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Aberrant methylation within *RUNX3* CpG island associated with the nuclear and mitochondrial microsatellite instability in sporadic gastric cancers. Results of a GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study

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Background: Gastric cancer (GC) development is a multistep process, during which numerous alterations accumulate in nuclear and mitochondrial DNA. A deficiency of repair machinery brings about an accumulation of errors introduced within simple repetitive microsatellite sequences during replication of DNA. Aberrant methylation is related to microsatellite instability (MSI) by the silencing of the *hMLH1* gene. The aim of this study is to investigate a possible relationship between the *RUNX3* promoter methylation, nuclear microsatellite instability (nMSI) and mitochondrial microsatellite instability (mtMSI), in order to clarify its biological role in GC.

Patients and methods: nMSI and mtMSI were evaluated in a consecutive series of 100 GC patients. For the analysis of the nMSI, we followed the National Cancer Institute guidelines. mtMSI was assessed by analyzing a portion of the displacement-loop region. The aberrant methylation of *RUNX3* was analyzed in 40 GC patients by methylation-specific PCR.

Results: Overall, 55% of GC demonstrated methylation of the *RUNX3* promoter; 82% of GC was classified as stable microsatellite instability, 5% as low-level microsatellite instability and 13% as high-level microsatellite instability (MSI-H); mtMSI was detected in 11% of GC. A significant association was found between mtMSI and tumor–node–metastasis staging, furthermore an interesting association between MSI-H status, mtMSI and *RUNX3* methylation.

Conclusion: These data suggest that *RUNX3* is an important target of methylation in the evolution of mtMSI and nMSI-H GC.

Key words: gastric cancer, methylation-specific PCR (MSP), mitochondrial microsatellite instability (mtMSI), nuclear microsatellite instability (nMSI), *RUNX3*

introduction

Gastric cancer (GC) is the second most common malignant neoplasm throughout the world and the second most frequent cause of cancer death [1]. GC development is a multistep process, during which numerous genetic and epigenetic alterations accumulate. In various human malignancies, alterations do not only involve nuclear but also mitochondrial DNA. Microsatellite instability (MSI) phenotype is characterized by the presence of errors during DNA replication, such as small deletions and or insertions, within simple repetitive microsatellite sequences [2]. In order for the cell to accurately copy its genome during cellular proliferation, the DNA mismatch repair (MMR) complex is essential. Deficiencies of this system result in a subsequent failure to repair errors introduced during replication of DNA [3]. The mitochondrial genome (mtDNA) is made up of 16 569 bp and encodes 13 polypeptides, 22 transfer RNAs and two ribosomal RNAs, all of which are essential for electron transport and ATP generation. All the mitochondrial genes are organized continuously, there being only two noncoding regions. The 1.1-kb displacement (D)-loop contains the main regulatory sequences for transcription and replication initiation [4]. A mononucleotide repetition of poly-C, (D310), has been identified among the nucleotides 16184 and 16193 and can be considered as a marker for the study of mitochondrial instability in GC [5]. For the analysis of the nuclear

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microsatellite instability (nMSI), we followed the guidelines provided by the National Cancer Institute (NCI) in 1997 recommending a panel of five microsatellite markers [6]. Nuclear MSI can be classified as high-level microsatellite instability (MSI-H), low-level microsatellite instability (MSI-L) or stable microsatellite instability (MSS). No correlation between nuclear and mitochondrial MSI was found in colorectal, breast and renal cancers [7]. Results involving GC, however, prove to be fairly controversial [8, 9, 32]. A further molecular defect described in gastric cancer is aberrant methylation of the cytosine within the CpG islands, a phenomenon associated with loss of gene expression by transcription repression [10]. Some GCs show a high degree of concordant methylation of CpG islands; these types of tumors are classified as high CpG island methylator phenotypes (CIMP) [9]. CIMP is closely related to MSI phenotypes; in fact in most sporadic GCs, nMSI is due to the silencing of *hMLH1*, which is a *MMR* gene [11–14]. In addition to aberrant methylation of the 5′ CpG islands of *hMLH1*, several other tumor-related genes such as *E-cadherin, APC, RASSF1A, DCC* and *PTEN* have also been described in GC [15–19]. Recently, *RUNX3* has been shown to be among the gene promoters that are most specifically methylated in CIMP-positive gastric cancer [20]. *RUNX3* is a tumor suppressor gene located on chromosome 1p36, a region that is thought to carry a tumor suppressor gene implicated in various types of cancers, especially those of the gastrointestinal tract [21]. Together with *RUNX1* and *RUNX2*, it belongs to the runt related transcription factors (RUNXs), which respond to the growth, differentiation and apoptosis signals induced by the TGF-β pathway [22–24]. *RUNX3* loss of expression by promoter hypermethylation is involved in roughly half of the cases of GC and is mostly cancer specific, although recent studies have demonstrated that promoter methylation occurs also in non-neoplastic cells during aging [25, 26]. No study to date has examined relationship between *RUNX3* promoter methylation, nMSI and mitochondrial microsatellite instability (mtMSI) in GC. The purpose of this study was to investigate a possible relationship between these events in order to clarify the biological role in GC and to associate the resulting data with available clinicopathological variables.

