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Original Article

Evaluation of Immunohistochemical Expression of Survivin and its Correlation with qRT-PCR Results as a Useful Diagnostic Marker in Gastric Cancer

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Abstract

Background: Today, survivin is known as one of the most specific cancer proteins; provide unique and practical study opportunities. Clinical value of survivin in gastric cancer (GC) is not yet appointed. To establish the expression level of survivin and its diagnosis value in Iranian patients with GC, we evaluated the association of survivin expression with clinicopathologic factors.

Methods: Overall, 60 matched-normal controls with 60 GC samples including 30 cases with evidence of metastasis at time of our study and 30 cases without evidence of metastasis were recruited, in Tehran, Iran during 2008 to 2018. Survivin expression was evaluated by quantitative Real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) study.

Results: Increased expression of survivin at mRNA and protein levels was found in 86.7% and 71.6% of cases, respectively. Evidence indicated a significant difference in survivin mRNA expression level between tumor and non-tumoral (marginal) tissues (P<0.001). The difference in expression of survivin mRNA was not significant between metastatic and non-metastatic tumor tissues (P=0.171). Positive immunoreactivity of survivin was observed to be predominantly in the nucleus of tumor cells. A significant difference in survivin protein expression was detected between tumor and non-tumoral tissues (P<0.001) and between metastatic and non-metastatic tumor tissues (P<0.001). There was no significant association between survivin mRNA expression and clinicopathological variables. However, survivin protein expression was significantly correlated with perineural involvement (P<0.018).

Conclusion: This data could be supportive of using survivin as a useful diagnostic marker in GC. Although, more research is needed in this area.

Keywords: Gastric cancer; Survivin; Scoring system; Reverse transcriptase polymerase chain reaction; Immunohistochemistry

Introduction

Gastric cancer (GC) is one of the most important cancers worldwide (1). The 5-year survival rate for GC patients is disappointing with 20% in Western countries and up to 60% in Asian

countries (2). Based on genetic characteristics, gastric cancers have recently been described as complex and heterogeneous diseases by different subtypes, each with specific molecular aspects and specific clinical behavior (3). Survivin is a cytoplasmic and nuclear protein shuttle with a molecular weight of 16.5 kD and 142 amino acids (wild type) encoded by the *BIRC5* gene on chromosome 17q25 (4, 5). In GC patients, survivin has been introduced as a specific protein with diagnostic and prognostic potential (6). The expression of survivin protein in GC is a very common occurrence as 88% of GC tissues have a positive expression of survivin (7, 8). It has a unique role in apoptosis and as a multifunctional protein, it participates in inhibiting differentiation, controlling cell division in most tumors, and responding to cellular stress (9, 10). Moreover, survivin involves in carcinogenesis, tumor progression, increase angiogenesis, malignant cell deformity, and abnormal P53 expression (4, 11-13). In differentiated adult tissues, survivin has a very low level of expression, but in the epithelium of the gastrointestinal tract, it helps to maintain gastric mucosal coherence and regulate the cell cycle (14).

Even though in previous studies (2, 5), the role of survivin in the diagnosis of GC patients and the relationship between its expression pattern and clinical features has been extensively assessed but it is still debated. The aim of the current study was to evaluate the expression of survivin at both RNA and protein levels and its association with clinicopathological data among patients GC.

Materials and Methods

Subjects

Clinical data and tumor specimens were provided by the Iran National Tumor Bank, funded by the Cancer Institute of Tehran University of Medical Sciences, Tehran, Iran. Sixty samples with diagnosis of gastric adenocarcinoma in patients who underwent gastrectomy and regional lymph nodes dissection between 2008 and 2018 were recruited. Thirty of the patients had already a history of proven metastasis based on their hospital records. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were selected by viewing original pathologic slides and choosing representative blocks that showed the tumor and non-tumoral (marginal) area for each patient. The representative area was marked by an expert pathologist and was punched for RNA extraction. Then it was freshly cut (4 µm) and mounted on aminopropyltriethoxysilane-coated slides.

