EMBO reports

scientific report

Human MRE11 is inactivated in mismatch repair-deficient cancers

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Received October 17, 2001; revised December 19, 2001; accepted January 4, 2002

Mutations of the *ATM* and *NBS1* genes are responsible for the inherited Ataxia-Telangiectasia and Nijmegen Breakage Syndrome, both of which are associated with a predisposition to cancer. A related syndrome, the Ataxia-Telangiectasia-like disorder, is due to mutations of the *MRE11* gene. However, the role of this gene in cancer development has not been established. Here we describe an often homozygous mutation of the poly(T)11 repeat within human *MRE11* intron 4 that leads to aberrant splicing, impairment of wild-type MRE11 expression and generation of a truncated protein. This mutation is present in mismatch repair-deficient, but not proficient, colorectal cancer cell lines and primary tumours and is associated with reduced expression of the MRE11–NBS1–RAD50 complex, an impaired S-phase checkpoint and abrogation of MRE11 and NBS1 ionizing radiation-induced nuclear foci. Our findings identify MRE11 as a novel and major target for inactivation in mismatch repair-defective cells and suggest its impairment may contribute to the development of colorectal cancer.

INTRODUCTION

Ataxia-Telangiectasia (A-T) and the Nijmegen Breakage Syndrome (NBS) are rare autosomal recessive diseases that are due to mutation of the *ATM* and *NBS1* genes, respectively. They are characterized by hypersensitivity to ionizing radiation, immunodeficiency and an increased predisposition to cancer (Shiloh, 1997). A milder form of A-T, the Ataxia-Telangiectasia-like disorder (AT-LD) has been linked recently to mutation of the human *MRE11* gene (Stewart *et al.*, 1999). Although clinical differences exist amongst these three syndromes, the striking similarity of the cellular phenotypes of A-T, NBS and AT-LD (Shiloh, 1997; Petrini, 2000) has lead to the hypothesis that the gene products ATM, NBS1 and MRE11 work in the same pathway. Indeed, in response to DNA damage induced by ionizing radiation, the ATM kinase phosphorylates NBS1 on serine 343, an event required for the activation of the S-phase checkpoint (Gatei *et al.*, 2000; Lim *et al.*, 2000) and the relocalization of the MRE11–NBS1–RAD50 (M–N–R) complex to ionizing radiation-induced nuclear foci (IRIF), which represent sites at which DNA is undergoing repair (Nelms *et al.*, 1998). As is clearly shown in yeast, the M–N–R complex, involving the NBS1 homologue Xrs2, participates in the DNA double-strand break (DSB) repair via the homologous recombination and the non-homologous end-joining pathways (Haber, 1998; Khanna and Jackson, 2001).

Mutations in *ATM* are firmly implicated in cancer (Shiloh, 1997; Stankovic *et al.*, 1999; Schaffner *et al.*, 2000), and NBS patients display an increased risk for lymphoproliferative disorders (Shiloh, 1997). Although *MRE11* mutations are also suspected to predispose to cancer via the inactivation of the M–N–R complex, this conclusion has been hampered by the cell-lethal phenotype of *MRE11* gene knockout (Xiao and Weaver, 1997) and the paucity of AT-LD families described so far (Petrini, 2000). Therefore, a direct link between MRE11 mutations and cancer has not been established yet. Here we report the identification of a splicing defect of the MRE11 precursor transcript linked to mutations of a poly(T)11 repeat within intervening sequence 4 (IVS-4). This mutation was found

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exclusively in mismatch repair (MMR)-deficient cancer cell lines and primary tumours, and is associated with a defective S-phase checkpoint and abrogation of MRE11 and NBS1 IRIF formation.

RESULTS AND DISCUSSION

To search for *MRE11* mutations, we PCR amplified the MRE11 transcript from a panel of cancer cell lines. An exponential phase amplification from SW48, LoVo, HCT116, MIP and LS174T colorectal cancer (CRC) cell lines produced extremely reduced levels of the 5′-fragment compared with other CRC cell lines (Figure 1A). The appearance of a second product suggested the presence of a second transcript in these cells (Figure 1B). Direct sequencing of the PCR products identified the higher molecular weight band as the wild-type (WT) MRE11 cDNA fragment (Figure 1C). The faster migrating band (defined as 484del88) contained a complete deletion of exon 5 (Figure 1D). The derived transcript is predicted to generate a truncated protein comprising the first 105 (of the 708) amino acids of the WT MRE11 followed shortly by a stop codon (Figure 1D). The skipping of exon 5 might be due to mutation of the splicing signal sequences. We noticed that the polypyrimidine tract of the 3′-splice site of the human *MRE11* IVS-4 is composed of 11 Ts (Figure 2A). Intriguingly, we found the aberrant 484del88 MRE11 transcript in MMR-deficient cell lines selectively; in contrast, the MMR-proficient cells only expressed high levels of WT MRE11 mRNA (Figure 1A and B; Table I). Therefore, we tested whether the partial splicing defect leading to the generation of the 484del88 transcript was due to alterations at the IVS-4 poly(T) tract. All the MMR-proficient cells were homozygous for the WT poly(T)11 allele (Figure 2B; Table I; data not shown). In contrast, we found deletions of one or two bases involving both *MRE11* alleles in LoVo, SW48, HCT116, LS174T and MIP. In agreement with the observation that shortening the length of a stretch of 11 continuous uridines reduces its strength as a signal sequence in splicing competition assays (Coolidge *et al.*, 1997), these data suggest that the shortening of the *MRE11* poly(T) tract strongly reduces, but does not abolish completely, the correct splicing process. The occurrence of alterations in this repeat and the appearance of the 484del88 transcript was not restricted to CRC cells; rather they appeared to be associated with a MMR-deficient phenotype, since they were also present in MMR-deficient prostate cancer Du145 and endometrial carcinoma AN3CA cells (Figure 1B; data not shown). To date, we have found no evidence for a mutation of this type in a number of MMR-proficient cancer cell lines (data not shown).

