Short Communication

Comparative Genomic Hybridization Analysis of Natural Killer Cell Lymphoma/Leukemia

Recognition of Consistent Patterns of Genetic Alterations

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Putative natural killer (NK) cell lymphoma/leukemia is a rare group of recently characterized hematolymphoid malignancies. They are highly aggressive and frequently present in extranodal sites, including the nasal area and the upper aerodigestive system, and nonnasal areas such as the skin and the gastrointestinal tract. According to clinicopathological features, they can be classified into nasal NK cell lymphoma, nasal-type NK cell lymphoma occurring in nonnasal areas, and NK cell lymphoma/leukemia. Genetic alterations in NK cell lymphoma/leukemia are not well defined. In this study, we have performed comparative genomic hybridization (CGH) on DNA extracted from fresh or frozen tissues of 10 patients with NK cell lymphoma/leukemia. They comprised four nasal NK cell lymphomas, one nasal-type NK cell lymphoma, and five NK cell lymphomas/leukemias. CGH showed frequent deletions at 6q16-q27 (four cases), 13q14-q34 (three cases), 11q22-q25 (two cases), 17p13 (two cases), and loss of the whole chromosome X (two cases). DNA amplification was observed in a majority of the chromosomes. Five cases showed DNA gains at region 1p32-pter. Frequent DNA gains were also found in chromosomes 6p, 11q, 12q, 17q, 19p, 20q, and Xp (three cases each). Interestingly, DNA gains were more frequent in nasal/nasal-type NK cell lymphomas than NK cell lymphoma/leukemia. These genetic alterations correlated well with karyotypic features found in some of the cases. The frequent DNA losses at 6q and 13q suggest that the presence of tumor suppressor genes at these regions is important in NK cell transformation. In addition to establishing novel patterns of genomic imbalances in these rare NK cell malignancies, which may be targets for future

molecular analysis, this study also provides important information on genetic alterations in NK cell lymphomas that may be useful in defining their positions in current lymphoma classification schemes, which are increasingly focusing on phenotypic and genotypic correlations. (*Am J Pathol* 1999, 155:1419–1425)

A group of highly aggressive hematolymphoid malignancies of putative natural killer (NK) cell origin have been recognized in recent years.^{1–5} This group of tumors is characterized phenotypically by the expression of CD2, cytoplasmic CD3ε (but not surface CD3 or the T-cell receptor), and CD56, and genotypically by the T-cell receptor gene in germline configuration, and by an almost invariable association with monoclonal Epstein-Barr virus infection.⁶ Clinically, these tumors may be classified into several categories according to the predominant sites involved. The majority of the tumors initially involve the nasal and nasopharyngeal areas and are referred to as nasal NK cell lymphoma. A minority involve primarily nonnasal areas, including the skin and the gastrointestinal tract, and are referred to as nasal-type NK cell lymphoma. Rarely, the lymphoma may be disseminated with a leukemic phase, in which case it is known as NK cell lymphoma/leukemia.^{3,4}

These putative NK cell malignancies are very rare diseases and show a predominant ethnic predilection, with a much higher incidence reported in Oriental as compared with Occidental populations.⁷ Of about 150 cases of NK cell lymphomas described, only fewer than 30 cases were reported in the West.^{1–5} In the recently published Revised European American Lymphoma

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(REAL) Classification scheme, NK cell malignancies were recognized as a provisional entity.⁸ However, in the proposed World Health Organization Classification for neoplastic diseases of the lymphoid tissues, these tumors are more clearly classified as nasal-type extranodal NK/T cell lymphoma and NK cell leukemia.⁹ In both classification systems, cytogenetic and molecular features are used to characterize lymphoma subtypes.^{8,9} Although NK cell lymphomas are recognized as distinct clinicopathological entities in both schemes, little is known of their cytogenetic and genetic changes. Therefore, there is an urgent need for data on genetic alterations in NK cell lymphomas, not only for the classification of these disorders, but also for a better understanding of their pathogenesis.

Recurrent chromosomal aberrations have been defined in a limited number of cases of NK cell lymphomas. Owing to the rarity of the tumor and the often marked tumor necrosis and the difficulty of obtaining adequate biopsy materials from the nasal areas, procurement of tissues for detailed karyotypic analysis has been problematic. Successfully karyotyped cases in fact were mostly derived from aggressive NK cell lymphoma/leukemia that involved the bone marrow, where enough cells could be obtained. Furthermore, conventional karyotyping studies are often hampered by difficulties in obtaining adequate cell growth and poor quality metaphases.

