

Alterations in *ATR* in nasal NK/T-cell lymphoma and chronic active Epstein–Barr virus infection

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Nasal natural killer (NK)/T-cell lymphoma (NKTCL) and chronic active Epstein–Barr virus infection (CAEBV) are relatively frequent, especially in Asia, and are poor in prognosis. Both diseases are proliferative diseases of NK/T cells that show highly complicated karyotypes, suggesting the involvement of chromosomal instability. *ATR* is an important gene for DNA damage response and chromosomal stability. To evaluate the role of *ATR* gene alterations in the pathogenesis of NKTCL and CAEBV, the whole coding region of the *ATR* gene was examined in cell lines derived from NKTCL and CAEBV, as well as tumor samples from patients. *ATR* alterations were detected in two of eight NKTCL and in one of three CAEBV lines. Most aberrant transcripts observed were deletions resulting from aberrant splicing. *ATR* alterations were also detected in four of 10 NKTCL clinical samples. Both NKTCL and CAEBV cell lines with *ATR* alterations showed a delay or abrogation in repair of ionizing radiation-induced DNA double-strand breaks and ultraviolet-induced DNA single-strand breaks, and both exhibited a defect in p53 accumulation. These findings show that alterations in the *ATR* gene result in an abnormal response to DNA double-strand break and single-strand break repair, suggesting a role for *ATR* gene alterations in NKTCL lymphomagenesis. (*Cancer Sci* 2006; 97: 605–610)

Generally, lymphoproliferative diseases of natural killer (NK)-cell lineage are rare; however, nasal NK/T-cell lymphoma (NKTCL) and chronic active EBV infection (CAEBV) are relatively frequent, especially in Asia. Both diseases are proliferative diseases of NK/T cells and are strongly associated with Epstein–Barr virus (EBV),^(1–4) and the clonal expansion of EBV-positive NK/T cells constitutes an essential part of the diseases. A recent study demonstrated the common cytological and cytogenetic features of nasal NKTCL and CAEBV cell clones, including highly complicated karyotypes with common 6q abnormalities, suggesting chromosomal instability in these cells.^(5,6) Histologically, NKTCL shows a polymorphous pattern of proliferation consisting of large lymphoid cells and normal inflammatory cells. Free radicals and oxidative stress generated in the inflammatory lesions could cause DNA damage, thus provide a basis for lymphomagenesis.

Maintenance of genome stability partly depends on the proper regulation of cellular responses to DNA damage and the integrity of DNA repair systems.⁽⁷⁾ In mammalian cells, two members of the phosphatidylinositol 3-kinase-related kinase (PI3KK) family, ataxia-telangiectasia mutated (*ATM*) kinase and Rad3-related (*ATR*) kinase, play a central role in DNA damage recognition and the initial phosphorylation

events.^(8–11) Previous studies suggested that *ATR* might participate in the signaling of ionizing radiation (IR)-induced and ultraviolet (UV)-induced DNA damage.^(8,9,12) Recently, O’Driscoll *et al.*⁽¹³⁾ reported that a aberrantly spliced transcript affecting *ATR* expression results in Seckel syndrome, an autosomal recessive disorder, and shares features with disorders showing impaired DNA-damage responses, such as Nijmegen breakage syndrome. Recent studies have shown that the *ATR* gene plays a role in the maintenance of chromosomal stability.⁽¹⁴⁾

In the present study, *ATR* gene alterations were examined in eight NKTCL and three CAEBV cell lines, as well as tumor biopsy samples obtained from NKTCL patients. Two representative cell lines from NKTCL and CAEBV with aberrant transcripts in the *ATR* gene were further analyzed to evaluate the functional consequences in DNA repair.

