DNA methylation profile in diffuse type gastric cancer: evidence for hypermethylation of the BRCA1 promoter region in early-onset gastric carcinogenesis

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NOVELTY AND IMPACT: We identified that hypermethylation of BRCA1 is associated with young age, suggesting a role in early-onset gastric carcinoma. The low frequency of hypermethylation of the p73 gene in normal adjacent mucosa suggests that it might play a role in the early stages of diffuse type gastric carcinoma.

ABSTRACT

Diffuse type gastric carcinoma is the most aggressive type of gastric cancer. This type of tumor is not preceded by precancerous changes and is associated with early-onset and hereditary syndromes. To test the hypothesis that DNA methylation profile would be useful for molecular classification of the diffuse type gastric carcinoma, DNA methylation patterns of the CpG Island of 17 genes were studied in 104 cases and 47 normal adjacent gastric mucosa by Methylation-specific PCR, Immunohistochemistry and Hierarchical clustering analysis. The most frequent methylated genes were FHIT, E-cadherin, BRCA1 and APC (>50%), followed by p14, p16, p15, p73, MGMT and SEMA3B (20-49%). Hierarchical clustering analysis reveals four groups with different clinical features. The first was characterized by hypermethylation of BRCA1 and younger age (<45 years old), and the second by hypermethylation of p14 and p16 genes, male predominance and Epstein-Barr virus infection. The third group was characterized by hypermethylation of FHIT and antrum located tumors and the fourth was not associated with any clinical variables. In normal adjacent mucosa only the p73 gene was significantly less methylated in comparison to tumor mucosa. DNA methylation identified subgroups of diffuse type gastric cancer. Hypermethylation of BRCA1 associated with young age suggests a role in early-onset gastric carcinoma.

Key terms: gastric cancer, diffuse type, early-onset, BRCA-1

INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide (Pisani et al., 2002). Although its incidence and mortality have fallen dramatically, gastric cancer remains a worldwide public health problem (Pisani et al., 2002; Rastogi et al., 2004). According to

Lauren's classification, there are two major histological types of gastric cancer, intestinal and diffuse (Lauren 1965). The diffuse type is the most aggressive form of gastric cancer and the mortality rate is increasing in spite of the decline of the intestinal type (Henson et al., 2004; Crew and Neugut 2006). In addition, the diffuse type is not preceded by sequential stages of precancerous changes, and tends to

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arise “*de novo*” (Vauhkonen et al., 2006). Furthermore, early-onset gastric cancer and hereditary diffuse gastric cancer (HDGC) are both associated with diffuse type histology (Dunbier and Guilford 2001; Milne et al., 2007).

Particular alterations at the genetic and epigenetic levels in oncogenes and tumor-suppressor genes have been associated with the multistage process of gastric cancer (Tahara 2004; Yasui et al., 2005). In the diffuse type, the best characterized genetic alteration is the loss of heterozygosity (LOH) of chromosome 17p and mutations of p53 and E-cadherin genes (Tamura 2006). In addition, amplification of K-sam and c-met, LOH at 1p and reduced p27 and nm23 expression have been associated with advanced stage disease and low survival rates (Yasui et al., 2005; Vauhkonen et al., 2006). In spite of these findings, no consistent gene alterations have been detected in diffuse type gastric cancer.

The identification and characterization of genes selectively hypermethylated in cancer may improve our understanding of gastric carcinoma (Esteller 2003). Several reports have shown frequent hypermethylation of tumor suppressor genes in the intestinal type of gastric cancer (Tamura 2006; Vauhkonen et al., 2006). However, besides hypermethylation of the promoter region of E-cadherin gene (Graziano et al., 2004), and more recently, PGP9.5 (Yamashita et al., 2006), no consistent information of the role of epigenetics in the diffuse type is currently available. In this study we used a candidate gene approach of 17 genes, covering all cellular pathways, to test the hypothesis that a hypermethylation profile would be useful for molecular classification of diffuse type gastric cancer. We also assessed the role of specific genes as molecular markers for the “*de novo*” precancerous changes in normal adjacent mucosa of paired tumor samples.

