Molecular Analysis of Oncogenes, ras Family Genes (N-ras, K-ras, H-ras), myc Family Genes (c-myc, N-myc) and mdm2 in Natural Killer Cell Neoplasms

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Natural killer (NK) cell neoplasms are rare diseases. Frequent abnormalities of the tumor suppressor genes Rb, p53, p15INK4B, p16INK4A and p14ARF have been reported. However, no oncogenes associated with tumorigenesis of NK cell neoplasms have been reported so far. We analyzed the status of oncogenes including N-ras, K-ras, H-ras, c-myc, N-myc and mdm2 by Southern blot, PCR-SSCP, western blot analysis and immunohistochemical staining. We analyzed four cell lines derived from NK cell neoplasms and 31 clinical samples with five subclasses of NK cell neoplasms. We found no point mutations of the ras family genes. We detected no mutations in the c-myc and N-myc genes. No overexpression of c-Myc protein was detected by western blot analysis. Although we found neither amplification nor rearrangement of the mdm2 gene, we found high expression of MDM2 protein in some cases by western blot analysis. Immunohistochemical staining confirmed the overexpression of MDM2 protein. We found 14 cases with overexpression of MDM2 protein out of 15 cases (93%) with four subclasses of NK cell neoplasms except chronic NK lymphocytosis. Our previous and these results suggested that the expression level of MDM2 protein is independent of the status of the mdm2 gene. We analyzed the overexpression of MDM2 protein. We found 14 cases with overexpression of MDM2 protein out of 15 cases (93%) with four subclasses of NK cell neoplasms except chronic NK lymphocytosis. Although the number of the cases we analyzed was not large, alterations of ras and myc family genes may rarely contribute to tumorigenesis in NK cell neoplasms. In contrast, overexpression of MDM2 might be associated with tumorigenesis of NK cell neoplasms, especially aggressive subclasses.

Key words: Southern blot analysis — Point mutations — PCR-SSCP — Western blot analysis — Immunohistochemical staining

Natural killer (NK) cell neoplasms are rare malignancies that occur predominantly in Asian countries, including Japan.1) The proliferating cells are derived from NK cells, and the disease is divided into six subtypes based on distinct morphological, histological and clinical features.1) The six subtypes are acute lymphoblastic leukemia with NK phenotype,1) myeloid/NK cell leukemia,2) blastic NK cell lymphoma/leukemia,3) nasal and nasal type NK cell lymphoma,4) aggressive NK cell leukemia/lymphoma5) and chronic NK lymphocytosis.6, 7)

Epstein-Barr (EB) virus are frequently detected in these neoplastic cells.8–11) It is, so far, not clear whether EB virus transforms NK cells or not. The etiology of NK cell neoplasms remains to be disclosed. Because of massive necrosis in the tissues of NK cell neoplasms, especially in nasal and nasal type NK cell lymphomas, karyotypes of these diseases are not always obtained.12, 13) However, some recurrent chromosomal abnormalities were reported.14–16) Recently, comparative genetic hybridization (CGH) analysis in these diseases was performed and the results suggested that many genetic changes exist in NK cell neoplasms.17)

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We and others have reported that the tumor suppressor genes, including Rb, p53, p15INK4B, p16INK4A and p14ARF are frequently mutated in NK cell neoplasms.18, 19) Recently, mutations of the c-kit and Fas genes have been reported.20, 21) In hematological malignancies derived from lineages other than NK cells, including myeloid leukemia, lymphoid leukemia, lymphoma and myeloma, abnormalities of the ras and myc family genes, and MDM2 protein have been reported.22) However, it has not been reported whether oncogenes are deregulated or mutated in NK cell neoplasms.

ras family genes, including N-ras, H-ras and K-ras are important oncogenes.23) Point mutations at codons 12, 13 and 61 in these three genes are frequently detected in a variety of human cancers, including hematological malignancies.23) The mutated ras molecule constitutively transduces the signal and promotes cellular proliferation.23)

myc family genes, including c-myc and N-myc are also oncogenes, amplification or rearrangement of which is frequently detected in human cancers.24) Structural alterations of these myc family genes result in overexpression, which leads to cellular transformation. In lymphomas, point mutations in the N-terminal regions of the c-myc gene have been reported to stabilize the c-Myc protein and to
enhance the function of c-Myc, contributing to cellular transformation.