Materials and Methods

Cell lines

Cell lines derived from five EBV-positive NKTCL (HANK-1,⁽¹⁵⁾ NK-92,⁽¹⁶⁾ SNK-6, SNT-8⁽¹⁷⁾ and YT⁽¹⁸⁾), three EBV-negative NKTCL KHYG-1,⁽¹⁹⁾ MOTN-1,⁽²⁰⁾ MTA⁽²¹⁾), three CAEBV (AIK-T4, SIS, SKN⁽²²⁾) and one lymphoblastoid cell line (IB4) were selected for this study. NK-92 and YT were purchased from Deutsche Sammlung Von Mikroorganismen und Zellkulturen (Braunschweig, Germany). MTA was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). Other cell lines were gifts from investigators listed in the Acknowledgments. All cell lines were incubated in RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 10–20% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Patients

Tumor biopsy samples from 10 patients with nasal NKTCL who were admitted to the Osaka University Hospital and affiliated hospitals during the period 1996–2000 were analyzed with informed consent in accordance with the Declaration of Helsinki. The age of the patients at admission ranged from 36 to 74 (median 62.5) years, with a male-to-female ratio of 8:2. Histological specimens obtained by biopsy were fixed in 10% formalin and were processed routinely for paraffin embedding or snap frozen for extraction of total RNA. Histological sections cut at 4 μm were stained with hematoxylin

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and eosin and immunoperoxidase procedures. Histological slides were reviewed by two of the authors (TT, KA) for diagnosis. All tumors were classified histologically as extranodal NK/T-cell lymphoma, nasal type, according to the World Health Organization classification.

Isolation of DNA and total RNA, reverse transcription-polymerase chain reaction and detection of alterations

DNA and total RNA were extracted from the cell lines with the TRIzol reagent (Invitrogen, Rockville, MD, USA) according to the manufacturer's instructions. Total RNA (5 µg) was reverse transcribed by random hexamer priming using the Superscript first strand synthesis system (Invitrogen). Semi-nested reverse transcription-polymerase chain reaction was carried out to amplify the *ATR* transcripts using four sets of primers spanning the whole open reading frame, as described previously.⁽²³⁾ Detection of aberrant *ATR* transcripts was carried out as described previously.⁽²³⁾

Western blotting

Western blotting was carried out as described previously.⁽²³⁾ Briefly, whole cells were lysed in 1 × sample buffer, separated using 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis, and then blotted to polyvinylidene difluoride (PVDF) membrane using a wet-blotting apparatus. The antibodies used to detect p53 were DO-7 (epitope; 19–26 amino acids of p53; DakoCytomation, Glostrup, Denmark) and Pab240 (epitope; 213–217 amino acids of p53; Calbiochem, San Diego, CA, USA). Anti-actin (Sigma-Aldrich, Steinheim, Germany) was used as a control. Signals were visualized with ECLplus chemiluminiscent reagents (GE Healthcare Bio-Sciences Corp., NJ, USA).

DNA double-strand break repair assays

More than 1 × 10⁵ cells from each cell line were embedded in agarose plugs as described previously.^(23,24) The plugs covered with RPMI-1640 medium were exposed to 20 Gy of IR on ice,

and kept at 37°C for up to 6 h to allow the cells to repair damaged DNA. The plugs were embedded into wells of a 0.8% agarose gel in 1 × TAE and subjected to pulsed-field gel electrophoresis (PFGE) in a CHEF apparatus (Bio-Rad, Hercules, CA, USA) at 3 V/cm, 14°C for 48 h with a pulse time of 45 s. Under these PFGE conditions, DNA fragments between 2 and 6 Mb migrate as a single band and form compression zones under each well, whereas most rejoined double-strand breaks (DSB) are too large to enter the gel. DSB were quantified as the fraction of DNA in the compression zone relative to that in the wells using FMBIO Analysis V8.0 (TaKaRa, Kusatsu, Japan).

DNA single-strand break repair assays

The alkaline single cell gel electrophoresis (Comet assay) was carried out as described previously with some modifications.⁽²³⁾ Briefly, more than 5000 cells mixed with 50 µL of 0.5% low melting point agarose was layered onto a microscope slide coated with 80 µL of 0.65% normal agarose in phosphate-buffered saline, and then exposed to 10 J/m² of UV. Electrophoresis was carried out for 40 min at a constant current of 300 mA in electrophoretic buffer (1 mM ethylenediamine tetraacetic acid, 300 mM NaOH [pH 13]) at 4°C. Fifty cells were analyzed based on the methods for collecting comet data, in which repair of DNA single-strand break (SSB) was quantitated and defined as the DNA migration length (DML), a ratio of the comet length to its width.