MATERIALS AND METHODS

Clinical Samples

We studied 104 formalin-fixed paraffin-embedded archival specimens of diffuse type gastric cancer. All cases were selected using

the histological criteria according to the Lauren’s classification (Lauren 1965). In 47 of these cases, normal gastric mucosa adjacent to the tumor was also available. Clinical characteristics of these cases are shown in Table 1. In this series, 66 (63.5%) cases were males with an age average of 58 years old and 17 (16.3%) patients were under 45 years of age. Forty-three (41.3%) tumors were located in cardia and 27 (26%) in the antrum. In 3 cases (2.9%) the location was not consigned. Fourteen (13.5%) cases were at early stage. Seventy-one (68.3%) of these cases were lymph node positive and 37 (35.6%) had signet-ring cell features. In this series, 30 (28.8%) cases were positive for EBV infection and had been reported previously (Corvalan et al., 2001). The date of the last follow-up and status (alive or dead) was available in 100 cases. The Institutional Review Boards of the Pontificia Universidad Católica de Chile and Hospital Clínico San Borja Arriarán, Santiago Chile approved this study.

DNA extraction

Five 15 μ m paraffin sections of representative areas of diffuse type gastric carcinoma (>70% tumor cells) were cut and placed into a 0.5 mL tube for DNA extraction. DNA extraction was performed in a 100 μ L extraction solution (1 M Tris pH 8.0, 50 mM EDTA, 0.5% and Tween 20) with 1 mg/mL Proteinase K (Sigma) for 12 hrs at 55°C. Proteinase K was inactivated by boiling at 100°C for 10 minutes and DNA was purified by phenol-chloroform extraction and ethanol precipitation according to standard protocols. DNA concentration was determined by spectroscopy using 1 OD260 for 50 μ g/mL.

DNA Methylation Assays

DNA was treated with sodium bisulphite as described previously (Riquelme et al., 2007). Briefly, 1 μ g of genomic DNA was denatured by incubation with 0.2 M NaOH for 10 min at 37°C. Aliquots of 10mM hydroquinone (30 μ L; Sigma Chemical Co., St. Louis, MO) and 3 M sodium bisulphite

(pH 5.0; 520 μ l; Sigma Chemical Co.) were added, and the solution was incubated at 50 °C for 16 h. Treated DNA was purified by use of the Wizard DNA Purification System (Promega Corp., Madison, WI), desulfonated with 0.3 M NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was stored at -80° C until used. The methylation status of 17 genes (APC, BRCA1, DAPK, ER, E-cadherin, FHIT, GSTP, hMLH1, MGMT, p14, p15, p16, p73, RAR β , SEMA3B, SOCS, TIMP3) was determined by methylation-specific polymerase chain reaction (MS-PCR) (Herman and Baylin 2003) and details on primer sequences and PCR conditions are available upon request. These genes were chosen because they are known to be tumor suppressor genes, methylation at these CpG sites is associated with gene silencing, they cover essential alterations in cell physiology that collectively dictate malignant growth and they had previously been described as

undergoing hypermethylation in other tumor types (Hanahan and Weinberg 2000; Virmani et al., 2000; Chan et al., 2002; Li et al., 2002; Oka et al., 2002; Sato et al., 2002; Kuroki et al., 2003; Miyamoto et al., 2003; Wild et al., 2003; Sarbia et al., 2004; Takahashi et al., 2004; Xu et al., 2004; Kim et al., 2005; Kim et al., 2005; Liu et al., 2005; Takahashi et al., 2005; Kawaguchi et al., 2006; Mori et al., 2006; Tamura 2006; Riquelme et al., 2007). Gene names, gene location and function for each gene selected in this study is summarized in Table 2. Only cases with positive unmethylated bands were considered informative for methylation status in this study. All reactions were done in triplicate. In vitro methylated SssI methyltransferase (New England Biolabs) and bisulfite-modified DNA from the MKN-45 cell line were used as a positive control. Samples without DNA template (water only) were included as negative control for each set of PCR reaction.

