Impairment of double-strand breaks repair and aberrant splicing of *ATM* and *MRE11* in leukemia–lymphoma cell lines with microsatellite instability

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(Received September 30, 2005/Revised December 2, 2005/Accepted December 13, 2005/Online publication March 1, 2006)

Mutations of DNA double-strand breaks (DSB) repair genes, *ATM*, *MRE11*, *RAD50*, *NBS1* and *ATR*, are postulated to play a role in the development of gastrointestinal malignancies with an impaired mismatch repair (MMR) function. In the present study, mutations of these genes together with the presence of microsatellite instability (MSI) were examined in 50 leukemia–lymphoma cell lines. MSI was detected in 13 (26%) lines. Mutations of intronic mononucleotide repeats in *ATM* and *MRE11* were found in nine and six lines, respectively, whereas mutations of mononucleotide repeats of *RAD50* were found in only one line, and none were found in either *NBS1* or *ATR*. Frequencies of *ATM* and *MRE11* mutations were significantly higher in MSI-positive than MSI-negative lines. These mutations generated aberrant splicing in both genes. The intensity of the aberrant transcript of *ATM* (497del22) was stronger in five lines harboring mononucleotide mutations of 2 bp or more than in the lines without or with a 1-bp mutation. The intensity of the aberrant transcript of *MRE11* (315del88) was stronger in four lines with mononucleotide mutations than in lines without. The expression levels of *ATM* and *MRE11* transcripts in MSI-positive lines were significantly higher than those in MSI-negative lines. MSI-positive cell lines showed delay or abrogation of DSB repair. The present study suggests that impairment of the MMR system causes aberrant transcripts in the DSB repair genes *ATM* and *MRE11*. This might result in inactivation of the DSB repair system, thus inducing an acceleration of genome instability and accumulation of genetic damage. (*Cancer Sci* 2006; 97: 226–234)

Exposure to hazardous agents in the environment may induce DNA damage that could be harmful to the maintenance of cell homeostasis and transmission of high-fidelity genetic information. Thus, cells have evolved several mechanisms for DNA repair to ensure genomic integrity and prevent mutations. The DNA MMR system corrects errors that might occur during DNA replication, whereas HR and non-homologous end joining are involved in the repair of DNA DSB.[1,2]

Double-strand breaks, which are extremely cytotoxic DNA lesions, activate an extensive array of responses that lead to repair of the damage and allow continuation of cellular life.[1,3] The nuclear protein kinase ATM is regarded as the primary activator of this network, phosphorylating key proteins in numerous signaling pathways.[4] The highly conserved MRN complex plays a role in DSB repair, particularly in the HR pathways.[5] While NBS1 is a target of ATM, recent observation has shown that the MRN complex itself contributes to the direct activation of ATM.[6] ATR, another protein kinase, has been reported to regulate responses to a broad range of damage, including DSB.[7,8] Whereas ATM is engaged primarily in DSB repair, several observations suggest that ATR has a critical role in virtually all cellular responses to the arrest of DNA replication forks, which are the DNA structures formed during replication.[8–10]

The increased rate of uncorrected replication errors at simple repeat sequences is known as MSI. Disruption of the MMR system, as revealed by MSI, is characterized by the accelerated accumulation of single nucleotide mutations and resultant alterations in the microsatellite DNA sequences, which affect the genome ubiquitously.[11] MSI is correlated with hereditary non-polyposis colorectal cancer, as well as a variety of sporadic cancers, including gastric, endometrial and colorectal cancers, through inactivation of several MMR target genes.[12,13] Although MSI was previously regarded to be uncommon in hematolymphoid malignancies,[14,15] recent studies have revealed it to be involved in the development of these malignancies, especially diseases involving the T-cell lineage.[16,17]

Recent studies have suggested a link between impaired MMR and mutations of DSB repair genes in cancers. In colorectal tumors with an impaired MMR system, mutations of intronic mononucleotide repeats in *ATM* and *MRE11* were found to result in aberrant splicing, followed by reduced expression of the wild-type protein.[18–20] Ottini *et al*. reported impaired MRE11 expression in gastric cancer with impaired MMR.[21] Meanwhile, mutations in coding mononucleotide repeats in *RAD50*, *NBS1* and *ATR* were reported in a subset of gastrointestinal cancers with MSI.[22,23] In the present study, we provide evidence that impaired MMR function may result in aberrant splicing of *ATM* and *MRE11* genes, which correlates with impairment of the DSB repair system in leukemia–lymphoma cell lines.

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Abbreviation used: ATM, Ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; DSB, double-strand break; HR, homologous recombination; IR, ionizing radiation; IVS, intervening sequence; MSI, microsatellite instability; MMR, mismatch repair; MRN, MRE11-RAD50-NBS1; ORF, open reading frame; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; RI, ratio of intensity; TAE, Tris-acetate/EDTA.