We also observed mutations at the (T)11 repeat in primary CRCs (Table II). Shortened (T)9 and/or (T)10 alleles were detected in 93% (14 out of 15) of the MMR-deficient colon cancers analysed. In >50% (eight out of 15) of the cases, the WT allele was absent or under-represented compared with the mutant alleles, indicating that the tumour may be homozygous for the alteration (Figure 3; Table II). In contrast, MRE11 mutations were never found in non-cancer colonic mucosa surrounding the MMR-deficient CRCs, in MMR-proficient CRCs or peripheral blood (Figure 3; Table II). Therefore, this newly described mutation is highly associated with MMR-deficient CRC *in vivo*. Furthermore, while we readily PCR amplified the WT MRE11 transcript in three out of three MMR-proficient primary CRCs, we failed to detect its expression in three out of three MMR-deficient CRC

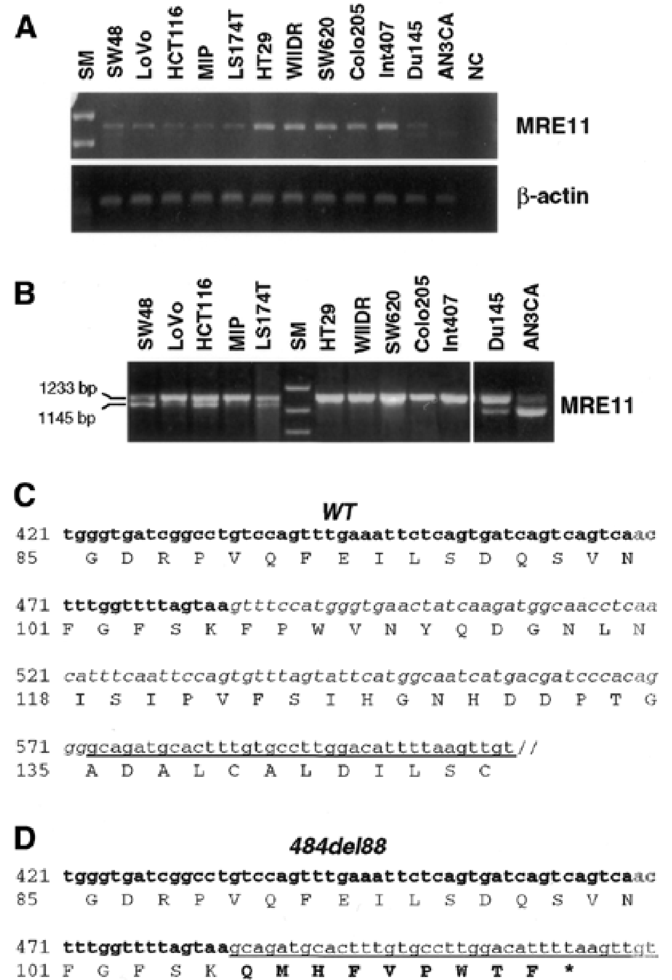

Fig. 1. Identification of the 484del88 mutant transcript in MMR-defective cell lines. (**A** and **B**) In a number of CRC (SW48, LoVo, HCT116, MIP and LS174T), prostate (Du145) and endometrial (AN3CA) MMR-deficient cancer cell lines, an exponential phase RT–PCR amplification of the MRE11 1233 bp 5′-fragment (A, 20 cycles) shows reduced expression of the WT transcript compared with MMR-proficient CRC cells and the appearance of a shorter band of 1145 bp that is even better appreciated in a plateau level amplification (B, 35 cycles). (**C**) By direct sequencing, we identified the 1233 bp amplicon as the WT transcript. Part of this fragment (*WT*) is shown in the figure to indicate the position of exons 4, 5 and 6 nucleotide sequences (represented in bold, italics and underlined plain text, respectively). (**D**) The 1145 bp fragment (*484del88*) missed 88 nucleotides (starting from nucleotide 484 of the human MRE11 mRNA sequences deposited under DDBJ/EMBL/GenBank accession No. NM_005591) corresponding to exon 5 of the human genomic *MRE11* sequence (DDBJ/EMBL/GenBank accession No. NT_009090). Skipping exon 5 generates a mutant transcript, which, due to an out-of-frame rejoining of exon 4 to exon 6, is predicted to code for the first 105 (of the total 708) amino acids of the WT MRE11 protein plus an additional nine amino acids followed by a stop codon.

cases (Figure 3B). In these samples, the expression of the 484del88 transcript and some WT MRE11 mRNA could only be revealed by a nested RT–PCR (data not shown). This suggests that the ratio between the WT and aberrant 484del88 transcript detected by PCR in MMR-defective cells could be affected by both impairment of WT splicing and selective degradation of the

