Int J Clin Exp Pathol 2016;9(8):8366-8372
www.ijcep.com /ISSN:1936-2625/IJCEP0023627

Original Article

A case report of superficially spreading gastric cancer with distinct molecular features: a novel approach for evaluating gastric molecular carcinogenesis

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Received January 9, 2016; Accepted March 22, 2016; Epub August 1, 2016; Published August 15, 2016

Abstract: A 62-year-old woman was admitted to our hospital because of epigastralgia. Upper gastrointestinal endoscopy revealed a superficial spreading type of gastric tumor (superficially spreading gastric cancer, SSGC) with a central depression in the lesser curvature of the pyloric antrum. Histologically, the tumor was diagnosed as a differentiated-type adenocarcinoma. The bottom of the depression was in muscularis propria, whereas the surrounding lesion was within mucosa. Neither p53 overexpression nor MLH1 expression was found in either layer of the lesion by immunostaining. However, proliferative activity was higher in the invasive area of the lesion than in the intramucosal area. Expression of gastric mucin was seen in both areas of the lesion. Isolated crypts were analyzed using PCR to assess loss of heterozygosity (LOH) at chromosomal loci, microsatellite instability (MSI), and methylation status in the tumor. In both the intramucosal and invasive areas of the lesion, multiple losses of heterozygosity (LOHs) and increased methylation were observed, while MSI was observed only in the invasive lesion. We hypothesized that multiple LOHs and extensive methylation are closely associated with the early development of a subset of differentiated-type SSGCs, whereas MSI plays an essential role in the progression of SSGC with gastric phenotype.

Keywords: Methylation, LOH, gastric cancer, superficially spreading tumor

Introduction

Gastric cancer is one of the most common cancers worldwide. As the incidence of early gastric cancer has been increased in Japan, several distinct clinicopathological features of early gastric cancers have been clarified. The superficial spreading type of early gastric cancer (SSGC) was initially characterized by its wide, superficial lateral spreading and relatively shallow vertical growth [1, 2], and has been defined as having lesions greater than 25 cm^2 in area [1-4]. Although histologically SSGC is composed of both differentiated-type and undifferentiated-type, most of it is differentiated-type [1-4]. Furthermore, although this cancer was first described more than 40 years ago, its underlying pathogenesis is still not fully understood.

Recent advances in molecular biology have enabled the identification of many genetic alterations, not only in human gastric cancers, but also in malignancies of various other organs [5]. According to the current model of carcinogenesis, there are two distinct molecular types: chromosomal instability (CIN) and microsatellite instability (MSI) [5]. This model has been developed for colorectal cancer and is thought to be true of other types of cancer, as well, including gastric cancer [6, 7]. CIN is characterized by multiple losses of heterozygosity (LOHs) and a high frequency of *p53* gene mutation [6, 7]. LOH is associated with mutations in *p53* and/or *Ki-ras* genes in malignant tumors [8]. In addition, LOH is believed to be a driving force for tumor progression [8]. On the other hand, MSI has been shown to be present in surgically resected specimens from 10 - 20% of patients