doi: 10.1111/j.1349-7006.2006.00165.x

Materials and Methods

Cell lines

A total of 50 leukemia–lymphoma cell lines derived from Burkitt lymphoma (BJAB, BL36, BL60, BL137, Daudi, Katata, NAB-2, Namalwa, Raji and Ramos), diffuse large B-cell lymphoma (OPL-1, OPL-2, OPL-3, OPL-4, OPL-5, OPL-7 and Pal-1),[24–26] precursor B-cell acute lymphoblastic leukemia (NALM-6 and REH), T-cell acute lymphoblastic leukemia (CCRF-CEM, HPB-ALL, MOLT-4, MOLT-14, Jurkat and Peer), adult T-cell leukemia (MT-1, MT-2, MT-4 and TL-Om1), NK/NKT-cell lymphoma (KHYG-1, MOTN-1, MTA, NK-92, SNK-6, SNT-8 and YT),[27] Hodgkin lymphoma (HDLM-2, HD-My-Z, KM-H2, L-428, L-540 and L-1236), non-B non-T acute lymphoblastic leukemia (P30/Ohkubo), pyothorax-associated lymphoma (PAL) with dual B/T-cell phenotype (Deglis),[28] acute myeloid leukemia (CTV-1, P31/Fujioka, HEL, HL-60 and KG-1), and chronic myeloid leukemia (K-562) were used in this study. OPL and Pal-1 were derived from tumor cells in PAL, a B-cell lymphoma of mostly large cell morphology that develops in long-standing inflammation of the pleura, usually consequent to lung tuberculosis. Two colorectal carcinoma cell lines (HCT 116 and LS 174T) known to harbor MSI were used as positive controls, whereas two lymphoblastoid cell lines (IB-4 and OS) were used as negative controls. Five normal peripheral blood samples as normal tissue controls were obtained from healthy volunteers.

BJAB, Daudi, Namalwa, Raji, Ramos, CCRF-CEM, MOLT-4, MOLT-14, Jurkat, Peer, MTA, P30/Ohkubo, P31/Fujioka, HL-60, KG-1 and K-562 were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). HDLM-2, HD-My-Z, KM-H2, L-428, L-540, L-1236, NALM-6, REH, HPB-ALL, YT, NK-92 and CTV-1 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). HCT 116 and LS 174T were purchased from American Type Culture Collection (Manassas, VA, USA). OPL-1, OPL-2, OPL-3, OPL-4, OPL-5, OPL-7 and OS were established in our laboratory.[24,25] Other cell lines were gifts from investigators listed in the Acknowledgments. All cell lines were cultured in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum and antibiotics at 37°C in 5% CO_2 in air.

Detection of MSI and mutations of *ATM*, *MRE11*, *RAD50*, *NBS1* and *ATR*

Genomic DNA was extracted from the cell lines by the sodium dodecylsulfate-proteinase K and phenol-chloroform extraction method. MSI was determined as present when mutations in both *BAT-25* and *BAT-26* were observed. Polymorphisms of both of these microsatellites have been described as being rare. Deletions of both of these markers have been shown to establish MSI status with an accuracy of > 99.5% without the requirement of matching normal DNA.[29–31]

Fragments containing poly$(T)_{15}$ nucleotide repeats in IVS 7 of *ATM*, poly$(T)_{11}$ nucleotide repeats in IVS 4 of *MRE11*, poly$(A)_9$ nucleotide repeats in the ORF of *RAD50*, poly$(A)_7$ nucleotide repeats in the ORF of *NBS1*, and poly$(A)_{10}$ nucleotide repeats in the ORF of *ATR* were amplified by PCR. Forward and reverse primer sequences were: *ATM*, 5′-AAATCCTTTTT-CTGTATGGGAT-3′ and 5′-(Hex)-GTTACTGAGTCTAAAA-CATGG-3′; *MRE11*, 5′-GGAGGAGAATTTTAGGGA-3′ and 5′-(Hex)-TGCTTTCCACAGACAAAC-3′; *RAD50*, 5′-GAGGCTGAGTTACAAGAAGTC-3′ and 5′-(Fam)-CATTT-CATCACGCCGCTTTTC-3′; *NBS*, 5′-CTACTTTCAGCCGT-CTACC-3′ and 5′-(Hex)-GCCACATCATCCATTTCC-3′; *ATR*, 5′-(Hex)-CCTTTCTCTGAACACGGAC-3′ and 5′-CAAGTTTT-ACTGGACTAGGTA-3′; *BAT-25*, 5′-(XRITC)-TCGCCTC-CAAGAATGTAAGT-3′ and 5′-CCACACTTCAAAATACATT-CTGC-3′; and *BAT-26*, 5′-(FITC)-TGACTACTTTTGACT-TCAGCC-3′ and 5′-AACCATTCAACATTTTTAACCC-3′. Briefly, 0.5 μL of each PCR product was mixed with 12.0 μL of deionized formamide and 0.5 μL of dye-labeled Internal Lane Size Standard (GeneScan 400 HD; Applied Biosystems, Foster City, CA, USA). The samples were run on an ABI PRISM 310 Genetic Analyzer, and the sizes of the PCR products were analyzed with GeneScan analysis software version 3.1 (Applied Biosystems). The experiment was repeated at least twice.

Detection of aberrant splicing in *ATM* (497del22) and *MRE11* (315del88)

RNA was extracted from the cell lines in the presence of TRIzol reagent (Life Technologies, Rockville, MD, USA), and was reverse transcribed by random hexamer priming. PCR amplification of the indicated fragments was carried out in a total volume of 15 μL consisting of 1 μL of cDNA template and 0.5 units of AmpliTaq Gold (Applied Biosystems), using the primer pairs as follows: *ATM* exon 8, 5′-CTCAGCAAC-AGTGGTTAG-3′ and 5′-GATATGATTTAGACCTGAAGAG-3′; and *MRE11* exon 5, 5′-CCAGGGGTTCTTGGAGAAG-3′ and 5′-CCAGCACAACTTAAAATGTC-3′. PCR was carried out at least twice for all samples. The PCR products (5 μL) were electrophoresed on a 1.5–2.5% agarose gel and visualized by ethidium bromide staining. The photographs taken under a UV transilluminator were scanned with an Epson™ ES-6000 HS scanner (Suwa, Japan). By using Adobe Photoshop™ version 3.0 J (San Jose, CA, USA), the intensity of the wild-type and aberrant bands on the scanned digital images were measured as follows: the degrees of average brightness for the same area of each band were quantified on a gray scale (the level ranging from 0 to 256) through the subtraction of the background brightness, then the ratio of average brightness of the aberrant band to that of the wild-type band was calculated. An intensity of less than 0.10 was regarded as negative.

