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Thorough Methylation Analysis of CpG Island Region outside the Putative Promoter of *CXCL12* Gene in Breast Cancer Cell Lines

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Abstract

Metastasis contributes to 90% of all breast cancer death. Several studies have highlighted the role of epigenetic events such as DNA methylation in promoter regions of genes as an important event in the process of metastasis in breast cancer. The promoter of the *CXCL12* gene, encoding a chemokine, is silenced by methylation in gastric, colon as well as in the breast cancer. The aim of this work was to map methylated regions flanking the promoter of *CXCL12* by cloning bisulfite treated DNA containing the distinct CpG regions and also correlate methylation pattern with the gene expression in different breast tumor cell lines. The results showed that the CpG islands 1, 3, 5 as well as the middle end of CpG 2 were more than 80% methylated in the cell lines that expressed the gene *CXCL12* (HB4a, PMC42 and MCF7). Expression analysis indicates strongly that these regions do not regulate this gene expression. However, CpG island 4 (CGI 4) located approximately 1550 bp away from the transcription start region and outside the putative promoter region, was differentially methylated and it seems to promote *CXCL12* gene silencing. In conclusion the CGI 4 is probably the last region to be methylated for silencing of *CXCL12* gene and could be a suitable DNA region for the diagnostic and prognostic to breast cancer studies.

Keywords: *CXCL12*; Chemokine; DNA methylation; Epigenetics; Breast cancer

Introduction

Despite advances in diagnosis and treatment, breast cancer is the most common type and the leading cause of cancer death among women worldwide [1]. However, it is not the primary tumor itself that causes death, but its spread to other secondary sites such as bones, lungs, liver, brain and lymph nodes [2]. In the study of breast cancer pair *CXCR4*/*CXCL12* is well defined as proteins that are part of the mechanism of metastases [3]. The *CXCR4* receptor is essential for the migration of tumor cells to organs where *CXCL12* chemokine is expressed; in addition, some studies indicate its involvement in the spread and progression of various tumors [3]. The *CXCL12* gene promoter has been studied intensively and started with results Garcia-Moruja et al. [4] who described the likely promoter located at -1010 and +122. Wendt et al. [5], evaluated the gene silencing *CXCL12* studying a promoter region containing a part of a CpG island (CGI) (-143 to +75) in colonic and breast tumor cell lines [5,6]. A recent study detailing the region 5' of the *CXCL12* gene identified five CGIs with emphasis on region 4 [7]. The silencing of the *CXCL12* was to highlight the CGI 4 that showed statistical significance with clinical pathological data, like metastasis and death, of these patients [7].

Approximately 70% of the promoters of annotated genes are associated with a CGI, making it the most common type of promoter in the genome of vertebrates [8]. Recent work has revealed a large class of CGI which are located remotely from the start of transcription (TSSs), but although the distance show evidence for a role as a promoter [9]. Moreover, Elango and Yi [10], report the importance of the size of CpG islands for regulation of gene expression.

This work aimed to study a large region of the *CXCL12* gene comprised TSS in 3447 bps containing high G + C content (≤ 0.6) and evaluate the dynamics of epigenetic silencing of a metastatic marker in the tumor breast cell lines model. This approach did not discussed in the literature before, might be used to determine CpG region that overlap with others in *CXCL12* gene expression.

Materials and Methods

Cell lines

Seven breast tumor cell lines were all obtained from the Ludwig Institute for Cancer Research (São Paulo, Brazil). The following cell lines were used: MDA-MB-231, MDA-MB-435, MDA-MB-436, MCF7, PMC42, HB4a (control immortalized normal cells) [11] and HB4aC3.6 [12]. The cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum (supplemented with 0.2 mM glutamine, and 40 µg/mL garamycin, 10 µg/mL insulin, if necessary) at 37°C in a humidified incubator with 5% CO_2.

DNA isolation and sodium bisulfite treatment

Genomic DNA was prepared from breast cancer cell lines by the phenol/chloroform protocol. They were then subjected to sodium bisulfite treatment using the EpiTect® Bisulfite Kit (Qiagen) according to the manufacturer's instructions.

