Short Communication

Specific Patterns of Gene Methylation in Natural Killer Cell Lymphomas

p73 Is Consistently Involved

Lisa Lai Ping Siu,^{*†} John Kwok Cheung Chan,[†]
Kit-Fai Wong,[†] and Yok-Lam Kwong^{*}

From the Department of Medicine,^{*} Queen Mary Hospital, Hong Kong, and Department of Pathology,[†] Queen Elizabeth Hospital, Hong Kong, People's Republic Of China

Aberrant methylation of promoter CpG regions is a putative mechanism whereby tumor suppressor genes are inactivated. We used a candidate gene approach to investigate the patterns and significance of this epigenetic change in natural killer (NK) cell malignancies. Thirty-three patients were studied for promoter methylation in five putative tumor suppressor genes by methylation-specific polymerase chain reaction (MSP), which has a sensitivity of 10^{-3} . The *p73* gene was methylated in 94% of cases, a frequency that is the highest known for any human malignancy. In the NK cell lymphoma line NK92, *p73* was also completely methylated, and the *p73* transcript was correspondingly not detectable by quantitative polymerase chain reaction. Treatment of the cell line with 5-azacytidine, a demethylation reagent, led to demethylation of the *p73* promoter and reinduction of *p73* gene expression. These results suggested that promoter CpG methylation might be an important mechanism in suppressing *p73* gene expression in NK cells. Other methylated genes included *bMLH1* (63%), *p16* (63%), *p15* (48%), and *RAR β* (47%). Methylation of two or more genes occurred in 88% of cases. With promoter methylation as a molecular marker, MSP identified two cases of occult marrow metastasis. Interestingly, the primary tumor and metastasis showed different methylation patterns, implying that separate clonal evolutions might have occurred at these sites. Furthermore, MSP also identified tumor infiltration in random oropharyngeal biopsies in a case where histological examination could not show evidence of tumor involvement. We conclude that NK cell malignancies show a specific pattern of promoter methylation, with *p73* being consistently involved.

These results suggest that *p73* may be an important target in the neoplastic transformation of NK cells, and the demonstration of its methylation may serve as a potential molecular tool for NK cell lymphoma detection. (*Am J Pathol* 2002, 160:59–66)

Natural killer (NK) cell lymphomas are a group of highly aggressive malignancies,^{1–3} characterized by a distinctive immunophenotype of CD2⁺, cytoplasmic CD3 ϵ ⁺, surface CD3⁺, CD56⁺, and T-cell receptor⁺. There is a near consistent association with Epstein-Barr virus (EBV) infection of the tumor cells. Clinically, NK cell lymphomas can be classified according to the initial sites of involvement. The majority of the tumors initially involve the nasal and nasopharyngeal areas, and are referred to as nasal NK cell lymphoma. A minority of tumors initially involve the non-nasal areas, including the skin and gastrointestinal tract, and are referred to as non-nasal NK cell lymphoma. Pathologically, nasal and non-nasal NK cell lymphomas have similar histological features, and are categorized as extranodal NK/T cell lymphoma, nasal type, under the World Health Organization (WHO) classification for lymphomas.⁴ Rarely, the lymphoma may present with multi-organ involvement and a leukemic phase, and is referred to as aggressive NK cell lymphoma/leukemia.^{1–4} These tumors are very rare diseases but show an interesting geographic predilection. They are reported mostly from Asia, Mexico, and South America, but are extremely rare in Western countries.⁵

Few genetic alterations are known in NK cell lymphomas. Recurrent chromosomal translocations have not been identified so far.⁶ Previous studies using comparative genomic hybridization⁷ and loss of heterozygosity analysis⁸ showed that deletions of chromosomes 6q,

Supported by the Kadoorie Charitable Foundation.

Accepted for publication October 12, 2001.

Address reprint requests to Dr. Y.L. Kwong, University Department of Medicine, Professorial Block, Queen Mary Hospital, Pokfulam Road, Hong Kong, PRC. E-mail: ylk Wong@hkucc.hku.hk.