Amplification of the *mdm2* gene is frequently observed in sarcomas. Amplification of the *mdm2* gene is frequently observed in sarcomas.25) MDM2 protein is an oncprotein, which binds p53 and impairs the function of p53,26) p53 is the most important tumor suppressor protein, sensing DNA damage and inducing cell cycle arrest or apoptosis to prevent cellular transformation. In osteosarcomas, mutations of the *p53* gene are rare and normal p53 protein is translated. However, MDM2 protein overexpressed by gene amplification impairs the function of p53 and induces tumorigenesis in these tumors. In other types of cancers, including breast cancers and lymphomas, overexpression of MDM2 protein is frequently detected without gene amplification.22, 28)

In this study we analyzed the status of these oncogenes in NK cell neoplasms of four cell lines and 31 clinical samples.

**MATERIALS AND METHODS**

**Cell lines, clinical samples and DNA extraction** Four cell lines derived from NK cell neoplasms were kindly provided by Drs. Robertson (NKL), Kagami (HANK1), Gong (NK92) and Tsuchiyama (NK-YS). Human embryonic kidney 293 cells were purchased from American Type Culture Collection (Manas-sas, VA). Peripheral blood or lymph-nodes or tumor biopsy samples were obtained from 31 cases with NK cell neoplasms, including three myeloid/NK cell leukemia/lymphoma, four blastic NK cell lymphoma/leukemia, six aggressive NK cell leukemias, six nasal and nasal-type NK cell lymphoma, 12 chronic lymphocytosis 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% in the samples, which were confirmed by flow cytometry analysis. High-molecular DNAs were extracted from the samples as previously reported. The DNAs were subject to Southern blot analysis and PCR.

**Probes and Southern blot analysis** Probes for Southern blot analysis were generated from normal human bone marrow cells using the following primers: *mdm2*-S, 5′-GGTA GAA ACC TGT GCA AT-3′; *mdm2*-AS, 5′-CCG ATG ATT CCT GCT GAT TG-3′. c-myc probe was generated from normal human bone marrow cells using the following primers: c-myc-S, 5′-ATG CCC CTC AAC GTT AGC TT-3′; and c-myc-AS, 5′-AGC TTC TCT GAG ACG AGC TT-3′. N-myc probe was generated from normal human DNA by PCR using the following primers: N-myc-S, 5′-CCA CCA TGC CGG GCA TGA TC-3′; N-myc-AS, 5′-GCC GGG CTG CCC CAC AGC TC-3′. Southern blot analysis of the *p14ARF* gene was performed as previously described. All probes used were ligated into pGEM-T vectors (Promega, Madison, WI) and the sequences were confirmed. Nucleotide sequences were determined using a Prism sequencing kit on a genetic analyzer 310 (Applied Biosystems, Foster City, CA). The probes were radio-labeled by random priming methods using Ready-to-go DNA labelling kit (Amersham Pharmacia, Uppsala, Sweden).

High-molecular DNAs (10 μg) were digested with restriction enzyme EcoRI (TaKaRa, Kyoto) and electrophoresed in 0.8% agarose gels. The fractionated DNAs were transferred onto nylon membranes (Hybond N+, Amersham Pharmacia Biotech) according to the manufacturer’s recommendations. Pre-hybridization, hybridization and washing were performed as previously described.

The membranes were exposed on an imaging plate and the signals were detected with the BAS 2500 imaging analyzer (Fuji, Tokyo).

