BRIEF ARTICLE

Clinical implication of 14-3-3 epsilon expression in gastric cancer

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Abstract

AIM: To evaluate for the first time the protein and mRNA expression of 14-3-3ε in gastric carcinogenesis.

METHODS: 14-3-3ε protein expression was determined by western blotting, and mRNA expression was examined by real-time quantitative RT-PCR in gastric tumors and their matched non-neoplastic gastric tissue samples.

RESULTS: Authors observed a significant reduction of 14-3-3ε protein expression in gastric cancer (GC) samples compared to their matched non-neoplastic tissue. Reduced levels of 14-3-3ε were also associated with diffuse-type GC and early-onset of this pathology. Our data suggest that reduced 14-3-3ε may have a role in gastric carcinogenesis process.

CONCLUSION: Our results reveal that the reduced 14-3-3ε expression in GC and investigation of 14-3-3ε interaction partners may help to elucidate the carcinogenesis process.

INTRODUCTION

Although gastric cancer (GC) rates have decreased substantially in most parts of the world, it is still the fourth most frequent cancer type and the second highest cause of cancer mortality worldwide. A total of 989 600 new

stomach cancer cases and 738 000 deaths are estimated to have occurred in 2008, accounting for 8% of the total cases and 10% of total deaths by cancer[1].

About 90% of stomach tumors are adenocarcinomas[2]. However, the etiology and disease evolution may vary among populations, primary tumor location, histological subtypes of adenocarcinoma, and other variables[3].

GC, as with other neoplasms, is a multifactorial disease that results from a combination of environmental factors and accumulation of generalized and specific genetic and epigenetic alterations. Chromosomal instability is characterized by changes in chromosome copy number (aneuploidy) and alterations in chromosomal regions, which may induce oncogene activation, tumor suppressor gene inactivation, or both[4]. The chromosomal aberrations that are constantly found in GC include gains of 3q, 7p, 7q, 8q, 13q, 17q, 20p and 20q and losses of 4q, 9p, 17p and 18q (for a review, see[5]). Our research group previously reported that the loss of one copy of *TP53* locus (17p13) is commonly found in gastric tumors of individuals from a Brazilian population[6], as well as in GC cell lines[7-9]. Although *TP53* is a key tumor suppressor gene in the carcinogenesis process[10], additional genes at 17p13 may play a role in gastric carcinogenesis.

The *YWHAE* gene is located at 17p13.3 and encodes the 14-3-3ε protein, one of the mammalian 14-3-3 protein family members that are highly conserved in eukaryotes. There are at least seven distinct *14-3-3* genes in vertebrates, giving rise to nine isoforms (α, β, γ, δ, ε, ζ, η, σ and τ/υ, with α and δ being phosphorylated forms of β and ζ, respectively)[11,12]. The 14-3-3 proteins are predominantly dimeric within the cell and bind either to multiple sites within single proteins or act as a bridge between two targets[13-15]. Up to now, > 300 proteins have been reported to interact with 14-3-3 proteins, including key signaling components, such as p53, Raf-1 kinase, Bcl-2 antagonist of cell death, protein kinase C, phophatidylinositol 3-kinase, and cdc25 phosphatase (RASGRF1)[12,13,15,16]. Although the exact 14-3-3 protein functions are not fully known, these proteins may act as a molecular scaffold, bringing together proteins that interact functionally and effecting phosphorylation-dependent cell regulation[12]. This protein family is involved in several biological processes and plays a regulatory role in processes such as apoptotic cell death, mitogenic signal transduction, and cell cycle control[13,17,18].

The isoform 14-3-3ε is the most highly conserved member of the 14-3-3 family, with conserved sequence in plants, yeast, and mammals[19,20]. Abnormal expression of 14-3-3ε has been found in some types of cancers. However, the role of 14-3-3ε in the carcinogenesis process is ambiguous and contradictory. Low expression of 14-3-3ε occurs in small cell lung cancer[21], laryngeal squamous cell carcinoma[22], and medulloblastoma[23], which suggest its role as a tumor suppressor gene. On the other hand, high expression of 14-3-3ε has been detected in renal carcinoma[24], astrocytoma[25], meningioma[26] and subependymomas[27], and, thus, probably it acts as a oncogene.

