Original paper

Increased Glypican-3 immunostaining is associated with longer survival outcomes in colorectal carcinoma

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Glypicans (GPC) are involved in the developmental morphogenesis and regulatory processes of cell signalling. Abnormal expression has been observed in different cancer types.
One hundred and thirty-seven colorectal carcinoma (CRC) and 44 nodal metastases were used to create tissue microarrays. Immunohistochemistry was done to detect and evaluate the impact of immunostaining patterns of GPC-3 protein in CRC.
GPC-3 immunostaining is increased in CRC and nodal metastasis ($p < 0.001$) and was not association with clinicopathological parameters. GPC-3 immunostaining was associated with longer disease-free survival ($p = 0.021$) and overall survival ($p = 0.05$).
For the first time, we show GPC-3 immunostaining association with survival outcomes in CRC. GPC-3 may be used as an independent prognostic factor for survival in CRC.

Key words: glypican-3, colorectal carcinoma, immunostaining, survival.

Introduction

Globally, colorectal cancer (CRC) is in third place in the rank of malignant tumours. In industrialised countries, CRC is second among causes of death related to malignancy [1]. The pathogenesis of CRC involves a multistep process through the accumulation of several genetic and epigenetic abnormalities. Aberrant activation of the Wnt signalling pathway, typically via mutation of APC, and mutations of other genes such as KRAS have been observed [2, 3, 4].

Glypicans (GPCs), constitute a family of heparan sulphate proteoglycans. Glypicans are attached to the plasma membrane by a glycosylphosphatidylinositol and form a complex with Wnt signalling pathway [5, 6]. GPC-3 has a role in cell signalling pathways, cell division and apoptosis. Interestingly, it has different effect in different signalling pathways; supressing effect in the Hedgehog pathway [7] but a stimulating effect in the Wnt pathway [8]. The immunohistochemistry expression of GPC3 in normal tissues differs between embryogenic and adult tissues. In adults, it was found to be expressed in few normal tissues (e.g., gastric glands, kidney tubules, placenta and testicular germ cells) while absent in many others including colon and liver tissue [9, 10]. Hard

Table I. Clinicopathological parameters of CRC

Parameter	Number (%)
Age	
< 60 years	72 (52.6)
≥ 60 years	65 47.4 ()
Sex	
Male	71 (51.8)
Female	66 (48.2)
Grade	
Well-differentiated	33 (24.1)
Moderately-differentiated	88 (64.2)
Poorly-differentiated	16 (11.7)
Tumour location	
Right colon	36 (26.3)
Left colon	89 (65.0)
Rectum	12 (8.8)
Tumour size	
< 5 cm	56 (40.9)
≥ 5 cm	81 (59.1)
Primary tumour	
T1	3 (2.2)
T2	21 (15.3)
T3	103 (75.2)
T4	10 (7.3)
Lymphovascular invasion	
Negative	114 (83.2)
Positive	23 (16.8)
Margin status	
Free	131 (95.6)
Involved	6 (4.4)
Nodal metastasis	
Negative	76 (55.5)
Positive	58 (42.3)
Distant metastasis	
Negative	101 (73.7)
Positive	36 (26.3)
Recurrence	
Negative	91 (66.4)
Positive	46 (33.6)
Survival	
Alive	106 (77.4)
Dead	31 (22.6)

T1 – tumour invades submucosa
T2 – tumour invades muscularis propria
T3 – tumour invades through the muscularis propria into the subserosa or into non-peritonealised pericolic or perirectal tissues
T4 – tumour directly invades other organs or structures, and/or perforates visceral peritoneum

work has been done by researchers to study the association between GPC-3 and different tumours especially hepatocellular carcinoma [11, 12, 13, 14, 15]. Only a few studies have been done to explore the association between GPC-3 and CRC [4, 16].

The present work was designed to investigate the immunostaining expression of GPC-3 in CRC and its relation to clinicopathological features as well as the prognostic significance of GPC-3 immunostaining.

Material and methods

Patients

The study is retrospective and included formalin-fixed, paraffin-embedded tumour tissue from 137 patients with CRC and 44 corresponding nodal metastases in the period 1995-2013. Blocks were retrieved from the archives of the Department of Pathology at King Abdulaziz University, Jeddah, Saudi Arabia. Clinicopathological characteristics of patients are listed in Table I. The study was approved by the Research Committee of the Biomedical Ethics Unit, Faculty of Medicine, King Abdulaziz University.

