

Frameshift Mutations of Human Gastrin Receptor Gene (*hGARE*) in Gastrointestinal Cancers with Microsatellite Instability

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SUMMARY: Gastrointestinal tumors with DNA mismatch repair (MMR) defects show microsatellite instability (MSI) and harbor frameshift mutations in coding mononucleotide repeats of cancer-related genes (targets). We assessed MSI status in 233 sporadic gastrointestinal tumors. We classified as MSI-H (high-frequency microsatellite instability) 15 (10%) of 150 colorectal cancers and 13 (16%) of 83 gastric cancers. We searched for frameshift mutations in a coding poly(T)₈ tract within the gastrin receptor gene (*hGARE*), which has a potential role in gastrointestinal carcinogenesis. To this purpose, we screened 43 unstable tumors (including 15 hereditary nonpolyposis colorectal cancer cases previously classified as MSI-H), 98 stable tumors, as well as 3 MMR-deficient and 4 MMR-proficient gastrointestinal cancer cell lines. We found mutations in 8 (19%) of the 43 MSI-H tumors but in none of the 98 stable cancers. *hGARE* mutation frequency was similar in gastric (23%) and colorectal cancers, including sporadic (13%) and hereditary (20%) cases. All mutated tumors proved to harbor frameshift mutations in other cancer-related genes that are considered as targets in MSI tumorigenesis. The MMR-deficient and gastrin-sensitive LoVo colorectal cancer cells also showed a *hGARE* heterozygous frameshift mutation, but expressed only the mutated allele. All detected mutations can be predicted to generate a truncated protein carrying amino acid changes. On the basis of genetic findings, we propose *hGARE* as a new candidate target gene in MSI tumorigenesis. Functional studies are warranted to elucidate the mechanism by which the *hGARE* mutation might contribute to gastrointestinal carcinogenesis. (*Lab Invest* 2002, 82:265–271).

The cancers arising in most patients with hereditary nonpolyposis colorectal cancer (HNPCC) and a subset (10–20%) of sporadic gastrointestinal tumors exhibit widespread genome instability that is manifested by increases in mutation rates and by alterations in the size of simple repeated sequences (Aaltonen et al, 1993; Ionov et al, 1993; Thibodeau et al, 1993). This tumor phenotype, called MSI (from microsatellite instability), is the result of alterations in DNA mismatch repair (MMR) genes, which play a crucial role in the maintenance of DNA-replication fidelity (reviewed by Peltomaki, 2001). Different molecular mechanisms have been found to inactivate MMR genes: germ line mutations, genomic rearrangements,

and unbalanced allelic expression are involved in hereditary tumors (Charbonnier et al 2000; Curia et al, 1999; Peltomaki and Vasen, 1997), whereas somatic mutations and promoter hypermethylation play a major role in sporadic tumors (Herman et al, 1998; Kane et al, 1997; Liu et al, 1995).

MMR-deficient tumors frequently harbor insertions/deletions in mononucleotide tracts within coding regions of genes involved in the control of cell proliferation and survival. Frameshift mutations of cancer-related genes (targets) have been proposed to be under positive selective pressure during the evolution of unstable tumors (Yamamoto et al, 1997). A number of target genes have been implicated in the pathogenesis of MMR-deficient gastrointestinal cancers (Duval et al, 2001) and some of these genes have been found to be frequently mutated in these tumors. These include the growth factor receptor genes *TGFβRII* (Markowitz et al, 1995) and *IGF1R* (Souza et al, 1996), the proapoptotic gene *BAX* (Rampino et al, 1997), the DNA-MMR genes *hMSH3*, *hMSH6* (Malkhosyan et al, 1996), and *MED1* (Bader et al, 1999; Riccio et al, 1999), the protease *CASP-5* gene (Schwartz et al,

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1999), the transcription factor *TCF-4* gene (Duval et al, 1999a), and the retinoblastoma protein interacting zinc finger *RIZ* gene (Piao et al, 2000). The identification of new targets might contribute to elucidating the progression pathway of MMR-deficient tumors and even provide clues for cancer treatment (Boland et al, 1998).