To the best of our knowledge, no study has evaluated the role of 14-3-3ε in gastric carcinogenesis until now. In the present study, we analyzed the *14-3-3ε* gene and protein expression in GC and matched non-neoplastic gastric samples. We also evaluated the possible associations between 14-3-3ε and clinicopathological characteristics.

MATERIALS AND METHODS

Tissue samples
14-3-3ε protein expression was evaluated in 20 pairs of GC samples and corresponding non-neoplastic gastric tissues (distant location of primary tumor). The mRNA expression was evaluated in 31 pairs of GC samples and corresponding non-neoplastic gastric tissues. Dissected tumor and paired non-neoplastic tissue specimens were immediately cut from stomach samples, frozen in liquid nitrogen, and stored at -80 ℃ until use for protein and RNA extraction. All the gastric samples were obtained surgically from João de Barros Barreto University Hospital (HUJBB) in Pará State, Northern Brazil. Informed consent with approval of the ethics committee of HUJBB was obtained. All patients had negative histories of exposure to either chemotherapy or radiotherapy before surgery and there was no other co-occurrence of diagnosed cancers. All samples were classified according to Laurén[28] and tumors were staged using standard criteria by TNM staging[29].

Protein and mRNA purification
Total protein and total mRNA were simultaneously isolated from gastric tissue samples using the AllPrep DNA/RNA/Protein Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To allow greatest solubilization, the protein pellet was dissolved in buffer containing 7 mol urea, 2 mol thiourea, 4% CHAPS, 50 mmol dithiothreitol, 1% Protease Inhibitor Cocktail (Sigma, St Louis, MO, United States), and 0.5% of each Phosphatase Inhibitor Cocktail 1 and 2 (Sigma-Aldrich, St Louis, MO, United States). Protein concentration was determined by the method of Bradford (Sigma-Aldrich). RNA concentration and quality were determined using a NanoDrop spectrophotometer (Kisker, Germany) and 1% agarose gels. Samples were stored at -80 ℃ until use.

