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BRIEF REPORT

Differential regulation of *LRRC37A2* in gastric cancer by DNA methylation

Fernanda Wisnieski [a,b,*], Jaqueline Cruz Geraldis [a,*], Leonardo Caires Santos [a,*], Mariana Ferreira Leal [a,c], Danielle Queiroz Calcagno [c], Carolina Oliveira Gigek [d], Elizabeth Suchi Chen [a], Ana Carolina Anauate [a], Ricardo Artigiani [d], Samia Demachki [c], Paulo Pimentel Assumpção [c], Laercio Gomes Lourenço [e], Carlos Haruo Arasaki [e], Julie Krainer [f], Stephan Pabinger [f], Rommel Rodriguez Burbano [c,g], and Marilia Arruda Cardoso Smith [a]

[a]Disciplina de Genética, Departamento de Morfologia e Genética, Universidade Federal de São Paulo, São Paulo, Brazil; [b]Disciplina de Gastroenterologia, Departamento de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil; [c]Universidade Federal do Pará, Belém, Brazil; [d]Departamento de Patologia, Universidade Federal de São Paulo, São Paulo, Brazil; [e]Disciplina de Gastroenterologia Cirúrgica, Departamento de Cirurgia, Universidade Federal de São Paulo, São Paulo, Brazil; [f]Center for Health & Bioresources, Austrian Institute of Technology, Vienna, Austria; [g]Laboratório de Biologia Molecular, Hospital Ophir Loyola, Belém, Brazil

ABSTRACT

Gastric cancer (GC) is one of the leading types of fatal cancer worldwide. Epigenetic manipulation of cancer cells is a useful tool to better understand gene expression regulatory mechanisms and contributes to the discovery of novel biomarkers. Our research group recently reported a list of 83 genes that are potentially modulated by DNA methylation in GC cell lines. Herein, we further explored the regulation of one of these genes, *LRRC37A2*, in clinical samples. *LRRC37A2* expression was evaluated by RT-qPCR, and DNA methylation was studied using next-generation bisulphite sequencing in 36 GC and paired adjacent nonneoplastic tissue samples. We showed that both reduced *LRRC37A2* mRNA levels and increased *LRRC37A2* exon methylation were associated with undifferentiated and poorly differentiated tumours. Moreover, *LRRC37A2* gene expression and methylation levels were inversely correlated at the +45 exon CpG site. We suggest that DNA hypermethylation may contribute to reducing *LRRC37A2* expression in undifferentiated and poorly differentiated GC. Therefore, our results show how some genes may be useful to stratify patients who are more likely to benefit from epigenetic therapy.

Abbreviations: AR: androgen receptor; 5-AZAdC: 5-aza-2'-deoxycytidine; *B2M*: beta-2-microglobulin; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; GC: gastric cancer; GLM: general linear model; *LRRC37A2*: leucine-rich repeat containing 37 member A2; SD: standard deviation; TFII-I: general transcription factor II-I; TSS: transcription start site; XBP1: X-box binding protein 1

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Introduction

Gastric cancer (GC) is the third leading cause of cancer-related death worldwide [1]. The major contributors to the high mortality rates of GC are late diagnosis and lack of effective therapies to combat disease heterogeneity [2]. The understanding of epigenomic regulation has provided further evidence for the application of epigenetic drugs for solid tumour treatment [3], with several agents progressing to clinical trials with gastrointestinal cancer patients [3,4]. In addition, the elucidation of gene expression regulatory mechanisms may help to identify patients who are more likely to benefit from the use of epigenetic drugs. In this context, our research group previously assessed the gene expression profile of GC cells [5] treated with the DNA demethylating agent 5-aza-2'-deoxycytidine (5-AZAdC) and identified 83 genes potentially modulated by DNA methylation [6]. *Leucine-rich repeat containing 37 member A2* (*LRRC37A2*) was one of the upregulated genes, with fold change>1.5-fold that presented CpG island near the TSS.