Table 1. Clinicopathological variables of the 100 GC patients

Clinicopathological variables	Patients (%)
Sex	
Male	65
Female	35
Age	
<60	29
60–69	36
>70	35
Tumor site	
Antrum	26
Corpus	30
Cardias or Fundus	29
Unknown	15
Tumor size (cm)	
<5	31
>5	68
Unknown	1
Tumor histotype	
Intestinal	55
Diffuse	24
Mixed	3
Unknown	18
Histological grade	
Well differentiated (G1)	7
Moderately differentiated (G2)	40
Poorly differentiated (G3)	51
Unknown	2
TNM staging	
I	27
II	34
III	29
IV	8
Unknown	2
Invasion	
PS–	37
PS+	25
Unknown	38
Lymph node status	
Negative	39
Positive	58
Unknown	3

GC, gastric carcinoma; TNM, tumor–node–metastasis.

patients and methods

clinicopathological variables

A prospective study was conducted on a consecutive series of 100 patients affected by GC who had undergone potentially curative surgical resection at the Department of Oncology of Palermo University between 1999 and 2007. The following clinicopathological variables were taken into account: sex, age, tumor site, tumor size, tumor histotype, histological grade, tumor–node–metastasis (TNM) staging, invasion, and lymph node status (Table 1). None of the patients had undergone radio or chemotherapy before surgery; curative resection included gastrectomy and wide dissection of the regional lymph nodes. The excised GC and the lymph nodes were examined and staged according to the most recent TNM classification. Histological sections of the 100 operated pieces were examined separately by two pathologists (Rosa Maria Tomasino and Vincenza Morello). Lauren's criteria were used in order to classify the tumors into intestinal or diffuse types. Differentiation was evaluated and classified as G1, G2 or G3.

sampling

After obtaining informed consent, multiple samples (from 3 to 10) were taken from the various representative areas of the primary GC within 30 min of surgical resection. Each tissue specimen was carefully analyzed to make sure that, as far as possible, all non-neoplastic material had been removed and also to eliminate nonvital areas. Furthermore, a sample of apparently healthy mucosa as far away as possible from the tumor was taken from each patient to be used as a standard control for the subsequent biomolecular investigation. After sampling, all tissues were immediately frozen and stored at −80°C.

tissue DNA extraction

DNA was extracted both from the tumoral gastric tissue and from the mucosa by means of a mini QIAamp DNA kit (Qiagen, Hilden, Germany), according to the manufacturers' instructions.

nMSI and mtMSI

For the analysis of the nMSI, we followed the NCI guidelines, which in 1997 recommended a panel of five microsatellite markers for detection of MSI, consisting of two mononucleotide-repeats (BAT-25 and BAT-26) and three dinucleotide repeats (D2S123, D5S345 and D17S250). This panel of markers is referred to as the Bethesda panel [6] and provides a uniform set of markers and criteria for tumor classification that has helped standardize MSI analysis. PCR was performed on a total volume of 15 μl with the use of ~30 ng of genomic DNA, 1.5 μl of 10× 25 mM $MgCl_2$ buffer, 0.6 μl of 25 mM $MgCl_2$, 0.6 μl of 10 mM deoxyribonucleotide triphosphate (dNTP), 0.12 μl of 5 U/μl AmpliTaq Gold (Applied Biosystems, Foster City, CA), 1 μl of 5 μM FAM or HEX-labeled forward primer and 1 μl of 5 μM unlabeled reverse primer.

