MYC Deregulation in Gastric Cancer and Its Clinicopathological Implications

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Abstract

Our study investigated the relationship between *MYC* alterations and clinicopathological features in gastric cancers. We evaluated the effect of *MYC* mRNA expression and its protein immunoreactivity, as well as copy number variation, promoter DNA methylation, and point mutations, in 125 gastric adenocarcinoma and 67 paried non-neoplastic tissues. We observed that 77% of the tumors presented MYC immunoreactivity which was significantly associated with increased mRNA expression ($p < 0.05$). These observations were associated with deeper tumor extension and the presence of metastasis ($p < 0.05$). MYC protein expression was also more frequently observed in intestinal-type than in diffuse-type tumors ($p < 0.001$). Additionally, *MYC* mRNA and protein expression were significantly associated with its copy number ($p < 0.05$). The gain of *MYC* copies was associated with late-onset, intestinal-type, advanced tumor stage, and the presence of distant metastasis ($p < 0.05$). A hypomethylated *MYC* promoter was detected in 86.4% of tumor samples. *MYC* hypomethylation was associated with diffuse-type, advanced tumor stage, deeper tumor extension, and the presence of lymph node metastasis ($p < 0.05$). Moreover, eighteen tumor samples presented at least one known mutation. The presence of *MYC* mutations was associated with diffuse-type tumor ($p < 0.001$). Our results showed that *MYC* deregulation was mainly associated with poor prognostic features and also reinforced the presence of different pathways involved in intestinal-type and diffuse-type gastric carcinogenesis. Thus, our findings suggest that *MYC* may be a useful marker for clinical stratification and prognosis.

Introduction

Gastric cancer is the fourth most frequent type of cancer and remains the second leading cause of cancer-related death worldwide [1]. This cancer is usually diagnosed at advanced stages and the single curative therapy available requires surgical resection [2]. Thus, gastric cancer is a serious public health problem in the world. An improved understanding of the biology of this neoplasm is critical and may be useful to guide patient management, as well as to develop new therapeutic options.

MYC is one of the most studied oncogenes stemming from its association with a large number of diseases [3]. MYC plays a role in several fundamental functions of cell biology, including the regulation of cell growth and proliferation, metabolism, differentiation, apoptosis, and angiogenesis (for review see [4,5]). Hence, MYC is an integrator of extracellular and intracellular signals, and its cellular phenotype is dependent on tissue location [6,7]. Not surprisingly, deregulation of MYC functions contributes to the tumor phenotype.

MYC deregulation due to gene amplification [8,9], chromosomal translocation or insertion [10,11], mutations [12], and epigenetic modifications [13,14], has been reported in different types of cancers, especially in gastric cancer. MYC expression is often elevated or deregulated in human neoplasms [4], and seems to be at the crossroad of several important pathways and processes involved in carcinogenesis [15], being a key event in gastric carcinogenesis [9]. Previously, our group demonstrated that *MYC* mRNA expression and copy number increases during the sequential steps of intestinal-type gastric carcinogenesis in a non-human primate model [16], suggesting that *MYC* may be involved in gastric tumor initiation and progression.

The understanding of *MYC* biology is of paramount importance to elucidate its role in the pathogenesis of gastric cancer. Up to date, there is no study correlating *MYC* mutation, amplification, protein/mRNA levels, and methylation in this neoplasia. Here, we evaluated the relationship between *MYC* alterations and clinicopathological features in gastric cancer. In addition, *MYC* mRNA expression and protein immunoreactivity, as well as several

molecular mechanisms previously related to its deregulation as copy number variation (CNV), mutation, and DNA methylation, were analyzed in the same set of gastric cancer samples.

Materials and Methods

Ethics Statement

All samples were derived with written informed consent and approval from the University Hospital (Belém, Pará, Brazil) ethical review boards (protocol number: 142004).

Clinical Samples

125 gastric adenocarcinoma and 67 corresponding non-neoplastic gastric tissues (control samples) were obtained surgically from patients of the João de Barros Barreto University Hospital in Pará State, Brazil. All subjects were not exposed to either chemotherapy or radiotherapy before surgery. Gastric tumors were classified according to Lauren [17] and tumors were staged using standard criteria by TNM staging [18]. The clinicopathological features are shown in table 1 and 2.

Dissected tumor and control specimens were quickly frozen in liquid nitrogen until nucleic acid purification. Another part of the same tissues was formalin-fixed and paraffin embedded. For the fluorescent *in situ* hybridization (FISH) assay, the remaining tumor sample was disaggregated as previously described [19].

MYC immunoreactivity

Immunohistochemical analyses for MYC protein were performed on 125 formalin-fixed, paraffin-embedded tumor sections. Immunohistochemical staining was performed according to Calcagno *et al.* [10]. Tumor tissue sections (3 or 4 mm-thick) were deparaffinized in xylene and rehydrated in a graded series of ethanol. After heat-induced epitope retrieval, the tissue sections were incubated with primary mouse monoclonal antibody against MYC (dilution 1:50; sc-40, Santa Cruz Biotechnology, USA and Zymed®, USA). A universal peroxidase-conjugated secondary antibody kit (LSAB System, DakoCytomation, USA) was used for the detection system. We used 3,30-diamino-benzidine/H_2O_2 (Dakocytomation, Denmark) as the chromogen and hematoxylin as the counterstain. Any nuclear stain with or without cytoplasmic staining was considered to be a positive result, irrespective of intensity. A MYC-positive case was defined as one having 10% or more tumor cells positive for this protein.

Nucleic acid extraction

The genomic DNA (gDNA) was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Total RNA was extracted with Tri-reagent® (Life Technologies, USA) according to the manufacturer's protocol. DNA and RNA concentration and quality were determined using the NanoDrop spectrophotometer (Kisker, Germany). RNA integrity was determined by gel electrophoresis (1% agarose gels). All samples were stored at −80°C until use.

MYC mRNA expression

To quantitate mRNA levels of *MYC*, total RNA was isolated from 49 paired normal and tumor tissues using Trizol (Life Technologies, USA). The RNA was reverse transcribed using the High-Capacity cDNA Archive kit according to the manufacturer's protocol (Life Technologies, USA). Complementary DNA was then amplified by real-time PCR using the TaqMan probes purchased as Assays-on-demand Products for Gene Expression (Life Technologies, USA) on a 7500 Fast Real Time PCR (Life Technologies, USA). *GAPDH* gene was selected as an internal

control for RNA input and reverse transcription efficiency. All real-time reverse transcription quantitative PCR (RT-qPCR) were performed in triplicate for both target gene (*MYC*: Hs00153408_m1) and internal control (*GAPDH*: NM_002046.3).

