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ATR alterations in Hodgkin's lymphoma

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Abstract. Hodgkin's lymphoma (HL) is characterized by the presence of neoplastic Hodgkin and Reed-Sternberg cells (HRSC) in a background of inflammatory cells. Free radicals and oxidative stress generated in the inflammatory lesions could cause DNA damage, thus providing a basis for lymphomagenesis. Ataxia-telangiectasia mutated (*ATM*) and Rad3-related (*ATR*) genes are responsive genes for DNA damage, therefore the potential involvement of the *ATR* gene in HL pathogenesis was examined in 8 HL cell lines and 7 clinical cases. *ATR* alterations were detected in 6 out of 8 HL lines. Most aberrant transcripts observed were heterozygous deletions, which may have resulted from aberrant splicing. *ATR* aberrant transcripts were also detected in 3 out of 7 clinical cases. Three alterations, del exon 4, deletion exon 29-34 and insertion of 137 bp in exon 46/47 were commonly observed in both cell lines and clinical samples. HL cells with *ATR* alterations except del exon 4 showed a delay/abrogation in repair for DNA double-strand breaks (DSBs) and single-strand break (SSB) as well as exhibiting a defect in p53 accumulation. These findings suggested the role of *ATR* gene alterations in HL lymphomagenesis.

Introduction

Hodgkin's lymphoma (HL) is characterized by the presence of neoplastic Hodgkin and Reed-Sternberg cells (HRSC) in a background of inflammatory cells (1). It is generally agreed that HRSC have a high degree of chromosomal instability, resulting in hyperdiploid complex karyotypes with chromosome breakpoints that are non-randomly distributed and increase in number as the clinical stage progresses (2). HRSC express mRNAs and proteins of various cytokines, implying an important role of cytokines in the pathophysiology of HL as a cytokine-producing tumor (3). However, it is not clear whether the unbalanced production of multiple cytokines causes the high degree of chromosomal instability and the predominance of hyperdiploid complex karyotypes.

Maintenance of the genome stability partly depends on the proper regulation of cellular responses to DNA damage and the integrity of DNA repair mechanisms (4). In mammalian cells, two members of the phosphatidylinositol 3-kinase-related kinase (PI3KK) family, ataxia-telangiectasia mutated (*ATM*) and *ATM* and Rad3-related (*ATR*) kinases, play a central role in DNA damage recognition and the initial phosphorylation events (5-8).

ATR is essential for cell proliferation. Previous studies suggested that ATR might participate in the signaling of ionizing radiation (IR)- and ultraviolet (UV)-induced DNA damage (5,6,9). Previously, O'Driscoll *et al* (10) reported that a splicing aberrant transcript affecting the expression of *ATR* results in Seckel syndrome, which shares features with disorders showing impaired DNA-damage responses such as Nijmegen breakage syndrome. Recent studies showed that the *ATR* gene plays a role in the maintenance of chromosomal stability in fragile sites (11).

In the present study, *ATR* gene alterations were examined in eight HL cell lines and HRSC isolated from tissues involved by HL using laser capture microdissection (LCM) microscopy. Three cell lines with heterozygous *ATR* aberrant transcripts were further analyzed to evaluate the functional consequences in DNA repair and p53 accumulation.

Materials and methods

Cell lines. Eight cell lines established from HL were used in the present study (Table I). KM-H2, L-1236, L-540, L-428, HD-MY-Z and HDLM-2 were purchased from Deutsche Sammlung Von Mikroorganismen und Zellkulturen (Braunschweig, Germany). DEV were established by Professor S. Poppema. HD-70 and the lymphoblastoid cell line IB4 were kindly provided by Dr M. Daibata (Kochi University, Japan) and Dr E. Kieff (Brigham and Women's Hospital, Boston, MA), respectively. OS was lymphoblastoid cell line established by us. All cell lines were incubated in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10-20% of heat-inactivated FCS at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

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Table I. *ATR* mutations and polymorphisms in HL lymphoma cell lines.