Sequencing analysis

Sequencing was carried out bythe dideoxy chain termination method using a DNA sequencing kit (Applied Biosystems) and the ABI PRISM 310 Genetic Analyzer as described previously.[32]

Real-time PCR for assessment of expression levels of DSB repair genes

Expression levels of *ATM*, *MRE11*, *RAD50*, *NBS1* and *ATR* were analyzed using the TaqMan Gene Expression Assays™ according to the protocol of the manufacturer (Applied Biosystems): Expression of combined wild-type and aberrant-type

genes was measured using the following primers: *ATM*, ID Hs00175892_m1, *MRE11*; Hs00271551_m1, *RAD50*; Hs00194871_m1, *NBS1*; Hs00159537_m1, *ATR*; Hs00169878_m1, β-actin; Hs99999903_m1 (Applied Biosystems). Standard curves for quantification of the molecules were constructed from the results of simultaneous amplifications of serial dilutions of cDNA from a lymphoblastoid cell line, IB-4. Real-time PCR and subsequent calculations were carried out with an ABI Prism 7700 Sequence Detector System (Applied Biosystems). To normalize the differences in RNA degradation and RNA loading for reverse transcription-PCR in individual samples, the expression level of each molecule was divided by that of β-actin in the same samples. All experiments were performed at least in duplicate. The expression level of each gene in IB-4 was defined as 100, and the relative gene expression levels in each cell line was compared.

DNA DSB repair assays

Ability of repair for DNA DSB induced by IR in each cell line was analyzed as described elsewhere.[33,34] Briefly, more than 1×10^5 cells from each cell line were embedded in agarose plugs as described previously.[33,34] 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. Untreated plugs containing the same concentration of cells were used as controls. The plugs were embedded into wells of a 0.8% agarose gel in 1 × TAE and subjected to 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 in size migrate as a single band and form a compression zone under each well. Much smaller DNA from apoptotic fragmentations could be detected as thin smears, but not bands. Rejoined DNA fragments were too large to enter the gel. After electrophoresis, DNA was stained with ethidium bromide and visualized with UV light. The signals from the wells and compression zones were measured using FMBIO Analysis V8.0 (TaKaRa, Kusatsu, Japan). DSB were quantified as the fraction of DNA in the compression zone relative to that in the wells.

Statistical analysis

The frequency of *ATM* and *MRE11* gene mutations between MSI-positive and MSI-negative lines was calculated using Fisher's exact test. *P*-values of less than 0.01 were considered to be statistically significant. The expression levels of *ATM*, *MRE11*, *RAD50*, *NBS1* and *ATR* and DSB repair ability were calculated using the Mann–Whitney test. *P*-values of less than 0.05 were considered to be statistically significant.

Results

MSI in leukemia–lymphoma cell lines

Microsatellite instability, as determined by the presence of mutations in both *BAT-25* and *BAT-26*, was observed in 13 of 50 leukemia–lymphoma cell lines, including 11 lymphoid and two myeloid lines (Table 1). Among the B-cell lineage, MSI was found in four lines: one of 10 Burkitt lymphomas (Daudi), one of seven diffuse large B-cell lymphomas (OPL-3), and two of two precursor B-cell acute lymphoblastic leukemia lines (NALM-6 and REH). Among the T-cell lineage, MSI was found in four lines derived from T-cell acute lymphoblastic leukemia (HPB-ALL, MOLT-4, MOLT-14 and Jurkat). MSI was found in one of seven NK/NKT-cell

Table 1. Summary of microsatellite instability and mutation of intronic mononucleotide repeats of *ATM* and *MRE11* in leukemia–lymphoma cell lines

Cell line	Microsatellite instability	Shortening		Mutation of intronic mononucleotide repeat		Aberrant transcript		Real-time expression value	
		BAT-25	*BAT-26*	*ATM*	*MRE11*	*ATM*	*MRE11*	*ATM*	*MRE11*
Leukemia–lymphoma									
OPL–3	MSI	–11	–12	–2	–1	+	+	12.62	18.32
Jurkat†	MSI	–4	–9	–4	–1	+	+	23.9	78.5
P31/Fujioka	MSI	–4	–3	–1	–1	+	+	185.75	487.37
MOLT-4	MSI	–2	–4	–1	0	+	+	349.65	513.83
NALM-6	MSI	–2	–4	0	0	+	+	7.5	13.75
CTV-1	MSI	–3	Deleted	–2	0	+	+	6.17	16.15
HPB-ALL	MSI	–1	–4	0	–1	+	+	42.4	101.4
REH	MSI	–1	–4	0	0	+	+	116.08	191.55
P30/Ohkubo	MSI	–4	–5	–2	0	+	–	290.36	406.28
Daudi	MSI	–3	–2	0	Het + 1	+	–	3.17	9.7
MOLT-14	MSI	–1	–1	–3	0	+	–	2.93	5.2
KM-H2	MSI	–1	1	0	0	+	–	1.2	1.66
SNK-6	MSI	1	–1	–1	0	+	–	2.2	5.87
OPL-4	MSS	–6	0	0	–1	+	–	4.5	4.9
Katata	MSS	0	0	Het + 1	0	+	–	30.5	17.3
Colorectal cancer (control)									
HCT 116†	MSI	–8	–13	–3	–2	+	+	3.3	1.62
LoVo†	MSI	–7	–1	–1	–1	+	+	ND	ND
LS 174T†	MSI	–6	–12	–3	–2	+	+	25.4	26.7

†Shortening/lengthening of mononucleotide repeat in *RAD50* was observed. Het, heterogenous; MSI, microsatellite unstable; MSS, microsatellite stable.

doi: 10.1111/j.1349-7006.2006.00165.x

Table 2. Mutations of mononucleotide repeats and microsatellite instability (MSI) in *ATM*, *MRE11*, *RAD50*, *NBS1* and *ATR*

	Total	*ATM*	*MRE11*	*RAD50*	*NBS1*	*ATR*
Total	50	9	6	1	0	0
MSI +	13	8*	5*	1	0	0
MSI –	37	1	1	0	0	0

*$P < 0.01$ Fisher's exact test.