14-3-3ε expression by western blotting
Reduced protein (30 μg) of each sample was separated on 12.5% homogeneous SDS-PAGE gel and electroblotted to a polyvinylidene fluoride (PVDF) membrane (Hybond-*P*; GE Healthcare, Uppsala, Sweden). The PVDF membrane was blocked with PBS containing 0.1% Tween 20, 5% low-fat milk, and incubated overnight at 4 ℃ with corresponding primary antibodies to anti-14-3-3ε (sc-31962, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, United States) and anti-β-actin (Ac-74, 1:3000; Sigma-Aldrich). After extensive washing, a peroxidase-conjugated secondary antibody was incubated for 1 h at room temperature. Immunoreactive bands were visualized using western blotting Luminol reagent and the images were acquired using an ImageQuant 350 digital image system (GE Healthcare, Uppsala, Sweden). The β-actin was used as a loading reference control.
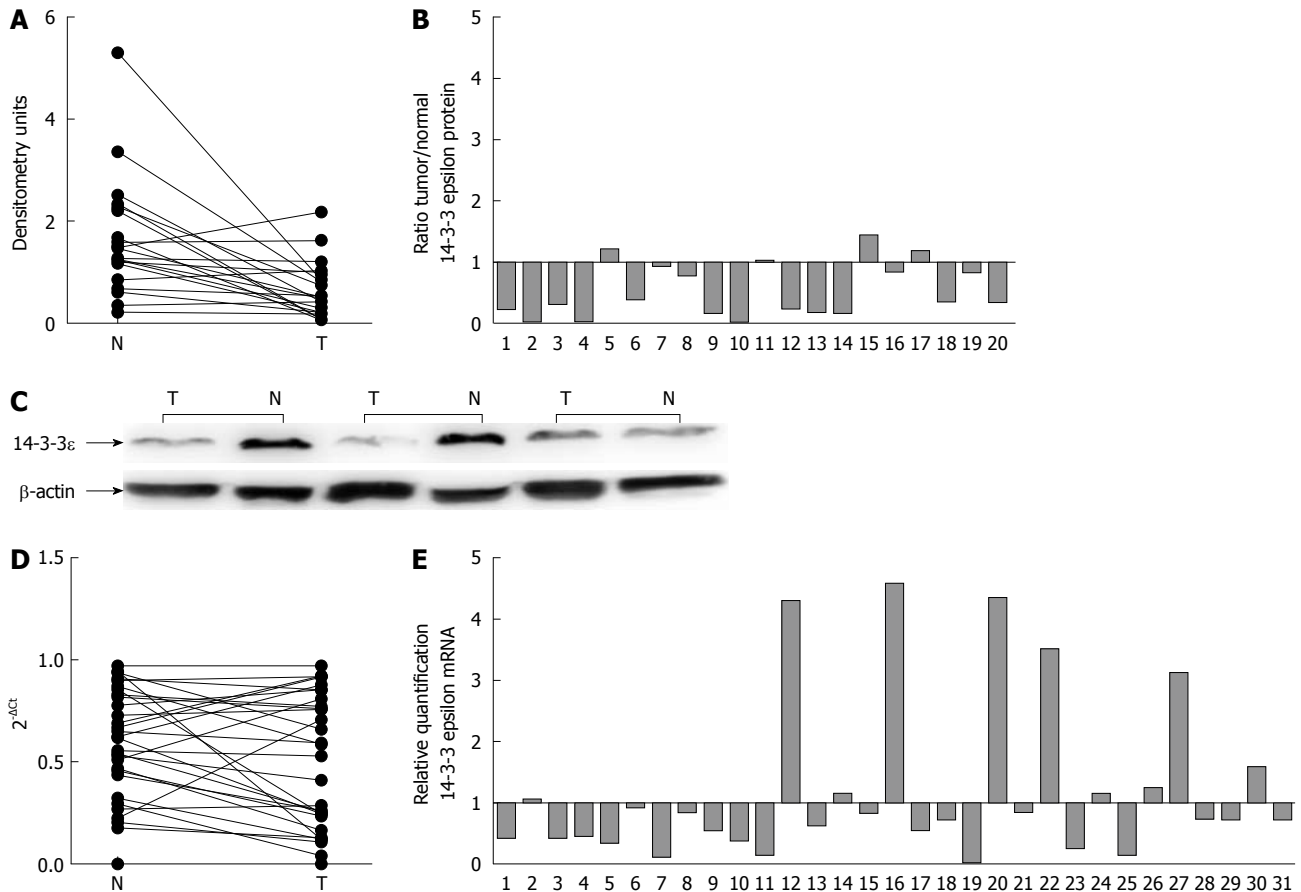
Figure 1 Expression of 14-3-3ε in tumor and non-neoplastic gastric tissue. A: Protein expression normalized by β-actin (ACTB); B: Ratio of protein expression between tumor and matched non-neoplastic gastric tissues; C: Western blotting using anti-14-3-3ε and anti-β-actin antibodies; D: mRNA expression normalized by the internal controls ACTB and glyceraldehyde-3-phosphate dehydrogenase; E: Relative mRNA quantification-gastric tumor samples normalized by matched non-neoplastic gastric tissues. T: Tumor samples; N: Non-neoplastic samples.

14-3-3ε mRNA expression by real-time quantitative RT-PCR

First, cDNA was synthesized using High-Capacity cDNA Archive kit (Applied Biosystems, Warsaw, Poland) according to the manufacturer's protocol. All real-time qRT-PCR reactions were performed in triplicate for both target gene (YWHAE: Hs00356749_g1, Applied Biosystems, United States) and internal controls (β-actin: Hs03023943_g1; glyceraldehyde-3-phosphate dehydrogenase: Hs99999905_m1; Applied Biosystems, United States). To compare 14-3-3ε mRNA expression between GC and non-neoplastic gastric samples, we converted ΔCt (ΔCt = Ct of *YWHAE* - Ct of internal controls) to linear form ($2^{-\Delta Ct}$). Relative quantification of the gene expression was calculated according to Pfaffl method[30]. Non-neoplastic gastric samples were designated as a calibrator of each paired tumor sample.

Statistical analysis

We first evaluated the normal distribution of all data using the Shapiro-Wilk normality test to determine subsequent use of appropriate tests for statistical comparison. Since 14-3-3ε mRNA and protein data did not present with a normal distribution, we performed parametric tests with bootstrapping, a re-sampling method. The re-sampling methods are relatively powerful and can control

a type I error (false positive), reducing over-fit bias and internally validating the accuracy estimates. Bootstrapping methods also produce confidence intervals (CIs) around the observed effects. Paired *t* test was performed to compare the mean of 14-3-3ε expression between neoplastic and matched non-neoplastic samples. The associations between clinicopathological parameters and the mean of 14-3-3ε expression were assessed using a *t* test for independent samples. The correlation among the 14-3-3ε mRNA and protein expression was analyzed by Pearson test. All the analyses performed in this article were based on 1000 bootstrap samples. In all analyses, the CI was 95% and $P < 0.05$ was considered significant.