			Mutations			Polymorphisms (nucleotide sites)					
Cell line	Patient/ sample	Subtype	Nucleotide change	Predicted effect	Frequency of mutation positive clones (%)	379,380[a] CGC→GCC (ARG→Ala)[c]	737[b] ATG→ACG (Met→Thr)[c]	1881[b] GGT→GGA (Gly→Gly)[c]	1920[b] GAT→GAC (Asp→Asp)[c]	5313[b] TAT→TAC (Tyr→Tyr)[c]	7980[a] CAA→CAG (Gln→Gln)[c]
KM-H2	37F PE	MC	WT			GC	T	T	T	C	A
L-428	37F PE	NS	WT			GC	C /T	T	T	C/T	A
L-1236	34M PB	MC	del exon 4 (878 bp)	Frameshift	40	CG	C /T	A/T	C/T	C/T	A
L-540	20F BM	NS	del exon 18 (131 bp)	Frameshift	30	GC	T	A/T	C/T	C	A
			del exon 29,30 (257 bp)	Frameshift	40						
HD-MY-Z	29F PE	NS	del exon 29-34 (867 bp)	In-frame	20	GC	C/T	A	T	T	A
			del exon 29,30 (257 bp)	Frameshift	20						
HDLM-2	74M PE	NS	del 121 bp (in exon 33)	Frameshift	30	GC	T	A	C	T	A
			exon 46/47 ins 137 bp	Frameshift	20						
HD70	69M PB	NS	del exon 4 (878 bp)	Frameshift	20	GC	C/T	A/T	C/T	T	A
DEV	50M PE	NLP	del exon 4 (878 bp)	Frameshift	50	GC	T	T	T	T	G/A
			del exon 29-34 (867 bp)	In-frame	20						

PE, pleural effusion; PB, peripheral blood; BM, bone marrow; MC, mixed cellularity; NS, nodular sclerosis; NLP, nodular lymphocyte predominant; WT, wild-type. [a]Ref. 16, (GenBank accession number U49844). [b]Ref.12. [c]Amino acid changes generated by nucleotide changes at indicated sites are shown in parentheses.

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Clinical samples from 7 patients with HL who were to the University Medical Center Groningen in the Netherlands were analyzed with informed consent in accordance with the Declaration of Helsinki: age of the patients at admission ranged from 11 to 57 (median 21) years, with a male to female ratio of 1:6. Histological specimens obtained by biopsy were fixed in 10% formalin and routinely processed for paraffin-embedding, or snap-frozen for extraction of total RNA. Histologic sections were cut at 4 μm and stained with hematoxylin and eosin and immunoperoxidase procedures for diagnosis and subclassification. All tumors were histologically classified according to the World Health Organization classification. Six cases were diagnosed as nodular sclerosis, and one as nodular lymphocyte predominant HL.

Isolation of DNA and total RNA, RT-PCR and detection of ATR aberrant transcripts. DNA and total RNA were extracted from the cell lines with the TRIzol reagent (Invitrogen, Inc., Rockville, MD) 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 RT-PCR was performed to amplify the *ATR* transcripts using 4 sets of primers spanning the whole open reading frame as described previously (12). Detection of ATR aberrant transcripts were performed as described previously (12).

LCM. Cryostat sections (8 μm) were cut and mounted on slides covered by polyethylene-naphthalate (PEN) membranes (P.A.L.M. Microlaser Technologies, Bernried, Germany) coated with 0.1% poly-L-Lysine (Sigma-Aldrich, St. Louis, MO). The slides were air dried at room temperature (RT) for 30 min and subsequently stained with hematoxylin. After a short rinse in RNase free water, the sections were dehydrated in 100% ethanol and air dried at RT for 30 min. LCM of HRSC was performed on a P.A.L.M. Laser Microbeam System (P.A.L.M. Microlaser Technologies). The microdissected HRSC were catapulted by a single laser shot into the lid of an adhesive cap (P.A.L.M. Microlaser Technologies). From each specimen, ~ 500 HRSC were collected. Three ATR fragments were amplified using the following primers: Delex 4F: GTGAGTGGAAGCCATGAG; Delex 4R: TCAGTCTG TTTTGGTGCTC; Delex29-34F: 5'-TCGCTCCAAAGCAT ACACAC-G-3'; Delex29-34R: 5'-GAACAATTAGTGCCT GGTG-3'. Delex46/47F: 5'-GACTTTTCTACATGATC CTC-3'; Delex 46/47R: 5'-GCATTACTTTTAGATTATTAA CAT-3'. *ATR* transcripts spanning the whole open reading frame using 8 to12 sets of primers. ß2-microglobulin was used as an RNA loading and quantity control.