TABLE 1

Clinical associations of clusters of gene methylation in diffuse type gastric cancer

Clinical variables		BRCA1 cluster		p14/p16 cluster		FHIT cluster		low MI cluster		p	Total	%
		N	%	N	%	N	%	N	%			
Gender	female	12	52.2	7	21.9	12	42.9	7	33.3	.02 ^a	38	36.5
	male	11	47.8	25	78.1	16	57.1	14	66.7		66	63.5
Age	<45 y.o.	8	34.8	4	12.5	4	14.3	1	4.8	.009 ^b	17	16.3
	>45 y.o.	15	65.2	28	87.5	24	85.7	20	95.2		87	83.7
Follow-up	alive	10	43.5	15	46.9	12	42.9	9	52.9	.02 ^c	46	46.0
	dead	13	56.5	17	53.1	16	57.1	8	47.1		54	54.0
Location	cardia	8	34.8	15	50.0	11	40.7	9	42.9	.02 ^c	43	41.3
	middle	10	43.5	10	33.3	4	14.8	7	33.3		31	29.8
	antrum	5	21.7	5	16.7	12	44.4	5	23.8		27	26.0
Tumor size	< 5 cm	7	30.4	6	20.0	9	34.6	7	35.0	NS	29	29.3
	> 5 cm	16	69.6	24	80.0	17	65.4	13	65.0		70	70.7
Stage	early	4	17.4	5	16.7	2	7.1	3	14.3	NS	14	13.5
	advanced	19	82.6	25	83.3	26	92.9	18	85.7		88	84.6
Signet-ring	negative	16	72.7	19	61.3	18	66.7	11	52.4	NS	64	61.5
	positive	6	27.3	12	38.7	9	33.3	10	47.6		37	35.6
Lymph nodes	negative	4	18.2	10	33.3	8	29.6	7	33.3	NS	29	27.9
	positive	18	81.8	20	66.7	19	70.4	14	66.7		71	68.3
EBV	negative	17	73.9	16	51.6	19	76.0	15	83.3	.008 ^d	67	64.4
	positive	6	26.1	15	48.4	6	24.0	3	16.7		30	28.8

^ap14/p16 cluster vs all others. ^b BRCA1 cluster vs all others. ^cFHIT cluster vs all others. ^dp14/p16 cluster vs all others.

TABLE 2

Summary data of genes tested for aberrant promoter hypermethylation in Diffuse-type gastric cancer

Gene Abbreviation	Gene Name	Location	Function
APC	Adenomatous polyposis coli gene	5q21	Signal transduction
BRCA1	Breast cancer 1 gene	17q2	DNA repair
DAPK	Death-associated protein kinase	9q34	Evasion of programmed cell death
CDH1	E-cadherin	16p	cell adhesion
ER	Estrogen Receptor	6q25.1	DNA binding, activation transcription
FHIT	Fragile histidine triad	3p14.2	Evasion of programmed cell death
GSTp1	Homologous to the MTG/ETO family of transcription factors	3p21.3	Cell cycle regulation
hMLH1	Human homologs of the MutL gene of bacteria	3p21.3	DNA repair (Mismatch repair genes)
MGMT	O-6-methylguanine-DNA methyltransferase	10q26	DNA repair
p14	Cyclin-dependent kinase inhibitor 2B	9p21	Cell cycle regulation
p15	Cyclin-dependent kinase inhibitor 2B	9p21	Cell cycle regulation
p16	Cyclin-dependent kinase inhibitor 2A	9p21	Cell cycle regulation
p73	TP73	1p36	Angiogenesis
RAR-b	Retinoic acid receptor beta 2 gene	3p24	DNA binding, activation transcription
SEMA3B	Semaphorin 3B	3p21.3	Evasion of programmed cell death
SOCS	ankyrin repeat and SOCS box-containing 11	Xp22.31	Suppressor of cytokine signalling
TIMP3	Tissue inhibitor of metalloproteinase-3	22q12	Tissue invasion and metastasis