**PCR-SSCP** Primers used for amplification of *ras* family genes were as follows: N-ras13-S, 5′-GAC TGA GTA CAA ACT GGT GG-3′ and N-ras13-AS, 5′-CTC TAT GGT GGG ATC ATA TT-3′ for codon 13 of the *N-ras* gene; N-ras61-S, 5′-GGT GAA ACC TGT TTG TTG GA-3′ and N-ras61-AS, 5′-ATA CAC AGA GGA AGC CTT CG-3′ for codon 61 of the *N-ras* gene; K-ras13-S, 5′-GAC TGA ATA TAA ACT TGT GG-3′ and K-ras13-AS, 5′-CTA TTG TTG GAT CAT ATT CG-3′ for codon 13 of the *K-ras* gene; K-ras61-S, 5′-TTG ATG GTC AGC GCA CTC TT-3′ and K-ras61-AS, 5′-AGA GTG TGT GAT CAT ATT CG-3′ for codon 13 of the *K-ras* gene; H-ras13-S, 5′-GAG GGA AGT AG-3′ and K-ras61-AS, 5′-CAC AAA GGA AGC CCT CCC CA-3′ for codon 61 of the *K-ras* gene; H-ras13-S, 5′-CAAG AGT GGA AGT AAT GTA GGT GGT GGT-3′ and H-ras13-AS, 5′-AGG AGG AGA GCC AGT GGT GAT CAT ATT CG-3′ for codon 13 of the *H-ras* gene; H-ras61-S, 5′-AGA CTT GCC GGC GGC TGT GCC ACA TC-3′ and H-ras61-AS, 5′-CGC ATG TAC TGG TCC CGG CA-3′ for codon 61 of the *H-ras* gene.

The N-terminal region of the *c-myc* gene was amplified using the primers, c-myc-S and c-myc-AS. The PCR was performed as previously described. Mutation in exons 5–8 of the *p53* gene were analyzed using PCR-SSCP as previously described.

The PCR products were electrophoresed in 0.5x Super-shift detection gel (Toyobo, Tokyo) at 300 V for 18 h according to the manufacturer’s recommendation. The PCR products of the N-terminal region of the *c-myc* gene were digested with *PstI* (TaKaRa) overnight at 37°C before electrophoresis. The gels were dried and exposed on an imaging plate, and the signals were detected with the BAS 2500 imaging analyzer for 24 h at room temperature.

**Western blot analysis** Neoplastic cells were lysed with lysis buffer and the concentration of protein 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% SDS-PAGE as previously described. The proteins were transferred onto nitrocellulose filters (Amersham Pharmacia). c-Myc proteins were detected using anti-c-Myc antibody (Cosmo Biotech, Tokyo), MDM2 proteins were detected with anti-MDM2 monoclonal antibody (SMP14, Santa Cruz Biotechnology, Santa Cruz, CA) and p53 were detected with anti-p53 monoclonal antibody (Clone BP 53-12, Sigma, St. Louis, MI). Anti-MDM2 monoclonal antibody (SMP14) is a mouse monoclonal IgG1 antibody specific for the epitope corresponding to amino acids 154–167 of MDM2 of human origin. The same membranes were incubated with anti-β-actin antibody (Sigma) to measure the applied protein amounts. Blocking and washing were performed as previously reported. C-Myc, MDM2 and β-actin proteins were detected with secondary horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin antibody (New England Bio Lab, Beverly, MA) and the signals were obtained on X-ray films using a West Pico Western Detection Kit (Pierce, Rockford, IL).

Densitometric analysis was performed using NIH Image software. MDM2/actin ratios were calibrated and compared, based on the ratio in K562 cell line taken as one. The samples with a value of more than half were evaluated as overexpressing MDM2.

**Immunohistochemical staining** Tissues or cell clumps were formalin-fixed and paraffin-embedded. Immunohistochemical staining were performed using the MDM2 K ImmunoCruz Staining system (Santa Cruz Biotechnology) according to the manufacturer’s protocol. Anti-MDM2 antibody was the same as that used in western blot analysis. Antigen retrieval was performed by pretreating the sections at 121°C for 10 min in an autoclave. MDM2 showed both nuclear and cytoplasmic immunostaining. The immunoreactivity was evaluated as strongly positive (++) when more than 50% of tumor cells were stained. Cases were regarded as negative for MDM2 immunoreactivity when less than 10% of the cells had clear immunoreactivity.

**RESULTS**

We analyzed ras family genes by PCR-SSCP and sequencing to screen the point mutations at codons 12, 13 and 61, where point mutations are detected in a variety of cancers. No point mutations were detected in these three genes.