RESULTS

14-3-3ε protein expression was significantly reduced in GC samples (densitometry units: 0.656 ± 0.552) compared to matched non-neoplastic gastric samples (1.656 ± 1.158) ($P = 0.005$, 95% CI: -1.59 to -0.53). 14-3-3ε protein was 1.5-fold lower in 60% of GC samples compared to their paired non-neoplastic gastric tissues. However, 14-3-3ε mRNA expression did not differ between GC ($2^{-\Delta Ct}$: 0.492 ± 0.325) and corresponding non-neoplastic gastric tissue (0.579 ± 0.272) ($P = 0.075$, 95% CI: -0.18 to 0.003) (Figure 1). Although mRNA and protein were simultaneously purified,

Table 1 Clinicopathological characteristics and 14-3-3ε expression in gastric cancer samples (mean ± SD)

Variable	14-3-3ε protein expression				14-3-3ε mRNA expression			
	Total	Ratio T/N	Adjusted *P*-value	95% CI	Total	Relative quantification	Adjusted *P*-value	95% CI
Gender								
Male	10	0.418 ± 0.41	0.210	-0.13 to 0.63	18	1.404 ± 1.43	0.279	-1.38 to 0.40
Female	10	0.667 ± 0.47			13	0.898 ± 1.09		
Onset (yr)								
< 45	6	0.303 ± 0.26	0.052	-0.68 to -0.05	8	0.583 ± 0.31	0.028[a]	-1.49 to -0.25
⩾ 45	14	0.646 ± 0.48			23	1.403 ± 1.45		
Tumor location								
Cardia	3	0.293 ± 0.11	0.067	0.03 to 0.56	4	0.796 ± 0.35	0.177	-0.12 to 1.23
Non-cardia	17	0.587 ± 0.47			27	1.250 ± 1.39		
Laurén classification								
Diffuse-type	4	0.244 ± 0.14	0.024[a]	0.12 to 0.65	13	1.119 ± 1.04	0.785	-0.69 to 1.07
Intestinal-type	16	0.618 ± 0.46			18	1.245 ± 1.49		
Stage								
Early	4	0.517 ± 0.64	0.921	-0.55 to 0.81	4	0.549 ± 0.38	0.046[a]	-1.41 to -0.16
Advanced	16	0.549 ± 0.41			27	1.287 ± 1.37		
Tumor invasion								
T1/T2	8	0.491 ± 0.49	0.674	-0.48 to 0.34	9	0.642 ± 0.33	0.052	-1.40 to -0.13
T3/T4	12	0.578 ± 0.43			22	1.417 ± 1.49		
Lymph node metastasis								
Absent	6	0.672 ± 0.57	0.510	-0.29 to 0.72	8	0.687 ± 0.53	0.069	-1.31 to -0.01
Present	14	0.488 ± 0.39			23	1.367 ± 1.45		
Distant metastasis								
Unknown/absent	16	0.557 ± 0.47	0.754	-0.42 to 0.47	23	0.978 ± 0.99	0.242	-2.25 to 0.51
Present	4	0.486 ± 0.37			8	1.807 ± 1.89		

Differentially expressed between groups, [a]$P < 0.05$. T: Tumor gastric samples; N: Non-neoplastic gastric samples.

no correlation was observed between 14-3-3ε mRNA and protein expression ($R = 0.236$, $P = 0.345$).

The associations between clinicopathological characteristics and 14-3-3ε expression are summarized in Table 1. A significant decrease in 14-3-3ε mRNA level was observed in early-onset GC (at age ⩽ 45 years[31]) compared to late-onset GC ($P = 0.028$, 95% CI: -1.49 to -0.25). Moreover, a tendency towards 14-3-3ε protein down-expression was also observed in early-onset compared to late-onset GC ($P = 0.052$, 95% CI: -0.69 to -0.05).

The 14-3-3ε mRNA level was reduced in early GC compared to advanced GC ($P = 0.046$, 95% CI: -1.41 to -0.16) and tended to present reduced levels with less invasive tumors ($P = 0.052$, 95% CI: -1.40 to -0.13). In contrast, these observations were not observed for protein level.