Table 1. Primer Sequences Used for MSP Analysis

Primer name	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
<i>p73</i> M _F	GGA CGT AGC GAA ATC GGG GTT C	60	68
<i>p73</i> M _R	ACC CCG AAC ATC GAC GTC CG		
<i>p73</i> U _F	AGG GGA TGT AGT GAA ATT GGG GTT T	69	59
<i>p73</i> U _R	ATC ACA ACC CCA AAC ATC AAC ATC CA		
<i>hMLH1</i> M _F	TTA ATA GGA AGA GCG GAT AGC	106	57
<i>hMLH1</i> M _R	CTA TAA ATT ACT AAA TCT CTT CG		
<i>hMLH1</i> U _F	TTA ATA GGA AGA GTG GAT AGT G	107	54
<i>hMLH1</i> U _R	TCT ATA AAT TAC TAA ATC TCT TCA		
<i>p16</i> M _F	TTA TTA GAG GGT GGG GCG GAT CGC	150	67
<i>p16</i> M _R	GAC CCC GAA CCG CGA CCG TAA		
<i>p16</i> U _F	TTA TTA GAG GGT GGG GTG GAT TGT	151	61
<i>p16</i> U _R	CAA CCC CAA ACC ACA ACC ATA A		
<i>p15</i> M _F	GCG TTC GTA TTT TGC GGT T	148	65
<i>p15</i> M _R	CGT ACA ATA ACC GAA CGA CCG A		
<i>p15</i> U _F	TGT GAT GTG TTT GTA TTT TGT GGT T	154	61
<i>p15</i> U _R	CCA TAC AAT AAC CAA ACA ACC AA		
<i>RARβ</i> M _F	GGA TTG GGA TGT CGA GAA C	93	68
<i>RARβ</i> M _R	TAC AAA AAA CCT TCC GAA TAC G		
<i>RARβ</i> U _F	AGG ATT GGG ATG TTG AGA ATG	95	55
<i>RARβ</i> U _R	TTA CAA AAA ACC TTC CAA ATA CA		

11q, 13q, and 17p might be important in the initiation and progression of this tumor. Although these genetic alterations may give useful information on lymphomagenesis, the techniques for their recognition cannot be used for the sensitive detection of the tumor cells. Therefore, the definition of minimal tumor involvement/infiltration of organs and the monitoring of treatment by molecular techniques has not been possible. Recently, epigenetic silencing of gene expression by CpG island hypermethylation has been shown to be important in cancer formation.⁹ Experimental data from the study of numerous tumors have shown that when these CpG islands are methylated, the expression of the corresponding genes is suppressed. Therefore, DNA methylation may be an alternative mechanism to mutations or deletions in disrupting tumor suppressor gene function in carcinogenesis.

In this study, we investigated the promoter methylation of five putative tumor suppressor genes in a series of NK cell lymphomas, with a view to defining the patterns of methylation, as well as exploring the use of aberrantly methylated genes as molecular markers for tumor detection.

Materials and Methods

Patients

A total of 33 (22 nasal, 6 non-nasal, 5 aggressive) cases of NK cell lymphomas were investigated. All tumors were CD2⁺, CD3⁺, CD3e⁺ and CD56⁺, with EBV demonstrable by *in situ* hybridization in all but one case (case 27). Two NK cell lymphoma lines, HANK1 and NK92, were also studied.

Bisulfite Conversion of DNA Samples

DNA was extracted from frozen tumor tissues with standard phenol-chloroform protocols. For archival paraffin sections, the QIAamp DNA mini kit (Qiagen, Hilden, Ger-

many) was used for DNA extraction. DNA was modified by the bisulfite reaction using the CpGenome DNA Modification Kit (Intergen, Purchase, NY). After completion of the reaction, all unmethylated cytosines are deaminated and converted to uracil, while methylated cytosines remain unchanged. Thus, methylated and unmethylated genomic regions following bisulfite conversion could be distinguished by sequence-specific polymerase chain reaction (PCR) primers.¹⁰

Methylation-Specific Polymerase Chain Reaction

The methylation status of the promoter region of the genes studied was determined by methylation specific polymerase chain reaction (MSP) using bisulfite modified DNA.¹⁰ Primer sequences for the methylated and unmethylated alleles were as published for *p15*, *p16*,¹⁰ *p73*,¹¹ *hMLH1*, and *RARβ*.¹² The forward and reverse primer sequences for the methylated (M_F and M_R) and the unmethylated alleles (U_F and U_R) were shown in Table 1. MSP was performed in a final volume of 50 μ l containing 5 μ l bisulfite modified DNA, 250 μ mol/L dNTP (Life Technologies, Gaithersburg, MD), 1 μ mol/L of each primer (Genosys, Cambridgeshire, UK), 1.5 to 3 mmol/L MgCl₂, 1 $\times$ PCR gold buffer, and 2U AmpliTaq Gold (PE Biosystems, Foster City, CA), in an MJ PTC-200 thermocycler (MJ Research, Waltham, MA) with the following cycling parameters: 95°C for 12 minutes, 32 to 40 cycles of 94°C for 1 minute, specific annealing temperature for 1 minute, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. PCR products were separated on 7.5% nondenaturing polyacrylamide gel and visualized by ethidium bromide staining. Normal and methylated DNA (Intergen) were used to optimize the MSP conditions, and included as normal and positive controls in every experiment.

