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Advances in Brief

Somatic Mutations in the DNA Damage-Response Genes *ATR* and *CHK1* in Sporadic Stomach Tumors with Microsatellite Instability[1]

Anna Menoyo,[2] Hafid Alazzouzi, Eloi Espín, Manel Armengol, Hiroyuki Yamamoto, and Simó Schwartz, Jr.[3]

Molecular Pathology Program, Centre d'Investigacions en Bioquímica i Biologia Molecular (CIBBIM), Barcelona 08035, Spain [A. M., H. A., S. S.]; Servei de Cirurgia General, Hospital General Universitari Vall d'Hebron, Barcelona 08035, Spain [E. E., M. A.]; and First Department of Internal Medicine, Sapporo Medical University, Sapporo 060-8543, Japan [H. Y.]

Abstract

Maintenance of genomic stability depends on the appropriate cellular responses to DNA damage and the integrity of the DNA repair systems. We analyzed stomach tumors with microsatellite instability (MSI) for frameshift mutations in several potential targets of the mutator phenotype involved in DNA damage-response pathways, such as the ataxia telangiectasia mutated protein-related protein (ATR)-CHK1-Cdc25c pathway, and DNA repair. High frequency of mutations was found within *ATR* [5 (21%) of 23], *MED1* [10 (43%) of 23], *hMSH3* [13 (56%) of 23], and *hMSH6* [10 (43%) of 23] genes. Also, a low frequency of mutations within the *CHK1* gene was detected in 9% (2 of 23) of tumors. No mutations of *hMLH3, ATM, BRCA1,* or *NBS1* genes were detected. These results confirm *ATR, MED1,* and *CHK1* as targets of the mutator pathway in stomach tumorigenesis, and also suggest a potential role of *MED1* increasing, together with *hMSH3* and *hMSH6,* the genomic instability in the mutator pathway as a secondary mutator. Furthermore, these results suggest that the inhibition of the ATR-CHK1 DNA damage-response pathway might be involved in the tumorigenesis of gastric cancer with microsatellite instability.

Introduction

Maintenance of the genome stability depends on the correct regulation of the cellular responses to DNA damage and the integrity of the DNA repair systems. Because of defects in DNA mismatch repair genes, sporadic stomach tumors of the mutator phenotype harbor hundreds of thousands of somatic mutations in repetitive sequences genomewide. According to the model (1), cells become tumorigenic when inactivating mutations occur in repetitive sequences within coding regions of cancer genes. The inactivation of these genes, known as targets of the mutator phenotype, give tumor cells growing advantage and positive selection, and become clonally perpetuated. ATR[4] is a member, together with the ATM and the DNA protein kinase (DNA-PK), of the phosphatidylinositol kinase family and activates after DNA damage (reviewed in Refs. 2 and 3). Also, ATR activates by phosphorylation the cell cycle checkpoint kinase CHK1, arresting cells into G_2-M through the inactivation of the Cdc25c kinase also by phosphorylation (4). Furthermore, it has been recently shown that ATR phosphorylates p53 and BRCA1 proteins after DNA damage (5, 6). Because *ATR* and *CHK1* genes have $(A)_{10}$ and $(A)_9$ repeats in their respective coding sequences, both are potential targets of the mutator phenotype. Frameshift mutations in the *ATR* or *CHK1* genes might compromise the ATR-CHK1-Cdc25c DNA damage-response pathway, avoiding the G_2-M cell cycle arrest of tumor cells. Also, inactivating mutations of the *ATR* or *CHK1* genes may inhibit the phosphorylation of p53 and BRCA1 proteins after DNA damage, compromising the p53 apoptotic activity or the repair activity of BRCA1 in these tumors. Indeed, BRCA1 together with ATM form a large protein repair complex with several other proteins such as the Mre11-Rad50-NBS1 repair complex and the hMLH1, hMSH2, and hMSH6 DNA repair proteins (7). Recently, two new repair proteins have also been reported to interact with hMLH1: MED1 and hMLH3 (8, 9). Because most of the genes encoding these repair proteins contain repetitive sequences within their coding regions, they are potential targets of the mutator phenotype and, therefore, secondary mutator candidates in the mutator pathway for gastrointestinal tumorigenesis.

