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Cell-type specificity of β-actin expression and its clinicopathological correlation in gastric adenocarcinoma

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Abstract

AIM: To investigate cell type specific distribution of β-actin expression in gastric adenocarcinoma and its correlation with clinicopathological parameters.

METHODS: β-actin is a housekeeping gene, frequently used as loading control, but, differentially expresses in cancer. In gastric cancer, an overall increased expression of β-actin has been reported using tissue disruptive techniques. At present, no histological data is available to indicate its cell type-specific expression and distribution pattern. In the present study, we analyzed β-actin expression and distribution in paired normal and tumor tissue samples of gastric adenocarcinoma patients using immunohistochemistry (IHC), a tissue non-disruptive technique as well as tissue disruptive techniques like reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting. Correlation of β-actin level with clinicopathological parameters was done using univariate analysis.

RESULTS: The results of this study showed significant overexpression, at both mRNA and protein level in tumor tissues as confirmed by RT-PCR (1.47 ± 0.13 *vs* 2.36 ± 0.16; $P < 0.001$) and western blotting (1.92 ± 0.26 *vs* 2.88 ± 0.32; $P < 0.01$). IHC revealed that β-actin expression is majorly distributed between epithelial and inflammatory cells of the tissues. Inflammatory cells showed a significantly higher expression compared to epithelial cells in normal (2.46 ± 0.13 *vs* 5.92 ± 0.23, $P < 0.001$), as well as, in tumor tissues (2.79 ± 0.24 *vs* 6.71 ± 0.14, $P < 0.001$). Further, comparison of immunostaining between normal and tumor tissues revealed that both epithelial and inflammatory cells overexpress β-actin in tumor tissues, however, significant difference was observed only in inflammatory cells (5.92 ± 0.23 *vs* 6.71 ± 0.14, $P < 0.01$). Moreover, combined expression in epithelial and inflammatory cells also showed significant increase (4.19 ± 0.15 *vs* 4.75 ± 0.14, $P < 0.05$) in tumor tissues. In addition, univariate analysis showed a positive correlation of β-actin level of inflammatory cells with tumor grade ($P < 0.05$) while epithelial cells exhibited negative correlation ($P > 0.05$).

CONCLUSION: In gastric cancer, β-actin showed an overall higher expression predominantly contributed by inflammatory or tumor infiltrating immune cells of the tissue microenvironment and correlates with tumor grade.

Key words: Gastric cancer; β-actin; Immunohistochemistry; Epithelial cells; Inflammatory cells; Tumor infiltrating immune cells; Adjacent mucosa; Resection margin

Core tip: Clinical implications of β-actin have been ignored despite the reports of its differential expression in cancer. The present study provides first histological evidence of an overall increase in β-actin expression in gastric cancer compared to histologically normal adjacent mucosa. Inflammatory and epithelial cells of tumor tissues showed differential pattern of β-actin expression and correlated with tumor grade. Further, overexpression of β-actin was predominantly contributed by inflammatory cells, suggesting further extensive studies to use β-actin as a diagnostic and prognostic biomarker and target of direct or indirect chemotherapeutic intervention.

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INTRODUCTION

Gastric cancer (GC) incidence and mortality is decreasing over several decades, however, it still remains the fourth most common type of cancer and the second leading cause of cancer related deaths worldwide[1]. In India, there are limited epidemiological studies on gastric cancer which also suffers from the juvenile state of cancer registries and under-reporting of cases. However, similar to global trend, Indian registries have also observed statistically significant reducing trend in stomach cancer cases in last 20-years with approximately 35675 estimated case in 2001; about 3.91% of global incidence[2,3]. A radical D2 gastrectomy and more recently radical surgery along with perioperative chemotherapy holds the best prospect of a cure in gastric cancer[4,5]. However, delayed presentation and thus diagnosis owing to the non-specific symptoms often preclude the possibility of a curative surgical resection making palliative chemotherapy and other measures as the treatment mainstay in these patients. The development of chemoresistance[6] is also an increasingly appreciated phenomenon contributing to the poor outcomes in the disease. Therefore, an improved understanding of GC molecular biology to ascertain new potential tumor biomarkers useful to guide patient management and develop new therapeutic options is essential.

β-actin is a housekeeping gene and an obligatory part of the cell cytoskeleton. It expresses in almost all eukaryotic cells and is involved in controlling basic housekeeping functions such as development and maintenance of cell shape, cell migration, cell division, growth and signaling. It also plays a critical role in transcriptional regulation, mRNA transport, mRNA processing and chromatin remodeling[7,8]. Further, β-actin is also one of the most commonly used endogenous reference loading controls in laboratory techniques to normalize gene and protein expressions as it is believed to have constant expression levels in different cellular, experimental and physiological conditions. However, growing evidences have demonstrated its differential expression in certain situations like growth, ageing, differentiation, developmental stages and diseases like asthma, Alzheimer's disease, congenital heart disease and cancer[9].