CXCL12 CpG island methylation analysis

In an attempt to determine all CpG Islands of *CXCL12* for cloning we used the identification of the promoter sequence as described [7]. The cloning strategies were performed using the Gardiner and Frommer CGI definition by CpG island as ≥ 200 bps of sequence with ≥ 50% C + G content and ≥ 0.6 CpG observed/CpG expected [13].

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The cloned regions were highlighted in the Figure 1 (this Figure was adapted from Ramos et al. [7]).

The primers design were made using Methprimer program (http://www.urogene.org/methprimer/index1.html>) and they were used in this study to amplify the CpG islands (CGIs) 1, 2, 3 e 5 from *CXCL12* gene. For each CGI region it was planned two pair of primers to use in the nested PCR as showed in the Table 1.

The CGIs were amplified from bisulfite-treated DNA samples using a nested-PCR protocol. The two sets of primers were used for the nested reactions at their appropriate annealing temperatures, and are shown in Table 1. The first PCR reactions were performed as described below: 1 cycle of 95°C for 10 min, 94°C for 3 min, the appropriate annealing temperature for 3 min, 72°C for 2 min; 5 cycles of 94°C for 3 min, annealing temperature for 3 min, 72°C for 2 min; 35 cycles of 94°C for 1 min, annealing temperature for 1 min, and 72°C for 5 min. Amplified products were purified using the Qiaquick Gel Extraction Kit (Qiagen) and cloned into a pGEM-T Easy cloning vector (Promega). Eight clones were sequenced for each CGI and each cell line using the universal or reverse primers. DNA sequencing reactions were performed using Big Dye Terminator technology (Applied Biosystems) on ABI 3100 sequencer (Applied Biosystems) according to the manufacturer's instructions. One hundred percent methylation was obtained if a methylated cytosine in the CpG dinucleotides was present in eight sequenced clones. The methylation percentage for each tumor cell line (global methylation pattern) was calculated by dividing the number of methylated CpG dinucleotides by the total number of CpGs analyzed.

Results

The CGIs fragments cloned in this work were 1, 2, 3 and 5 and are showed in the Figure 1. The *CXCL12* expression pattern in breast

DNA cloned region	Primers	Anneling Temperature (°C)	Fragment (bp)
PCR 1 for CGI-1	1-F1I1 TTGATTTTAAGTTGGTTGTA	*43, 45, 47*	*684*
	2-R1I1 CTTTACTACAACTCTCATTCTA		
PCR2 for CGI-1	3-FNI1 TTTGTTTGTTTATTTTTTATTTATTGT	*47, 49, 51*	*454*
	4-RNI1 ATCCCTAAACCTCTCTCAAATAAAC		
PCR1 for CGI-2F	5-FFI2 GGGAAGAGTTTTTTGGATTTAGAAG	*41, 43, 45*	*696*
	6-RFI2 TTATTATCCCTATAACAAAACC		
PCR2 for CGI-2F	7-FNFI2 FNGAGTTTTTAGTTTTTTGGGT	*44, 46, 48,5*	*511*
	8-RNFI2 CAATAAAAAACAAACAAATTAATC		
PCR1 for CGI-2I	9-FII2 GGATTAATTTGTTTGTTTTTTATTG	*52, 54, 56*	*711*
	10-RII2 AACTACCTCCACCCCCACTATAT		
PCR2 for CGI-2I	11-FNII2 GGGGTTTTGTTATAGGGATAATAA	*54, 56, 58*	*595*
PCR1 for CGI-3	12-FI3 GGATTTTTTAGGGATAGGGA	*44, 46, 48*	*453*
	13-RI3 TCTAACAAACAAACAATACTCA		
PCR2 for CGI-3	14-FNI3 GTTGGGAAGGATATAGAGAGGTGGTT	*46, 48, 50*	*271*
	15-RNI3 AAACTAAAAACTCCCCCACCCC		
PCR1 for CGI-5	16-FI5 AATGGTTTGGTTTGATGGT	*46, 48, 50*	*668*
	17-RI5 ACACCCACTATATACTAAACCT		
PCR2 for CGI-5	18-FNI5 ATTTAGGTTGGAGTGTAG	*46, 48, 50*	*306*
	19-RNI5 ACAAACCTCACAATTTTA		

Table 1: Summary of primer sequences, used for nested-PCR cloning.