Protein expression assays

To establish the association between CpG island hypermethylation and gene silencing we determined protein expression by immunohistochemistry assay on tissue microarray (TMA) slides. Tissue microarrays were performed using a Manual Tissue Arrayer II instrument (Beecher Instruments, Silver Spring, Maryland, USA). Archival tumor tissue blocks from 104 tumors were selected, cut and stained with hematoxylin and eosin for the best tumor area identification. After whole-section glass slide evaluation, tumor area was selected for placement into the TMA by a circling on the glass slide and identified in the corresponding paraffin block. Six hundred μ m stylets in inner diameter were used to take three cylindrical core biopsies from each tumor tissue block

(donor block), with subsequent arraying into a new recipient paraffin block. In this way, all 104 cases were held in the 3 recipient blocks. An adequate case was defined as a tumor occupying more than 10% of the core area. Immunohistochemistry was performed on 4- μ m-thick section TMA blocks. Sections were dewaxed in xylene, rehydrated through graded alcohol, and placed in an endogenous peroxide block for 15 minutes. Antigen retrieval was performed in a citrate buffer (10% citrate buffer stock in distilled water, pH 6.0) and microwaved for 10 minutes. Non-reactive staining was blocked by 1% horse serum in Tris-buffered saline, pH6.0 for 3 minutes. After primary incubation, antibody binding was detected using two-stage visualization systems based on an enzyme-conjugated polymer backbone carrying secondary antibody

molecules (EnVision™ Systems, Dako Cytomation). Protein expression of E-cadherin, p16 and p73 on TMA was determined in which cell membrane (E-cadherin) and nuclear (p16 and p73) staining from 10% or more tumor cells was considered positive for expression (Fig. 2).

Data Analysis

In order to identify clinically relevant groups based on to the DNA methylation pattern, we performed hierarchical clustering analysis in a similar fashion to cDNA expression microarrays in breast tumors and lymphomas (Alizadeh et al., 2000; van 't Veer et al., 2002) or more recently, DNA methylation signature in neuroblastoma (10 genes) or hepatocellular carcinoma (18 genes) (Alaminos et al., 2004; Nishida et al., 2007). TIGR MultiExperiment Viewer was applied to the DNA methylation dataset using unsupervised hierarchical clustering analysis with Pearson correlation and complete linkage to cluster the tumors. In addition, we analyzed this data using the Methylation index, defined as the number of methylated genes, divided by the total number of genes analyzed, as a way to compare the methylation status of each cluster. Clinical variables and follow-up data of cases from each particular cluster were compared using c2 tests. Survival analysis was performed using the Kaplan-Meier method and differences among groups were compared with the Log-rank testing and Cox regression models (Stata 8.0, Stata Corporation; College Station, TX). For all tests, probability values of $p < 0.05$ were regarded as statistically significant.

RESULTS

Frequency of the DNA methylation in diffuse type gastric cancer

As shown in Fig. 1A, the most frequent methylated genes (>50% cases) were FHIT, E-cadherin, BRCA1, APC. Genes with intermediate frequency of methylation (20-

49%) were p14, p16, p15, p73, MGMT and SEMA3B. Genes with a low level of methylation (<20%) were RARbeta, hMLH1, DAPK, ER, TIMP3, GSTP and SOCS. Representative examples are shown in Fig. 1B.

Correlations between DNA methylation and loss of protein expression

To establish the association between DNA methylation and gene silencing, we determined protein expression of three genes that were methylated in a relatively large number of cases (E-cadherin, p16 and p73). This analysis was performed in all 104 tested cases. For all three genes, the presence of CpG island methylation was associated with loss of protein expression. Conversely, in those cases harbouring an unmethylated CpG island, each gene was expressed. Representative examples are shown in Fig.2.

DNA methylation patterns and clinical variables

To explore the relationship among DNA methylation patterns and clinical variables, we performed unsupervised hierarchical clustering analysis. As shown in Fig. 3, clustering analysis revealed two different clusters, both further subdivided into two major branches. The most upper branch was characterized by hypermethylation of BRCA1 gene ($p = 0.0006$). The second branch was characterized by hypermethylation of p14 and p16 ($p < 0.0001$). The upper branch of the second major cluster was characterized by hypermethylation of FHIT ($p < 0.0001$) and the lower branch was not associated with hypermethylation of any specific gene. Accordingly, methylation index analysis revealed that this cluster was significantly less methylated in comparison to all other clusters ($p < 0.0001$) (data not shown). Subsequently, clinical variables of cases from each cluster were compared and are shown in Table 1. The most upper branch (BRCA1 cluster) was associated with patients of younger age (age <45 y.o.) ($p = 0.009$). The second branch (p14/p16 cluster) was associated with male

predominance ($p=0.002$) and Epstein-Barr virus infection ($p=0.008$). The upper branch of the second major cluster (FHIT cluster) was associated with antrum as the predominant location ($p=0.02$). Finally, the lower branch of the second major group (low methylation index cluster) was not related to any specific clinical variables.