In comparison to normal, an overall differential expression of β-actin is reported in multiple cancers[10-16]. The methodologies used in earlier tissue based studies make it difficult to answer, which specific cell type out of the heterogeneous population of cells in a tissue, is responsible for altered expression of β-actin in cancer. To date, no histological studies have been conducted to provide informations about the pattern of β-actin expression and distribution in different cell types of the normal and tumor tissues. Such information of β-actin expression in a tissue will provide a better understanding of its role in carcinogenesis, its correlation with clinicopathological parameters and its potential to be used as a tumor biomarker or therapeutic target. β-actin polymerization or remodeling plays a crucial role in a cell's physiology and drugs altering the dynamics of β-actin have been studied as potential chemotherapeutic agent, however, clinical implications of these drugs are yet to be established[17-19]. The present study aimed to provide histological evidence of β-actin expression and distribution in specific cell types of gastric adenocarcinoma and its correlation with clinicopathological parameters. A total 31 paired (from the same patient) tumor and corresponding adjacent histopathologically normal mucosa tissue samples were analyzed using reverse transcription polymerase chain reaction (RT-PCR), western blotting and immunohistochemistry (IHC). We report, an overall higher expression of β-actin in gastric cancer at both mRNA and protein level. Further, as per the best of our knowledge, IHC analysis revealed it for the first time that overall higher expression of β-actin in gastric cancer is majorly contributed by tumor inflammatory cells (5.92 ± 0.23 *vs* 6.71 ± 0.14, $P < 0.01$), though, tumor epithelial cells (2.46 ± 0.13 *vs* 2.79 ± 0.24, $P > 0.05$) also showed overexpression. Moreover, univariate analysis showed a positive correlation between β-actin levels of inflammatory cells and tumor grade ($P < 0.05$) while epithelial cells exhibited a negative correlation ($P > 0.05$).

Table 1 Scoring system for β-actin immune-staining

Percent positivity of stained cells	IHC score	Staining intensity	IHC score
0%	0	None	0
< 25%	1	Weak	1
25%-50%	2	Moderate	2
50%-75%	3	Strong	3
75%-100%	4		

Total IHC score = IHC score of percent positivity + IHC score of staining intensity
Average total IHC score = (Total IHC score of EC + Total IHC score of IC)/2

EC: Epithelial cells; IC: Inflammatory cells; IHC: Immunohistochemistry.

MATERIALS AND METHODS

Tissue samples and histopathological analysis

Surgically resected fresh tissues of 5 and formalin-fixed paraffin-embedded tissue blocks of 26 gastric adenocarcinoma patients were collected from ICMR-tumor tissue repository of Tata Memorial Hospital, Mumbai, India. Surgically resected tissues were frozen immediately in liquid nitrogen, and then stored at -80 ℃ until required for experimental use. Form each patient, tumor and apparently normal adjacent gastric mucosa proximal and distal to the tumor was collected, however, only either one of the mucosa was used in the study depending upon their maximum resection-margin distance from the tumor site. All tumor samples had more than 60% tumor content, as confirmed by a blinded specialist gastrointestinal pathologist. The adjacent mucosa was confirmed to be free of tumor for all surgically resected fresh tissues and 24 (out of 26) formalin-fixed paraffin-embedded tissues on histopathological analysis. Surgically resected fresh tissues (n = 5) were used for RT-PCR and western blot analysis while formalin-fixed paraffin-embedded tissues were used for IHC analysis and correlational study. The protocol was reviewed and approved by institutional review board and ethics committee. All patients provided a written informed consent.

Cell lines and culture conditions

Gastric cancer cell lines AGS (ATCC® Number: CRL-1739™; moderately differentiated) and KATO Ⅲ (ATCC® Number: HTB-103™; signet ring cell carcinoma) was used. AGS and KATO Ⅲ cells were cultured in RPMI1640 (Invitrogen) and F12K (Himedia) media respectively at 37 ℃ with 5% CO_2 supplemented with 10% FBS, 100U/ml penicillin, 100 mg/mL streptomycin (Sigma). For trypsinization, 0.05% trypsin-EDTA (Sigma) was used for both the cell lines.

Total RNA isolation and RT-PCR

Total RNA from 25 mg of tissues was extracted (Thermo scientific, 0731) and 10 μg of which was used for cDNA synthesis (Fermentas life sciences, K1632). RT-PCR amplification was done using specific primers for β-actin (F: 5' AGAAAATCTGGCACCACACC 3' and R: 5' CCATCTCTTGCTCGAAGTCC 3') and 18S rRNA (F: 5' AAACGGCTACCACATCCAAG 3' and R: 5' CCTCCAATGGATCCTCGTTA 3') with an initial denaturation step at 95 ℃ for 2 min, followed by 20 cycles of denaturation at 95 ℃ for 45 min, primer annealing at 55 ℃ for 30 s, primer extension at 72 ℃ for 30 s and a final extension at 72 ℃ for 10 min. Each reaction was performed in triplicate. Amplified products were resolved on 1% agarose gels and visualized by Ethidium bromide staining.