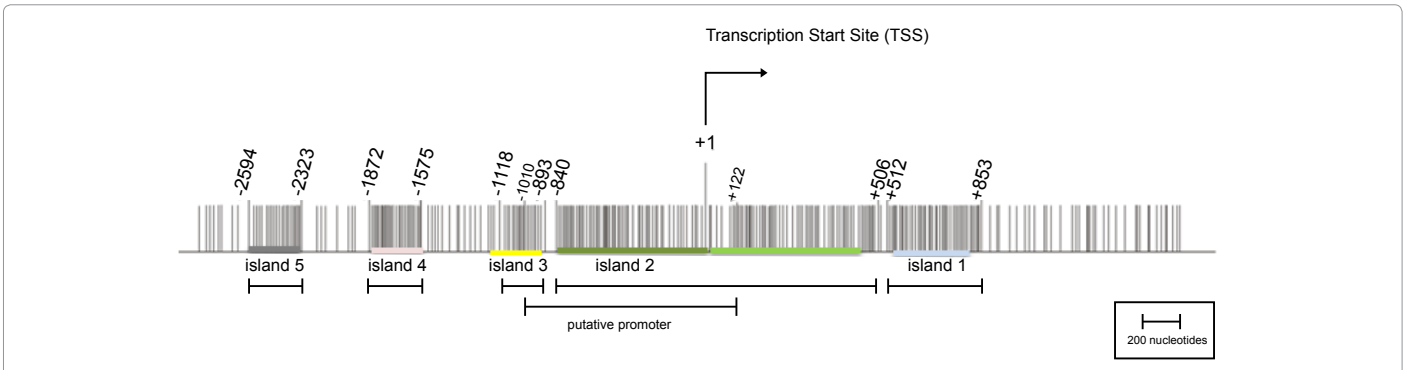

Figure 1: The CGIs in the 5' region from *CXCL12* gene. The CGIs is inside an area from -2594 to +853. The vertical lines correspond to the CpG dinucleotides. The numbers above correspond to the distance in relation to the +1 (TSS). The CGI 1 is represented in blue, the CGI 2I in light green, the CGI 2F in dark green, the CGI 3 in yellow, CGI 4 in pink and CGI 5 in gray. This figure was adapted from Ramos et al. [7].

Breast cell line	*CXCL12* Expression*
HB4a	+
HB4aC3.6	+
MCF7	+
PMC-42	+
MDA-MB-435	–
MDA-MB-436	–
MDA-MB-231	–

*This data were collected from [7].

Table 2: *CXCL12* expression in breast cancer cell lines.

cancer cell lines was studied by Ramos et al. [7] and it was adapted here as a table (see the Table 2).

Sequencing data of CGI 4 was showed in the Figures 2 and 3 and it was obtained from Ramos et al. [7] and was used here for comparison with the CGIs studied here. The CGI 1 localized at +512 to +853 was cloned in a fragment containing 454 bp with 41 CpGs. The methylation pattern for CGI 1 showed that breast cancer cell lines that do not express the gene *CXCL12*, MDA-MB-435, 436 and 231, appeared highly methylated (Figure 2, in blue) with percentages of 97.5%, 94.2% and 90.8%, respectively (Figure 3). These results illustrated a correlation between the high density of methylation and the lack of gene *CXCL12* expression. The two cell lines immortalized (HB4a and HB4aC3.6) expressing the gene *CXCL12* showed low levels of methylation, 38.7% and 6.7% respectively (see Figure 2, in blue and Figure 3). These data are consistent with the low methylation to gene expression. However, two breast cell lines (MCF7 and PMC42) which also express the gene showed high levels of methylation throughout the CGI 1, 95.4% and 86.0%, respectively (Figure 2, in blue and Figure 3). At first, this result was not expected and became evident that differences in the methylation pattern along the promoter may indicate the regions involved in epigenetic regulation of *CXCL12* expression.

