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ORIGINAL ARTICLE

hTERT methylation and expression in gastric cancer

Carolina Oliveira Gigek[1], Mariana Ferreira Leal[1], Patricia Natalia Oliveira Silva[1], Luara Carolina Frias Lisboa[1], Eleonidas Moura Lima[2], Danielle Queiroz Calcagno[3], Paulo Pimentel Assumpção[4], Rommel Rodriguez Burbano[3], and Marilia de Arruda Cardoso Smith[1]

[1]Disciplina de Genética, Departamento de Morfologia e Genética, Universidade Federal de São Paulo, SP, Brasil, [2]Departamento de Biologia, Campus Ministro Reis Velloso/Parnaíba, Universidade Federal do Piauí, PI, Brasil, [3]Laborátorio de Citogenética Humana e Genética Toxicológica, Departamento de Biologia, Centro de Ciências Biológicas, Universidade Federal do Pará, PA, Brasil, and [4]Serviço de Cirurgia, Hospital Universitário João de Barros Barreto, Universidade Federal do Pará, PA, Brasil

Abstract
Gastric cancer is the second most prevalent cause of cancer death worldwide. DNA methylation is a common event in gastric carcinogenesis. *hTERT* seems to be the rate-limiting determinant of telomerase activation, which is responsible for stability and life span. *hTERT* hypermethylation has been associated with telomerase expression. In the present study, we investigated the promoter methylation status and hTERT protein expression in gastric cancer and normal mucosa samples. One hundred and nine gastric cancer and 53 normal mucosa samples were investigated through methylation-specific PCR. Immunohistochemistry was analysed using peroxidase in 55 gastric cancer and 18 normal gastric mucosa samples. This is the first study evaluating *hTERT* methylation status in gastric carcinogenesis. We did not observe hTERT protein expression in normal gastric mucosa. Moreover, hTERT expression was observed in 80% of tumours and was associated with gastric cancer ($p < 0.0001$). Partial methylation was the most frequent pattern in gastric samples, even in normal mucosa. The frequency of specimens presenting hypermethylation was significantly higher in tumours than in normal mucosa samples ($p = 0.0002$), although the presence of hypermethylated promoter was not associated with a higher frequency of hTERT expression. A low correlation between hTERT protein expression and methylation was verified in gastric cancer samples. There was a clear difference in the frequency of hTERT expression and methylation within tumoral and non-tumoral tissues. Methylation status and telomerase expression may be useful for the diagnosis of gastric cancer and may have an impact on the anti-telomerase strategy for cancer therapy.

Keywords: *Gastric cancer;* hTERT*; IHC; methylation; MSP; protein expression*

Introduction

Gastric cancer is the second most prevalent cause of cancer death worldwide (Li et al. 2008), and it is two to three times more frequent in developing countries. In the state of Pará, Northern Brazil, the gastric cancer mortality rates are higher than the national average rate (Resende et al. 2006).

Gastric cancer is the result of environmental, including diet and infectious agents, genetic and epigenetic factors (Shang & Pena 2005). Epigenetic events play a key role in development and progression of cancer. DNA methylation is the most common epigenetic alteration and occurs by addition of a methyl radical on a deoxycytosine, frequently found in cytosine-phosphate-guanine (CpG) sites (Richardson 2003). Gene promoters are CpG-rich sites and contain transcriptional sites. Indeed, methylation of CpG islands is usually associated with gene silencing (Zinn et al. 2007). Moreover, gene promoter methylation is observed in carcinogenesis processes, including gastric cancer (Lima et al. 2007, 2008, Leal et al. 2007, Moura Lima et al. 2008, Guimaraes et al. 2007).

