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Absence of *CIP1/KIP1* Hypermethylation in Gastric Cancer Patients from Northern Brazil

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Abstract. *The aim of this study was to investigate the protein expression and methylation pattern of $P21^{CIP1}$ and $P27^{KIP1}$ genes. Patients and Methods: Twenty samples of gastric tumor and non-tumoral tissues of patients from Pará state, Brazil were collected. Methylation patterns were assessed by bisulfite sequencing, and protein expression evaluated with immunohistochemical analysis. Results: None of the analyzed samples showed methylation in both genes. Immunohistochemistry analysis demonstrated mislocalization or absence of expression of $P21^{CIP1}$ and $P27^{KIP1}$ in 7/20 and 6/20 of the studied samples, respectively. No correlations regarding protein expression and clinicopathological characteristics were observed; down-regulation of expression of $P21^{CIP1}$ with low (I-II) tumor stage (p=0.0777), and older age (>50 years old) with negative or mislocalization of $P27^{KIP1}$ (p=0.0922) were of borderline the statistical significance. Conclusion: Our results suggest that hypermethylation does not contribute to $P21^{CIP1}$ and $P27^{KIP1}$ silencing in gastric cancer, and that the role of these genes in the gastric tumorigenesis pathways should be studied further in the Pará state population.*

The cell cycle comprises numerous events that can lead to cell proliferation, senescence or apoptosis (1). Its progression is regulated by the interactions of different cyclins with their respective CDK (cyclin-dependent kinase) subunits (1, 2).

The CDK inhibitors (CDKIs) are a group of negative regulators of cell cycle progression, especially in the transition from the G_1 to S phase. They regulate CDK activity by physically blocking activation or substrate/ATP access (3). Moreover, additional functions have been postulated for CDKIs, suggesting they could be good markers for carcinogenesis (4). This group is subdivided into two: the INK4 family (comprising $P16^{INK4a}$, $P15^{INK4b}$, $P18^{INK4c}$ and $P19^{INK4d}$) and the CIP/KIP family, which includes $P21^{CIP1}$, $P27^{KIP1}$ and $P57^{KIP2}$ genes (2, 4). CIP/KIP inhibitory proteins bind and inactivate multiple cyclin D, E, A/CDK complexes during all phases of the cell cycle, especially in the G_1/S transition (5).

Few genetic alterations, such as mutations and gene deletions, have been described for these genes, especially $P21^{CIP1}$ and $P27^{KIP1}$, suggesting that other mechanisms, such as epigenetic ones, regulate gene expression at transcriptional, translational and post-translational levels (1, 6). Promoter hypermethylation may act as a possible mechanism for molecular inactivation of $P21^{CIP1}$ and $P27^{KIP1}$; however few studies have analyzed the influence of this mechanism in $P21^{CIP1}$ and $P27^{KIP1}$ regulation. In this work, we evaluated the protein expression and promoter region of these two genes for the presence of hypermethylation in tumor and normal samples in gastric cancer patients from Pará state, from northern Brazil.

Patients and Methods

Patients and DNA extraction. Samples of tumoral and non-tumoral gastric tissues of 20 patients with no previous chemo- or radiotherapic treatment were collected between 1999 and 2001 at Ofir Loiola and João de Barros Barreto hospitals. Tumor cells were isolated through microdissection and classified according to the Làuren criteria (7). Genomic DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Mainz, Rheinland-Pfalz, Germany). All procedures were approved by the Ethical Committee of the involved hospitals.

DNA methylation analyses. For methylation analyses, DNA was modified using sodium bisulfite (8). A fragment with 14 CpGs and 17 CpGs of the $P21^{CIP1}$ and $P27^{KIP1}$ promoter regions was amplified by polymerase chain reaction (PCR), using the primers described elsewhere (9) and sequenced using an ABI3130 automatic sequencer (Applied Biosystems, Foster City, CA, USA). All obtained sequences were aligned with BioEdit v7.0.5 (10), and samples with more than 20% of CpG sites methylated were considered hypermethylated.

