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LETTER TO THE EDITOR

CDKN1A histone acetylation and gene expression relationship in gastric adenocarcinomas

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Abstract *CDKN1A* is a tumor suppressor gene involved in gastric carcinogenesis and is a potential target for histone deacetylase inhibitor-based therapies. Upregulation of *CDKN1A* is generally observed in several cell lines after histone deacetylase inhibitor treatment; however, little is known about the histone acetylation status associated with this gene in clinical samples, including gastric tumor tissue samples. Therefore, our goal was to quantify the H3K9 and H4K16 acetylation levels associated with three *CDKN1A* regions in 21 matched pairs of gastric adenocarcinoma and corresponding adjacent non-tumor samples by chromatin immunoprecipitation and to correlate these data with the gene expression. Our results demonstrated that the −402, −20, and +182 *CDKN1A* regions showed a significantly increased acetylation level in at least one of the histones evaluated ($p < 0.05$, for all comparisons), and these levels were positively correlated in gastric tumors. However, an inverse correlation was detected between both H3K9 and H4K16 acetylation at the −402 *CDKN1A* region and mRNA levels in gastric tumors ($r = -0.51$, $p = 0.02$; $r = -0.60$, $p < 0.01$, respectively). Furthermore, increased H4K16 acetylation at the −20 *CDKN1A* region was associated with gastric tumors of patients without lymph node metastasis ($p = 0.04$). These results highlight the complexity of these processes in gastric adenocarcinoma and contribute to a better understanding of *CDKN1A* regulation in carcinogenesis.

Keywords *CDKN1A* · Histone acetylation · Gene expression regulation · Gastric cancer

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Dear Editor,

CDKN1A is a potent inhibitor of cell proliferation and DNA replication through p53-dependent and p53-independent mechanisms. This protein plays additional roles, including regulation of transcription, apoptosis, DNA repair, and cell motility [1, 2]. CDKN1A has dual behavior, acting as a tumor suppressor or as an oncogene, depending on the cell type and on the cellular localization [2]. Our research group previously demonstrated that CDKN1A protein and mRNA expression were reduced in gastric adenocarcinoma (GAC) tissue samples [3, 4], which suggests that CDKN1A acts as a tumor suppressor in this neoplasia.

Histone acetylation is the result of the balance of two specific enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). The levels of histone acetylation play a crucial role in chromatin remodeling and in the regulation of gene transcription [4]. The presence of

Table 1 Oligonucleotide sequences for the ChIP-qPCR analysis of *CDKN1A*

Amplicon[a]	Forward primer	Reverse primer
−402	CCTGATCTTTTCAGCTGCATTG	GCCCCCTTTCTGGCTCA
−20	TATATCAGGGCCGCGCTG	GGCTCCACAAGGAACTGACTTC
+182	CGTGTTCGCGGGTGTGT	CATTCACCTGCCGCAGAAA

[a] The amplicon name indicates the position of the central base pair of the amplicon relative to the transcription start site of *CDKN1A* (base pairs) [10, 11]

acetylated lysine residues on the amino-terminal tails of histones is generally associated with gene transcription activation [5]. Recently, our research group showed increased *CDKN1A* expression associated with the reduction in GAC cell lines proliferation after trichostatin A (TSA) treatment [4]. TSA is a HDAC inhibitor (HDACi) that increases *CDKN1A* expression by acetylating histones at the promoter regions in a p53-independent manner [6]. Thus, in addition to its tumor suppressor role in GAC, our results demonstrated that *CDKN1A* is a potential target for anticancer therapies.

Upregulation of *CDKN1A* is observed in several cell lines after HDACi treatment, but little is known about the histone acetylation status associated with this gene in clinical samples, including GAC tissue samples. Therefore, our goal was to quantify the histone acetylation level associated with *CDKN1A* regions in matched pairs of GAC and corresponding adjacent non-tumor samples by chromatin immunoprecipitation (ChIP) and to correlate these data with gene expression. Possible associations of *CDKN1A* gene expression and histone acetylation levels with clinicopathological features were also evaluated.

Matched pairs of GAC and corresponding adjacent non-tumor samples were obtained from 21 patients 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 before surgery, and there was no co-occurrence of diagnosed cancers. Written informed consent with approval of the ethics committees of HUJBB and HSP was obtained from all patients prior to sample collection.