Tumor staging was in accordance with the American Joint Committee on Cancer staging system (15). Patient and tumor characteristics including sex, age at the time of surgery, tumor size, location, focality, perineural and lymphovascular invasion, and tumor stage were all obtained by hospital records and reviewed. Overall survival (OS) was measured from the date of primary diagnosis to death, or the last follow-up date. Disease-free survival (DFS) time was calculated from the date of primary diagnosis to disease recurrence, death, or the last follow-up date.

The study design was assessed and confirmed by Ethics Committee of Science and Research Branch, Islamic Azad University, Tehran, Iran (ethical confirmation code: IR.IAU.SRB.REC.1397.109).

RNA extraction and cDNA synthesis

Deparaffinization was performed using xylene (cat. no. 247642, Navid Tejarat). Total RNA was isolated from FFPE sections using a solution of RiboExTM, according to the manufacturer's instructions of the RNA extraction kit (cat. no. 305-101, Gene all). The extracted RNAs were quantified through Nanodrop (Thermo Fisher, USA). cDNA was synthesized with 100 ng of purified total RNA in a total 20 µl reaction mixture and using random hexamer primers in accordance with the manufacturer's instructions of the cDNA synthesis kit (cat. no. YT4500, Yekta taghiz).

qRT-PCR analysis

Real-time PCR was done and duplicated for each sample with specific primers described previously

(16,17). β -2 Microglobulin (*β2m*) gene was used as an internal control. The amplification was carried out in a total volume of 20 µl, including 200 ng of cDNA, 10 µl Hot Start 2X RT-PCR Master Mix Green-No ROX (cat. no. A323402-25, Ampliqon), and 15 pmol from each forward and reverse primer specific. The qRT-PCR program initiated with denaturation in 15 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 61 °C in 30 sec, and extension at 72 °C for 30 sec by the Bio-Rad (CFX-96 C1000 Touch Real-Time System) thermal cyclers.

Data analysis of qRT-PCR

The relative expression of each gene was accomplished using a comparative threshold cycle according to $2^{-\Delta\Delta Ct}$ method (18). The mean threshold cycle (mCT) was acquired from duplicated amplicon during the exponential phase of amplification. To achieve ΔCt for each tumor or matched normal tissues, subtracted mCT value of the reference gene from mCT value of survivin gene. Fold changes were presented in the relative quantification over the control group. Melting curve analysis approved accuracy of the qRT-PCR amplification curves.

Immunohistochemistry staining

IHC staining was implemented on 4 μm sections after antigen retrieval in Tris-EDTA-citrate buffer pH 8.0 for 20 min. The endogenous peroxidase activity was blocked by immersion in 0.3% hydrogen peroxide for 10 min at room temperature (RT). After washing with phosphate-buffered saline, sections were incubated in 10% normal rabbit serum for 5 min to block non-specific antibody binding. A monoclonal antibody to survivin (clone EP119, Abcam, USA) was used as a primary antibody (for 60 min at RT). Antimouse immunoglobulin G (cat. no. 41020, Seville Spanish) labeled with horseradish peroxidase-conjugated (HRP) was added as a secondary antibody for the detection of primary antibodies and the samples were incubated for 30 min at RT. Immunostain visualization advanced with 3,3'-diaminobenzidine (DAB) chromogen solution (2 min, at RT). Fragments of skin tissue with invasive melanoma were used as positive controls, and incubation with the primary antibodies was omitted for the negative controls.

Immunoassay analysis

Immunohistochemical staining was evaluated by two independent pathologists who were blinded to the clinical information. Protein expression was found in the nuclei of the tumor cells, and graded separately in an identical manner. Expression was determined using the semiquantitative method named as immunoreactive score (IRS) (19, 20) and defined with regard to the intensity of staining (0, absent; 1, weak; 2, moderate; and 3, strong) and the percentage of positive tumor cells. The percentage of positive cells was rated as follows: 0%-10% positive cells [1]; 11%-25% positive cells [2]; 26%-50% positive cells [3]; 51%-75% positive cells [4]; and >75% positive cells [5]. Scores for the percentage of positive cells and scores for the expression intensities were multiplied to calculate the immunoreactive score 0=no staining, 1-3=positive, mild expression, 4-8=positive, moderate expression, 9-12=positive, strong expression. The study group was divided into negative cases (IRS score <1) and positive cases (IRS score ≥1).