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Immunohistochemical staining. Deparaffinized tissue sections (4 μm) were incubated with primary monoclonal antibody to $P21^{CIP1}$ and $P27^{KIP1}$ (DO-7, dilution 1:50; DakoCytomation, CA, USA) and secondary antibody followed by streptavidin-biotin-peroxidase complex (DakoCytomation) as described previously (11). Slides were visualized with diaminobenzidine-H_2O_2 and counterstained with Harry's hematoxylin. The results were interpreted using the criteria of Ozturk *et al*. (12). Positive $P21^{CIP1}$ and $P27^{KIP1}$ expression was defined as clear nuclear staining, whereas negative immunostaining was considered when there were fewer than 25% weakly stained tumor cells. A breast adenocarcinoma sample with known $P21^{CIP1}$ and $P27^{KIP1}$ immunoreactivity was used as positive control and normal gastric mucosa as negative control. Two pathologists evaluated the immunostaining results independently.

Statistical analyses. Associations among gene expression and clinicopathological features were performed using Fisher's exact test, with a significance level (α) of 0.05. All statistical analyses were calculated in BioEstat software, v5.0 (13).

Results

The mean age of the studied patients was 55.8 years (SE: 2.4372 years), ranging from 35 to 75 years. Seven samples were of the intestinal type and 13 of the diffuse type of gastric cancer (Table I), all of them were in advanced tumor stage (T2-T4).

For *P27KIP1* gene, only one CpG site in a tumor sample was methylated. For the promoter region of *P21CIP1* gene, no samples showed any sign of methylation in any of the CpG sites analyzed (Figure 1).

Immunohistochemistry analysis demonstrated mislocalization (cytoplasmatic expression), or down-regulated expression of $P21^{CIP1}$ and $P27^{KIP1}$ in 7/20 and 6/20 of the studied samples (tumoral *vs*. non-tumoral comparison). One patient had simultaneous mislocalization of $P21^{CIP1}$ and $P27^{KIP1}$, however simultaneous absence of both proteins was not observed (Table I).

Correlations between protein expression and age at diagnosis, histological tumor type, nodal invasion and microsatellite instability of *P53* marker (D17S153) were tested. *P*-values close to statistical significance were obtained for down-regulation of $P21^{CIP1}$ protein with low (I-II) tumor stage (p=0.0777) and for older age (>50 years old) with negative or mislocalization of P27KIP1 protein (p=0.0922).

Table I. *Samples studied in the present work, with their respective clinicopathological characteristics and P21CIP1 and P27KIP1 protein expression*.

Sample	*P21*	*P27*	Age (years)	Gender	Histo-logical type	TNM	Tumor site	Tumor stage
1N								
1T	N		59	M	Intestinal	T4N1Mx	Antral	IV
2N								
2T			50	M	Diffuse	T3N0Mx	Antral	II
3N								
3T		N	65	M	Diffuse	T3N1Mx	Non-antral	IIIA
4N								
4T	N		52	F	Diffuse	T2N0	Antral	IB
5N								
5T			43	M	Diffuse	T3N1	Antral	IIIA
6N								
6T			54	M	Diffuse	T3N1Mx	Non-antral	IIIA
7N								
7T			57	M	Intestinal	T3N1	Antral	IIIA
8N								
8T	ML		55	M	Intestinal	T3N1	Antral	IIIA
9N								
9T			60	M	Intestinal	T3N2	Antral	IIIB
10N								
10T	N		55	M	Intestinal	T3N1	Antral	IIIA
11N								
11T		N	55	M	Diffuse	T3N2	Non-antral	IIIB
12N								
12T			35	F	Diffuse	T4N1Mx	Non-antral	IV
13N								
13T		ML	74	M	Diffuse	T3N2	Antral	IIIB
14N								
14T	ML	ML	75	M	Intestinal	T3N0	Non-antral	II
15N								
15T			42	M	Diffuse	T3N1	Antral	IIIA
16N								
16T	ML		52	F	Diffuse	T2N1Mx	Antral	II
17N								
17T		ML	72	F	Intestinal	T3N2	Antral	IIIB
18N								
18T	ML		48	F	Diffuse	T2N0	Antral	IB
19N								
19T			45	M	Diffuse	T3N0	Antral	II
20N								
20T		ML	68	M	Diffuse	T4N2M0	Antral	IV

N, Negative for protein expression; ML, mislocalization (cytoplasmatic) of the studied proteins; M, male; F, female.

Discussion

The methylation status of *P21CIP1* and *P27KIP1* in gastric cancer has to our knowledge only been evaluated in three papers. Ying *et al*. (14), in the United Kingdom, studied gastric cell lines and tumor samples checking the methylation status of *P21CIP1* using methylation-specific PCR (MSP) and bisulfite sequencing, also reporting negative results for promoter hypermethylation.