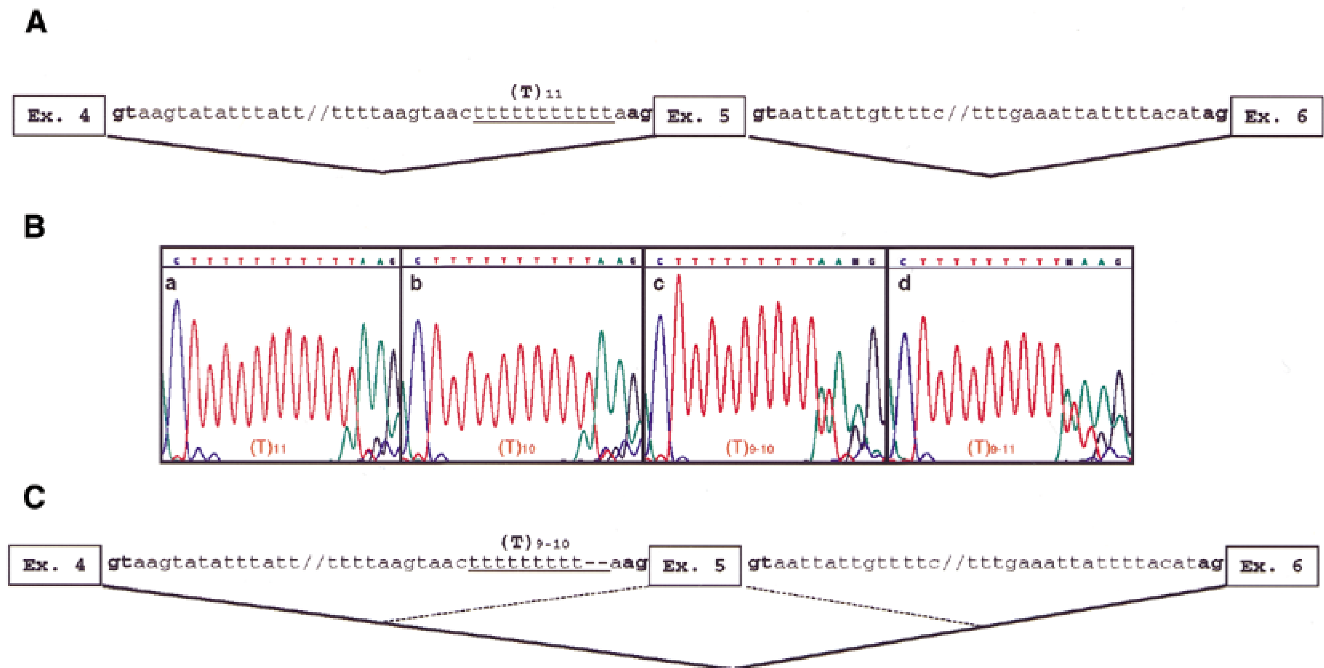

Fig. 2. Mutations at the IVS-4 (T)11 mononucleotide repeat of the human *MRE11* gene in MMR-deficient CRC cell lines. (**A**) Schematic representation of the genomic sequence including IVS-4 and -5 splicing consensus sequences [underlined text, pyrimidine stretches; bold, gt/ag splicing signals; continuous lines, predominant (WT) splicing process]. (**B**) Electropherograms showing the appearance of the MRE11 poly(T)11 repeat obtained by direct sequencing of the amplicons obtained from all CRC cells listed in Table I: (a) WT (T)11 repeat representative of all MMR proficient cell lines; (b) SW48 and LoVo; (c) LS174T and HCT116; and (d) MIP cell lines. (**C**) In the presence of the poly(T)11 repeat mutation, the correct rejoining of exon 4 to exon 5 is impaired (dashed line), while the rejoining of exon 4 to exon 6, skipping exon 5 (continuous line), occurs.

Table I. Relevant features of the CRC cell lines including MMR status, *MRE11* alleles at the IVS-4 poly(T)11 and *RAD50* alleles at the coding sequence poly(A)9

Cell line	MMR[a]	*MRE11* (T)11 alleles	*RAD50* (A)9 alleles
Int407	+	(T)11	(A)9
HT29	+	(T)11	(A)9
WIDR	+	(T)11	(A)9
SW620	+	(T)11	(A)9
Colo205	+	(T)11	(A)9
SW48	–	(T)10	(A)9
LoVo	–	(T)10	(A)8/(A)9
HCT116	–	(T)9/(T)10	(A)8/(A)9
MIP	–	(T)9/(T)10/(T)11	(A)9
LS174T	–	T(9)/T(10)	(A)9/(A)10

[a]According to the literature and confirmed in our laboratory.

484del88 transcript. In fact, an aberrant MRE11 transcript was found recently to be degraded by nonsense-mediated decay (NMD), a process requiring active translation (Maquat, 2000; Pitts *et al.*, 2001). To test whether 484del88 is also degraded through NMD, we studied the effects of cycloheximide on the expression of MRE11 transcripts. Supporting our hypothesis, cycloheximide induced a consistent reduction in the ratio between the WT and aberrantly spliced transcript in SW48, HCT116 and LoVo cells compared with untreated controls (Figure 3C). To prove finally that the expression of the WT MRE11 is impaired by the described mutation at the *MRE11* IVS-4 splicing site, we analysed MRE11 expression by northern and western blotting. We observed a strongly reduced expression of MRE11 at either the mRNA (Figure 3D) or protein (Figure 4A) level in MMR-deficient cells compared with the MMR-proficient CRC cells lines and the normal colonic mucosa cell line Int407.

