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ORIGINAL ARTICLE

The impact of DNA demethylation on the upregulation of the *NRN1* and *TNFAIP3* genes associated with advanced gastric cancer

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Abstract

Gastric cancer (GC) is the third leading cause of cancer-related death worldwide. Very few therapeutic options are currently available in this neoplasia. The use of 5-Aza-2′-deoxycytidine (5-AZAdC) was approved for the treatment of myelodysplastic syndromes, and this drug can treat solid tumours at low doses. Epigenetic manipulation of GC cell lines is a useful tool to better understand gene expression regulatory mechanisms for clinical applications. Therefore, we compared the gene expression profile of 5-AZAdC-treated and untreated GC cell lines by a microarray assay. Among the genes identified in this analysis, we selected *NRN1* and *TNFAIP3* to be evaluated for gene expression by RT-qPCR and DNA methylation by bisulfite DNA next-generation sequencing in 43 and 52 pairs of GC and adjacent non-neoplastic tissue samples, respectively. We identified 83 candidate genes modulated by DNA methylation in GC cell lines. Increased expression of *NRN1* and *TNFAIP3* was associated with advanced tumours ($P < 0.05$). We showed that increased *NRN1* and *TNFAIP3* expression seems to be regulated by DNA demethylation in GC samples: inverse correlations between the mRNA and DNA methylation levels in the promoter of *NRN1* ($P < 0.05$) and the intron of *TNFAIP3* ($P < 0.05$) were detected. Reduced *NRN1* promoter methylation was associated with III/IV TNM stage tumours ($P = 0.03$) and the presence of *Helicobacter pylori* infection ($P = 0.02$). The identification of demethylated activated genes in GC may be useful in clinical practice, stratifying patients who are less likely to benefit from 5-AZAdC-based therapies.

Key messages

Fernanda Wisnieski, Leonardo Caires Santos and Danielle Queiroz Calcagno contributed equally to this work.

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- Higher expression of *NRN1* and *TNFAIP3* is associated with advanced gastric cancer (GC).
- *NRN1* promoter hypomethylation contributes to gene upregulation in advanced GC.
- *TNFAIP3* intronic-specific CpG site demethylation contributes to gene upregulation in GC.
- These findings may be useful to stratify GC patients who are less likely to benefit from DNA demethylating-based therapies.

Keywords Cancer therapy · DNA methylation · 5-Aza-2′-deoxycytidine · *NRN1* · *TNFAIP3*

Introduction

Gastric cancer (GC) is the third leading cause of cancer-related death worldwide. The absence of specific symptoms in the early tumour stages contributes to the diagnosis at advanced stages and the poor response to the currently available therapeutic options [1]. Even after curative resection and adjuvant therapy, approximately 35% of patients still develop recurrences [2].

Currently, no molecular biomarkers have been widely applied in clinical practice, which limits the successful management of patients [3]. The numerous GC histological and molecular classifications currently available reflect the complexity and heterogeneity of this cancer (see review [4]). Although recent high-throughput studies have classified gastric tumours into subgroups with clinical relevance [5], the underlying mechanism of gastric carcinogenesis should be further elucidated for the identification of novel biomarkers and the development of new therapeutic strategies.

Epigenetic alterations play an important role in the cellular transformation to cancer, and these alterations have strong application potential for cancer detection, diagnosis, and therapy. Two epigenetic drugs, 5-Azacytidine and 5-Aza-2′-deoxycytidine (5-AZAdC), have Food and Drug Administration (FDA) approval for the treatment of myelodysplastic syndromes and can treat solid tumours at low doses. Several other epigenetic agents are in clinical and preclinical studies [6].

The analysis of epigenetic markers may elucidate the regulatory mechanism of drivers of gastric carcinogenesis and contribute to the discovery of biomarkers that may help to identify patients who are more likely to benefit from the use of epigenetic drugs. Therefore, in this study, we first identified genes modulated by DNA methylation in GC by assessing the gene expression profile modified by treatment with the epigenetic 5-AZAdC drug in cell lines. To further explore epigenetically regulated genes involved in GC, we selected two differentially expressed genes (DEGs): *neuritin 1* (*NRN1,* also referred to as *CPG15*) and *tumour necrosis factor alpha induced protein 3* (*TNFAIP3*, also referred to as *A20*) for analysis of mRNA and methylation levels in GC and paired non-neoplastic tissue samples. We showed that the expression of these two genes is associated with advanced GC, and using next-generation sequencing of bisulfite-converted DNA, we observed DNA demethylation in CpG sites around the transcription start sites (TSSs) of genes in tumour samples.

Materials and methods

Cell lines and 5-AZAdC treatment

ACP02 and ACP03 cell lines were previously established by our research group from primary gastric adenocarcinomas [7]. A cell culture of non-neoplastic gastric mucosa cells pooled from 10 patients without GC (MNP01) was also used to evaluate *NRN1* and *TNFAIP3* gene expression. All cell lines were cultured in RPMI 1640 media (GIBCO, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (GIBCO) and 100 μg/mL kanamycin (GIBCO).

To evaluate the best dosage and the period of treatment with 5-AZAdC, ACP02, and ACP03 cells were seeded in duplicate on 96-well plates and then treated with 2 μM, 5 μM, or 10 μM of 5-AZAdC (Sigma-Aldrich, St. Louis, MO, USA) for 24, 72, or 120 h. Untreated cells were used as controls. Viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Invitrogen, Eugene, OR, USA) assay [8]. Both cell lines showed decreased cell viability of approximately 70% when treated with 5 μM of 5-AZAdC for 120 h (Supplementary Fig. 1). Therefore, this dose and this period of treatment were selected for further evaluation of the 5-AZAdC effect.