Mitochondrial instability was assessed by analyzing a portion of the D-loop, the mononucleotide tract of poly-C (D310) made up of 12–18 C interrupted by a T at bp10, following the same conditions for the PCR of nMSI but with the use of specific primers for the D310 region: FAM-labeled forward primer 5′-ACA GCC ACT TTC CAC ACA G-3′ and reverse primer 5′-TGG TTA GGC TGG TGT TAG GG-3′. Most of the alterations generally occur in the upstream portion of T.

The reaction was assessed on the GeneAmp 9700 (Applied Biosystems). Amplification was started with 12 min at 95°C, followed by 10 cycles composed of 15 s at 94°C, 15 s at 55°C and 30 s at 72°C. This was followed by 25 cycles composed of 15 s at 89°C, 15 s at 55°C and 30 s at 72°C. Approximately 1–2 ng of each PCR product were mixed with a 1:40 dilution of formamide-Gene scan 400 HD standard size (Applied Biosystems), denatured at 95°C for 2 min and visualized on an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems) Applied Biosystems. DNA alterations were detected using a GeneMapper software version 3.5. Samples were considered positive for MSI when alternate-sized bands were present in the tumor DNA but absent in mucosa DNA. Tumors showing nuclear instability were classified as MSI-H when two or more markers showed allelic shifts, as MSI-L if only one marker shows an allelic shift and as MSS when no marker showed an allelic shift. Tumors showing mtMSI were confirmed by direct sequencing on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The results were analyzed with the use of the software Sequencing Analysis 5.1 in order to detect the possible coexistence of several different variants (mutated or wild type) along the mononucleot tract (heteroplasmy).

Methylation Specific PCR

DNA methylation of *RUNX3* was determined by methylation-specific PCR (MSP). MSP distinguishes unmethylated (UM) from M alleles of a given gene based on DNA sequence alterations after bisulfite treatment of DNA, which converts UM but not M, cytosines to uracils. Subsequent PCR using primers specific to sequences corresponding to either M or UM DNA sequences is then performed. Bisulfite modification of genomic DNA was carried out essentially as described previously [27]. Briefly, 2 μg of genomic DNA was denatured with 5.5 μl of 2 M NaOH, freshly prepared for 10 min at 37°C. We then added and mixed 30 μl of freshly prepared 10 mM hydroquinone and 520 μl of freshly prepared 3 M sodium bisulfite at pH 5. The samples were then incubated in mineral oil for 16 h at 50°C. The modified DNA was purified with a Wizard DNA clean-up system (Promega, Madison, WI). After purification, the samples were treated with NaOH (0.3 M) for 5 min at 37°C. The DNA was then ethanol precipitated and resuspended in 20 μl of water for MSP analysis. The bisulfite-induced changes affecting UM and M alleles were detected by PCR. Primer sequences for the UM reaction were 5′-TTATGAGGGGTGGTTGT ATGTGGG-3′(forward) and 5′-AAAACAACCAACACAAACACCTCC-3′ (reverse), and primer sequences for the M reaction were 5′-TTACGAGGGGCGGTCGTACGCGGG-3′ (forward) and 5′-AAAACGACCGACGCGAACGCCTCC-3′ (reverse). The primers, which amplify the proximal regional (- 218 and - 69) of the initial transcription site, were designed by taking into consideration the whole sequence of the gene codifying RUNX3, containing also the P2 promoter sequence (GeneBank Access number AL023096), where there is a long CpG (4.2 kb) island. The PCR was performed in a total volume of 50 μl, using 5 μl of 10× 25 mM $MgCl_2$ buffer, 1.5 μl of dNTP 10 mM, 2.5 μl of forward primer (UM and M) 10 μM, 2.5 μl of reverse primer (UM and M) 10 μM, 0.3 μl of 5U/μl AmpliTaq Gold (Applied Biosystems) and about 30 ng of bisulfite-modified DNA. The amplification was performed on GeneAmp 9700 thermocycler (Applied Biosystems) under the following conditions: predenaturation at 95°C for 10 min, 35 amplification cycles (denature at 94°C for 30 s, annealing at 66°C for 40 s and extension at 72°C for 1 min) and final extension at 72°C for 7 min. The product indicative of an UM *RUNX3* allele and of a M *RUNX3* allele were analyzed on 2% agarose gels and photographed. CpG Universal M DNA (Chemicon International, CA) was used as the positive control for M alleles, CpG Universal UM DNA (Chemicon International), as a negative control for M genes, 100 bp DNA ladder as size marker. MSP assay for each sample was repeated at least twice.

