	+	NE	unknown
23	65/M	chronic	NE	–	–[c]	NE	AWD
24	61/F	chronic	germ	–	–[c]	NE	AWD
25	65/F	myeloid	germ	–	+	NE	dead
26	86/M	myeloid	NE	–	+	NE	unknown
27	86/F	myeloid	NE	NE	+	NE	unknown

nasal, nasal NK cell lymphoma; aggressive, aggressive NK cell leukemia/lymphoma; blastic, blastic NK cell lymphoma/leukemia; chronic, chronic NK lymphocytosis; myeloid, myeloid/NK cell precursor acute leukemia; TCR, status of the T cell receptor β gene; germ, germ line; EBV, status of EB virus; ISH, presence of EBV was confirmed by *in situ* hybridization using EBER1 probe; TR, presence of EBV was confirmed by Southern blot analysis using the EBV terminal repeat region as a probe; NE, not examined; AWD, alive with disease.

a) Determined by clinical reports.

b) Diagnosed by the clinical history reported.

c) CD16 positive.

then suspended in hybridization buffer (Vysis) and denatured at 73°C for 5 min, followed by annealing with denatured normal metaphase spreads (Vysis) at 37°C for 3–5 days. The spreads were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in anti-fade solution (Vysis). The color images were captured and analyzed on a CytoVision digital imaging system (Applied Imaging, Santa Clara, CA). At least 17 metaphases were analyzed in each sample.

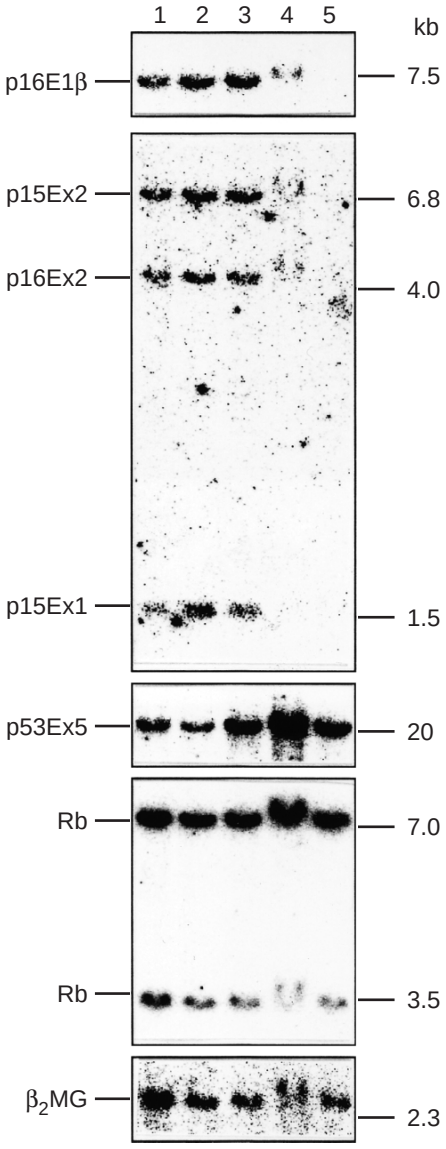

Fig. 1. Southern blot analysis. Representative cases are presented. In lanes 4 and 5, homozygous deletions of *p15*, *p16* and *p14ARF* (*p16*E1β) are seen. In lane 4, amplification of the *p53* gene is detected. *p16*E1β, exon 1 of the *p14ARF* gene; *β2MG*, β2 microglobulin gene as an internal control.

RESULTS

The characteristics of these cases are listed in Table I. In most cases, lack of rearrangement of the *TCR* genes was confirmed by Southern blot analysis. All cases, except one, of chronic NK lymphocytosis, the outcomes of which are known, were healthy with disease. Fourteen patients died of NK cell neoplasms and one was cured without recurrence.