Tissue microarray

Paraffin-embedded CRC samples were retrieved and used to create TMA recipient blocks. Tumour areas were marked on haematoxylin and eosin-stained slides. Areas of necrosis and any defect were avoided. The marked areas were correlated with selected paraffin blocks, and two cores of tissue – each 1.5 mm in diameter – were punched within the automated TMA instrument (Master 3D Histech) [17, 18].

Immunohistochemistry

Paraffin blocks of tumour were cut at 4 μm thickness, and mounted on positive-charged slides (Leica Microsystems Plus Slides). Sections were deparaffinized in xylene and rehydrated in an automated immunostainer (BenchMark XT, Ventana® Medical Systems Inc., Tucson, AZ, USA). Pre-treatment was done using CC1 (prediluted cell conditioning solution) for 60 min. Mouse monoclonal anti-*glypican-3* antibody (1G12 from Cell Marque) was incubated at 37°C for 20 min. Ventana® I-view DAB detection kit was used according to kit manufacturer instructions. Subsequently, slides were washed, counterstained with Mayer's haematoxylin and mounted. Negative control (substitution of primary antibody with Tris buffered saline) and positive control slides (normal liver tissue previously known to be positive to *glypican-3*) were included.

Table II. Categories of glypican-3 immunostaining in primary CRC and nodal metastasis

Tissue	Glypican-3 immunostaining		p value
	Low	High	
Colorectal carcinoma (137)	41 (29.9%)	96 (70.1%)	< 0.001#
Lymph node metastasis (44)	13 (29.6%)	31 (70.4%)	< 0.001#