Total protein lysate preparation and western blotting

Total cell lysate was prepared from 100 mg of tissue using Lysis buffer (20 mmol/L Tris-Cl pH 8, 2 mmol/L EDTA pH 8, 10 mmol/L EGTA, 5 mmol/L $MgCl_2$, 0.1% Triton X-100, 1 mmol/L Sodium orthovandate, 1 mmol/L Sodium fluoride, 20 mmol/L β-Glycerophosphate, 1 mmol/L DTT, 1 mmol/L PMSF, 10 ug/mL Leupeptin, 10 ug/mL Aprotinin). Tissues were powdered in liquid nitrogen, homogenized in 2 mL of lysis buffer and then kept at 4 ℃ for 30 min with intermittent mixing. Further, the total cell lysate from gastric cancer cell lines AGS and KATO Ⅲ was prepared using MKK lysis buffer[20]. The homogenate was then centrifuged at 100000 xg and supernatant was collected as total cell lysate and stored at -20 ℃. For western blotting, total cell lysate was first estimated using Bradford method and then 75 μg of protein was loaded on 10% SDS-PAGE and transferred to PVDF membrane. Anti-β-actin antibody (Sigma, A5316) was used at the dilution of 1:10000.

Immunohistochemistry

Immunohistochemical staining using VECTASTAIN® ABC kit (Vector Lab, P6200) was performed. Formalin-fixed paraffin-embedded tissue blocks were sectioned at a thickness of 5 μm and mounted on poly-L-lysine coated glass slides. The sections were deparaffinized through a graded series of xylene and rehydrated through a graded series of absolute alcohol to distilled water. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature for 30 min in dark. Microwave antigen retrieval was carried out with 0.01 mol/L Sodium citrate buffer (pH 6.0). Anti-β-actin monoclonal antibody (Sigma, A5316) was applied for 16 h at 4 ℃ at the dilution of 1:1000. Immunoreactive proteins were chromogenically detected with Diaminobenzidine (DAB) (Sigma, D5537). The sections were counterstained with Harris's hematoxylene and then dehydrated and mounted. In parallel, control staining was performed without adding primary antibody.

Evaluation of Immunohistochemistry

The cytoplasmic immunohistochemical staining of β-actin was scored semi-quantitavely for epithelial and inflammatory cells as described in a previous study by Yip *et al*[21]. "IHC score", "Total IHC score" and "Average Total IHC score" were calculated by taking the account into

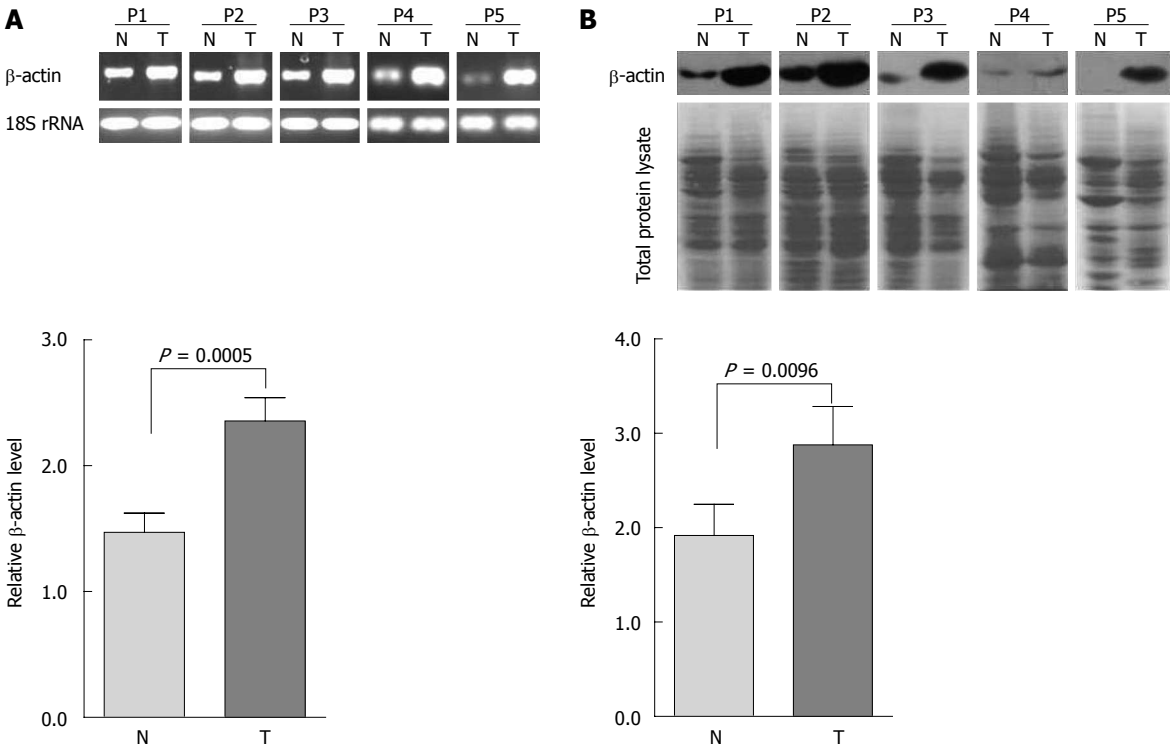

Figure 1 Comparison of overall β-actin level in gastric normal and tumor tissue (*n* = 5). A: Reverse transcription polymerase chain reaction analysis of β-actin and 18S rRNA was used as an internal loading control (upper panel). Band intensities of β-actin mRNA were normalized with 18S rRNA band intensity of respective lanes and obtained values were plotted (lower panel); B: Western blot analysis of β-actin (upper panel). Band intensity of blot was normalized with the total protein lysate intensity of respective lanes and obtained values were plotted (lower panel). Statistical significance was tested using "paired *t*-test". N: Normal; T: Tumor.