CDKN1A mRNA expression was evaluated as previously described [4] and was analyzed using the ΔCt method, in which the target mRNA level was normalized to the geometric mean of the reference gene expression. We used *GAPDH* and *B2M* as suitable reference genes, as previously described by our research group [7].

ChIP assays were performed by adapting the methodology previously described [8] to gastric samples. Briefly, each sample was lysed, fragmented, and immunoprecipitated using antibodies against acetyl-H3K9 and acetyl-H4K16 (Millipore, Temecula, CA, USA). Negative controls were obtained using a non-antibody pull-down. Next, input and ChIP-enriched DNA fractions were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Three regions from transcription start site (TSS) of *CDKN1A* were selected for ChIP analysis: The −402 bp is a promoter region that has been identified as one of the motifs for regulatory factors [9]; the −20 bp is an enhancer element [10]; and the +182 bp is a coding region associated with transcriptional elongation after promoter escape by RNA polymerase II [10]. These regions are important for *CDKN1A* transcription and have been evaluated in previous studies [10, 11]. The oligonucleotide sequences for amplification of selected *CDKN1A* regions are described in Table 1. Input and ChIP-enriched DNA fractions were amplified in duplicate reactions using the Applied Biosystems 7500 fast real-time qPCR system (Foster City, CA, USA) and the SYBR Green reagent (Qiagen, Hilden, Germany). The ChIP-qPCR data were analyzed in the same way as the mRNA data, except that the ChIP levels were normalized to the corresponding input DNA level.

For statistical analysis, we first evaluated the distribution of data using the Shapiro–Wilk test. Analyses of the mRNA and histone acetylation levels between matched pairs of GAC and adjacent non-tumor samples were performed by the repeated measures general linear model (GLM). Possible associations of the mRNA and histone acetylation levels with clinicopathological features were evaluated by GLM. The effect size was based on partial eta squared (η_p^2), in which 0.15 and below was determined as a small effect size, 0.16–0.40 as a medium effect size, and above 0.40 as a large effect size. Analyses of not normally distributed data were performed by Wilcoxon's test, and the effect size was based on Cohen's *r*, in which 0.10 and below was determined as a small effect size, 0.11–0.49 as a medium effect size, and above 0.50 as a large effect size. Pearson or Spearman tests were used to correlate the gene expression and histone acetylation levels in tumor samples, in which a value below 0.40 was determined as a weak correlation, 0.40–0.59 as a moderate correlation, 0.6–0.79 as a strong correlation, and ≥0.80 as a very strong correlation. In all analyses, differences were considered significant at $p < 0.05$.

A significantly reduced *CDKN1A* expression in GAC compared with corresponding adjacent non-tumor samples was confirmed in the studied tissue samples (Fig. 1a). We