Survival Analysis

The impact in prognosis of clusters and individual genes was examined by survival

analysis using Kaplan-Meier method, Log-rank testing, and Cox regression models. The average follow-up period was 64 months (Standard deviation = 48, range 1-146). Univariate analysis, using all four branches or individual genes, demonstrated that no clusters or single gene was significantly correlated with worse prognosis. Multivariate Cox regression analysis showed that only the lymph node metastasis was prognostic determinant ($HR=3.06$, 95% $CI=1.07-8.7$; $p=0.03$)(data not shown).

A

B

Fig. 1: Methylation-specific polymerase chain reaction (MS-PCR) analysis of 104 diffuse-type gastric carcinomas. (A) Histogram representing the percentage of tumors showing methylation for the 17 genes as indicated. (B) Representative results. M PCR product with primers specific for methylated DNA, U PCR product with primers for unmethylated DNA; CM positive control for methylated DNA; CU positive control for unmethylated DNA.

Fig. 2 Lack of protein expression at low (A, C and E) and high (B, D and F) magnification of E-cadherin (A-B), p16 (C-D) and p73 (E-F) on tissue microarray of diffuse-type gastric cancer. Positive controls for each antibody are shown in the corresponding inserts.

Fig. 3: Unsupervised Hierarchical Clustering analysis of 104 diffuse-type gastric cancer showing two different clusters, both further subdivided into two major groups. Each row represents a tumor and each column a single gene. Red indicates positive, black negative and gray not determined.

DNA methylation patterns in normal adjacent mucosa from diffuse type gastric cancer

The frequency of DNA methylation patterns of the CpG Island of 8 genes was studied in a subgroup of 47 paired tumor and normal adjacent mucosa. The most representative genes of each cluster (BRCA1, p14/p16 and FHIT) and 4 non-cluster related genes (APC, MGMT, p15 and p73 genes) were included in this analysis. Case-informative incidence ranged from 8 to 39 samples. As shown in Fig 4A, only the p73 gene was significant less methylated in normal adjacent mucosa in comparison to tumor mucosa ($p=0.006$). Representative examples are shown in Fig. 4B.

DISCUSSION

In order to define the DNA methylation signature in diffuse type gastric carcinoma, the most aggressive and increasing form of gastric cancer, we used the candidate gene approach by searching the hypermethylation profile of CpG Island of 17 genes. To identify associations between genes and clinical variables, we performed hierarchical clustering analysis in a similar fashion to cDNA expression microarrays in breast tumors and lymphomas (Alizadeh et al., 2000; van 't Veer et al., 2002) or DNA methylation signature in Neuroblastoma (Alaminos et al., 2004) or Hepatocellular carcinoma (Nishida et al., 2007). Here, we found that DNA methylation is a frequent event in diffuse type gastric cancer and clustering analysis reveals different branches associated with hypermethylation of specific genes. Interestingly, these branches were associated with distinct clinical variables. For example, BRCA1 was frequently more methylated in a group of tumors associated with young age (<45 years old). Tumors at this age or less have been considered early-onset gastric cancer (Milne et al., 2007) and although they represent less than 10% of gastric carcinoma, they have unique clinico-pathological features including diffuse type histology (Milne et al., 2007). Although