Non-parametric χ^2

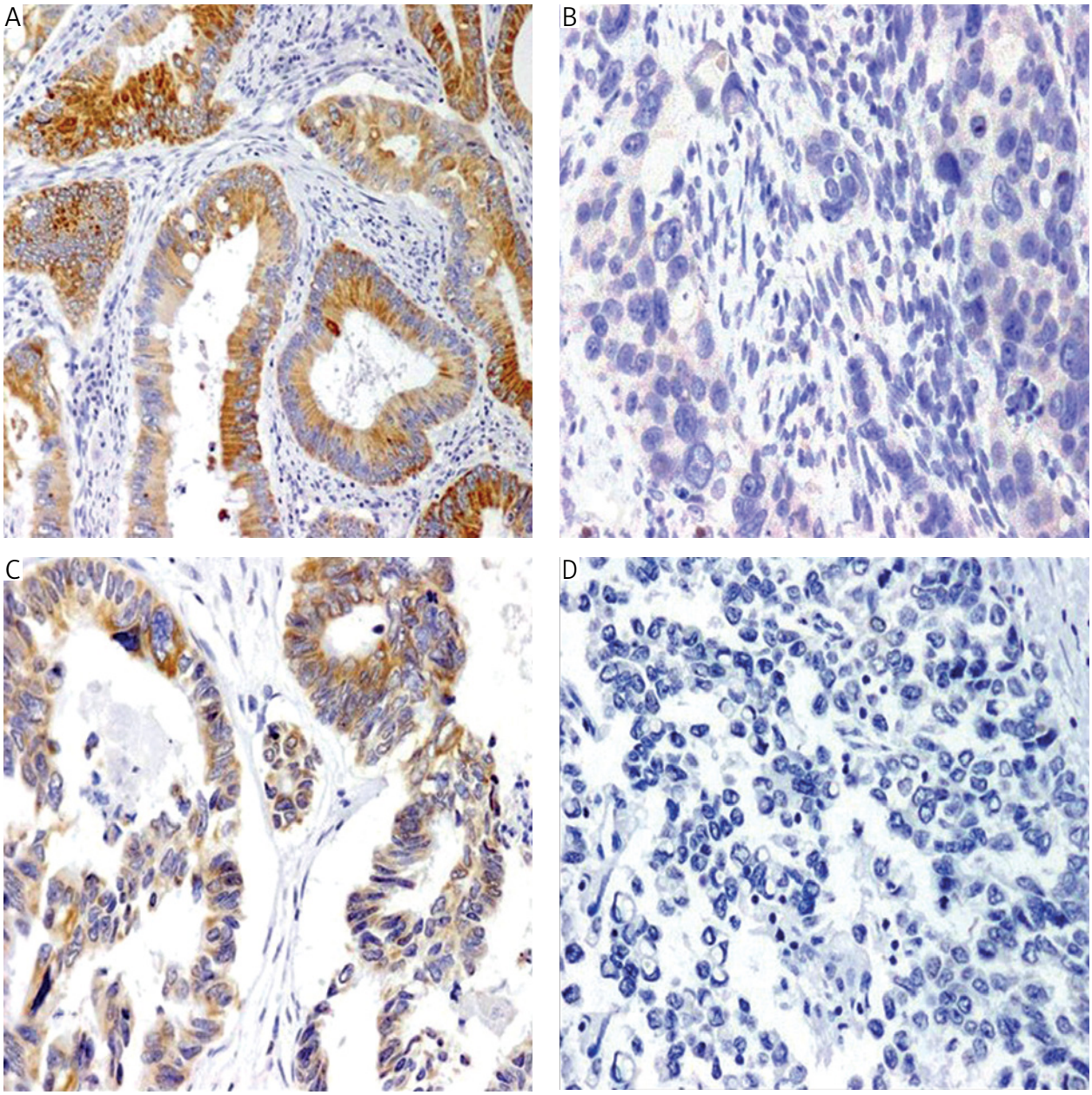

Fig. 1. GPC-3 Immunostaining. Immunostaining of GPC-3 in colorectal carcinoma using immunohistochemical labelling with anti- GPC-3 with diaminobenzidine used as the chromogen and haematoxylin as counterstain. A) Positive cytoplasmic immunostaining in a primary moderately differentiated CRC (100×). B) A primary poorly differentiated CRC with no immunostaining (200×). C) Positive cytoplasmic immunostaining in a nodal metastatic moderately differentiated CRC (200×). D) No immunostaining in a nodal metastatic moderately differentiated CRC (200×)

Table III. Distribution of Glypican-3 immunostaining in relation to clinicopathological parameters of CRC

Parameter	Glypican-3 immunostaining		p value
	Low	High	
Age			
> 60 years	23 (16.8%)	49 (35.8%)	0.709#
≥ 60 years	18 (13.1%)	47 (34.3%)	
Sex			
Male	25 (18.2%)	46 (33.6%)	0.193#
Female	16 (11.7%)	50 (36.5%)	
Grade			
Well-differentiated	8 (5.8%)	25 (18.2%)	0.376*
Moderately-differentiated	27 (19.7%)	62 (45.3%)	
Poorly-differentiated	7 (5.1%)	9 (6.6%)	
Tumour location			
Right colon	11 (8.0%)	25 (18.2%)	0. 927*
Left colon	27 (19.7%)	32 (23.4%)	
Rectum	3 (2.2%)	9 (6.6%)	
Tumour size			
< 5 cm	13 (9.5%)	43 (31.4%)	0.186#
≥ 5 cm	28 (20.4%)	53 (38.7%)	
Depth of invasion (pT)			
T1	0 (0%)	3 (2.2%)	0.114*
T2	5 (3.6%)	16 (11.7%)	
T3	30 (21.9%)	73 (53.3%)	
T4	6 (4.4%)	7 (5.1%)	
Lymphovascular invasion			
Negative	31 (22.6%)	83 (60.6%)	0138#
Positive	10 (7.3%)	13 (9.5%)	
Margin status			
Free	39 (28.5%)	92 (67.2%)	0.998#
Involved	2 (1.5%)	4 (2.9%)	
Nodal metastasis			
Negative	24 (17.5%)	53 (38.7%)	0.499#
Positive	17 (12.4%)	41 (29.9%)	
Distant metastasis			
Negative	32 (23.4%)	69 (50.4%)	0.298#
Positive	9 (6.6%)	27 (19.7%)	
Recurrence			
Negative	30 (21.9%)	61 (44.5%)	0.186$^{\mu}$
Positive	11 (8%)	35 (25.5%)	

** Kruskal-Wallis test*

 # χ^2 test

T1 – tumour invades submucosa

T2 – tumour invades muscularis propria

T3 – tumour invades through the muscularis propria into the subserosa or into non-peritonealised pericolic or perirectal tissues

T4 – tumour directly invades other organs or structures, and/or perforates visceral peritoneum

Interpretation of glypican-3 immunostaining

Scoring was performed independently by two pathologists (AH, WG) without prior knowledge of clinicopathological data. GPC-3 immunostaining was semi-quantitatively evaluated and the percentage of tumour cells showing cytoplasmic GPC-3 staining was recorded. GPC-3 immunostaining was considered as low when less than 5% of tumour cells were stained. When 5% or more of tumour cells were stained, GPC-3 immunostaining was considered as high GPC-3 [19].