Table 2. Clinicopathological Features and Promoter Methylation in 33 Cases of NK Cell Malignancy

Case	Sex/age	Type	Tumor sample	Promoter methylation					Remarks
				<i>p73</i>	<i>hMLH1</i>	<i>p16</i>	<i>p15</i>	<i>RARβ</i>	
1	M/32	Nasal	Primary	+	N	+	N	+	No marrow infiltration; died of systemic dissemination; MSI – ve
2	M/46	Nasal	Marrow Primary	+	N	N	N	N	Occasional marrow cells EBER + ve, died of systemic dissemination
3	M/45	Nasal	Marrow Primary	+	N	+	+	+	No marrow infiltration, treatment failure due to local relapse, MSI – ve
4	F/45	Nasal (relapsed)	Marrow	N	N	N	N	N	MSI – ve
5	M/57	Nasal (relapsed)	Ovarian relapse	+	+	+	+	+	
6	F/50	Nasal	Cheek relapse	+	+	+	+	+	MSI – ve
7	M/60	Nasal	Primary	+	+	+	+	+	
8	M/41	Nasal	Primary (A)	+	N	+	N	+	MSI – ve
9	M/46	Nasal	Primary (A)	+	+	+	+	+	
10	M/66	Nasal	Primary (A)	+	+	+	N	N	
11	M/60	Nasal	Primary (A)	+	+	+	+	+	
12	M/36	Nasal	Primary (A)	+	+	N	N	N	
13	F/63	Nasal	Primary (A)	N	+	N	N	N	
14	M/46	Nasal	Primary (A)	+	+	N	F	N	
15	M/50	Nasal	Primary (A)	+	+	N	N	N	
16	M/71	Nasal	Primary (A)	+	N	N	F	N	
17	M/68	Nasal	Primary (A)	+	+	+	N	N	
18	M/56	Nasal	Primary (A)	+	+	N	F*	N*	
19	M/64	Nasal	Primary (A)	+	+	N	+	N*	
20	M/81	Nasal	Primary (A)	+	+	+	N*	N*	
21	M/78	Nasal	Primary (A)	+	+	N	F*	N*	
22	M/53	Nasal	Primary (A)	+	+	+	F*	N*	
23	M/77	Nonnasal	Buttock	+	N	+	+	+	MSI – ve
24	F/87	Nonnasal	Skin primary	+	+	+	+	+	
25	M/74	Nonnasal (relapsed)	Skin relapse	+	N	+	N	+	MSI – ve
26	F/11	Nonnasal	Lymph node	+	N	+	+	+	EBV – ve, MSI – ve
27	F/87	Nonnasal	Small intestine (A)	N	+	N	F	N	
28	M/26	Nonnasal (relapsed)	Pharynx (A)	N	ND	ND	ND	ND	No definite tumor cells localized in the biopsied samples
			Tongue base (A)	+	ND	ND	ND	ND	Relapse ultimately occurred in the Waldeyer's ring
			Oropharynx (A)	+	ND	ND	ND	ND	No leukemic phase
			Ventricular cord (A)	N	ND	ND	ND	ND	
			Tonsil (A)	+	ND	ND	ND	ND	
			Postcricoid (A)	N	ND	ND	ND	ND	
29	M/37	Aggressive	Marrow	+	N	+	N	+	
30	M/71	Aggressive	Marrow	+	+	+	+	+	
31	F/55	Aggressive	Marrow	+	N	+	N	+	
32	M/44	Aggressive	Marrow	+	N	+	+	+	
33	F/34	Aggressive	Marrow	+	N	N	N	N	
			Peripheral blood	N	N	N	N	N	
		Cell line, nonnasal	HANK1	+	N	+	N	+	
		Cell line, aggressive	NK92	+	N	+	+	N	
		Normal control	Blood	N	N	N	N	N	
		Normal control	Tonsil (A)	N	N	N	N	N	
		Normal control	Appendix (A)	N	N	N	N	N	
		Normal control	Appendix (A)	N	N	N	N	N	

A, archival paraffin specimen; +, methylated; N, not methylated; F, failed reaction; MSI, microsatellite instability; +ve, positive; –ve, negative; *, done once only; ND, not done because of insufficient materials.

