The Study of Survivin Expression by Immunohistochemistry in Gastric Carcinoma

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ABSTRACT

Background*: Gastric cancer is still one of the most common malignant tumors worldwide. Survivin; a novel member of the inhibitor of apoptosis protein (IAP) family is a unique inhibitor of apoptosis that has been found in various human cancers. Its expression is associated with tumor progression and adverse outcome.* ***Aim of the work****: The current study was conducted to evaluate the expression pattern of Survivin in gastric carcinoma by immunohistochemistry and to correlate the relationship between its expression with various clinicopathological parameters and the patients' survival.* ***Patients and methods****: 30 patients with gastric carcinoma were included in this study. The expression pattern of Survivin protein was evaluated by immunohistochemistry in formalin-fixed paraffin-embedded blocks from 30 surgically resected gastric cancer tissues cases.* ***Results****: The positive expression of Survivin in gastric cancer tissues was demonstrated in 66.7% (20 out of 30) of cases. Positive staining pattern of Survivin protein was associated significantly with tumors larger than 5cm and undifferentiated carcinomas (P<0.05). Survivin expression was more frequent in diffuse tumors (72.7%) than in intestinal (66.7%) and mixed type (60%) carcinomas. There was no significant association between Survivin expression and the invasion depth of the tumor, lymph node involvement and tumor stage (P>0.05). The overall survival was 60% for Survivin positive patients as compared to 80% for Survivin negative patients (P>0.05).* ***Conclusion****: Our results suggest that Survivin may be a useful biomarker of prognosis in patients with gastric cancer and consequently increased expression of Survivin would be expected to predict a poor prognosis. Survivin may aid in a more accurate prediction of the clinical outcome of gastric cancer and may also be a novel therapeutic target.*

Key words: *Survivin –Immunohistochemistry – Gastric cancer.*

INTRODUCTION

Gastric cancer is still one of the most common malignant tumors worldwide. Surgical resection remains the mainstay and only potentially curative treatment but many patients eventually die of disease recurrence.[1] Despite aggressive treatment, prognosis of patients with advanced gastric cancer remains poor.[2] It is attributed not only to the high disease stage but also to the biologic aggressiveness of the individual disease which is characterized by a high potential for metastasis and resistance to anticancer therapy.[3] Although the pathologic tumor-node-metastasis stage and the possibility of curative surgery are the most powerful prognostic factors of gastric cancer, each patient with same stage gastric cancer has different risks of recurrence and survival. Therefore along with the development of more aggressive surgery and newer anti-neoplastic agents, efforts have been directed toward the identification of patients who have a higher risk of poor prognosis and the selection of patients who have a greater likelihood of responding to adjuvant treatments.[4] Regulation of apoptosis or programmed cell death, is crucial to the preservation of homeostasis and morphogenesis of human tissue.[5] Disturbance of this process by aberrantly extending cell viability or favouring accumulation of transforming mutation is thought to contribute to carcinogenesis.[6] The inhibitors of apoptosis proteins (IAPs) comprise a family of highly conserved cell death inhibitors that have been found in yeast, invertebrates and vertebrates.[7] Survivin, a novel member of the IAP family, is a unique inhibitor of apoptosis usually expressed in the embryonic lung and fetal organs in the developmental stages but undetectable in normal

adult tissues other than the thymus, placenta, CD34, stem cells and basal colonic epithelial cells.[8] However Survivin seems to be selectively expressed in transformed cells and in most human cancers including lung, breast, pancreatic and colon carcinomas, soft- tissue sarcomas, brain tumors, melanoma, neuroblastoma and hematologic malignancies.[9] Survivin, the smallest member of the IAP family, is a 142- amino acid, 16.5- KDa protein coded by a single-copy gene located on the human 17q25 chromosome.[10] Survivin inhibits apoptosis by binding specifically to the terminal-effected cell death proteases, Caspase-3 and Caspase-7 thereby inhibiting Caspase activity and apoptosis in cells exposed to diverse apoptotic stimuli. Also it regulates the cell cycle in the G2/M phase by interacting with spindle microtubules.[11]

The aim of this study is to evaluate the expression pattern of Survivin in gastric carcinoma by immunohistochemistry and to correlate the relationship between its expression with various clinicopathological parameters and the patients' survival.