Relative quantification (RQ) of the gene expression was calculated according to Livak and Schmittgen [20]. The corresponding control sample was designated as a calibrator from each tumor.

MYC copy number

FISH and qPCR were used to evaluate *MYC* copy number in a subset of 49 tumors, the same used in the study of the expression. FISH was performed according to the protocol of Pinkel *et al.* [21] with modifications introduced by Calcagno *et al.* [22]. Cells were hybridized with *Spectrum Orange* Probe (LSI Vysis/Abbott, Inc., IL) for the *MYC* gene region (8q24.12–q24.13) and nuclei were counterstained with 4′,6-diamidino-2-phenylindole antifade. Fluorescence was detected using an Olympus BX41 fluorescence microscope (Olympus, Japan) with excitation filters for 4′,6-diamidino-2-phenylindole (260 nm) and rhodamine (570 mn). For each case, 200 interphase nuclei were analyzed using an ASI image analysis system (Applied Spectral Imaging, Israel). Positive *MYC* gene signals appeared as red spots in nuclei and were scored using the criteria of Hopman *et al.* [23]. To avoid misinterpretation due to technical error, normal lymphocyte nuclei and normal gastric tissue were used as a control. The FISH results were presented as the percentage of *MYC* amplification by a cell, in which we calculated the percentage of cells showing 3 or more signals for the *MYC* probe by cell.

qPCR was performed using quantitative TaqMan CNV assays (Life Technologies, USA) for the *MYC* gene (Hs01764918_cn) and for the internal control *RNAse P* (#4403326). Multiplex qPCR reactions were performed in quadruplicate with gDNA according to the manufacturer's protocol and cycling conditions in 7500 Fast Real-Time PCR (Life Technologies, USA). The relative copy number was estimated for each sample using the Copy Caller Software V1.0 (Life Technologies, USA). Commercial human gDNAs (G1521 and G1471; Promega, USA) were used for calibration.

MYC methylation

The methylation pattern and frequency of the *MYC* promoter were evaluated in 125 tumors and 67 matched control samples by Methyl-specific PCR (MSP) as previously described [24]. gDNA (2 μg) of all samples was modified by bisulfite treatment, converting unmethylated cytosines to uracils and leaving methylated cytosins unchanged [25]. Specific primers for the *MYC* promoter were as follows: F5′-TAGAATTGGATTGGGG-TAAA-3′ and R5′-CCAACCAAAAATCAACATGAAT-3′ for the unmethylated reactions (expected product size of 291 bp); F5′-TAGAATTGGATCGGGGTAAA-3′ and R5′-CGACC-GAAAATCAACGCGAAT-3′ for the methylated reactions (expected product size of 290 bp), as previous described [26].

PCR reactions were carried out with 0.1 μmol/L of dNTPs, 2 μmol/L of $MgCl_2$, 0.5 μmol of primers, 1.25 U of Taq DNA polymerase, and 100 ng of bisulfite-modified DNA. After initial denaturation for 5 min at 94°C, 40 cycles at 9 4°C for 45 s, 52.4°C for 45 s, and 72°C for 30 s were carried out, followed by a final extension for 5 min at 72°C. PCR products were directly loaded onto 3% agarose gels and electrophoresed. The gel was stained with SYBR® Safe DNA Gel Stain (Life Technolgies, USA) and directly visualized under UV illumination. As a positive control of all MSP reactions, a gDNA sample was completely methylated using CpG Methylase (SssI, New England Biolabs,

Table 1. Clinicopathological characteristics, MYC immunoreactivity, DNA methylation and point mutations in gastric cancer samples.

Variable (N)	Protein immunoreactivity					DNA methylation					Point mutations				
	Positive [N(%)]*	Negative [N(%)]	p-value	OR	CI 95%	Hypomethylated [N(%)]*	Partial methylated [N(%)]	p-value	OR	CI 95%	Present [N(%)]*	Absent [N(%)]	p-value	OR	CI 95%
Gender															
Female (40)	29 (23.2%)	11 (8.8%)	0.641	1.241	0.501–3.079	33 (26.4%)	7 (5.6%)	0.480	1.468	0.506–4.260	6 (4.8%)	34 (27.2%)	0.247	0.501	0.156–1.614
Male (85)*	67 (53.6%)	18 (14.4%)				75 (60%)	10 (8%)				7 (5.6%)	78 (62.4%)			
Onset (years)															
≤45 (18)	10 (8%)	8 (6.4%)	0.026**	3.276	1.152–9.315	14 (11.2%)	4 (3.2%)	0.257	2.066	0.590–7.236	2 (1.6%)	16 (12.8%)	0.915	0.917	0.186–4.526
>45 (107)*	86 (68.8%)	21 (16.8%)				94 (75.2%)	13 (10.4%)				11 (8.8%)	96 (76.8%)			
Tumor location															
Non-cardia (73)	58 (46.4%)	15 (12%)	0.262	0.604	0.250–1.457	63 (50.4%)	10 (8%)	0.929	0.953	0.333–2.732	7 (5.6%)	66 (52.8%)	0.732	1.225	0.385–3.899
Cardia (52)*	38 (30.4%)	14 (11.2%)				45 (36%)	7 (5.6%)				6 (4.8%)	46 (36.8%)			
Histologic subtype															
Diffuse-type (54)	31 (24.8%)	23 (18.4%)	<0.001**	7.856	2.803–22.013	52 (41.6%)	2 (1.6%)	0.007**	0.117	0.025–0.556	12 (9.6%)	42 (33.6%)	0.004**	0.046	0.006–0.373
Intestinal-type (71)*	65 (52%)	6 (4.8%)				56 (44.8%)	15 (12%)				1 (0.8%)	70 (56%)			
Stage															
Early (8)	4 (3.2%)	4 (3.2%)	0.593	1.546	0.312–7.652	4 (3.2%)	4 (3.2%)	0.033**	6.602	1.162–37.501	2 (1.6%)	6 (4.8%)	0.135	0.204	0.025–1.643
Advanced (117)*	92 (73.6%)	25 (20%)				104 (83.2%)	13 (10.4%)				11 (8.8%)	106 (84.8%)			
Tumor invasion															
T1/T2 (30)	16 (12.8%)	14 (11.2%)	0.045**	2.975	1.027–8.623	21 (16.8%)	9 (7.2%)	0.022**	4.752	1.257–17.965	3 (2.4%)	27 (21.6%)	0.978	1.023	0.205–5.101
T3/T4 (95)*	80 (64%)	15 (12%)				87 (69.6%)	8 (6.4%)				10 (8%)	85 (68%)			
Lymph node metastasis															
Absent (12)	0 (0%)	12 (9.6%)	0.998	<0.001	0.000	7 (5.6%)	5 (4%)	0.032**	5.120	1.149–22.814	0 (0%)	12 (9.6%)	0.999	<0.001	0.000
Present (113)*	96 (76.8%)	17 (13.6%)				101 (80.8%)	12 (9.6%)				13 (10.4%)	100 (80%)			
Distant metastasis															
Absent (66)	39 (31.2%)	27 (21.6%)	<0.001**	17.682	3.914–79.882	55 (44%)	11 (8.8%)	0.439	1.537	0.517–4.571	3 (2.4%)	63 (50.4%)	0.032**	4.492	1.141–17.679
Present (59)*	57 (45.6%)	2 (1.6%)				53 (42.4%)	6 (4.8%)				10 (8%)	49 (39.2%)			
Protein immunoreactivity															
Negative (29)	-	-	-	-	-	22 (17.6%)	7 (5.6%)	0.151	2.283	0.739–7.056	2 (1.6%)	27 (21.6%)	0.486	0.562	0.112–2.835
Positive (96)*	-	-				86 (68.8%)	10 (8%)				11 (8.8%)	85 (68%)			
DNA methylation															
Partial methylated (17)	10 (8%)	7 (5.6%)	0.142	2.303	0.755–7.025	-	-	-	-	-	0 (0%)	17 (13.6%)	0.998	<0.001	0.000
Hypomethylated (108)*	86 (68.8%)	22 (17.6%)				-	-				13 (10.4%)	95 (76%)			