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Telomerase activity is observed in about 85% of cancer samples and is associated with cellular immortality and acquisition of malignancy (Hu et al. 2004). Telomerase is responsible for maintenance of telomeres, which are responsible for stability and life span. Telomerase is composed by two main components: a RNA template (*hTER*) and the catalytic subunit (*hTERT*) (Li et al. 2008). *hTERT* expression is highly specific to cancer cells and associated with telomerase activity, while the other subunits are constitutively expressed in normal and cancer cells (Kyo et al. 2008). Chen et al. (2005) reported that higher *hTERT* expression was associated with gastric cancer in comparison to other telomerase subunits. In addition, expression of *hTERT* has been associated with gastric carcinogenesis (Nowak et al. 2003, Chen et al. 2005, Hu et al. 2004, Li et al. 2008, Wang et al. 2004).

A number of transcriptional factors have been identified at the *hTERT* promoter regulation pathway. Chromatin structure via DNA methylation or histone alterations has also been suggested to be important for regulation of *hTERT* promoter (Kyo et al. 2008). In *hTERT* promoter, a 4-kb CpG island (−1800 to +2000) with CG content up to 70% was identified in the 5′-region, including the core promoter (−310 to −20) (Choi et al. 2007).

Some studies revealed an association of *hTERT* promoter methylation and decreased expression of this gene (Lopatina et al. 2003, Liu et al. 2004). On the other hand, a positive correlation between *hTERT* hypermethylation and mRNA expression has been described, in contrast to the current model in which the methylated cytosines lead to transcription silencing (Guilleret et al. 2002). Either absence or partial correlation between this gene methylation and expression has also been reported (Dessain et al. 2000). However, gene methylation status and regulation is frequently tissue-specific and disease-specific (Nagase & Ghosh 2008). To our knowledge, no study in literature has evaluated *hTERT* methylation status in gastric carcinogenesis.

The aim of this study was to investigate the protein expression and methylation pattern of *hTERT* in gastric cancer and their correlation with clinicopathological characteristics.

Materials and methods

Samples

The hTERT protein expression was evaluated in formalin-fixed, paraffin-embedded tissues of 55 patients with sporadic gastric adenocarcinoma. Eighteen samples (33%) had non-neoplastic and non-infiltrated gastric mucosa. *hTERT* methylation pattern was evaluated in 162 samples of gastric tissue. Among these gastric samples, 53 were non-neoplastic gastric mucosa of patients (distant location of primary tumour) and 109 sporadic gastric adenocarcinomas. *hTERT* methylation and protein expression were both evaluated in 52 gastric cancer samples and 18 normal gastric mucosa. All samples were classified according to Laurén (Lauren 1965) and tumours were staged using standard criteria by TNM staging.

In this study, all gastric samples were obtained surgically from João de Barros Barreto University Hospital (HUJBB), Belém, Pará State. The human population is composed of interethnic crosses among three main origin groups: European (mainly represented by Portuguese), Africans and Amerindians (Batista dos Santos et al. 1999). Informed consent with approval of the ethics committee of HUJBB was obtained. All patients had negative histories of exposure to either chemotherapy or radiotherapy before surgery and there were no other co-occurrent diagnosed cancers.

Immunohistochemical staining

Antigen retrieval was performed by microwave treatment 20 min at 900 W in a citrate buffer, pH 6.0. After cooling, sections were immersed in 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min to block endogenous peroxidase activity. Sections were then incubated in a humid chamber overnight with hTERT primary antibody (clone 44F12, dilution 1:50, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). After the PBS rinse, slides were incubated with secondary antibody and then with streptavidin–biotin–peroxidase complex, both for 30 min at room temperature with a PBS wash between each step. Slides were visualized with diaminobenzidine–hydrogen peroxide and counterstained with Harry's haematoxylin.