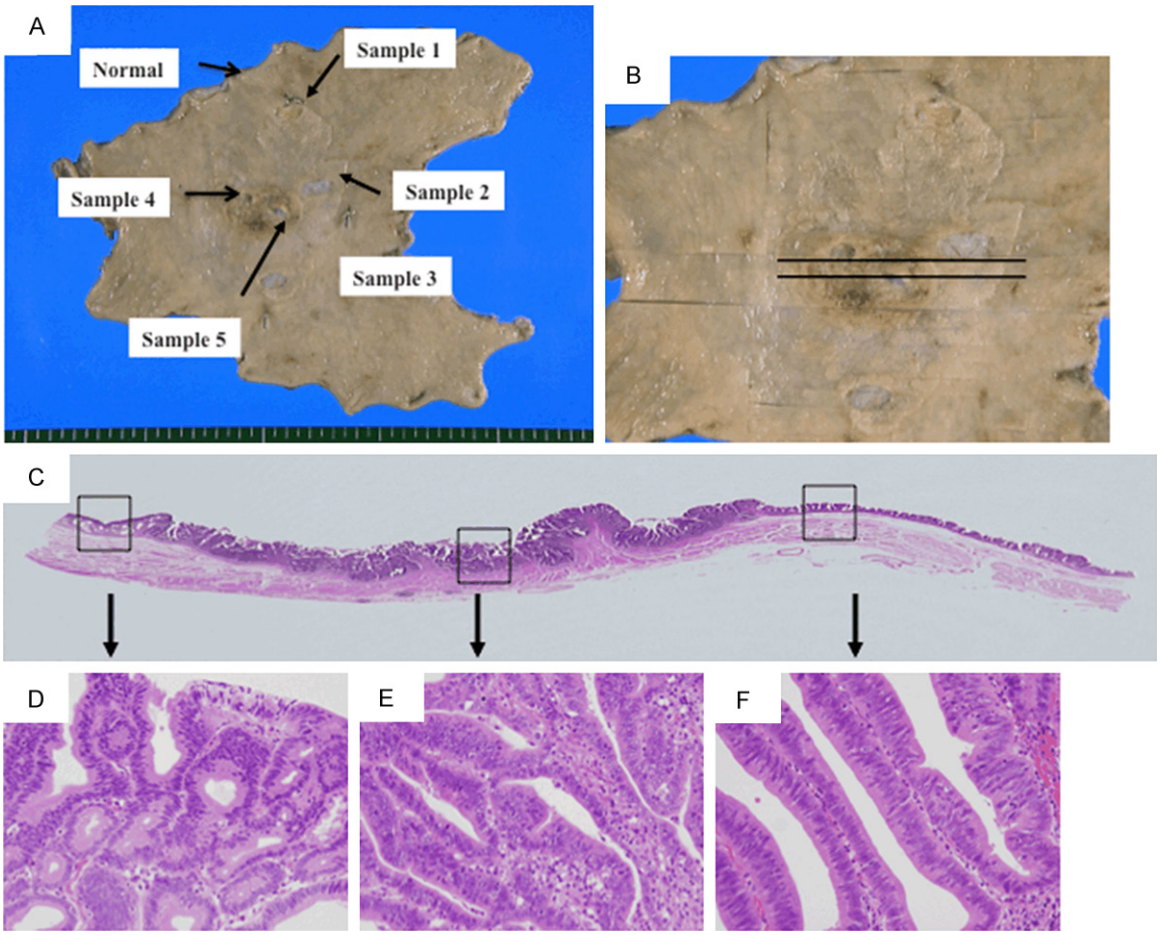

Figure 1. Resected stomach and histological features. A. Large superficial spreading tumor with a central invasive area is seen in the resected stomach. Sampling sites are indicated on the resected stomach (normal and tumor samples). Two samples were taken from the central area of the tumor (samples 4 and 5). The other 3 samples were obtained from the surrounding peripheral tumor tissue (samples 1, 2, and 3). The normal sample was taken from the greater curvature of normal stomach. B. Magnification of the depressed area. The black lines drawn on the area represent a specimen that is stained with H and E. C. Low-power view of the histological section. The three squares indicate different histological sections. D. Distal section of intramucosal lesion. E. Invasive lesion. F. Proximal section of intramucosal lesion.

with sporadic gastric cancers [9, 10]. In addition, MSI occurs at an early phase of gastric carcinogenesis [11, 12]. MSI has also been observed more frequently in differentiated-type adenocarcinomas and in patients with multiple tumors than in those with a single tumor [13-15]. In a recent study it was suggested that the CpG island methylator phenotype (CIMP) is a general increase in methylation throughout the genome [16]. Although CIMP and MSI have common clinicopathological features, such as mucin formation, presence in poorly differentiated type cancers, and gastric mucin expression [11, 12, 17], whether these types of molecular alterations are associated with superficial spreading gastric cancer (SSGC) remains unknown.