percentage of immunostained cells and staining intensity (Table 1). Total IHC score of 2 and above was considered as positive immunoreactivity. Total IHC score ranges from 2 to 7 and further grouped into: low (score 2 and 3), intermediate (score 4 and 5) and high (score 6 and 7). The immunohistochemical staining was examined by two independent researchers one of whom is a senior consultant pathologist to ensure the evaluations were performed properly and accurately. Both the researchers were blinded to all clinicopathological and outcome variables.

Statistical analysis

To test the statistical significance of β-actin differential expression between normal and tumor paired tissue samples by RT-PCR or western blotting and IHC, paired t-test with one-tailed *P*-value and Wilcoxon matched pair test with two-tailed *P*-value was applied respectively. To establish statistical correlation between clinicopathological parameters and β-actin expression level Mann-whitney and Krukal-wallis test with two-tailed *P*-value was applied. Wherever applicable, data is presented as mean ± SE and $P < 0.05$ was considered as statistically significant.

RESULTS

Overexpression of β-actin in tumor compared to normal gastric tissue

To detect an overall relative mRNA and protein expression of β-actin between gastric normal and tumor tissues, RT-PCR and western blot was performed on curatively resected fresh tissues from 5 randomly selected gastric cancer patients. Relative β-actin mRNA and protein levels were expressed after normalizing their intensities with the intensity of 18S rRNA and total protein respectively. Intensities were calculated by using ImageJ software[22]. Compared to normal, RT-PCR and western blot analysis showed a significant higher expression of β-actin level in tumor tissues both at mRNA (1.47 ± 0.13 *vs* 2.36 ± 0.16; $P < 0.001$) and protein level (1.92 ± 0.26 *vs* 2.88 ± 0.32; $P < 0.01$) as confirmed by paired *t*-test (Figure 1A and B).

Overexpression of β-actin in tumor tissue is predominantly contributed by inflammatory cells

After confirming an overall higher expression of β-actin in tumor compared normal gastric tissues, distribution of β-acting expression was studied in different cell types of the tissues on formalin-fixed paraffin-embedded tissue blocks using IHC. Study was carried out in paired normal and tumor tissues from 24 gastric adenocarcinoma patients. Analysis of immunostained tissue sections revealed that the β-actin immunostaining was majorly distributed between epithelial and inflammatory cells (Figure 2A). "Total IHC score" for β-actin immunostaining was calculated for both epithelial and inflammatory cells as mentioned in Table 1 and frequency of tissue sample for a particular total IHC score was determined (Table 2). For both normal and tumor tissues, analysis of frequency table showed that the most of the samples scored low to