(SNK-6), one of six Hodgkin lymphoma (KM-H2), and one of one non-B non-T acute lymphoblastic lymphoma (P30/Ohkubo) cell lines. These results showed the frequency of MSI to be highest in acute lymphoblastic leukemia lines irrespective of immunophenotype. Among the myeloid cell lines, MSI was found in two acute myeloid leukemia lines (CTV-1 and P31/Fujioka). No MSI was found in five normal blood samples.

Mutations of mononucleotide repeats in *ATM*, *MRE11*, *RAD50*, *NBS1* and *ATR*

Mutations of the intronic poly$(T)_{15}$ nucleotide repeats in IVS 7 of *ATM* were found in nine lines (Table 2): homozygous shortening in eight lines with MSI (OPL-3, MOLT-4, MOLT-14, Jurkat, SNK-6, P30/Ohkubo, CTV-1 and P31/Fujioka) and 1-bp heterozygous lengthening in an MSI-negative cell line (Katata). Shortening by 4 bp was found in Jurkat, 3 bp in MOLT-14, 2 bp in OPL-3, P30/Ohkubo and CTV-1, and 1 bp in MOLT-4, SNK-6 and P31/Fujioka. No mutations were observed in five normal blood samples. The frequency of *ATM* mutation was significantly higher in MSI-positive than MSI-negative lines ($P < 0.01$).

Mutations in the intronic poly$(T)_{11}$ nucleotide repeats in IVS 4 of *MRE11* were found in six lines (Table 2): five mutations in MSI-positive lines (OPL-3, Daudi, HPB-ALL, Jurkat and P31/Fujioka) and one mutation in an MSI-negative line (OPL-4). All but one (Daudi) of these lines showed shortening by only 1 bp. Except for the 1-bp heterozygous lengthening found in the Daudi cell line, all mutations were homozygous. No mutations were observed in five normal blood samples. The frequency of *MRE11* mutation was significantly higher in MSI-positive than MSI-negative cell lines ($P < 0.01$).

As for the ORF of *RAD50*, 1-bp heterozygous lengthening was found in the poly$(A)_9$ nucleotide repeats in the Jurkat cell line. No mutation was found in the poly$(A)_7$ nucleotide repeats in the ORF of *NBS1* or poly$(A)_{10}$ nucleotide repeats in the ORF of *ATR*. It is noteworthy that the MSI-positive Jurkat line showed mutations in *ATM*, *MRE11* and *RAD50*.

Aberrant splicing in *ATM* (497del22) and *MRE11* (315del88)

The intronic mononucleotide repeat mutations were expected to generate aberrant splicing in the next exon of *ATM* (497del22) and *MRE11* (315del88), as previously reported in colorectal cancer cell lines (Fig. 1A).[18,19] PCR amplification of the cDNAs for fragments containing exon 8 of *ATM* and exon 5 of *MRE11* was carried out using primer pairs as mentioned before. The electrophoresis revealed that aberrant bands in *ATM* were observed in two of five normal blood samples, suggesting the presence of transcript at a lesser intensity in normal controls (Fig. 1B). Aberrant bands were observed in 13 of 13 (100%) MSI-positive lines and in 21 of 37 (56.8%) MSI-negative lines. Among the 13 MSI-positive lines, eight (61.5%) showed intronic mononucleotide mutations, whereas only one of the 21 MSI-negative lines with aberrant bands (4.8%) showed an intronic mutation. The RI was stronger in five MSI-positive lines (P30/Ohkubo, Jurkat, OPL-3, CTV-1 and MOLT-14) harboring intronic mononucleotide mutations of 2 bp or more (RI, mean 1.03 ± 0.35) compared to MSI-positives lines without mutations or with only 1-bp mutations (RI, mean 0.32 ± 0.15) (Fig. 1B). The RI in one MSI-negative line (Katata) and two normal blood samples in which aberrant bands were observed was 0.13 ± 0.07, 0.21 ± 0.04 and 0.29 ± 0.03, respectively.

The aberrant bands for *MRE11* were found in eight of 13 (61.5%) MSI-positive and two of 37 (5.4%) MSI-negative lines. Aberrant bands were not observed in normal blood samples. Among the eight MSI-positive lines with aberrant bands, 1-bp intronic mononucleotide mutations were found in four lines (Jurkat, OPL-3, HPB-ALL and P31/Fujioka), which also showed a stronger RI of the aberrant to wild-type bands (RI, mean 0.80 ± 0.46) as compared to lines without intronic mononucleotide mutations (RI, mean 0.20 ± 0.12). The RI of two MSI-negative lines with aberrant bands was 0.11 ± 0.01 for CCRF-CEM and 0.19 ± 0.09 for Namalwa.

As representatives, the aberrant bands found in the OPL-3 line were cloned and sequenced, revealing a 22-bp deletion in exon 8 of *ATM* (497del22, GenBank accession no. U33841) and the exclusion of the whole exon 5 (which is normally included in mRNA) of *MRE11* (315del88, GenBank accession no. NM_005591) (data not shown). Adenine within the start codon was defined as nucleotide number 1.