In AT-LD cell lines, the expression of MRE11 mutant transcripts is associated with reduced expression of the three proteins of the M–N–R complex, impaired ionizing radiation-induced S-phase checkpoint [measured as radio-resistant DNA synthesis (RDS)] and defective IRIF formation (Stewart *et al.*, 1999). To explore the phenotypic consequences of the newly described MRE11 mutation, we also analysed the levels of NBS1 and RAD50 proteins in all CRC cells. Intriguingly, the expression of the $p95^{NBS1}$ and RAD50 proteins was reduced severely in all MMR-deficient cells also (Figure 4A). In contrast, the levels of all three M–N–R proteins were similar to those of the untransformed colonic mucosa Int407 and lymphoblastoid cell lines in all MMR-proficient CRC cells (Figure 4A; data not shown). Among them, only HT29 provided some evidence of reduced expression of $p95^{NBS1}$ (Figure 4A). Although the molecular base of this finding is still unclear, the M–N–R complex is functional in these cells (see below). This is consistent with the absence of a detectable phenotype in NBS heterozygous carriers, although their cells show reduced $p95^{NBS1}$ levels (Shiloh, 1997).

Table II. *MRE11* alleles at the IVS-4 poly(T)11 and *RAD50* alleles at the coding sequence poly(A)9 in CRC primary tumours

Sample	MMR[a]	Familial forms, sporadic cancers	MMR mutant[b]	*MRE11* alleles normal tissue[c]	*MRE11* alleles tumour tissue	*RAD50* (A)9 tumour tissue
C226	–	hnpcc-like	MSH2	(T)11	(T)9< (T)10 >>(T)11	(A)9
C313	–	spor	MLH1	ND	(T)10<< (T)11	(A)9
C337	–	hnpcc-like	MLH1	(T)11	(T)9 = (T)10 >>(T)11	(A)9
C359	–	spor	WT	(T)11	(T)10 >>(T)11	(A)9
C418	–	spor	ND[d]	(T)11	(T)9< (T)10 >>>(T)11	(A)9
C430	–	spor	ND	(T)11	(T)10 >>>(T)11	(A)9
C446	–	hnpcc	MSH2	(T)11	(T)9 >(T)10 >>(T)11	(A)8/(A)9
C459	–	hnpcc	WT	(T)11	(T)9 = (T)10 >>>(T)11	(A)8/(A)9
C469	–	spor	WT	(T)11	(T)10 = (T)11	(A)9
CRC5	–	spor	WT	(T)11	(T)9< (T)10 >>>(T)11	(A)9
CRC10	–	spor	MSH2	(T)11	(T)9 >(T)10	(A)8/(A)9
CRC13	–	spor	WT	(T)11	(T)10	(A)9
CFS185	–	hnpcc	WT	ND	(T)9 = (T)10 >>(T)11	(A)9
CFS200	–	hnpcc-like	MLH1	(T)11	(T)9< (T)10 >>(T)11	(A)9
CFS268	–	hnpcc-like	ND	(T)11	(T)9<< (T)10 >(T)11	(A)9
C393	+	spor	ND	ND	(T)11	ND
C396	+	spor	ND	ND	(T)11	ND
C424	+	spor	ND	ND	(T)11	ND
C450	+	spor	ND	ND	(T)11	ND
C460	+	spor	ND	ND	(T)11	ND
C494	+	spor	ND	ND	(T)11	ND
C503	+	spor	ND	ND	(T)11	ND
CRC4	+	spor	ND	ND	(T)11	ND
CRC8	+	spor	ND	ND	(T)11	ND
CRC11	+	spor	ND	ND	(T)11	ND
CRC14	+	spor	ND	ND	(T)11	ND
CRC17	+	spor	ND	ND	(T)11	ND
CRC19	+	spor	ND	ND	(T)11	ND
CRC20	+	spor	ND	ND	(T)11	ND

[a]MMR status was defined according to the definition of the National Cancer Institute.
[b]MSH2 and MLH1 genes were screened for mutation as reported (Viel *et al.*, 1997).
[c]Normal tissue adjacent to the malignant area or, when unavailable, peripheral blood from the same patient.
[d]ND, not done.

We then assessed the integrity of the S-phase checkpoint in the CRC cell lines. Following exposure of cells to 10 or 20 Gy γ rays, DNA synthesis was reduced markedly in MMR-proficient cells (Figure 4B) expressing high levels of M–N–R complex proteins. In sharp contrast, the MMR-defective cells expressing minimal levels of M–N–R complex proteins, such as the LS174T, LoVo and MIP cell lines (Figure 4A), were significantly more resistant to DNA synthesis inhibition (Figure 4B) and they still showed between 69 and 75% of DNA synthesis after irradiation with 10 Gy, and between 58 and 65% after 20 Gy, compared with unirradiated controls. Thus, they displayed an RDS phenotype similar to AT-LD cell lines, whose DNA synthesis was reported to be reduced to only 70–80% and 55–65% after exposure to 10 and 20 Gy, respectively (Stewart *et al.*, 1999). Furthermore, following radiation, MRE11 and NBS1 IRIFs were observed in the majority of WIDR, HT29 and Int407 cells (Figure 4C; data not shown). In contrast, there was no significant formation of IRIFs in MIP, LoVo, LS174T and HCT116 cells (Figure 4C; data not shown). These results indicate that at least two known functions of the M–N–R complex are defective in mutated *MRE11*, but not WT CRC, cell lines.