PATIENTS AND METHODS

This study was conducted on 30 patients with gastric carcinoma who underwent gastric resection at the Department of Surgery, Medical Research Institute Hospital, Alexandria University, Egypt between April 2011 and August 2014. The patients were 18 males and 12 females with a male to female ratio (3:2) with a mean age of 52.56±11.83 years (range 34-82 years).

Twenty three patients were subjected to subtotal gastrectomy and the remaining seven patients underwent total gastrectomy. None of the patients included in this study received pre-operative chemotherapy or radiotherapy.

Every patient included in this study was subjected to full history taking, a thorough clinical examination, routine laboratory investigations, radiological investigations including abdominal US and CT scan and upper GIT endoscopy with a biopsy taken from the tumor to establish the pathological diagnosis before surgery.

Clinicopathological information and survival data were obtained from hospital records, and pathology and oncology departments.

Tumor Samples:

Samples from the gastric carcinoma specimens were taken from the representative cancerous lesions as well as adjacent non-cancerous mucosa for microscopic examination, tissues were routinely fixed in formalin before being embedded in paraffin. A 5 µm section from each specimen block was stained with Hematoxylin and Eosin (H&E) for histological evaluation. Adjacent normal gastric mucosa was used as an internal control.

Tumors were staged at the time of surgery by the standard for TNM staging system using the American Joint Committee on cancer.[12] By Lauren system tumors were classified into intestinal and diffuse types.[13]

Immunohistochemical Staining:

The Hematoxylin and Eosin stained slides of tumor specimens were reviewed and the tissue blocks that included the tumor area for formalin–fixed and paraffin-embedding were selected. Antibodies for Survivin (1:800, monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for immunohistochemical analysis.

Immunostaining was performed using the Avidin-Biotin Complex (ABC) method.[14] Representative paraffin blocks were cut consecutively at 4micron thickness. Sections were deparaffinized in xylene and treated with 0.3% hydrogen peroxide in methanol for 20 minutes to block endogenous peroxidases activity. Sections were subjected to 3,3'-diaminobenzidine tetra-hydrochloride (Sigma) used as the chromogen.

Sections were counterstained with Hematoxylin and mounted. Negative controls were treated similarly with the exception that primary antibodies were omitted. Sections from a block of infiltrating breast carcinoma were stained as a positive control for Survivin.

Evaluation of immunostaining:

Immunostained slides were evaluated; scoring for staining intensity was done as follows: 0, no appreciable staining in tumor cells; 1, barely detectable staining in cytoplasm and/or nucleus as compared with stromal elements; 2, readily appreciable brown staining distinctly marking the tumor cell cytoplasm and/or nucleus; and 3, dark brown staining in tumor cells completely obscuring the cytoplasm and/or nucleus. The percentage of stained tumor cells was scored as 0 (none), (1% to 10%), 2(11% to 50%), or 3(51% to100%). Immunoreactivity was considered low if

the sum of the proportion score and the intensity score was between 0 and 1, moderate if the sum was between2 and 4 and finally marked immunoreactivity if the sum was between 5 and 6.

Adjuvant Therapy:

After surgery patients were referred to the Oncology department for post-operative adjuvant therapy. All patients in this study were subjected to adjuvant treatment (5- Flurouracil 425 mg /m^2/d and Leucoverin 20 mg /m^2/d for 5 days). Treatment was initiated on day 1 and was followed by concurrent chemo-radiotherapy beginning 28 days after the start of the initial cycle of chemotherapy.

Chemo-radiotherapy consisted of 4500 GY of radiation at 180 c GY per day, five days per week for 5 weeks with 5 FU (400 mg /m^2 /d) and Leucoverin (20 mg /m^2/ d) on the first four and the last three days of radiotherapy. One month after completion of radiotherapy (5FU 425 mg /m^2/d + Leucoverin 20 mg /m^2/d) from D1-D5 for 2 cycles one month apart.(15)

Follow up:

All patients were followed-up for 24 months. During follow-up patients were subjected to physical examination, laboratory investigations including tumor markers (CEA, CA19-9) and radiological imaging (Chest x ray, and abdominal US) every 3 months for a total of 24 months. Patients underwent upper GIT Endoscopy 6 months after surgery and every 12 months thereafter. Abdominal & Pelvic CT scan was performed for corroborative evidence of relapse.