Concerning the 14-3-3ε protein, we observed that the diffuse-type GC presented reduced expression compared to intestinal-type GC ($P = 0.024$, 95% CI: 0.12-0.65). However, a significant difference was observed between non-neoplastic gastric tissues and intestinal-type GC ($P = 0.045$, 95% CI: -1.55 to -0.29) and diffuse-type GC ($P = 0.024$, 95% CI: -2.22 to -1.14).

DISCUSSION

Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis[32]. The 14-3-3 proteins continue to generate intense interest due to their roles in signal transduction pathways that control cell cycle checkpoints, mitogen-activate protein kinase activation, apoptosis, and regulation of gene expression. 14-3-3 stabilizes non-native conformations of bound ligands to promote their interactions with downstream targets, or facilitates their subsequent modification by kinases and phosphatases[18].

In the present study, we evaluated the 14-3-3ε mRNA and protein expression in gastric carcinogenesis. To the best of our knowledge, no study has evaluated 14-3-3ε expression in this neoplasm. Here, we observed that 14-3-3ε protein expression was reduced in GC samples compared to matched non-neoplastic gastric tissue. Low expression of 14-3-3ε has also been reported in small cell lung cancer[21], laryngeal squamous cell carcinoma[22], and medulloblastoma[23], suggesting a tumor suppressor function. The reduced 14-3-3ε expression may be in part due to the loss of its locus (17p13), which is a common finding in gastric tumors of individuals from the studied population[6].

Little is known about the role of 14-3-3ε in carcinogenesis. One of the major partners of 14-3-3ε is the CDC25 protein. CDC25 protein is virtually inactive during interphase, but undergoes a strong activation at mitosis due to phosphorylation of its N-terminal regulatory domain[33]. 14-3-3ε acts as a negative regulator of CDC25[33,34]. The overexpression of CDC25 proteins has previously been described in GC[35-37]. Thus, the reduced expression of 14-3-3ε in GC cells may enhance the ability of CDC25 to induce mitosis, contributing to the gastric carcinogenesis process.

Several classification systems have been described for

GC. According to the Laurén classification, one of the most used, gastric adenocarcinoma is classified mainly into intestinal and diffuse types[28]. Intestinal-type GC progresses through a number of sequential steps, beginning with atrophic gastritis followed by intestinal metaplasia, intraepithelial neoplasia, and carcinoma[38]. In contrast, diffuse-type GC generally does not evolve from precancerous lesions[39,40]. In the present study, we observed that the expression of 14-3-3ε protein was lower in diffuse-type than in intestinal-type GC, confirming that these two histological GC subtypes follow different genetic pathways and may be two distinct entities[39].

MYC deregulation is a frequent finding in GC[41]. Moreover, MYC immunoreactivity seems to be more frequently detected in intestinal-type than diffuse-type GC[42,43]. Gene amplification is the main mechanism of MYC deregulation in GC[41], and we have previously described a higher frequency of *MYC* locus amplification in intestinal-type than diffuse-type GC[44,45]. Interestingly, one of the MYC target proteins is CDC25, which is negatively regulated by 14-3-3ε. A correlation between MYC and CDC25 has previously been reported in GC[46]. Here, we hypothesize that the relative increase of 14-3-3ε in the subset of tumors of the intestinal-type may be a compensatory mechanism to control the increase of cell proliferation due to MYC and CDC25 action. Moreover, MYC overexpression also induces the production of reactive oxygen species, which may lead to double-stranded DNA breaks and point mutations resulting in genomic instability[47]. Thus, the relative increase in 14-3-3ε in the intestinal-type GC may be also a compensatory response to other oncogenic mutations accumulated in cancer cells, which lead to genomic instability and initiate a DNA damage check-point in cells that contain some functional p53 alleles, as already proposed for other 14-3-3 isoforms[18].

Here, we also described a decrease in 14-3-3ε expression in early-onset compared to late-onset GC. Early-onset GC is observed in < 10% of GC patients, and only 10% of these patients have a positive family history[31]. Most young patients present at an advanced clinical stage similar to elderly patients, so the prognosis in both age groups is poor. However, early-onset GC shows different clinicopathological and molecular profiles compared to late-onset GC, suggesting that they represent a separate entity within gastric carcinogenesis, with genetic factors probably presenting a more important role in early-onset GC patients[48-50]. 14-3-3ε deregulation may have a direct or indirect function in early-onset GC because this protein interacts with several others.