Shin *et al*. (4), working with gastric cancer cell lines using MSP, also reported negative results for promoter methylation in both *P21CIP1* and *P27KIP1* genes, suggesting that other epigenetic mechanisms, such as histone deacetylation, play the major role in inactivation, especially in *P21CIP1*. Regarding *P27KIP1*, no inactivating mechanism in gastric cancer was proposed, as *P27KIP1* was expressed in the studied cell lines, and it seems to be essential for cell survival (4).

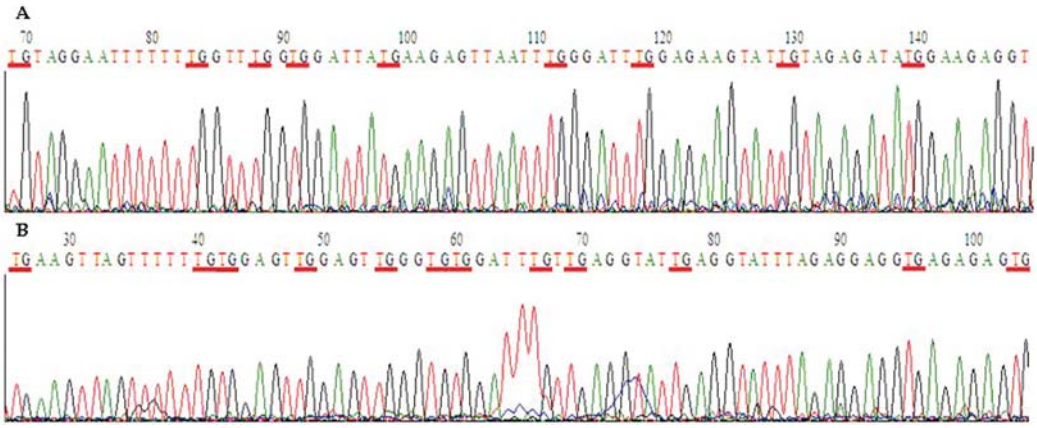

Figure 1. *Chomatogram of bissulfite-modified tumoral tissues showing absence of methylation in the analyzed samples. A: $P27^{KIP1}$ gene promoter; B: $P21^{CIP1}$ gene promoter.*

Other authors have suggested that the main mechanisms responsible for $P27^{KIP1}$ inactivation in primary tumors are at transcriptional, translational and post-translational levels, including protein stability, complex association and its localization (6). Recently, Tahara *et al*. (15) reported promoter methylation in one gastric cancer patient (1.1%), suggesting that methylation may have a minor contribution to $P21^{CIP1}$ expression.

Despite the results of the studies cited above, the real influence of methylation on $P21^{CIP1}$ and $P27^{KIP1}$ inactivation has not been completely explained, as in some tumor types, such as odontogenic, hepatocellular and adenoid cystic carcinomas, these genes are frequently methylated (16-18).

Low P21 and P27 expression has been reported in several tumor types, including both types of gastric cancer, commonly associated with a poorer prognosis (19, 20).

Immunostaining assays revealed high rate of low or absence of $P21^{CIP1}$ and $P27^{KIP1}$ protein expression, varying from 20 to 84% for $P21^{CIP1}$, and from 32.7 to 73% for $P27^{KIP1}$, in Asians, Italians and another Brazilian population (Ribeirão Preto, São Paulo) (21-23).

However, Mattioli *et al*. (24) in Italians and Kaye *et al*. (25) studying $P21^{CIP1}$ in South Africans found rates of mislocalization and down-regulated protein expression of 27.7% and 25%, respectively, values similar to those we found.

The small number of samples with low or absence of expression obtained by us may be influenced by other factors, such as ethnic contribution, *Helicobacter pylori* infection, which alters $P21^{CIP1}$ and $P27^{KIP1}$ protein expression, and disruption of other regulatory pathways, including phosphoinositide 3-kinases/protein kinase B/Mammalian target of rapamycin (PI3K/Akt/mTOR) and deregulation of ubiquitin-proteasome degradation.

Our results are in accordance with other studies (25-30) which did not find any correlation between clinicopathological features and P21 and P27 immunoexpression, suggesting that their role in diagnosis and prognosis are still controversial.

In conclusion, our results are in agreement with the literature which suggests that promoter methylation is not the major mechanism controlling the expression of $P21^{CIP1}$ and $P27^{KIP1}$ genes in gastric cancer, and that the role of these genes in the gastric tumorigenesis pathways should be studied further in Pará state population.

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