For the gene expression profile, cells were seeded in 75-cm^2 cell culture flasks in triplicate and treated with 5 μM 5-AZAdC (Sigma-Aldrich) for 120 h. Untreated GC cells were used as controls.

Clinical samples

According to the studied genes, the sample size varied from 43 to 52 matched pairs of GC and the corresponding adjacent non-neoplastic tissues (control group). These samples 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, from 2009 to 2018. None of the patients had a history of exposure to either chemotherapy or radiotherapy prior to surgery or the co-occurrence 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).

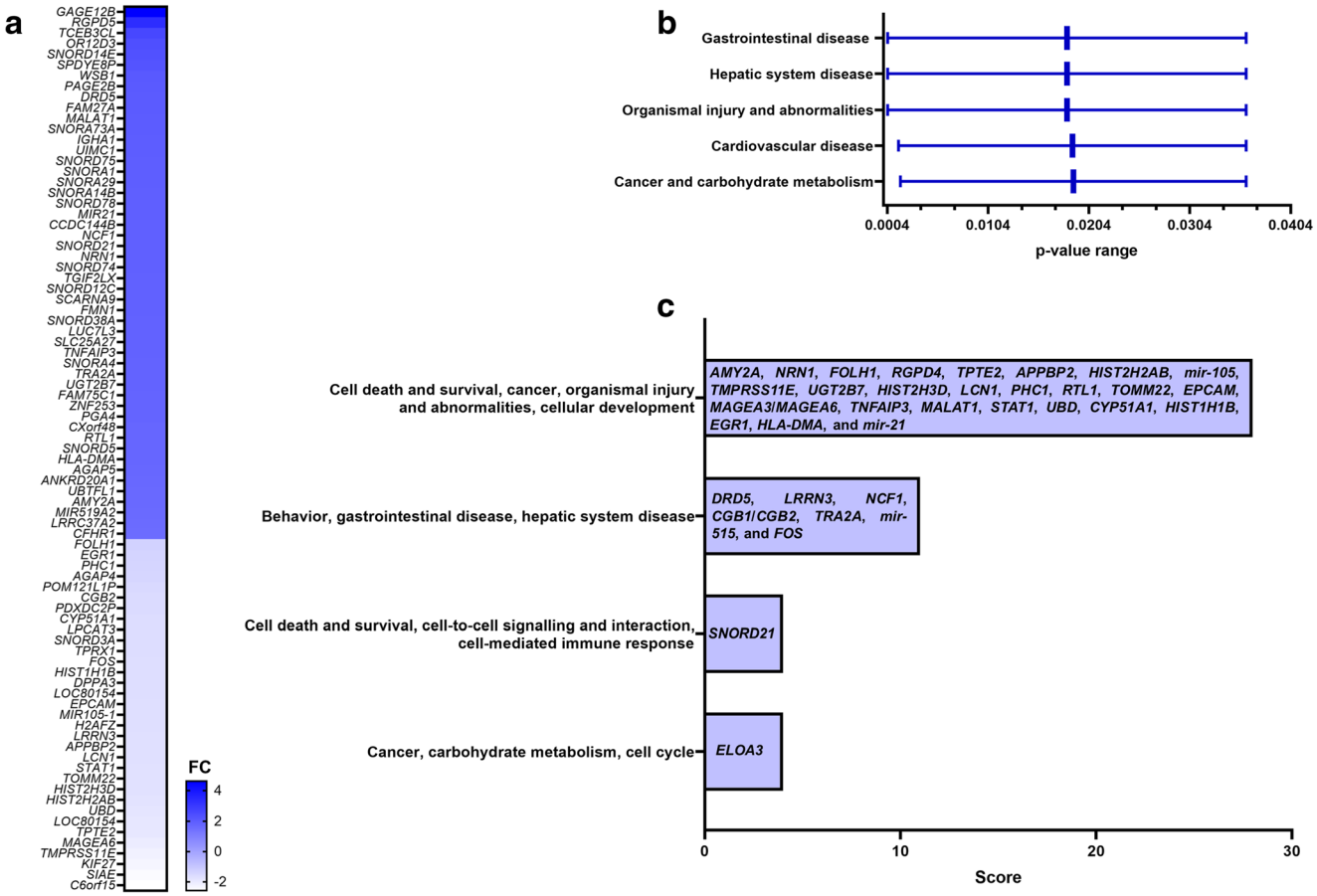


Fig. 1 Identification of candidate genes regulated by DNA methylation in GC cell lines. **a** List of 83 DEGs by comparing 5-AZAdC-treated and non-treated GC cell lines. **b** Diseases and disorders associated with DEGs by IPA. **c** Functional categories of the DEGs by IPA. The calculated score for each functional category is derived from a *p* value and indicates that the probability of genes has been found in a functional category and not by chance. A score of 2 or more has at least 99% confidence to not be generated by chance. FC; fold change

All of the samples were classified according to Lauren [9] and TNM staging criteria [10]. The presence of *Helicobacter pylori*, a class I carcinogen, and the cagA virulence factor in gastric samples were detected by PCR as previously described [8].

DNA and RNA extraction

Total RNA was isolated from cell lines using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Only samples with an RNA integrity number of ≥ 7 based on a Bioanalyzer (Agilent 2100 Bioanalyzer; Agilent Technologies, Waldbronn, Germany) and no DNA contamination were used in the microarray hybridization.