Mutations of mononucleotide repeats within coding sequences are frequent in MMR-deficient cells (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Thibodeau *et al.*, 1993), and they

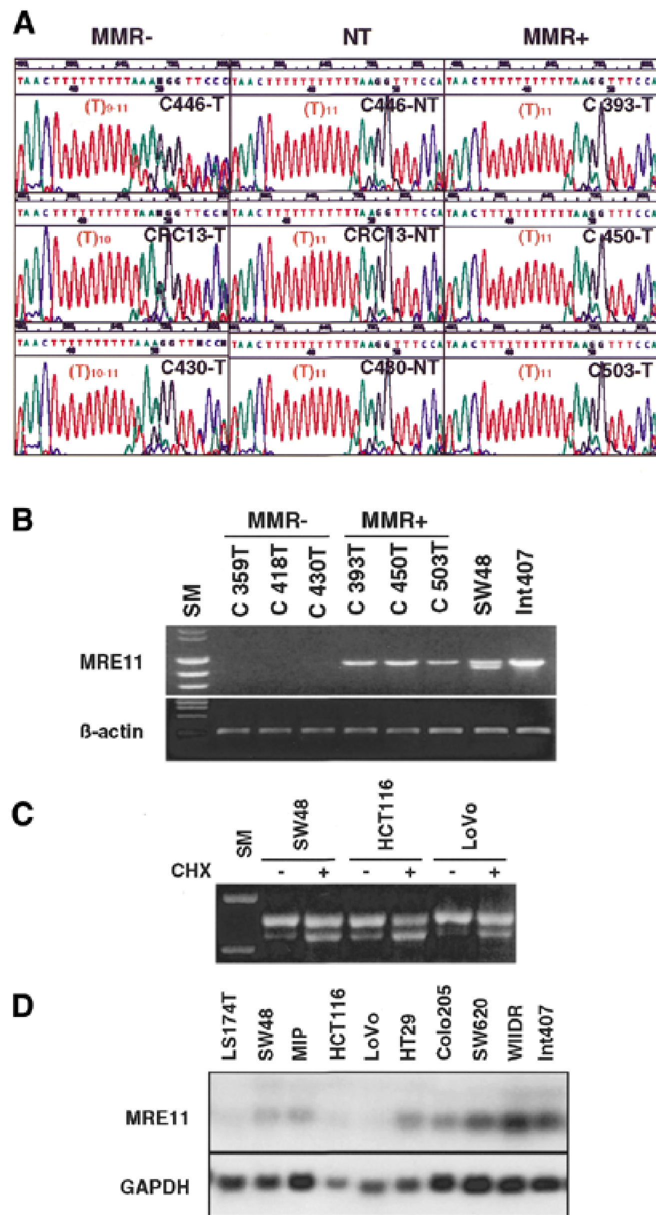

Fig. 3. Mutations of the IVS-4 (T)11 repeat of the human *MRE11* gene cause an altered expression of MRE11 in MMR-deficient colorectal primary tumours. (**A**) Electropherograms representative of the appearance of the *MRE11* poly(T)11 repeat in some of the primary MMR-deficient CRC samples (MMR–), the corresponding normal tissue (NT) and MMR-proficient CRC samples (MMR+; described in Table II). (**B**) RT–PCR amplification of the 5′-fragment of the human MRE11 transcript in MMR-deficient and -proficient primary CRC samples. Int407 and SW48 cell lines are controls for the amplification of the WT fragment and the 484del88 mutant form, respectively. β-actin amplification from the same RT reaction provides a quality and loading control. (**C**) Treatment of SW48, HCT116 and LoVo CRC cells with cycloheximide increased the absolute amount of the 484del88 transcript and the ratio between 484del88 versus WT transcript expression in all cell lines tested, confirming that the 484del88 form is subjected to NMD. (**D**) Northern blot analysis of total RNA showing a decreased amount of MRE11 transcript in 484del88 expressing and MMR-deficient LS174T, SW48, MIP, HCT116 and LoVo cells, but not in CRC cells expressing the WT MRE11 mRNA only (HT29, Colo205, SW620, WIDR and Int407).