We analyzed sporadic stomach tumors and cell lines with MSI for frameshift mutations within the *MED1*, *hMLH3*, *hMSH3*, *hMSH6*, *ATM*, *BRCA1*, *NBS1*, *ATR*, and *CHK1* genes. Frequent frameshift mutations in *ATR*, *CHK1*, *MED1*, *hMSH3*, and *hMSH6* were found. These results confirm *ATR*, *CHK1*, and *MED1* as target genes of the mutator phenotype in gastric cancer, which suggests the inhibition of the ATR-CHK1 DNA damage-response pathway in stomach tumorigenesis. A potential role of *MED1* as secondary mutator together with *hMSH3* and *hMSH6* in the mutator pathway is also suggested.

Materials and Methods

Tumor Samples and Cell Lines. Tumors were obtained from the National Cancer Center Research Institute (Tokyo, Japan), from the Sapporo Medical University (Sapporo, Japan) and from the Centre d'Investigacions en Bioquimica I Biologia Molecular Vall d'Hebron (CIBBIM; Barcelona, Spain). Sample collection was carried out in accordance with previously established ethical protocols. Collected tumors were immediately frozen in liquid nitrogen for further analysis. Human cancer cell lines were obtained from the American Type Culture Collection and also from the Japanese Cancer Research Resources Bank in Osaka, Japan. Genomic DNA was extracted with phenol-chloroform according to standard procedures.

Analysis of Instability in Repetitive Sequences within Microsatellites and Target Genes. Stomach tumors were analyzed for genomic MSI by PCR using two mononucleotide repeats, $(A)_{18}$ of APΔ3 and $(A)_{26}$ of BAT26, and the dinucleotide repeat D1S158, as described previously (10–12). Frameshift mutations in target genes were also analyzed. Briefly, PCR was carried out with Vent DNA polymerase (New England Biolabs, Beverly, MA) in the presence of 0.2 mCi of [α-^{32}P]dCTP as follows: one cycle at 94°C for 4 min., followed by 35 cycles at 94°C for 30 s, 55°C for 30 s., and 72°C for 30 s. PCR products were analyzed on a 6% denaturing polyacrylamide gel and subjected to autoradiography. The PCR primers for *ATM*, *BRCA1*, *NBS1*, *hMSH3*, and *hMSH6* have been described elsewhere (13, 14). The corresponding primers for *ATR* were 5′-GCC ACT TCT CAA CAT GAA TG-3′ and 5′-GCA AGT TTT ACT GGA CTA GG-3′; for *CHK1*, 5′-AAT TGC CAT GGG ACC AAC C-3′ and 5′-CTA GAG GAG CAG AAT CGA TT-3′; for *MED1*, 5′-TGC

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[2] Present address: Institut National de la Sante et de la Recherche Medicale (INSERM), Hôpital Henri Mondor, 94010 Criteil, France.

[3] To whom requests for reprints should be addressed, at Molecular Pathology Program, CIBBIM, Passeig Vall d'Hebron 119-129, Barcelona 08035, Spain. Phone: 34-93-489-4060; Fax: 34-93-489-4064; E-mail: schwartz@hg.vhebron.es.

[4] The abbreviations used are: ATM, ataxia telangiectasia mutated protein; ATR, ATM-related protein; MSI, microsatellite instability; MMP, microsatellite mutator phenotype.

ATT TCT GAT GCT GGA GC-3′ and 5′-TGA TGC CAG AAG TTT TTT GTT C-3′; and for *hMLH3*, 5′-ATG CTA CTG AAG TGG GAT GCC A-3′ and 5′-CAG TGG AAC ATA ATT TAA CTC G-3′. All primers amplified coding sequences that comprise the mononucleotide tracts of $(A)_7$ in *NBS1*; $(T)_7$ in *ATM*; $(A)_8$ in *BRCA1* and *hMSH3*; $(C)_8$ in *hMSH6*; $(A)_9$ in *hMLH3* and *CHK1*; and $(A)_{10}$ in *ATR* and *MED1* genes.

Results

Frequent Frameshift Mutations within the *ATR* and *CHK1* Genes of the DNA Damage-Response Pathway in Stomach Tumors of the Mutator Phenotype. MSI was detected in 13% (23 of 175) sporadic stomach tumors from our collection. These cases were analyzed for frameshift mutations in repetitive sequences within the DNA damage-response genes *ATR* and *CHK1*, from the ATR-CHK1-Cdc25c-Cdc2 damage-response pathway (Fig. 1). Mutations within the $(A)_{10}$ repeat of *ATR* were found in 21% (5 of 23) of cases. All of the cases showed deletions of one adenine and were heterozygous (Table 1). Frameshift mutations were also detected in the endometrial tumor cell lines with MSI as follows: HEC-1A, HEC-1B, SK-UT-1, and SK-UT-1B. These frameshift mutations generate, from the codon 774 of *ATR*, two alternative reading frames with stop codons at the 776 codon for the insertions, and the 777 codon for the deletions. These truncated forms of ATR lack the last two-thirds of the protein, which include the functional ATR phosphatidylinositol 3-kinase signature at the COOH termini (reviewed in Ref. 3).