We present a patient with a differentiated-type SSGC with a gastric phenotype that is expected to exhibit distinct molecular features.

Case report

A 62-year-old Japanese woman was admitted to Iwate Medical University Hospital, Japan, because of epigastralgia. An upper gastrointestinal series revealed a large filling defect in the middle gastric body. Upper gastrointestinal endoscopy demonstrated a large, flat, elevated lesion with a central deep, depressed region in the middle gastric body. A distal gastrectomy was performed because the tumor biopsies revealed adenocarcinoma. The stomach was not adherent to the peritoneal wall, there was

Table 1. The list of the antibodies used in the case

Antibody	Clone	Supplier	Dilution
p53	DO-7	Novocastra	1:100
MLH-1	G168-15	BD Pharmingen	1:20
Ki-67	MIB1	Dako	1:100
MUC2	Ccp58	Novocastra	1:100
CD10	56C6	Novocastra	1:100
MUC5AC	CLH2	Novocastra	1:100
MUC6	CLH5	Novocastra	1:100

no swelling observed in the surrounding lymph nodes, and no metastases were found. The postoperative course was uneventful. Finally, hereditary nonpolyposis colorectal cancer was excluded.

Pathological findings

The tumor was 90 x 64 mm in size and was a flat, elevated lesion characterized by lateral and circumferential extension. The central area of the tumor was deeply depressed. The tumor was located in the middle gastric body, as seen in **Figure 1A**. The tumor was cut for paraffin embedding and further histological analysis as shown in **Figure 1B**.

A well differentiated or papillary adenocarcinoma was found within the central depressed area (**Figure 1C**). There was invasion of tumor cells into the muscularis proper. There were no lymphatic or venous invasions seen within the invasive lesion. In addition, no necrosis was observed. In the surrounding area, a well differentiated adenocarcinoma that appeared to be more differentiated than the central depressed lesion was seen (**Figure 1E**). The depth of the tumor in the surrounding area was within the mucosa propria (**Figure 1D** and **1F**). The histological criteria used to make histological diagnoses in this gastric tumor were based on our hospital criteria, which are modifications of the criteria of the Japanese Research Society for Gastric Cancers [19]. Based on the macroscopic and histological findings, we diagnosed this cancer as differentiated-type superficial spreading gastric cancer.

Multiple samples from the invasive and surrounding noninvasive lesion and normal mucosa (normal sample and tumor samples [1-5] were obtained to assess molecular alterations (**Figure 1A**).

Immunohistochemical analysis of tissue sections

The antibodies used are listed in **Table 1**. Immunostaining after microwave treatment (in citrate buffer, pH 6.0, x 3 for 5 min at 750 W) (H2500, Microwave Processor, Bio Rad, USA) was performed using the DAKO Envision+ detection system, as previously described [18]. Immunohistochemical analysis was performed on tissue sections of the resected stomach adjacent to the regions sampled for molecular analysis.

There was no p53 overexpression or loss of MLH1 expression seen in either the intramucosal or invasive areas of the tumor. The percentages of Ki-67-positive tumor cell nuclei were higher in the invasive areas of the lesion than in the intramucosal area. Although there was no expression of MUC2 or CD10 in either area of the lesion, MUC5AC, a marker for gastric foveolar epithelium, was positive in both areas. Areas of cancerous tissue stained positive for MUC6.

Analyses of genetic and epigenetic alterations using isolated tumor glands

For accurate molecular analysis, glands were isolated from the resected tumor. To perform crypt isolation, fresh tumor and normal tissue samples were obtained from the resected gastric cancer. Crypt (or gland) isolation was performed according to the method previously reported [20]. DNA was extracted from isolated non-neoplastic and tumor tissues as reported previously [6].

LOH on chromosomes 3p, 5q, 17p, and 18p was examined in paired tumor and normal DNA samples obtained from 5 tumor sites and one nonneoplastic site using 9 highly pleomorphic microsatellite markers (D3S2402, D3S1234, D5S107, D5S346, D5S299, D5S82, TP53, D18S487, and D18S34).