Western blotting. Western blotting was performed as described previously (12). Briefly, whole cells were lyzed in 1 X sample buffer, separated with 10% SDS-PAGE for p53 protein and 5-20% gradient SDS-PAGE for ATR and then blotted to PVDF membrane using a wet-blotting apparatus. The antibody that used was N-19 (SantaCruz Biotechnology, Inc., CA, USA) for detection of ATR protein, DO-7 (epitope; 19-26 amino acids of p53) (Dako Cytomation, Glostrup, Denmark) and/or Pab240 (epitope; 213-217 amino acids of p53) (Calbiochem, San Diego, CA) for p53. Anti-actin (Sigma-Aldrich, Steinheim, Germany) were used as a control. Signals were visualized with ECLplus chemiluminescent reagents (Amersham Pharmacia Biotech, UK).

DNA double-strand break (DSB) repair assays. More than $1x10^5$ cells from each cell line were embedded in agarose plugs as described previously (12,13). The plugs on ice were exposed to 20 Gy of IR, covered with RPMI-1640 medium 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 X TAE and subjected to pulsed-field gel electrophoresis (PFGE) in a CHEF apparatus (Bio-Rad, Hercules, CA) at 3 V/cm at 14˚C for 48 h with a pulse time of 45 sec. Under these PFGE conditions, DNA fragments between 2 and 6 megabase sizes migrate as a single band and form compression zones under each well, whereas most DSB rejoining results in a size too large to enter the gel. DSBs 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 (SSB) repair assays. The alkaline single cell gel electrophoresis (Comet assay) was performed as previously described with some modifications (12,14,15). Briefly, more than 5,000 cells were layered onto a microscope slide coated with 80 μl of 0.65% normal agarose in PBS and then exposed to 10 J/m^2 of UV. After UV exposure, a top layer of 80 μl of a low melting point agarose was added and incubated for up to 18 h at 37˚C in the culture medium. Thereafter, the slides were rinsed in the lysis solution (2.5 M NaCl, 10 mM Tris-HCl, 100 mM EDTA and 1% Triton X-100, pH 10.0) at 4˚C for 1 h. Electrophoresis was performed for 40 min at a constant current of 300 mA in the buffer (1 mM EDTA, 300 mM NaOH, pH 13.0) at 4˚C to allow unwinding of DNA. The slides were washed in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) and stained with ethidium bromide. Fifty cells were analyzed based on the methods for collecting comet data, in which the repair of DNA 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 <0.05 were taken as statistically significant.

Results

ATR aberrant transcripts and protein expression in the HL cell line. Aberrant transcripts of the *ATR* gene were observed in 6 out of 8 HL cell lines (75%) (Table I). Most aberrant transcripts observed were heterozygous deletions, which may have resulted from aberrant splicing. Deletion of exon 4 (878 bp) was observed in three cell lines (L-1236, HD-70 and DEV), deletion of exons 29 and 30 (257 bp) in two cell lines (L-540, HD-MY-Z), deletion of exons 29-34 (867 bp) in two cell lines (HD-MY-Z, DEV) and the deletion of exon 18 in one cell line (L-540). A 121 bp deletion in exon 33 was detected in HDLM-2. An insertion of 137 bp from intron 46 was found between exon 46 and 47 in HDLM-2. The deletion of exon 29-34 (HD-MY-Z, DEV) and the deletion of 121 bp

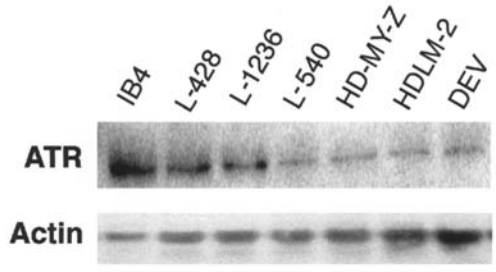

Figure 1. ATR protein expression examined by Western blotting. Cells harboring an ATR aberrant transcript except L-1236 (del exon 4) showed an impaired expression. Equivalence of the loading of cell lysates is shown by anti-actin labeling.