Although the LRR superfamily is composed of a heterogeneous group of proteins involved in the differentiation and development of normal nervous tissues [7] and the immune

CONTACT Fernanda Wisnieski f.wisnieski@unifesp.br Disciplina de Gastroenterologia, Departamento de Medicina, Universidade Federal de São Paulo, Rua Loefgreen, 1726, São Paulo 04040002, Brazil

*These authors have contributed equally to the work.

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response [8], studies have reported that deregulation of LRR genes is critical in tumorigenesis [7,9]. In particular, the role of *LRRC37A2* and its underlying mechanism of regulation remain unknown in GC.

Here, we report a detailed analysis of the *LRRC37A2* DNA methylation profile in a panel of GC and matched control tissue samples using next-generation sequencing of bisulphite-converted DNA. Moreover, we provide further evidence that DNA methylation impacts the transcript level of *LRRC37A2* in GC, especially in undifferentiated and poorly differentiated tumours.

Materials and methods

Subjects and sample preparation

Thirty-six matched pairs of GC and adjacent nonneoplastic tissues (control group) were obtained from patients with gastric adenocarcinoma who underwent gastric resection in João de Barros Barreto University Hospital (HUJBB) and São Paulo Hospital (HSP), Brazil. None of the patients had a history of exposure to either chemotherapy or radiotherapy prior to surgery or the cooccurrence of diagnosed cancers. Written informed consent with the approval of the ethics committees of HUJBB and HSP was obtained from all patients before sample collection (Ethics Committee number 0511/09).

The genomic DNA and RNA from fresh snap-frozen tissues in liquid nitrogen were isolated and quantified as previously reported, and RNA conversion to cDNA was performed [6].

Quantitative polymerase chain reaction

TaqMan gene expression assays (Thermo Fisher Scientific, Waltham, MA, USA) were used to evaluate the expression of *LRRC37A2* (Hs03805446_mH) in triplicate. Expression data were normalized to *GAPDH* (Hs99999905_m1) and *B2M* (Hs00984230_m1), as described previously by our research group [10]. The mRNA levels were analysed using the ΔCt method.

Next-generation sequencing

Primers were designed by using MethPrimer software [11] for the *LRRC37A2* CpG island, which includes 8 CpG sites surrounding the transcription start site (TSS):5'-AAGAGTGTTTTGGTGATATGGAGTA G-3', and 3'-CCACAATAATTACCACATAAAAA AAA-5' (Figure 1a).

DNA samples were bisulphite-converted using EpiTect Fast DNA Bisulphite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A 201 bp fragment corresponding to the *LRRC37A2* CpG island was PCR amplified at an annealing temperature of 58°C.

Sequencing was performed using the IonTorrent PGM^{TM} platform (Thermo Fisher Scientific) and data were processed as described previously [6]. In detail, Bismark version 0.16.3 [12] was used to align the reads to the human reference genome (hg19) restricted to the target region. A minimum of 100 CpG measurements across samples was required for the region set. Non-CpG methylation was used as an internal upper-bound estimate of the inefficiency of bisulphite conversion. All samples presented less than 1% methylation in the CHG and CHH contexts.

Statistical analysis

The Shapiro-Wilk normality test was used to evaluate the distribution of all data. Data were not normally distributed and were transformed (z-score). Repeated-measures or univariate general linear models (GLMs), and Pearson correlation tests were employed to analyse the data. Two-sided tests were performed, and 95% confidence intervals were reported.

Results

LRRC37A2 is downregulated in gastric cancer

To first confirm that *LRRC37A2* may have a role in gastric carcinogenesis, we evaluated its mRNA expression in a panel of clinical samples (Supplementary Table 1). The expression of *LRRC37A2* was reduced in GC tissue samples compared with the controls ($P< 0.01$; Figure 1b). Undifferentiated and poorly differentiated GC