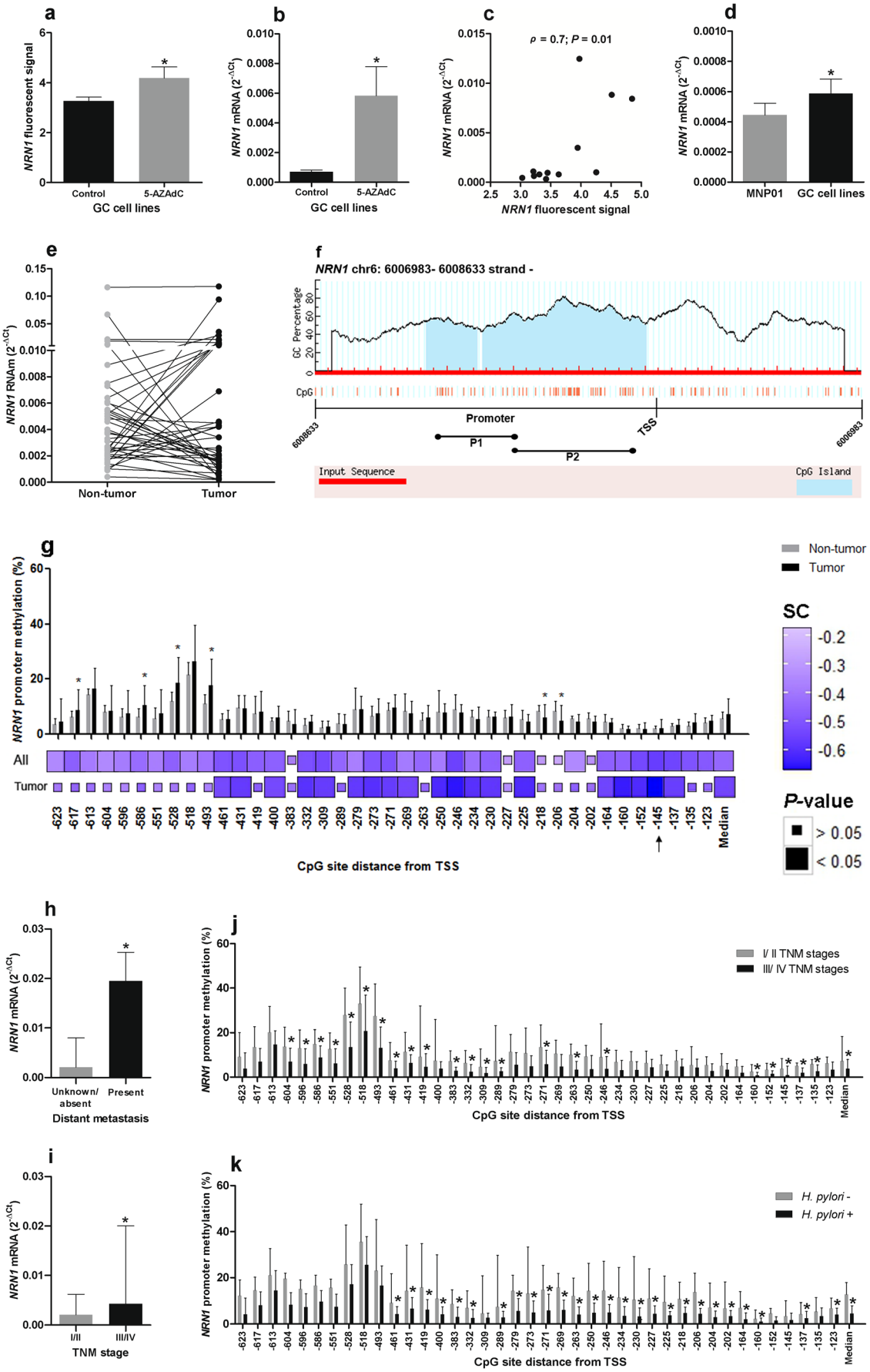
Total DNA and RNA were isolated from tissue samples using the AllPrep DNA/RNA/Protein Kit (Qiagen) according to the manufacturer's instructions.

Microarray and bioinformatics analyses

The gene expression profile was assessed using the Affymetrix Human Gene 1.0 ST array (which covers 36,079 transcripts) following the manufacturer's instructions. Data were deposited in the ArrayExpress database [11] at the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) (https://www.ebi.ac.uk/arrayexpress/) under accession number E-MTAB-7880. Gene expression values were obtained using the three-step robust multiarray average preprocessing method implemented in the Affy package from R/Bioconductor (http://www.bioconductor.org/). The RankProd method [4] was employed for the selection of DEGs (FDR < 5%) [12]. Pathway analysis was carried out using the Ingenuity® Pathway Analysis (IPA®, http://www.ingenuity.com).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

cDNA was synthesized using High-Capacity® cDNA Reverse Transcription (Thermo Fisher Scientific, Waltham, MA, USA). TaqMan gene expression assays (Thermo Fisher Scientific) were used to evaluate the expression of *NRN1* (Hs00213192_m1) and *TNFAIP3* (Hs00234713_m1) together