We used the GenBank database to search for mononucleotide repeats within the coding regions of growth factor and growth factor receptor genes possibly related to gastrointestinal tumorigenesis. We found a (T)₈ repeat in exon 5 of the human gastrin receptor gene (*hGARE*, or *CCKBR*). The peptide hormone gastrin represents a recognized growth factor for gastrointestinal tumor cells (Iwase et al, 1997; Kusyk et al, 1986; Seva et al, 1994; Singh et al, 1994; Stepan et al, 1999). In addition, gastrin and CCK-B receptor, the expression of which is low or absent in normal colon (Monstein et al, 1996; Wank, 1998), have been proposed to be involved in maintaining auto-crine/paracrine growth pathways in gastrointestinal cancer cells (McWilliams et al, 1998). To verify whether unstable tumors harbor *hGARE* frameshift mutations, we analyzed the (T)₈ repeat of exon 5 in a panel of both stable and unstable gastric and colorectal cancers, as well as in MMR-deficient/proficient cell lines derived from gastrointestinal tumors.

Results

We assessed MSI status in 233 sporadic gastrointestinal tumors. On the basis of recently established consensus criteria (Boland et al, 1998), we classified as MSI-H 15 (10%) of 150 colorectal cancers and 13 (16%) of 83 gastric cancers.

We analyzed MSI-H tumors for insertions/deletions within coding repeats of *TGFβRII*, *CASP-5*, *BAX*, *hMSH3*, *hMSH6*, and *IGFIIR* target genes. As expected, somatic mutations proved to occur in all of these genes, although with different frequencies. The prevalence of gene mutations are reported in Table 1.

We searched for insertion/deletion mutations of *hGARE* gene in 43 unstable and 98 stable tumors, as well as in 3 MMR-deficient and 4 MMR-proficient cell lines derived from gastrointestinal tumors. Unstable tumors included 13 gastric and 15 sporadic colorectal cancers that we identified during this survey, as well as 15 HNPCC cases previously classified as MSI-H. Stable tumors included 28 colorectal and 70 gastric cancers. Genomic DNA were used to amplify the

hGARE exon-5 sequence encompassing the (T)₈ repeat. We analyzed polymerase chain reaction (PCR) products either by sequencing gel or by capillary electrophoresis. To avoid misclassifications, PCR products from each tumor and corresponding normal tissue were analyzed in parallel.

Among cancer cell lines, only MMR-deficient LoVo cells showed an *hGARE* heterozygous mutation due to a 1-bp deletion within the (T)₈ repeat. Seven 1-bp deletion and one 1-bp insertion mutations were found in 8 (19%) of 43 MSI-H gastrointestinal tumors, but in none of the 98 stable cancers. The frequency of mutation was similar in gastric (3 of 13, 23%) and colorectal cancers of both sporadic (2 of 15, 13%) and hereditary (3 of 15, 20%) types. All tumors showing frameshift mutations also showed a wild type allele. This indicates that neoplastic cells were heterozygous for the observed mutations and/or that non-neoplastic cells contributing the normal allele were present in the tissue sections used for DNA extraction. Figure 1 shows 1-bp deletion mutations as detected by capillary (A) and gel electrophoresis (B).

DNA sequencing confirmed the deletion of 1 bp within the (T)₈ repeat of *hGARE* exon 5 in the LoVo cells (Fig. 2) and in seven MSI-H tumors. This mutation, leading to a premature stop at codon 377, is predicted to generate a truncated protein carrying amino acid changes. Sequencing also confirmed the insertion of 1 bp, causing a stop mutation at codon 350, in one gastric cancer. In a gastric cancer harboring a (T)₇ repeat, DNA-sequencing revealed a missense mutation (G → A transition; Val → Ile) at codon 339. By cloning the PCR product from genomic DNA and by sequencing different clones, we assessed that frameshift and missense mutations were located on different alleles (Fig. 3). The functional significance, if any, of the missense mutation remains unclear. Among the eight *hGARE* mutated tumors, six also harbored a frameshift mutation of *TGFβRII* gene, four of *BAX*, three of *hMSH3*, four of *hMSH6*, and three of *IGFIIR*.