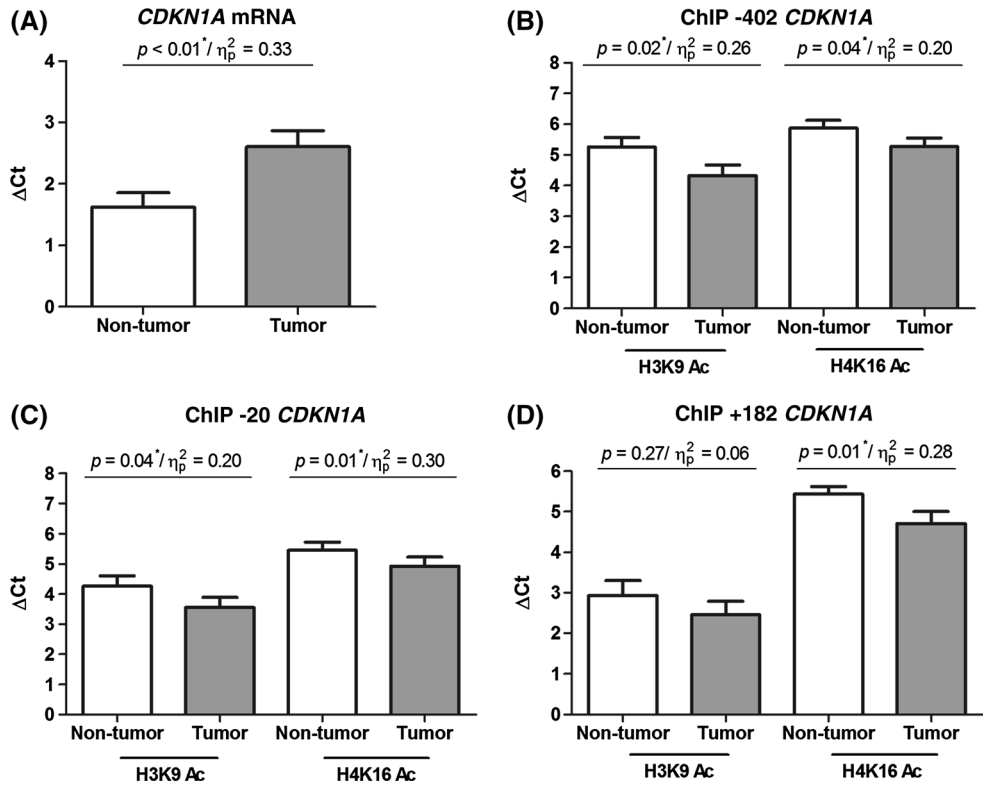

Fig. 1 *CDKN1A* mRNA and histone acetylation levels in matched pairs of gastric adenocarcinoma and corresponding adjacent non-tumor samples. **a** *CDKN1A* mRNA, **b** histone acetylation at the −402 *CDKN1A* region, **c** histone acetylation at the −20 *CDKN1A* region, **d** histone acetylation at the +182 *CDKN1A* region. Data are expressed as the mean ± standard deviation. *Significant difference between groups by repeated measures GLM test, $p < 0.05$; η_p^2: effect size based on partial eta squared; *Ac* acetylated; gene expression and histone acetylation levels are inversely proportional to the ΔCt values

did not detect significant associations between the mRNA level and clinicopathological features (Table 2).

ChIP-qPCR data revealed that H3K9 and H4K16 acetylation levels at the −402 and −20 *CDKN1A* regions were significantly increased in GAC compared with corresponding adjacent non-tumor samples (Fig. 1b, c, respectively). Significantly increased H3K9 acetylation at the +182 *CDKN1A* region was also observed in GAC compared with corresponding adjacent non-tumor samples; however, no statistical difference in H4K16 acetylation was detected at this site (Fig. 1d). Additionally, the H3K9 and H4K16 acetylation levels associated with the three *CDKN1A* regions studied here were positively correlated (Table 3). Conversely, an inverse correlation was detected between both H3K9 and H4K16 acetylation at the −402 region and *CDKN1A* mRNA levels (Table 3).

In contrast to our results, a previous study described histone hypoacetylation in *CDKN1A* associated with its reduced mRNA expression in gastric carcinoma [12]. Some methodology aspects may have influenced these contradictory results. In contrast to our study, the authors evaluated the total H3 and H4 acetylation levels independently of specific lysines, as well as different *CDKN1A* regions.

Although histone acetylation is associated with a more relaxed chromatin state, allowing accessibility of transcriptional regulatory proteins to chromatin templates, studies have demonstrated that this correlation is not exclusive [13–15]. In addition, there is little information about the cause-and-effect relationship between histone acetylation and transcriptional activity or about the underlying molecular mechanisms [5]. To explain the results observed in our study, three possible non-exclusive hypotheses are raised. Firstly, the levels of acetylation are not strictly static, and acetyl groups may be added or removed in a dynamic way [16]. The dynamic site-specific acetylation of nucleosomal histones is a key mechanism to the switch between a permissive and repressive chromatin structure, and the targeting of enzymes that modulate chromatin histone acetylation status is a key process of gene regulation [17]. The level of acetylation must always be balanced to obtain appropriate gene expression. HATs and HDACs are required by the promoters of active genes to facilitate transcription [16]. In neoplastic cells, there is a weakening of the balance between acetylation and deacetylation, which affects the regulation of gene expression [18]. We have recently reported a deregulation

Table 2 Associations between clinicopathological features, *CDKN1A* mRNA, and histone acetylation levels