*Reference group for logistic regression analysis;
**Differentially expressed between groups, p<0.05.
N: number of samples; OR: odds ratio; CI: confidence interval.
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Table 2. Clinicopathological characteristics, *MYC* mRNA expression, copy number and percentage of amplification in gastric cancer samples.

Variable (N)	RQ				Copy number				% of amplification (FISH)			
	Mean±SD	*p*-value	η²	OP	Mean±SD	*p*-value	η²	OP	Mean±SD	*p*-value	η²	OP
Gender												
Female (16)	3.42±1.03	0.410	0.015	0.129	4.50±1.55	0.402	0.015	0.132	70.69±8.03	0.658	0.004	0.072
Male (33)	3.37±0.92				4.48±1.30				72.77±7.81			
Onset (years)												
≤45 (5)	2.63±0.82	0.060	0.073	0.472	3.2±0.45	0.025*	0.103	0.622	60.4±5.21	<0.001*	0.257	0.976
>45 (44)	3.47±0.93				4.63±1.37				73.42±6.98			
Tumor location												
Non-cardia (28)	3.57±0.93	0.108	0.055	0.362	4.75±1.53	0.102	0.057	0.372	72.52±8.36	0.585	0.007	0.084
Cardia (21)	3.14±0.93				4.14±1.06				71.52±7.31			
Histologic subtype												
Diffuse-type (21)	3.35±0.67	0.723	0.003	0.064	3.81±0.75	0.009*	0.139	0.762	68.55±8.77	0.037*	0.091	0.557
Intestinal-type (28)	3.41±1.12				5.00±1.52				74.75±5.98			
Stage												
Early (2)	2.09±0.14	0.202	0.035	0.245	3.00±0.00	0.546	0.008	0.091	54.5±6.36	0.037*	0.091	0.558
Advanced (47)	3.44±0.93				4.55±1.37				72.84±7.04			
Tumor invasion												
T1/T2 (11)	2.55±0.84	0.006*	0.152	0.801	3.55±0.69	0.200	0.035	0.246	60.82±6.36	<0.001*	0.401	1.000
T3/T4 (38)	3.63±0.84				4.76±1.40				75.36±4.53			
Lymph node metastasis												
Absent (5)	2.27±0.32	0.023*	0.107	0.632	3.20±0.45	0.143	0.046	0.308	36.60±6.94	0.179	0.039	0.267
Present (44)	3.51±0.91				4.64±1.37				73.06±7.43			
Distant metastasis												
Absent (23)	2.50±0.48	<0.001*	0.788	1.000	3.61±0.66	<0.001*	0.356	0.999	68.20±7.29	0.001*	0.221	0.942
Present (26)	4.16±0.41				5.27±1.37				75.54±6.75			
Protein immunoreactivity												
Negative (11)	2.56±0.44	0.003*	0.178	0.870	3.27±0.47	0.003*	0.177	0.869	65.45±8.37	0.009*	0.139	0.759
Positive (38)	3.63±0.92				4.84±1.35				74.01±6.66			
DNA methylation												
Hypomethylated (42)	3.40±0.91	0.802	0.001	0.057	4.45±1.37	0.283	0.025	0.186	72.40±7.40	0.806	0.001	0.057
Partial methylated (7)	3.33±1.24				4.71±1.5				70.21±10.73			
Point mutations												
Present (7)	3.73±0.79	0.107	0.055	0.363	4.43±0.79	0.614	0.006	0.079	72.00±10.83	0.216	0.033	0.233
Absent (42)	3.33±0.97				4.5±1.45				72.11±7.42			

*Differentially expressed between groups, p<0.05.
RQ: relative quantification; N: number of samples; SD: standard deviation; η²: effect size base on Eta Squared; OP: observed power.
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USA) following the manufacturer's instructions. Furthermore, the primers for wild-type were used to monitor complete conversion of DNA obtained in the bisulfite reaction.

Samples were stratified as: 1) hypomethylated samples when positive amplification product was detected only in the PCR with specific primers for unmethylated sequences; 2) hypermethylated samples when positive amplification was detected only in the PCR with specific primers for methylated sequences; 3) partial methylated samples when positive amplification was detected in the PCR with the two primer sets.

MYC Genotyping

The three exons of the *MYC* gene were selected for mutation analysis in all 125 gastric cancer samples. The following primers were designed for PCR amplification and sequencing: exon 1 F5′-TTTATAATGCGAGGGTCTGGA-3′ and R5′-GCATTC-GACTCATCTCAGCA-3′ (expected product size of 654 bp); exon 2 F5′-CTGCCTCCCGCTTTGTGT-3′ and R5′-TTTGATGAAGGTCTCGTCGT-3′ (expected product size of 423 bp), F5′-TGGGAGGAGACATGGTGAA-3′ and R5′-TGCCAATGAAAATGGGAAAG-3′ (expected product size of 507 bp); exon 3 F5′-TGTCCAGAGACCTTTCTAACGTAT-3′ and 5′-CCGTAGCTGTTCAAGTTTGTG-3′ (expected product

size of 663 bp), 5′-TGTCCGTCCAAGCAGAGG-3′ and 5′-TGATGAAAACAAACAGGGATG-3′ (expected product size of 639 bp).