***p53* analysis** We analyzed the *p53* gene by Southern blotting and detected gene amplification in one nasal NK cell lymphoma (Fig. 1, Table I). Subsequently, we performed

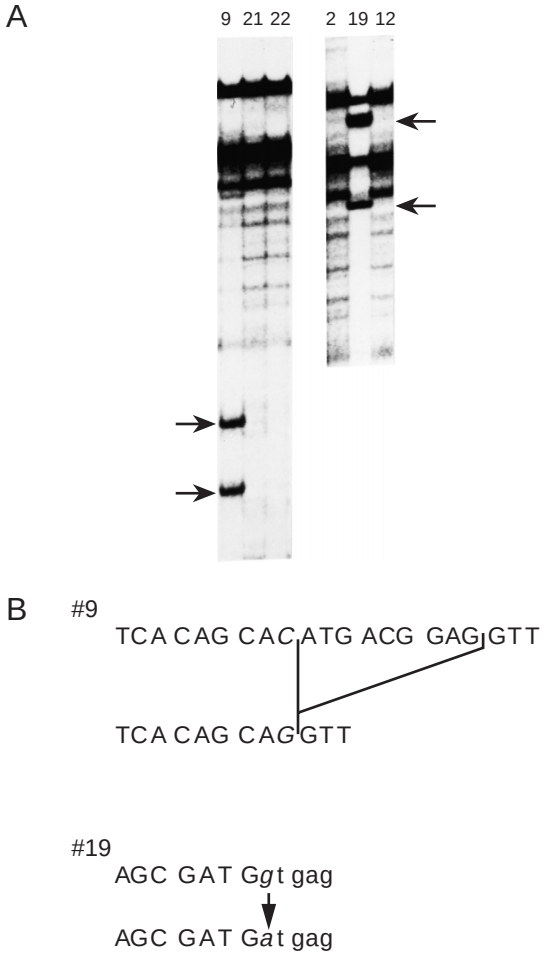

Fig. 2. PCR-SSCP and mutations of the *p53* gene. A) PCR-SSCP of exon 5 of the *p53* gene. Shifted bands are indicated by arrows. Cases 9 and 19 demonstrate the shifted bands. B) Sequence analysis of the shifted bands. Case 9 (upper) reveals a nine nucleotide deletion and nucleotide substitution of guanine for cytosine. Case 19 (lower) reveals the nucleotide substitution of adenosine for guanine at the exon-intron boundary. Capital letters indicate coding sequences and lower letters indicate intron sequences.

PCR-SSCP to detect point mutations in exons 5 to 8, in which point mutations are frequently detected in human cancers.[16] We detected shifted bands in 2 cases (Fig. 2). One was chronic NK lymphocytosis (case 19) and the other was blastic NK cell lymphoma/leukemia (case 9) (Table II). Sequence analysis revealed the nucleotide substitution of guanine for cytosine (CAC: His → CAG: Glu), a nine nucleotide deletion (case 9), and the substitution of adenosine for guanine at the exon-intron boundary (case 19) (Fig. 2). In the former case, the mutation led to the impairment of DNA binding activity of *p53*. In the latter case, the mutation at the exon-intron boundary resulted in abnormal splicing of the *p53* gene and impairment of the production of normal p53 protein.

***p15, p16* and *p14* analysis** Subsequently, we analyzed *p15*, *p16* and *p14* genes by Southern blot analysis. The status of the *p14* gene was detected by *p16* exon 1 β probe. We detected homozygous deletions in 5 cases

Table II. Genetic Alteration in NK Cell Neoplasms

Sample	Southern blot						*p53* mutation	Rb protein
	p14	*p16*E×2	*p15*E×1	*p15*E×2	*Rb*	*p53*		
Cell lines								
HANK1	+/+	+/+	+/+	+/+	+/+	+/+	−	++
NK-YS	+/+	+/+	+/+	+/+	+/+	+/+	−	−
NK-92	+/+	+/+	+/+	+/+	+/+	+/+	−	+[a]
NKL	+/+	+/+	+/+	+/+	+/+	+/+	−	+
Clinical samples								
Patient No.								
1	+/+	+/+	+/+	+/+	+/+	+/+	−	−
2	+/+	+/+	+/+	+/+	+/+	+/+	−	−
3	+/+	+/+	+/+	+/+	+/+	+/+	−	ND
4	+/+	+/+	+/+	+/+	+/+	+/+	−	ND
5	−/−	−/−	−/−	−/−	+/+	+/+	−	−
6	−/−	−/−	−/−	−/−	+/+	+/+	−	−
7	−/−	−/−	−/−	−/−	+/+	+++[b]	−	−
8	+/+	+/+	+/+	+/+	+/+	+/+	−	−
9	+/+	+/+	+/+	+/+	+/+	+/+	+	−
10	−/−	−/−	−/−	−/−	+/−	+/+	−	ND
11	+/+	+/+	+/+	+/+	+/+	+/+	−	−
12	−/−	−/−	−/−	−/−	+/+	+/+	−	−
13	+/+	+/+	+/+	+/+	+/+	+/+	−	−
14	+/+	+/+	+/+	+/+	+/+	+/+	−	ND
15	+/+	+/+	+/+	+/+	+/+	+/+	−	ND
16	+/+	+/+	+/+	+/+	+/+	+/+	−	ND
17	+/+	+/+	+/+	+/+	+/+	+/+	−	−
18	+/+	+/+	+/+	+/+	+/+	+/+	−	ND
19	+/+	+/+	+/+	+/+	+/+	+/+	+	−
20	+/+	+/+	+/+	+/+	+/+	+/+	−	−
21	+/+	+/+	+/+	+/+	+/+	+/+	−	ND
22	+/+	+/+	+/+	+/+	+/+	+/+	−	−
23	+/+	+/+	+/+	+/+	+/+	+/+	−	−
24	+/+	+/+	+/+	+/+	+/+	+/+	−	−
25	+/+	+/+	+/+	+/+	+/+	+/+	−	+
26	+/+	+/+	+/+	+/+	+/+	+/+	−	+
27	+/+	+/+	+/+	+/+	+/+	+/+	−	ND

