RESEARCH ARTICLE

Hypermethylation Status of E-Cadherin Gene in Gastric Cancer Patients in a High Incidence Area

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Abstract

Gastric cancer (GC) is the fourth most prevalant cancer and the second leading cause of cancer-related mortality worldwide. As in other cancers gastric carcinogenesis is multifactorial involving environmental, genetic and epigenetic components. Epigenetic silencing due to hypermethylation of tumour suppressor genes is one of the key events in gastric carcinogenesis. This study was aimed to analyse the hypermethylation status of the E-Cadherin (*CDH1*) gene promoter in GCs in the ethnic Kashmiri population. In this study a total of 80 GC patients were recruited. Hypermethylation in tumour tissue was detected by methylation specific PCR (MS-PCR). Hypermethylation of *CDH1* promoter was observed in 52 (65%) of gastric carcinoma cases which was significantly much higher than adjacent normal tissue [p≤0.0001]. Further the frequency of *CDH1* promoter methylation was significantly different with intestinal and diffuse types of gastric cancer [55.7% vs 82.1%; p<0.05]. Moreover females and cases with lymph node invasion had higher frequencies of *CDH1* hypermethylation [P≤0.05]. Thus the current data indicate a vital role of epigenetic alteration of *CDH1* in the causation and development of gastric cancer, particularly of diffuse type, in our population.

Keywords: Gastric cancer - hypermethylation - *CDH1* - MSP - kashmiri population

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Introduction

Gastric cancer is the most prevalent malignancy in the world (Jemal et al., 2011; Zhu et al., 2012). Despite reduction in frequency and mortality rates in recent decades, it is still the fourth most common cancer and the second leading cause of cancer-related fatality globally (Parkin et al., 2005; Seigel et al., 2014). In India among males it is the fifth most common cancer and among females the seventh most frequent malignancy (Rao et al., 1998). In the Kashmir region in India, however, this malignancy has been reported to exceed 40 % of all cancers, with incidences up to 3-6 times higher than those reported in various other metropolitan areas in India. As per a recent hospital based study conducted by Pandith et al. gastric cancer was found to be the leading cancer with an average frequency of 19.2 % in Kashmir valley (Pandith et al., 2012).

Although numerous environmental factors, including *Helicobacter pylori* infection, bile reflux, N-nitroso compounds, excessive salt intake and a deficiency of antioxidants, have been associated with the development of gastric cancer, its exact cause is still inadequately understood (Correa et al.,1992). As in other malignancies, gastric carcinogenesis involves steady accretion of various genetic and epigenetic alterations, leading to gain-of-function in oncogenes and loss-of-function in tumor suppressor genes. Accumulating data has indicated that silencing of tumor suppressor genes, chiefly caused by hypermethylation of CpG islands in promoters, is critical to gastric carcinogenesis (Wani et al., 2012; Hu et al., 2013., Yu et al., 2013; Yousuf et al., 2014).

Cadherin-1 (*CDH1*) or E-dadherin is a member of cell-cell adhesion molecules family encoded by the *CDH1* gene located on chromosome 16 (Bussemakers et al., 1993; Van et al. 2014). This transmembrane homodimeric protein has three major domains, i.e 5 extracellular tandem repeats, a single transmembrane region, linked to cytoplasmic tail which is highly consrved. It is pivotal for maintaining cellular polarity, cell adhesion and the normal architecture of epithelial tissues (Takeichi et al., 1990; Karayiannakis et al., 1998). Expression of *CDH1* is frequently reduced or lost in a range of epithelial tumors. This loss of normal intercellular junctions results in enhanced tumour invasion and metastasis and has been associated with numerous types of malignancies including GC (Zheng et al., 1999; Humar et al., 2009; Oliveira et al., 2009). Besides mutation in *CDH1* gene, several studies have suggested

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hypermethylation of the *CDH1* gene to be crucial for loss of or reduced *CDH1* expression. Though hypermethylation of *CDH1* gene has been well studied, data from this ethnic Kashmiri population is lacking. Moreover there are reports demonstrating that methylation profile of genes differs among different etnicities (Enokida et al., 2005; Galanter et al., 2015). It has been suggested that this difference in the methylation may be due to the fact that different ethnic groups have different environmental exposure (Galanter et al., 2015). The aim of the present study was thus to estimate the frequency and influence of *CDH1* gene promoter hypermethylation in the pathogenesis of gastric cancer in ethnic Kashmiri population.