contribute to carcinogenesis through the inactivation of cancer-related genes, including TGFβ-RII, IGF-IIR and Bax. In this paper, we have shown that mutations of an intronic repeat in the human *MRE11* gene result in aberrant splicing, giving rise to the unstable 484del88 transcript and an associated reduced expression of all components of the M–N–R complex. Examples of MRE11 mutations have been reported in breast and hematological malignancies (Fukuda *et al.*, 2001). However, their functional consequences had not been investigated. In contrast, we showed that CRC cells expressing the 484del88 aberrant MRE11 transcript are defective in the radiation-induced S-phase check-point and IRIF relocalization. The occurrence of a monoallelic inactivation of RAD50 in ~30% of MMR-deficient CRC cell lines and primary tumours (Tables I and II; Kim *et al.*, 2001) might add a further contribution to the inactivation of the M–N–R complex, although this aspect needs to be investigated further. However, the MRE11 alteration we described appears to be sufficient to impair both the S-phase checkpoint and M–N–R complex relocalization to IRIF in the MMR-defective MIP cell line, where we failed to detect mutations in the *RAD50* (A)9 repeat and *NBS1* gene (Table I; data not shown). Inactivation of other components of the S-phase checkpoint, such as ATM, BRCA1 and CHK2, is associated with cancer (Bell *et al.*, 1999; Falck *et al.*, 2001; Xu *et al.*, 2001). Intriguingly, the M–N–R complex is likely to work in the very early events of the radiation response, since its activity is required for CHK kinase activation and RAD9 phos-phorylation in yeast (Grenon *et al.*, 2001). The M–N–R complex also plays a role in DNA repair and M–N–R relocalization to IRIF (the sites of DNA damage) is thought to reflect this (Nelms *et al.*, 1998). It is likely that abrogation of either of these key protective mechanisms will result in further genome instability. Indeed, it is a common observation that in addition to the microsatellite instability causing subtle but diffuse genetic alterations, MMR-defective tumour cells show an increased degree of chromosomal aberration compared with non-neoplastic cells, but not the gross aneuploidy observed in 'chromosomally instable' tumours bearing mutations in the APC or mitotic checkpoint genes. This mild form of chromosomal instability is compatible with a DNA DSB repair defect. Finally, since the M–N–R complex is also involved in the maintenance of telomere integrity, loss or impairment of additional functions exerted by this complex might provide a further advantage to cancer cells.

Our data implicate human MRE11 in cancer development and indicate that it is a major target for inactivation in MMR-deficient CRC and other cells. Together with the identification of other mutations affecting this complex (Fukuda *et al.*, 2001; Kim *et al.*, 2001; Varon *et al.*, 2001; and the present paper), they also suggest that inactivation of the M–N–R complex is likely to be a frequent event in sporadic cancer.

METHODS

RT–PCR amplification of the 1235 bp 5′-fragment of MRE11 from CRC cell lines was performed on total RNA (1 μg) using the Gene Amp Gold RNA PCR Core kit (PE-Applied Biosystems, Warrington, UK). An aliquot (5 μl) of the RT reaction was subjected to 20–35 PCR cycles, with the MRE11-specific primers MRE11-19233 (5′-GAGTGCATTTTCTGACATTTGAGTAC-3′) and MRE11-H (5′-CTGGCTAAAGCGAAGAACAC-3′). The DNA fragment for the analysis of the IVS-4 (T)11 repeat was PCR