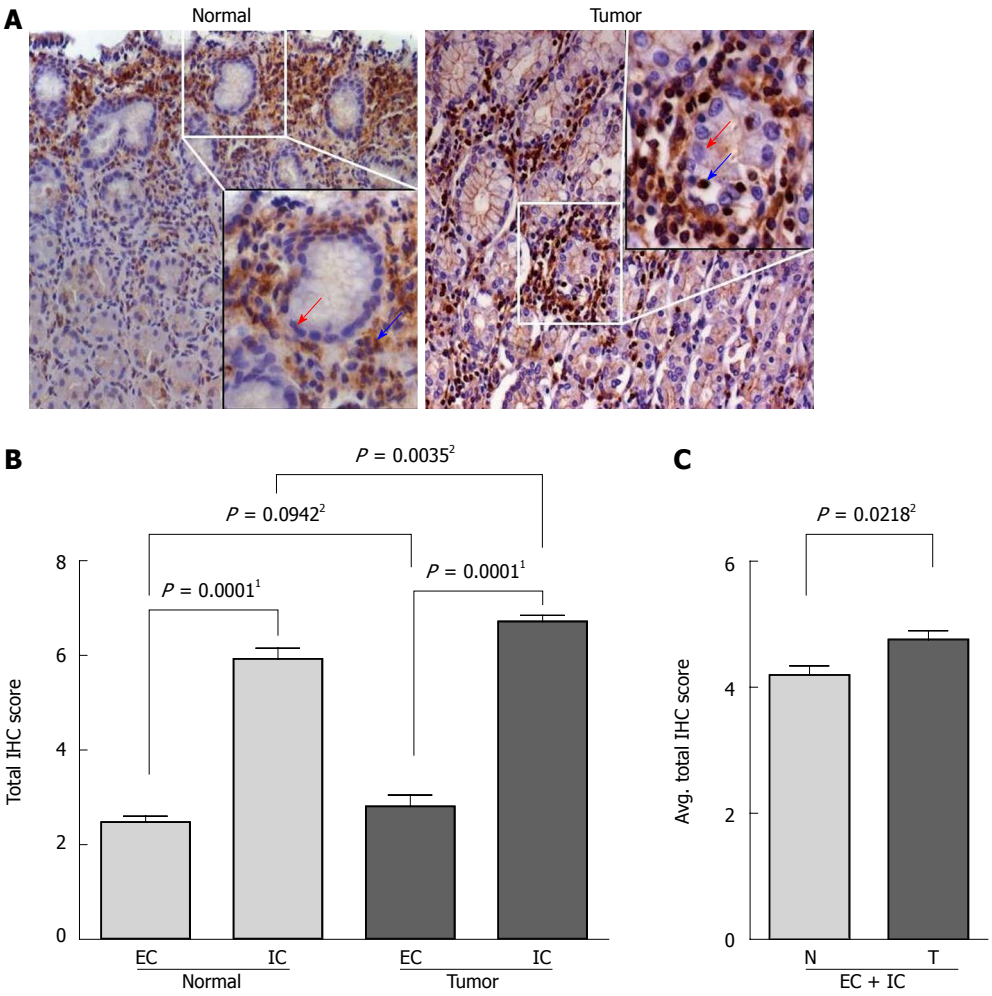

Figure 2 Histological analysis of β-actin in gastric normal and tumor tissues (*n* = 24). "Total IHC score" and "Average total IHC score" were calculated as described in Table 1. A: Representative pictures of β-actin immuno-staining of normal (left panel) and tumor (right panel) tissues showed β-actin expression is majorly distributed between epithelial (red arrow) and inflammatory (blue arrow) cells. The image is taken at 20 × magnification; B: "Total IHC score" of EC and IC of normal (N) and tumor (T) tissues were plotted; C: "Average total IHC score" for normal and tumor tissues were plotted. [1]Mann-Whitney test; [2]Wilcoxon matched pair test. IHC: Immunohistochemistry; EC: Epithelial cells; IC: Inflammatory cells.

Table 2 Frequency of samples with respect to total immunohistochemistry score of β-actin *n* (%)

β-actin immune-positive cells in tissues		Total IHC score (*n* = 24)					
		Low		Intermediate		High	
		2	3	4	5	6	7
Epithelial cells	Normal tissue	15 (63)	7 (29)	2 (8)	0 (0)	0 (0)	0 (0)
	Tumor tissue	16 (67)	2 (8)	3 (13)	3 (13)	0 (0)	0 (0)
Inflammatory cells	Normal tissue	0 (0)	0 (0)	3 (13)	7 (29)	3 (13)	11 (46)
	Tumor tissue	0 (0)	0 (0)	1 (4)	0 (0)	4 (17)	19 (79)

IHC: Immunohistochemistry.

intermediate "total IHC score" for β-actin immunostaining of epithelial cells while in case of inflammatory cells most of the samples scored Intermediate to high "total IHC score".

Comparison of "total IHC scores" showed that inflammatory cells express significantly higher level of β-actin compared to the epithelial cells in both normal (2.46 ± 0.13 *vs* 5.29 ± 0.23, $P < 0.001$) and tumor (2.76 ± 0.24 *vs* 6.70 ± 0.14, $P < 0.001$) tissues as confirmed by Mann-whitney test (Figure 2B). Furthermore, tumor tissues express relatively higher level of β-actin compared to normal in both epithelial and inflammatory cells, however, difference between epithelial cells was not significant (2.46 ± 0.13 *vs* 2.79 ± 0.24, $P > 0.05$) whereas inflammatory cells differed significantly (5.92 ± 0.23 *vs* 6.71 ± 0.14, $P < 0.01$) as confirmed by Wilcoxon matched-pair test (Figure 2B).

As overall β-actin level in a tissue will be a combined

Table 3 Univariate analysis of β-actin immunostaining with clinicopathological parameters *n* (%)

Clinicopathological parameters	Groups		Epithelial cells (total IHC score) *P* value	Inflammatory cells (total IHC score) *P* value	Epithelial + Inflammatory cells (avg. total IHC score) *P* value
Age (yr)					
	≤ 50	11 (42)	0.4933[1]	0.2724[1]	0.2941[1]
	> 50	15 (58)			
Sex					
	Male	20 (77)	0.9721[1]	0.2724[1]	0.5275[1]
	Female	6 (23)			
Tumor grade					
	WD	0 (0)	0.6089[2]	0.0168[2]	0.8393[2]
	MD	4 (15)			
	PD	14 (54)			
	Mucinous	0 (0)			
	SRC	8 (31)			
Depth of invasion[3]					
	T1	2 (8)	0.5446[2]	0.6618[2]	0.8804[2]
	T2	2 (8)			
	T3	13 (52)			
	T4	8 (32)			
Lymph Node status[3]					
	N0	6 (24)	0.7510[2]	0.6293[2]	0.5426[2]
	N1	8 (32)			
	N2	8 (32)			
	N3	3 (12)			
Treatment Modality[3]					
	Surgery	14 (56)	0.3542[1]	0.8135[1]	0.2910[1]
	NACT + surgery	11 (44)			