Positive hTERT expression was defined as clear nuclear staining in 50% or more of the cells, whereas negative hTERT immunostaining was considered when no positive cells were observed or, in rare cases, less than 25% weakly stained tumour cells (Figure 1). Normal gastric mucosa was used as the internal control.
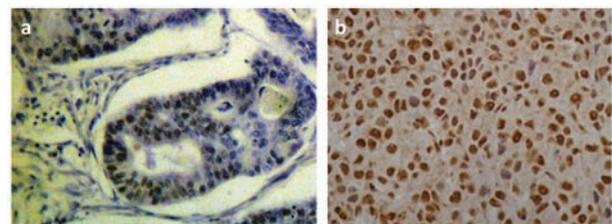

Figure 1. Immunohistochemical detection of hTERT protein. (a) Intestinal type gastric adenocarcinoma; (b) diffuse type gastric adenocarcinoma. A positive reaction was shown in the nuclei of cancer cells. Magnification: x100.

Two pathologists evaluated the immunostaining results independently.

Methylation- specific polymerase chain reaction

Genomic DNA (200 ng) of gastric tissue samples underwent bisulfite modification using EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Specific primers for methylation-specific polymerase chain reaction (MSP), located within the hTERT promoter, were as follows: 5′- TTGAGAATTTGTAAAGAGAAATGATG-3′ (sense) and 5′- ACTAAAAACAAACCCAAAAACACA-3′ (antisense) for the unmethylated reactions; 5′-TTGAGAATTTGTAAAGAGAAATGAC-3′ (sense) and 5′- TAAAAACGAACCCGAAAACG-3′ (antisense) for the methylated reactions, with PCR products of 133 bp and 131 bp, respectively (Silva et al. 2008). Briefly, PCR reaction was carried out in a 25 μl volume with 200 μmol l⁻¹ of $MgCl_2$, 100 ng of DNA, 200 pmol l⁻¹ of primers and 1.25 units of Taq (LGC, Rio de Janeiro, Brazil). After initial denaturing for 5 min at 94°C, 40 cycles at 94°C for 45 s, at 55°C for 45 s, and at 72°C for 30 s were carried out, followed by a final extension for 5 min at 72°C. Results were scored when there was a clear and visible band on the electrophoresis gel with the methylated and unmethylated primers (Herman et al. 1996). Hypermethylation was considered only in the presence of the methylated band, partial methylation was considered in the presence of methylated and unmethylated bands and hypomethylation was considered in the presence of only unmethylated band for *hTERT* promoter (Figure 2). Samples with unmethylated and partial methylated results were grouped for statistical analyses due to the low frequency of unmethylated samples.

Statistical analyses

Statistical analyses were performed using the χ^2 test or Fisher's exact test to assess associations between the
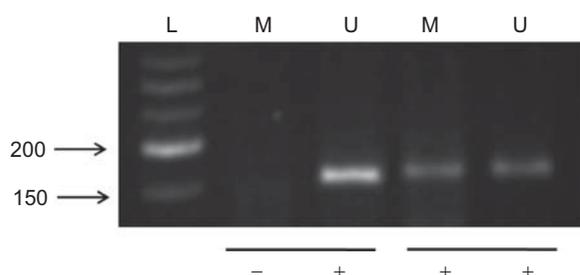

Figure 2. Methylation analysis by methylation-specific PCR of *hTERT* promoter showing unmethylated and partially methylated samples. L, size marker; M, methylated; U, unmethylated; +, positive PCR amplification; -, no PCR amplification.

expression or methylation status and clinicopathological characteristics. The χ^2 test (Phi correlation) was performed to evaluate the relationship between *hTERT* methylation and its protein expression. The non-parametric Mann–Whitney *U* test was used to compare patient age and methylation status or protein. *p*-Values less than 0.05 were considered significant.