To evaluate *hGARE* expression, we performed RT-PCR experiments on LoVo and HCT116 MMR-deficient colon cancer cell lines. By using 30 amplification cycles and specific primers encompassing *hGARE* exons 4 and 5, we could detect a single PCR product in LoVo but not in HCT116 cells (Fig. 4a); a PCR product was detectable in HCT116 cells only by reaching 50 amplification cycles. Direct sequencing of

Table 1. Frameshift Mutations at Coding Repeats of Target Genes in High-Frequency Microsatellite Instability Gastrointestinal Tumors

	<i>n</i>	<i>TGFβRII</i> <i>n</i> (%)	<i>CASP-5</i> <i>n</i> (%)	<i>BAX</i> <i>n</i> (%)	<i>hMSH3</i> <i>n</i> (%)	<i>hMSH6</i> <i>n</i> (%)	<i>IGFIIR</i> <i>n</i> (%)
Colorectal cancers	30 ^a	24 (80)	16 (53)	14 (46)	6 (20)	8 (26)	6 (20)
Gastric cancers	12 ^b	9 (75)	nd	2 (16)	2 (16)	6 (50)	4 (33)

nd, not determined.

^a Includes: 15 sporadic and 15 hereditary tumors.

^b DNA from one case was not available.

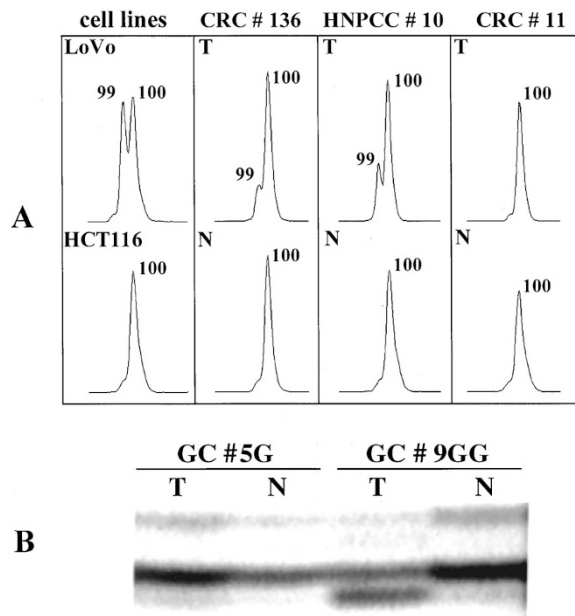


Figure 1.

hGARE frameshift mutations. *T*, tumor DNA; *N*, DNA from normal tissue. A, Electropherograms of fluoresceinated PCR-products from colon cancer cell lines (*LoVo* and *HCT116*) and from sporadic (*CRC#136* and *CRC#11*) and hereditary (*HNPCC#10*) colorectal cancers. Numbers indicate the length of the amplicons (in bp). Frameshift mutations are present in *LoVo* cells and in tumor DNA from samples *CRC#136* and *HNPCC#10*. B, Gel electrophoresis of radiolabelled PCR-products from gastric cancers (*GC*). A tumor sample (*GC#5G*) with a wild-type (T_8) repeat, and a tumor sample (*GC#9GG*) harboring a (T_7) repeat are shown.

the product we obtained from *LoVo* cells demonstrated the presence of only one of the two isoforms that are known to be generated by exon 4 alternative splicing (Fig. 4c) (Song et al, 1993). Interestingly, this isoform corresponded with the *hGARE* frameshift-mutated allele and has been characterized by a (T_7) repeat in exon 5 (Fig. 4b).

Discussion

We identified frameshift mutations within the *hGARE* exon 5 coding repeat in approximately 20% of the MSI-H gastrointestinal tumors. This value is similar to values obtained for coding repeats of *hMSH3*, *hMSH6*, and *IGFIIR* target genes in colorectal cancers, and for coding repeats of *hMSH3*, *BAX*, and *TCF-4* target genes in gastric cancers (see Table 1, and Duval et al, 1999b).

Mutation frequencies of coding repeats within genes that are potentially implicated in human carcinogenesis have recently been evaluated in a large series of MSI-H colorectal tumors. Using a statistical method, Duval et al (2001) separated genes into two groups, with a cut-off value for mutation frequency of about 12%. Genes showing higher mutation frequencies have been proposed to represent valid "targets," the mutation of which is selected for and plays a role during tumorigenesis. Genes with lower mutation frequencies have been proposed to represent "bystanders," the mutation of which is probably due to the high

background of instability present in MMR-deficient cells. In this respect, *hGARE* cancer-related gene is likely to represent a new target, the mutation of which may have functional significance.