Specificity and Sensitivity of MSP

MSP was performed in duplicate, except in cases with insufficient DNA (Table 2). As controls, amplifications for the unmethylated/methylated alleles on bisulfite-modified

normal/methylated DNA, and no template control were performed. To test the sensitivity of the MSP, the NK92 DNA showing complete methylation at the *p73* locus was serially diluted in normal DNA, bisulfite treated, and amplified with primers for the methylated allele.

Table 3. Primer and TaqMan Probe Sequences for *p73* Gene Expression

Primer/probe	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
<i>p73</i> Q _F	TGG AGA CGA GGA CAC GTA CTA CC	70	60
<i>p73</i> Q _R	TTT CAG CTT CAT CAG GAT CTC AAA		
<i>p73</i> Probe	6-Fam-TTC TCC CGG CCT CGC ACC TGA-Tamra	226	60
<i>GAPDH</i> Q _F	GAA GGT GAA GGT CGG AGT C		
<i>GAPDH</i> Q _R	GAA GAT GGT GATGGG ATT TC		
<i>GAPDH</i> probe	6-Fam-CAA GCT TCC CGT TCT CAG CC-Tamra		

Sequencing Analysis

PCR products were electrophoresed in 2% agarose gel. The specific band was excised and purified by the Qiaex II gel extraction kit (Qiagen). PCR products were sequenced directly in both directions with the same primers used for MSP. Each sequencing reaction contained 5 μ l of purified DNA, 3.2 pmol of primer, 4 μ l of sequencing mix (dRhodamine Terminator Cycle Sequencing Ready Reaction Kit, PE Biosystems) in a final volume of 10 μ l, and was performed according to the manufacturer's instructions. Sequencing products were purified by the DyeEx Spin Kit (Qiagen) and analyzed by an automated DNA sequencer (ABI Prism 377, PE Biosystems).

5-Azacytidine Treatment of NK92 Cell Line

The NK92 lymphoma cell line was cultured in RPMI 1640 medium with 10% fetal bovine serum (Life Technologies) supplemented with interleukin-2 (IL-2) (300 U/ml). The demethylation agent 5-azacytidine (5-AC) (Sigma, St. Louis, MO) was used to reinduce the expression of methylated genes. NK92 cell lines were cultured in the absence and presence of 5-AC at 1 μ mol/L and 3 μ mol/L for 3 to 9 days.

Quantification of *p73* Gene Expression

Total cellular mRNA was extracted from the cultured NK92 cell pellets with the QIAamp RNeasy kit (Qiagen). The expression of the *p73* gene was quantified by a real-time quantitative PCR (Q-PCR) technique with an automated DNA sequence detector (ABI Prism 7700 Sequence Detector, PE Biosystems). The primer sequences for the *p73* gene (*p73* Q_F and *p73* Q_R), and the TaqMan probe (*p73* probe) dual-labeled at the 5' end with 6-carboxyfluorescein (FAM) and the 3' end with 6-carboxytetramethylrhodamine (TAMRA), were designed by the Primer Express software (PE Biosystems), to span at least one intron to prevent amplification of contaminating DNA (Table 3). The gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was similarly amplified as an internal control for RNA amount and integrity (Table 3). First strand cDNA synthesis and the subsequent Q-PCR were performed by the Taqman EZ RT-PCR kit (PE Biosystems). Real-time PCR amplification data were collected continuously and analyzed with the Sequence Detection System (ABI Prism 7700 Sequence Detector, PE Biosystems). The threshold cycle (C_T) at which a significant increase in fluorescence signal was first detected was set

at a minimum of 10 standard deviations above the mean baseline fluorescence. The copy number of target sequences in the tested samples was inversely proportional to and hence could be deduced from the C_T .

Results

Specificity and Sensitivity of MSP

The specificity of MSP was shown by the inclusion of appropriate positive and negative controls (Figure 1). DNA methylation was only detected in methylated DNA but not in normal DNA samples. Sequencing of the PCR products also confirmed the expected patterns of bisulfite-induced changes (Figure 1). When performed in duplicate, all of the patient samples gave concordant results. Finally, the MSP had a sensitivity of 10^{-3} (Figure 1).