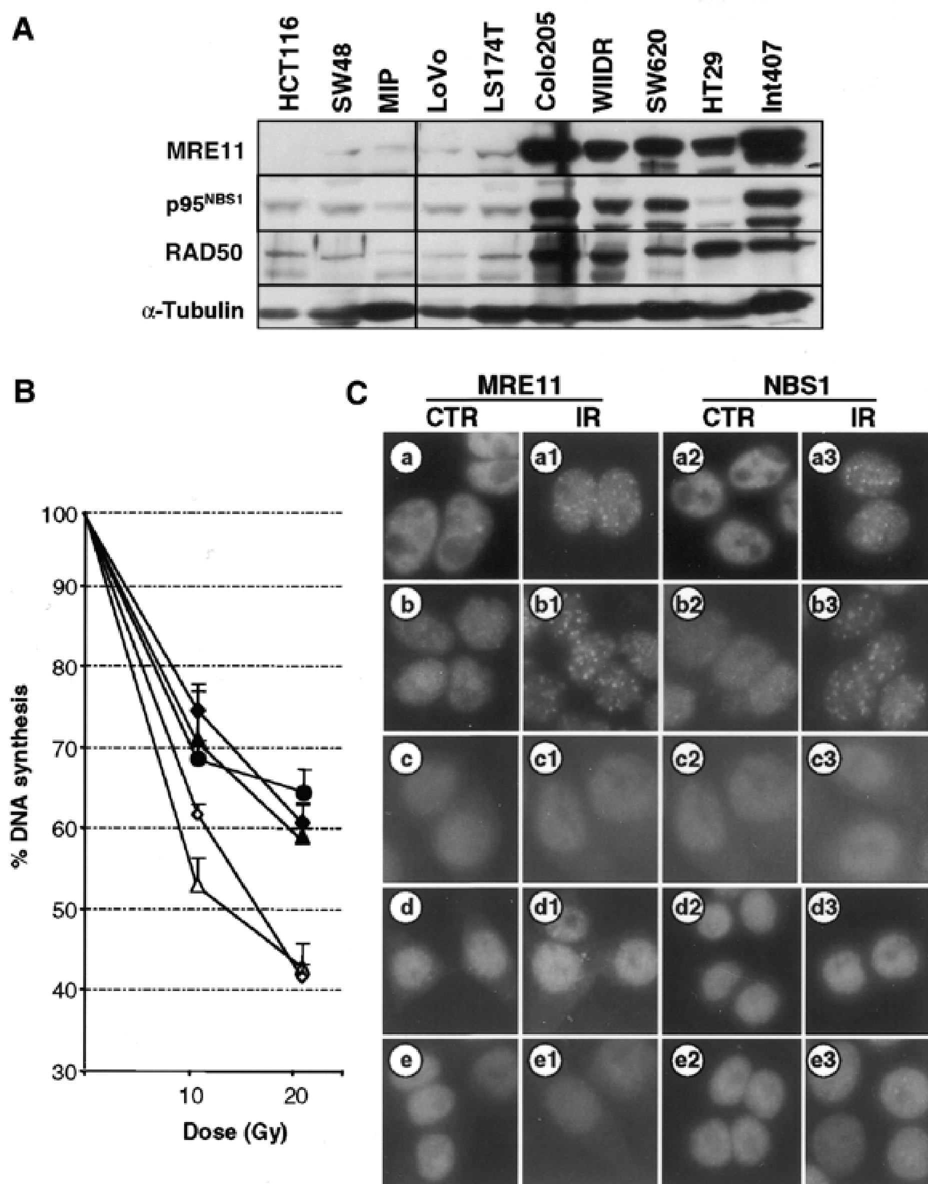

Fig. 4. M–N–R protein expression and response to ionizing radiation in CRC cell lines. (**A**) MRE11, p95[NBS1] and RAD50 expression in CRC cell lines evaluated by western blotting on total cell extracts. (**B**) Examination of RDS in CRC cell lines. HT29 (open triangles), WIDR (open diamonds), LoVo (filled diamonds), LS174T (filled circles) and MIP (filled triangles) cell lines were treated with γ-rays and subjected to a [^{3}H]thymidine pulse before harvesting and counting. The results show the percentage of DNA synthesis relative to unirradiated controls. The data represent the mean value ± SD (bars) of six replicate wells for each point. These results are represententative of at least three separate experiments. The overall variations between MMR-defective and MRE11-mutated cell lines (LoVo, LS174 and MIP) compared with MMR-proficient cells (HT29 and WIDR) are significant at both doses of irradiation (P <0.0001). Variance analysis also indicated a significant modification of the slope (F value, 5.61; Pr>F, 0.0210) between the two groups. (**C**) MRE11 and NBS1 IRIF formation in CRC cell lines. WIDR (a), HT29 (b), MIP (c), LoVo (d) and LS174T (e) CRC cell lines were irradiated at 12 Gy and fixed 8 h post irradiation. Either control unirradiated (CTR) or irradiated (IR) cells were then stained for immunofluorescence with anti-MRE11 and anti-NBS1 polyclonal antibodies. Note that MRE11 staining is partially delocalized to the cytoplasm in MIP, LoVo and, to a lower extent, LS174T, while it is totally nuclear in WIDR and HT29 cells.

amplified from genomic DNA (200 ng) through 35 PCR cycles with the primers MRE11-100F (5′-AATATTTTGGAGGAGAATCT-TAGGG-3′) and MRE11-L (5′-AATTGAAATGTTGAGGTTGCC-3′). Purified fragments were directly sequenced by an ABI PRISM 377 DNA Sequencer (PE-Applied Biosystems).

Northern blot analysis was performed on total RNA (20 µg) blotted to nylon membranes (Gene Screen Plus, NEN Life Science Product, Boston, MA) and hybridized to randomly primed cDNA probes.

The expression of MRE11, NBS1 and RAD50 proteins was evaluated by western blotting. Each protein lysate (40 µg) was separated on 8% SDS–PAGE gels, blotted onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) and probed with rabbit anti-p95[NBS1], anti-MRE11, anti-RAD50 and

anti-tubulin (Novus Biologicals, Littleton, CO) polyclonal antibodies. Immunoreactive bands were visualized by enhanced chemoluminescence (Pierce Chemical Co., Rockford, IL).

For thymidine incorporation assays, cells were seeded at 2×10^4 cells/well in 96-well plates and, after 18–24 h, were γ-irradiated at 3 Gy/min using a ^{60}Co source at room temperature, labelled with 1 μCi/well [^{3}H]thymidine and incubated for 4 h under standard conditions. Cells were then harvested and counted in a Top Count NST scintillation counter (Packard Instruments, Downers Grove, IL).

For estimation of IRIF formation, after γ-irradiation with 12 Gy, cells were recovered in fresh complete medium for 8 h followed by fixation and permeabilization in 4% paraformaldehyde with 0.25 Triton X-100 or prepared as described recently (Mirzoeva and Petrini, 2001). After blocking in 10% normal goat serum (NGS), cells were then washed and incubated with rabbit anti-p95^{NBS1} or anti-MRE11 anti-sera, and diluted 1/600 and 1/300 in 3% NGS, respectively. After washing, dishes were incubated with FITC-conjugated goat anti-rabbit secondary antibody (Cappel, distributed by ICN, Costa Mesa, CA) at 1/50 dilution in 3% NGS for 30 min.

ACKNOWLEDGEMENTS

This work was partially supported by grants from the Italian Association for Cancer Research, the Ministries of Health and Research and University, the National Research Council Biotechnology and Oncology Projects, the Neuroblastoma Association, the MURST-CNR 'Biomolecole per la Salute Umana' Program and the Pasteur Institute Cenci-Bolognetti Foundation.