Then we performed Southern blot analysis using c-myc and N-myc probes to detect amplifications and rearrangements of these genes. We found neither amplification nor rearrangement of these genes. Further, we performed PCR-SSCP on the N-terminal region of the c-myc gene and found no mutations. We also performed western blot anal-

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**Table I. Genetic Status of the mdm2 Gene and MDM2 Protein Levels by Western Blotting Analysis**

| Rearrangement | Amplification | Protein overexpression |
|---------------|---------------|------------------------|
| Myeloid       | 0/3           | 0/3                    | 3/3                    |
| Blastic       | 0/4           | 0/4                    | 3/3                    |
| Aggressive    | 0/4           | 0/4                    | 1/1                    |
| Nasal         | 0/4           | 0/4                    | 2/3                    |
| Chronic       | 0/12          | 0/12                   | 0/6                    |
| Cell line     | 0/4           | 0/4                    | 3/4                    |
ysis using anti-c-Myc antibody to analyze protein expression levels of c-Myc. We found no overexpression of c-Myc proteins.

We performed Southern blot analysis using the mdm2 probe and found neither amplification nor rearrangement of the gene (Fig. 1C, Table I). However, we found overexpression of MDM2 proteins in three out of four NK cell lines (HANK-1, NK92, NKL) by western blot analysis (Fig. 1B, Table I). In clinical samples, we found that nine cases overexpressed MDM2 proteins, i.e., three myeloid/NK-cell precursor acute leukemias, three blastic NK cell lymphoma/leukemias, one aggressive NK cell leukemia/lymphoma, and two nasal NK cell lymphomas (Fig. 1C, Table I). In our study, overexpression of MDM2 proteins was not detected in chronic NK lymphocytosis. The expression levels were confirmed by densitometric analysis (Table II).

To analyze the association between overexpression of MDM2 proteins and the status of other tumor suppressor genes, including the p14ARF, Rb, and p53 genes, we compared the results in this study with the status of these tumor suppressor genes and protein expression levels of p53 and Rb in these cases (Fig. 1, B, C and Table II). While anti-p53 antibody detected the p53 protein in human embryonic kidney 293 cells (Fig. 1A), we detected only trace levels of expression of p53 protein in NK neoplasms (Fig. 1). In some cases overexpressing MDM2 proteins, we detected no mutations of p53 and p14ARF genes (Fig. 2). There was no apparent association of the status of these tumor suppressor genes and the expression levels of MDM2 proteins (Table II).

Since we could not obtain enough tissue samples to perform western blot analysis in some cases with NK neoplasms, we performed immunohistochemical staining of MDM2 proteins on seven available paraffin-embedded samples (Fig. 3). MDM2 immunoreactivity, usually limited to the nuclei and nucleoli, but sometimes also in cytoplasm, was observed in cases strongly positive for MDM2. We detected strong positivity for MDM2 proteins in 7 cases by immunohistochemical staining. Overexpression of MDM2 proteins were also confirmed by western blot-