Frequency of Promoter CpG Methylation

Of the five genes examined, *p73* was the most frequently methylated, occurring in 31 of 33 (94%) of cases (Table 2). Both NK cell lymphoma lines, HANK1 and NK92, showed methylation of the *p73* gene. The two other more commonly methylated genes were the DNA mismatch repair gene *hMLH1* (20 of 32, 63%) and the cyclin-dependent kinase gene inhibitor *p16* (20 of 32, 63%). *p15* and *RAR β* were less frequently methylated (12 of 25, 48% and 15 of 32, 47% respectively).

Reinduction of *p73* Expression with Promoter Demethylation

The effect of promoter methylation on gene expression was tested for *p73*, since it was the most frequently methylated gene and therefore an important candidate in NK cell lymphomagenesis. The NK92 cell line was fully methylated at the *p73* gene, as MSP did not show any PCR product with the unmethylated primers (Figure 2 A). Accordingly, no *p73* transcript could be detected with Q-PCR (Figure 2, B and C). Treatment of the cell line with 5-AC for 1 to 3 days resulted in demethylation of the *p73* gene, as shown by appearance of positive amplification with the unmethylated primers (Figure 2A). There was also reinduction of *p73* gene expression as shown by Q-PCR (Figure 2, B and C), confirming the functional importance of methylation in epigenetic silencing of the *p73* gene expression in NK lymphoma cells. Furthermore, the growth rate of the NK92 cell line was retarded after

Figure 1. MSP results of NK cell malignancies. **A:** Sequencing of PCR product obtained with primers for the methylated allele of *p73*. The 3' end of the product is shown, with the sequence of the wild-type *p73* aligned for comparison. In the MSP product, methylated cytosine residues remaining unchanged are underlined in blue, and the unmethylated cytosine residues that have become uracil/thymidine are underlined in red. **B:** Sensitivity of MSP, showing detection of the methylated *p73* at 10⁻³. **Lane MW:** molecular weight marker; **lanes 1 to 6:** serial dilution of bisulfite-modified DNA of NK92 cell line from 10⁰ to 10⁻⁵; **N:** bisulfite-modified normal DNA; **W:** water control. **C:** MSP of *p73*. PCR product in **lane U** (unmethylated) indicates the presence of the unmethylated allele, and in **lane M** (methylated) the methylated allele. MW: molecular weight marker; W: water control; N: normal control DNA; P: methylated control DNA. Case number is indicated at the top. *p73* methylation was found in cases 33 and 2, but not in case 13. U, 69 base pairs (bp); M, 60 bp. **D:** MSP of *bMLH1*, showing gene methylation in cases 12 and 20, but not in case 2. U, 107 bp; M, 106 bp. **E:** MSP of *p16*, showing gene methylation in cases 25 and 3, but not in case 33. U, 151 bp; M, 150 bp. **F:** MSP of *p15*, showing gene methylation in cases 32 and 4, but not in case 33. U, 154 bp; M, 148 bp. **G:** MSP of *RARβ*, showing gene methylation in cases 2 and 31, but not in case 12. U, 95 bp; M, 93 bp.

Figure 2. Reinduction of *p73* gene expression in NK92 cell line by 5-AC at 1 μmol/L and 3 μmol/L. **A:** MSP of *p73* on the NK92 cell line before and after 5-AC treatment. With the untreated cell line (day 0), PCR product was obtained only from the methylated primers (lane M) but not from the unmethylated primers (lane U). This indicates that the NK92 cell line is fully methylated at the *p73* gene. Treatment of the cell line with 5-AC for 1 to 3 days resulted in de-methylation of the *p73* gene. Consequently, PCR product was obtained from the unmethylated primers (lane U) as well. Amplification with the methylated primers (lane M) was weak, indicating that the *p73* gene was de-methylated in the majority of the cells. MW: molecular weight marker. **B:** Amplification plot of real-time Q-PCR on *p73* gene expression using the NK92 cell line before and after 5-AC treatment at 1 μmol/L. The y axis represents the change in Rn, which is a ratio of the fluorescence signal of the reporter dye (FAM) to that of the passive reference dye (ROX). The x axis is the threshold cycle (C_T). The copy number of the target sequences in the tested sample is inversely proportional to the C_T value. The *p73* gene expression in the untreated cell line was undetectable (below the baseline). On addition of 1 μmol/L 5-AC, the C_T values were 28.5, 27.5, and 26.5 for day 3, day 6, and day 9, respectively. This shows that 5-AC reinduces the expression of *p73*. The amplification profile of *GAPDH*, however, was similar before and after 5-AC treatment at around C_T 17. **C:** Amplification plot of real-time Q-PCR on *p73* gene expression using the NK92 cell line before and after 5-AC treatment at 3 μmol/L. The *p73* gene expression in the untreated cell line was undetectable. On addition of 3 μmol/L 5-AC, the C_T values were 27, 25.5, and 26.5 for days 3, 6, and 9, respectively.