PCR was carried out using a DNA autosequencer (Applied Biosystems 373A sequencer; Applied Biosystems, CA, USA) as previously described [20]. PCR analysis was performed using GeneScan software (Applied Biosystems) for allele scoring and assessment of allelic loss as described previously [21]. MSI analysis was performed according to previous criteria [12]. The methylation status of the genes we examined (*MLH1*, *MGMT*, *p16*, *HPP1*, *RUNX3*, and

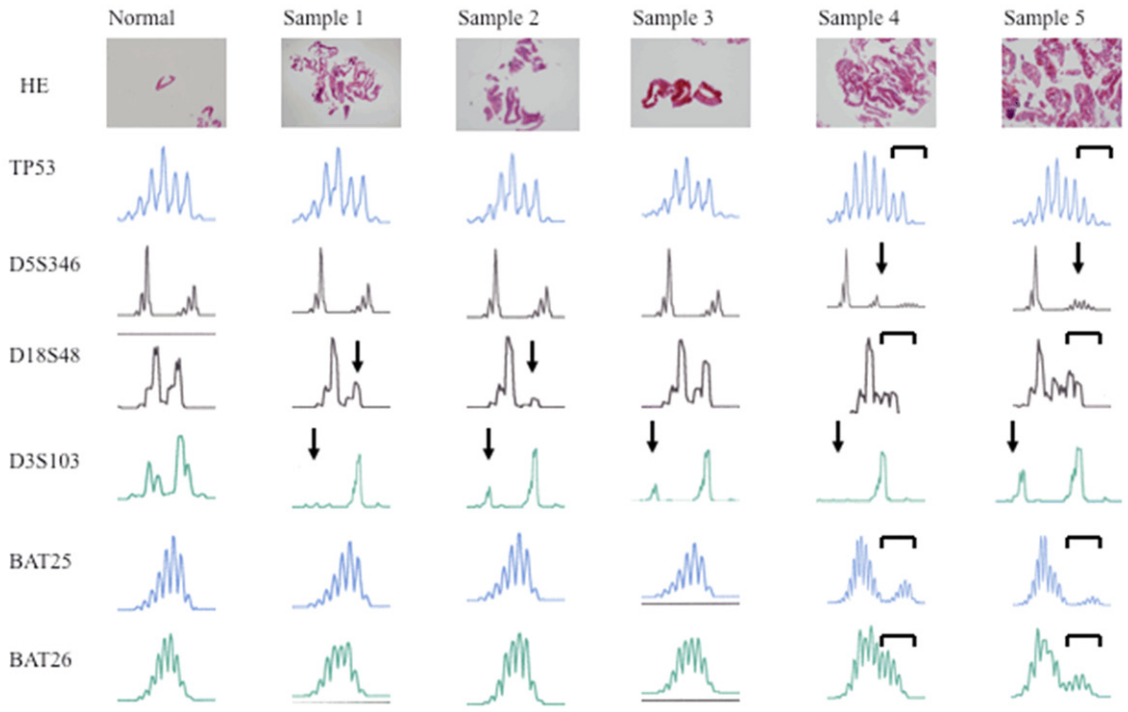

Figure 2. Genetic analysis of the isolated tumor glands from samples 1-5. Arrowheads indicate allelic loss. Parentheses indicate MSI. D2S123 and 17S250 were not seen in this figure. There were no allelic losses seen at the TP53 locus on chromosome 17p in samples 1 to 3; however, the loss could not be judged in samples 4 and 5 because of MSI. Only samples 4 and 5 showed 5q allelic loss. Although the isolated tumor glands in samples 1 and 2 had 18q allelic losses, they did not show 18q allelic losses in sample 3. Determination of 18q allelic loss in samples 4 and 5 was excluded because the two samples showed MSI. 3q allelic losses were common alterations occurring in all samples. MSI-high was detected only in samples 4 and 5.