Results

Tables 1 and 2 shows the clinicopathological characteristics, immunohistochemistry and methylation results of the studied samples. No normal gastric mucosa presented hTERT protein, but it was observed in 80% of tumours. hTERT expression was significantly associated with gastric cancer samples ($p < 0.0001$). The frequency of hTERT expression did not differ between diffuse and intestinal type gastric cancer (0.7667 vs 0.84, $p = 0.7363$). Gastric cancer samples with lymph node metastasis

Table 1. Clinicopathological and immunohistochemistry results of gastric tissue samples.

| | | Immunohistochemistry | | |
| | | Positive, | Negative, | |
Variable	Total, *n*	*n* (%)	*n* (%)	*p*-Value
Gender				0.7296
Male	36	28 (77.8)	8 (22.2)	
Female	19	16 (84.2)	3 (15.8)	
Tissue				< 0.001*
NGM	18	0	18 (100)	
GC	55	44 (80)	11 (20)	
Onset (years)				1
≤ 45	4	3 (75)	1 (25)	
> 45	51	41 (80.4)	10 (19.6)	
Helicobacter pylori				0.9968
Present	41	33 (80.5)	8 (19.5)	
Absent	14	11 (78.6)	3 (21.4)	
Lauren classification				0.7363
Diffuse	30	23 (76.7)	7 (23.3)	
Intestinal	25	21 (84)	4 (16)	
Tumour location				0.4297
Cardia	11	10 (90.9)	1 (9.1)	
Non-cardia	44	34 (77.3)	10 (22.7)	
Stage				0.0985
I/II	3	1 (33.3)	2 (66.7)	
III/IV	52	43 (82.7)	9 (17.3)	
Lymph node metastasis				0.0985
Present	52	43 (82.7)	9 (17.3)	
Absent	3	1 (33.3)	2 (66.7)	
Distant metastasis				0.1333
Present	19	18 (94.7)	1 (5.3)	
Absent	34	26 (76.5)	8 (23.5)	
Unknown	2			

NGM, normal gastric mucosa; GC, gastric cancer.

presented hTERT expression more frequently. Protein expression was also more frequent in samples at the III/IV stage than the I/II stage. There was no clear association between the frequency of hTERT expression and age, gender, tumour location, *Helicobacter pylori* infection, tumour extension and presence of distant metastasis (Table 1).

Partial methylation was the more common pattern in gastric cancer (55.96%) and in normal mucosa (86.79%) samples. Hypomethylated *hTERT* promoter was observed in 2.76% and 1.89% of gastric cancer and normal mucosa samples, respectively. The frequency

Table 2. Clinicopathological and methylation frequency in gastric tissue samples.

| | | Methylation-specific PCR | | |
| | | U/M+U, | | |
Variable	Total, n	n (%).	M, n (%)	p-Value
Gender				0.9264
Male	72	42 (58.3)	30 (41.7)	
Female	37	22 (59.5)	15 (40.5)	
Tissue				0.0002*
NGM	53	47 (88.7)	6 (11.3)	
GC	109	64 (58.7)	45 (41.3)	
Onset (years)				0.9017
≤ 45	14	9 (64.3)	5 (35.7)	
> 45	94	55 (58.5)	39 (41.5)	
Helicobacter pylori				0.4128
Present	73	45 (61.4)	28 (38.6)	
Absent	36	19 (52.8)	17 (48.2)	
Lauren classification				0.8822
Diffuse	56	33 (58.9)	23 (41.1)	
Intestinal	53	31 (58.5)	22 (41.5)	
Tumour location				1
Cardia	22	13 (59.1)	9 (40.9)	
Non-cardia	84	49 (58.3)	35 (41.7)	
Stage				0.5843
I/II	15	10 (66.7)	5 (33.3)	
III/IV	93	54 (58.1)	39 (41.9)	
Lymph node metastasis				0.1498
Present	94	53 (56.4)	41 (43.6)	
Absent	14	11 (78.6)	3 (21.4)	
Distant metastasis				$p = 0.3604$
Present	30	21 (70)	9 (30)	
Absent	62	36 (58.1)	26 (41.9)	
Unknown				

U, unmethylated; M+U, partial methylated; M, methylated; NGM, normal gastric mucosa; GC, gastric cancer.

of specimens presenting hypermethylation was significantly higher in tumours than in normal mucosa samples (0.4128 vs 0.1132, $p = 0.0002$). *hTERT* methylation pattern did not differ between diffuse and intestinal type (0.4107 vs 0.4151, $p = 0.8822$). There was no clear association between the frequency of only methylated *hTERT* promoter sequences and age, gender, tumour location, *H. pylori* infection, tumour extension, presence of lymph node and distant metastasis and tumour staging (Table 2).