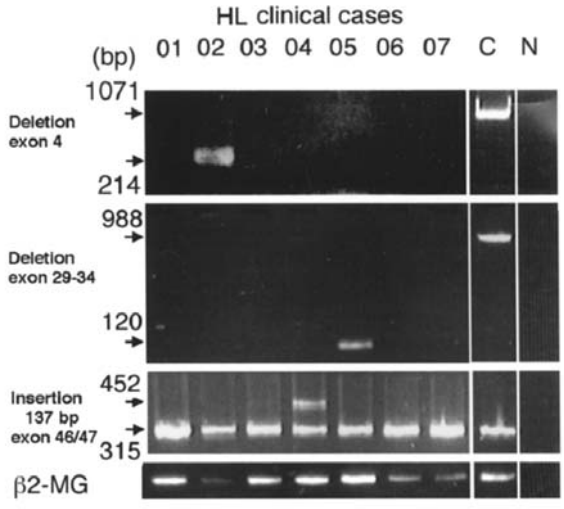

Figure 2. Aberrant transcripts of *ATR* were examined in HRSC obtained from 7 clinical samples with a laser capture microdissection technique, using the primer pairs flanking exon 4, exons 29-34 and a boundary between exon 46 and 47, respectively. Three out of 7 cases had aberrant transcripts in either region.

(nt 5723-5843) in exon 33 (HDLM-2) might generate an in-frame aberrant transcript. Other deletion/insertion aberrant transcripts may generate a frameshift, resulting in a premature stop codon. Two different aberrant transcripts in addition to the wild transcript were detected in four lines (L-540, HDLM-2, HD-MY-Z and DEV). In the cell lines with aberrant *ATR* transcripts, sequences around the skipping exons were analyzed, but no alterations were detected in the acceptor, donor splice site, or around the insertion sequence (data not shown). In lymphoblastoid cell lines, IB-4 and OS, no alterations were observed.

ATR protein expression was examined by Western blotting (Fig. 1). IB4 and L-428 expressed the ATR protein at the same level. The cell lines with *ATR* transcripts except for L-1236 showed a reduced expression of ATR protein. No additional band was detected in any of the cell lines examined.

Polymorphisms and alternative splicing within the ATR gene. Dinucleotide and single nucleotide changes were observed at one and five loci of the *ATR* gene, respectively (Table I). Polymorphisms of these loci have already been reported

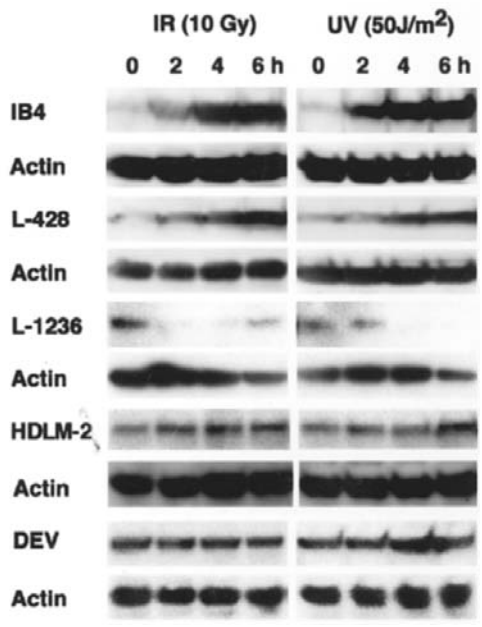

Figure 3. Accumulation of p53 protein. Equal numbers of cells were exposed to 10 Gy of IR and 50 J/m^2 of UV light, respectively and incubated for up to 6 h. Whole cells were lyzed and an equal volume of protein extracts were subjected to Western blot analysis using anti-p53 antibody. Accumulation of p53 protein in L-1236, HDLM-2 and DEV cells was defective after IR and UV exposure. In L-428 and IB4 without ATR alterations, p53 protein accumulated as expected after IR and UV exposure. Equivalence of loading of cell lysates is shown by anti-actin labeling.

(12,16). Nucleotide changes at 379-380 and 737 result in amino acid alterations, Arg→Ala and Met→Thr, respectively, while other nucleotide changes do not result in amino acid changes. Skipping of exon 6, which was reported to be an alternative splicing (17), was not detected.