Comparative genomic hybridization (CGH) is a recently developed molecular cytogenetic technique that permits scrutinization of the entire genome of a tumor for chromosomal copy number aberrations in a single hybridization.¹⁰ The method is based on cohybridization of differentially fluorochrome-labeled tumor DNA and normal DNA to normal metaphase chromosomes. Differences in tumor *versus* normal control fluorescence intensities along the chromosomes reveal over- and underrepresentations of DNA sequences in the entire tumor genome. Digital image analysis provides a quantitation of the copy number changes. In this study, we have used CGH to analyze the genetic aberrations of 10 cases of NK cell lymphoma/leukemia with DNA from fresh or frozen tumor tissues.

Materials and Methods

Case Selection

Ten patients with CD2-positive (+ve), CD3-negative (-ve)/CD3 ϵ +ve, CD56+ve NK cell lymphoma/leukemia were studied by CGH. The cases were selected by 1) the availability of fresh or frozen tissue for DNA extraction and 2) the presence of a significant proportion (over 50%) of tumor cells in the tissue sample. The specimens included peripheral blood or bone marrow aspirates (seven cases) and snap-frozen biopsy samples from the tumors, including the nasopharynx (three cases) and the ovary (one case). In one patient (case 4), frozen materials were available from both the nasopharynx and marrow. Cytogenetic analysis was performed in four cases. The

clinicopathological features of these cases were summarized in Table 1.

Comparative Genomic Hybridization

High-molecular-weight DNA was extracted from the tumor by standard protocols. Normal reference DNA was obtained from peripheral blood of karyotypically normal males and females. CGH was performed using direct fluorochrome conjugation of tumor and control DNA. Briefly, tumor DNA was labeled by nick translation with SpectrumGreen dUTP and normal reference DNA (matching the sex of the patient) with SpectrumRed dUTP. The fragment lengths of the labeled probes were confirmed to range from 500 to 2000 bp by agarose gel electrophoresis. All reagents used in the nick translation, including the fluorochromes, were obtained from Vysis (Naperville, IL). Labeled tumor and normal DNA (450 ng each) were ethanol precipitated in the presence of 36 μ g Cot-1 DNA (Gibco BRL, Gaithersburg, MD), dried, and resuspended in hybridization buffer (Vysis). The hybridization mixture was denatured at 73°C for 5 minutes and allowed to preanneal at 37°C for 30 minutes. Before hybridization, normal metaphase spreads (Vysis) were denatured in 70% formamide/2 $\times$ standard saline citrate (SSC) at 72°C for 5 minutes and dehydrated in ice-cold 70%, 85%, and 100% ethanol. Hybridization was performed in a light-tight humid chamber at 37°C for 3–5 days. The slides were washed in 0.4 $\times$ SSC/0.3% Igepal at 73°C for 2 minutes, followed by a 1-minute wash in 2 $\times$ SSC/0.1% Igepal at room temperature. The slides were briefly dehydrated in 70% and 85% ethanol for 30 seconds each, then left to dry in the dark before being counterstained with 125 ng/ml 4',6-diamidino-2-phenylindole (DAPI) in anti-fade solution (Vysis).

All tumor DNAs were reversely labeled with Spectrum-Red, except cases 8 and 9, because of an inadequate amount of DNA. This "inverse" labeling CGH served as a control for the differences in hybridization between the SpectrumGreen-labeled and SpectrumRed-labeled probes. Chromosomal aberrations were recorded when the two fluorochrome-labeled probes showed similar CGH profiles.

Digital Image Analysis

Metaphases that showed uniform and intense hybridization and contained well-separated chromosomes were captured with a cooled charge-coupled device (CCD) camera mounted on a Leitz DM RBE (Leica, Wetzlar, Germany) fluorescence microscope. A 100 $\times$ Plan fluotar objective (NA 1.30, oil) was used to capture the images. Three-color digital images (green for SpectrumGreen, red for SpectrumRed, and blue for DAPI) were acquired from at least 10 metaphases per hybridization, using a filter wheel containing excitation filters appropriate for SpectrumGreen, SpectrumRed, and DAPI fluorochromes. The CytoVision digital imaging system (version 3.1; Applied Imaging, Santa Clara, CA) was used for calculation of the green-to-red fluorescence ratio for each

Table 1. Clinical Features and Cytogenetic and CGH Findings of 10 Cases of NK Cell Lymphoma/Leukemia