The CGI 2 is the greatest of all, and contains 1346 bp (Figure 2, in green) comprising the region -840 to +506. This region contains the transcription start of the gene and it is most likely promoter between positions -1010 to +122 (Figure 1). The cloning step of this region

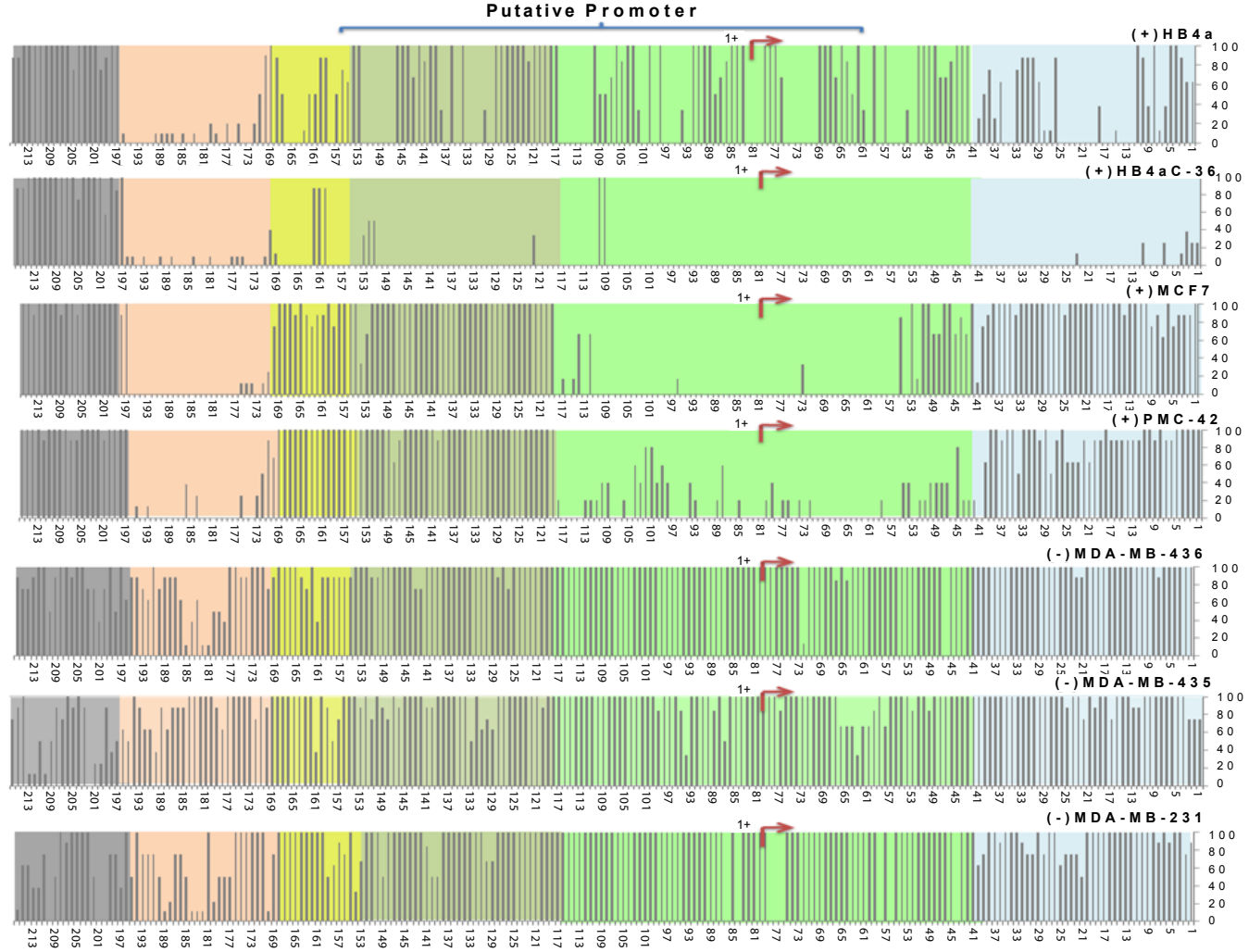

Figure 2: Methylation % of CGIs, 1, 2I, 2F, 3, 4 and 5 from *CXCL12* gene in breast cancer cell lines. Each graphic represents one breast tumor cell line. The percentage of methylation from eight clones are represented by bars and numbered 1 to 216 CG. The CGI 1 is represented in blue, the CGI 2I in light green, the CGI 2F in dark green, the CGI 3 in yellow, CGI 4 in pink and CGI 5 in gray. The red arrow indicates the TSS and the key shows the putative promoter from CGs +157 to +61. The expression pattern for each breast cancer cell line is indicate by + or –. The sequencing data for CGI 4 was obtained for Ramos et al. [7].