ATR alterations in clinical samples. Alterations of *ATR* were examined in HRSC isolated from 7 clinical samples using LCM. In the HL cell lines, deletion of exon 4, exons 29 and 30, aberrant splicing between exons 29 and 34 and an insertion of 137 bp from intron 46 between exons 46 and 47 were observed in more than 2 cell lines. Thus, occurrence of these aberrant splicings in the clinical HL samples was examined using PCR with the primer pairs flanking exon 4, exons 29 to 34 and boundary between exon 46 and 47, respectively. Three out of 7 cases were found to have aberrant transcripts in either region (Fig. 2). Direct sequencing revealed the fragments corresponding to the aberrant splicing expected (data not shown). The whole ATR genes were amplified after subdividing into 8 to 12 regions and sequenced. No point mutations were observed (data not shown).

Functional consequence of ATR alterations. Three alterations, del exon 4, deletion exon 29-34 and insertion of 137 bp in exon 46/47 were commonly observed in both the cell lines and the clinical samples. To assess the functional consequence of the *ATR* alterations for DNA-damage response and p53 regulation, 3 lines that carry heterozygous *ATR* alterations (L-1236; exon 4, HDLM-2; insertion of 137 bp in exon 46/47 plus deletion exon 33, DEV; exon 29-34 plus exon 4) were subjected to the functional studies shown below.

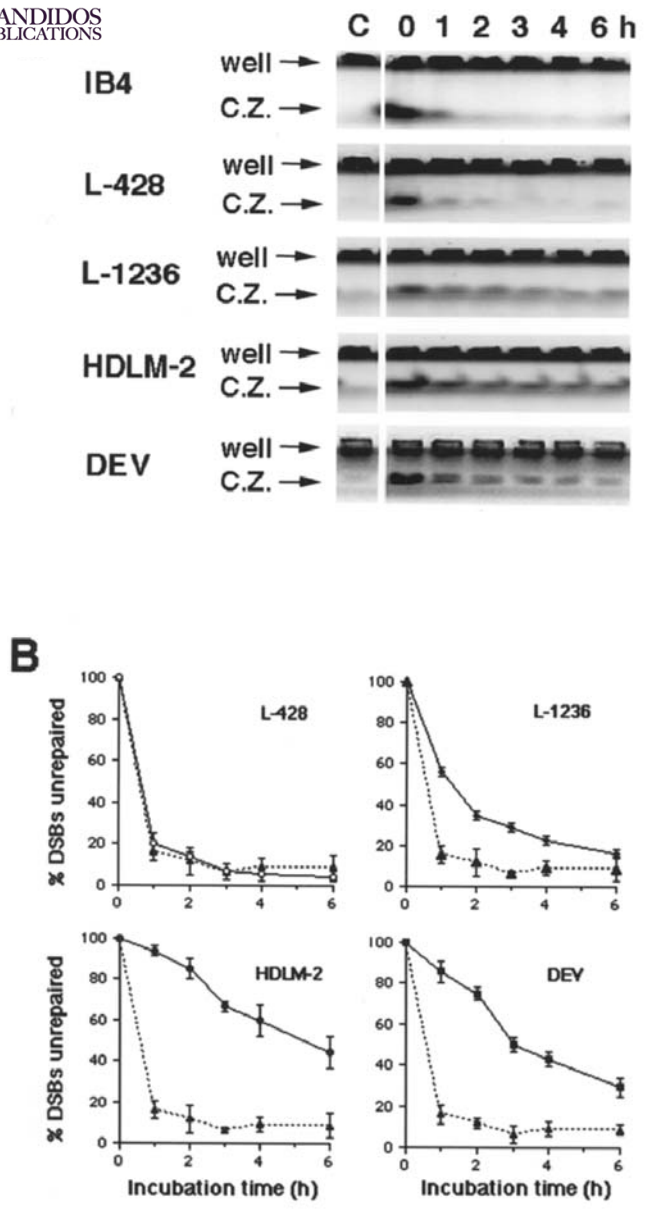

Figure 4. Induction and repair of DNA DSBs after IR exposure determined by PFGE. (A) Ethidium bromide-stained gel after PFGE. Equal numbers of IB4, L-428, L-1236, HDLM-2 and DEV cells were embedded in 0.8% agarose plugs and exposed to 20 Gy of IR. Cells were allowed to repair DSBs for 0-6 h at 37˚C, then lyzed to extract DNA, which was subjected to PFGE. The untreated control is shown in the first lane. The upper bands derived from undamaged DNA or repaired DNA in wells. The lower bands show the compression zones (C.Zs) consisting of DSBs DNA. (B) Quantitation of DSB repair assays was performed. The percentage of unrepaired DSBs was calculated by ratio of signal from DSBs DNA in the C.Zs to DNA in the corresponding well. Percentage of unrepaired DSBs at 0-h incubation was set at 100%. Repair of DNA DSBs after IR exposure was delayed in HDLM-2 and DEV compared to L-428 and IB4 without ATR alteratons. In L-1236 cells, repair of DNA DSBs was only slightly delayed compared to L-428 and IB4. The means (symbol) and standard deviation (error bar) from three experiments is depicted. Symbols represent cell lines: IB4 (▲), L-428 (○), L-1236 (◆), HDLM-2 (◆), DEV (■).