early onset also has unique molecular features (lack of microsatellite instability, infrequent LOH, low COX2 expression, infrequent loss of TFF1 expression, no loss of RUNX3, gains at chromosomes 17q, 19q and 20q) (Milne et al., 2007), DNA methylation has not been extensively explored in this type of gastric cancer. Only Kim et al (Kim et al., 2005) assayed the hypermethylation status in genes associated with the APC-beta-catenin axis and the mismatch repair system (hMLH1, TIMP3, THBS1, DAPK, GSTP1, APC, and MINT2) and found that hypermethylation is a frequent phenomenon in early-onset gastric carcinoma. However, no specific genes were hypermethylated. Thus, to our knowledge this is the first report that has identified hypermethylation of CpG island of the BRCA1 gene in association with early-onset gastric carcinoma. Interestingly, Varis et al (Varis et al., 2003) identified increases of DNA copy number at chromosome 17q, the location of the BRCA1 gene, in 52% of 22 cases of early-onset gastric cancer and Semba et al (Semba et al., 1998) described LOH on chromosome 17q12-21 with several neighbouring markers in this region, while no mutation was found in the BRCA1 gene. In addition, although associated with hereditary and not early-onset gastric cancer, studies exploring additional tumors on relatives of BRCA1 carriers identified gastric cancer as one of the most common sites for malignancies (Gallardo et al., 2006). Taken together, these findings suggest that alterations of the BRCA1 gene should be included as one of the molecular features of early-onset gastric carcinoma.

An association of hypermethylation of p14 and p16 and the presence of Epstein-Barr virus infection were characteristic of the lower cluster of the upper branch in the clustering analysis. This association has been described previously (Koriyama et al., 2004). However, our male gender association is contrary to previous studies for p16 methylation (Vauhkonen et al., 2006). Hypermethylation of FHIT has been associated with antrum as a predominant location. Interestingly, recent data showed that *FHIT* knock-out mice develop tumors

in the forestomach and small intestines (Fujishita et al., 2004). These findings suggest that *FHIT* plays an important role in the integrity of gastrointestinal mucosal structures. Chang et al (Chang et al., 2002) described frequent LOH at the *FHIT* locus and loss of Fhit protein expression in a series of 7 signet-ring cell gastric cancer. Although these authors have proposed that alteration of the *FHIT* gene might be the

hallmark of signet-ring cell gastric cancer, we did not confirm this.

The finding that seven out of 8 tested genes (with the only exception being the *p73* gene) were hypermethylated in a similar frequency in normal adjacent mucosa in comparison to tumor mucosa, suggests the presence of an epigenetic field for cancerization (Ushijima 2007). Epigenetic field for cancerization has been

Fig. 4: Methylation-specific polymerase chain reaction (MS-PCR) analysis of 47 paired tumor and non-tumor adjacent mucosa of diffuse-type gastric cancer. (A) Histogram representing the percentage of cases showing methylation for the most representative genes as indicated. Black indicates tumor mucosa and white indicates normal adjacent mucosa. B) Representative results. M PCR product with primers specific for methylated DNA, U PCR product with primers for unmethylated DNA; CM positive control for methylated DNA; CU positive control for unmethylated DNA.

demonstrated for Barrett's esophagus, liver, lung and urothelial cancers (Eads et al., 2000; Wistuba et al., 2002). Since changes in DNA methylation status are specific for each tumor, it is likely that specific genes are methylated according to unique carcinogenic factors (Ushijima 2007). The p73 gene was the only one not hypermethylated in normal adjacent mucosa in comparison to tumor mucosa. Diffuse type gastric cancer does not have sequential stages of precancerous changes, as does intestinal-type gastric cancer, and consequently is considered to arise "*de novo*" (Vauhkonen et al., 2006). Thus, this finding suggests that hypermethylation of p73 might play an important role in the early stages of diffuse type gastric carcinoma. Extremely low levels of p73 expression has been observed in gastric cancer cell lines, although reports have shown that mutations of p73 are rare in primary human cancers (Pillozzi et al., 2003). These findings suggest that p73 could be a target of epigenetic regulation in gastric carcinogenesis.

In summary, we found that in DNA, methylation is a frequent event in diffuse type gastric cancer. Clustering analysis reveals specific association between genes and clinical variables, in particular BRCA1 to early-onset gastric carcinoma. The finding that the p73 gene was significantly less methylated in normal adjacent mucosa suggests that it may play a role in the early stages of diffuse type gastric carcinoma.

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