In addition, no correlation was observed between 14-3-3ε mRNA and protein expression, corroborating a previous study of laryngeal squamous cell carcinoma[22]. The lack of correlation between 14-3-3ε protein and mRNA expression patterns indicates the post-translational regulation mechanism involved in this protein expression, and highlights the complexity of the relationship between protein and mRNA expression.

In conclusion, our data suggest that, for the first time, reduced 14-3-3ε may have a role in gastric carcinogenesis, mainly in diffuse-type and early-onset GC. Moreover, further investigations are necessary to understand which proteins interact with 14-3-3ε in these subtypes of GC.

COMMENTS

Background
Gastric cancer (GC) is the fourth most frequent cancer type and the second highest cause of cancer mortality worldwide. Although this neoplasm is a serious public health problem due to its high incidence and mortality, little is known about the molecular events involved in gastric carcinogenesis.

Research frontiers
GC, as with other neoplasms, is a multifactorial disease that results from a combination of environmental factors and the accumulation of generalized and specific genetic and epigenetic alterations. The 14-3-3 protein family has been recently associated with carcinogenesis, but not in the stomach. In this study, the authors evaluated mRNA and protein expression of 14-3-3ε in gastric neoplasms and corresponding non-neoplastic samples.

Innovations and breakthroughs
No previous study has evaluated the gene and protein expression of 14-3-3ε in gastric carcinogenesis. This study demonstrates that 14-3-3ε has a role in gastric carcinogenesis, especially in diffuse-type and early-onset GC.

Applications
14-3-3ε may have a role in gastric carcinogenesis as a tumor suppressor protein.

Terminology
The *YWHAE* gene is located at 17p13.3, a region frequently deleted in gastric neoplasms. This gene encodes the 14-3-3ε protein, one of the mammalian 14-3-3 protein family members that are highly conserved in eukaryotes. This protein family is involved in several biological processes and plays a regulatory role in processes such as apoptotic cell death, mitogenic signal transduction, and cell cycle control.

Peer review
The results report for the first time the expression of 14-3-3ε in GC, both at the protein and mRNA level, and the data obtained reveal reduced 14-3-3ε protein expression in GC. The data contribute to the biology of GC with possible future clinical implications to be tested in a subsequent study in a larger number of GC patients.

REFERENCES

1   **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90

2   **Crew KD**, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; **12**: 354-362

3   **Shah MA**, Ajani JA. Gastric cancer--an enigmatic and heterogeneous disease. *JAMA* 2010; **303**: 1753-1754

4   **Ottini L**, Falchetti M, Lupi R, Rizzolo P, Agnese V, Colucci G, Bazan V, Russo A. Patterns of genomic instability in gastric cancer: clinical implications and perspectives. *Ann Oncol* 2006; **17** Suppl 7: vii97-vi102

5   **Panani AD**. Cytogenetic and molecular aspects of gastric cancer: clinical implications. *Cancer Lett* 2008; **266**: 99-115

6   **Khayat AS**, Guimarães AC, Calcagno DQ, Seabra AD, Lima EM, Leal MF, Faria MH, Rabenhorst SH, Assumpção PP, Demachki S, Smith MA, Burbano RR. Interrelationship between TP53 gene deletion, protein expression and chromosome 17 aneusomy in gastric adenocarcinoma. *BMC Gastroenterol* 2009; **9**: 55

7   **Leal MF**, Martins do Nascimento JL, da Silva CE, Vita Lamarão MF, Calcagno DQ, Khayat AS, Assumpção PP, Cabral IR, de Arruda Cardoso Smith M, Burbano RR. Establishment and conventional cytogenetic characterization of three gastric cancer cell lines. *Cancer Genet Cytogenet* 2009; **195**: 85-91

8   **Ribeiro HF**, Alcântara DF, Matos LA, Sousa JM, Leal MF, Smith MA, Burbano RR, Bahia MO. Cytogenetic characterization and evaluation of c-MYC gene amplification in PG100, a new Brazilian gastric cancer cell line. *Braz J Med Biol Res* 2010; **43**: 717-721

9   **Leal MF**, Calcagno DQ, Borges da Costa Jde F, Silva TC, Khayat AS, Chen ES, Assumpção PP, de Arruda Cardoso Smith M, Burbano RR. MYC, TP53, and chromosome 17 copy-number alterations in multiple gastric cancer cell lines and in their parental primary tumors. *J Biomed Biotechnol* 2011; **2011**: 631268