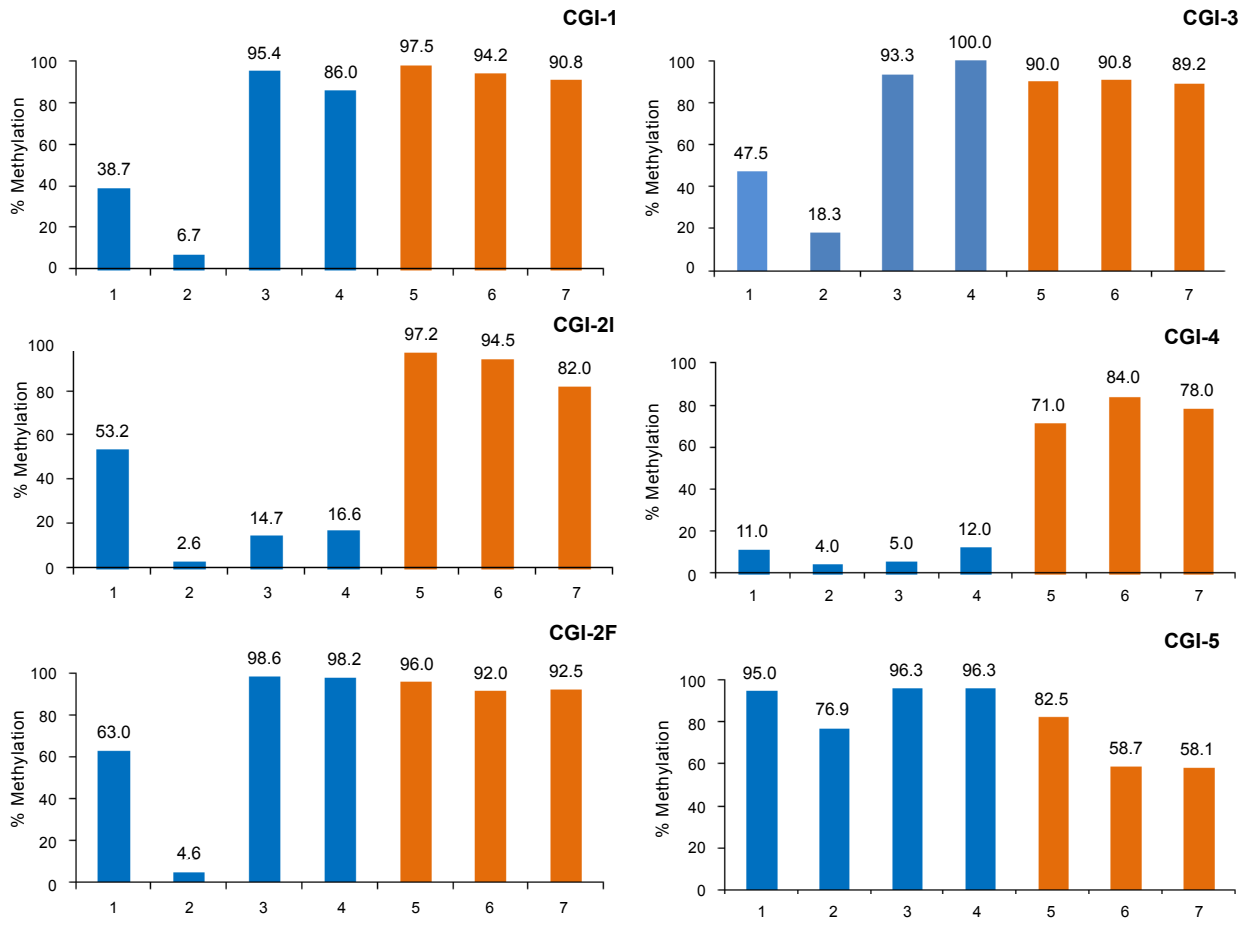

Figure 3: Global Methylation % of CGIs, 1, 2I, 2F, 3, 4 and 5 from *CXCL12* gene in breast cancer cell lines. Each graphic represents each CGI (1, 2I, 2F, 3, 4 and 5). The percentage of methylation from each breast cancer cell line are represented by bars numbered, 1 (HB4a), 2 (HB4aC3.6), 3 (MCF7), 4 (PMC42), 5 (MDA-MB-436), 6 (MDA-MB 435) and 7 (MDA-MB-231). The bars in blue represent positive and red negative expression of *CXCL12* respectively. The sequencing data for CGI 4 was obtained for Ramos et al. [7].

was facilitated by dividing it into 2 parts, starting on the region which contains 595 bp with 77 CpGs (CGI 2I) and finalizing the island on 511 bp and it has 36 CpGs (CGI 2F). The sequence data revealed that the HB4aC3.6 lines, MCF7 and PMC42 showed low levels of methylation localized near of the transcription start (CGI 2I) (Figure 2, in light green) and overall methylation were 2.6%, 14.7% and 16.6%, respectively (Figure 3). Other strains which do not express the gene MDA-MB-436, 435 and 231 demonstrated high levels of methylation (97.2%, 94.5% and 82.0%, respectively) (Figure 3). These results are consistent with the lack of *CXCL12* gene expression in these strains. However, HB4a (the normal cell line) which also expressed the gene, showed high density methylation (53.7%) throughout the CGI 2I region (Figure 2, light green and Figure 3). The sequence data of CGI 2F for