a
NRN1 fluorescent signal
Control
5-AZAdC
GC cell lines
b
NRN1 mRNA (2^-ΔCt)
Control
5-AZAdC
GC cell lines
c
ρ = 0.7; P = 0.01
NRN1 mRNA (2^-ΔCt)
NRN1 fluorescent signal
d
NRN1 mRNA (2^-ΔCt)
MNP01
GC cell lines
e
NRN1 RNAm (2^-ΔCt)
Non-tumor
Tumor
f
NRN1 chr6: 6006983- 6008633 strand -
GC Percentage
CpG
Promoter
TSS
P1
P2
6008633
6006983
Input Sequence
CpG Island
g
NRN1 promoter methylation (%)
Non-tumor
Tumor
SC
-0.2
-0.3
-0.4
-0.5
-0.6
All
Tumor
P-value
> 0.05
< 0.05
-623 -617 -613 -604 -596 -586 -551 -528 -518 -493 -461 -431 -419 -400 -383 -332 -309 -289 -279 -273 -271 -269 -263 -250 -246 -234 -230 -227 -225 -218 -206 -204 -202 -164 -160 -152 -145 -137 -135 -123 Median
CpG site distance from TSS
h
NRN1 mRNA (2^-ΔCt)
Unknown/ absent
Present
Distant metastasis
j
NRN1 promoter methylation (%)
I/ II TNM stages
III/ IV TNM stages
CpG site distance from TSS
i
NRN1 mRNA (2^-ΔCt)
I/II
III/IV
TNM stage
k
NRN1 promoter methylation (%)
H. pylori -
H. pylori +
CpG site distance from TSS

◀ **Fig. 2** *NRN1* mRNA and methylation levels in GC, **a** Significantly increased fluorescent signal of *NRN1* in 5-AZAdC-treated GC cell lines compared with non-treated GC cells detected by a microarray assay. **b** Validation of microarray results showing increased *NRN1* mRNA in 5-AZAdC-treated GC cell lines compared with non-treated GC cells by RT-qPCR. **c** Strong positive correlation between *NRN1* gene expression assessed by a microarray assay and RT-qPCR. **d** Increased *NRN1* mRNA in GC cell lines compared with non-neoplastic gastric mucosa cells, MNP01. **e** Absence of a significant difference in *NRN1* mRNA between GC and the corresponding adjacent non-neoplastic tissue samples. **f** Schematic diagram of CpG dinucleotide density across the *NRN1* locus and the location of the investigated CpG dinucleotides. **g** significant differences in *NRN1* methylation levels at specific promoter CpG sites comparing GC and the corresponding adjacent non-neoplastic tissue samples. The purple squares represent the correlation between *NRN1* mRNA and DNA methylation in all gastric tissue samples (tumour and control samples) and in tumour samples. **h** Significantly increased *NRN1* mRNA in tumours of patients with distant metastasis. **i** Significantly increased *NRN1* mRNA in tumours of patients with III/IV TNM stages. **j** Consistent hypomethylation throughout the 40 evaluated CpG sites of the *NRN1* promoter region in tumours of patients with III/IV TNM stages. **k** Consistent hypomethylation throughout the 40 evaluated CpG sites of the *NRN1* promoter region in tumours of patients positive for *H. pylori* infection. Cell line data are expressed as the mean ± standard deviation; tissue sample data are expressed as the median ± interquartile range. *Significant difference between groups by *t* test, Wilcoxon test or Mann-Whitney test. The arrow indicates the CpG binding site of the HIF-1 transcription factor TSS; transcription start site. SC; Spearman correlation coefficient

with *B2 M* (Hs00984230_m1), *ACTB* (Hs03023943_g1), or *GAPDH* (Hs99999905_m1) in triplicate. *ACTB* + *B2M* and *GAPDH* + *B2M* were used for the normalization of target gene expression in GC cell lines and tissue samples, respectively, as determined previously by our research group [13]. The mRNA levels were analysed using the ΔCt method according to the manufacturer's instructions.

DNA methylation analysis

The methylation profiles of *NRN1* and *TNFAIP3* CpG islands were evaluated by next-generation sequencing. Bisulfite treatment was carried out using 500 ng of DNA and the EpiTect Fast DNA Bisulfite kit (Qiagen, Hilden, Germany) in duplicate, in which only unmethylated cytosines are converted into uracils. Primers were designed by using MethPrimer software [14] to amplify sequences spanning the CpG island region (Figs. 2f and 3f). Supplementary Table 1 shows the primer sequences and PCR reaction conditions.

Sequencing was performed in duplicate using IonTorrent technology. Libraries were prepared using the Ion XpressTM Plus Fragment Library Kit (Thermo Fisher Scientific) and sequenced on an Ion Torrent™ PGM sequencer (Thermo Fisher Scientific) by using the Ion PGMTM Hi-QTM Sequencing kit (Thermo Fisher Scientific) and the Ion 318 Chip kit v2 (Thermo Fisher Scientific), following the manufacturer's instructions.

Data were processed using a custom version of TABSAT [15, 16]. In detail, Bismark version 0.16.3 [17] using the TMAP mapper (version 5.2.25) was used to align the reads to the human reference (hg19) restricted to the target regions. Only reads with a range of 50–400 bp were used, and a maximum of one mismatch was tolerated. Short reads and low-coverage region sets were filtered out to reduce background noise [18]. After alignment, the methylation information was extracted and aggregated for the analysed samples. A minimum of 1000 CpG measurements across samples was required for each region set.

The percentage of methylation in CpG sites was determined as the percentage of cytosine reads from the total of cytosine and thymine reads of each CpG site. To quantify the percentage of methylation in a functional genomic region, the median percentage of methylation across CpG sites for each region was calculated. The median of the medians for the entire studied CpG island was also obtained.

Commercial non-methylated gDNA (Thermo Fisher Scientific) was used as an internal control to estimate the inefficiency of bisulfite conversion. More than 1% methylation was detected in seven (for *NRN1*) and five (for *TNFAIP3*) CpG sites, which were excluded from further analysis.