The PCR reactions were carried out with 0.1 μmol/L of dNTPs, 2 μmol/L of MgCl$_2$, 0.5 μmol/L of primers, 1 U of Taq polymerase, and 100 ng of DNA. The PCR conditions were 95°C for 10 min, followed by 35 cycles of 1 min of denaturation at 95°C, 1 min of annealing temperature (ranging from 59 to 61°C), and 1 min of extension at 72°C. The amplicons were separated on a 2% agarose gel stained with SYBR® Safe DNA Gel Stain (Life Technolgies, USA) and directly visualized under UV illumination.

Amplicons were sequenced using the Sanger method [27]. Direct sequencing was carried out using the Big Dye® Terminatorv3.1 Cycle Sequencing kit (Life Technologies, USA) and analyzed on an ABI PRISM® 3130 Genetic Analyzer (Life Technologies, USA) using Pop 7 polymer. The in silico mutation search was performed using the Chromas Pro 1.5 (Technelysium Pty Ltd, Australia). The reference sequence was Gene ID: 4609 (NCBI). Variants with less than 1% minor allele frequency were reported. Pathogenicity of missense mutations was assessed by in silico analysis using PolyPhen (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org).

Statistical analysis

MYC methylation, mutation, or its products' immunoreactivity odds ratio (OR) for clinicophatological features was estimated by logistic regression. The age at gastric tissue sampling was defined as covariate in the regression model.

The normality of distribution for quantitative variables was tested by the Shapiro-Wilk's test. Data that were not normally distributed were transformed (z-score transformation) into a normal distribution for analysis. Analysis of MYC mRNA expression and copy number were performed by the General Linear Model (GLM) with adjustment for age, which provides the effect size and observed power (OP) of each analysis. The effect size for GLM analyses was based on Eta Squared (η^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.

The correlation between mRNA expression and copy number was analyzed by the Pearson test, in which a value of its correlation coefficient (r) below 0.30 was determined as a weak correlation, 0.30–0.70 as a medium correlation, and above 0.70 as a strong correlation.

In all analyses, the confidence interval was 95% and p values less than 0.05 were considered significant.

Results

MYC amplification is known to be related to high protein/mRNA expression in gastric carcinogenesis [28], however, to the best of our knowledge, few studies have been performed to describe the role of methylation and MYC mutations in this process. To reach this goal, we analyzed 125 cases in which 68% were males and 32% were females. The mean age of our sample set was 62 years (range of 26–89 years). A slightly higher frequency of intestinal-type (56.8%) and non-cardic (58.4%) tumors were observed (Table 1 and 2).

MYC nuclear protein was found positive in 76.8% (96/125) of gastric tumors (Figure 1). MYC protein expression was more frequently observed in intestinal-type than diffuse-type tumors ($p<0.001$, OR = 7.856, CI 95% = 2.803–22.013) (Table 1). MYC immunostaining was also associated with late-onset (p = 0.026, OR = 3.276; CI 95% = 1.152–9.315), deeper tumor extension ($p = 0.045$, OR = 2.975, CI 95% = 1.027–8.623), and the presence

of distant metastasis ($p<0.001$, OR = 17.682, CI 95% = 3.914–79.882) (Table 1). Futhermore, MYC immunoreactivity was associated with increased mRNA expression ($p = 0.003$, $\eta^2 = 0.178$, OP = 0.870) and MYC copy number by FISH ($p = 0.009$, $\eta^2 = 0.139$, OP = 0.759) and by qPCR ($p = 0.003$, $\eta^2 = 0.177$, OP = 0.869) (Table 1).

The expression level of MYC mRNA was higher in all tumor samples than their paired controls (RQ = 3.39±0.14; range of 1.57–5.18). An increased MYC mRNA expression was associated with deeper tumor extension ($p = 0.006$, $\eta^2 = 0.152$, OP = 0.801), presence of lymph node metastasis ($p = 0.023$, $\eta^2 = 0.107$, OP = 0.632), and distant metastasis ($p<0.001$, $\eta^2 = 0.788$, OP = 1) (Table 2). Additionally, the mRNA level was directly correlated to the MYC copy number ($p<0.01$; r = 0.716).

Gain of MYC copies was found in all gastric adenocarcinoma samples by FISH and qPCR assays. By FISH, the mean percentage of cells presenting MYC amplification was 72.1% (range of 50 to 83.5% cells with amplification) (Figure 2 A and B). The mean of MYC copies by qPCR was 4.5 (range 3 to 9 copies) (Figure 2 C).

FISH and qPCR analyses showed that increased MYC copy number was associated with late-onset ($p<0.001$; $\eta^2 = 0.257$, OP = 0.976; $p = 0.025$, $\eta^2 = 0.103$, OP = 0.662, respectively), intestinal-type cancer ($p = 0.037$; $\eta^2 = 0.091$, OP = 0.557; $p = 0.009$; $\eta^2 = 0.139$, OP = 0.762, respectively), and the presence of distant metastasis ($p = 0.001$; $\eta^2 = 0.221$, OP = 0.942; $p<0.001$; $\eta^2 = 0.356$, OP = 0.999, respectively). In addition, MYC amplification by FISH was associated with advanced tumor stages ($p = 0.037$; $\eta^2 = 0.091$, OP = 0.558), however, only two early tumors were analyzed in this subset of samples (Table 2).

All gastric cancer samples presented positive amplification with an unmethylated primer set. Interestingly, 86.4% of cancer samples were hypomethylated. On the other hand, the presence of unmethylated sequences at the MYC promoter was observed in 28.4% of control samples (partial methylated samples), suggesting the loss of methylation in these samples (Figure 3). The primers's specificity and MSP results were confirmed using the bisulfite sequencing PCR (BSP) approach [29] in which we randomly selected five hypomethylated samples; five hypermethylated samples and five partial methylated samples (data not shown). MYC hypomethylation was more frequently observed in the diffuse-type as compared to the intestinal-type gastric cancer ($p = 0.007$; OR = 8.554; 95% CI = 01.798–40.695, using diffuse-type as reference group). In addition, MYC hypomethylation was associated with advanced tumor stages ($p = 0.033$; OR = 6.602; 95% CI = 1.162–37.501), deeper tumor extension ($p = 0.022$; OR = 4.752; 95% CI = 1.257–17.965), and the presence of lymph node metastasis ($p = 0.032$; OR = 5.12; 95% CI = 1.149–22.814) (Table 1).