bisulfite sequencing

In order to verify MSP, bisulfite sequencing was performed by automated sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit and the model ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

statistical analysis

Associations between biomolecular and clinicopathological variables were evaluated by the chi-square test with Yates correction, where appropriate. A P value <0.05 was considered statistically significant.

results

methylation status of *RUNX3* in gastric cancers

To determine aberrant CpG island methylation of *RUNX3*, we analyzed the promoter region spanning the transcription start site of 40 gastric cancers using MSP. In total, 55% (22/40) of GC demonstrated complete methylation of the *RUNX3* promoter. M GC always displayed evidence of an accompanying UM PCR product (Figure 1). These UM *RUNX3* alleles most likely reflect either the unavoidable presence of normal cells in the cancer specimen or heterogeneity of the methylation event within the tumor cell population itself. Methylation observed is relatively cancer specific since only two of 40 cases showed evidence for aberrant *RUNX3* methylation in the normal mucosa of older patients (data not shown). Bisulfite direct sequencing was performed on all of these normal and tumor samples to confirm the accuracy of the MSP results (Figure 2).

nuclear MSI

None of the 100 patients analyzed had a family history of gastric cancer. Overall, 82% of GC was classified as MSS, 5%

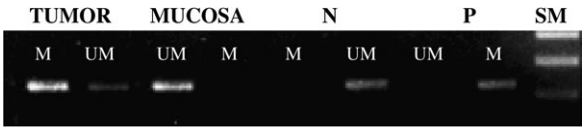

Figure 1. Representative examples of *RUNX3* promoter methylation by MSP in gastric cancer. Methylated (M), unmethylated (UM), negative control (N), positive control (P), size marker (SM).

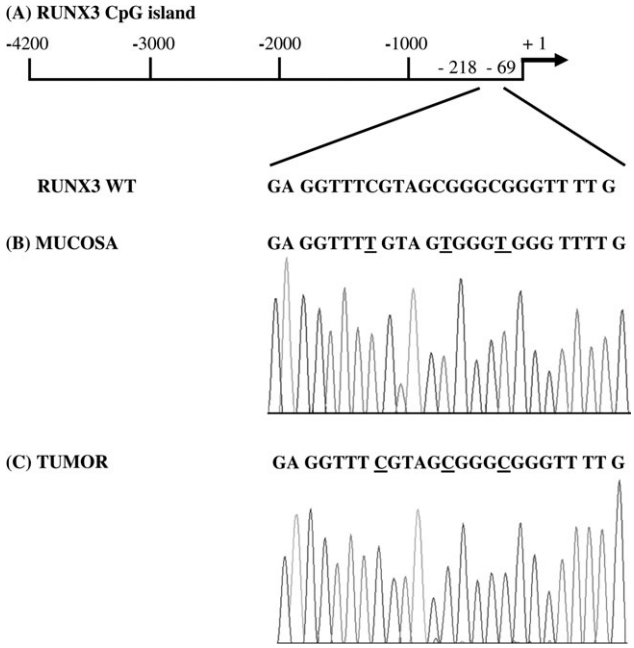

Figure 2. (A) Representative illustrations of *RUNX3* CpG island. (B) Electropherogram of unmethylated *RUNX3* promoter. The underlined T indicates that it was converted from C by bisulfite treatment, suggesting that the residue was not methylated. (C) Sequence chromatogram of methylated *RUNX3* promoter. The underlined C indicates that it was resistant to bisulfite treatment, suggesting that the residue was methylated.