Statistical analysis

Statistical analysis was performed using SPSS software (ver. 26.0) (IBM Corp., Armonk, NY, USA). Analysis of Continuous variables was presented as means (±standard deviation, range). Student's *t*-test, Fisher's exact test, Chi-square test, Mann-Whitney u test and Monte Carlo test were used as appropriate for data analysis. The patient OS and DFS were calculated by the Kaplan-Meier method and comparison between different subgroups was analyzed using Log-rank test. The hazard ratio (HR) from univariate and multivariate analysis was accomplished using Cox regression model. A *P*-value<0.05 was considered significant.

Results

Quantification of survivin expression at the mRNA level

Increased expression of survivin mRNA was found in 86.7% of tumors and 13.3% of marginal- matched tissues. The expression was significantly different between marginal tissues and non-metastatic (*P*=0.001) and metastatic (*P*=0.001) tumor tissues (Fig. 1). No significant difference (*P*=0.171) was found in the expression of survivin mRNA between metastatic and non-metastatic tumor tissues (Fig. 2). No significant association was identified between clinicopathologic parameters and survivin mRNA expression. According to receiver operating characteristic curve (ROC curve) analysis, the expression of survivin mRNA can differentiate the marginal matched control from the metastatic tumor tissues with a sensitivity of 75.8% and a specificity of 76.75% at the cut-off point of 5.10 (area under the curve (AUC): 0.759, 95% CI: 0.640-0.878, *P*<0.001). Similarly, marginal matched control area from the non-metastatic tumor tissues can be differentiated with a sensitivity of 72.7% and a specificity of 45.5% at the cut-off point of 3.15 (AUC: 0.694, 95% CI: 0.568-0.821, *P*=0.007). The metastatic tumor can be discriminated from the non-metastatic tumor tissues, at the cut-off point of 2.15 with a sensitivity of 72.7% and a specificity of 64.5% (AUC: 0.830, 95% CI: 0.727-0.902, *P*< 0.001). (Fig. 3).

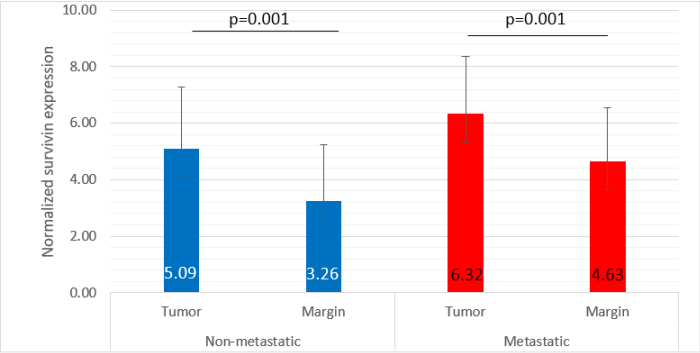

Fig. 1: Comparison of survivin mRNA expression between tumor with the margins of the respective tissues. Data are represented by the expression of a normalized gene with an endogenous β2m reference gene

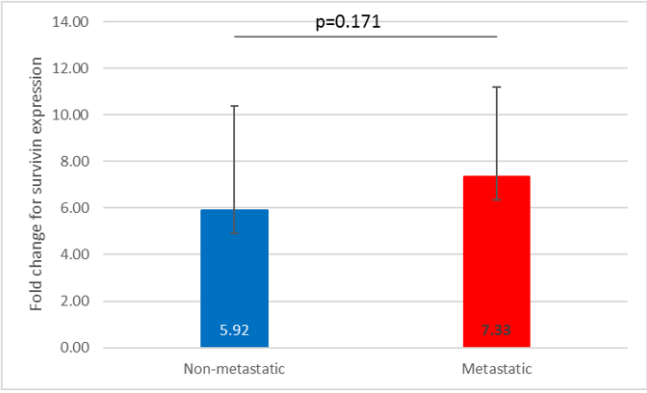

Fig. 2: The relative expression level of survivin mRNA. Increased expression of mRNA survivin was significantly seen in tumor samples compared to marginal matched. Each qRT-PCR test was repeated at least twice. The expression levels of survivin mRNA were normalized to the expression level of β2m in the relevant tissues. Relative change of mRNA expression calculated using equation 2 –ΔΔCt