On the other hand, the analysis of a number of noncoding (intronic) mononucleotide tracts has demonstrated that there is great variation in the prevalence of mutations among repeated sequences (Zhang et al, 2001). Some of the investigated tracts proved to be altered in less than 5% of the MMR-deficient tumors, whereas other tracts were altered in nearly 50% of the cases (Zhang et al, 2001). Very recently, it has been found that a large number of noncoding microsatellite mutations can accumulate in phenotypically normal MMR-deficient cells, even before the occurrence of a gatekeeper gene mutation, and in the absence of abnormal clonal expansion (Vilkki et al, 2001). Taken together these findings indicate that microsatellite instability in MSI-H tumors is a complex mechanism depending on different factors including size, nature, and localization of sequences, as well as functional significance. Therefore, the implication of a mutated gene as a target may present a difficulty in the absence of evidence that coding microsatellite mutations are functionally relevant in tumorigenesis (Boland et al, 1998; Zhang et al, 2001).

Frameshift mutations in the *hGARE* (T_8) repeat lead to major changes in the highly conserved structure of the CCKB receptor, a member of the superfamily of G-protein-coupled receptors possessing seven transmembrane domains (Wank, 1998). The VI transmembrane domain is modified in its amino acid sequence, whereas the VII domain and the intracellular C-terminus are completely lost. It has been shown that the truncation of the carboxyl terminus of a G-protein-coupled receptor can result in constitutive activity (Matus-Leibovitch et al, 1995; Thomas et al, 1995). In particular, a rat CCKB receptor mutant, lacking the 44 carboxyl-terminal amino acids and closely resembling the putative receptor originating by *hGARE* frameshift mutations, has recently been shown to retain binding properties, G-protein coupling, and signal transduction activity (Pohl et al, 1997). Thus, the identified *hGARE* mutations might lead to a gain rather than a loss of the receptor function. Preliminary evidence in support of this concept derives from expression studies we performed on *LoVo* and *HCT 116* MSI-H cancer cell lines. *LoVo* cells, which harbor an *hGARE* heterozygous mutation, are known to be responsive to the gastrin peptides growth stimulatory effect, whereas *HCT 116* are nonresponsive (Stepan et al, 1999). We easily detected *hGARE* mRNA in gastrin-responsive *LoVo* cells, but not in *HCT 116* cells. In addition, *LoVo* cells proved to express only mRNA molecules corresponding to the frameshift mutated allele. Accordingly, *LoVo* have recently been shown to express at the cell surface only a gastrin receptor isoform of 40 kDa (Watson et al, 1998), a molecular weight corresponding to that predicted for a truncated protein originating from the frameshift mutated *hGARE* gene.