Statistical analysis

Comparisons were made using the Student's *t*-test (unpaired); *P*-values less than 0.05 were taken as statistically significant.

Results

ATR alterations expressed in NK/TCL and CAEBV cell lines

Aberrant transcripts of the *ATR* gene were observed in two of five cell lines derived from EBV-positive NK/TCL, zero of three EBV-negative NK/TCL, and one of three CAEBV cell

Table 1. *ATR* alterations in nasal natural killer (NK)/T-cell lymphoma (NK/TCL) and chronic active Epstein-Barr virus infection (CAEBV) cell lines

Cell line	Patient/sample	Original disease	EBV	Nucleotide change	Predicted effect	Mutation-positive clones (%)	Reference no.
NK/TCL							
HANK-1	46F LN	Nasal-type (at diagnosis)	+	WT			15
NK-92	50 M PB	NHL with LGL cells (at diagnosis)	+	del exon 29–34 (867 bp)	In-frame	50	16
SNK-6	62 M TU	Nasal-type	+	del exon 29–34 (867 bp)	In-frame	50	17
				del 65 bp (in exon 41)	Frameshift	30	
SNT-8	48F TU	Nasal-type	+	WT			17
YT	15 M PF	ALL + thymoma (at relapse)	+	WT			18
KHYG-1	45F PB	Aggressive NK-cell leukemia (at diagnosis)	–	WT			19
MOTN-1	63F PB	(T-LGL/T-CLL) in chronic phase	–	WT			20
MTA	58F PB	Aggressive NK-like T-cell leukemia/lymphoma	–	WT			21
CAEBV							
AIK-T4	9F PB	Severe chronic active EBV infection	+	del exon 18 (131 bp)	Frameshift	50%	22
SIS	1 M PB	Severe chronic active EBV infection	+	WT			22
SKN	6 M PB	Severe chronic active EBV infection	+	WT			22

ALL, acute lymphoblastic leukemia; F, female; LN, lymph node; M, Male; NHL, non-Hodgkin lymphoma; PB, peripheral blood; PF, pericardial fluid; T-CLL, T-cell chronic lymphocytic leukemia; T-LGL, T-cell large granular lymphocyte leukemia; TU, tumor tissue; WT, wild type.

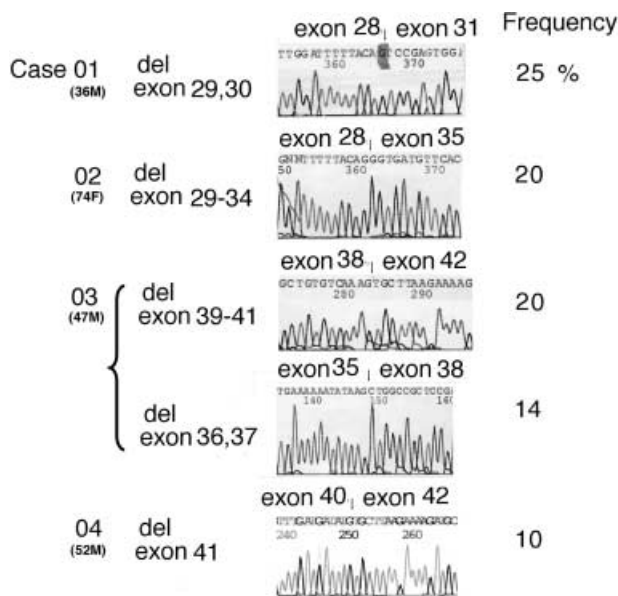


Fig. 1. *ATR* gene alterations detected in biopsy samples from nasal natural killer (NK)/T-cell lymphoma (NKTCL) patients. Four cases had alterations due to aberrant splicing. Deletion of exons 29 and 30 (257 bp) was found in case 1, exons 29–34 (867 bp) in case 2, exons 36 and 37 (241 bp) plus exons 39–41 (489 bp) in case 3, and exon 41 (144 bp) in case 4. Deletion of exons 29–34, 39–41 and 41 might generate an in-frame alteration. Other deletions might generate a frameshift mutation, resulting in a premature stop codon.