Case	Sex and age	Type of NK lymphoma/leukemia	Specimen	Conventional cytogenetics	Comparative genomic hybridization	
					Copy number losses	Copy number gains
1	F/45	Nasal	Ovary (relapse)	Not available	11q23-q25, 13q14-q34	1pter-q32, 6p21, 7q, 11q14-q21, 19p13, 20
2	F/50	Nasal	Nasopharynx	Not available	X, 6q16-q25	—
3	M/32	Nasal	Nasopharynx	Not available	Normal	Normal
4a	F/48	Nasal	Marrow (relapse)	46, XX	X, 6q25-q27, 7p15-p22, 13q14-q21	1q23-q31, 2p11-p16, 4p15-q25, 5p12-p15, 6pter-q22, 7q11-q31, 14q21-q23, 17q21-q25, 20
4b			Nasopharynx	Not available	13q21-q22	1p32-pter, 3p21, 6p21, 9q, 11q12-q13, 11q23, 12q23-q24, 14q24-q32, 15q23-q24, 16, 17, 19, 20q11-q12, 22
5	M/73	Nasal-type	Blood	Not available	—	1p33-pter, 6p21, 11q12-q13, 11q23-q24, 12q23-q24, 16p12-p13, 17p12-q22, 19, 20q, 22
6	M/37	Aggressive NK cell lymphoma/leukemia	Marrow	46,XY,del(6)(q21q25), del(13)(q14q22) [11]/46, idem, del(17)(p12)[2]/46, idem, i(17)(q10)[3]/46,XY[4]	6q23-q25, 13q14-q21*, 17p13	Xp22
7	M/71	Aggressive NK cell lymphoma/leukemia	Blood	46,XY,del(6)(q16.2q22.2)[9]/46,Y,ins(X,?)(p11;?),del(6)(q16.2q22.2)[7]/46,XY,del(6)(q16.2q22.2), add(15)(p13)[16]/46,XY[6]	6q21	Xp21-p22
8	M/17	Aggressive NK cell lymphoma/leukemia	Marrow	Not available	Normal	Normal
9	M/35	Aggressive NK cell lymphoma/leukemia	Blood	Not available	9p21-p24, 11q22-q25	Xp11-p21, Xq21-q27, 1p34-pter, 3p24-pter, 12q23-q24, 17q11-q22
10	M/44	Aggressive NK cell lymphoma/leukemia	Marrow	46,XY	1p13-p31, 10p13-p15, 17p13	1p36, 8

*Confirmed by fluorescence *in situ* hybridization with a mixture of an *Rb* gene probe mapping to 13q14 (labeled with SpectrumGreen) and a DNA Probe mapping to 21q22.13-q22.2 (labeled with SpectrumOrange) (LSI 13/21, Vysis). Ninety-nine of 200 interphase cells analyzed showed one green and two red hybridization signals, confirming deletion of the 13q14 region, as shown by CGH.

chromosome. Chromosomes were identified on the reverse DAPI banding images. The calculated average ratios were plotted along ideograms of their corresponding chromosomes in a relative copy number karyotype. The ratio values of 1.25 and 0.75 were used as upper and lower thresholds for the identification of chromosomal imbalances.¹¹ The Cot-1 DNA included in the hybridization inhibited binding of the labeled DNA to the centromeric and heterochromatic regions, and thus these regions were not analyzed. Chromosomes that were heavily bent or overlapping or had overlying artifacts were excluded from the analysis. Cautious interpretation was made in telomeric and heterochromatic regions, 1p32-pter, 16p, 19, and 22, as recommended by Kallioniemi et al.¹²

Controls

Negative control included hybridization of a SpectrumGreen-labeled normal male DNA against Spectrum-Red-labeled normal female DNA and monitoring of the green-to-red ratio of the X chromosome in relation to that of the autosomes. This could help the examination of the dynamic range of the hybridization. The tumor cell line

MPE-600 (Vysis), an immortalized breast cancer cell line that was known to have DNA losses in 1pter, 9p, 11q14-q25, and 16q and DNA gain at 1q, was included as a positive control. The conditions of CGH experiments were optimized so that the genetic alterations of MPE-600 could be detected unequivocally.

Results

CGH analysis was abnormal in eight of 10 cases, with significant DNA gains or losses observed. The results are summarized in Table 1 and Figure 1. Deletions of 6q and 13q were the commonest findings, being found in four and three cases, respectively. The CGH profile showed loss of DNA to involve a segment spanning 6q16 to 6q27. The deleted region on chromosome 13 involved regions q14 to q34. Two cases showed loss of the entire chromosome X. Other regions of DNA losses included 1p, 7p, 9p, 10p, 11q, and 17p. In contrast to DNA losses, gains of DNA were more extensive and involved different genomic regions. Frequent DNA gains were located at regions 1p (five cases), 6p, 11q, 12q, 17q, 19p, 20q, and Xp (three cases in each region). A partial CGH karyotype

Figure 1. Summary of genetic imbalances detected by CGH in 10 cases of NK cell lymphoma/leukemia. Chromosome losses are indicated by red lines on the left of each ideogram, and green lines on the right represent chromosome gains. Each line represents an individual tumor where the aberration was identified. Two samples from case 4 were analyzed and are represented separately in this figure.

of case 10 was shown in Figure 2. A CGH image of the ovarian relapse in case 1 was shown in Figure 3.

