Epigenetic Alterations in a Gastric Leiomyoma

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Leiomyomas constitute 2.5% of all resected neoplasms of the stomach. They are usually asymptomatic, but may present mucosal ulceration. Aberrant DNA methylation is a well-defined epigenetic change in human neoplasms; however, gene-acquired methylation may not necessarily be related with a malignant phenotype. In this report we analyzed in a gastric leiomyoma, the methylation status of 84 CpGIs in tumor suppressor and DNA repair genes. We analyzed the tumor center (TC) and tumor periphery (TP) separately. We found aberrant methylation in 2/84 CpGI in the TC portion, that is, MLH1 and MSH3, and 5/84 CpGI in the TP, that is, MLH1, MSH3, APC, MSH6, and MGMT. The gene with the highest methylation percentage in the TC and TP was MLH1. Given that MLH1 methylation has been associated with microsatellite instability, we analyzed the status of the microsatellite Bat-26. We found that neither the TC nor the TP presented instability. The methylation of MLH1, MGMT, and APC has been described in GISTs, but to the best of our knowledge this is the first time that the methylation of these genes has been associated with gastric leiomyoma. Further research should be conducted to identify reliable molecular markers that could differentiate between GISTs and gastric leiomyomas.

1. Introduction

Gastric leiomyomas account for 2.5% of gastric neoplasms. Although most of them are asymptomatic, patients may present upper gastrointestinal hemorrhage [1, 2]. Endoscopically, gastric leiomyomas appear as a large submucosal lesion, and generally endoscopic biopsies are not deep enough to be of any diagnostic value [3]. Pathologically, most of these tumors are composed of spindle cells and display smooth muscle differentiation. Leiomyomas are defined as being desmin and actin positive and c-Kit (or CD117) negative tumors [4].

Tumorigenesis is the result of a multistep process characterized by the accumulation of genetic and epigenetic alterations leading to uncontrolled growth. Among epigenetic modifications, the most studied event in human neoplasms is the deregulation of methylation of DNA, giving rise to widespread changes in the methylome patterns during tumor progression [5]. The epigenome of tumors is characterized by global DNA hypomethylation and by gene-specific hypermethylation. Gene silencing by CpG islands (CpGIs) hypermethylation in gene promoters can modulate pathways that control the basic function of the cell by acting directly on tumor suppressor genes and caretaker genes and indirectly on oncogenes through their regulators [5].

Gene expression profile studies have demonstrated that some genes are hypermethylated in gastric GISTs (gastrointestinal stromal tumors) [6–8], but, to our knowledge, there is no information of the methylation profile of gastric leiomyomas.

The objective of this study was to analyze by methyl specific-multiplex ligation probe amplification (MS-MLPA) the methylation status of tumor suppressor and DNA repair genes in a gastric leiomyoma.
2. Case Report

A 63-year-old Hispanic female presented with history of melena for two days. Upon clinical examination the patient mentioned that she was treated for *H. pylori* infection 15 years before. No other constitutional symptoms were present. Upper gastrointestinal endoscopy was performed, showing an ulcerated submucosal tumor localized on the cardiac region. Upper endoscopic ultrasonography (EUS) evidenced a submucosal lesion, of about 50 mm with decreased echogenicity and homogeneous structure, and no necrotic areas. The lesion belonged to the muscular layer, and EUS suggested GIST (gastrointestinal stromal tumor). Multisided tomography revealed that the lesion was 5 cm and showed no evidence of other lesions in the abdominal area. The patient was scheduled for laparoscopic surgery and was discharged from the hospital eleven days after the operation.