reinduction of *p73* expression with 5-AC (data not shown).

Patterns of Methylation and Detection of Occult Disease

There was no discernable difference in gene methylation in the different subtypes of NK cell malignancies. However, tumors at different sites were available for the analysis of methylation patterns in five cases. A differential

pattern of gene methylation was found in the primary and metastatic lesions in two cases (cases 1 and 2). In both cases, the marrow was morphologically normal, but occasional EBV positive cells were found in case 2. MSP showed aberrant gene methylation in both cases, suggesting occult infiltration by lymphoma cells; which in fact became clinically manifest later in the course of disease (Table 2). In both cases, *p73* was methylated in the primary tumor and marrow. On the other hand, *p16* and *RARβ* were methylated only in the primary tumor but not in the marrow in case 1, and *RARβ* methylated only in the primary tumor and *p16* in the marrow in case 2. In another two cases (cases 3 and 33), the marrow and peripheral blood were negative by MSP. Case 3 never developed any disease outside the nose, and case 33 did not show involvement of the peripheral blood at any stage of the illness. In the last case (case 28), the patient was referred for treatment of a relapse at the oropharyngeal region. After chemotherapy and radiotherapy, morphological examination in a series of random biopsies could not definitely show the presence of tumor, due to the extensive necrosis in the small biopsies. However, *p73* methylation could be detected unambiguously in the tonsil, oropharynx, and the tongue base, suggesting persistent disease. The lymphoma ultimately relapsed in the Waldeyer's ring, at sites where *p73* methylation was detected.

Discussion

Genomic abnormalities in NK cell malignancies have remained largely unknown, owing to the rarity of the disease. To define the genetic aberrations in NK cell lymphomas, we have used a candidate gene approach¹³ to investigate a set of putative tumor suppressive genes that may undergo epigenetic inactivation via promoter CpG methylation. Such an approach has demonstrated that characteristic profiles of gene hypermethylation exist for different malignancies, in which some genes are shared while others might be cancer type-specific.¹³

In this study, we have shown that *p73* is methylated in over 90% of NK cell lymphomas. This frequency is the highest known for any human malignancy.¹³ The *p73* protein shares substantial homology and functional similarity with *p53*,¹⁴ both being involved in gene transactivation and induction of apoptosis or cell cycle arrest.¹⁵ It has been shown that while E2F1 activates *p53* indirectly via *CDKN2A*, *p73* can be regulated directly by E2F1 through the recognition and transactivation of the *p73* promoter, leading to activation of *p53*-responsive target genes and apoptosis. It has therefore been postulated that *p73* is a candidate tumor suppressor gene.¹⁴ Interestingly, mutation or structural alterations of the *p73* gene locus are rare in hematological malignancies.¹⁶ Limited data have shown, however, that promoter methylation may be a mechanism of inactivation of *p73* gene function. In one study, *p73* methylation was reported in approximately 30% of acute lymphoblastic leukemia and Burkitt's lymphoma.¹¹ In two other studies where lymphoid leukemia or lymphoma cell lines instead of primary tumor materials were examined, *p73* methylation was found in

16% to 32% of cell lines investigated.^{16,17} Hence, although extensive studies have not been performed, existing data indicate that *p73* methylation is found only in about one third of lymphoid malignancies, in contrast to our findings of *p73* methylation in almost all of NK cell malignancies. Furthermore, we have shown that in the NK lymphoma cell line NK92, complete *p73* methylation led to abolition of gene expression, and demethylation of *p73* resulted in reinduction of gene expression. Therefore, promoter methylation may represent a critical mechanism for the inactivation of *p73* in NK cells. The very high frequency of *p73* methylation found in NK cell lymphomas strongly suggests that it may be a major target gene in NK cell transformation.