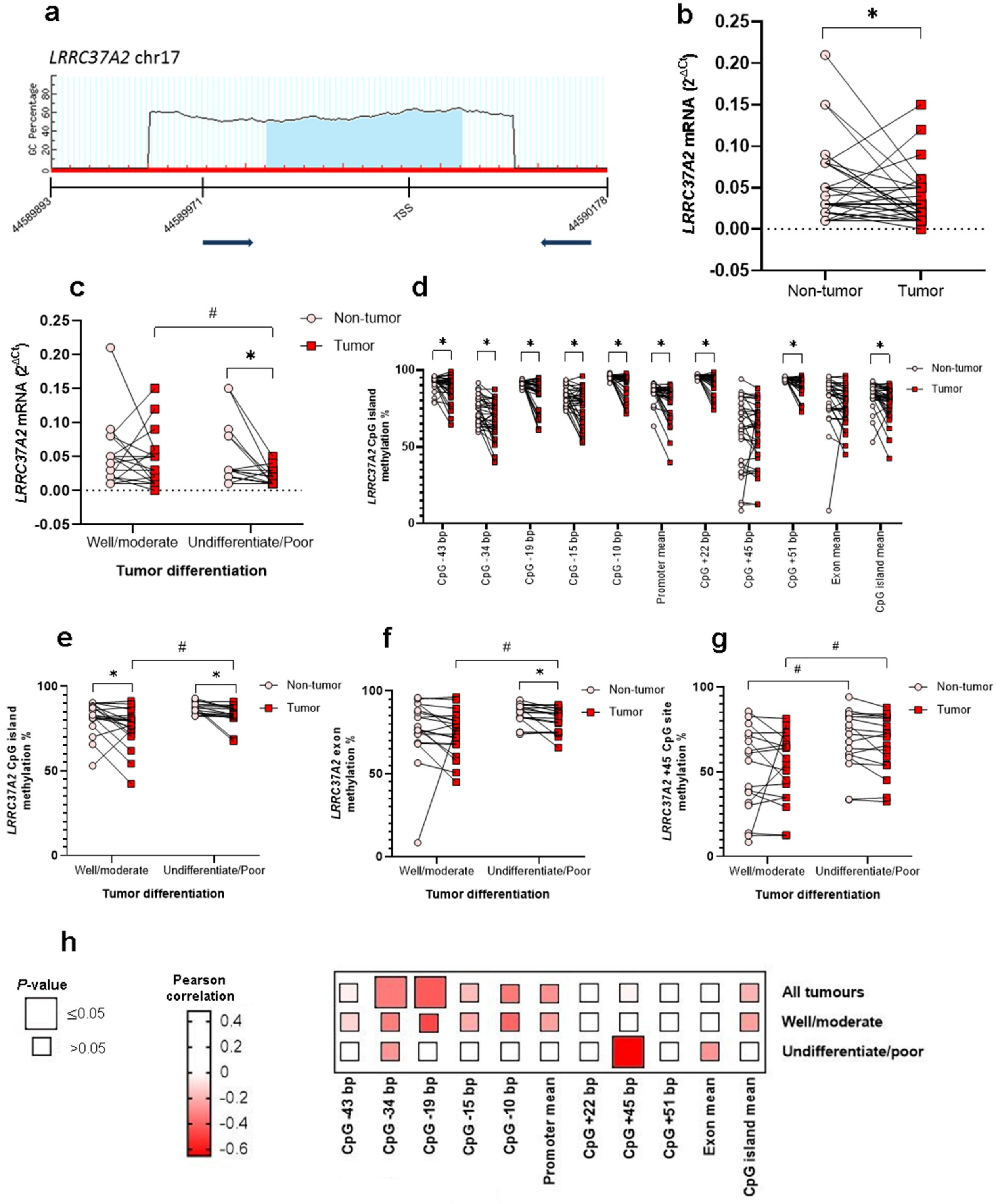

Figure 1. *LRRC37A2* mRNA and methylation levels in GC. a: schematic diagram of CpG dinucleotide density across the *LRRC37A2* locus and the location of the investigated CpG dinucleotides. b: reduced *LRRC37A2* mRNA in GC compared with paired adjacent nonneoplastic tissue samples. c: reduced *LRRC37A2* mRNA levels associated with undifferentiated and poorly differentiated GC. In this subset of tumours, significantly reduced *LRRC37A2* mRNA levels were also observed compared with paired adjacent nonneoplastic tissue samples. d: significant differences in *LRRC37A2* methylation levels across CpG sites between GC and the corresponding adjacent nonneoplastic tissue samples. e: increased *LRRC37A2* CpG island methylation associated with undifferentiated and poorly differentiated tumours. f: increased *LRRC37A2* exon methylation associated with undifferentiated and poorly differentiated tumours. In this subset of tumours, significantly increased *LRRC37A2* mRNA levels were also observed compared with paired adjacent

showed reduced *LRRC37A2* expression in comparison with well and moderately differentiated tumours (*P*= 0.05; Figure 1c and Supplementary Table 1). In addition, low *LRRC37A2* mRNA levels were detected in undifferentiated and poorly differentiated tumours compared with the respective controls (*P*< 0.01; Figure 1c).