[1]Mann Whitney Test; [2]Kruskal Wallis Test; [3]TNM staging and Treatment modality information was available for only 25 (out of 26) patients. $P < 0.05$ indicates statistically significant difference. IHC: Immunohistochemistry; MD: Moderately differentiated; PD: Poorly differentiated; SRC: Signet ring cell carcinoma.

result of its expression in all cell types of the tissue, therefore, we asked, whether our IHC analysis corroborates with our RT-PCR and western blot data showing an overall higher expression of β-actin in tumor tissues? To answer this, we compared "average total IHC score" (average of "total IHC scores" of epithelial and inflammatory cells) of normal and tumor tissue. IHC analysis supports the results of RT-PCR and western blotting and also showed a significant increase of β-actin expression in tumor tissues (4.19 ± 0.15 *vs* 4.75 ± 0.14, $P < 0.05$) compared to normal (Figure 2C).

Correlation of β-actin expression with clinicopathological parameters

A total 26 non-metastatic gastric adenocarcinoma cases were examined and analyzed. Although, only inflammatory cells showed significant increase in β-actin level of tumor tissues; for correlational studies, epithelial cells were also considered because they have also shown an increase in tumor compared to normal tissues (Figure 2B). Univariate analysis was performed to correlate "total IHC score" and "average total IHC score" of epithelial and inflammatory cells for β-actin immunostaining with clinicopathological parameters like age, sex, tumor grade, depth of invasion, lymph node status and mode of treatment. The associations between β-actin expression and clinicopathological parameters are shown in Table 3. Epithelial and overall level of β-actin did not show any significant correlation with any of the clinicopathological parameters while β-actin level of inflammatory cells showed significant correlation with tumor grade or WHO classification ($P < 0.05$). Further, identification of pattern and statistical significance of β-actin level in inflammatory cells of tumor tissues of different tumor grades: moderately differentiated (MD), poorly differentiated (PD) and signet ring cell carcinoma (SRC) was carried out. The results showed a positive correlation of β-actin level with tumor grade (Figure 3A) with significantly higher level in PD (6.25 ± 0.22 *vs* 6.79 ± 0.21, $P < 0.05$) and SRC (6.25 ± 0.22 *vs* 6.88 ± 0.14, $P < 0.05$) compared to MD; however, PD to SRC difference was not significant (6.79 ± 0.21 *vs* 6.88 ± 0.14, $P > 0.05$). In addition, low level of β-actin in signet ring cell carcinoma (a type of poorly differentiated cell) cell line KATO Ⅲ compared to moderately differentiate gastric adenocarcinoma cell line AGS (Figure 3B) attracted us to look for the pattern of β-actin expression of tissue epithelial cells with tumor grade. β-actin level in tissue epithelial cells followed a similar pattern of cell lines and decreases from MD to PD and to SRC (Figure 3C), a negative correlation with tumor grade, though insignificant.

The SRC is a type of poorly differentiated adenocarcinoma, therefore, SRC and PD was combined together and analyzed for their β-actin expression in epithelial and inflammatory cells compared to MD (Figure 3A and C). The significance of differential expression of β-actin in-