Expression levels of *ATM*, *MRE11*, *RAD50*, *NBS1* and *ATR*

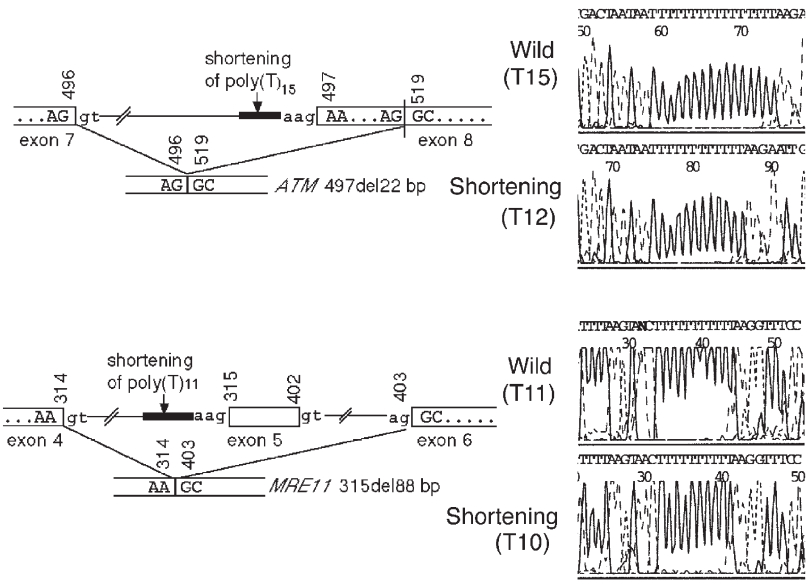
In the real-time PCR, the expression levels of *ATM and MRE11* in 13 MSI-positive lines (mean 80.30 ± 120.19 and 142.28 ± 195.27, respectively) were significantly higher than those in 37 MSI-negative lines (mean 30.07 ± 41.44 and 57.86 ± 91.81, respectively; $P < 0.05$) (Fig. 2A). In contrast, no significant differences were found in the expression levels of *RAD50*, *NBS1* and *ATR* between MSI-positive and MSI-negative lines (mean 33.03 ± 50.52 and 26.54 ± 35.18, 106.83 ± 183.28 and 51.79 ± 77.82, 64.63 ± 85.03 and 37.82 ± 49.02, respectively).

The expression levels of *ATM*, *MRE11* and *NBS1* were higher in cell lines with *ATM* mutations (mean 100.45 ± 137.82, 172.09 ± 225.61 and 137.08 ± 215.73, respectively) than in lines without mutations (mean 30.57 ± 42.30, 59.64 ± 90.61 and 50.46 ± 74.43, respectively; $P < 0.05$) (Fig. 2B). Mutation of *MRE11* upregulated the expression of *MRE11* itself, whereas the expression of other genes was not affected (data not shown).

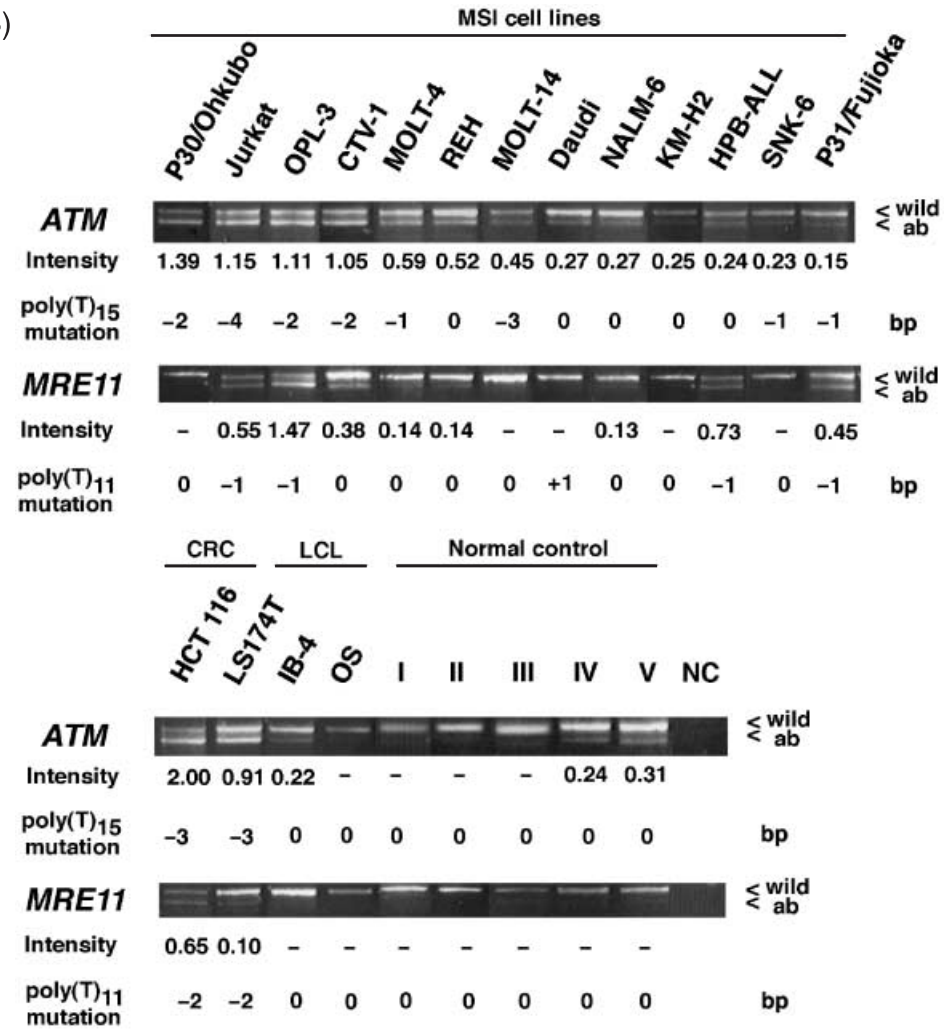
DSB repair assays

To evaluate whether defects in MMR function may correlate with impairment of the DSB repair system in leukemia–lymphoma cell lines, kinetics of DNA DSB repair were examined using the PFGE. More than 80% of the DSB in IB-4, a lymphoblastoid cell line derived from healthy individuals, were repaired within 2 h of IR exposure (Fig. 3A). Kinetics of DNA DSB repair was examined in 22 cell line selected