LRRC37A2 is methylated in gastric cancer

We next evaluated the *LRRC37A2* methylation pattern in CpG sites in the promoter and exon portions. Overall, high *LRRC37A2* methylation (~80%) was observed in the studied specimens; however, GC samples presented significantly decreased methylation in the CpG island (*P*< 0.01) and promoter regions (*P*< 0.01) compared with controls (Figure 1). A detailed analysis of *LRRC37A2* methylation revealed significantly decreased methylation at all the specific CpG sites (*P*< 0.01) in the GC compared with controls, except at the +45 location (Figure 1d).

By grouping the patients according to clinicopathological features, we observed that *LRRC37A2* CpG island and exon methylation percentages were increased in patients with undifferentiated and poorly differentiated tumours (*P*= 0.03 and *P*= 0.04, respectively; Supplementary Table 1, Figure 1e,Figure 1f). *LRRC37A2* CpG island, promoter, and exon methylation percentages were also increased in patients with lymph node metastasis (*P*= 0.04, *P*= 0.05, and *P*= 0.03, respectively; Supplementary Table 1). Furthermore, *LRRC37A2* CpG and promoter methylation percentages were increased in patients with TNM stage III and IV tumours (*P*= 0.01 and *P*= 0.02, respectively; Supplementary Table 1). Last, *LRRC37A2* exon methylation was increased in patients with early onset (*P*= 0.03; Supplementary Table 1).

The impact of DNA methylation on *LRRC37A2* gene expression

To provide further evidence that *LRRC37A2* is regulated by epigenetic processes, we performed correlation analysis between mRNA and methylation levels. Overall, *LRRC37A2* gene expression and DNA methylation at the specific −34 (r = −0.34, *P*= 0.05) and −19 (r = −0.42, *P*= 0.02) promoter CpG sites were inversely correlated (Figure 1h and Supplementary Table 2).

Considering the consistently reduced *LRRC37A2* gene expression and increased DNA methylation in undifferentiated and poorly differentiated tumours, we analysed gene expression and methylation data correlation in this subset of tumours. We observed that *LRRC37A2* expression and DNA methylation at the specific +45 exon CpG site were inversely correlated (r = −0.65, *P*< 0.01; Figure 1h and Supplementary Table 2). In well and moderately differentiated tumours, no significant correlation between gene expression and DNA methylation data was observed (Figure 1h).

We also observed that undifferentiated and poorly differentiated tumours and their paired controls presented increased levels of methylation specifically at the +45 CpG site in relation to well and moderately differentiated tumours (*P*= 0.03 and *P*= 0.01, respectively; Figure 1g).

Discussion

In this study, we assessed in clinical samples the expression and methylation of *LRRC37A2*, a candidate target modulated by DNA methylation originally identified by our research group in GC cell lines [6]. Our results showed reduced *LRRC37A2* mRNA levels in GC, especially in undifferentiated and poorly differentiated GC, a subset of GC that tend to grow quickly and show a more invasive phenotype than well and moderately differentiated GC [13]. Therefore, we demonstrated that *LRRC37A2* is downregulated in

nonneoplastic tissue samples. g: increased methylation at the +45 CpG exon site in undifferentiated and poorly differentiated tumours and the corresponding adjacent nonneoplastic samples compared with well and moderately differentiated tumours and adjacent nonneoplastic samples. h: correlation analysis between *LRRC37A2* gene expression and methylation levels.

Data are expressed as the mean ± SD* Significant difference between groups by repeated-measures GLM# Significant difference between groups by univariate GLMTSS: transcription start site.