The cyclin-dependent kinase gene inhibitors *p16* and *p15* were methylated in 63% and 48% of cases, which was in close concordance with those observed in lymphomas¹³ and other hematological malignancies.¹⁸ The DNA mismatch repair gene *hMLH1* was methylated in 63% of cases. This frequency is much higher than the 6% reported in leukemia,¹³ although data for lymphoma are lacking. In solid tumors, methylation of *hMLH1* is associated with loss of gene expression and microsatellite instability (MSI).¹⁹ However, MSI is rare in hematological malignancies, and, accordingly, methylations or mutations of *hMLH1* are rarely found.²⁰ Furthermore, nine of the 32 cases included in this study have been investigated in a previous study of loss of heterozygosity with microsatellite markers,⁸ and MSI was not observed (Table 2). Further investigations will therefore be required to elucidate the functional significance of *hMLH1* methylation in NK cell lymphomas. The *RAR β* gene is important in the growth regulation of epithelial cells, and its aberrant methylation has been observed in cancers of the breast and lung.^{21,22} In this study, *RAR β* gene methylation was found in nearly half of the cases, implying that dysregulation of *RAR β* function may also be important in NK cell transformation.

Whether promoter hypermethylation initiates or is merely a reflection of gene inactivation²³ remains debatable. This controversy notwithstanding, CpG methylation may still be a sensitive molecular marker independent of its functional implications.²⁴ To test this potential application, we examined the marrow (cases 1 to 3) and peripheral blood (case 33) by MSP in some patients. Aberrant gene methylation correctly detected/ruled out occult involvement in these cases. Furthermore, an interesting observation was that gene methylation appeared to be different between the primary lesion and the marrow in cases 1 and 2. *p73* was methylated in the primary tumor and marrow in both cases. However, *p16* and *RAR β* were methylated only in the primary tumor but not in the marrow in case 1, and *RAR β* methylated only in the primary tumor and *p16* in the marrow in case 2. This difference could be a reflection of progressive methylation of different genes in a multi-step carcinogenic process,²⁵ with separate clonal evolutions at different sites from a common tumor progenitor cell. That *p73* methylation was commonly found in the primary/metastatic site suggested that this might be an early step in NK cell transformation. In case 28, morphological examination of

biopsies at different sites failed to definitively localize tumor cells. This is a common problem in the diagnosis of NK cell lymphoma, where zonal necrosis in the tumor sometimes makes morphological examination difficult even for the experienced pathologist. Owing to the small size of the biopsies, we had only performed MSP for the *p73* gene. *p73* methylation could be detected in the tongue base, oropharynx, and tonsil, which were subsequently the sites of disease recurrence. The results showed that gene promoter methylation could be a sensitive tool for the detection of residual tumor in NK cell malignancies, and may therefore be a useful ancillary tumor marker for the definition of minimal tumor involvement before and after treatment.

In conclusion, aberrant gene methylation occurs commonly and with a specific pattern in NK cell malignancies. Methylation of *p73* is nearly always observed, implying that functional perturbation of *p73* may be important in malignant NK cell transformation, and that it may serve as a useful molecular marker for disease detection.

Acknowledgments

We thank Dr. Y. Kagami (Aichi Cancer Center Hospital, Nagoya, Japan) for providing the HANK1 cell line and valuable advice, and Eunice Chan and Alfa Bai for technical assistance.