(A)



(B)



doi: 10.1111/j.1349-7006.2006.00165.x

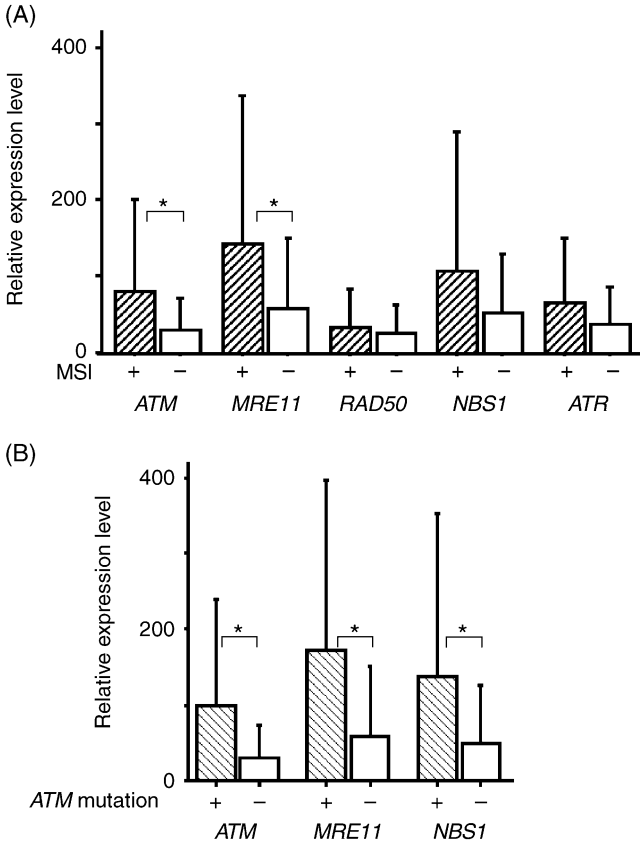


Fig. 2. (A) Gene expression levels of *ATM* and *MRE11* were significantly higher in microsatellite instability (MSI)-positive than MSI-negative leukemia–lymphoma cell lines, whereas there was no significant difference in the expression of *RAD50*, *NBS1* or *ATR*. (B) In cell lines with a mutation in the intronic poly(T)$_{15}$ nucleotide repeats in intervening sequence (IVS) 7 of *ATM*, the expression levels of *MRE11* and *NBS1* were also significantly different from those in lines without the mutation. The expression level of each gene in IB-4 was defined as 100, and the relative gene expression levels in each cell line was calculated. *$P < 0.05$.

randomly, comprising eight MSI-positive and 14 MSI-negative cell lines (Fig. 3B). DSB that were unrepaired after 2 h of incubation were then compared. Degrees of delay or abrogation of DSB repair, as shown by unrepaired DSB in eight MSI-positive lines (mean 0.42 ± 0.25), were significantly higher than those in 14 MSI-negative lines (mean 0.16 ± 0.10; $P < 0.05$) (Fig. 3C).

Discussion

Thirteen (26.0%) of 50 leukemia–lymphoma cell lines showed MSI, as determined by mutations in both *BAT-25* and *BAT-26*. Among these 13 lines, mutations of intronic mononucleotide repeats in *ATM* and *MRE11* were found in eight (61.5%) and five (38.5%) lines, respectively. MSI status in leukemia–lymphoma cell lines were also reported by Takeuchi *et al*.[35] (12 of 49 lines; 24.5%), Inoue *et al*.[17] (11 of 91 lines; 12.1%) and Matheson and Hall[36] (five of nine lines; 55.6%). Several cell lines showed different MSI status among these studies. The REH cell line was MSI-positive in the present study as well as in those of Matheson and Hall[36] and Takeuchi *et al*.,[35] but was MSI-negative in the study by Inoue *et al*.[17] The Daudi cell line, which was MSI-positive in the present study, was previously reported as MSI-negative by Inoue *et al*.[17] and by Matheson and Hall.[36] These discrepancies might be caused by differences in the methods and definitions used in each study. The criteria in hematolymphoid malignancies has not been established as yet. In the present study, the presence of MSI was determined when mutations of both *BAT-25* and *BAT-26* were observed at GeneScan analysis. This criteria has been validated in defining high-frequency of MSI in colorectal neoplasms,[11] and was used in hematolymphoid malignancies.[31]

The same mutation of *MRE11* has been reported in MSI-positive colorectal, endometrial, ovarian and stomach tumors,[19–21] whereas the information for mutation of *ATM* is limited to colorectal cancer cell lines.[18] The mutations in mononucleotide repeats result in splicing abnormalities, which generate aberrant transcripts of *ATM* and *MRE11*. The RI of the aberrant *ATM* transcripts was higher in lines harboring a mutation of 2 bp or more in the intronic mononucleotide repeats than in those without the mutations. The mononucleotide repeats, especially polypyrimidine tracts together with GU and AG dinucleotides at the exon–intron and intron–exon junctions, respectively, function as splicing regulatory elements to ensure high accuracy of pre-mRNA splicing site selection.[37] The present data provided evidence that a defective MMR system affects the polypyrimidine tracts, thus resulting in a disorder of the splicing mechanism.