2.1. Histopathology. The histopathological diagnosis for the submucosal lesion was gastric leiomyoma, characterized by a proliferation of bland, spindle-shaped cells with elongated nuclei and eosinophilic fibrillary cytoplasm without necrosis and atypia (Figure 1). The mitotic index (number of mitoses per 50 high-power fields, HPF) was <2/50 HPF. The immunohistochemistry assay indicated diffuse positivity for vimentin, smooth muscle actin, and desmin and negative staining for c-Kit/CD117, CD34, cytokeratins AE1/AE2, and S-100. The morphology of the lesion along with the immunohistochemical features supported the diagnosis of leiomyoma.

2.2. Methylation Analysis. MS-MLPA assay was performed by kits ME001, ME002, ME003, ME0024, and ME011 according to manufacturer’s, MRC-Holland, Amsterdam, The Netherlands, instructions (http://www.mlpa.com/) on DNA obtained from the formalin fixed embedded tumor. The methylation status of 84 CpGIs in 41 cancer related genes was assessed (Table 1). We have established a cut-off threshold by considering a region to show methylation if the dosage ratio was >8% [9]. Due to tumor heterogeneity and in order to analyze if there were epigenetic differences between different regions of the tumor, we analyzed separately the tumor center (TC) and the tumor periphery (TP). We found aberrant methylation in 2/84 CpGIs in the TC portion, that is, one site localized at 485 nt before the transcription start site (TSS) of *MSH3* and one site at 382 nt before TSS of *MLH1*. Even though both genes presented methylation percentages above the established cut-off level, it is interesting to mention that they differed significantly: 11.6% and 52.9%, respectively (Figure 2, green bars). The peripheric portion of the tumor presented a different profile: 5/84 CpG sites were aberrantly methylated, from which 2 sites were shared with the center portion, that is, *MSH3* and *MLH1*, and 3 sites were exclusively methylated.
Table 1: List of genes analyzed in the gastric leiomyoma. Gene names and gene symbols are according to the HGNC database.

| Gene symbol | Gene name                                      |
|-------------|------------------------------------------------|
| APC         | Adenomatous polyposis coli                     |
| ATM         | Ataxia telangiectasia mutated                 |
| BCL2        | B-cell CLL/lymphoma                            |
| BNI1P3      | BCL2/adenovirus E1B 19 kDa interacting protein 3 |
| BRCA1       | Breast cancer 1                                |
| BRCA2       | Breast cancer 2                                |
| CACNA1A     | Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit |
| CACNA1G     | Calcium channel, voltage-dependent, P/Q type, alpha 1B subunit |
| CADM1       | Cell adhesion molecule 1                       |
| CCND2       | Cyclin D2                                      |
| CD44        | CD44 molecule                                  |
| CDH13       | Cadherin 13                                    |
| CDKN1B      | Cyclin-dependent kinase inhibitor 1B           |
| CDKN2A      | Cyclin-dependent kinase inhibitor 2A           |
| CHFR        | Checkpoint with forkhead ring finger domains   |
| CREM        | cAMP responsive element modulator              |
| DAPK1       | Death-associated protein kinase 1              |
| DLC1        | DLC1 Rho GTPase activating protein             |
| ESR1        | Estrogen receptor 1                            |
| FHIT        | Fragile histidine triad                        |
| GATA5       | GATA binding protein 5                         |
| GSTP1       | Glutathione S-transferase pi 1                 |
| H2AFX       | H2A histone family, member X                   |
| HIC1        | Hypermethylated in cancer 1                    |
| HLTF        | Helicase-like transcription factor             |
| ID4         | Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein |
| MGMT        | O-6-Methylguanine-DNA methyltransferase        |
| MLH1        | MutL homolog 1                                 |
| MLH3        | MutL homolog 3                                 |
| MSH2        | MutS homolog 2                                 |
| MSH3        | MutS homolog 3                                 |
| MSH6        | MutS homolog 6                                 |
| PAH         | Phenylalanine hydroxylase                      |
| PAX5        | Paired box 5                                   |
| PAX6        | Paired box 6                                   |
| PMS2        | PMS2 postmeiotic segregation increased 2       |
| PRDM2       | PR domain containing 2, with ZNF domain        |
| PTCH1       | Patched 1                                      |
| PTEN        | Phosphatase and tensin homolog                 |
| PYCARD      | PYD and CARD domain containing                 |
| RARB        | Retinoic acid receptor beta                    |
| RASSF1      | Ras association domain family member 1A        |
| RBI         | Retinoblastoma 1                               |