Discussion

Conventional cytogenetic analysis often yields important information on tumorigenesis. However, it has a number of inherent limitations.¹³ First, conventional karyotyping is limited to dividing cells and thus excludes a large number of nondividing cells. It is possible that dividing cells represent only a subclone of the main neoplastic population. Second, normal cells may sometimes outgrow the tumor cells, thus giving normal metaphases. Third, chromosomal banding is difficult in solid tumors, where the morphology of the metaphases is often poor. Finally, karyotypic analysis depends on the availability of fresh tissues and cannot be carried out in archival materials. Comparative genomic hybridization (CGH) is a novel molecular cytogenetic tool that permits the evaluation of genetic

changes throughout the whole genome of tumors in a single experiment using a small amount of DNA.^{10,14} Fluorescence ratio changes representing gains or losses of parts or the whole of the chromosomes are presented as copy number karyotype. CGH can be performed on DNA extracted from archival materials, or even formalin-fixed paraffin-embedded specimens, sometimes after whole genome amplification if the DNA yield is too low.¹⁵ It is thus especially helpful in the investigation of genetic changes underlying solid tumors.¹⁶

In this study, we have used CGH to investigate NK cell lymphoma/leukemia and defined a characteristic pattern of copy number aberrations. In cases that showed DNA losses, frequently deleted chromosomal regions included 6q and 13q. Four cases (cases 2, 4, 6, and 7) showed loss of DNA at region 6q16 to 6q27. The smallest area of deletion involved band 6q21, and the largest area of deletion spanned region 6q16 to 6q25. Among the deleted regions, the region at 6q25 was most commonly

Figure 2. Partial fluorescence ratio profile of case 10. The central line represents a ratio value of 1.0. Green lines to the right indicate ratio values of 1.25 and 1.5, and red lines to the left indicate ratio values of 0.75 and 0.5. The chromosome numbers are given below the individual ideograms (n = number of homologues examined). The CGH profile suggests DNA losses at 1p13-p31, 10p13-p15, and 17p13, and DNA gains at 1p36 and chromosome 8.

involved. These results correlated closely with those of conventional cytogenetics in the two karyotyped cases (cases 6 and 7). The results also confirmed molecularly the previous observations of conventional cytogenetics that deletion of 6q (del(6q)) was the commonest recurrent aberration in NK cell lymphoma/leukemia.¹⁷⁻¹⁹ It is noteworthy that del(6q) is also commonly seen in acute lymphoblastic leukemia and non-Hodgkin's lymphomas (NHLs).²⁰ Previously, candidate tumor suppressor genes have been proposed to be present at regions 6q21, 6q23, and 6q25-q27.²¹ Recently, a region in distal 6q deleted in many tumor types, including non-Hodgkin's lymphoma, has also been mapped and cloned.²² Together with these reports, our findings suggest that there may be critical tumor suppressor gene(s) present in this region of 6q.

Loss of DNA at 13q was observed in three cases (cases 1, 4, and 6). The regions involved band 13q13 to 13q34. The minimal commonly deleted region was located at 13q14 to 13q21. Karyotyping showed a corresponding aberration del13(q13q22) in one case (case 6) but was normal in another one (case 4). Again, our results confirmed previous cytogenetic reports of del(13q) observed in NK cell lymphoma/leukemia.¹⁸ Del (13q) has also been reported in B-cell chronic lymphocytic leukemia,²³ mantle cell lymphoma,²³ and acute lymphoblastic leukemia.²⁴ In this chromosomal region, in addition to the retinoblastoma (*Rb*) gene, other tumor suppressor genes have also been proposed to be present.²³⁻²⁵ In one of our cases showing DNA loss at 13q14-q21 (case 6), we have used dual-color fluorescence *in situ* hybridization with locus-specific probes for the *Rb* gene and the region

Figure 3. (A) CGH image of hybridized chromosomes in case 1. Green regions represent gains, and red regions highlight the losses. Uninvolved regions appear yellow. (B) Reverse DAPI profile of the same metaphase.

21q22.13-q22.2 (as an internal control) to confirm deletion of the region 13q14 (Table 1).