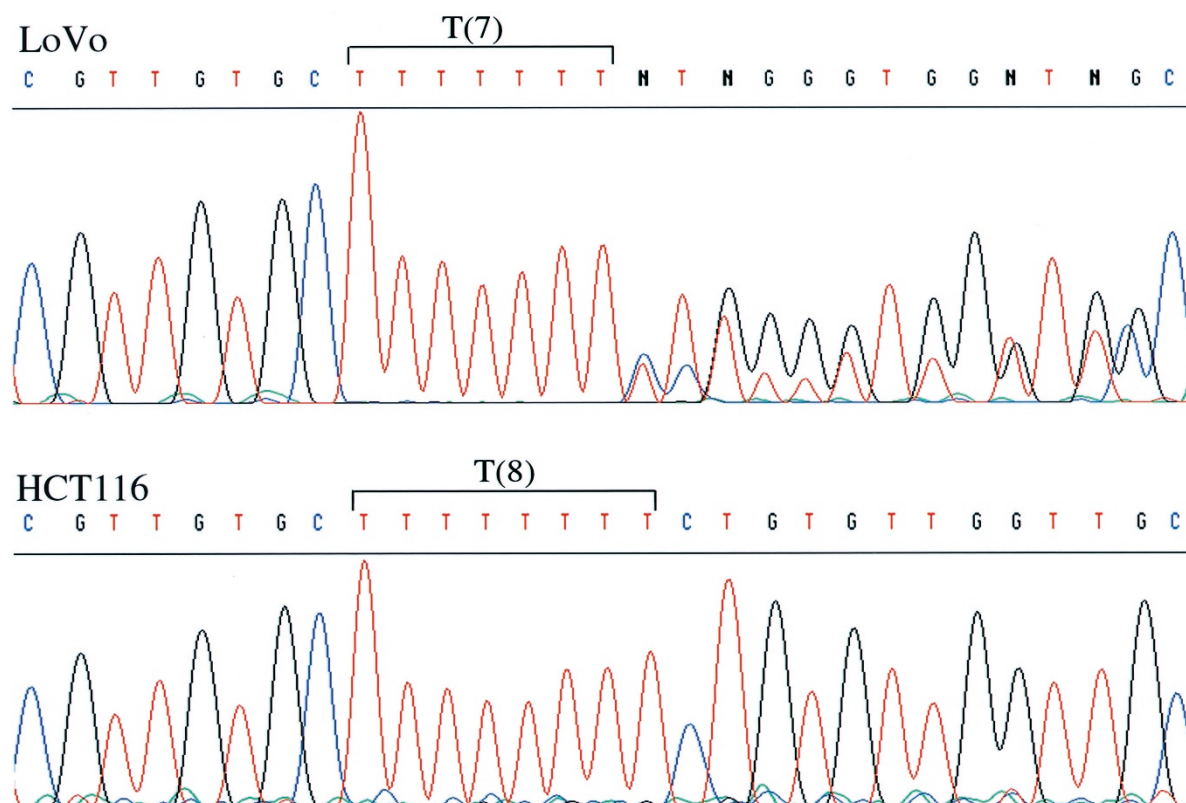


Figure 2.

DNA sequence of *hGARE* PCR products from LoVo and HCT116 cell lines. A frameshift mutation (1-bp deletion) within the (T)₈ repeat is detectable in LoVo cells, whereas a wild-type repetitive tract is present in HCT116 cells.

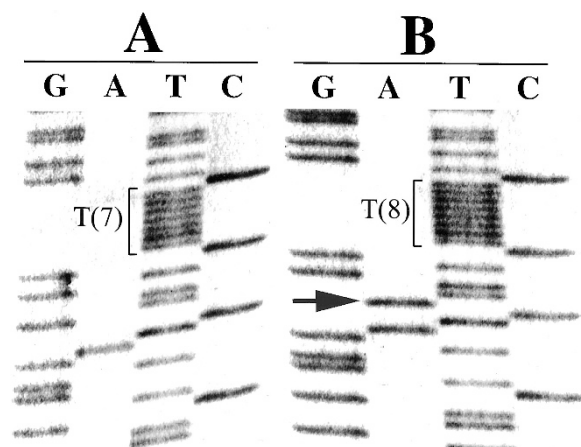


Figure 3.

DNA sequences of *hGARE* cloned PCR products from GC#10G gastric cancer sample. A (T)₇ repeat is present in Clone A; a G → A transition at codon 339 is present with a (T)₈ repeat in Clone B. The arrow indicates the G → A transition detected at codon 339 in Clone B.

Although a large body of data exists, further studies are needed on normal and tumor gastrointestinal tissues to definitely assess the role of gastrin and gastrin-receptors in both cell differentiation and proliferation. This makes it difficult to precisely evaluate the functional significance of the frameshift-mutated *hGARE* gene in unstable tumors. However, the presence of only mutated mRNA molecules in LoVo cells,

which are known to grow in response to the gastrin, is consistent with a role of the putative truncated receptor in mediating a trophic effect. Taken together, our findings makes *hGARE* a new candidate target gene playing a role in the tumorigenesis of a fraction of unstable tumors.

Materials and Methods

Cell Lines and Tumor Samples

MMR-deficient cells from colon cancers (LoVo and HCT116) and from gastric cancers (SNU1), as well as MMR-proficient cells from colon cancers (HT29, FINCO1, FINCO2, and SW480), were kindly provided by Dr. G. Marra (Institute for Medical Radiobiology, Zurich, Switzerland) or purchased from the American Type Culture Collection (Manassas, Virginia).

The cancer panel included 150 consecutive colorectal cancers surgically resected at the Istituto Clinico Humanitas of Milan between 1997 and 1999 and 83 gastric cancers referred for histologic diagnosis to the Department of Pathology of the University of Pavia. Fifteen colorectal cancers from HNPCC patients, already classified as MSI-H, were obtained from the Department of Human Genetics of the University Hospital of Basel.