To determine whether the presence of *hTERT* methylation leads to its protein expression, we compared the presence of hypermethylated and partially methylated *hTERT* promoter with the protein expression in gastric cancer specimens and their corresponding normal mucosa. None of the samples with protein expression data presented hypomethylated *hTERT* promoter. No samples of normal mucosa presented hypermethylated promoter. In gastric cancer samples, the presence of hypermethylated promoter was not correlated with a higher frequency of hTERT expression ($r = 0.2377$) (Table 3).

Discussion

Most human normal somatic cells lack telomerase activity due to transcriptional repression of *hTERT*, leading to telomere loss and eventually trigger senescence. hTERT expression and subsequent activation of telomerase are important to the stabilization of telomere length and, thus, are required for malignant cells to erase senescence checkpoints and acquire the capacity to proliferate unlimitedly. hTERT assessment has been demonstrated as a useful diagnostic and prognostic marker in many types of human malignancies, including gastric cancer (Li et al. 2008).

Transcriptional regulation of *hTERT* is the major mechanism for cancer-specific activation of telomerase. *hTERT* promoter contains a cluster of CpG sites, and therefore many researchers suggested a DNA methylation-based regulation (Kyo et al. 2008).

We first observed that no normal gastric mucosa and 80% of gastric cancer samples presented hTERT protein expression. hTERT expression frequency in gastric cancer samples was similar to two previous studies (78% and 90%) (Li et al. 2008, Wang et al. 2004). Other studies also demonstrated that hTERT mRNA had differential

Table 3. *hTERT* promoter methylation and protein expression in tumour and normal gastric samples.

Immunohistochemistry assay	Tumour, n (%)			Normal, n (%)		
	U/M+U	M	p-Value	U/M+U	M	p-Value
Positive	29 (87.9)	13 (68.4)	0.1424	0 (0)	0 (0)	1
Negative	4 (12.1)	6 (31.6)		18 (100)	0 (0)	

U, unmethylated; M+U, partial methylated; M, methylated

expression in gastric cancer samples and in corresponding normal mucosa (Suzuki et al. 2000, Chen et al. 2005, Hu et al. 2004). Conversely, a recent study observed no correlation between the presence of hTERT transcript and gastric cancer due to the presence of hTERT mRNA in both normal and gastric cancer samples (Li et al. 2008). These findings confirmed previous studies (Kawai et al. 2005, Nowak et al. 2003), suggesting that hTERT expression is detectable in normal and malignant gastric tissue. However, there is a consensus that telomerase can be an efficient cancer marker in this type of carcinoma and for cancer therapeutic purposes. Our findings support this evidence and confirm that detection of hTERT expression by immunohistochemistry can be a useful tool for gastric cancer diagnosis.

We also verified that hTERT expression was more frequent in samples with lymph node metastasis and higher stage. In a Chinese population, Wang et al. (2004) reported a significant association between hTERT and lymph node metastasis and clinical stage. Therefore, hTERT expression might be implicated in a poor prognosis. The *hTERT* promoter region was previously described as hypomethylated in normal tissues and hypermethylated in telomerase-positive cancer cells. Guilleret and Benhattar (2004) analysed a variable number of tissues but did not investigate gastric cancer cells. An unusual distribution of DNA methylation in CpG islands is associated with *hTERT* expression (Guilleret & Benhattar 2004). Our data showed no correlation between hypermethylation of the studied CpG island and protein expression.