Also, an insertion and a deletion of one adenine in the *CHK1* gene were found in tumor cases V155 and V158, respectively. The low intensity of the mutant band of case V158 (Fig. 1) is attributable to the high amount of contaminating normal tissue present in the tumor sample, according to the bat26 microsatellite analysis (not shown). Therefore, a low frequency of slippage mutations within *CHK1* characterizes stomach tumors with MSI. No mutations of *ATR* or *CHK1* were found in stomach tumors and tumor cell lines without MSI (Fig. 1). Interestingly, when we confronted cases with mutations within these target genes, we found that most tumors with *ATR* mutations (4 of 5) also harbor concomitant mutations of the *hMSH6* gene. Indeed, 17% (4 of 23) of tumor cases show simultaneous mutations of *ATR* and *hMSH6*, whereas the expected ratio of concomitant *ATR-hMSH6* mutations was only 9%. However, this association did not reach statistical significance because of the low number of cases. No similar associations were observed when the other target genes were confronted, and the yielded values were always in accordance with the expected ratios in all cases.

***MED1* as a Target Gene of the Mutator Phenotype in Gastric Tumors: High Incidence of Frameshift Mutations in Secondary Mutators.** Frameshift mutations at the DNA repair genes *ATM*, *BRCA1*, *NBS1*, *MED1*, *hMLH3*, *hMSH3* and *hMSH6*, were also analyzed (Fig. 1; Table 1). Mutations within the $(A)_{10}$ tract of *MED1* were found in 43% (10 of 23) of the tumors (Table 1). All these positive cases had deletions of one adenine and were heterozygous. No mutations were found within the repetitive tracts of *ATM*, *BRCA1*, *NBS1*, or *hMLH3* genes, although a deletion of one adenine within *hMLH3* was found in the MSI+ stomach tumor cell line SNU-1. Also, mutations in the $(A)_8$ and $(C)_8$ tracts from the *hMSH3* and *hMSH6* secondary mutators were found in 56% (13 of 23) and 43% (10 of 23), respectively, of tumors. Furthermore, all positive cases for *hMSH3* mutations had deletions of one adenine, whereas tumors with *hMSH6* mutations had preferentially insertions of one or two cytosines. Also, cases V156, J53, and J85 were homozygous for mutations in *hMSH6* and cases V22, J71, J85, J89, J94, and J99 showed concomitant mutations of both genes (Table 1). No mutations of *MED1*, *hMLH3*, *hMSH3*, or *hMSH6* were found in additional stomach tumors and tumor cell lines without MSI (Fig. 1). According to our results, a high incidence of frameshift mutations of the *MED1*, *hMSH3*, and *hMSH6* DNA repair genes characterizes stomach tumors with MSI. These results also suggest a potential role of *MED1*, together with *hMSH3* and *hMSH6*, as a secondary mutator of the mutator phenotype pathway for gastrointestinal tumorigenesis.

Discussion

We have shown frequent frameshift mutations of the ATR and a low frequency of slippage mutations of the CHK1 kinases in gastric tumors with MSI. ATR and CHK1 proteins are checkpoint kinases of the cellular response pathway to DNA damage, which induce cellular arrest in G_2-M after DNA damage through the inactivation of Cdc25c