Materials and Methods

Patients and specimens

A total of 160 gastric tissue specimens, comprising 80 tumor tissues and 80 corresponding adjacent normal tissues from patients who underwent surgical resection in the department of General & Minimal Invasive Surgery of the Sher-i-Kashmir Institute of Medical Sciences (SKIMS) were included in this study. None of the patients enrolled for this study received pre-operative radiation or chemotherapy. Histopathological grades and clinical stages were evaluated according to standard criteria by two pathologists independently. Only histopathologically confirmed cases were included in this study. Written informed consent was obtained from all the subjects included in this study. The study was approved by the Ethics Committee of SKIMS.

DNA extraction

Tissue samples (both tumor and adjacent normal) were immediately snap-frozen and stored at -70°C until use. DNA was extracted from the tissues using a Purelink genomic DNA mini kit (Invitrogen,Van Allen Way Carlsbad, CA) according to the manufacturer's protocol.

Methylation-specific PCR

The methylation status of the CpG island of the *CDH1* gene promoter was determined for each of the patient samples using methylation-specific PCR (MSP). To this end, 1-2 µg genomic DNA isolated from gastric cancer tissues and corresponding normal tissues were modified with sodium bisulfite using the Imprint DNA Modification Kit (Sigma Aldrich). The modified DNA was immediately used for MSP. The PCR reaction was carried out in a 25 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 1 U of Taq polymerase, 100 mM of each dNTP (Biotools, Valle de Tobalina, 52-Nave 39 28021, Madrid, Spain) and 0.2 µM of each forward and reverse primer as described previously (MF-TTAGGTTAGAGGGTTATCGCGT;MR-TAACTAAAAATTCACCTACC GAC, UF-TAATTTTAGGTTAGAGGGTTATTGT;UR-CACAACCAATCAACAACACA) (Biotools, Valle deTobalina, 52-Nave 39 28021, Madrid, Spain) (Kang et al., 2003). The reaction was heated at 95°C for 10 min, then amplified for 40 cycles (95°C for 30 s, 56°C for 30 s, and 72°C for 30 s), followed by a final 10 min extension at 72°C. Aliquots of PCR products were separated on 4% agarose gels. The gels were stained with ethidium bromide and photographed under UV illumination (Figure 1).

Results

Promoter hypermethylation of the *CDH1* gene were assessed in all 80 gastric cancer tissues and their adjacent normal tissues. The clinicopathological characteristics of the studied subjects are listed in Table 1.

The promoter region of the *CDH1*gene was found to be hypermethylated in 65 % (52 of 80) of the gastric cancer samples. Out of these 52 gastric cancer cases that showed promoter hypermethylation, 20 (38.46 %) samples showed both methylated and unmethylated bands in the tumor tissues, while 32 (61.5 %) samples showed only methylated bands. Of these 52 gastric cancer cases, 8 (15.38%) showed methylation in both tumor tissues and adjacent normal tissues, while the other 44 (84.6%) cases showed methylation in the tumor tissues only. The frequency of *CDH1*hypermethylation was significantly higher in tumor tissues compared to adjacent normals Table 2. We also tested whether E-cad promoter hypermethylation correlated with various clinicopatholgic characteristics in the 80 cases included. Interestingly, we found that gastric cancer patients with Lymph node involvement had higher frequency of *CDH1*

Table 1. Clinicopathologic Characteristics of the Gastric Cancer Patients used for Analysis of Promoter Hypermethylation ofthe E-CAD gene (n=80)

Variables	No. of Patients
Age group	
<60 yrs	32
≥ 60 yrs	48
Gender	
Male	60
Female	20
Smoking status	
smoker	42
Non smoker	38
Dwelling	
Urban	23
Rural	57
Salt tea intake	
< 4 cups	35
≥ 4 cups	45
Histopathological differentiation	
Well	26
Moderate/Poor	54
Lymph node Invasion	
Yes	41
No	39
Stage	
I/II	30
III/IV	50

Table 2. *CDH1* Promoter Hypermethylation in Normal and Tumour Tissue

	Normal	Tumour	P-value
Methylated	8	52	<0.0001
Unmethylated	72	28	

Table 3. Association of *CDH1* Promoter Hypermethylation with Various Clinicopathological Characteristics of Gastric Cancer Patients

Variables	Methylation state of the tumor		P- value	OR(95% CI)
	Methylated n= 52 (65%)	Unmethylated n=28(35%)		
Age group				
<60 yrs	23	9	0.34	1.64 (0.58-4.92)
> 60 yrs	29	19		
Gender				
Male	35	25	0.03	0.25(0.05-1.04)
Female	17	3		
Smoking status			0.64	
Smoker	26	16		0.75(0.27-2.09)
Non smoker	26	12		
Dwelling			0.32	
Urban	17	6		1.78(0.55-5.99)
Rural	35	22		
Salt tea intake			0.81	0.85(0.30-2.36)
<4 cups	22	13		
≥4 cups	30	15		
Histopathological differentiation			0.63	1.32(0.44-4.06)
Well	18	8		
Moderate/Poor	34	20		
Lymph Node Invasion			0.02	3.24(1.01-7.63)
Yes	25	6		
No	27	21		
Lauren's Classificatio			0.01	3.65(1.08-12.9)
Intestinal	29	23		
Diffuse	23	5		
Clinical Staging			0.46	0.65(0.23-1.85)
I/II	17	12		
III/IV	35	16		