REFERENCES

Aaltonen, L.A. *et al*. (1993) Clues to the pathogenesis of familial colorectal cancer. *Science*, **260**, 812–816.

Bell, D.W. *et al*. (1999) Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. *Science*, **286**, 2528–2531.

Coolidge, C.J., Seely, R.J. and Patton, J.G. (1997) Functional analysis of the polypyrimidine tract in pre-mRNA splicing. *Nucleic Acids Res.*, **25**, 888–896.

Falck, J., Mailand, N., Syljuasen, R.G., Bartek, J. and Lukas, J. (2001) The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature*, **410**, 842–847.

Fukuda, T. *et al*. (2001) Alterations of the double-strand break repair gene MRE11 in cancer. *Cancer Res.*, **61**, 23–26.

Gatei, M. *et al*. (2000) ATM-dependent phosphorylation of nibrin in response to radiation exposure. *Nature Genet.*, **25**, 115–119.

Grenon, M., Gilbert, C. and Noel, F.L. (2001) Checkpoint activation in response to double-strand breaks requires the Mre11/Rad50/Xrs2 complex. *Nature Cell Biol.*, **3**, 844–847.

Haber, J.E. (1998) The many interfaces of Mre11. *Cell*, **95**, 583–586.

Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D. and Perucho, M. (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature*, **363**, 558–561.

Khanna, K.K. and Jackson, S.P. (2001) DNA double-strand breaks: signaling, repair and the cancer connection. *Nature Genet.*, **27**, 247–254.

Kim, N.G., Choi, Y.R., Baek, M.J., Kim, Y.H., Kang, H., Kim, N.K., Min, J.S. and Kim, H. (2001) Frameshift mutations at coding mononucleotide repeats of the *hRAD50* gene in gastrointestinal carcinomas with microsatellite instability. *Cancer Res.*, **61**, 36–38.

Lim, D.S., Kim, S.T., Xu, B., Maser, R.S., Lin, J., Petrini, J.H. and Kastan, M.B. (2000) ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature*, **404**, 613–617.

Maquat, L.E. (2000) Nonsense-mediated RNA decay in mammalian cells: a splicing-dependent means to down-regulate the levels of mRNAs that prematurely terminate translation. In Sonenberg, N. and Hershey, J.W.B. (eds), *Translational Control of Gene Expression*, Vol. 462. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 849–868.

Mirzoeva, O.K. and Petrini, J.H. (2001) DNA damage-dependent nuclear dynamics of the Mre11 complex. *Mol. Cell. Biol.*, **21**, 281–288.

Nelms, B.E., Maser, R.S., MacKay, J.F., Lagally, M.G. and Petrini, J.H. (1998) *In situ* visualization of DNA double-strand break repair in human fibroblasts. *Science*, **280**, 590–592.

Petrini, J.H. (2000) The Mre11 complex and ATM: collaborating to navigate S phase. *Curr. Opin. Cell Biol.*, **12**, 293–296.

Pitts, S.A. *et al*. (2001) hMRE11: genomic structure and a null mutation identified in a transcript protected from nonsense-mediated mRNA decay. *Hum. Mol. Genet.*, **10**, 1155–1162.

Schaffner, C., Idler, I., Stilgenbauer, S., Dohner, H. and Lichter, P. (2000) Mantle cell lymphoma is characterized by inactivation of the ATM gene. *Proc. Natl Acad. Sci. USA*, **97**, 2773–2778.

Shiloh, Y. (1997) Ataxia-telangiectasia and the Nijmegen breakage syndrome: related disorders but genes apart. *Annu. Rev. Genet.*, **31**, 635–662.

Stankovic, T., Weber, P., Stewart, G., Bedenham, T., Murray, J., Byrd, P.J., Moss, P.A. and Taylor, A.M. (1999) Inactivation of ataxia telangiectasia mutated gene in B-cell chronic lymphocytic leukaemia. *Lancet*, **353**, 26–29.

Stewart, G.S. *et al*. (1999) The DNA double-strand break repair gene *hMRE11* is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell*, **99**, 577–587.

Thibodeau, S.N., Bren, G. and Schaid, D. (1993) Microsatellite instability in cancer of the proximal colon. *Science*, **260**, 816–819.

Varon, R., Reis, A., Henze, G., von Einsiedel, H.G., Sperling, K. and Seeger, K. (2001) Mutations in the Nijmegen Breakage Syndrome gene (NBS1) in childhood acute lymphoblastic leukemia (ALL). *Cancer Res.*, **61**, 3570–3572.

Viel, A. *et al*. (1997) Characterization of MSH2 and MLH1 mutations in Italian families with hereditary nonpolyposis colorectal cancer. *Genes Chromosomes Cancer*, **18**, 8–18.

Xiao, Y. and Weaver, D.T. (1997) Conditional gene targeted deletion by Cre recombinase demonstrates the requirement for the double-strand break repair Mre11 protein in murine embryonic stem cells. *Nucleic Acids Res.*, **25**, 2985–2991.

Xu, B., Kim, S. and Kastan, M.B. (2001) Involvement of Brca1 in S-phase and G(2)-phase checkpoints after ionizing irradiation. *Mol. Cell. Biol.*, **21**, 3445–3450.

DOI: 10.1093/embo-reports/kvf044