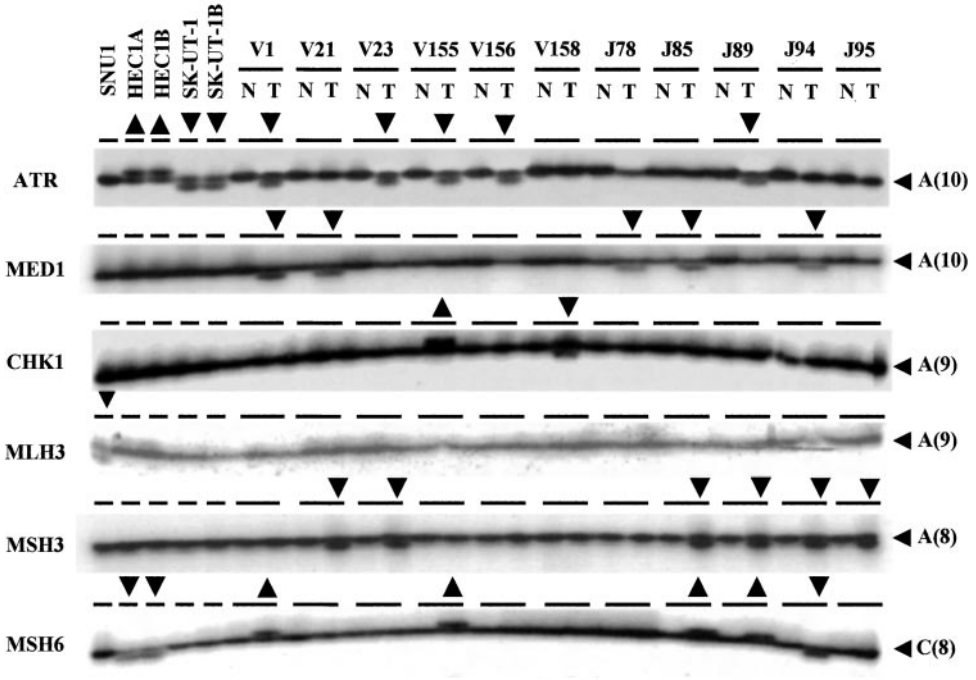

Fig. 1. Frameshift mutations in stomach tumors and tumor cell lines of the mutator phenotype. *On the left*, genes. *At the top*, tumor cell lines and tumor samples. *Arrowheads on the right*, the wild-type PCR product and the size of the repetitive sequence; *arrowheads* pointing up or down, insertions and deletions, respectively, of one base. No mutations were detected in 50 additional stomach tumors without MSI. Additional tumor cell lines without MSI were also negative for mutations, including seven cell lines from the stomach (MKN1, MKN28, MKN45, MKN74, KATOIII, NUGC3, and AZ521), four from the colon (SW628, CaCo2, HT-29, and SW403), and three from endometrium (Hs34.T, Hs248.T, and Hs825.T).

Table 1 *Target genes in stomach tumors with MSI*

Tumor cases	Analysis of microsatellites		Target Genes					
	Bat26	APΔ3	*hMSH3* (A)8[a]	*hMSH6* (C)8[a]	*CHK1* (A)9[a]	*hMLH3* (A)9[a]	*ATR* (A)10[a]	*MED1* (A)10[a]
Stomach tumors, percentage of positive cases[b]			56% (13/23)	43% (10/23)	9% (2/23)	0%	21% (5/23)	43% (10/23)
V1	+[c]	+	−	+u	−	−	+d	+d
V6	+	+	−	−	−	−	−	−
V21	+	+	+d	−	−	−	−	+d
V22	+	+	+d	+u	−	−	−	−
V23	+	+	+d	−	−	−	+d	−
V152	+	+	+d	−	−	−	−	−
V153	+	+	−	−	−	−	−	+d
V154	+	+	+d	−	−	−	−	+d
V155	+	+	−	+u	+u	−	+d	−
V156	+	+	−	+u,uu	−	−	+d	−
V157	+	+	−	−	−	−	−	+d
V158	+	+	−	−	+d	−	−	−
J53	+	+	−	+u,uu	−	−	−	−
J60	+	+	+d	−	−	−	−	−
J62	+	+	−	−	−	−	−	+d
J71	+	+	+d	+u	−	−	−	+d
J78	+	+	−	−	−	−	−	+d
J85	+	+	+d	+u,d	−	−	−	+d
J89	+	+	+d	+u	−	−	+d	−
J94	+	+	+d	+d	−	−	−	+d
J95	+	+	+d	−	−	−	−	−
J99	+	+	+d	+d	−	−	−	−
J106	+	+	+d	−	−	−	−	−

[a] The size of the repeat.
[b] All tumors and tumor cell lines were negative for mutations within the DNA repair genes *ATM* $(T)_7$, *NBS1* $(A)_7$, and *BRCA1* $(A)_8$.
[c] +, mutant; −, wild type; +u, 1-bp insertion; +u,uu, homozygous insertions of 1 and 2 bp; +d, 1-bp deletion.

and Cdc2 by phosporylation (4). ATR plays a central role in the regulation of this response pathway, and its inactivation in gastric tumors clearly suggests that the inhibition of the ATR/CHK1 pathway in stomach cancer may lead to tumor cell progression.