Statistical analysis

Association between two variables was tested using the χ^2 test. To test the association in three groups of patients for one independent variable, the Kruskal Wallis test was used. The non-parametric χ^2 test was used to test variance along one variable. The Kaplan-Meier procedure was used to calculate the survival probabilities and the log rank test was used to compare the difference between survival rates. The end point for patients was last seen or death. Disease-free survival (DFS) was calculated as the time from diagnosis to the appearance of recurrent disease (or date last seen disease-free). Overall survival was calculated from the time of diagnosis to the date last seen or death. Statistical procedures were performed using SPSS® Release 16.0. Statistical significance was determined at p value of ≤ 0.05 and was 2-sided.

Results

Immunostaining for GPC-3 was seen in the cytoplasm of malignant cells both in primary tumours and nodal metastatic deposits. High GPC-3 immunostaining was statistically significantly higher than low GPC-3 in both the primary tumour and nodal metastasis. In primary CRC, high GPC-3 immunostaining was observed in 70.1% while low GPC-3 was seen in 29.9% ($p < 0.001$). In nodal metastasis, high GPC-3 immunostaining was reported in 70.4% while low GPC-3 immunostaining in 29.6% (< 0.001). There was no difference in GPC-3 immunostaining between primary CRC and nodal metastasis ($p = 0.744$). Data are shown in Table II. Representative examples of GPC-3 immunostaining are shown in Figure 1 (A-D).

GPC-3 immunostaining in CRC showed not association with any of the clinicopathological parameters (data are shown in Table III). On the other hand, in the Kaplan–Meier survival analysis, patients with high GPC-3 immunostaining in primary CRC showed a significant longer disease-free survival than in patients with low immunostaining ($p = 0.021$ log rank [Mantel-Cox] = 5.311). Also, there was longer overall survival in patients with high GPC-3 immunostaining than those with low immunostaining ($p = 0.05$ and Log Rank [Mantel-Cox] = 3.674).

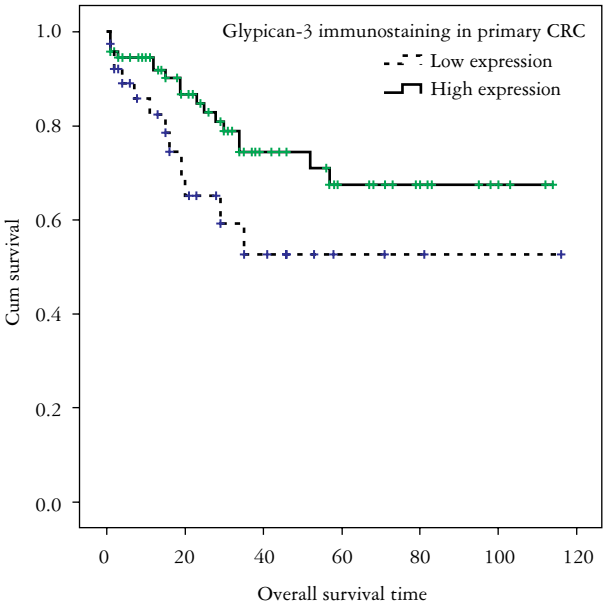

Fig. 2. Overall survival curve (Kaplan-Meier) according to GPC-3 immunostaining in colorectal carcinoma (1: low GPC-3 immunostaining; 2: High GPC-3 immunostaining [log-rank; Mantel-Cox] = 3.674, p = 0.05)