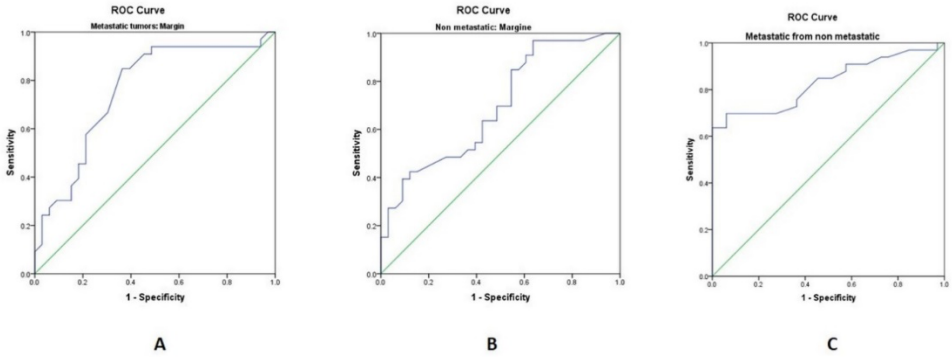

Fig. 3: ROC curve to evaluate the expression potential of survivin mRNA. It distinguishes A: marginal matched of metastatic tumor tissues. B: marginal matched of non-metastatic tumor tissues. C: metastatic from non-metastatic tumor tissues.

Assessment of immunoreactivity

The stained slides were reviewed by an expert pathologist. Positive immunoreactivity of survivin was observed to be predominantly in the nucleus of tumor cells compare to the cytoplasm as brown sedimented granules, similar to the control positive sample. However, it was rare and scattered in the adjacent normal cells (Fig. 4). Overall, positive expression was observed in 43 out of 60 (71.6%) tumor tissues. The intensity of survivin expression was classified as weak, moderate and strong for 16 out of 60 (26.6%), 18 out of 60 (30%) and 9 out of 60 (15%) of tumor tissues, respectively.

Moderate and strong staining intensity was more common among metastatic compared with non-metastatic tumors. Although, no significant association in staining intensity was found between metastatic and non-metastatic tumor tissues (P=0.300). A considerable difference was shown in the distribution pattern of cell staining percentage between metastatic and non-metastatic tissues (P=0.033). In metastatic and non-metastatic tumors, 40% and 6.7% of cells were stained more than 50%, respectively. In other words, in non-metastatic tumors, 93.3% of cells stain up to 50%. Moreover, no significant difference was identified between the marginal normal tissues in the two tumor groups by the IRS (P<0.313), (Table 1).

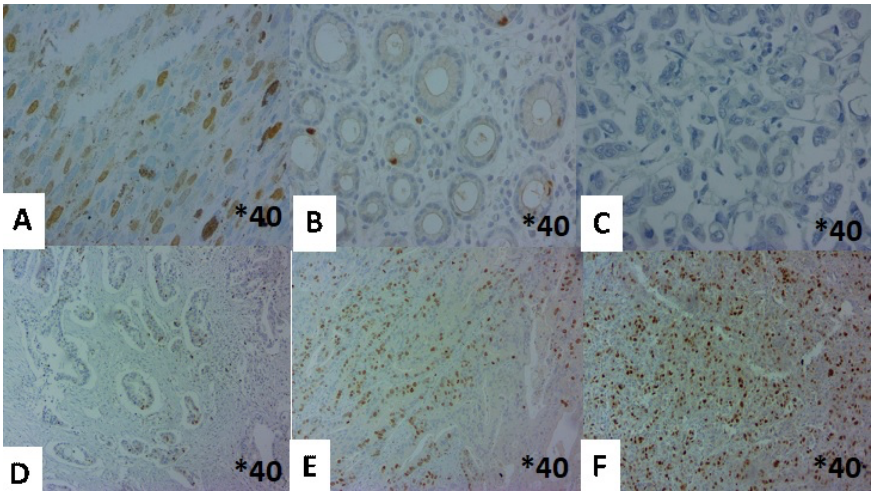

Fig. 4: IHC staining of survivin protein in patients with GC. A: skin melanoma cells (positive control); B: marginal matched control cells; C: tumor cells with score 0; D: tumor cells with score 1-3; E: tumor cells with score 4-8; F: tumor cells with score 9-12