With regards to gene sequencing, no novel mutation was detected in gastric tumors. Thirteen (10.4%) tumor samples presented at least one known mutation, with variants on less than 1% minor allele frequency. In total, 18 mutations were identified, with 4 samples exhibiting co-occurring mutations. In exon 1, five with GG and four with CG at rs117856857; one with GG at rs73707292; and four with CT at rs4645949. In exon 2, concerning missense mutations, two tumors harbored a mutation at codon 47 resulting in a change from tyrosine to histidine (rs114570780; SIFT prediction = deleterious; PolyPhen prediction = probably damaging), and two at codon 72 resulting in a change from proline to serine (rs28933407; SIFT prediction = tolerated; PolyPhen prediction = probably damaging). All tumor with a mutation in exon 2 presented one or two known mutation in exon 1. Exon 2 mutations were only detected in diffuse-type
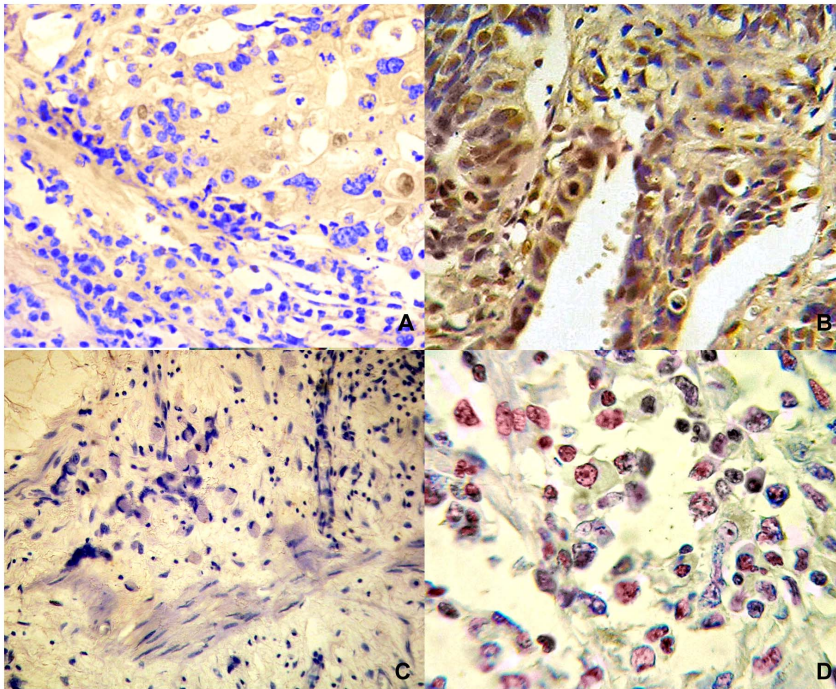
Figure 1. MYC protein expression. A) intestinal-type gastric cancer without MYC immunoreactivity (400×); B) intestinal-type gastric cancer presenting MYC immunoreactivity (400×); C) diffuse-type gastric cancer without MYC immunoreactivity (400×); D) diffuse-type gastric cancer presenting MYC immunoreactivity (400×).
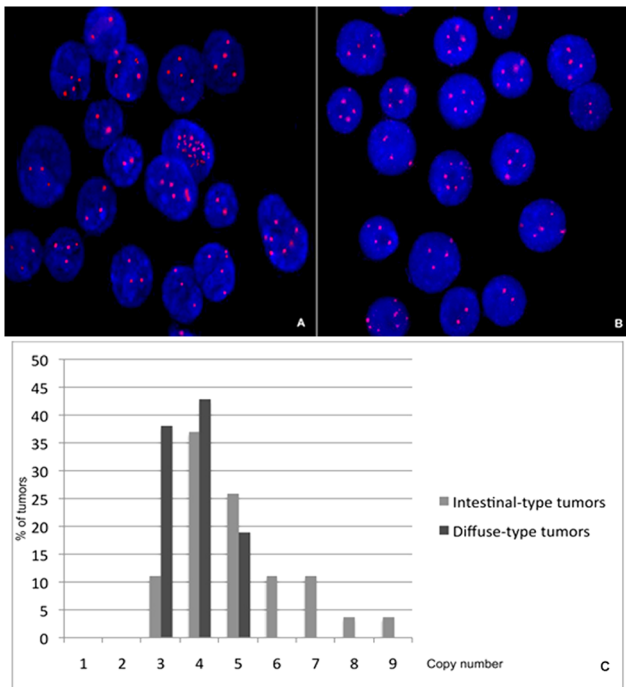doi:10.1371/journal.pone.0064420.g001

tumors in advanced stage. No mutation was identified in exon 3. The presence of known *MYC* mutations was associated with diffuse-type tumors ($p = 0.004$, OR = 21.717, 95% CI = 2.678–176.111; using diffuse-type as reference group) and the presence of distant metastasis ($p = 0.032$, OR = 4.492, 95% CI = 1.141–17.679).

Discussion

The MYC protein has an effect on about 15% of the genes in the human genome [30]. Thus, MYC deregulation may result in alterations in different biological pathways involved in cancer initiation and progression [5]. However, up to date, the relationship between *MYC* alterations and clinicopathological parameters has not been well understood. Our samples presented a male-female ratio of 2:1 and the majority of the patients were
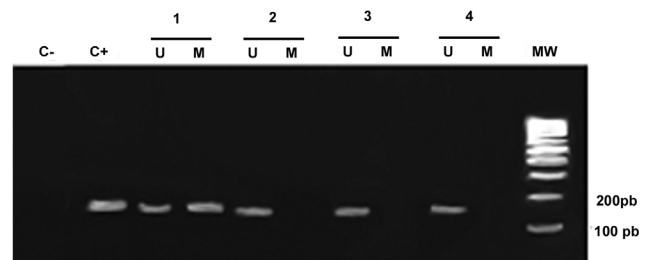

Figure 2. *MYC* amplification in gastric cancer. A) interphase nuclei presenting *MYC* amplification (red) in intestinal-type gastric cancer; B) interphase nuclei presenting *MYC* amplification (red) in diffuse-type gastric cancer; C) *MYC* copy number distribution by qPCR in intestinal-type and diffuse-type tumors.
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Figure 3. Methylation analysis of the *MYC* promoter showing methylated and unmethylated bands. Sample 1 presented partial methylation. Samples 2, 3 and 4 presented a hypomethylated promoter. C-: blank; C+: positive control, gDNA sample completely methylated; U: unmethylated; M: methylated: MW: molecular weight marker; bp: base pairs.
doi:10.1371/journal.pone.0064420.g003

older than fifty-five. Moreover, the intestinal-type gastric cancer was more frequent than the diffuse-type and tumors were more frequent in non-cardia. These epidemiological data are in accordance with previous studies [28,31–33].