Variable	*N* (%)	mRNA		−402 H3K9 Ac		−402 H4K16 Ac		−20 H3K9 Ac		−20 H4K16 Ac		+182 H3K9 Ac		+182 H4K16 Ac	
		ΔCt (mean ± SD)	*p*	ΔCt (mean ± SD)	*p*	ΔCt (mean ± SD)	*p*	ΔCt (mean ± SD)	*p*	ΔCt (mean ± SD)	*p*	ΔCt (mean ± SD)	*p*	ΔCt (mean ± SD)	*p*
Sex															
Male	11 (52)	2.44 ± 1.30	0.63	4.85 ± 1.39	0.12	5.89 ± 1.15	0.02*	4.03 ± 1.39	0.13	5.52 ± 1.61	0.04*	2.72 ± 1.43	0.41	5.31 ± 0.93	0.03*
Female	10 (48)	2.71 ± 1.15		3.76 ± 1.66		4.60 ± 1.10		3.04 ± 1.52		4.27 ± 0.92		2.17 ± 1.62		4.05 ± 1.46	
Onset (years)															
≤45	5 (24)	2.46 ± 1.42	0.78	4.21 ± 0.81	0.98	5.50 ± 1.04	0.38	3.82 ± 1.12	0.50	5.40 ± 1.66	0.23	2.84 ± 1.15	0.88	4.50 ± 2.04	0.91
>45	16 (76)	2.60 ± 1.19		4.37 ± 1.79		5.20 ± 1.37		3.47 ± 1.63		4.78 ± 1.40		2.45 ± 1.64		4.78 ± 1.13	
Tumor location															
Cardia	1 (5)	4.21 ± 0.00	0.14	3.89 ± 0.00	0.53	4.46 ± 0.00	0.20	4.01 ± 0.00	0.99	4.5 ± 0.00	0.45	2.68 ± 0.00	0.98	4.07 ± 0.00	0.21
Non-cardia	20 (95)	2.53 ± 1.19		4.27 ± 1.64		5.36 ± 1.32		3.61 ± 1.54		5.00 ± 1.50		2.54 ± 1.53		4.93 ± 1.11	
Histopathological type[a]															
Intestinal type	15 (71)	2.72 ± 1.26	0.31	4.21 ± 1.45	0.34	5.30 ± 1.37	0.66	3.51 ± 1.41	0.56	4.98 ± 1.54	0.85	2.43 ± 1.37	0.74	4.88 ± 1.09	0.65
Diffuse type	6 (29)	2.19 ± 1.08		4.63 ± 2.02		5.20 ± 1.13		3.66 ± 1.86		4.78 ± 1.28		2.53 ± 1.97		4.28 ± 1.90	
Stage															
Early	2 (10)	3.44 ± 1.61	0.36	4.70 ± 2.80	0.09	4.67 ± 1.87	0.92	3.47 ± 2.36	0.65	4.55 ± 1.32	0.75	2.58 ± 2.69	0.68	4.33 ± 1.53	0.73
Advanced	19 (90)	2.48 ± 1.18		4.29 ± 1.53		5.34 ± 1.26		3.57 ± 1.49		4.96 ± 1.48		2.45 ± 1.46		4.75 ± 1.36	
Tumor invasion															
T1/T2	5 (24)	2.45 ± 1.37	0.66	4.89 ± 1.56	0.10	5.19 ± 1.59	0.43	3.99 ± 1.43	0.16	5.00 ± 1.17	0.33	3.26 ± 1.71	0.08	4.86 ± 1.22	0.21
T3/T4	16 (76)	2.61 ± 1.20		4.16 ± 1.61		5.30 ± 1.23		3.42 ± 1.55		4.90 ± 1.55		2.21 ± 1.41		4.66 ± 1.41	
TNM stage[b]															
I/II	12 (57)	2.76 ± 1.30	0.43	4.21 ± 1.69	0.74	4.93 ± 1.37	0.17	3.36 ± 1.53	0.54	4.52 ± 1.35	0.14	2.51 ± 1.59	0.84	4.55 ± 1.05	0.59
III/IV	9 (43)	2.31 ± 1.10		4.50 ± 1.53		5.73 ± 1.05		3.82 ± 1.52		5.46 ± 1.47		2.39 ± 1.49		4.92 ± 1.70	
Lymph node metastasis															
Absent	8 (38)	3.03 ± 1.13	0.21	3.85 ± 1.90	0.46	4.56 ± 1.02	0.09	2.90 ± 1.65	0.21	4.02 ± 1.12	0.04*	2.18 ± 1.73	0.65	4.25 ± 0.86	0.42
Present	13 (62)	2.28 ± 1.21		4.63 ± 1.36		5.71 ± 1.26		3.96 ± 1.32		5.48 ± 1.37		2.63 ± 1.40		4.99 ± 1.52	
Distant metastasis															
Unknown/absent	19 (90)	2.59 ± 1.24	0.79	4.25 ± 1.64	0.43	5.19 ± 1.32	0.29	3.48 ± 1.56	0.43	4.87 ± 1.51	0.56	2.33 ± 1.52	0.22	4.61 ± 1.37	0.22
Present	2 (10)	2.35 ± 1.27		5.15 ± 0.87		6.07 ± 0.04		4.33 ± 0.65		5.44 ± 0.007		3.72 ± 0.74		5.70 ± 0.06	
H. pylori infection[c]															
Absent	6 (29)	2.48 ± 1.25	0.74	4.13 ± 1.44	0.99	5.12 ± 1.30	0.82	3.48 ± 1.22	0.83	4.88 ± 0.94	0.67	2.35 ± 1.50	0.99	4.88 ± 0.84	0.32
Present	15 (71)	2.60 ± 1.23		4.41 ± 1.69		5.34 ± 1.31		3.59 ± 1.64		4.94 ± 1.63		2.50 ± 1.57		4.64 ± 1.52	