Table 1: Comparison the IRS in metastatic and non-metastatic tumor with its marginal tissues

	Metastatic %			***Non-metastatic%***			***Tumor%***		
Score	Margin	Tumor	*P*-value	Margin	Tumor	*P*-value	Non-metastatic	Metastatic	*P*-value
0	100	23.3	<0.001	90	33.3	<0.001	33.3	23.3	<0.001*
1-3	0	23.4	*	10	30	*	30.0	23.4	
4-8	0	30		0	30		30.0	30	
9-12	0	23.3		7	6.7		6.7	23.3	

Clinicopathological features

The medical records of all 60 patients were carefully examined. Most patients (73.3%) were male and 26.7% were female. The mean age of the patients was 60.72 ± 13.14 yr old (range: 24 to 87 yr). The mean tumor size was 4.45 ± 2.92 cm (range: 1 to 19 cm). No significant association was found between clinicopathologic parameters and survivin protein expression. A significant relationship was noted between survivin protein expression and perineural involvement (P<0.018).

Survival analysis

The results of survival analyses by the Kaplan-Meier method are presented in Fig. 5. During the study period (36 months), 38 patients (63.3%) eitherrelapsed or died. The mean OS of the subjects was 17.58 (95% CI: 13.62-21.53) months.

The mean DFS was 17.19 (95% CI: 13.16-21.21) months. OS and DFS were longer in patients with tumor size less than or equal to 6 cm, the location of the tumor in the middle or lower parts, tumor stage I or II, intestinal tissue type, tumors without metastasis and without lymphovascular invasion and these differences were significant (P<0.05). These results were also confirmed in the Cox regression model, in the univariate mode. The results of the Cox regression model in multivariate mode were shown that OS was reduced among those with a tumor stage of III/IV versus I/II, as well as those with metastasis versus no metastasis tumors, with an HR equal to 6.45 (95% CI: 1.81-23.27, P=0.004) and 2.45 (95% CI: 1.1-5.95, P=0.04), respectively.

Moreover, DFS was reduced in patients with a tumor stage of III/IV versus I/II, with an HR equal to 6.6 (95% CI: 1.72-21.44, P=0.005), in the multivariate mode. As well as, the OS and DFS were less in patients with positive expression compared with those with negative expression of survivin, but these differences were not significant (P=0.28 and P=0.32, respectively).

Discussion

Several studies have focused on the role of genes involved in the development and progression of GC (2,3). The role of survivin in GC is still well not studied and there is no reported publication in Iranian cases yet. First-degree relatives are approximately three-fold increase in a risk of developing gastric carcinoma (20). In the current research, the expression level of survivin mRNA was increased in 86.7% of tumors and 13.3% of marginal-matched tissues. In agreement with other studies, in patients with gastric carcinoma, the expression of survivin mRNA was higher in 98.6% of peripheral blood samples compared to the normal controls.