Table 1: Continued.

| Gene symbol | Gene name                      |
|-------------|--------------------------------|
| RUNX3       | Runt-related transcription factor 3 |
| SCGB3A1     | Secretoglobin, family 3A, member 1 |
| SFRP4       | Secreted frizzled-related protein 4 |
| SFRP5       | Secreted frizzled-related protein 5 |
| STK11       | Serine/threonine kinase II      |
| TGIF1       | TGFβ-induced factor homeobox 1  |
| THBS1       | Thrombospondin 1                |
| TIMP3       | TIMP metallopeptidase inhibitor 3 |
| TP53        | Tumor protein p53               |
| TP73        | Tumor protein p73               |
| TWIST1      | Twist family bHLH transcription factor 1 |
| VHL         | Von Hippel-Lindau tumor suppressor |
| WT1         | Wilms tumor 1                   |

Figure 2: Genes methylated in a gastric leiomyoma. Grey bars represent the genes methylated in the tumor periphery and green bars represent the genes methylated in the tumor center.

In the tumor periphery, that is, MSH6, MGMT, and APC (Table 2). As a control, MS-MLPA assay was performed in normal tissue (i.e., leucocytes of healthy patients); we determined that there was no aberrant methylation in the regions analyzed. The percentages of methylation in the TP were 10.8%, 77%, 14.3%, 9.5%, and 27.7%, respectively (Figure 2, grey bars). Even though the methylated genes differed between the TC and the TP, it is interesting to mention that MLH1 was shared by both and presented the highest percentage of methylation (52.9% and 77%, resp.). To analyze whether methylation affected gene expression, we performed qRT-PCR assays on 2 cell lines (MDA-MB231 and MCF-7) which presented different percentages of APC promoter methylation (0% and 52%, resp.); we confirmed that the methylation of APC on the CpG site −21nt before TSS reduces gene expression (data not shown). The methylation of MLH1 at 382nt before TSS has been previously shown to provoke downregulation of gene expression [10].

Given that MLH1 methylation is associated with microsatellite instability (MSI) in sporadic endometrial and...
Table 2: List of methylated genes in the gastric leiomyoma studied. Gene names and gene symbols are according to the HGNC database; CpG site locations are mentioned based on the MRC-Holland data sheets. TP indicates tumor periphery and TC tumor center.

| Gene symbol | Name                                | CpG location | Sample | Methylation % |
|-------------|-------------------------------------|--------------|--------|---------------|
| APC         | Adenomatous polyposis coli          | 21 nt before TSS | TP     | 277           |
|             |                                     |              | TC     | 0             |
| MGMT        | O-6-Methylguanine-DNA Methyltransferase | 233 nt after TSS | TP     | 9.5           |
|             |                                     |              | TC     | 0             |
| MSH6        | mutS homolog 6                      | 317 nt before TSS | TP     | 14.3          |
|             |                                     |              | TC     | 0             |
| MLH1        | MutL Homolog 1                      | 382 nt before TSS | TP     | 77.6          |
|             |                                     |              | TC     | 59.2          |
| MSH3        | mutS homolog 3                      | 485 nt before TSS | TP     | 10.8          |
|             |                                     |              | TC     | 11.6          |

TSS: transcription start site. nt: nucleotide.

colorectal cancers [11, 12], we decided to analyze MSI in the gastric leiomyoma. To evaluate MSI, we analyzed by PCR the status of the mononucleotide microsatellite Bat-26 in the tumor center and periphery. This microsatellite has been shown to be highly efficient and sensitive to determine MSI-H when used as a single marker [13, 14]. Interestingly, Bat-26 was stable in the TC as well as in the TP portion of the gastric leiomyoma analyzed. When we tested Bat-26 status in 5 nontumoral tissues and in peripheral blood of healthy patients, none of these tissues presented Bat-26 instability.