lines (Table 1). All alterations were heterozygous deletions, which might have resulted from aberrant splicing. Deletion of exon 18 (131 bp) was observed in AIK-T4, exons 29–34 (867 bp) in NK-92, and exons 29–34 (867 bp) and a 65 bp deletion (nucleotides 7082–7146) in exon 41 in SNK-6. Deletion of exons 29–34 might generate an in-frame alteration. Other deletion alterations might generate a frameshift mutation, resulting in a premature stop codon. In addition to the wild-type transcript, two aberrant transcripts of different types were detected in SNK-6. In these lines, sequences around the skipping exon were also analyzed, but no alterations were detected in the acceptor/donor splice site, or around the insertion sequence (data not shown).

Alteration analysis of clinical samples

Alterations in *ATR* were observed in four of 10 nasal NKTCL clinical samples (Fig. 1). Four alterations resulted from aberrant splicing. Deletion of exons 29 and 30 (257 bp) was found in case 1, exons 29–34 (867 bp) in case 2, exons 36 and 37 (241 bp) plus exons 39–41 (489 bp) in case 3, and exon 41 (144 bp) in case 4. Deletion of exons 29–34, 39–41 and 41 might generate an in-frame alteration. Other deletions might generate a frameshift mutation, resulting in a premature stop codon. In addition to the wild-type transcripts, two different aberrant transcripts were detected in case 3. The clinical samples contained 20–40% neoplastic cells, therefore it was not possible to determine whether the mutations were homozygous or heterozygous. In these cases, sequences around the skipping exon were analyzed, but no alterations were detected in the acceptor/donor splice site, or around the insertion sequence (data not shown).

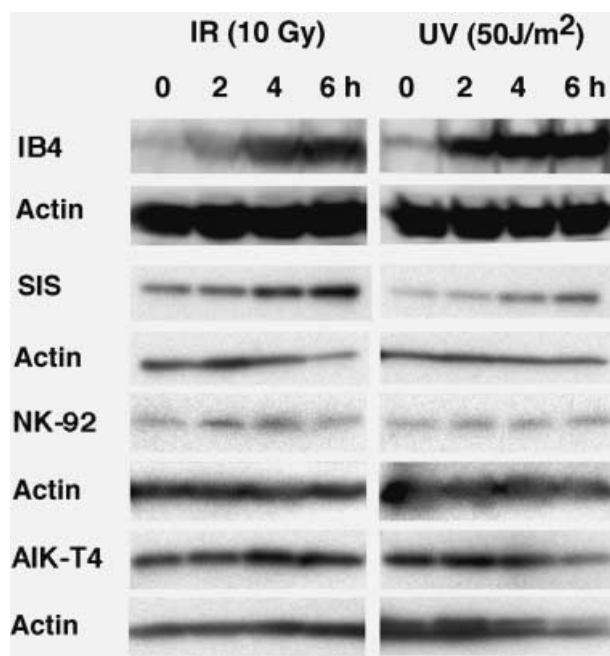


Fig. 2. Accumulation of p53 protein. Equal numbers of cells were exposed to 10 Gy of irradiation (IR) and 50 J/m² of ultraviolet (UV) light and incubated for up to 6 h. Whole cells were lysed and equal volumes of protein extracts were subjected to western blot analysis using anti-p53 antibody. Accumulation of p53 protein in NK-92 and AIK-T4 cells was defective after IR and UV exposure as expected. Equivalence of loading cell lysates is shown by anti-actin labeling.