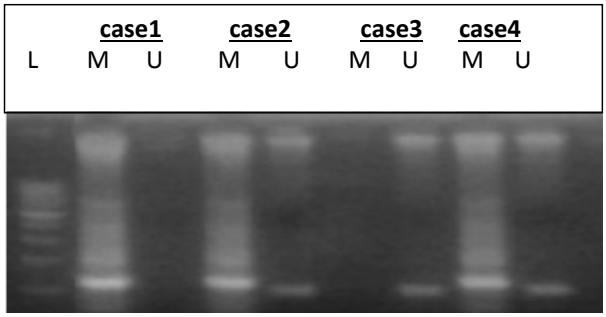

Figure 1. Representative picture of promoter hypermethyaltion of E-Cadherin by MSP ; L: 100 bp DNA marker, M: (115bp) indicates presence of methylated E-Cadherin U: (97 bp) indicates presence of unmethylated E-Cadherin Cases 1–4: gastric cancer cases

hypermethylation compared to patients with no lymp node involment. This difference was found to be statistically significant (P<0.05: OR=3.24, 95%CI=1.01-7.63) Table 3. Moreover a similar association was found with diffuse type GC with an OR=3.65 (95 % CI=1.1-12.9), and this difference between the groups of Lauren's Classification was significant (P<0.05). Further in this study we observed methylation of *CDH1* more frequent in females than males (P<0.05, O.R=0.25 (95% CI=0.05-1.04)). When compared between tumor grades *CDH1* hypermethylation was found more often in higher grade tumors (moderately/poorly differentiated) than in lower grade tumors. However this association was not statistically significant (P<0.05) Table 3. No other clinical feature showed any association with the methylation status of the gene.

Discussion

The development and progression of gastric cancer involves a number of genetic and epigenetic alterations of tumor suppressor and tumor-related genes (Zourdis et al., 2005; Wang et al., 2014). Aberrant methylation of CpG islands in promoter regions can permanently inactivate tumor-suppressor genes, as mutations and chromosomal abnormalities do. In gastric cancer, genes are inactivated more frequently by aberrant methylation than by mutations (Choi et al., 2005). A great number of genes inactivated by hypermethylation have been reported in gastric cancer (Gaun et al., 2013; Du et al., 2013; Yousuf et al., 2014). The knowledge of epigenetic alterations associated with the development and progression of cancer is vital for effective diagnosis, treatment and prevention of cancer. A distinctive characteristic of epigenetic alteration is its reversibility; therefore aberrant DNA methylation may represent promising tool for the epigenetic therapy. This study therefore focussed on elucidating the role of epigenetic alteration of *CDH1* gene in the pathogenesis of gastric carcinoma in ethnic Kashmiri population. Hypermethylation of *CDH1* promoter resulting in gene silencing is present in both diffuse- and intestinal-type gastric tumors. The frequency of *CDH1* promoter hypermethylation in sporadic gastric cancers ranges from 11% to 75% (Machado et al., 2001; Lee et al., 2002). In our study hypermethylation was seen in 65% of primary gastric cancer cases. This is thus consistent with other previous studies. Further, hypermethylation of *CDH1*was much more predominant in diffuse type than intestinal type cancers (82.1% *vs* 55.7%). Previous studies also reported a higher preponderance of *CDH1* hypermethylation in diffuse type gastric cancer (Machado

et al., 2001; Graziano et al., 2005). Moreover in the current study we observed a positive correlation between *CDH1* hypermethylation and lymph node metastasis. This finding seen in our series of gastric cancer patients is consistent with other reports showing correlation with aggressiveness and metastasis of gastric cancer (Yi et al 2007; Zhang et al., 2008). In this study we found a higher frequency of *CDH1* hypermethylation in female patients than males. Similar finding was reported in the study of Tahara et al (Tahara et al., 2010). None of the other clinicopathological parameters showed any significant association with the methylation status of *CDH1*.

Thus the present study highlights the importance of epigenetic alteration of *CDH1* gene in the development and progression of gastric carcinoma in this population. The current data thus indicates that hypermethylation of E-cadherin gene occurs frequently in gastric cancer particularly in the diffuse type in our population.

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