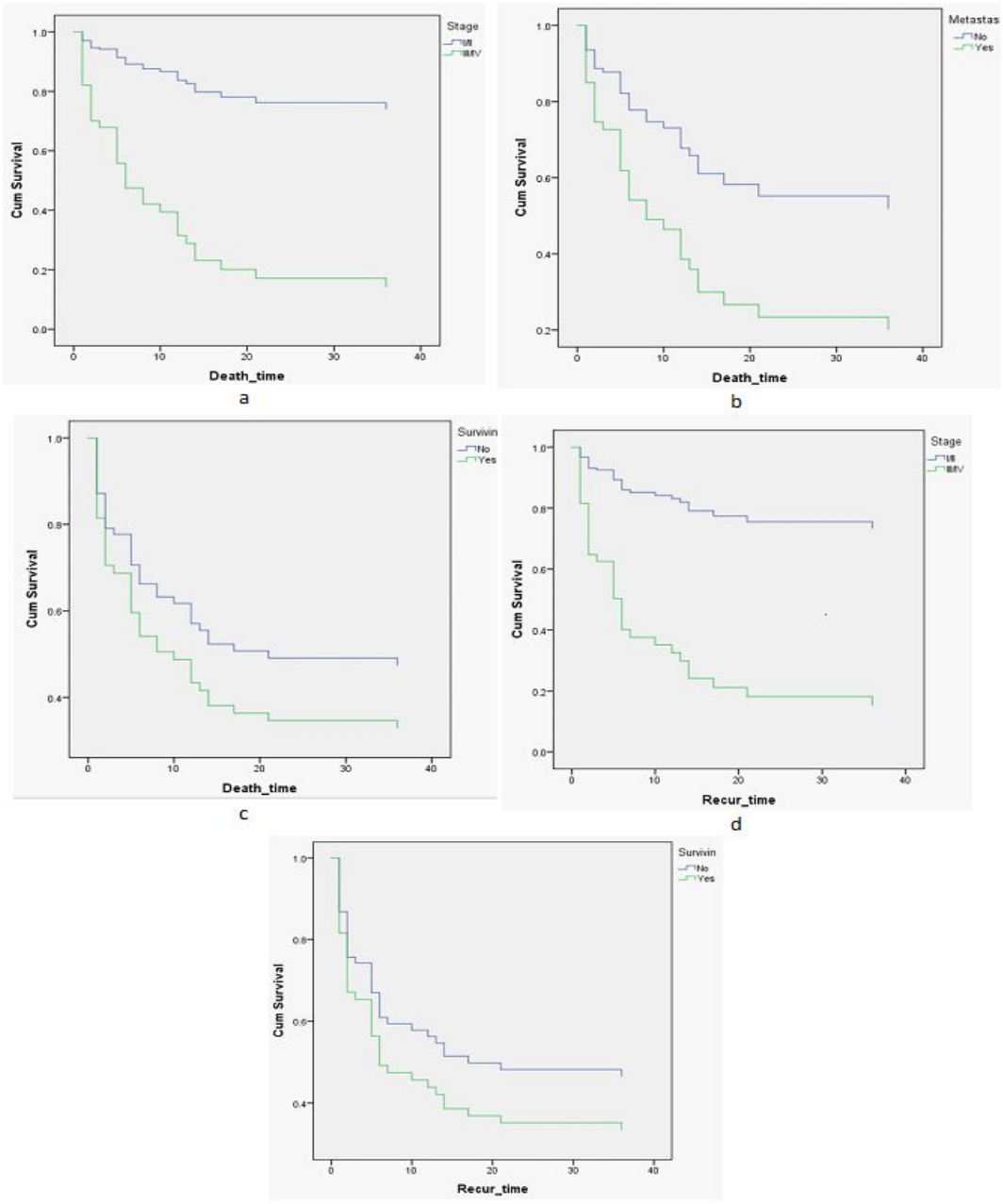

Fig. 5: Kaplan-Meier curves for overall-survival (OS) and disease-free survival (DFS). (a) Kaplan-Meier curves for OS according to ypTNM stage. (b) KaplanMeier curves for OS according to metastatic status. (c) Kaplan-Meier curves for OS according to survivin expression. (d) Kaplan-Meier curves for DFS according to ypTNM stage. (e) Kaplan-Meier curves for DFS according to survivin expression

The survivin mRNA level was as an independent prognostic factor, and high expression was associated with the worst prognosis only in primary tumors and not in the metastatic group (21). Survivin mRNA expression is higher in the mucosa of these individuals than in the control group. Therefore, measuring survivin mRNA in the mucosa of susceptible patients may be considered as a way to identify these patients (22). Although, survivin mRNA expression did not detect in marginal-matched areas when the corresponding cancerous tissues were negative

(20). However, they observed its expression in 68% of all 5 gastric cell lines and 22% of the adjacent non-tumor samples. They also did not find any correlation between survivin mRNA expression level and patient demographic information, tumor staging, lymph node involvement, and tumor subtypes consistent with our study (20).