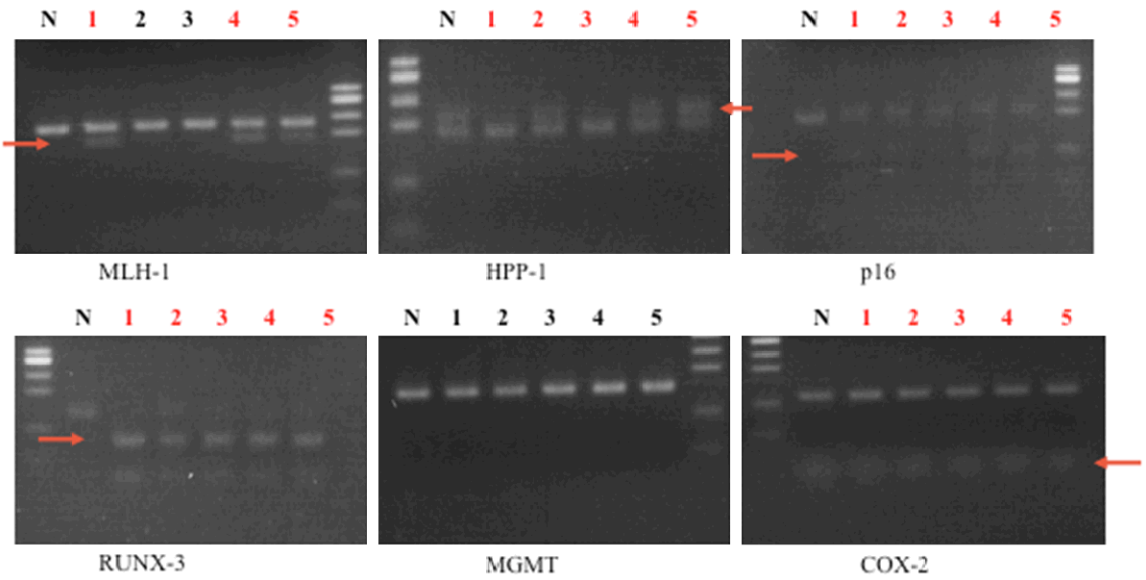

Figure 3. Methylation analysis of the promoter regions of *MLH1*, *HPP1*, *p16*, *RUNX*3, *MGMT* and *COX2* genes. Arrowheads indicate methylated bands. Promoters of *HPP1*, *p16*, *RUNX3*, and *COX2* were methylated in all samples. In contrast, no methylation of the *MGMT* gene promoter was seen in any sample. On the other hand, the *MLH1* gene promoter was methylated in samples 1, 4, and 5.

Table 2. Summary of molecular alterations of isolated tumor glands

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
p53	N	N	N	N	N
17p	N	N	N	MSI	MSI
5q	N	N	N	P	MSI
18q	P	P	N	MSI	MSI
3p	P	P	P	P	P
MSI	N	N	N	P	P
p16	M	M	M	M	M
MLH-1	M	N	N	M	M
RUNX3	M	M	M	M	M
HPP1	M	M	M	M	M
COX2	M	M	M	M	M
MGMT	N	N	N	N	N

COX2) was determined by COBRA (combined bisulphate restriction analysis) as described previously [22, 23].

The results of molecular analysis of isolated glands obtained from multiple samples are shown in **Figures 2** and **3**. Whereas multiple allelic losses were found in the intramucosal neoplastic areas, MSI was detected in the invasive areas. There was an increase in methylation in both areas. These findings are summarized in **Table 2**.