10   **Szymańska K**, Hainaut P. TP53 and mutations in human cancer. *Acta Biochim Pol* 2003; **50**: 231-238

11   **Aitken A**. 14-3-3 proteins: a historic overview. *Semin Cancer Biol* 2006; **16**: 162-172

12   **Fu H**, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol* 2000; **40**: 617-647

13   **van Hemert MJ**, Steensma HY, van Heusden GP. 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. *Bioessays* 2001; **23**: 936-946

14   **Pozuelo Rubio M**, Geraghty KM, Wong BH, Wood NT, Campbell DG, Morrice N, Mackintosh C. 14-3-3-affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking. *Biochem J* 2004; **379**: 395-408

15   **Satoh J**, Nanri Y, Yamamura T. Rapid identification of 14-3-3-binding proteins by protein microarray analysis. *J Neurosci Methods* 2006; **152**: 278-288

16   **Rajagopalan S**, Sade RS, Townsley FM, Fersht AR. Mechanistic differences in the transcriptional activation of p53 by 14-3-3 isoforms. *Nucleic Acids Res* 2010; **38**: 893-906

17   **Tzivion G**, Avruch J. 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. *J Biol Chem* 2002; **277**: 3061-3064

18   **Yaffe MB**. How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the molecular anvil hypothesis. *FEBS Lett* 2002; **513**: 53-57

19   **Aitken A**. Functional specificity in 14-3-3 isoform interactions through dimer formation and phosphorylation. Chromosome location of mammalian isoforms and variants. *Plant Mol Biol* 2002; **50**: 993-1010

20   **Jones DH**, Ley S, Aitken A. Isoforms of 14-3-3 protein can form homo- and heterodimers in vivo and in vitro: implications for function as adapter proteins. *FEBS Lett* 1995; **368**: 55-58

21   **Konishi H**, Nakagawa T, Harano T, Mizuno K, Saito H, Masuda A, Matsuda H, Osada H, Takahashi T. Identification of frequent G(2) checkpoint impairment and a homozygous deletion of 14-3-3epsilon at 17p13.3 in small cell lung cancers. *Cancer Res* 2002; **62**: 271-276

22   **Che XH**, Chen H, Xu ZM, Shang C, Sun KL, Fu WN. 14-3-3epsilon contributes to tumour suppression in laryngeal carcinoma by affecting apoptosis and invasion. *BMC Cancer* 2010; **10**: 306

23   **Cvekl A**, Zavadil J, Birshtein BK, Grotzer MA, Cvekl A. Analysis of transcripts from 17p13.3 in medulloblastoma suggests ROX/MNT as a potential tumour suppressor gene. *Eur J Cancer* 2004; **40**: 2525-2532

24   **Liang S**, Xu Y, Shen G, Liu Q, Zhao X, Xu Z, Xie X, Gong F, Li R, Wei Y. Quantitative protein expression profiling of 14-3-3 isoforms in human renal carcinoma shows 14-3-3 epsilon is involved in limitedly increasing renal cell proliferation. *Electrophoresis* 2009; **30**: 4152-4162

25   **Cao L**, Cao W, Zhang W, Lin H, Yang X, Zhen H, Cheng J, Dong W, Huo J, Zhang X. Identification of 14-3-3 protein isoforms in human astrocytoma by immunohistochemistry. *Neurosci Lett* 2008; **432**: 94-99

26   **Liu Y**, Tian RF, Li YM, Liu WP, Cao L, Yang XL, Cao WD, Zhang X. The expression of seven 14-3-3 isoforms in human meningioma. *Brain Res* 2010; **1336**: 98-102

27   **Lukashova-v Zangen I**, Kneitz S, Monoranu CM, Rutkowski S, Hinkes B, Vince GH, Huang B, Roggendorf W. Ependymoma gene expression profiles associated with histological subtype, proliferation, and patient survival. *Acta Neuropathol* 2007; **113**: 325-337

28   **Lauren P**. 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* 1965; **64**: 31-49

29   **Sobin LH**, Wittekind CH. TNM classification of malignant tumours. 6th ed. New York: Wiley-Liss, 2002

30   **Pfaffl MW**. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; **29**: e45