References

1. Chan JK, Sin VC, Wong KF, Ng CS, Tsang WY, Chan CH, Cheung MM, Lau WH: Non-nasal lymphoma expressing the natural killer cell marker CD56: a clinicopathologic study of 49 cases of an uncommon aggressive neoplasm. *Blood* 1997, 89:4501-4513
2. Kwong YL, Chan ACL, Liang R, Chiang AKS, Chim CS, Chan TK: CD56+ NK lymphomas: clinicopathologic features and prognosis. *Br J Haematol* 1997, 97:821-829
3. Cheung MMC, Chan JKC, Lau WH, Foo W, Chan PT, Ng CS, Ngan RKC: Primary non-Hodgkin's lymphoma of the nose and nasopharynx: clinical features, tumor immunophenotype, and treatment outcome in 113 patients. *J Clin Oncol* 1998, 16:70-77
4. Jaffe ES, Harris NL, Stein H, Vardiman JW (eds): World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. IARC Press, Lyon, France, 2001, pp 189-230
5. Chan JKC: Natural killer cell neoplasms. *ASCP Rev Pathol (Anat Pathol)* 1998, 3:77-145
6. Wong KF, Chan JKC, Kwong YL: Identification of del(6)(q21q25) as a recurring chromosomal abnormality of putative NK cell lymphoma/leukaemia. *Br J Haematol* 1997, 98:922-926
7. Siu LLP, Wong KF, Chan JKC, Kwong YL: Comparative genomic hybridization analysis of natural killer cell lymphoma/leukemia: recognition of consistent patterns of genetic alteration. *Am J Pathol* 1999, 155:1419-1425
8. Siu LLP, Chan V, Chan JKC, Wong KF, Liang R, Kwong YL: Consistent patterns of allelic loss in natural killer cell lymphoma. *Am J Pathol* 2000, 157:1803-1809
9. Baylin SB, Herman JG: DNA hypermethylation in tumorigenesis. *Trends Genet* 2000, 16:168-174
10. Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996, 93:9821-9826
11. Corn PG, Kuerbitz SJ, van Noesel MM, Esteller M, Compitello N, Baylin SB, Herman JG: Transcriptional silencing of the *p73* gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5' CpG island methylation. *Cancer Res* 1999, 59:3352-3356

12. Ueki T, Toyota M, Sohn T, Yeo CJ, Issa JPJ, Hruban RH, Goggins M: Hypermethylation of multiple genes in pancreatic adenocarcinoma. *Cancer Res* 2000, 60:1835–1839
13. Esteller M, Corn PG, Baylin SB, Herman JG: A gene hypermethylation profile of human cancer. *Cancer Res* 2001, 61:3225–3229
14. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, Mckeon F, Caput D: Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* 1997, 90:809–819
15. Soengas MS, Lowe SW: p53 and p73: seeing double? *Nat Genet* 2000, 26:391–392
16. Kawano S, Miller CW, Gombart AF, Bartram CR, Matsuo Y, Asou H, Sakashita A, Said J, Tatsumi E, Koeffler HP: Loss of p73 gene expression in leukemias/lymphomas due to hypermethylation. *Blood* 1999, 94:1113–1120
17. Liu M, Taketani T, Li R, Takita J, Taki T, Yang HW, Kawaguchi H, Ida K, Matsuo Y, Hayashi Y: Loss of p73 gene expression in lymphoid leukemia cell lines is associated with hypermethylation. *Leuk Res* 2001, 25:441–447
18. Baur AS, Shaw P, Burri N, Delacrétaz F, Bosman FT, Chaubert P: Frequent methylation silencing of *p15^{INK4b}* (*MTS2*) and *p16^{INK4a}* (*MTS1*) in B-cell and T-cell lymphomas. *Blood* 1999, 94:1773–1781
19. Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JPJ, Markowitz S, Willson JKV, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, Baylin SB: Incidence and functional consequences of *hMLH1* promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA* 1998, 95:6870–6875
20. Auner HW, Olipitz W, Hoeffler G, Bodner C, Konrad D, Crevenna R, Linkesch W, Sill H: Mutational analysis of the DNA mismatch repair gene *hMLH1* in myeloid leukaemias. *Br J Haematol* 1999, 106:706–708
21. Widschwendter M, Berger J, Hermann M, Muller HM, Amberger A, Zeschnick M, Widschwendter A, Abendstein B, Zeimet AG, Daxenbichler G, Marth C: Methylation and silencing of the retinoic acid receptor- $\beta 2$ gene in breast cancer. *J Natl Cancer Inst* 2000, 92:826–832
22. Virmani AK, Rath A, Zochbauer-Muller S, Sacchi N, Fukuyama Y, Bryant D, Maitra A, Heda S, Fong KM, Thunnissen F, Minna JD, Gazdar AF: Promoter methylation and silencing of the retinoic acid receptor- β gene in lung carcinomas. *J Natl Cancer Inst* 2000, 92:1303–1307
23. Fearon ER: BRCA1 and E-cadherin promoter hypermethylation and gene inactivation in cancer: association or mechanism? *J Natl Cancer Inst* 2000, 92:515–517
24. Baylin SB, Belinsky SA, Herman JG: Aberrant methylation of gene promoters in cancer: concepts, misconcepts, and promise. *J Natl Cancer Inst* 2000, 92:1460–1461
25. Kang GH, Shim YH, Jung HY, Kim WH, Ro JY, Rhyu MG: CpG island methylation in premalignant stages of gastric carcinoma. *Cancer Res* 2001, 61:2847–2851