All *p* values were adjusted for sex

Gene expression and histone acetylation levels are inversely proportional to the ΔCt values

N number of samples, *SD* standard deviation, *Ac* acetylated

* Significant difference between groups by GLM test, $p < 0.05$

[a] According to the Lauren classification [43]

[b] According to AJCC [44]

[c] According to Wisnieski et al. [4]

Table 3 Correlation between *CDKN1A* mRNA, *p53* mRNA, and histone acetylation levels

	CDKN1A mRNA	*p53* mRNA	−402 H3K9 Ac	−402 H4K16 Ac	−20 H3K9 Ac	−20 H4K16 Ac	+182 H3K9 Ac	+182 H4K16 Ac
CDKN1A mRNA								
r	1	0.50*	−0.51*	−0.60*	−0.37	−0.33	−0.35	−0.31
p	–	0.03	0.02	<0.01	0.10	0.15	0.12	0.18
p53 mRNA								
r	0.50*	1	−0.38	−0.39	−0.28	−0.20	−0.24	−0.33
p	0.03	–	0.11	0.10	0.24	0.42	0.32	0.17
−402 H3K9 Ac								
r	−0.51*	−0.38	1	0.81*	0.91*	0.62*	0.87*	0.73*
p	0.02	0.11	–	<0.01	<0.01	<0.01	<0.01	<0.01
−402 H4K16 Ac								
r	−0.60*	−0.39	0.81*	1	0.77*	0.83*	0.65*	0.84*
p	<0.01	0.10	<0.01	–	<0.01	<0.01	<0.01	<0.01
−20 H3K9 Ac								
r	−0.37	−0.28	0.91*	0.77*	1	0.79*	0.90*	0.74*
p	0.10	0.24	<0.01	<0.01	–	<0.01	<0.01	<0.01
−20 H4K16 Ac								
r	−0.33	−0.20	0.62*	0.83*	0.79*	1	0.55*	0.76*
p	0.15	0.42	<0.01	<0.01	<0.01	–	<0.01	<0.01
+182 H3K9 Ac								
r	−0.35	−0.24	0.87*	0.65*	0.90*	0.55*	1	0.68*
p	0.12	0.32	<0.01	<0.01	<0.01	<0.01	–	<0.01
+182 H4K16 Ac								
r	−0.31	−0.33	0.73*	0.84*	0.74*	0.76*	0.68*	1
p	0.18	0.17	<0.01	<0.01	<0.01	<0.01	<0.01	–

r = Pearson's correlation coefficient; * $p < 0.05$; *Ac* acetylated

of HATs and HDACs genes in GAC samples [4]. Therefore, we hypothesize that this deregulation may contribute to the *CDKN1A* hyperacetylation observed in our study.