31   **Kokkola A**, Sipponen P. Gastric carcinoma in young adults. *Hepatogastroenterology* 2001; **48**: 1552-1555

32   **Hanahan D**, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57-70

33   **Kumagai A**, Yakowec PS, Dunphy WG. 14-3-3 proteins act as negative regulators of the mitotic inducer Cdc25 in Xenopus egg extracts. *Mol Biol Cell* 1998; **9**: 345-354

34   **Kumagai A**, Dunphy WG. Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25. *Genes Dev* 1999; **13**: 1067-1072

35   **Kudo Y**, Yasui W, Ue T, Yamamoto S, Yokozaki H, Nikai H, Tahara E. Overexpression of cyclin-dependent kinase-activating CDC25B phosphatase in human gastric carcinomas. *Jpn J Cancer Res* 1997; **88**: 947-952

36   **Zhang XM**, Yao GY, Zhang BY, Wang LL, Zhao M. [Study on the expression and significance of Galectin-3 and CDC25B mRNA in human gastric carcinoma]. *Zhonghua Yixue Yichuanxue Zazhi* 2009; **26**: 288-292

37   **Takahashi H**, Murai Y, Tsuneyama K, Nomoto K, Okada E, Fujita H, Takano Y. High labeling indices of cdc25B is linked to progression of gastric cancers and associated with a poor prognosis. *Appl Immunohistochem Mol Morphol* 2007; **15**: 267-272

38   **Correa P**. Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* 1992; **52**: 6735-6740

39   **Tahara E**. Genetic pathways of two types of gastric cancer. *IARC Sci Publ* 2004; 327-349

40   **Smith MG**, Hold GL, Tahara E, El-Omar EM. Cellular and molecular aspects of gastric cancer. *World J Gastroenterol* 2006; **12**: 2979-2990

41   **Calcagno DQ**, Leal MF, Assumpcao PP, Smith MA, Burbano RR. MYC and gastric adenocarcinoma carcinogenesis. *World J Gastroenterol* 2008; **14**: 5962-5968

42   **Kozma L**, Kiss I, Hajdú J, Szentkereszty Z, Szakáll S, Ember I. C-myc amplification and cluster analysis in human gastric carcinoma. *Anticancer Res* 2001; **21**: 707-710

43   **Yang GF**, Deng CS, Xiong YY, Gong LL, Wang BC, Luo J. Expression of nuclear factor-kappa B and target genes in gastric precancerous lesions and adenocarcinoma: association with Helicobactor pylori cagA (+) infection. *World J Gastroenterol* 2004; **10**: 491-496

44   **Burbano RR**, Assumpção PP, Leal MF, Calcagno DQ, Guimarães AC, Khayat AS, Takeno SS, Chen ES, De Arruda Cardoso Smith M. C-MYC locus amplification as metastasis predictor in intestinal-type gastric adenocarcinomas: CGH study in Brazil. *Anticancer Res* 2006; **26**: 2909-2914

45   **Calcagno DQ**, Leal MF, Taken SS, Assumpção PP, Demachki S, Smith Mde A, Burbano RR. Aneuploidy of chromosome 8 and C-MYC amplification in individuals from northern Brazil with gastric adenocarcinoma. *Anticancer Res* 2005; **25**: 4069-4074

46   **Xing X**, Chen J, Chen M. Expression of CDC25 phosphatases in human gastric cancer. *Dig Dis Sci* 2008; **53**: 949-953

47   **Prochownik EV**, Li Y. The ever expanding role for c-Myc in promoting genomic instability. *Cell Cycle* 2007; **6**: 1024-1029

48   **Carvalho R**, Milne AN, van Rees BP, Caspers E, Cirnes L, Figueiredo C, Offerhaus GJ, Weterman MA. Early-onset gastric carcinomas display molecular characteristics distinct from gastric carcinomas occurring at a later age. *J Pathol*

2004; **204**: 75-83

49   **Milne AN**, Carvalho R, Morsink FM, Musler AR, de Leng WW, Ristimäki A, Offerhaus GJ. Early-onset gastric cancers have a different molecular expression profile than conventional gastric cancers. *Mod Pathol* 2006; **19**: 564-572

50   **Milne AN**, Sitarz R, Carvalho R, Carneiro F, Offerhaus GJ. Early onset gastric cancer: on the road to unraveling gastric carcinogenesis. *Curr Mol Med* 2007; **7**: 15-28