as MSI-L and 13% as MSI-H. The representative examples of MSI-H and MSI-L GC are shown in Figure 3. Of the GCs presenting MSI-H, 10 demonstrated instability in all five microsatellite markers (Figure 3A), two in BAT 25, BAT 26, D2S123 and D5S346 (Figure 3B) and one in BAT 25, BAT 26 and D2S123 (Figure 3C). It is interesting to note that instability in D2S123 was detected in all the five tumors with MSI-L (Figure 3D).

mitochondrial MSI

mtMSI was detected in 11% (11/100) of GC. Seven (64%) of the changes were deletions and four (36%) were insertions. Most of the deletions and insertions involved 1–2 bp. The largest alteration was a 4-bp deletion seen in two cases. In one case there was a 3 bp insertion. An example of the DNA fragment and sequencing analysis is shown in Figure 4. After analysis of chromatograms obtained by DNA sequencing, seven of 11 (64%) of alterations were evaluated to be heteroplasmic and four of 11 (36%) homoplasmic.

relationship between mtMSI, nMSI, RUNX3 methylation status and clinicopathological variables

The clinicopathological variables of GC patients are summarized in Table 1. No significant association was found between biological variables analyzed and sex, age, tumor site, tumor size, tumor histotype histological grade, invasion or lymph node status (data not shown). The only significant association was detected between mtMSI and TNM staging ($P < 0.025$) (Table 2). Statistical analysis highlighted an interesting association between MSI-H status, mtMSI and *RUNX3* methylation (Tables 2 and 3).

discussion

Tumor-related genes tend to be preferentially inactivated by specific genetic or epigenetic mechanisms. Accumulating evidence suggests that epigenetic silencing of tumor suppressor genes due to aberrant hypermethylation of gene promoter regions play an important role in gastric cancer pathophysiology [28]. Concordant methylation of multiple genes (termed CIMP) has been reported in carcinomas from diverse sites. The methylation affects several critical molecular pathways leading to cell immortalization and transformation, including alterations in cell cycle regulation, metabolic detoxification enzymes, cellular adherence and the DNA damage response pathways [29]. Recently, a causal relationship has been identified between aberrant methylation of the promoter of *RUNX3* and the incidence of gastric cancer in humans [20]. The results obtained from this study demonstrated that 55% (22/40) of human GCs presented hypermethylation of the promoter region of the gene and only two of 40 cases also showed methylation of the *RUNX3* promoter in the nonmalignant gastric tissue. Our results indicate that *RUNX3* promoter methylation is a cancer-specific event. A possible explanation for detecting methylation in normal gastric tissues is that the methylation of *RUNX3* has been attributed to the aging process, and this is similar to previous findings in which methylation was observed in people >70 years of age [26]. The data reported in literature show differences in methylation frequency of the gene *RUNX3*. In a previous study, Waki et al. [26] reported that *RUNX3* methylation was observed in 45% of GCs; other investigators reported that *RUNX3* methylation was observed in 71% of GCs [30]. These data can be explained by the fact that different regions were examined within the *RUNX3* CpG island. Homma et al. [31] studied the whole *RUNX3* CpG island and showed that the 5′ outskirt region is not informative. In contrast, methylation spanning the transcription start site within the *RUNX3* promoter CpG island is predominantly cancer specific and is critical for gene silencing. In our study of sporadic GCs, the association between *RUNX3* promoter methylation, nMSI and mtMSI showed significant associations, suggesting that these cancers have acquired a 'methylator' phenotype. The mechanism of cancer development proposed for sporadic MSI-H GCs is the aberrant promoter methylation of *hMLH1* with the consequent functional loss of *MMR* genes [14]. All the mtDNA repair machinery is encoded by nDNA, and the corresponding gene