The CpG island chosen for this study contains the main regulator sites of *hTERT* transcription, like Myc, Sp1 and CTCF. *MYC,* located at 8q24, is a transcription factor involved in cell cycle regulation, differentiation, apoptosis and neoplasic transformation. *MYC* is an oncogene involved in several carcinogenesis processes in humans, expressed in over 40% of gastric cancer (Calcagno et al. 2008). In our sample, *MYC* amplification and expression were observed in all gastric cancer samples, including early-onset (Calcagno et al. 2005, 2006, Costa Raiol et al. 2008). Myc seems to be a major activator of *hTERT* promoter by binding to DNA. It was demonstrated that its binding is affected by methylation status (Guilleret et al. 2002). Moreover, strong expression of transcriptional factor Sp1 was observed in cancer cell lines, suggesting a close correlation between Myc and Sp1 expression and hTERT transcription levels (Kyo et al. 2000). CTCF binds preferentially to CG-rich DNA regions, exhibiting an inhibitory effect when bound downstream of a transcriptional start site. Therefore, CTCF binding might be influenced by methylation of CpGs sites (Renaud et al. 2007).

Our group previously demonstrated that *hTERT* exhibits differential methylation in ageing and Alzheimer's

disease. We observed a hypermethylation in young controls and in patients in comparison to healthy elderly. Thus, we speculate that hypermethylation could result in telomerase activity and would increase longevity (Silva et al. 2008). However, *hTERT* hypermethylation was not associated with ageing in the current study, which can be explained either by tissue-specific *hTERT* regulation or age homogeneity of gastric cancer patients.

In this study, methylation status of *hTERT* promoter was evaluated for the first time in gastric cancer. We observed a strong association between *hTERT* hypermethylation and gastric cancer. However, we were not able to associate methylation status with protein expression. The absence of association can be explained in part due to the high frequency of *hTERT* methylation. In all normal mucosa samples that underwent *hTERT* methylation and expression analyses, a partially methylated *hTERT* promoter was observed. Moreover, only 2% of samples presented a hypomethylated *hTERT* promoter.

Although different frequencies of *hTERT* methylation in normal tissue (0% and 26.6%) was observed in other studies, no correlation between *hTERT* hypermethylation and mRNA expression was detected in cervical cancer, confirming our results (Widschwendter et al. 2004, Oikonomou et al. 2007). Lack of association between *hTERT* hypermethylation and expression was also observed in ovarian cancer samples (Widschwendter et al. 2004). On the other hand, the degree of *hTERT* methylation exhibited an impact on telomerase activity in B-cell lymphocytic leukaemia, colorectal and pancreatic carcinoma (Bechter et al. 2002, Choi et al. 2007, Kumari et al. 2009). In hepatocellular carcinoma, it was proposed that *hTERT* is regulated by DNA methylation and other epigenetic mechanism, such as histone modification (Iliopoulos et al. 2009). Taken together, these findings suggest that other factors might contribute to *hTERT* regulation in gastric carcinogenesis.

Further investigations in gastric carcinogenesis processes are necessary to verify whether partial methylation in normal mucosa implicates a risk of malignancy by subsequent hTERT expression. This partial methylation observed by MSP can be due to a heterogeneous methylation pattern in different cell populations in normal mucosa, as well as all cells presenting methylation at one allele. Dessain et al. (2000) suggested that the *hTERT* CpG island is under continuous pressure to become increasingly methylated in cells with high *de novo* methylase activity. A recent study showed that *de novo* DNA methyltransferases (DNMT3a and b) expression increase during gastric carcinogenesis (Ding et al. 2008).

Considering the immunohistochemistry and MSP results, about 5% of gastric cancer samples presented negative expression and partially methylated *hTERT* promoter, and could be considered a false-negative

result using these assays. Although poor correlation between hTERT protein expression and methylation was observed in gastric cancer samples, the clear difference in the frequency of *hTERT* expression and methylation between cancerous and non-cancerous tissues still might be useful for diagnosis of gastric cancer and may have an impact on the anti-telomerase strategy for cancer therapy.

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