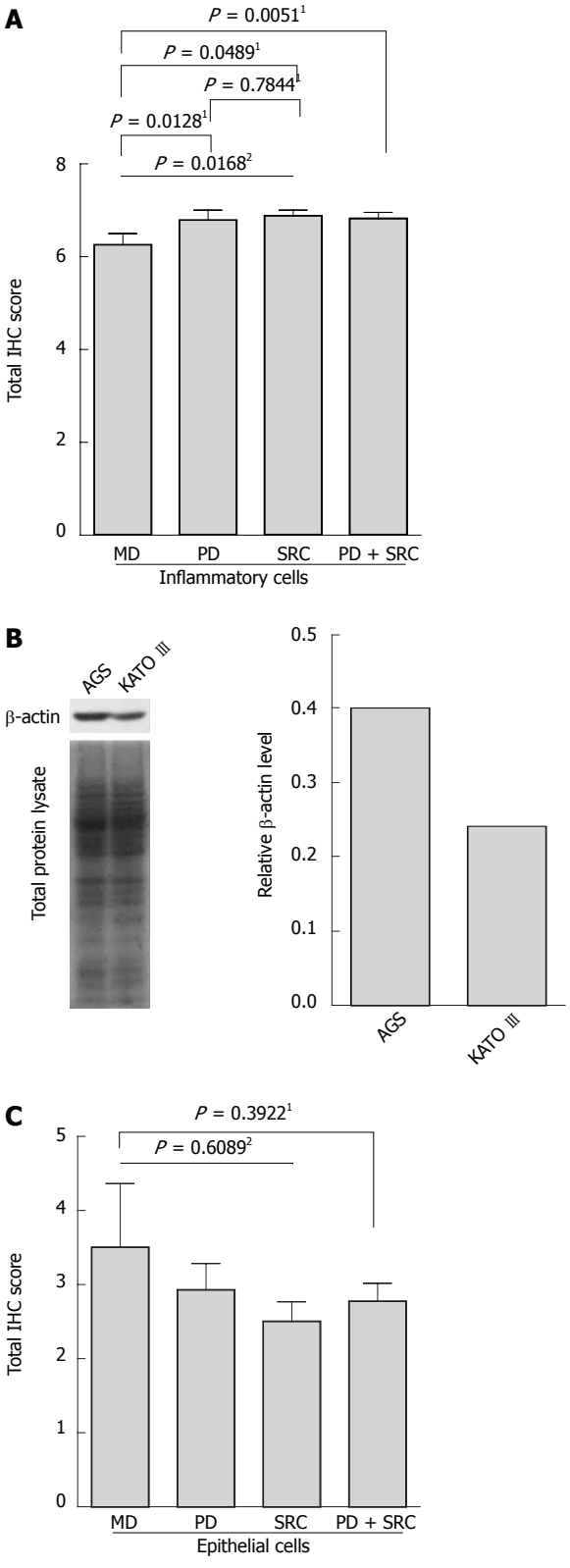

Figure 3 Correlation of β-actin expression with tumor grade. A: "Total IHC scores" of β-actin immunostaining in inflammatory cells were correlated with tumor grade; B: β-actin expression between gastric cancer cell lines AGS and KATO Ⅲ was analyzed using western blotting (right panel). Blot intensities were normalized with the intensity of total protein lysate of respective lanes and obtained values from three independent experiments were plotted (left panel); C: "Total IHC scores" of β-actin immunostaining in epithelial cells were correlated with tumor grade. [1]Mann-Whitney test; [2]Kruskal-Wallis test.

creased both in case of inflammatory cells ($P = 0.0168$ to $P = 0.0051$) and epithelial cells ($P = 0.6089$ to $P = 0.3922$), further confirming the association of β-actin expression with tumor grade in gastric adenocarcinoma.

DISCUSSION

β-actin has been reported to be differentially expressed in multiple cancers[10-16] and suggested as a possible target for chemotherapy[17-19]. These studies signify the potential of β-actin to be considered as a tumor biomarker. Till date, only overall level of varying expression of β-actin in cancer has been reported at the mRNA and protein level by "tissue disruptive techniques", where whole tissue with heterogeneous population of cells crushed and lysed, therefore, observed differential level of β-actin can not be attributed to a specific cell type. The present study, along with tissue disruptive techniques (RT-PCR and western blotting) provides histological evidences (IHC) of differential expression and distribution of β-actin in different cell types of gastric adenocarcinoma.

β-actin overexpression in tumor compared to normal tissues at mRNA level was most consistent and significant as evident by comparing *P*-values of RT-PCR (1.47 ± 0.13 *vs* 2.36 ± 0.16; $P < 0.001$) and western blot (1.92 ± 0.26 *vs* 2.88 ± 0.32; $P < 0.01$) analysis (Figure 1A and B). Therefore, the significant overexpression of β-actin at mRNA level in gastric cancer suggests its deregulation at the level of transcription or mRNA turnover. Earlier reports have also shown β-actin overexpression in colorectal, pancreatic, esophageal, hepatic and gastric cancers patients using tissue disruptive techniques. Molecular mechanism of β-actin transcription control is still unclear, however, CpG island hypermethylation of β-actin promoter has been found to be a negative regulator of expression[23]. Further, rapid upregulation in β-actin transcription in response to mitogenic stimuli including epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and platelet derived growth factor[24-26] have also been reported. In addition, miR-145, miR-206 and miR-466a are known to target and degrade β-actin mRNA, therefore, playing a critical role in altering its mRNA turnover[27-30]. Functionally, β-actin plays a predominant role in cell migration as its overexpression is observed in cells with metastatic potential compared to non-metastatic or cells with less metastatic potential; for example, metastatic variants of human colon adenocarcinoma cell line LS180[15], hepatoma morris 5123[31] and human invasive melanoma cells[32] overexpress β-actin. Collectively, our results along with the existing literature suggest, β-actin transcription is tightly regulated in a normal cell, required for its diverse and critical functions in cell's physiology and its deregulation may have an important role in carcinogenesis.

Immunohistochemistry analysis ($n = 24$) shows an overall increase (4.19 ± 0.15 *vs* 4.75 ± 0.14, $P < 0.05$) in β-actin expression in tumor compared to normal gastric

adenocarcinoma tissues (Figure 2C), this is in conjunction with β-actin profile observed by western blotting (Figure 1B). Further, the expression of the β-actin is mainly distributed between epithelial and inflammatory cells of the tissues with significantly higher level in inflammatory cells than their corresponding epithelial cells both in normal (2.46 ± 0.13 *vs* 5.92 ± 0.23, P <0.001) and tumor tissues (2.79 ± 0.24 *vs* 6.71 ± 0.14, P < 0.001) (Figure 2A and B). Both epithelial and inflammatory cells of tumor overexpressed β-actin compared to normal tissues, however, only inflammatory cells showed significant increase (5.92 ± 0.23 *vs* 6.71 ± 0.14, P < 0.01). The increased expression of β-actin of inflammatory cells is in strong correlation with chronic inflammation in gastric cancer[33] which leads to the homing of large number of inflammatory cells with higher level of β-actin required for immediate cytoskeleton rearrangement for the formation of membrane protrusions at the time of their migration[34-36]. This observation is important as inflammation is a key component of the tumor microenvironment, promotes tumor development and being considered as a hallmark of cancer[37,38].