Functional consequences of *ATR* alterations

To assess the functional consequence of *ATR* alterations for DNA-damage response and p53 accumulation, functional studies on two cell lines (NK-92, AIK-T4) with heterozygous *ATR* alterations were carried out. IB4 and SIS, which carry no alterations within *ATR*, were used as negative controls.

p53 protein

Compared to unexposed cells, increased expression of p53 was found in IB4 and SIS cells after IR and UV exposure (Fig. 2). In contrast, p53 expression levels in NK-92 and AIK-T4 cells did not change after IR and UV exposure. These results indicate that IR and UV exposure did not induce p53 accumulation in these cell lines.

Repair of DNA double-strand breaks induced by irradiation

To evaluate whether *ATR* gene alterations result in impaired DNA DSB repair, the kinetics of DNA DSB repair was examined using PFGE (Fig. 3a). More than 87.0% of the DSB in IB4 and SIS lines were repaired within 1 h of IR exposure (Fig. 3b). In contrast, approximately 69.9% and 81.8% of DSB in NK-92 and AIK-T4 lines, respectively, were repaired after 1 h, reaching 92.5% and 91.5% after 6 h incubation. The difference in the rate of DSB repair 1 h after IR exposure between NK-92, AIK-T4 and IB-4 was significant ($P < 0.05$). These results show that repair of DNA DSB after IR exposure is delayed in NK-92 and AIK-T4 lines.