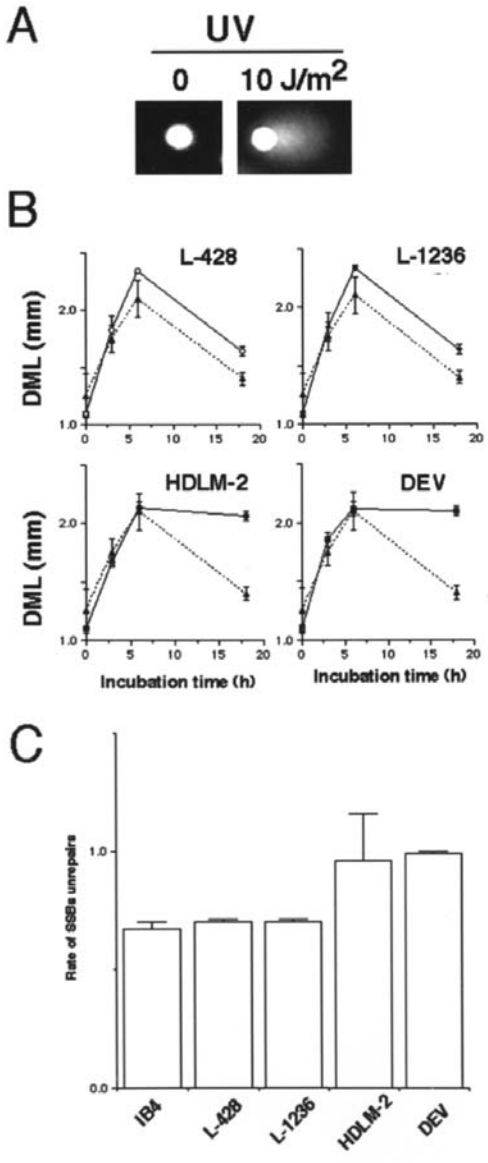

Figure 5. Repair of DNA SSBs after UV exposure determined by Comet assay. (A) The representative pattern of cell DNA migration (comet tails) produced by Comet assay. (B) IB4, L-428, L-1236, HDLM-2, and DEV cells were exposed to 10 J/m^2 of UV, incubated at 37˚C for 0-18 h and assayed as described in Materials and methods. Compared to IB4 and L-428 without ATR alterations, HDLM-2 and DEV cells showed a failure and inability to repair DNA SSBs after UV exposure. Repair ability of DNA SSBs after UV exposure in L-1236 cells was similar to L-428. The means (symbol) and standard deviation (error bar) from the three experiments is depicted. Symbols represent cell lines: IB4 (▲), L-428 (○), L-1236 (◆), HDLM-2 (□), DEV (■). (C) Ratio of the DML at 18- to 6-h incubation after UV exposure, which represented the rate of unrepaired DNA SSBs. Rates in HDLM-2 and DEV cells were higher than that in IB4 and L-428. Whereas the rate of unrepaired DNA SSBs in L-1236 was similar to that in the control cell lines. DML indicates DNA migration length.

L-428 and IB4, which carry no alterations within *ATR*, were also included in the studies.

p53 protein expression. Compared to the unexposed cells, an increased expression of p53 was found in the L-428 and IB4 cells after IR and UV exposure (Fig. 3). In contrast, the p53 expression level in L-1236, HDLM-2 and DEV cells did not change after IR and UV exposure. These results indicate that IR and UV exposure did not induce p53 accumulation properly in HL cell lines, with the exception of L-428.