In this study, the results of the IHC were strongly consistent with the results of the qRT-PCR test. We calculated the positive expression of survivin protein in 71.6% and 5% of the tumor and marginal-matched tissues, respectively. Similarly, the positive expression of survivin retained in 72% and 5% of the tumor and normal tissues, respectively (22). The range of survivin protein expression varied from 50% to 88% in GCs (14). The survivin protein expression in 55.4% of gastric carcinoma and the expression level of survivin was significantly higher in patients with lymph node metastasis than those without metastasis (23). Moreover, the survivin protein expression evaluated in 62.5% and 12.5% of the tumor and the adjacent non-cancer mucosa samples by IHC (20). In addition, the serum levels of survivin were significantly higher in cancer patients than healthy subjects (12). Survivin protein did not observe in the normal gastric mucosa (24). Unlike qRT-PCR, the IHC test results showed a significant difference in the expression of survivin between metastatic and non-metastatic tumor tissues. Since, metastatic groups showed higher levels of the IRS, it can be considered as an accurate factor for distinction of metastatic from non-metastatic tumor tissues.

In addition to the expression level of the survivin, its expression site may be also effective in prognosis (25). The survivin - ΔEx3 splicing variant is localized in the nucleus, whereas wild-type survivin and survivin-2B splicing have been detected in the cytoplasm (11, 24). Currently, there is no antibody that can specifically identify the splicing variants (24). Cytoplasmic survivin expression as an inhibitor of apoptosis is associated with poor prognosis. While nuclear survivin is necessary to complete the cell cycle, it does not significantly affect the overall survival (13, 26). Similar to Yu et al (20), we mainly observed the survivin protein in the nucleus of tumor cells with only scattered staining in the cytoplasm. This study did not find any relation between the clinical features of patients and cell location of survivin expression. Cytoplasmic and membranous staining was detected in Yusufu et al study (22). These may be related to differences in tissue reagents and clinical stages. In a meta-analysis, a heterogeneity was seen in all studies according to the method of identification, different scoring methods, and the affinity of polyclonal antibodies compared to monoclonal antibodies in IHC (2). However, there is disagreement to this extent and more research is needed.

In the present study, no significant association between clinicopathologic parameters and survivin protein expression level was detected. A significant relationship only was observed between survivin expression and perineural involvement ($P<0.018$). Moreover, the survival rate of the patients was significantly associated with tumor size less than or equal to 6 cm, the location of the tumor in the middle or lower parts, tumor stage I or II, intestinal type, and tumors without metastasis and without lymphovascular invasion. In contrast, Yusufu et al reported an association of the survivin expression with metastatic lymph nodes, gross type, depth of invasion, vascular invasion, distant metastasis, necrosis tumor, metastasis stage, poorer survival, and an increased risk of recurrence in most tumors (22). In a meta-analysis, only the association between positive expressions of survivin with the presence of lymph node metastases (2).

Overall, the positive expression of survivin alone may not predict poor prognosis, but it may be useful in identifying subtypes of patients who may benefit from targeted therapy in advanced GC (2). Moreover, data analysis has not yet established the correlation of patient survival with increasing survivin expression; larger sample size is needed in this area. However, sample size, incomplete patient's medical records and

inadequate tumor tissue in the FFPE blocks were our limitations in this research.

Conclusion

To the best of our knowledge, this study is novel and there is no documented report on ours studied population as well. The current study showed an expression difference of survivin protein between metastatic and non-metastatic samples. The high expression rate of survivin in tumor tissues, especially among those with metastasis, has revealed a possible role of survivin in cancer progression and also its application as a potential molecular marker for diagnosis and therapy. One of the limitations of our study was the small sample size. We did not have access o blood and fresh samples.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

All authors have no conflict of interest to declare.

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