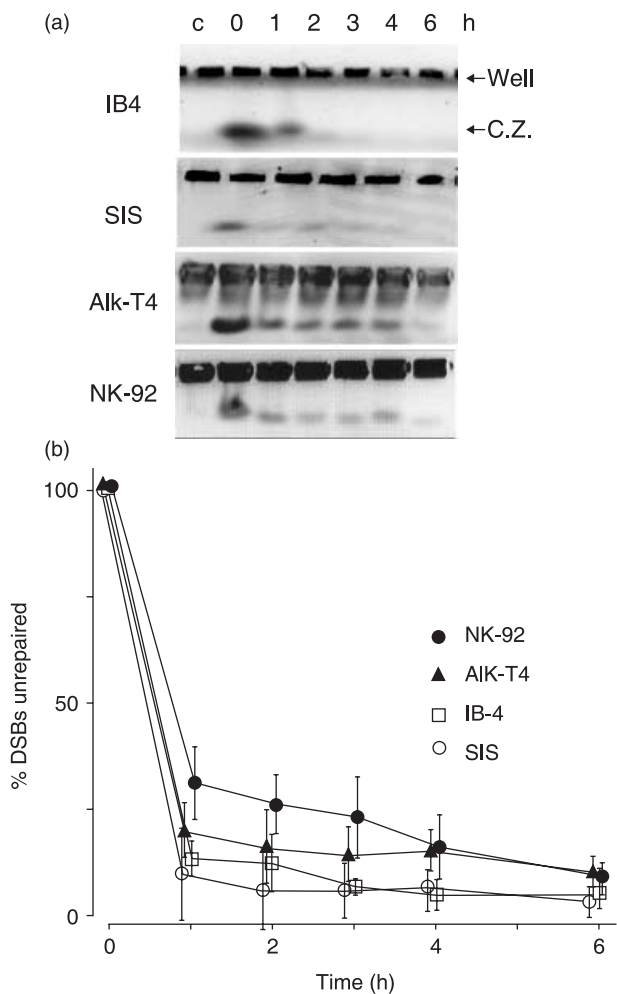


Fig. 3. Induction and repair of DNA double-strand breaks (DSB) after irradiation (IR) exposure determined by pulsed-field gel electrophoresis (PFGE). (a) Ethidium bromide-stained gel after PFGE. Equal numbers of IB4, SIS, NK-92 and AIK-T4 cells were embedded in 0.8% agarose plugs and exposed to 20 Gy IR. Cells were allowed to repair DSB for 0–6 h at 37°C, and were then lysed for DNA extraction and subjected to PFGE. Untreated control is shown in the first lane. The upper bands are derived from undamaged DNA or repaired DNA in wells. The lower bands show the compression zones (CZ) consisting of DSB DNA. (b) Quantification of DSB repair assays was performed. The percentage of unrepaired DSB was calculated using the ratio of signal from DSB DNA in the CZ-to-DNA in the corresponding well. The percentage of unrepaired DSB at 0 h incubation was set at 100%. Repair of DNA DSB after IR exposure was slightly delayed in NK-92 and AIK-T4 compared to control cell lines IB4 and SIS. The means (symbol) and standard deviation (error bar) from three experiments are depicted. Symbols represent cell lines: IB4 (□), SIS (○), NK-92 (●), and AIK-T4 (▲).

Repair of DNA single-strand breaks induced by UV light

To evaluate whether alterations in the *ATR* gene result in impaired DNA SSB repair, NK-92 and AIK-T4 cell lines with *ATR* alterations together with IB4 and SIS cells were exposed to 10 J/m² of UV, and repair of DNA SSB was evaluated using the Comet assay. After UV exposure, the comet tails were observed in all cell lines, indicating that DNA SSB had occurred (Fig. 4a). The maximum DML of IB4 was observed after an 6-h incubation and a significant decrease in DML was observed at 18 h after exposure (Fig. 4b). The

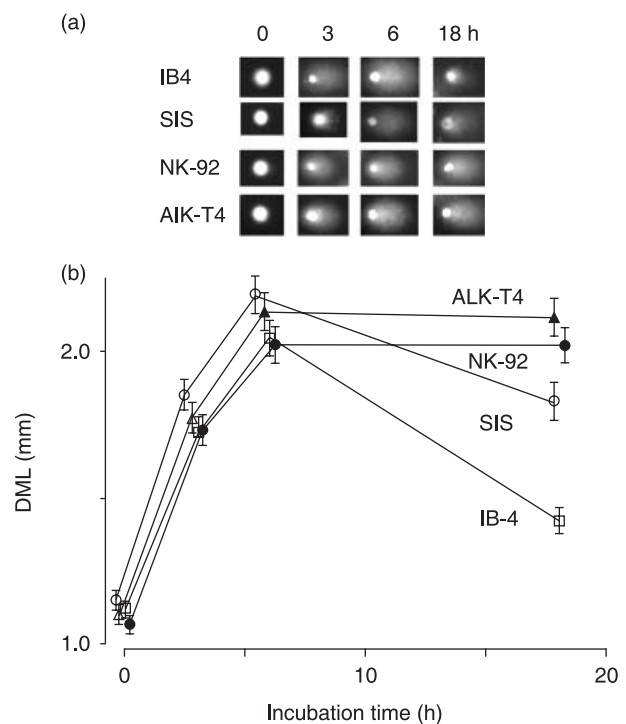


Fig. 4. Induction and repair of DNA single-strand breaks (SSB) after ultraviolet (UV) exposure determined using the Comet assay. IB4, SIS, NK-92 and AIK-T4 cells were exposed to 10 J/m² of UV, incubated at 37°C for 0–18 h, then lysed and subjected to Comet assay. (a) The representative pattern of DNA migration (comet tails) from the Comet assay. (b) Quantification of SSB repair assays was carried out as described in Materials and Methods. Compared to control cell lines, IB4 and SIS, NK-92 and AIK-T4 cells showed failure and inability to repair DNA SSB after UV exposure. The means (symbol) and standard deviation (error bar) from three experiments is depicted. Symbols represent cell lines: IB4 (□), SIS (○), NK-92 (●), and AIK-T4 (▲).

DML of SIS decreased, whereas the DML of NK-92 and AIK-T4 cells did not show an obvious decrease in DML after 18 h incubation. These results indicate that the NK-92 and AIK-T4 lines failed to repair DNA SSB after UV exposure.