Chromosomal translocations in the *MYC* locus is very commun in haematopoietic cancers. However, in solid human tumours like gastric cancer, *MYC* alterations are commonly due to gene amplification [34]. Furthermore, *MYC* is recognized to be the most frequently amplified protein-coding gene across all cancer types [35]. In the present study, we observed three or more *MYC* gene copies in all gastric tumors studied, corroborating with previous studies in primary gastric tumors of individuals from our population [10,28,36–39], as well as from Eastern Asia and Europe [40–42]. Also, *MYC* amplification was observed in plasma [43] and in gastric cancer cell lines established in our group [44–47]. Additionally, our group had observed that clonal high amplification of *MYC* is less frequent in diffuse-type than in intestinal-type primary gastric cancer [10,22,28], and this was reinforced by this study.

MYC overexpression is described as ranging from 15.6% to 100% in primary gastric cancers [9]. *In vitro* studies with knocked-down MYC expression in gastric cancer cell lines demonstrated the crucial role of MYC expression in gastric tumor cell growth, survival, and the maintenance of tumor cell parameters that may contribute to malignant potential [48]. Moreover, Mehndiratta *et al.*, [49] showed a significant decrease (40%) in MYC expression of both mRNA and protein and its downstream targets using siRNA.

In the present study, 76.8% of gastric tumors showed MYC immunoreactivity and all tumors, intestinal and diffuse type, presented increased mRNA expression compared to their paired controls. Furthermore, we observed that MYC immunoreactivity and increased mRNA expression were associated with deeper tumor extension and presence of metastasis. These findings suggest that MYC has a role in the tumor invasiveness, metastasis, and thus aggressiveness, corroborating a previous study [37]. Although some analyses presented a small effect size, these findings are also in agreement with a previous study of our group in non-human primates, in which we demonstrated a continuous increase of *MYC* mRNA expression and copy number during the sequential steps of intestinal-type gastric carcinogenesis in N-methyl-nitrosourea (MNU)-treated non-human primates [16]. On the other hand, any association between MYC and histological grade, tumor location, lymph node metastasis, or pathological stage was detected in a gastric cancer study developed in a Chinese population [50], reinforcing that the ethnicity of the afflicted population may lead to biologically and clinically gastric cancer subsets [51].

Although, gene amplification is not necessarily associated or required for its overexpression, our study shows that MYC immunoreactivity, *MYC* mRNA levels, and copy number were directly correlated.

DNA methylation is a potent mechanism of transcriptional repression. Proper genomic methylation-patterns become profoundly altered in cancer cells: both gains (hypermethylation) and losses (hypomethylation) of methylated sites have been observed [52,53]. Hypomethylation at specific promoters can activate the aberrant expression of oncogenes and loss of imprinting in some loci [44]. So far, few studies discussed the relationship between the methylation pattern of *MYC* and its effect on gene expression and on carcinogenic processes. *MYC* hypomethylation was previously associated with the oncogenic progression and metastasis induction in a rat model of liver cancer [54] and in human colorectal cancer samples [55]. In addition, *MYC* hypomethylation was also associated with *MYC* expression in gastric tumors [26,56–58] and

cell lines [48]. However, we were unable to observe a significant association between *MYC* hypomethylation and its expression in our samples. Among other factors, this lack of association may be due to the presence of *MYC* amplification in all tumors with hypomethylated promoters (86.4% of samples) and thus, masking the possible effect of this epigenetic modification on *MYC* transcriptional regulation.

Suzuki *et al.* [59] previously showed that a high level of hypomethylation was an indicator of poor prognosis in both gastric and colon cancer, and epigenetic alterations were age dependent, occuring before genetic alterations. In the present study, some controls already presented unmethylated sequences in the *MYC* promoter. In addition, we demonstrated that *MYC* hypomethylation was associated with a more aggressive phenotype (tumor aggressiveness, presence of lymphnode metastasis, and histological types of cancer). These findings suggest that *MYC* demethylation may be accumulated during tumor progression and this could be a common event in gastric carinogenesis [60,61], since this mechanism has been observed for other genes in several tumor types [62,63]. As already proposed for DNA hypermethylation [64], the promoter hypomethylation may be used as a new generation of biomarkers and holds diagnostic and prognostic promise for clinicians.

To the best of our knowledge, *MYC* gene exons have never been completely sequenced in human gastric tumors. Here, we sequenced the three exons of *MYC*: exon 1 is a non-coding protein, exons 2 and 3 are protein-coding [for review see (Pelengaris & Khan, 2003)]. We did not find any new mutation, however, we observed that 4 tumors presented missense mutations (rs114570780 and rs28933407) on exon 2. These mutations were in an evolutionary conserved sequence of *MYC*: the transactivation domain [4,65]. In addition, both identified mutations were considered as probably damaging according to the PolyPhen software. The change of proline to serine at codon 72 was also previously reported as a pathogenic variant in the NCBI dbSNP database (http://omim.org/). Thus, both mutations at exon 2 may affect MYC activity in gastric tumors. In addition, the presence of *MYC* mutations was associated with distant metastasis. However, further investigations are necessary to clarify if *MYC* mutations have a role in the metastatic process.

According to Laurén classification, gastric adenocarcinoma is classified mainly into intestinal and diffuse types [17]. Intestinal-type gastric cancer progresses through a number of sequential steps, beginning with atrophic gastritis followed by intestinal metaplasia, intraepitelial neoplasia, and carcinoma [66]. On the other hand, the diffuse-type generally does not evolve from precancerous lesions [67,68]. In the present study, we observed that the intestinal-type presented more frequent MYC immunoreactivity, as well as a higher number of *MYC* copies than diffuse-type tumors, which corroborates with previous studies of our group [10,22,28,38]. In addition, *MYC* hypomethylation and point mutations were more frequently observed in diffuse-type as compared to intestinal-type tumors. Thus, our findings support that these two histological subtypes follow different genetic pathways and may be two distinct entities [67].

In conclusion, our data suggest that *MYC* overexpression and promoter hypomethylation may have a role in the gastric carcinogenesis process. *MYC* deregulation was associated mainly to poor prognostic features. Our results also reinforce the presence of different pathways involved in intestinal-type and diffuse-type gastric carcinogenesis. Thus, our findings suggest that MYC may be a useful marker for clinical stratification and prognosis.

Author Contributions

Conceived and designed the experiments: CS ML DC PA MS RB. Performed the experiments: CS ML DC ES BB HR. Analyzed the data: CS ML RM RB. Contributed reagents/materials/analysis tools: CS BB AS SS PA MS RB. Wrote the paper: CS ML DC BB RM RB.