Discussion

The tumor we studied was characterized by lateral and circumferential extension and large size. A colorectal tumor showing such macroscopic features is well known as a lateral spreading tumor (LST) [24, 25]. Colorectal LSTs are, in general, classified into 2 types: LST-granular (LST-G) and LST-nongranular (LST-NG) [25, 26]. The two tumor types are reported to differ in their clinicopathological and molecular findings [30, 31]. For example, whereas the LST-G type has a high frequency of *Ki-ras* mutations and is usually located in the left colon [26], the LST-NG type has a low frequency of *Ki-ras* mutations and frequently develops in the right colon [26]. In addition, the LST-NG type has a higher malignant potential than the LST-G type [25]. It is well known that the LST found in the colorectum can also be found in the stomach [1, 2], where it is termed a superficial spreading gastric cancer. However, the pathogenesis of the gastric tumor that is macroscopically similar to colorectal LST has not been well studied. Although SSGC is defined by Japanese investigators as a tumor with characteristic lateral extension and greater than 5 x 5 cm in size [1, 2], the molecular tumorigenesis of gastric LST has not been elucidated. In this report, the molecular characteristics of differentiated-type SSGC were examined.

The histological diagnosis of "differentiated-type intramucosal adenocarcinoma" used by Western pathologists is different from that used by Japanese pathologists [18]. The differences between Japanese and Western pathologists are due to discrepancies in the histological criteria assigned to intramucosal differentiated-type neoplastic lesions [18]. The tumor described here, an intramucosal tumor exhibiting well differentiated glandular features, may be diagnosed as a gastric adenoma (or low grade dysplasia) by most Western pathologists. However, our molecular study demonstrated the presence of multiple genetic alterations in the intramucosal well-differentiated-type area of the lesion. This finding suggests that well differentiated intramucosal tumor cells acquire multiple genetic alterations. In addition, this finding supports our histological criteria.

In many gastric cancers, a striking heterogeneity in histological patterns and genetic alterations has been reported [27, 28]. The multifocal analysis used in the present case using a panel of 9 microsatellite and 6 methylation markers demonstrated that the heterogeneous foci of a single gastric cancer had the same extent of allelic loss (3p allelic loss). However, the patterns of microsatellite alterations and methylation obtained from multiple foci differed. This finding shows that the tumor contains multiple heterogeneous clones. In addition, MSI was only observed in the invasive areas of this tumor. These findings indicate that MSI is a late event in gastric tumorigenesis, and this appears to be in conflict with previous studies showing that MSI is primarily seen in early differentiated-type gastric cancer [11, 12]. On the other hand, multiple LOHs were also detected in the surrounding, noninvasive area of the tumor. A recent study has found that tumors that exhibit MSI rarely show LOH, which is considered to be the other independent major genetic pathway

[29]. Therefore, LOH and MSI are believed to be mutually exclusive genetic events in human cancers. However, both LOH and MSI were present in the tumor in this case. Furthermore, extensive methylation, which is termed CIMP by Toyota et al. [16], was observed in the present tumor. These findings suggest that multiple LOHs and extensive methylation (CIMP) of tumor cells play a major role in the development of intramucosal cancer, although MSI is associated with tumor progression in some types of gastric cancer [30]. The findings define a novel type of gastric cancer and will be useful for pathological evaluation of gastric tumors.

In examining tumor development and progression, we could trace the ancestry of individual samples within a single tumor according to their microsatellite alteration patterns. These classifications are based on the principle that a genetically altered cell transmits the alteration in the next round of cell division, thereby generating a permanent genetic alteration within daughter cells if the genetic alteration is not repaired [28]. According to this theory, a common genetic alteration among the multiple samples is expected to be the first genetic alteration in a tumor. A possible order in which genetic alterations occurred in the tumor is sample 3→sample 2→sample 1→sample 4 or 5. We were able to determine the order of tumor expansion and progression within the same tumor. Our results also showed that acquisition of various molecular alterations is required for the expansion or progression of tumor cells within one tumor.

In conclusion, it is hypothesized that multiple LOHs and extensive methylation are associated with early development of differentiated-type SSGC with gastric mucin phenotype but that MSI plays a major role in late carcinogenesis. In addition, the tumor in this study was used to chart the process of tumor progression by analyzing multiple samples in combination with the crypt isolation method.

Disclosure of conflict of interest

None.

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