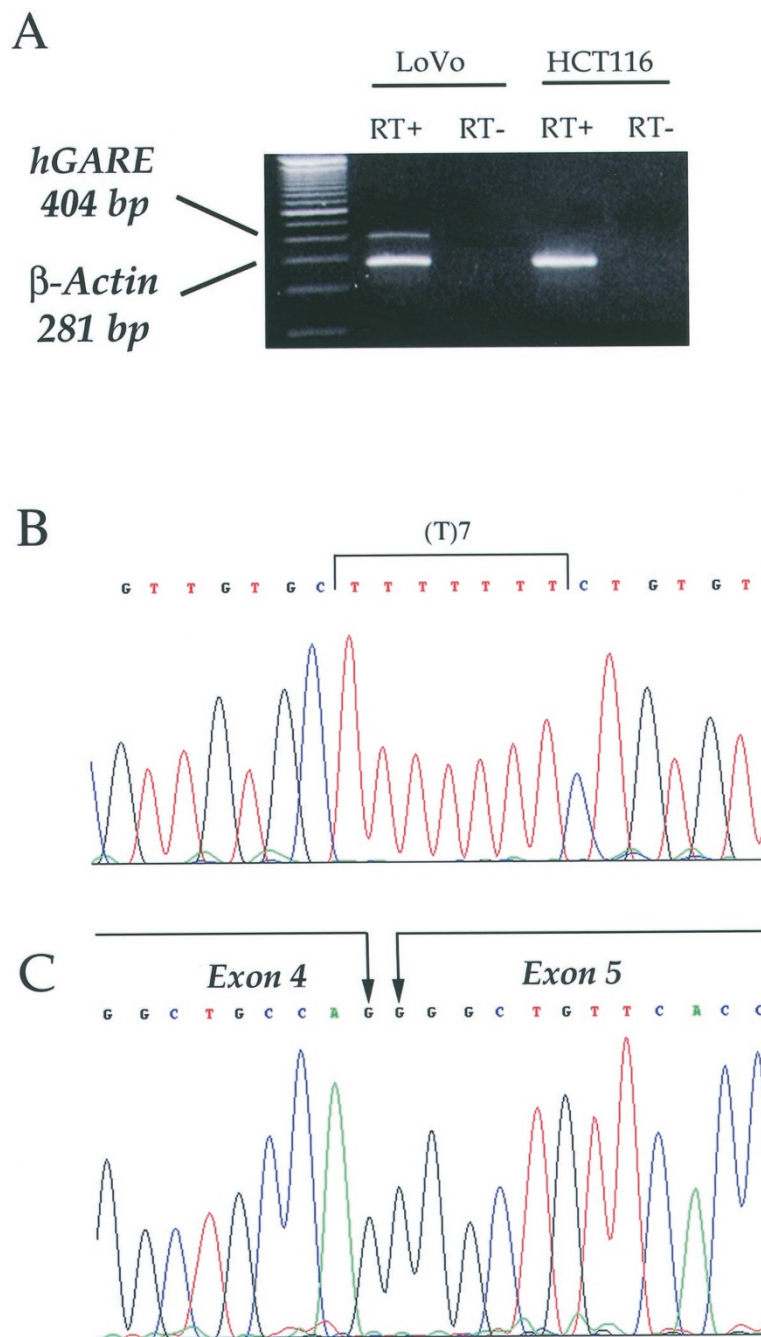


Figure 4.

hGARE mRNA expression in cancer cell-lines. A, A 404 bp *hGARE* RT-PCR product is detectable in *LoVo*, but not in *HCT116* cells, whereas a 281-bp β -actin RT-PCR product is present in both cell lines. RT+: , RT-: negative control. B and C, Direct sequencing of the *LoVo hGARE* RT-PCR product. As evident, only the exon 5 (T)₇ mutated allele is transcribed (B), and only the short exon 4 splice variant isoform accounts for the *hGARE* expression (C).