For Southern blots, +/+, normal allele; +/−, hemizygous deletion; −/−, homozygous deletion. For *p53* mutations, −, no mutations were found; +, mutations were found. Levels of Rb proteins were quantified by western blotting. −, none or trace level of expression; +, moderate level of expression; ++, high level of expression; ND, not done.

a) Aberrant migration.

b) Gene amplification.

(Fig. 1, Table II); 3 were nasal NK cell lymphoma and 2 were blastic NK cell leukemia/lymphoma. In these cases, the genes were simultaneously deleted.

***Rb* analysis** Next, we analyzed the *Rb* gene by Southern blotting and detected hemizygous deletion in one blastic NK cell lymphoma/leukemia (Table II). We also performed western blot analysis using anti-Rb antibodies to analyze protein expression levels of Rb (Fig. 3). We found increased expression of Rb protein in one cell line, HANK1 and two myeloid/NK cell precursor acute leukemias (Fig. 3, Table II). We also found complete loss of expression of Rb protein in one cell line, NK-YS and an aberrant migration pattern of Rb protein in another cell line, NK-92 (Fig. 3).

The results of our study are summarized in Table II. Aggressive NK cell leukemia/lymphomas and chronic NK

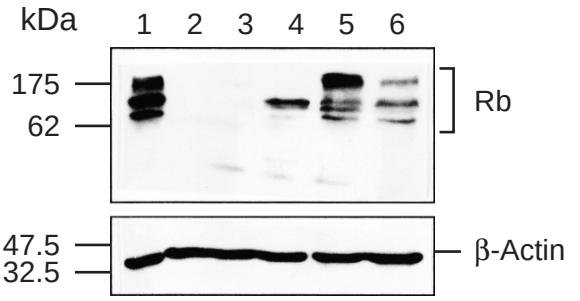

Fig. 3. Western blot analysis of Rb protein. Results for mononuclear cells (MNC) of peripheral blood from a normal volunteer and cell lines are presented. 1, Jurkat (T-cell line); 2, MNC; 3, NK-YS; 4, NK-92; 5, HANK1; 6, NKL. The same membrane was reacted with anti-β-actin antibody for quantification of loaded protein amounts (lower panel).