Indeed, it has been shown that heterozygous mutations of *CHK1* enhance tumor formation in WNT-1 transgenic mice, which directly implies an involvement of the ATR/CHK1 pathway in tumor suppression (4). Also, homozygous inactivation of *ATR* in mice results in embryonic lethality (15), and overexpression of a kinase-inactive form of ATR in human fibroblasts reduces cell viability by 2–5 times (16, 17). These studies show a clear dominant-negative effect of the inactive-ATR form, capable of inducing cell death independently of the presence of wild p53. In accordance with this data, our results show heterozygous mutations of *ATR* and *CHK1* in the tumors and tumor cell lines analyzed, which suggests a possible dominant-negative effect of the ATR mutations in stomach cancer also. The presence of heterozygous mutations of *ATR* in the endometrial tumor cell lines HEC1A/B and SK-UT-1/B will allow functional studies of this protein and will provide the field with a cellular model for further research. These studies should clarify whether the mutant forms of ATR, originated by slippage mutations, also have a dominant-negative effect in these tumors.

Recently, ATR has been shown to phosphorylate the Ser15 of p53, promoting the transactivation of p53 and the phosporylation of p21, which in turn inhibits the CdK2 kinase and also arrests the cell cycle (Ref. 5; reviewed in Ref. 2). It should be note also, that phosphorylation of p53 by CHK1 has been reported recently by Shieh *et al.* (18). However, although this pathway may also lead cells to apoptosis after DNA damage, it is not clear yet whether the inactivation of ATR and CHK1 in stomach tumors will prevent tumor cells from undergoing apoptosis. Nonetheless, we suggest that frameshift mutations in *ATR* and *CHK1* might lead to the inactivation of the p53-cell cycle arrest pathway in stomach tumors with MSI, thus contributing to the progression of tumor cells in gastric cancer of the mutator phenotype.

Furthermore, our results also agree with the haploinsufficiency model of the mutator phenotype, recently proposed by Perucho *et al.* (19). According to the model, monoallelic mutations in several target genes that belong to the same tumor-related pathway accumulate in cancer cells during tumor progression and inactivate the pathway. Hence, our results suggest that monoallelic mutations in *ATR* and *CHK1* genes may cause the inactivation of the ATR/CHK1 DNA damage-response pathway in gastrointestinal tumors of the mutator phenotype.

On the other hand, because the integrity of the cellular genome also depends on specific DNA repair protein complexes, we extended our analysis to other targets involved in DNA repair. It is known that ATR and ATM, another checkpoint kinase that is also involved in pathways similar to those of ATR, can activate BRCA1 by phosphorylation. BRCA1 together with ATM forms a large repair protein complex with the DNA repair Mre11-Rad50-NBS1 complex and hMLH1, hMSH2, and hMSH6 proteins. This complex, known as BASC (for BRCA1-associated genome surveillance complex), acts as a sensor of DNA damage and is involved in the repair of double-strand brake lesions (7). Also, hMLH1, hMSH2, and hMSH6, together with hMSH3, MED1, and hMLH3 proteins are involved in the recognition complexes and repair of mismatch replication errors. Interestingly, most of these proteins have repetitive sequences within the coding region of their respective genes and are candidate targets of the mutator phenotype. Indeed, previous studies have already shown mutations in *hMLH1*, *hMSH2*, *MED1*, *Rad50*, *hMSH3*, and *hMSH6* in gastrointestinal tumors of the mutator phenotype (12–14, 20). Accordingly, our results show frequent mutations of *hMSH3*, *hMSH6*, and *MED1* genes in gastrointestinal tumors with MSI. Although it is not known yet whether mutations in MED1 can increase the genomic instability that characterizes these tumors, our results suggest MED1 as a potential secondary mutator of the mutator pathway, although further analyses are needed to confirm this possibility.

An adequate regulation and activity of specific cellular DNA dam-

age-response pathways and DNA repair systems are responsible for the maintenance of the genome stability and integrity in human cells. Alterations of these central molecular regulatory pathways may lead cells to became tumoral. We have shown that because of an exacerbated genomic instability, gastric tumors of the mutator phenotype harbor frameshift mutations in genes involved in main cell-cycle regulatory and DNA repair pathways. Accordingly, inactivating mutations in several repair genes such as *hMSH3*, *hMSH6*, and *MED1*, and also the inactivation of the ATR/CHK1 DNA damage-response pathway, might contribute to the tumor progression of the gastric cancer with MSI. Further analyses are required to show whether the inactivation of these DNA repair/damage-response genes might act also as cause-contributors of the mutator phenotype instability.

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