MSI-H Assessment and Target Gene Mutation Analysis

DNA was purified by standard procedures from cancer cell lines and from paraffin sections of formalin-fixed specimens or from frozen tissue fragments of gastrointestinal tumors and matched normal mucosa. We searched for instability in tumor samples by screening a standard panel of microsatellite loci (Boland et al, 1998) according to the conditions reported by Dietmaier et al (1997). We amplified microsatellite sequences by using either radiolabeled or fluoresceinated specific

primers. Accordingly, we analyzed PCR products either by standard polyacrylamide DNA sequencing gel or by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, California). PCR products from tumor and corresponding control DNA were analyzed in parallel. We followed the recently assessed criteria for the definition of MSI-H (Boland et al, 1998).

Frameshift mutations at coding mononucleotide repeats within *TGF β R2*, *BAX*, *hMSH3*, *hMSH6*, *IGF1R*,

and *CASP-5* genes were investigated by PCR. Reactions were carried out with either radiolabeled or fluoresceinated specific primers (Malkhosyan et al, 1996; Markowitz et al, 1995; Rampino et al, 1997; Schwartz et al, 1999; Souza et al, 1996). PCR products were analyzed as reported above.

hGARE Gene Mutation Analysis

A 100-bp fragment of *hGARE* exon 5, encompassing the (T)₈ repeat (GenBank sequence ID L10822), was amplified with the primers 5'-GGCCAAGCTGCTGGCTAAGAA-3' (forward) and 5'-GCCACGTGTGGCACTATAAAC-3' (backward). PCR was carried out on a 25- μ l volume containing 50–100 ng of template DNA; 0.2 μ M of each primer; 200 μ M of dGTP, dCTP, dTTP, and dATP; 1.5 mM of MgCl₂; 1 unit of *Taq* "GOLD" polymerase (PE Applied Biosystems); 1x buffer supplied with the enzyme. The reaction was carried out as follows: 1 cycle consisting of 10 minutes at 95° C and 35 cycles each consisting of 30 seconds at 94° C, 30 seconds at 55° C, and 30 seconds at 72° C. Alternatively, PCR was carried out in a 50- μ l volume containing 50–100 ng of template DNA; 0.2 μ M of each unlabeled primer; 100 μ M of dGTP, dCTP, and dTTP; 12.5 μ M of dATP; 5 mCi [α -³⁵S] dATP; 1.5 mM of MgCl₂; 1 unit of *Taq* "GOLD" polymerase; and 1x buffer supplied with the enzyme. The reaction was performed as above.

PCR products were analyzed in denaturing conditions, either by capillary electrophoresis (ABI PRISM 310 Genetic Analyzer; PE Applied Biosystems) or by 7-M urea/6% polyacrylamide sequencing gel electrophoresis. Amplicons with abnormal electrophoretic patterns were subjected to direct sequencing, using cycle-sequencing, either manually (Thermo Sequenase Cycle Sequencing Kit; Amersham Pharmacia Biotech, Uppsala, Sweden) or automatically (ABI PRISM Big Dye Terminator Cycle Sequencing Kit; PE Applied Biosystems, Warrington, United Kingdom).

PCR product from a case of interest was subcloned in a pCR Vector (TA Cloning Kit; Invitrogen Corporation/Novex, Carlsbad, California) following the manufacturer's instructions; cloned fragments were sequenced as above reported.

Detection of hGARE mRNA in Cell Lines

Total RNA was extracted from LoVo and HCT116 cell lines by using TRIZOL (Life Technologies, Paisley, United Kingdom) according to the manufacturer's instructions. After treatment with DNAase (Amersham Pharmacia Biotech), RNA was reverse-transcribed (RT) using oligo-dT_{12–18} primers and SuperScript II reverse-transcriptase (Life Technologies), according to manufacturer's recommendations. *hGARE* mRNA level was assessed by 30-cycle PCR (one cycle 94° C for 30 seconds, 58° C for 30 seconds, 72° C for 40 seconds) with specific primers mapping in exons 4 (forward, 5'-CTCGCGAGCTCTACTTAGGG-3') and 5 (backward, 5'-GTGAATGAAGGAGATAGGAGCAC-3'). To allow *hGARE* expression level assessment, β -*actin*

and *hGARE* mRNA were coamplified. The β -*actin* primers were: 5'-ACCAACTGGGACGACATGGA-3' (forward); and 5'-GCATACCCCTCGTAGATGGG-3' (backward). RT-PCR products were run on 2% agarose gel and visualized by ethidium bromide staining. The specific *hGARE* RT-PCR product was characterized by direct cycle sequencing.

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