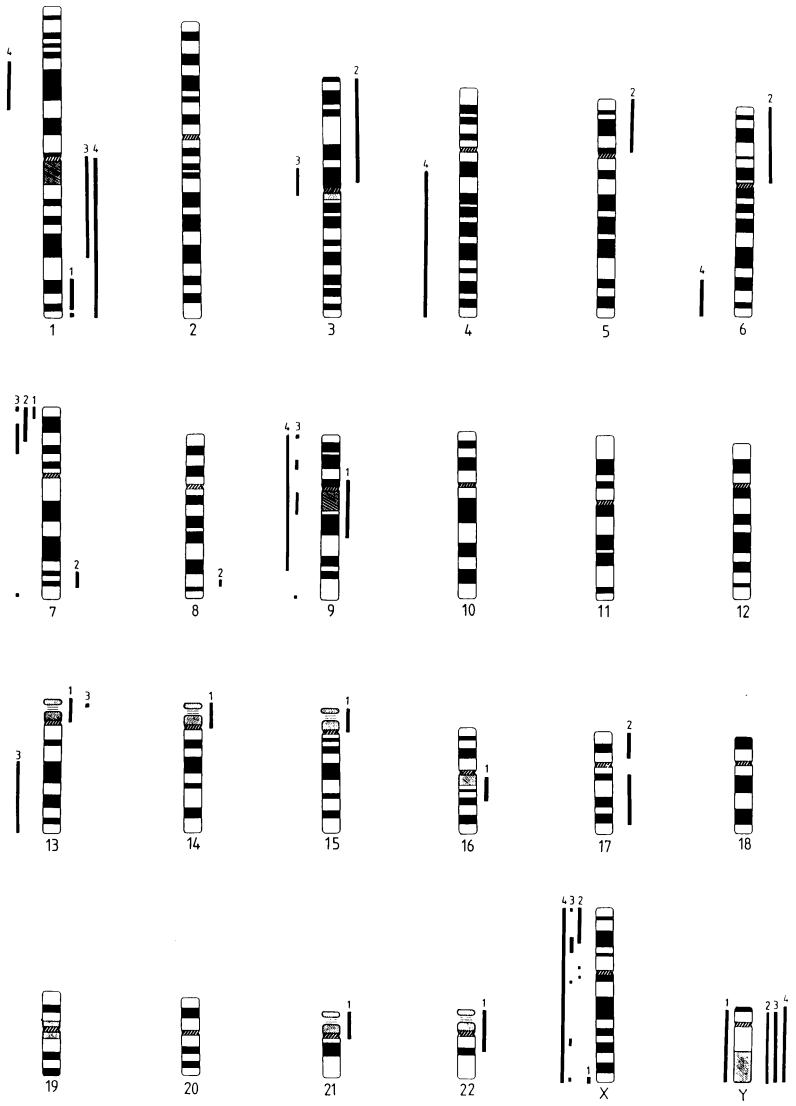

Fig. 4. Comparative genomic hybridization. On four cases with homozygous deletions of the *p15*, *p16* and *p14* genes, CGH was performed. Bars on the left side of each chromosome indicate deleted regions. Bars on the right side of each chromosome indicate amplifications. Numbers on each bar indicate sample numbers: 1, case 6; 2, case 7; 3, case 10; 4, case 12. Deletions of 9p21, on which the *p15*, *p16* and *p14* genes reside were detected in two cases (numbers 3, 4).

lymphocytosis except case 19 showed no abnormalities of the genes analyzed. High incidences of homozygous deletions of the *p15*, *p16* and *p14* genes were detected in nasal NK cell lymphomas, and blastic NK cell lymphoma/leukemias.

CGH It is not clear whether these homozygous deletions of the *p15*, *p16* and *p14* genes could be identified by karyotyping since chromosomal analysis could not be performed on these cases. Consequently, we performed CGH on four of the cases with homozygous deletions of the *p15*, *p16* and *p14* genes. CGH revealed loss of the long arm of chromosome 13, on which the *Rb* gene resides, in one blastic NK cell lymphoma/leukemia, and gain of the short arm of chromosome 17, on which the *p53* gene resides, in one nasal NK cell lymphoma (Fig. 4). These abnormalities were also detected by Southern blot analysis (Fig. 1, Table II). We found loss of 9p21, on which the *p15*, *p16* and *p14* genes reside, in two cases (Fig. 4).

DISCUSSION

NK cell neoplasms are very rare diseases, but occur in Asian countries, including Japan.[1)] The genetic changes in oncogenes and anti-oncogenes of these disease have not been fully analyzed. Overexpression of p53 protein and point mutations of the *p53* gene in these diseases have been reported.[19, 34)] In our study, we found two mutations of the *p53* gene. We might have underestimated the frequency of point mutations of the *p53* gene in NK cell neoplasms, since there might be point mutations outside the regions we analyzed in the *p53* gene,[16)] and PCR-SSCP might have failed to detect mutations in some cases, as previously reported.[35)]

We found mutation of the *p53* gene in one case of chronic NK lymphocytosis. In that case, the shifted bands of PCR-SSCP were strong, suggesting that most of the proliferating cells had identical mutations and monoclonality. The medical history of the patient did not suggest that this patient had Li-Fraumeni syndrome, one of the cancer-prone syndromes, in which mutations of the *p53* gene are inherited.[36)] The mutation was thought to be acquired and specific to the proliferating cells. Chronic NK lymphocytosis is an indolent disease and frequently lacks chromosomal abnormalities.[1)] So, it is not clear whether proliferating cells in this disease are monoclonal or polyclonal. Our results suggest that, at least in some cases with chronic NK lymphocytosis, proliferating cells are monoclonal.