References

1. Jemal A, Center MM, DeSantis C, Ward EM (2010) Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol Biomarkers Prev 19: 1893–1907.
2. Nardone G (2003) Review article: molecular basis of gastric carcinogenesis. Aliment Pharmacol Ther 17 Suppl 2: 75–81.
3. Huppi K, Pitt JJ, Wahlberg BM, Caplen NJ (2012) The 8q24 gene desert: an oasis of non-coding transcriptional activity. Front Genet 3: 69.
4. Pelengaris, S & Khan M (2003) Arch Biochem Biophys 416(2): 129–136.
5. Lutz W, Leon J, Eilers M (2002) Contributions of Myc to tumorigenesis. Biochim Biophys Acta 1602: 61–71.
6. Pelengaris S, Khan M, Evan GI (2002) Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. Cell 109: 321–334.
7. Pelengaris S, Littlewood T, Khan M, Elia G, Evan G (1999) Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion. Mol Cell 3: 565–577.
8. Dalla-Favera R, Wong-Staal F GR (1982) Onc gene amplification in promyelocytic leukaemia cell line HL-60 and primary leukaemic cells of the same patient. Nature 299: 61–63.
9. Calcagno DQ, Leal MF, Assumpcao PP, Smith MAC, Burbano RR (2008) MYC and gastric adenocarcinoma carcinogenesis. World J of Gastroenterol 14: 5962–5968.
10. Calcagno DQ, Guimarães AC, Leal MF, Seabra AD, Khayat AS, et al. (2009) MYC insertions in diffuse-type gastric adenocarcinoma. Anticancer Res 29: 2479–2483.
11. Liu Y, Gong L, Dong X, Liu H (2012) Detection of C-MYC oncogene translocation and copy number change in the normal-dysplasia-carcinoma sequence of the larynx by fluorescence in situ hybridization. Diagn Cytopathol doi: 10.1002/dc.22879 [In press]
12. Tuupanen S, Yan J, Turunen M, Gylfe AE, Kaasinen E, et al. (2012) Characterization of the colorectal cancer-associated enhancer MYC-335 at 8q24: the role of rs67491583. Cancer Genet 205: 25–33.
13. Amente S, Lania L, Majello B (2011) Epigenetic reprogramming of Myc target genes. Am J Cancer Res 1: 413–418.
14. Tsai KW, Liao YL, Wu CW, Hu LY, Li SC, et al. (2011) Aberrant hypermethylation of miR-9 genes in gastric cancer. Epigenetics 6:1189–1197.
15. Dang C V (2012) MYC on the path to cancer. Cell 149: 22–35.
16. Da Costa JDFFB, Leal MF, Silva TCR, Andrade Junior EF, Rezende AP, et al. (2011) Experimental gastric carcinogenesis in Cebus apella nonhuman primates. PloS One 6: e21988.
17. Lauren P (1965) The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 64: 31–49.
18. Sobin L, Wittekind C (2002) TNM: Classification of malignant tumours. 6th ed. New York: Wiley-Blackwell.
19. Stamouli MI, Ferti AD, Panani AD, Raftakis J, Consoli C, et al. (2002) Application of multiplex fluorescence in situ hybridization in the cytogenetic analysis of primary gastric carcinoma. Cancer Genet Cytogenet 135: 23–27.
20. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2($-$Delta Delta C(T)) Method. Methods 25: 402–408.
21. Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83: 2934–2938.
22. Calcagno DQ, Leal MF, Taken SS, Assumpção PP, Demachki S, et al. (2005) Aneuploidy of chromosome 8 and C-MYC amplification in individuals from northern Brazil with gastric adenocarcinoma. Anticancer res 25: 4069–4074.
23. Hopman AH, Ramaekers FC, Raap AK, Beck JL, Devilee P, et al. (1988) In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. Histochemistry 89: 307–316.
24. Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93: 9821–9826.
25. Xiong Z, Laird P (1997) COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 25: 2532–2534.
26. Fang JY, Cheng ZH, Chen YX, Lu R, Yang L, et al. (2004) Expression of Dnmt1, demethylase, MeCP2 and methylation of tumor-related genes in human gastric cancer. World J Gastroenterol 10: 3394–3398.
27. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467.
28. Calcagno DQ, Leal MF, Seabra AD, Khayat AS, Chen ES, et al. (2006) Interrelationship between chromosome 8 aneuploidy, C-MYC amplification and increased expression in individuals from northern Brazil with gastric adenocarcinoma. World J Gastroenterol 12: 6207–6211.
29. Borges B, Santos E, Bastos C, Pinto L, Anselmo N, et al. (2010) Promoter polymorphisms and methylation of E-cadherin (CDH1) and KIT in gastric cancer patients from northern Brazil. Anticancer Res 30: 2225–2233.
30. Fernandez PC, Frank SR, Wang L, Schroeder M, Liu S, et al. (2003) Genomic targets of the human c-Myc protein. Genes Dev 17:1115–1129.
31. Li F, Zhong MZ, Li JH, Liu W, Li B (2012) Case-control Study of Single Nucleotide Polymorphisms of PSCA and MUC1 Genes with Gastric Cancer in a Chinese. Asian Pac J Cancer Prev 13: 2593–2596.
32. Chang MS, Uozaki H, Chong JM, Ushiku T, Sakuma K, et al. (2006) CpG island methylation status in gastric carcinoma with and without infection of Epstein-Barr virus. Clin Cancer Res 12: 2995–3002.
33. Shibata D, Hawes D, Stemmermann GN, Weiss LM (1993) Epstein-Barr virus-associated gastric adenocarcinoma among Japanese Americans in Hawaii. Cancer Epidemiol Biomarkers Prev 2: 213–217.
34. Meyer N, Penn LZ (2008) Reflecting on 25 years with MYC. Nat Rev Cancer 8: 976–990.
35. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, et al. (2010) The landscape of somatic copy-number alteration across human cancers. Nature 463: 899–905.
36. Assumpção PP, Ishak G, Chen ES, Takeno SS, Leal MF, et al. (2006) Numerical aberrations of chromosome 8 detected by conventional cytogenetics and fluorescence in situ hybridization in individuals from northern Brazil with gastric adenocarcinoma. Cancer Genet Cytogenet 107: 45–49.
37. Burbano RR, Assumpção PP, Leal MF, Calcagno DQ, Guimarães AC, et al. (2006) C-MYC locus amplification as metastasis predictor in intestinal-type gastric adenocarcinomas: CGH study in Brazil. Anticancer Res 26: 2909–2914.
38. Calcagno DQ, Leal MF, Demachki S, Araújo MTF, Freitas FW, et al. (2010) MYC in gastric carcinoma and intestinal metaplasia of young adults. Cancer Genet Cytogenet 202: 63–66.
39. Costa Raiol LC, Figueira Silva EC, Mendes da Fonseca D, Leal MF, Guimarães AC, et al. (2008) Interrelationship between MYC gene numerical aberrations and protein expression in individuals from northern Brazil with early gastric adenocarcinoma. Cancer Genet Cytogenet 181: 31–35.
40. Fan B, Dachrut S, Coral H, Yuen ST, Chu KM, et al. (2012) Integration of DNA copy number alterations and transcriptional expression analysis in human gastric cancer. PLoS One 7: e29824.
41. Zhang D, Wang Z, Luo Y, Xu Y, Liu Y, et al. (2011) Analysis of DNA copy number aberrations by multiple ligation-dependent probe amplification on 50 intestinal type gastric cancers. J Surg Oncol 103: 124–132.
42. Buffart TE, Van Grieken NCT, Tijssen M, Coffa J, Ylstra B, et al. (2009) High resolution analysis of DNA copy-number aberrations of chromosomes 8, 13, and 20 in gastric cancers. Virchows Archiv 455: 213–223.
43. Park KU, Lee HE, Park DJ, Jung EJ, Song J, et al. (2009) MYC quantitation in cell-free plasma DNA by real-time PCR for gastric cancer diagnosis. Clin Chem Lab Med 47: 530–536.
44. Leal MF, Martins Do Nascimento JL, Da Silva CEA, Vita Lamarão MF, Calcagno DQ, et al. (2009) Establishment and conventional cytogenetic characterization of three gastric cancer cell lines. Cancer Genet Cytogenet 195: 85–91.
45. Costa Guimarães A, Gonçalves Quintana L, Ferreira Leal M, Satomi Takeno S, Pimentel Assumpção P, et al. (2006) Aneuploidy of chromosome 8 detected by fluorescence in situ hybridisation in ACP01 cell line gastric adenocarcinoma. Clin Exp Med 6: 129–133.
46. Leal MF, Calcagno DQ, Borges da Costa JDFF, Silva TCR, Khayat AS, et al. (2011) MYC, TP53, and chromosome 17 copy-number alterations in multiple gastric cancer cell lines and in their parental primary tumors. J Biomed Biotechnol 2011: 631268.
47. Ribeiro HF, Alcântara DFA, Matos LA, Sousa JMC, Leal MF, et al. (2010) Cytogenetic characterization and evaluation of c-MYC gene amplification in PG100, a new Brazilian gastric cancer cell line. Braz J Med Biol Res 43: 717–721.
48. Zhao Y, Li JS, Guo MZ, Feng BS, Zhang JP (2010) Inhibitory effect of S-adenosylmethionine on the growth of human gastric cancer cells in vivo and in vitro. Chin J Cancer 29: 752–760.
49. Mehndiratta M, Palanichamy JK, Pal A, Bhagat M, Singh A, et al. (2011) CpG hypermethylation of the C-myc promoter by dsRNA results in growth suppression. Mol Pharm 8: 2302–2309.
50. Liu X, Cai H, Huang H, Long Z, Shi Y, et al. (2011) The prognostic significance of apoptosis-related biological markers in Chinese gastric cancer patients. PloS One 6: e29670.
51. Shah M, Ajani J (2010) Gastric cancer-an enigmatic and heterogeneous disease. JAMA 303: 1753–1754.
52. De Smet C, Loriot A (2013) DNA hypomethylation and activation of germline-specific genes in cancer. Adv Exp Med Biol 754: 149–166.
53. Portela A, Esteller M (2010) Epigenetic modifications and human disease. Nat Biotechnol 28: 1057–1068.