### Table II. No Relationship between MDM2 Protein Levels and Status of the p14ARF/p16, p53, Rb Genes

| Case no. | Diagnosis | p14ARF/p16 | p53 | Rb protein | MDM2 protein |
|----------|-----------|------------|-----|------------|--------------|
| 1        | Myeloid   | Wt          | Wt  | +          | ++ (0.67)    |
| 2        | Myeloid   | Wt          | Wt  | +          | ++ (4.04)    |
| 3        | Myeloid   | Wt          | Wt  | NE         | ++ (0.59)    |
| 4        | Blastic   | Wt          | Mt  | –          | ++ (0.60)    |
| 5        | Blastic   | Del         | Wt  | NE         | ++ (0.84)    |
| 6        | Blastic   | Wt          | Wt  | –          | NE           |
| 7        | Blastic   | Del         | Wt  | –          | ++ (0.68)    |
| 8        | Aggressive| Wt          | Wt  | –          | NE           |
| 9        | Aggressive| Wt          | Wt  | –          | ++ (1.14)    |
| 10       | Aggressive| Wt          | Wt  | NE         | NE           |
| 11       | Aggressive| Wt          | Wt  | NE         | NE           |
| 12       | Nasal     | Del         | Wt  | –          | ++ (2.65)    |
| 13       | Nasal     | Del         | Wt  | –          | ++ (2.49)    |
| 14       | Nasal     | Del         | Wt  | –          | NE           |
| 15       | Nasal     | Wt          | Wt  | –          | ± (0.20)     |
| 16       | Chronic   | Wt          | Wt  | –          | ± (0.04)     |
| 17       | Chronic   | Wt          | Wt  | NE         | NE           |
| 18       | Chronic   | Wt          | Wt  | NE         | NE           |
| 19       | Chronic   | Wt          | Wt  | NE         | ± (0.13)     |
| 20       | Chronic   | Wt          | Wt  | –          | NE           |
| 21       | Chronic   | Wt          | Wt  | NE         | NE           |
| 22       | Chronic   | Wt          | Mt  | –          | ± (0.28)     |
| 23       | Chronic   | Wt          | Wt  | –          | ± (0.10)     |
| 24       | Chronic   | Wt          | Wt  | NE         | ± (0.14)     |
| 25       | Chronic   | Wt          | Wt  | –          | ± (0.13)     |
| 26       | Chronic   | Wt          | Wt  | –          | NE           |
| 27       | Chronic   | Wt          | Wt  | –          | NE           |

For p14ARF/p16, Wt, wild type; Del, homozygous deletion. For p53, Wt, wild type; Mt, mutations were found. For Rb and MDM2 protein, levels of Rb and MDM2 proteins were quantified by western blotting. ±, none or trace level of expression; +++, high level of expression; NE, not examined. ( ), calibrated MDM2/actin ratio (see “Materials and Methods”).

Fig. 2. Representative cases of normal status of the p53 and p14ARF genes. A) PCR-SSCP analysis of exons 5–8 of the p53 gene. Some of the cases overexpressing MDM2 protein presented the normal pattern of migration bands in PCR-SSCP. B) Southern blot analysis of the p14ARF genes. Some of the cases overexpressing MDM2 protein presented intact genomic status of the p14ARF gene by Southern blot analysis. β2MG: the β2 microglobulin gene as an internal control.
Taking 3 cases out of these 7 cases. Taking the results of western blotting and immunohistochemical staining together, we detected high expression of MDM2 proteins in 14 out of 21 clinical cases (66%), comprising three myeloid/NK cell precursor acute leukemia, three blastic NK cell lymphoma/leukemia, three aggressive NK cell leukemia/lymphoma, and five nasal NK cell lymphoma (Table III).

**DISCUSSION**

NK cell neoplasms are very rare diseases. The associated genetic changes in oncogenes and anti-oncogenes have not been fully analyzed. High frequencies of mutation of tumor suppressor genes, including Rb, p53, p16INK4A, p15INK4B and p14ARF have been reported. So far, the status of oncogenes in NK cell neoplasms has not been reported. For the first time, we analyzed the status of ras and myc family genes, and the mdm2 gene in NK cell neoplasms.

In our study, we found no mutations among the ras family genes. Although the number of cases we analyzed was not large, it appears that point mutations of the ras family genes rarely contribute to tumorigenesis of NK cell neoplasms.

We found no structural alteration of the c-myc and N-myc genes. In the c-myc gene, no point mutations were detected in the N-terminal region, where point mutations were frequently detected in lymphomas. Structural alteration of the c-myc and N-myc genes could be uncommon in NK cell neoplasms. In human cancers, overexpression of oncoproteins is detected even when structural alteration is not found. Thus, we performed western blot analysis to evaluate the protein expression levels of c-Myc. We found no overexpression of c-Myc protein, which suggested that c-Myc protein is rarely associated with tumorigenesis of NK neoplasms.