We also found gene amplification of the *p53* gene in one case of nasal NK cell lymphoma. However, significance of this amplification is not clear at present. In this case, no point mutations of the *p53* gene were detected in the regions we analyzed (Table II).

We analyzed the *Rb* gene and its protein expression levels by Southern and western blot analyses, respectively. We detected one hemizygous deletion in blastic NK cell lymphoma/leukemia. Since PCR-SSCP analysis was not performed on the *Rb* gene, it was unclear as to whether the intact allele of the *Rb* gene had mutations or not in this case. As previously reported, abnormalities of the *Rb* gene frequently lead to decreased expression or abnormal size of the protein.[28)] Unfortunately, we were not able to perform western blot analysis on this case.

In cell lines, we found complete loss of Rb protein in NK-YS, an aberrant migration pattern of Rb protein in NK92, and overexpression in HANK1. In these cell lines, abnormalities of Rb protein expression might be associated with tumorigenesis. Increased expression of Rb protein was detected in three cases, including one of the cell lines. In normal peripheral white blood cells, expression of Rb protein was very low (Fig. 3). We could not conclude that the high expression of Rb involved abnormality of the *Rb* gene. Abnormalities in other gene(s) might have resulted in the increase in Rb protein levels. Interestingly, clinical cases with high expression of Rb protein were myeloid/NK cell precursor acute leukemia. High expression of Rb protein might be characteristic of myeloid/NK cell precursor acute leukemia as distinct from any other entity of NK cell neoplasms.

The *p15*, *p16* and *p14* genes are also tumor suppressor genes and abnormalities of these gene are frequently detected in a variety of human cancers.[20)] Homozygous deletions of these genes represent dominant types of mutation in human malignancies and point mutations of these genes constitute minor types of mutations, although nucleotide substitutions of these gene have occasionally been reported.[20)] Therefore, we performed Southern blot analysis only, and not PCR-SSCP analysis on these genes.

Of the 31 NK cell neoplasms, five showed homozygous deletions, including 3 nasal NK cell lymphoma and 2 blastic NK cell lymphoma/leukemia. Three out of the 4 nasal NK cell lymphoma cases revealed homozygous deletions. In addition, out of the 4 cases with blastic NK cell lymphoma/leukemia, 2 revealed homozygous deletions. Although the numbers of cases with nasal NK cell lymphoma and blastic NK cell lymphoma/leukemia were not large, this high incidence of homozygous deletion of these genes seems significant. In these two entities, the mechanisms of tumorigenesis might be similar.

Among primary solid tumors, high frequencies of abnormalities in the *p16* gene have been detected in lung cancers (9–67%), bladder cancers (19%) and glioblastoma (67%).[20)] Among hematological malignancies, abnormalities of *p15*, *p16* gene have been detected in T-cell acute lymphoblastic leukemia at high frequencies (25–83%). In B-cell lineage leukemia and non-Hodgkin's lymphoma derived from B-cell, abnormalities of the *p15*, *p16* genes are not so frequent (6–21%).[20)] Thus, the frequency of abnormalities of the *p16* gene in nasal NK cell lymphoma

and blastic NK cell lymphoma/leukemia are as high as in T-cell leukemia, lung cancer and glioblastoma.

We performed CGH on 4 samples with homozygous deletions of the *p15*, *p16* and *p14* genes. However, only two case revealed loss of 9p21, on which these genes reside. This finding suggested that the homozygous deletions of the *p15*, *p16* and *p14* genes could be subtle deletions not detectable by CGH or standard karyotyping in some cases of nasal NK cell lymphoma or blastic NK cell lymphoma/leukemia.

In summary, our results suggest that abnormalities of the *Rb*, *p53*, *p15*, *p16* and *p14* genes are associated with tumorigenesis in NK cell neoplasms. In particular, homozygous deletions of the *p15*, *p16* and *p14* genes might be important for tumorigenesis in nasal NK cell lymphoma, and blastic NK cell lymphoma/leukemia. These findings indicate that it might be possible to develop gene therapy for potentially fatal NK cell neoplasms using cell cycle-associated genes, including the *Rb*, *p53*, *p15*, *p16* and *p14* genes.

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