Repair of DNA DSBs induced by IR. To evaluate whether *ATR* gene aberrant transcripts result in impaired DNA DSBs repair, kinetics of DNA DSBs repair were examined using PFGE (Fig. 4A). More than 80% of the DSBs in L-428 and IB4 lines

were repaired within 1 h after IR exposure (Fig. 4B). In contrast, about 6.6, 14.4, and 43.8% of DSBs in HDLM-2, DEV and L-1236 lines, respectively, were repaired after 1 h and about 54.1, 70.6, and 83.6% of DSBs after 6-h incubation. These results demonstrate that the repair of DNA DSBs after IR exposure in HDLM-2, DEV and L-1236 is delayed.

Repair of DNA SSBs induced by UV. To evaluate whether aberrant transcripts of *ATR* gene result in impaired DNA SSBs, HL cell lines with *ATR* aberrant transcripts together with L-428 and IB4 cells were exposed to 10 J/m^2 of UV and repair of DNA SSBs was evaluated with the Comet assay. After UV exposure, the comet tails were observed in all cell lines examined, showing that DNA SSBs occurred (Fig. 5A). The maximum DNA migration length (DML) was observed after a 6-h incubation period in all cells (Fig. 5B). The ratio of the DML at 18- to 6-h incubation periods after UV exposure was calculated, representing the ratio of unrepaired DNA SSBs (Fig. 5C). In the L-428, L-1236 and IB4 cell lines, a decrease in the DML was observed at 18 h after exposure. After 18 h of incubation, a slight decrease of DML was observed in HDLM-2 and DEV cells. These results indicate that the HDLM-2 and DEV line failed to repair DNA SSBs after UV exposure.

Discussion

ATM alterations are involved in the development of HL (18,19), while information on the role of *ATR* gene alterations in lymphomagenesis has been limited until present (12). In the present study, heterozygous aberrant transcripts affecting the PI3K domain of *ATR*, thus indicative of the loss-of-function mutation (20), were detected in 6 out of 8 HL cell lines. HRSC in the clinical samples expressed the aberrant transcripts in 3 out of 7 cases.

Three alterations in *ATR*, del exon 4, deletion exon 29-34, and insertion of 137 bp in exon 46/47 were commonly observed in both the cell lines and the clinical samples. The cell lines with deletion exon 29-34 and insertion of 137 bp in exon 46/47 showed a delay in repair for DSBs, an abrogation in repair for SSBs and exhibited a defect in p53 accumulation after IR and UV exposure, suggesting that the alterations of the *ATR* gene results in functional defects in these cell lines. Whereas, cell line (L-1236) with del exon 4 exhibited only a partial or no defect in the assays and the expression level of ATR protein was comparable to that of the control cell line. Del exon 4 may yield small truncated protein of 115 amino acids. At present, it is not clear whether this ATR alteration is functionally significant *in vivo* or not.

Previous studies showed the *ATR* alterations in cancers of the stomach (21), uterus (22), urinary bladder (23) and colorectum (24) with mismatch repair phenotypes, in which deletion/insertion mutation in poly A tracts within exon 10 were found. However, deletion mutations as observed in the present study were never found. The differences in the kinds of cell lineages used and the methods employed for investigation of *ATR* alterations between these studies might be causes for different results, i.e. mRNA were used in the present study, but genomic DNA in the previous studies. Because large deletion and exon skipping is difficult to detect with genomic DNA, further study is necessary to clarify the extent of *ATR* alterations in cancers and hematopoietic malignancies.

The frequency of genetic alterations in the HL cell lines ranged from 20 to 50%, indicating the heterozygous mutations. *ATR* is an essential gene required for cell proliferation, therefore cells with homozygous ATR mutation is lethal (25,26). O'Driscoll *et al* (10) reported that heterozygous splicing transcripts reduced the expression of wild-type ATR protein which may result in Seckel syndrome, an autosomal recessive disorder. They demonstrated that the cells derived from the 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 showed an abnormality in DNA repairs as well as cell cycle checkpoints.

HRSC are recognized as cytokine producing neoplastic cells (3). Many inflammatory cells migrate towards and surround the HRSC and synergistically produce chemical mediators. In these circumstances, hazardous effects of chemical mediators such as superoxide and free radicals to the genome may occur. Because *ATR* is activated not only by UV-induced SSBs but also other forms of DNA damage and replication blocks (5-7,9), abrogation of ATR in HRSC may facilitate the accumulation of genomic abnormalities. The present study suggests the possibility that *ATR* alterations are involved in the initiation and/or progression of HL.

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