3. Discussion

The onset and progression of tumorigenesis involves a cascade of genetic and/or epigenetic events. Results from recent investigations have shown that DNA methylation profiles contain tumor type-specific signatures which, in the future, could serve as biomarkers for clinical outcome [15]. Hypermethylation at the promoter region of several genes has been shown to be an important mechanism in gene silencing. However, gene-acquired methylation may not necessarily be related with malignant phenotype [16, 17]. In this report, we show that a gastric leiomyoma, considered a benign disease, presents methylation in 2–5 of 84 analyzed CpGIs, varying in different tumor parts.

The methylation percentage in the overall sample varied widely (from 9.5% to 77%) for the different genes (Figure 1). This wide-ranging methylation levels could be indicating that the aberrant hypermethylation occurs at different sites and times, and, therefore, probably the genes with higher methylation levels (such as MLH1 or APC) were the first ones to be epigenetically altered during tumor progression. Considering that MLH1 presents a high methylation percentage, we hypothesize that its methylation could be an initial epigenetic event during gastric leiomyoma formation.

Given that MLH1 methylation is associated with MSI in several tumors [11, 12], we analyzed the status of the mononucleotide microsatellite Bat-26. As we mentioned before, neither the TC nor the TP portion presented Bat-26 instability. We speculate that the gastric leiomyoma analyzed is not MSI-H due to Bat-26 stability, but we cannot discard MSI-L or MSI-S. It is interesting to mention that the fact that MLH1 is methylated and MSI is stable has been also described by other authors and several hypotheses have been proposed. For example, studies performed by Esteller and colleagues on endometrial atypical hyperplasia concluded that MLH1 promoter methylation is an early event and in some cases may precede a detectable MSI phenotype [18]. Therefore, a possible explanation could be that the leiomyoma presents MLH1 methylation as an early event, lacking yet MSI signs. In another work performed by Kanaya et al. the authors studied the region 700 bp upstream of MLH1 promoter region covering 48 CpG sites; they classified the methylation status as full (over 80% of CpGs were methylated), partial (10–80%), or nonmethylated (less than 10%). The authors concluded that the degree, rather than region-specific methylation of CpG islands, is critical for MSI phenotype [19].

The genes found methylated in the gastric leiomyoma play different functions in the cell: MLH1, MSH3, MGMT, and MSH6 participate in DNA repair functions whereas APC gene participates in cell proliferation. The methylation of MLH1, MGMT, and APC has been previously described in GISTs, but to the best of our knowledge this is the first time that the methylation of these genes has been associated with gastric leiomyoma, which is considered a benign disease. Interestingly, we also found that there were differentially methylated genes between GISTs and the gastric leiomyoma. For example, in a study performed by House et al., the authors determined that the most frequently methylated genes in GISTs (in decreasing frequency) are MGMT, p16, RASSFIA, E-cadherin, and MLH1 [6]. In another report performed by Saito et al., the authors determined that, besides MLH1 and MGMT, GISTs also presented methylation in MINT2, p73, p16, E-cadherin, MINT1, p15, and MINT3. Moreover, the methylation of RASSFIA progressively increased from small to malignant GISTs and p16 was specifically methylated in malignant-prone and malignant GIST [8]. Note that neither RASSF1, p16, nor p73 is methylated in the studied gastric leiomyoma. These epigenetic differences between malignant GISTs and gastric leiomyoma have not been described before.
To the best of our knowledge this is the first report showing the methylation profile of a gastric leiomyoma. Further research should be conducted to identify reliable and accurate molecular markers that could help in the differentiation between GISTs and gastric leiomyomas.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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