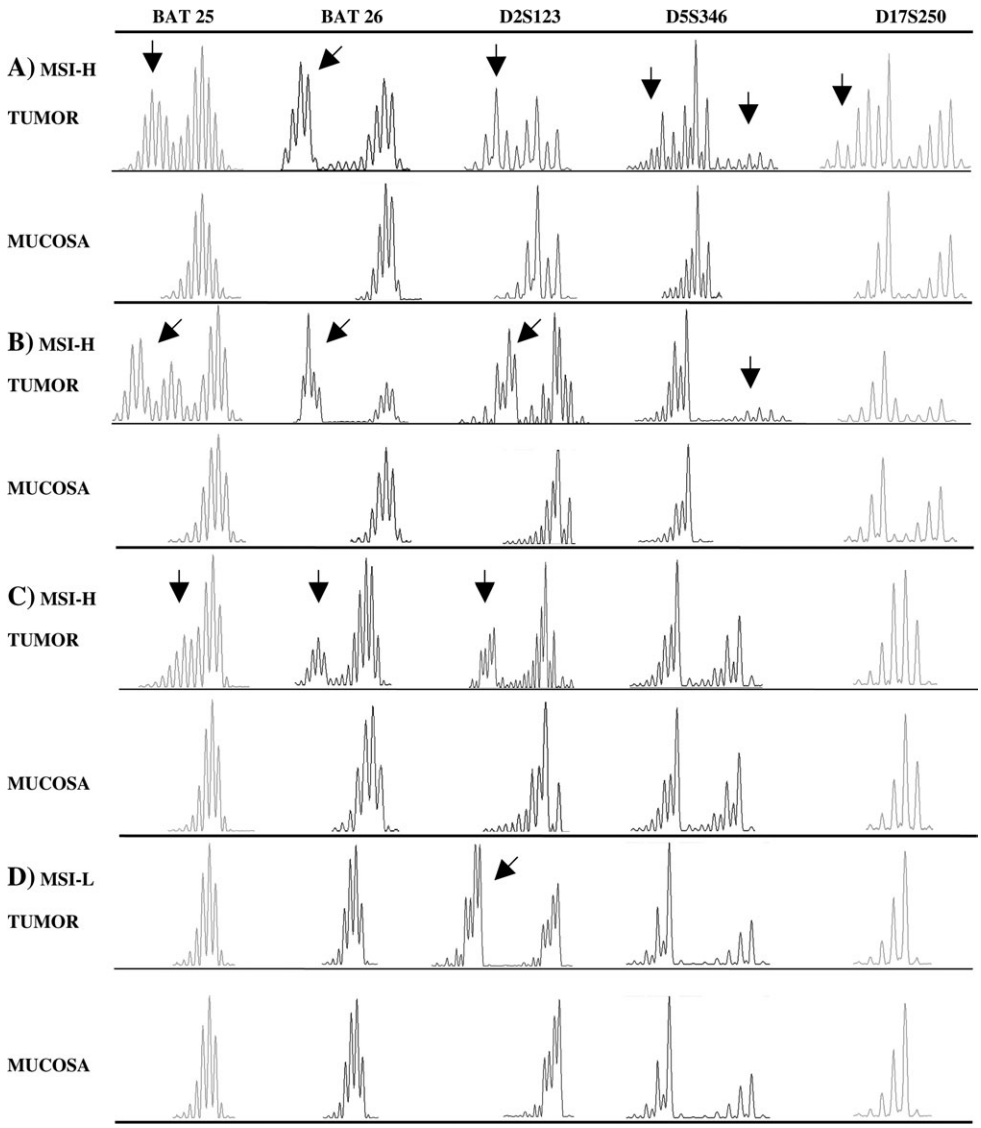

Figure 3. Representative allelic profiles of the five mononucleotide markers used for the determination of nMSI status. New alleles present in MSI-H and MSI-L samples were indicated with an arrow.

Table 2. Association between *RUNX3* methylation, nMSI and mtMSI in gastric cancers

	RUNX3 unmethylated, fraction (%)	*RUNX3* methylated, fraction (%)	*P* value
Total: 40			
nMSI			
MSI-S	18/18 (100)	9/22 (41)	≤0.01
MSI-L	0/18 (0)	0/22 (0)	
MSI-H	0/18 (0)	13/22 (59)	
mtMSI			
mtMSI	0/18 (0)	11/22 (50)	≤0.01
mtMSS	18/18 (100)	11/22 (50)	

MSI, microsatellite instability; nMSI, nuclear MSI; mtMSI, mitochondrial MSI; MSI-S, stable MSI; MSI-L, low-level MSI; MSI-H, high-level MSI; mtMSS, mitochondrial stable MSI.