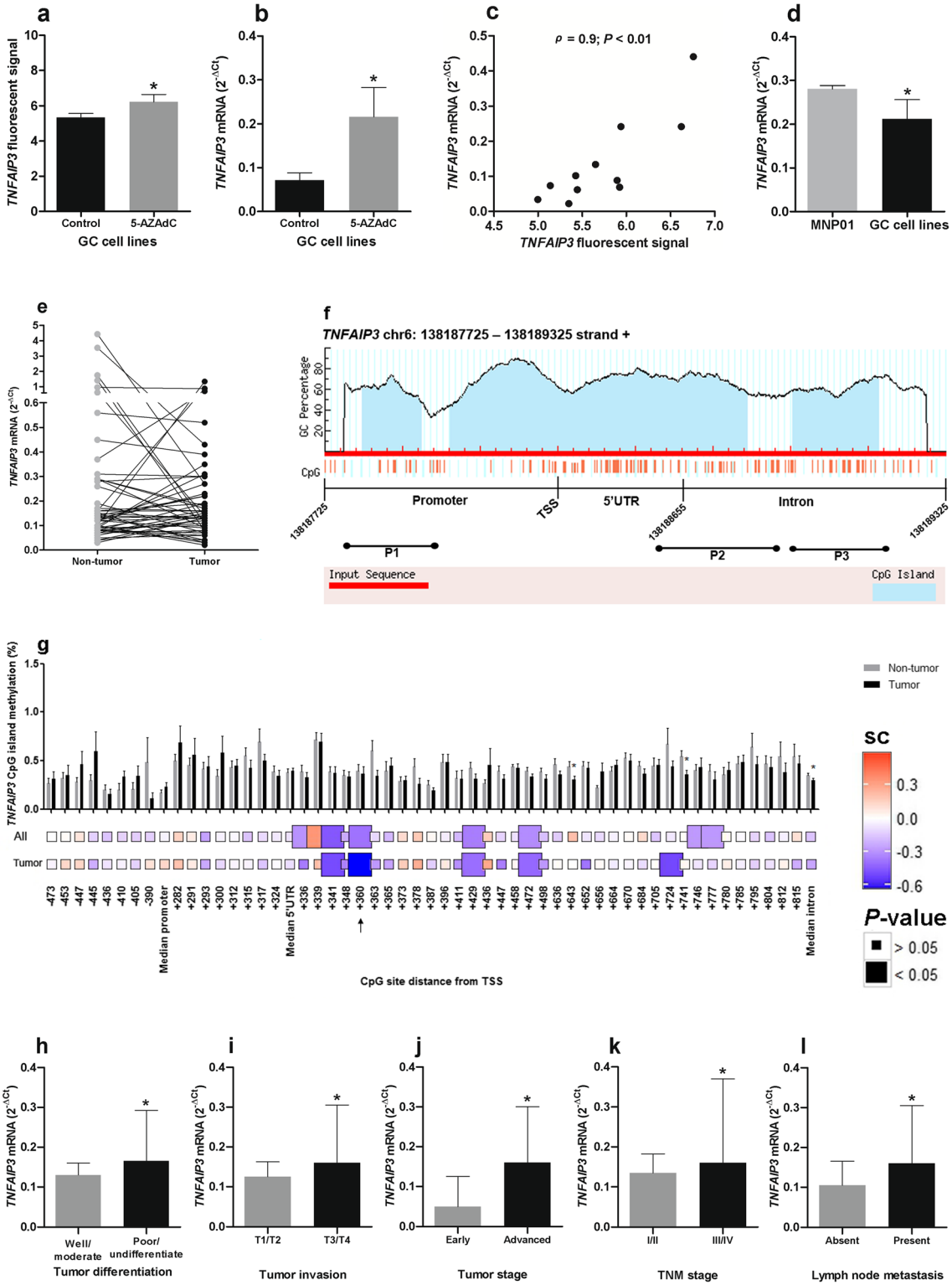
Statistical analysis of gene expression and DNA methylation

The Shapiro-Wilk normality test was used to evaluate the distribution of all data. Gene expression data from cell lines were normally distributed and analysed using *t* tests and Pearson correlation tests. Gene expression and DNA methylation data from paired tissue samples were not normally distributed and were analysed by using the Wilcoxon test and Spearman test. Clinicopathological associations were assessed by the Mann-Whitney test. *P*-values were two-sided, and all confidence intervals were at the 95% level. Statistical tendencies ($0.05 < P \leq 0.07$) were considered significant when at least a medium effect size was detected by partial eta square ($\eta_p^2 > 0.16$) or based on Cohen's r ($r > 0.11$).

Results

Identification and selection of candidate genes regulated by DNA methylation in gastric cancer

We identified 83 DEGs, 50 upregulated and 33 downregulated, when comparing 5-AZAdC treated and untreated GC cell lines (Fig. 1a and Supplementary Table 2). Most of the DEGs have functions that are associated with gastrointestinal disease (Fig. 1b), and four relevant functions were enriched: (a) cell death and survival, cancer, organismal injury and abnormalities, and cellular development; (b) behaviour, gastrointestinal


a
TNFAIP3 fluorescent signal
Control
5-AZAdC
GC cell lines
b
TNFAIP3 mRNA (2^-ΔCt)
Control
5-AZAdC
GC cell lines
c
ρ = 0.9; P < 0.01
TNFAIP3 mRNA (2^-ΔCt)
TNFAIP3 fluorescent signal
d
TNFAIP3 mRNA (2^-ΔCt)
MNP01
GC cell lines
e
TNFAIP3 mRNA (2^-ΔCt)
Non-tumor
Tumor
f
TNFAIP3 chr6: 138187725 – 138189325 strand +
GC Percentage
CpG
Promoter
TSS
5'UTR
Intron
138187725
138188655
138189325
P1
P2
P3
Input Sequence
CpG Island
g
TNFAIP3 CpG island methylation (%)
Non-tumor
Tumor
SC
0.3
0.0
-0.3
-0.6
P-value
> 0.05
< 0.05
All
Tumor
Median promoter
Median 5'UTR
Median intron
CpG site distance from TSS
h
TNFAIP3 mRNA (2^-ΔCt)
Well/ moderate
Poor/ undifferentiate
Tumor differentiation
i
TNFAIP3 mRNA (2^-ΔCt)
T1/T2
T3/T4
Tumor invasion
j
TNFAIP3 mRNA (2^-ΔCt)
Early
Advanced
Tumor stage
k
TNFAIP3 mRNA (2^-ΔCt)
I/II
III/IV
TNM stage
l
TNFAIP3 mRNA (2^-ΔCt)
Absent
Present
Lymph node metastasis