Despite the fact that both *ATM* and *MRE11* were frequently mutated and aberrantly spliced in the MSI-positive cell lines, the present data also showed differences. Eight of 13 MSI-positive lines showed aberrant *MRE11* transcripts, whereas only two MSI-negative lines and no normal controls showed aberrant *MRE11* transcripts. Furthermore, the DNA repair

Fig. 1. (A) Schematic representation of a 22-bp deletion of exon 8 of *ATM* (497del22) and the skipping of exon 5 of *MRE11* (315del88). Length variation of the poly(T)$_{15}$ nucleotide repeats in intervening sequence (IVS) 7 of *ATM* was expected to cause aberrant splicing, which results in a 22-bp deletion in exon 8. Length variation of the intronic poly(T)$_{11}$ nucleotide in IVS 4 of *MRE11* was expected to cause aberrant splicing, which results in the skipping of exon 5. Nucleotide numbering starts with the adenine residue in the start codon. (B) Aberrant transcripts of *ATM* and *MRE11*. Polymerase chain reaction amplification of the cDNA of 13 microsatellite instability (MSI)-positive leukemia–lymphoma cell lines for fragments containing exon 8 of *ATM* and exon 5 of *MRE11* were carried out. Aberrant bands for *ATM* and *MRE11* were observed in 13 (100%) and eight (61.5%) lines, respectively. Two lymphoblastoid cell lines (IB-4 and OS) and five peripheral lymphocytes from healthy volunteer were used as controls, whereas two colorectal carcinoma cell lines (HCT 116 and LS 174T) known to harbor MSI were used as positive controls. The ratios of intensity (RI) of the aberrant to wild-type band was measured using Adobe Photoshop™. A ratio of less than 0.10 was regarded as negative. The intensity of the *ATM* aberrant transcript was stronger in five lines (P30/Ohkubo, Jurkat, OPL-3, CTV-1 and MOLT-14) harboring a mutation of 2 bp or more in intronic poly(T)$_{15}$ nucleotide repeats in IVS 7 of *ATM* (RI, mean 1.03 ± 0.35) than in those lines without a mutation or with only a 1-bp mutation (RI, mean 0.32 ± 0.15). Of the lines with a detectable *MRE11* aberrant transcript, strong intensity was observed in four lines (Jurkat, OPL-3, HPB-ALL and P31/Fujioka) with mutations in the intronic poly(T)$_{11}$ nucleotide repeats in IVS 4 of *MRE11* (RI, 0.80 ± 0.46) compared to those without mutation (RI, 0.20 ± 0.12). ab, aberrant band; CRC, colorectal cancer cell line; NC, negative control; wild, wild-type band. *Length variation was detected as heterozygous.