Table 3. Association between mtMSI, TNM staging and nMSI in gastric cancers

	mtMSS, fraction (%)	mtMSI, fraction (%)	*P* value
TNM staging			
I	19/78 (24)	7/11 (64)	≤0.025
II	28/78 (36)	0/11 (0)	
III	24/78 (31)	4/11 (36)	
IV	7/78 (9)	0/11 (0)	
nMSI			
MSI-S	77/80 (96)	1/11 (9)	≤0.01
MSI-L	0/80 (0)	1/11 (9)	
MSI-H	3/80 (4)	9/11(82)	

MSI, microsatellite instability; mtMSI, mitochondrial MSI; TNM, tumor–node–metastasis; nMSI, nuclear MSI; mtMSS, mitochondrial stable MSI; MSI-S, stable MSI; MSI-L, low-level MSI; MSI-H, high-level MSI.

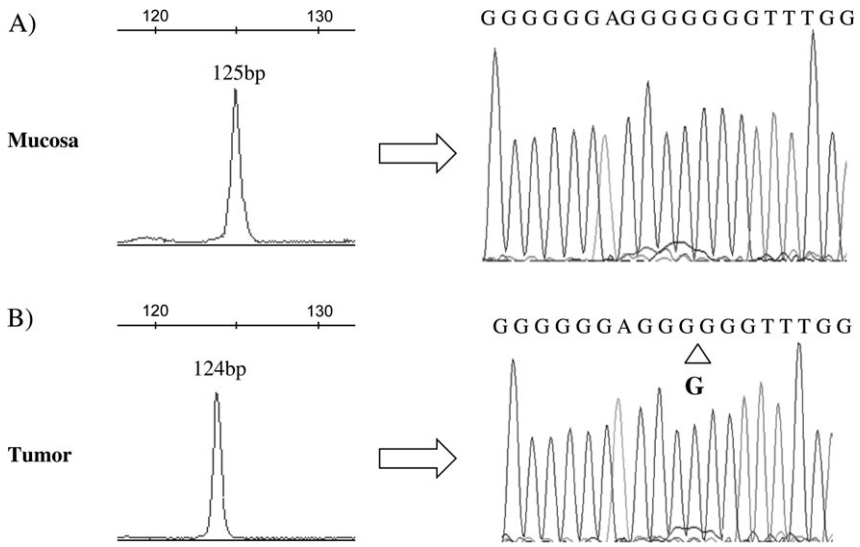

Figure 4. Representative examples of the DNA fragment and sequencing analysis of the D310 region. (A) Fragment analysis of D310 in mucosa and corresponding sequencing result. (B) Frameshift mutation (1 bp deletion) in GC patient and corresponding sequencing result which reveals the deletion. Electropherogram refers to reverse direction.

products are subsequently transported into the mitochondria. This indicates that there might be a common cause for the onset of mtMSI and nMSI. In the present study, mitochondrial and nuclear instability were examined in 100 GCs. The prevalence of mtMSI and nMSI-H in gastric cancer varies among different studies, and the relationship between the occurrence of these alterations remains controversial [8, 9, 32]. We found that 11% of the gastric cancer patients had alterations in the D310 poly-C tract in the D-loop; 13% of the cases showed MSI-H, 5% MSI-L and 82% MSS. These data are in concordance with previous studies [8, 9]. The mtMSI was significantly associated with nMSI, in fact, nine of 11 cases positive for mtMSI showed MSI-H. In several studies, the MSI-H, mtMSI and *RUNX3* promoter methylation in GC have been associated with several clinicopathological variables, although several reports come to different conclusions [30–34]. In the present study, no association was found with any of the clinicopathologic variables; mitochondrial instability only proved to be associated with the TNM staging. Up to now, no study had ever examined the relationship between *RUNX3* promoter methylation, nuclear and mitochondrial MSI in GC. Our data highlight a significant association between these events in gastric cancer, suggesting that *RUNX3* is an important target of methylation in the evolution of mtMSI and nMSI-H GC.

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