54. Du Y, Peng J, Sun A, Tang Z, Ling W, et al. (2009) Assessment of the effect of betaine on p16 and c-myc DNA methylation and mRNA expression in a chemical induced rat liver cancer model. BMC Cancer 9: 261.

55. Sharrard RM, Royds JA, Rogers S, Shorthouse AJ (1992) Patterns of methylation of the c-myc gene in human colorectal cancer progression. Br J Cancer 65: 667–672.

56. Fang JY, Zhu SS, Xiao SD, Jiang SJ, Shi Y, et al. (1996) Studies on the hypomethylation of c-myc, c-Ha-ras oncogenes and histopathological changes in human gastric carcinoma. J Gastroenterol Hepatol 11: 1079–1082.

57. Fang JY, Xiao SD, Zhu SS, Yuan JM, Qiu DK, et al. (1997) Relationship of plasma folic acid and status of DNA methylation in human gastric cancer. J Gastroenterol 32: 171–175.

58. Weng YR, Sun DF, Fang JY, Gu WQ, Zhu HY (2006) Folate levels in mucosal tissue but not methylenetetrahydrofolate reductase polymorphisms are associated with gastric carcinogenesis. World J Gastroenterol 12: 7591–7597.

59. Suzuki K, Suzuki I, Leodolter A, Alonso S, Horiuchi S, et al. (2006) Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage. Cancer Cell 9: 199–207.

60. Frigola J, Solé X, Paz MF, Moreno V, Esteller M, et al. (2005) Differential DNA hypermethylation and hypomethylation signatures in colorectal cancer. Hum Mol Genet 14: 319–326.

61. Ehrlich M (2002) DNA methylation in cancer: too much, but also too little. Oncogene 21: 5400–5413.

62. Papaggeli PC, Kortsaris AC, Matsouka PT (2003) Aberrant methylation of c-myc and c-fos protooncogenes and p53 tumor suppressor gene in myelodysplastic syndromes and acute non-lymphocytic leukemia. J BUON 8: 341–350.

63. Shen L, Qui D, Fang J (1997) Correlation between hypomethylation of c-myc and c-N-ras oncogenes and pathological changes in human hepatocellular carcinoma. Zhonghua Zhong Liu Za Zhi 19: 173–176.

64. Li M, Chen WD, Papadopoulos N, Goodman SN, Bjerregaard NC, et al. (2009) Sensitive digital quantification of DNA methylation in clinical samples. Nat Biotechnol 27: 858–863.

65. Ryan KM, Birnie GD (1996) Myc oncogenes: the enigmatic family. Biochem J 314 (Pt 3): 713–721.

66. Correa P (1992) Human gastric carcinogenesis: a multistep and multifactorial process–First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. Cancer Res 52: 6735–6740.

67. Tahara E (2004) Genetic pathways of two types of gastric cancer. IARC scientific publications: 327–349.

68. Smith MG, Hold GL, Tahara E, El-Omar EM (2006) Cellular and molecular aspects of gastric cancer. World J Gastroenterol 12: 2979–2990.