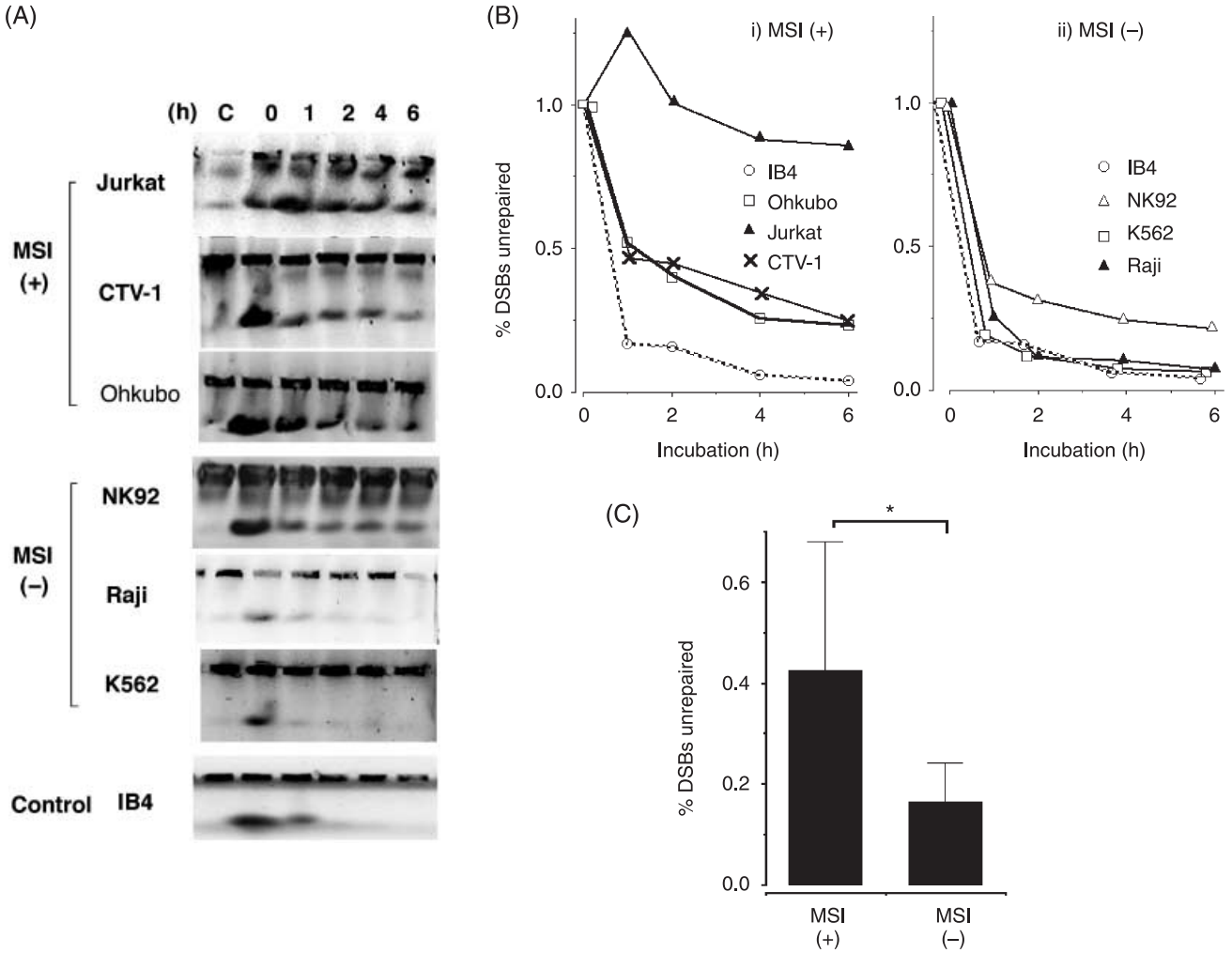


Fig. 3. Induction and repair of DNA double-strand breaks (DSB) after ionizing radiation (IR) exposure determined by pulsed-field gel electrophoresis (PFGE). (A) Ethidium bromide-stained gel after PFGE. Equal numbers of cells were embedded in 0.8% agarose plugs and exposed to 20 Gy IR. Cells were allowed to repair DSB for 0–6 h at 37°C, they were then lysed to extract DNA 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 using the results of representative PFGE. The percentage of unrepaired DSB was calculated by the ratio of signal from DSB DNA in the CZ to DNA in the corresponding well. The percentage of unrepaired DSB at 0 h of incubation was set at 100%. Symbols represent cell lines: (i) IB-4 (○), Ohkubo (□), Jurkat (▲), CTV-1 (×) (ii) IB-4 (○), NK-92 (△), K-562 (□), Raji (▲). (C) Repair of DSB after 2 h incubation were compared between MSI-positive and MSI-negative cell lines. Delay or abrogation of DSB repair as shown by unrepaired DSB in eight MSI-positive lines (mean 0.42 ± 0.25) was significantly higher than that in 14 MSI-negative lines (mean 0.16 ± 0.10) ($P < 0.05$).

function was much more impaired in those lines harboring *MRE11* mutations than those without the mutations. These findings confirmed that *MRE11* is a target gene for MMR inactivation in leukemia–lymphoma cell lines as well as in other malignancies reported previously.[19–21]

As for *ATM*, strong expression of aberrant *ATM* transcripts were detected in lines with the MSI phenotype. Ejima *et al*. described the same aberrant splicing (497del22) exclusively in four colorectal cell lines with polypyrimidine tract mutations within the preceeding intron.[18] Aberrantly spliced transcripts with lesser intensities were also observed in half of the MSI-negative lines (56.8%) and in normal controls (40.0%). Recent studies have demonstrated that *ATM* has multiple functions other than DSB repair, such as cell-cycle checkpoint, telomere maintenance and induction of apoptosis.[1,3,4,6,7] The significance of the aberrant *ATM* transcripts is not clear as yet, because of the limited number of studies pertaining to its function.

Gene expression levels of *ATM* and *MRE11* were significantly higher in MSI-positive lines than MSI-negative lines in the present study, which is in contrast to the studies of Ejima *et al*. and Giannini *et al*. using colorectal cancer cell lines.[18,19] The present experiment included the same two cell lines (HCT 116 and LS 174T) used in their studies. Expression levels of all genes examined in these lines were apparently lower than the mean expression levels of MSI-positive leukemia–lymphoma cell lines. Therefore the difference in gene expression levels found between our study and that of Giannini *et al*. might be due to the origin of the tumors. *NBS1* expression was increased in the cell lines with *ATM*

doi: 10.1111/j.1349-7006.2006.00165.x

mutations, although there was no mutation in the *NBS1* gene. ATM phosphorylates NBS1 on serine-343, which may change the kinetics of the NBS1 protein as well as NBS1 mRNA. This might be a reason why *NBS1* expression was increased in cell lines with *ATM* mutations even though there was no mutation in the *NBS1* gene.[3,4]

Other genes engaged in DNA repair, *RAD50*, *NBS1* and *ATR*, did not show mutations in any mononucleotide repeats except in one cell line. Expression levels of these genes did not differ between MSI-positive and MSI-negative leukemia–lymphoma cell lines. The sensitivity of detecting the mutations in the present study would be adequate because the mutations detected in the two cell lines (HCT116 and LS174T) of this study were identical to those reported in a previous study (data not shown). ATR and RAD50 were reported to be necessary not only for DNA damage repair but also for normal growth of the individual cells.[8–10,38] The absence of mutations in mononucleotide repeats in *ATR* and *RAD50* might be due to their essential role for cell survival.

The present study suggests that impairment of the MMR system generates aberrant transcripts in DSB repair genes, especially *ATM* and *MRE11* but not *RAD50*, *NBS1* or *ATR*, in hematolymphoid malignancies. This might result in inactivation of the DSB repair system, thus inducing a harmful chain reaction such as an acceleration of genome instability and accumulation of genetic damage.

Acknowledgments

We thank Dr T. Al Saati (Laboratoire d'Anatomie Pathologique CHU Purpan, Toulouse, France) for providing the Deglis cell line, Dr G. W. Bornkamm (GSF-Research Center for Environment and Health, Munich, Germany) for BL36, BL60 and BL137, Dr E. Kieff (Brigham and Women's Hospital, Boston, MA, USA) for IB-4, Dr P. W. Tucker (University of Texas, Austin, TX, USA) for NAB-2, Drs M. Daibata and S. Imai (Kochi Medical School, Nangoku, Japan) for Pal-1 and Katata, Drs M. Matsuoka and Y. Taniguchi (Kyoto University, Kyoto, Japan) for MT-1, MT-2, MT-4 and TL-Om1, Dr Y. Matsuo (Hayashibara Biochemical Laboratories, Okayama, Japan) for MOTN-1 and HEL, Dr N. Shimizu (Tokyo Medical and Dental University, Tokyo, Japan) for SNT-8 and SNK-6, and Dr M. Yagita (Tazuke-Kofukai Medical Research Institute, Osaka, Japan) for the KHYG-1 cell line. This work was supported by grants from the Ministry of Education, Science, Culture and Sports, Japan (15406013, 16390105, 40244933).

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doi: 10.1111/j.1349-7006.2006.00165.x