Discussion

Maintenance of genome stability depends on the appropriate response to DNA damage.⁽⁷⁾ This response is based on complex networks of signaling pathways that activate numerous processes and lead ultimately to damage repair and cellular survival and apoptosis. The protein kinases ATM and ATR are master controllers of some of these networks.^(8–11) These two kinases act either in concert or separately to orchestrate the response to specific types of DNA damage or stalled replication. ATM responds mainly to DNA DSB, whereas ATR is activated by DNA SSB and stalled DNA replication forks.

The greatest frequency of genetic alteration encountered in any of the cell lines was 50%, indicating heterozygous mutations. *ATR* is an essential gene required for cell proliferation, therefore homozygous *ATR* mutation is lethal.^(25,26) O'Driscoll *et al.*⁽¹³⁾ reported that a heterozygous aberrantly spliced transcript affecting *ATR* expression results in Seckel syndrome, an

autosomal recessive disorder. They demonstrated that the cells derived from patients with Seckel syndrome had an abnormal response to DNA breaks. Lewis *et al.*⁽²⁷⁾ demonstrated that cells with heterozygous truncating *ATR* mutations in exon 10 show abnormalities in DNA repair as well as cell cycle checkpoints.

In the present study, heterozygous aberrant transcripts affecting the PI3K domain of *ATR* were detected in two of eight NKTCL cell lines and one of three CAEBV cell lines. In the clinical samples, alterations in *ATR* were also detected in four of 10 cases. The cell lines with *ATR* alterations showed a delay in the repair of DSB, an abrogation in the repair of SSB, and exhibited a defect in p53 accumulation after IR and UV exposure. These findings indicate that the alterations in *ATR* result in functional deficiencies in the cell lines.

In the present study, two cell lines with heterozygous *ATR* aberrant transcripts showed abnormal responses to DNA damage induced by UV and IR exposure. Because UV-induced SSB mainly triggers *ATR* but not *ATM*, the abnormal response after UV exposure may result from abrogation of *ATR*, whereas IR-induced DSB mainly trigger *ATM*. Tibbetts *et al.*⁽⁸⁾ suggested that *ATM* and *ATR* might be induced sequentially by IR. They proposed that, in response to DSB, *ATM* is activated first, phosphorylating specific substrates, and then *ATR* kicks in as a maintenance enzyme to prolong phosphorylation of the substrates targeted initially by *ATM*. Delay of DSB repair as observed in NK-92 and AIK-T4 lines may be consistent with their hypothesis.

Histologically, NKTCL usually shows a polymorphous pattern of proliferation of large lymphoid cells intermingling with small lymphocytes, plasma cells, macrophages and, less frequently, eosinophils.⁽²⁸⁾ Free radicals secreted by the infiltrating inflammatory cells might cause DNA damage. Recent studies have demonstrated the common cytological and cytogenetic features of nasal NKTCL and CAEBV cell clones, including highly complicated karyotypes, indicative of chromosomal instability in these cells.^(5,6) Alterations in *ATR*, the gene respon-

sive to DNA damage, might facilitate accumulation of structural abnormalities of chromosomes as well as mutations of the genes.

ATR alterations were observed in cancers of the stomach,⁽²⁹⁾ endometrium,⁽³⁰⁾ urothelium⁽³¹⁾ and colorectum⁽³²⁾ with mismatch repair phenotypes. In these cases, 10 repeats of mononucleotide A in exon 10 were changed, which resulted in frameshift mutations. The same kind of mutation was not observed in either the present study or our previous study on 50 cell lines derived from hematopoietic malignancies.⁽³³⁾ Although the deletion mutations observed in the present study have not been reported in cancers, this might be due to differences in the cell lineages used, and in the methods employed for investigation of *ATR* alterations (i.e. mRNA was used in the present study, whereas previous studies on cancers used genomic DNA. Because detection of large deletions and exon skipping is difficult using genomic DNA, further study is necessary to clarify the extent of *ATR* alteration in cancers and hematopoietic malignancies.

The present study showed that alterations in *ATR* were relatively frequent in NKTCL, suggesting a role for *ATR* alterations in the initiation and progression of nasal NKTCL.

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