Secondly, changes in the promoter structure that accompany transcriptional activation are not a direct result of acetylation. These changes occur due to the synergistic actions of several factors, including other covalent modifications and the rearrangement of histones/nucleosomes relative to the DNA by nucleosome remodeling factors [17]. We hypothesize that H3K9 and H4K16 acetylation associated with the studied *CDKN1A* regions is not sufficient to establish the full chromatin accessibility in our samples. *CDKN1A* is one of the better-characterized transcriptional targets of p53, which is upregulated, often in a p53-dependent manner, in response to many physiologic stresses and results in a transient or sustained cell cycle arrest [19]. Our research group previously reported p53 mRNA and protein downregulation and gene deletion in GAC tissue samples [20, 21], in some preneoplastic gastric lesions [22] and in GAC cell lines [23] from the Brazilian population. We also reported p53 mRNA and protein downregulation in a non-human primate model of gastric carcinogenesis [24]. Moreover, we observed that the reduced *CDKN1A* and *p53* mRNA levels were positively correlated in our GAC samples (Table 3). This result demonstrated that *CDKN1A* mRNA may be regulated in a p53-dependent manner in these samples. Because histone acetylation at the promoter region is also required for p53-dependent *CDKN1A* transcriptional activation [19], other histone residues and/or promoter sites that were not evaluated in this study may be involved in *CDKN1A* transcription regulation in our samples.

Finally, other downstream molecular mechanisms may hinder the potential activation of histone acetylation. The transcriptional repression of *CDKN1A* by MYC plays a role in the development of tumors in which MYC is overexpressed [25]. Our research group previously reported that MYC mRNA and protein overexpression and its gene amplification is a common finding in GAC samples [21, 26–35], GAC cell lines [23, 36], and some preneoplastic gastric lesions [22] from the Brazilian population, as well as in a non-human primate model of gastric carcinogenesis [37]. We suggest that MYC may inhibit the *CDKN1A* mRNA level, independently of the histone acetylation status of our GAC samples. Moreover, we previously demonstrated that TSA treatment in GAC cell lines leads to *CDKN1A* upregulation concomitantly with *MYC* downregulation [4]. A previous study in HeLa cells demonstrated that TSA induced MYC downregulation and its release from the *CDKN1A* promoter, contributing to *CDKN1A* transcriptional activation [38]. Gene expression alterations may also result from TSA indirect effects, which may explain the identification of up- and downregulated genes after cell line treatment [39].

In addition to transcriptional control of *CDKN1A*, posttranslational modifications are equally important for cellular protein levels. It has been described that CDKN1A ubiquitination promotes its proteolysis through the proteasome at specific stages in an unperturbed cell cycle. Several proteins involved in this process are upregulated in a variety of human tumors, suggesting that CDKN1A downregulation may account for some of the oncogenic properties of these proteins [25]. Moreover, the phosphorylation was described to regulate CDKN1A stability and localization in the cell [25, 40]. This posttranslational modification may also leads to CDKN1A ubiquitination and subsequent proteolysis, promoting cellular progression during S phase of the cell cycle [25, 41]. Therefore, these events may also contribute to significant variations in CDKN1A levels, with consequent relevant influence on tumor behavior.

Our results also demonstrated that the level of H4K16 acetylation at the −20 *CDKN1A* region was significantly increased in the GAC samples of patients without lymph node metastasis compared to GAC samples of patients with lymph node metastasis (Table 2). By comparing GAC patients without lymph node metastasis and corresponding adjacent non-tumor samples, we observed significant *CDKN1A* downregulation and increased H4K16 acetylation at −20 *CDKN1A* region in this GAC sample subset (Fig. 2a, b, respectively). We also observed a tendency to significant inverse correlation between H4K16 acetylation at the −20 region and the *CDKN1A* mRNA levels in GAC patients without lymph node metastasis ($r = -0.70$, $p = 0.06$). Conversely, no significant differences were observed in *CDKN1A* mRNA and H4K16 acetylation at the −20 region when comparing GAC samples of patients with lymph node metastasis and corresponding adjacent non-tumor samples (Fig. 2c, d, respectively). Furthermore, no significant correlation was detected between H4K16 acetylation at the −20 region and the mRNA levels in GAC of patients with lymph node metastasis.