Although we found no structural alteration of the mdm2 genes in NK cell neoplasms we studied, we found overexpression of the MDM2 protein in three myeloid/NK cell precursor acute leukemias, three blastic NK cell lymphoma/leukemias, three aggressive NK cell leukemia/lymphomas, five nasal NK cell lymphomas. The protein expression level is regulated by translation from messenger RNA and protein stability. Expression of mdm2 transcripts is regulated by p53 protein, so that neoplastic cells having mutations in the p53 gene frequently express low amounts of MDM2 protein. P14ARF is an anti-oncoprotein which cannot bind the MDM2 protein and induce its degradation. P14ARF is translated from the p16/p14ARF gene, which is an important tumor suppressor gene, and is homozygously deleted in a variety of human cancers. We compared the expression level of MDM2 protein with the status of other tumor suppressor genes and found no

| Table III. High Expression of MDM2 Protein Is Frequently Detected in NK Cell Neoplasms except Chronic NK Lymphocytosis |
|-----------------|--------|
| Myeloid         | 3/3    |
| Blastic         | 3/3    |
| Aggressive      | 3/3    |
| Nasal           | 5/6    |
| Chronic         | 0/6    |
| Total           | 14/21  |

Fig. 3. Immunohistochemical staining of MDM2 protein. Left: normal bone marrow. Faint staining of MDM2 protein was detected in nuclei of several cells. Middle: bone marrow infiltrated by aggressive NK leukemia/lymphoma cells. Expression of MDM2 protein is detected in nuclei of most leukemic cells. Right: metastatic lymph node tissue of nasal NK cell lymphoma. Expression of MDM2 proteins is detected in nuclei of most lymphoma cells. MDM2 immunoreactivity, usually limited to the nuclei and nucleoli, sometimes also in cytoplasm, was observed in cases strongly positive for MDM2.
association between the level of MDM2 protein and the status of these tumor suppressor genes. This might suggest that the expression level of MDM2 protein is independent of the status of the p14ARF and p53 genes in these cases (Table II). We could not detect any structural abnormalities of the mdm2 gene. Mechanisms of overexpression of MDM2 protein are not clear at present. Some transcriptional factor(s) might up-regulate the transcription of the mdm2 gene or/and some protein(s) might block the degradation of MDM2 protein. Overexpression of MDM2 protein might independently contribute to carcinogenesis of NK cell neoplasms. Recently, it has been reported that MDM2 directly regulates the transcription of NFκB protein, which is a transcriptional factor in a p53-independent pathway.40) In NK neoplasms overexpressing MDM2 protein, MDM2 might activate NFκB.

Immunohistochemical staining of MDM2 protein is useful method to evaluate the expression level of MDM2 in NK cell neoplasms, especially in cases in which enough sample tissue could not be obtained for western blot analysis. This method could make it possible to evaluate the expression level of MDM2 protein in biopsy samples of NK cell neoplasms.

Interestingly, we found no overexpression of MDM2 protein in chronic NK lymphocytosis although the number of cases we analyzed was not large. In contrast, high levels of MDM2 protein were detected in aggressive NK cell leukemia/lymphoma. It is sometimes difficult to distinguish these two subclasses in terms of the phenotypes of proliferating cells. However, the clinical courses of these two subclasses are completely different: aggressive NK cell leukemia/lymphoma is fatal without treatment, in contrast to chronic NK lymphocytosis, which is frequently indolent and requires no treatment. Analysis of expression levels of MDM2 protein might help to distinguish these two subclasses.

Protein overexpression of MDM2 has been reported in some human cancers.22) In chronic lymphocytic leukemia, an indolent neoplastic disease, overexpression of MDM2 protein was reported.40) Interestingly, chronic NK lymphocytosis, resembling chronic lymphocytic leukemia in some clinical features, did not overexpress MDM2. In cases with acute lymphoblastic leukemia, overexpressing MDM2, the leukemic cells were resistant to chemotherapy and the prognosis of these cases was very poor.40) These clinical features are very similar to those of aggressive types of NK cell neoplasms.

In summary, our results suggest that abnormalities of ras and myc family genes rarely contribute to tumorigenesis in NK cell neoplasms. On the other hand, although the mechanism of deregulation of the mdm2 gene is not clear at present, we found 14 cases with overexpression of MDM2 protein out of 15 cases (93%) with four subclasses of NK cell neoplasms, except chronic NK lymphocytosis. The clinical features of these four subclasses are very aggressive. MDM2 could be associated with tumorigenesis in NK cell neoplasms except chronic NK lymphocytosis.

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