HB4a, MCF7 and PMC42 which express the gene had high percentage of methylation, 63.0%, 98.6% and 98.2%, respectively (Figure 3). Curiously, the sequence data of CGI 2F, for HB4aC3.6, which expresses the gene showed low levels of methylation (4.6%). Strains that do not express the gene, MDA-MB-436, 435 and 231 had the methylation percentage of 96.0%, 92.0% and 92.5%, respectively (Figure 3).

For analysis of the CGI 3 were cloned fragments of 271 bp, comprising the CpG dinucleotides region -1118 to -893 with 15 CpGs. This region is heavily methylated and it was presented in six of the seven strains of this study (Figure 2 in yellow). The correlation with the expression of the gene was observed only for HB4aC3.6 cell line (18.3% overall methylation) (Figure 3).

The CGI 4 was cloned and sequenced by Ramos et al. [7] and we used here for comparisons with the results of this study. This DNA region was the only that has correlation with the reduction of the *CXCL12* expression and the methylation pattern of this gene (Figure 2 in pink). The cell lines HB4a, HB4aC3.6, MCF7 and PMC42 which this gene is expressed had low levels of methylation (11.0%, 4.0%, 5.0% and 12.0%, respectively) (Figure 3). This scenario is entirely the opposite as it was observed in these cell lines MDA-MB-436, 435 and 231 which no *CXCL12* expression were detected (Figure 2 in pink) and the gene is hypermethylated (71.0%, 84.0% and 78.0%, respectively) (Figure 3).