◀ **Fig. 3** *TNFAIP3* mRNA and methylation levels in GC. **a** Significantly increased fluorescent signal of *TNFAIP3* in 5-AZAdC-treated GC cell lines compared with non-treated GC cells detected by a microarray assay. **b** Validation of microarray results showing increased *TNFAIP3* mRNA in 5-AZAdC-treated GC cell lines compared with non-treated GC cells by RT-qPCR. **c** Very strong positive correlation between *TNFAIP3* gene expression assessed by a microarray assay and RT-qPCR. **d** Reduced *TNFAIP3* mRNA in GC cell lines compared with non-neoplastic gastric mucosa cells, MNP01. **e** Absence of a significant difference in *TNFAIP3* mRNA between GC and the corresponding adjacent non-neoplastic tissue samples. **f** Schematic diagram of CpG dinucleotide density across the *TNFAIP3* locus and the location of the investigated CpG dinucleotides. **g** Significant differences in overall methylation and at specific CpG sites of *TNFAIP3* intronic region comparing GC and the corresponding adjacent non-neoplastic tissue samples. The colourful squares represent the correlation between *TNFAIP3* mRNA and methylation levels in all gastric tissues (tumour and control samples) and in tumour samples. **h** Significantly increased *TNFAIP3* mRNA in tumours of patients with poor cell differentiation. **i** Significantly increased *TNFAIP3* mRNA in tumours of patients with T3/T4 invasion. **j** Significantly increased *TNFAIP3* mRNA in tumours of patients with advanced stages. **k** Significantly increased *TNFAIP3* mRNA in tumours of patients with III/IV TNM stages. **l** Significantly increased *TNFAIP3* mRNA in tumours of patients with lymph node metastasis. Cell line data are expressed as the mean ± standard deviation; tissue sample data are expressed as the median ± interquartile range. *Significant difference between groups by *t* test, Wilcoxon test, or Mann-Whitney test. The arrow indicates the CpG binding site of the SMARCA4 transcription factor. TSS; transcription start site. SC; Spearman correlation coefficient

disease, and hepatic system disease; (c) cell death and survival, cell-to-cell signalling and interaction, and cell-mediated immune response; (d) cancer, carbohydrate metabolism, and the cell cycle (Fig. 1c).

For the selection of candidate DEGs to be evaluated in the next step of the study, we established the following criteria: (a) upregulated DEGs to ensure a direct effect of 5-AZAdC; (b) fold change> 1.5-fold; (c) association with the main IPA functional category; and (d) presence of the CpG island near the TSS. Based on these criteria, we selected *NRN1* and *TNFAIP3* to be validated in the same RNA samples assessed by the microarray assay and to be evaluated for gene expression and DNA methylation levels in gastric tissue samples.

NRN1 and *TNFAIP3* gene expression validation in gastric cancer cell lines

NRN1 and *TNFAIP3* were increased in 5-AZA-treated GC cell lines compared with non-treated GC cell lines by microarray assay ($P < 0.01$, Fig. 2a; and $P < 0.01$, Fig. 3a, respectively) and mRNA levels detected by RT-qPCR ($P = 0.04$, Fig. 2b; and $P = 0.04$, Fig. 3b). Validating the microarray results, *NRN1* and *TNFAIP3* gene expression levels assessed by these two methodologies were strongly correlated ($\rho = 0.7$, $P = 0.01$, Fig. 2c; and $\rho = 0.9$, $P < 0.01$, Fig. 3c, respectively).

NRN1 and *TNFAIP3* gene expression in gastric cancer

The expression of *NRN1* was increased and *TNFAIP3* was reduced in GC cell lines in relation to non-neoplastic gastric mucosa cells MNP01 ($P = 0.06$, $\eta_p^2 = 0.43$, Fig. 2d; and $P = 0.01$, Fig. 3d).

As expected, the expression of these two genes was widely heterogeneous among GC tissue samples compared to the controls, with 58% of the tumours (25/43) showing reduced *NRN1* levels (Fig. 2e) and 50% (26/52) showing increased *TNFAIP3* levels (Fig. 3e).

NRN1 and *TNFAIP3* methylation in gastric cancer

To better characterize the levels of DNA methylation and to understand its impact on gene expression regulation, the analysis of *NRN1* and *TNFAIP3* CpG island methylation was performed at CpG sites individually, as well as by grouping these CpG sites in functional gene regions (Figs. 2g and 3g).

Gastric tissue samples presented an *NRN1* promoter methylation median of approximately 5% (Fig. 2g). Even so, GC tissue samples presented significantly increased methylation at the − 617 ($P = 0.03$), − 586 ($P = 0.05$, $r = -0.37$), − 528 ($P = 0.02$), and − 493 ($P = 0.03$) CpG sites and reduced methylation at the − 218 ($P = 0.07$, $r = -0.34$), and − 206 CpG sites ($P = 0.03$) compared with the controls (Fig. 2g).