Several regulatory factors are described to mediate their effects on *CDKN1A* expression via the proximal region of the promoter (−210 to +1 bp relative to the TSS) [9]. In addition, the GC-rich region in the *CDKN1A* promoter, located upstream of the TATA box, acts as an important regulatory element responsive to various agents, including an HDACi [42]. We suggest that the histone hyperacetylation observed at the −20 *CDKN1A* region in lymph node-negative GAC may be an attempt to increase gene expression, although other molecular pathways may be involved in *CDKN1A* downregulation in our samples. In conclusion, our study describes increased H3K9 and H4K16 acetylation levels at specific *CDKN1A* regions in GAC tissue samples, despite *CDKN1A* downregulation. The levels of acetylated H3K9 and H4K16 at the −402

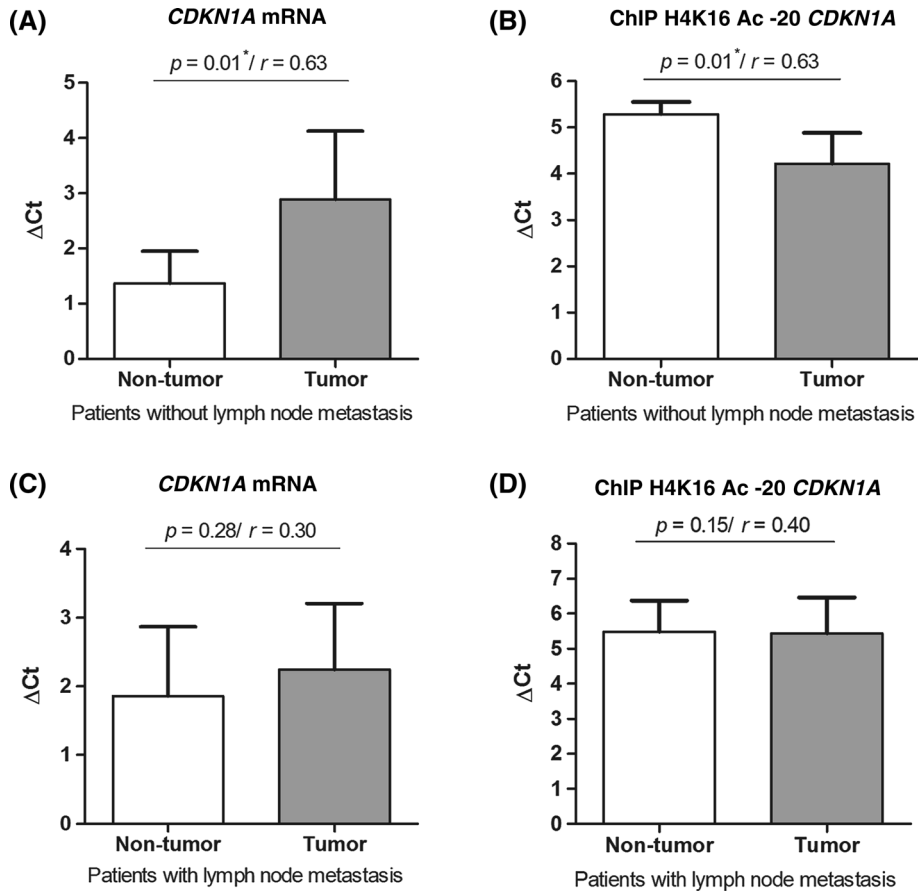

Fig. 2 *CDKN1A* mRNA and H4K16 acetylation levels in matched pairs of gastric adenocarcinoma and corresponding adjacent non-tumor samples. **a** *CDKN1A* mRNA and **b** H4K16 acetylation at the −20 *CDKN1A* region in the gastric samples of patients without lymph node metastasis, and **c** *CDKN1A* mRNA and **d** H4K16 acetylation at the −20 *CDKN1A* region in gastric samples of patients with lymph node metastasis. Data are expressed as the median ± interquartile range. *Significant difference between groups by Wilcoxon's test, $p < 0.05$; *r*: effect size based on Cohen; *Ac* acetylated; gene expression and histone acetylation levels are inversely proportional to the ΔCt values

CDKN1A region are inversely correlated with mRNA in this neoplasia. In addition, the increased level of acetylated H4K16 at the −20 *CDKN1A* region is associated with lymph node-negative GAC. Although our sample size does not reflect all heterogeneity of GAC, our results highlight the complexity of *CDKN1A* regulation in this neoplasia. Further studies are necessary to understand how different pathways coordinately regulate CDKN1A gene and protein expression in carcinogenesis. This knowledge could provide new insights into how *CDKN1A* for potential therapeutic applications can be explored.

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Compliance with ethical standards

Conflict of interest None.

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