DNA copy losses mapping to the localization of known tumor suppressor genes were also observed at regions 17p13 (*p53*) and 9p21 (*p15* and *p16*), whereas in other regions of DNA losses including 1p13, 7p15, 10p13, and 11q24, no known tumor suppressor genes have yet been identified. Therefore, among the eight cases that showed genetic aberrations, DNA losses were observed in all but one cases, with many showing multiple regions of deletion. These findings indicate that dysregulation or deletions of putative tumor suppressor genes, possibly at multiple loci, might be an important pathogenetic step in NK cell transformation.

Compared with DNA losses, gains of DNA were more frequent and involved a large number of regions in the genome. DNA gains were commonly found in chromosomes 1p, 6p, 11q, 12q, 14q, 17q, 19p, 20q, and Xp. Interestingly, our result showed that DNA gains were more frequently observed in nasal/nasal-type NK cell lymphoma (cases 1, 4, and 5) than in aggressive NK cell lymphoma/leukemia (cases 6, 7, 9, and 10) (Table 1). This is illustrated in the nasopharyngeal biopsy of case 4,

where DNA amplifications were mapped to 14 different genomic regions. On the other hand, DNA gains in Xp were more commonly observed in aggressive NK cell lymphoma/leukemia than in nasal/nasal-type NK cell lymphoma. DNA gains might represent amplifications of putative oncogenes. Therefore, the chromosomal regions gained in our cases, particularly Xp, which was also seen in conventional karyotyping as reported by us and other groups,¹⁷⁻¹⁹ may be further targets for the identification of putative proto-oncogenes.

In two cases (cases 1 and 4), materials other than the primary tumor were investigated. In case 4, where both nasal biopsy and the involved marrow were studied, some of the genetic changes seen in the involved marrow were absent from the nasopharyngeal biopsy (Table 1). The most likely explanation is clonal evolution. However, both samples showed deletion at 13q and amplification at chromosomes 6p, 14q, 17q, and 20q, indicating close clonal relationships between these lesions. The normal karyotype seen in the involved marrow probably represented mitoses of nonmalignant cells. Extensive genetic alterations were noted in the ovarian relapse in case 1 (Figure 3), which occurred 2 years after a primary nasal NK cell lymphoma. These genetic alterations may be related to the tumor progression and the associated genomic instability. It is interesting to note that part of the genetic alterations defined by CGH in this case, ie, +7q and deletion at 11q and 13q, were also observed cytogenetically in one case of nasal NK cell lymphoma with CNS involvement.¹⁷

Our data represented novel findings of genetic alterations in several rare types of NK cell malignancies. Cheng et al²⁶ have reported, with the use of degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) on microdissected formalin-fixed paraffin-embedded tumor tissues, CGH results of 18 cases of nasal NK/T cell lymphoma. They found DNA losses in 17p, 1pter, 16p, 9qter, and 19p. Besides DNA loss at 17p, these changes were not observed in our series, or in conventional karyotypic studies of NK cell lymphomas.¹⁷⁻¹⁹ As these regions also involved the well-recognized problematic areas that require cautious interpretation in CGH,^{12,27} as well as the fact that DOP-PCR might not give a true representation of genomic imbalances, their results might have to be interpreted with caution. In our study, CGH was performed on frozen tumor samples. At the same time, we have included inverse labeling CGH²⁸ to further improve accuracy. This would help to correct for minor fluctuations of the green-to-red fluorescence ratio, making interpretation of genetic changes with borderline amplitude much more reliable.²⁹

Although CGH is a powerful tool in the investigation of DNA copy number changes, it has a number of limitations. Gain of DNA (amplicon size times level of amplification) has to be at least 2 Mb for it to become detectable by CGH. The smallest size of deletions detectable is expected to be around 3-5 Mb.¹² Thus, low-level DNA amplification or small deleted regions may be missed. CGH is also not capable of detecting balanced chromosomal translocations, when there is no change in the relative DNA copy number. Thus, CGH might have failed

to detect genetic aberrations in cases 3 and 8 in our series.

In conclusion, our results showed a consistent pattern of DNA gains or losses in NK cell lymphoma/leukemia, with a strong correlation between CGH results and those of conventional karyotyping, particularly del(6q), del(13q), del(17p), and partial gain of Xp. It also showed that chromosomal aberrations are very frequent in NK cell lymphoma/leukemia. Further molecular studies in these regions may help to identify putative tumor suppressor genes or proto-oncogenes that are of pathogenetic importance in this group of rare but clinically aggressive tumor. Finally, these genetic alterations may contribute to defining the positions of NK cell malignancies in current or future lymphoma classification schemes, which are increasingly focusing on phenotypic and molecular features for definitive identification of lymphoma subtypes.

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