Gastric tissue samples also presented a methylation median of the studied portion of the *TNFAIP3* CpG island of approximately 0.3% (Fig. 3g). Even so, GC tissue samples presented significantly reduced methylation of the *TNFAIP3* intron region ($P = 0.04$), as well as at the specific intron + 643 ($P = 0.03$) and + 741 ($P = 0.04$) CpG sites compared with controls (Fig. 3g).

Impact of *NRN1* and *TNFAIP3* gene expression and methylation on clinicopathological features

NRN1 gene expression was increased in tumours of patients with distant metastasis ($P = 0.05$, $r = -0.30$; Fig. 2h and Supplementary Table 3) and III/IV TNM stages ($P = 0.07$, $r = -0.30$; Fig. 2i and Supplementary Table 3).

NRN1 promoter methylation was reduced in tumours of patients at the III/IV TNM stage ($P = 0.03$; Fig. 2j and Supplementary Table 3). This reduced methylation was consistent throughout the 40 evaluated CpG sites of the promoter; 22 of them showed significantly reduced methylation levels ($0.05 < P \leq 0.07$ and $r > 0.11$; Fig. 2j). In addition, *NRN1* promoter methylation was reduced in tumours of patients positive for *H. pylori* infection ($P = 0.02$; Fig. 2k and Supplementary Table 3). This reduced methylation was consistent throughout the 40 evaluated CpG sites of the promoter; 26 of them showed significantly reduced methylation levels ($0.05 < P \leq 0.07$ and $r > 0.11$; Fig. 2k).

TNFAIP3 gene expression was increased in tumours of patients with poor cell differentiation ($P = 0.06$, $r = -0.26$; Fig. 3h and Supplementary Table 4), T3/T4 invasion ($P = 0.02$; Fig. 3i and Supplementary Table 4), advanced stages ($P < 0.01$; Fig. 3j and Supplementary Table 4), III/IV TNM stages ($P = 0.02$; Fig. 3k and Supplementary Table 4), and lymph node metastasis ($P < 0.01$; Fig. 3l and Supplementary Table 4). *TNFAIP3* methylation showed no relevant clinicopathological associations (Supplementary Table 4).

Role of *NRN1* and *TNFAIP3* methylation in gene expression regulation

In general, considering GC and control samples, *NRN1* expression and overall promoter methylation, as well as at specific CpG sites, were inversely correlated ($P < 0.05$ for each correlation; Fig. 2g and Supplementary Table 5). Some of these correlations were stronger in GC samples, even in sample size detriment ($P < 0.05$ for each correlation; Fig. 2g and Supplementary Table 5). In GC samples, the most significant inverse correlation was between *NRN1* expression and methylation at the promoter − 145 CpG site ($\rho = -0.68$, $P < 0.01$; Fig. 2g and Supplementary Table 5). Using the Open Regulatory Annotation database (OregAnno) [19], we identified this CpG site as a HIF-1 transcription factor binding site.

Similarly, *TNFAIP3* expression and methylation at specific CpG sites in the intronic region were significantly correlated considering GC and control samples ($P < 0.05$ for each correlation; Fig. 3g and Supplementary Table 6). Some of these correlations were strongly inversed in GC samples, even in sample size detriment ($P < 0.05$ for each correlation; Fig. 3g and Supplementary Table 6). In GC samples, the most significant inverse correlation was between *TNFAIP3* expression and methylation at the intronic + 360 CpG site ($\rho = -0.63$, $P < 0.01$; Fig. 3g and Supplementary Table 6). Using OregAnno, we identified this CpG site as a SMARCA4 transcription factor binding site.

Discussion

In the present study, we initially focused on the screening of genes modulated by DNA methylation using GC cell lines. For this, we used cell line models established directly from primary GC [7] and treated them with a demethylation agent. This approach allowed us to identify 83 genes possibly regulated by DNA methylation. We then selected the *NRN1* and *TNFAIP3* genes to be further investigated in a panel of GC and matched controls to better elucidate the effect of DNA methylation in a heterogeneous population of tumours.

NRN1 has been associated with the development of tumours [20] through its role in hypoxia, angiogenesis, apoptosis, and proliferation [21]. However, very little is known about the function of *NRN1* in GC. Yuan and collaborators [22] previously described increased NRN1 mRNA and protein levels in GC in relation to adjacent non-neoplastic samples. Although we observed that *NRN1* is most highly expressed in GC cell lines in comparison to a cell culture of non-neoplastic gastric mucosa cells, our study revealed a very heterogeneous pattern of *NRN1* gene expression in tumour samples in relation to controls. Further interrogating our population, we observed that tumours of patients with distant metastasis and III/IV TNM stages had the highest expression of *NRN1*. Therefore, our results suggest that the upregulation of *NRN1* may play an important role in advanced GC.

To provide more evidence that *NRN1* is regulated by epigenetic factors, we evaluated multiple CpG sites by next-generation sequencing. The *NRN1* promoter was overall hypomethylated in gastric tissue samples; however, as expected, the DNA methylation status varied depending on the position of the CpG site. Most notably, four CpG sites more distant from TSS (approximately 550 bp upstream from TSS) were significantly less hypomethylated and two other CpG sites near TSS (approximately 200 bp upstream from TSS) were significantly more hypomethylated in GC samples in relation to controls. In addition, we identified an inverse correlation between *NRN1* mRNA and overall promoter methylation levels and the 145 CpG site upstream of the TSS, which is a HIF-1 transcription factor binding site. HIF-1 constitutes an important mediator of cellular adaptation to hypoxia. HIF-1 transcriptionally upregulates several genes that play pivotal roles in the central features of cancer pathogenesis, such as angiogenesis, invasion, metastasis, and anti-apoptosis effects [23]. Supporting the relationship of *NRN1* with poor GC prognosis, we also observed *NRN1* promoter hypomethylation in tumours of patients at III/IV TNM stages. Taking together gene expression and promoter methylation results, as well as the inverse correlation between these parameters, our study shows for the first time that the loss of DNA methylation in the *NRN1* promoter region may be a mechanism of gene activation in III/IV TNM stage GC.