GC and may be involved in tumour cell differentiation.

To evaluate whether DNA methylation is the mechanism implied in the reduction of *LRRC37A2* mRNA levels in GC, we analysed CpG sites surrounding the TSS individually and grouped by promoter and exon portions. We first observed decreased methylation in the CpG island and promoter regions, as well as at all the specific CpG sites (except at the +45 location) in the GC compared with controls. Although promoter CpG islands are typically devoid of methylation, studies have demonstrated hypermethylation of CpG islands in nonneoplastic gastric mucosae in relation to GC [14–16]. Chronic inflammation and ageing seem to be closely associated with increased methylation in these samples [17].

Our results also showed high levels of methylation in the entire CpG island and in the specific exon region associated with undifferentiated and poorly differentiated tumours. Gene expression and methylation correlation analysis reinforced these results and revealed the +45 site as a possible methylated exon CpG site involved in *LRRC37A2* downregulation in undifferentiated and poorly differentiated GC. These findings corroborate a previous study in which DNA methylation downstream of the TSS (first exon) was shown to be the most critical for transcriptional silencing [18].

To predict transcription factors binding the +45 exon CpG site, we used the PROMO server with TRANSFAC version 8.3 [19,20], and we found that this CpG site falls within the recognition sequence of general transcription factor II–I (TFII-I), X-box binding protein 1 (XBP1), and androgen receptor (AR). These findings suggest that methylation at the +45 exon CpG site may inhibit the binding of transcription factors and repress *LRRC37A2* expression.

Taking the gene expression and methylation results together, our study shows that increased methylation of *LRRC37A2*, more specifically at the +45 exon CpG site, may be a mechanism of gene silencing in undifferentiated and poorly differentiated GC. Thus, our results show how specific genes may be useful to stratify patients who are more likely to benefit from epigenetic therapy.

Our study is the first to investigate the possible involvement of DNA methylation in the regulation of *LRRC37A2* transcription; however, there are three limitations that should be addressed. First, *LRRC37A2* is contained in a core duplication region on chromosome 17 [8], and the commercial assay available for gene expression analysis also detects another LRR family member, the *LRRC37A* gene. Furthermore, the methylation results were only specific for *LRRC37A2*, once the alignment of reads was restricted to the region of interest. Second, a correction for multiple comparisons (multiple CpG sites being evaluated simultaneously) was not carried out in the analysis of DNA methylation data. Because no similar study has been published previously, we chose to prioritize the biological effect rather than reject the involvement of an epigenetic event in GC due to statistical rigour (type II error). Third, it is known that the traditional bisulphite sequencing technique does not distinguish between 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) [21,22]. Although the presence of 5hmC in the mammalian genome is less abundant than 5mC [23], and a previous study, which had performed genome-wide profiling of 5hmC in GC, did not identify our CpG island as a differentially hydroxymethylated region [24–27], the quantification of 5hmC at *LRRC37A2* CpG island and the determination of its impact on gene expression in our samples would contribute to our findings.

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Disclosure statement

The authors declare that they have no conflict of interest.

Author contribution

Conception and design: FW, MFL, RRB, and MACS. Sample collection: FW, LCS, DQC, JCG, COG, ACA, ESC, PPA, LGL, and CHA. Pathological analysis: RA and SD. Molecular experiments: FW, JCG, LCS, MFL, DQC,

COG, ESC, and ACA. Data analysis: FW, JCG, LCS, SP, and JK. Writing, review, and/or revision of the manuscript: all authors. Administrative, technical, or material support: MACS and RRB.

Ethical Statements

Written informed consent with the approval of the ethics committees of João de Barros Barreto University Hospital and São Paulo Hospital was obtained from all patients before sample collection (Ethics Committee number 0511/09).

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ORCID

Fernanda Wisnieski http://orcid.org/0000-0003-4963-3194
Elizabeth Suchi Chen http://orcid.org/0000-0002-3573-0164
Ana Carolina Anauate http://orcid.org/0000-0001-6158-3412
Julie Krainer http://orcid.org/0000-0002-2953-7982
Marilia Arruda Cardoso Smith http://orcid.org/0000-0002-1441-1033

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