Further, univariate analysis showed β-actin level of tumor inflammatory cells positively correlates (P < 0.05) with tumor grade or poorer differentiation of gastric cancer while epithelial cells showed an inverse correlation (P > 0.05) (Figure 3A and C). The insignificant correlation of epithelial cells can be attributed to low number of moderately differentiated gastric adenocarcinoma tissue samples (n = 4) with high range of "total IHC score" (3.5 ± 1.5). This correlation indicates toward an important role of β-actin in tumor dedifferentiation. The chronic inflammation in gastric cancer, predominantly caused by *Helicobacter pylori* infection, is known to promote poorer tumor differentiation and CpG-island hypermethylation[33,39,40] and β-actin promoter hypermethylation downregulates the gene expression[25]. Therefore, the positive correlation of β-actin level of tumor inflammatory cells with tumor grade may be due to the persistent inflammation in tumor micro-environment. On the other hand, hypermethylation of β-actin promoter may be a cause of negative correlation of β-actin level of tumor epithelial with tumor grade. Low level of β-actin in gastric adenocarcinoma cell line KATO Ⅲ (signet ring cell carcinoma, a type of poorly differentiated cell) compared to AGS (moderately differentiated) (Figure 3B), further strengthens the observation that β-actin level of tumor epithelial cells negatively correlates with poorer tumor differentiation.

In summary, to the best of our knowledge, present study provides first histological evidence of cell type specific distribution of β-actin in normal and tumor gastric tissues. The significant increase in β-actin expression in tumor tissues is due to inflammation, an initial characteristic in the stage of gastric cancer progression and positively correlates with tumor grade. Therefore, β-actin may represent a promising biomarker in early diagnosis and prognosis of gastric cancer. However, further studies are needed to explore the relationship of cell type specific differential expression of β-actin with its functional implications in carcinogenesis and to be used as a chemotherapeutic target.

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COMMENTS

Background

On one side β-actin has been a renowned internal equal loading control for RNA and protein expression studies, on the other side reports of its differential expression in growth, ageing, differentiation, development as well as diseases like asthma, Alzheimer's disease, congenital heart disease and cancer is increasing progressively. Further, there is an emerging view of the use of β-actin as a potential direct or indirect target for chemotherapy. Therefore, the study of this "so called" housekeeping gene in cancer becomes as important as any other molecule involved in this critical disease.

Research frontiers

Validation of housekeeping genes as an internal loading control, role of actin in biological process important in carcinogenesis, investigation of actin binding proteins specifying its function and identifying new chemotherapy targets affecting actin cytoskeleton directly or indirectly are the major research areas which is related to the article.

Innovations and breakthroughs

Differential expression of β-actin has been reported in a number of physiological conditions along with its overexpression in multiple cancers. Now days, oncology research is emphasizing on tumor microenvironment, the present study provides first histological proof of β-actin overexpression but differentially in different cell types in gastric cancer. The histology based investigation provides evidence that β-actin overexpression in gastric cancer is predominantly contributed by the infiltrating inflammatory cells in between tumor epithelial cells. In addition, a significant correlation was observed between β-actin expression and tumor grade which emphasizes the role of β-actin in carcinogenesis.

Applications

The findings of the present study strengthen the area of actin biology and emphasize on the fact that conventional housekeeping genes should not be chosen as internal loading control without validation. This article provides impetus to further study of β-actin expression in different cancers and implicate the findings to understand the role of β-actin in carcinogenesis. It also encourages us to find prognostic and diagnostic value of β-actin in cancer along with as a direct or indirect target for chemotherapeutic intervention similarly as other cytoskeletal element such as microtubules.

Terminology

Tissue disruptive and non-disruptive techniques: A tumor tissue is comprised of heterogeneous population of cells. Therefore, crush and/or homogenizing a tissue for genomics, proteomic and expression studies is defined as tissue disruptive technique. This technique does not give specific information about the type of cells contributing to the results and therefore can be misleading. On the other hand, tissue non-disruptive techniques like histology based immunohistochemistry provide information at the level of specific cell type.

Peer review

In the present study, the authors revealed an overall increase in β-actin expression in gastric cancer compared to histologically normal adjacent mucosa. They revealed that inflammatory and epithelial cells of tumor tissues showed differential pattern of β-actin expression and correlated with tumor grade. Overexpression of β-actin was predominantly contributed by inflammatory cells. According to the results, they concluded that β-actin might be a promising biomarker of gastric cancer and chemotherapeutic target. They showed interesting and valu-

able data in this paper.

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