It is known that *H. pylori* induces chronic inflammation that may lead to gene promoter hypermethylation [24] and gene type-specific methylation profiles involved in the multistep process of carcinogenesis [25].

Although the increased *NRN1* mRNA levels in *H. pylori* positive tumours were not statistically significant in our results, the *NRN1* promoter hypomethylation observed in these samples may be one of the mechanisms associated to increased *NRN1* mRNA levels, allowing *H. pylori* to escape immune response. This hypothesis can be supported by a previous study that described the role of NRN1 in recruiting certain immune suppressive cells to escape immune surveillance [26].

Both TNFAIP3 overexpression [26–32] and downregulation [33, 34] were described in several tumour types. Our study reinforces this very heterogeneous pattern of *TNFAIP3*

expression in GC, probably related to its multiple roles in cancer development acting in apoptosis [35–40] and cell proliferation [36, 37, 41–43] pathways. Most notably, we observed that increased *TNFAIP3* mRNA levels were associated with poor clinical patient outcomes, supporting a previous study in GC [43]. However, it is worth highlighting that CG cell lines showed lower expression of *TNFAIP3* than normal cells. TNFAIP3 is a ubiquitin-editing enzyme originally identified as a nuclear factor-κB (NF-κβ) protein inhibitor, protecting cells from TNF-induced cytotoxicity [35] and preventing excessive inflammation via its deubiquitinase activity [36–38], but it also acts in TNF-independent inflammatory signalling [36] and acts outside the immune system [38]. This paradoxical role of TNFAIP3 may explain its heterogeneity in GC samples as well the discrepancy in relation to GC cell lines, because its expression must be modulated by the tumour microenvironment. The microenvironment along with external stimuli likely contributes to gene expression regulation, as well as changes in epigenetic markers [44].

Different mechanisms of *TNFAIP3* transcription regulation have been described [45], including its activation by two NF-κβ binding sites in the promoter region [46] and by regulators of cell-intrinsic energy homeostasis, such as oestrogen-related α (ERRα), linking energy homeostasis to cell activation [47]. However, no study has reported the methylation pattern of *TNFAIP3* in tumours, including GC. Thus, our study is the first to observe an inverse correlation between mRNA and specific CpG sites located in the studied *TNFAIP3* intronic region, indicating that this gene may also be regulated epigenetically. Despite overall *TNFAIP3* CpG island demethylation in gastric tissue samples, GC presented differences in DNA methylation in relation to controls, which depended on the genomic positions. Only the studied *TNFAIP3* intronic region presented a significantly marked demethylation in GC compared with controls. Interestingly, our data also demonstrated a significant inverse correlation between *TNFAIP3* mRNA CpG methylation at the intronic 360 bp downstream of TSS. This CpG site is a binding site of the SMARCA4 transcription factor, which is an ATPase subunit of the SWI/SNF chromatin remodelling complex. A study demonstrated that SMARCA4 is mostly overexpressed in many types of tumours [48] and has been associated with activated genes [49]. Therefore, we suggest that intronic CpG site demethylation may play a role in *TNFAIP3* gene expression activation in GC, underscoring the importance of evaluating epigenetic markers in non-promoter CpG sites.

Our study is the first to investigate the possible involvement of DNA demethylation in *NRN1* and *TNFAIP3* activation in GC; however, there are two limitations that could be addressed here. First, it is worth noting that the 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 reject the null hypothesis to prioritize the biological effect rather than reject the involvement of an epigenetic event in GC due to statistical rigour (type II error).

The second limitation concerns our sample size. Because we reported in this study *NRN1* and *TNFAIP3* gene expression heterogeneity (some GC tissues presented gene upregulation and others presented gene downregulation), it would be interesting compare DNA methylation between these subgroups of samples, as well as to associate DNA methylation with clinicopathological features within each of these two subgroups. To analyse DNA methylation in a subdivided tissue sample according gene expression, it would be necessary a larger sample size.

In summary, our study demonstrated that higher expression of *NRN1* and *TNFAIP3* was associated with advanced CG. Using the bisulfite DNA sequencing standard gold methodology for methylation analysis, we originally demonstrated that *NRN1* promoter hypomethylation may contribute to gene upregulation in GC, especially in advanced GC. In addition, *TNFAIP3* intronic-specific CpG site demethylation may contribute to gene upregulation in GC. The identification of demethylated activated genes in GC may be applicable in the future, stratifying patients who are less likely to benefit from 5-AZAdC-based therapies.

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Author contribution Conception and design: FW, MFL, RRB, and MACS. In vitro experiments: FW and DQ. Sample collection: FW, LCS, DQC, JCG, COG, ACA, ESC, PPA, LGL, and CHA. Pathological analysis: RA and SD. *H. pylori* diagnosis: LTR and SLMP. Molecular experiments: FW, LCS, DQC, JCG, COG, ACA, ESC, and MFL. Data analysis: FW, LCS, JCG, SP, and JK. Writing, review, and/or revision of the manuscript: all authors. Administrative, technical, or material support: MACS and RRB.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent The study was approved by medical ethics committee of each study center. Written informed consent was obtained from each patient.

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