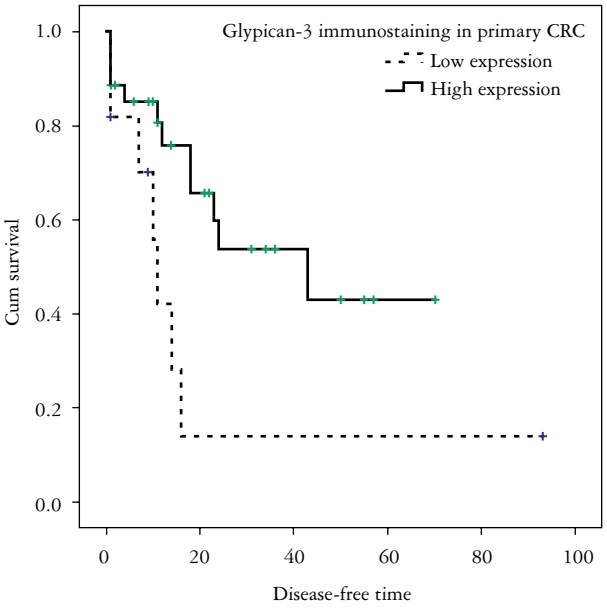

Fig. 3. Disease-free survival curve (Kaplan Meier) according to GPC-3 immunostaining in colorectal carcinoma (1: low GPC-3 immunostaining; 2: High GPC-3 immunostaining [log-rank; Mantel-Cox] = 5.311, p = 0.021)

Discussion

Colorectal carcinoma is one of the most common cancers worldwide with high morbidity and mortality. The postsurgical outcome varies among patients. Multiple factors are considered in management including tumour stage, lymph nodes involvement, lymphovascular invasion, tumour budding and others. The status of different mutation is now a well-known predictive factor that plays a role in the oncologist decision when it comes to treatment options. It has been observed that patients with CRC at the same stage may have different outcomes. Therefore, lots of efforts are being made to understand the pathogenesis, prognostic and predictive factors for the purpose of improving and personalising the management.

Given the role of GPC3 in the cell cycle, studies have been performed to understand its role in tumorigenesis and tumour progression in different organs. Trials to highlight its role and diagnostic and prognostic values have been conducted on various tumours including hepatocellular carcinoma, urothelial carcinoma, ovarian carcinoma, endometrial carcinoma, breast carcinoma, gastric carcinoma, pancreaticobiliary carcinoma and colon carcinoma [16, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35]. Different methods have been used to detect expression of glypican proteins in the tissues. These methods include mass spectrometry [31, 33], functional genomic mRNA profiling [29, 34] and immunohistochemistry. Poor prognosis was observed to be associated with higher expression of GPC3 in hepatocellular carcinoma [24, 36] and pancreatic adenocarcinoma [28]. A subtype of gastric adenocarcinoma with expression of GPC3 was found to have poor prognosis [37]. GPC-3 expression has been found to be increased in some regenerative processes as well [34, 38]. Some researchers have investigated GPC3 as potential therapeutic target in some tumours [22, 39, 40].

There are not enough data about GPC-3 and CRC and few studies have investigated GPC3 expression in CRC. In previous studies, immunostaining of GPC-3 was higher in cancer tissue in comparison to non-cancerous tissue [16] and also a significant increase in the expression of the GPC3 gene was revealed in about half of CRC tumours tissues as compared to the normal adjacent tissues [29]. In contrary, another study found however that CRC tissue had lower expression of GPC3 in comparison to normal tissue [31]. Also, GPC-3 was higher non-mucinous cancer in comparison to mucinous CRC [30]. In the present study, high GPC-3 immunostaining was associated with shorter survival outcome (disease-free survival and overall survival. This is a new finding and may help in patient stratification for post-operative therapeutic modalities.

In the current study, immunohistochemical staining method was chosen to detect GPC-3 expression in CRC as it is an easy way, with less limitation and available for most laboratories worldwide. The variability in conclusions among the scant studies conducted over the years might be related to different patient populations, aetiology, risk factors, mutations status, methods used to detect GPC-3 expression and other factors. Although only one other study was found that have investigated the prognostic values of GPC-3 in CRC, it showed no correlation between GPC-3 expression and CRC prognosis [30]. This difference might be related to the sample size, duration for which the patients were followed up, or other factors.

A limitation of the current study is not including normal colorectal tissues or adenomatous tissue. This may be further studied in a prospective study.

In conclusion, we showed for the first time the prognostic value of GPC-3 in relation to survival outcome in CRC in a good cohort of patients. It is an interesting and potentially promising independent prognostic factor. Further investigations are required to confirm its value.

The authors declare no conflict of interest.

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