The CGI 5 cloned fragments were 306 bp, comprising the CpG dinucleotides region -2594 to -2323 and it has 20 CpGs. This region of DNA showed up heavily methylated in all cell lines studied (Figure 2 in gray). It was observed global methylation of 95.0%, 76.9%, 96.3%, 96.3%, 82.5%, 58.7% and 58.1% corresponding to each strain under study (Figure 3).

Discussion

The great interest to study the chemokine *CXCL12* it is metastatic function [3] and also its interesting 5 CpG rich regions [7]. Our previous article [7] showed that the CpG Island 2 was not important to the tumor progression because it showed no statistical significance with any tumor variables studied, despite the other positive studies with tumor samples [5-6]. Ramos et al. [7] also studied CpG Island 4 which results showed statistical correlation with variables related with metastasis. The exam of these two regions (CGI 2 and CGI 4) and their different association results got our attention to study the full CpG island region outside the putative promoter of *CXCL12* gene, especially because we did not know if other different regions would have the same methylation pattern that can switch gene off.

Therefore, in our study design we used breast tumor cell lines which methylation of CGIs occurs more intensely [14]. This feature was exploited in our work to study the dynamics of gene promoter methylation of *CXCL12* in particular the distribution of methylation along the 5 CGIs and their correlation with gene expression. Our results showed that the sequencing of DNA region contained in the CGI 1 was hypermethylated in PMC42 and MCF7 cell lines that expressed the gene. This data illustrated that there are regions that dominate the process of regulation gene expression considering epigenetics mechanisms. In this case it seems that this region may be hypermethylated and it does not affect the expression of *CXCL12* gene in these cell lines.

When was analyzed the CGI 2 it was observed that cell line HB4a was hypermethylated at CGI 2I region. Ramos et al. [7] used MSP (methylation specific PCR) technique with forward primer designed in the CpG dinucleotides region 69 and reverse primer for others CpG dinucleotides 79, 80 and 81 (primers designed by [5]), and they did not detect methylated bands in HB4a cell line. Conversely in this work, it was adopted sequencing data which demonstrated unmethylation in that primer-specific localization of CpGs but even so, the CGI 2I region had 53.2 % of global hypermethylation (Figure 2 in light green). This result could indicate that CGI 2I is really irrelevant for *CXCL12* silencing by DNA methylation, simultaneously with the absence of correlation between this region and the breast cancer clinic-pathological data [7]. Consequently it demonstrated the real importance to complete thorough methylation analysis of *CXCL12* gene promoter region.

The sequence data from the CGI 1, 2F, 3 and 5 showed that these regions are heavily methylated in most strains of this study therefore the regions appear not to be important to regulate gene expression in *CXCL12* breast tumor cell lines. Moreover, the CGI 4 showed to be the characteristic region of *CXCL12* gene silencing considering that it was the only region almost completely demethylated in the strains expressing *CXCL12*, and densely methylated in that breast cell lines silenced. The methylation pattern observed in this CGI 4 region has potential importance in the *CXCL12* dynamics of epigenetic silencing particularly when it was analyzed with other CGI 1, 2, 3 and 5 regions.

The CGI 4 is located at 1575 bp from the transcription start and 565 bp from the end of the probable promoter (Figure 1). In spite of the CGI 4 localization, Deaton and Bird [15] also pointed about a large class of CGIs that are remote from the start of transcription (TSSs), but although the distance shows evidence for a role as a promoter [10].

The detailed study of the 5' region of a gene that contains multiple regions rich in CpG dinucleotides, such as gene *CXCL12*, may show the importance of regulatory site distant and it reinforces the role of CpG Island 4 as a principal DNA region involved with the *CXCL12* gene expression.

Apparently the CGI 4 is the last region to be methylated for silencing of *CXCL12* gene and reflects the dynamic of DNA methylation mechanism in the breast cancer cell lines. The CGI 4 could be the best DNA region in diagnostic and prognostic to breast cancer studies.

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