Statistical analysis:

It was carried out using SPSS program version 15. The correlation between the expression of Survivin and clinicopathological parameters was analyzed for statistical significance by x^2 (Chi square) test and Monte Carlo test. P value ≤0.05was considered statistically significant. The survival cures were plotted according to the Kaplan- Meier method and checked by log-rank test

RESULTS

The current study was undertaken on 30 patients with gastric cancer. Gastric carcinoma cases were 18 males &12 females with a male to female ratio (3:2). The mean age of the studied gastric carcinoma cases was 52.56 ± 11.83 years ranging from 34-82 years.

In the present study Survivin expression was mainly in the cytoplasm of the gastric carcinoma cells. Different patterns of Survivin expression were shown in (Fig. 1).

Survivin was frankly positively expressed in 66.7% of gastric carcinoma, specimens (20 out of 30 cases). Marked expression of Survivin protein was detected in 25% (5 out of 20 cases), moderate expression was detected in 40% (8 out of 20 cases) and weak expression was detected in 35% (7 out of 20 cases). Survivin was negatively expressed in 33.3% (10 out of 30 cases) of gastric cancer. The clinicopathological features of our cases and results from the analysis of correlation between Survivin expression and various clinicopathological parameters are presented in table (1).

Regarding patient demographic data, there was no association between Survivin expression and patients age or sex (p>0.05). As regards tumor size, positive Survivin expression was observed in 60% of gastric carcinoma specimens (12 out of 20 cases) in tumors having ≤ 5 cm in size while in tumors more than 5 cm in size Survivin was expressed in 80% of cases (8 out of 10 cases). The rate of Survivin expression in tumors more than 5cm in size was significantly higher than that in tumors ≤5cm in size (P≤0.05).

Concerning the tumor grade, the histopathological examination of the gastric carcinoma specimens revealed that differentiated tumors with varying degrees of differentiation (well, moderate and poorly) were the most frequent among gastric cancer patients (18 patients) accounting for 60% of cases, while the undifferentiated tumors were observed in (12 patients) accounting for 40% of cases. Survivin expression was detected in 10 cases (5 weak (+), 5 moderate (++) out of 18) differentiated tumors accounting for 55.5 %. While it was expressed in 10 cases (2 weak (+), 3 moderate (++) and 5 marked (+++) out of 12), undifferentiated tumors accounting for 83.3%. Survivin expression was significantly higher with undifferentiated adenocarcinoma than that of differentiated tumors.

Furthermore, and according to the histological classification of Lauren, Survivin expression was more frequent in diffuse tumors (8 out of 11 cases) (72.7%), than in intestinal type of cancer (6 out of 9 cases) (66.7%), and mixed type (6 out of 10 cases)(60%), however no significant

association was found between Survivin expression and Lauren classification (P>0.05).

Table (1) showed no significant association could be verified between expression of Survivin protein and the invasion depth of the tumor: T_1 (3out of 4 cases), T_2, (6 out of 8 cases),T_3 (9 out of 13 cases) and T_4 (2 out of 5 cases) (75%,75%,.69.2% and 40%) respectively.

As regards the lymph node involvement, their histologic examination, revealed that Survivin was expressed in 75% (3 out of 4 cases), 57.1% (4 out of 7 cases), 71.4% (5 out of 7 cases) and 66,7% (8 out of 12 cases) in N_0, N_1, N_2 and N_3 respectively. No significant correlation was observed between Survivin expression and lymph node involvement.

According to the TNM classification at the time of surgery 10 patients (33.3%) were stage II, 13 patients (43.3%)were stage III, and 7 patients (23,3%) were stage IV. Survivin expression was found in 70% (7 out of 10 cases), 69.2% (9 out of 13 cases) and 57.1% (4 out of 7 cases) in stages II, III & IV respectively. There was no statistical significant association between Survivin expression and tumor stage (P> 0.05).

Survival analysis:

Duration of follow-up was up to 24 months, by the end of this period; the disease free survival was 60% (6 out of 10 patients) for Survivin –ve patients and 40% (8 out of 20 patients) for Survivin +ve patients. Relapse was seen as either local recurrence, lymph nodes and/or multiple liver metastases. The overall survival was 60% (12 out of 20 patients) for Survivin +ve patients as compared to 80% (8 out of 10 patients) for Survivin –ve patients. Kaplan-Meier curves were constructed to compare the survival of patients with Survivin +ve expression versus patients with Survivin –ve expressions. Patients with +ve expression of Survivin appeared to have a shorter survival time as compared to those who have a negative Survivin expression although the difference was not statistically significant (P> 0.05). (Fig 2&3).

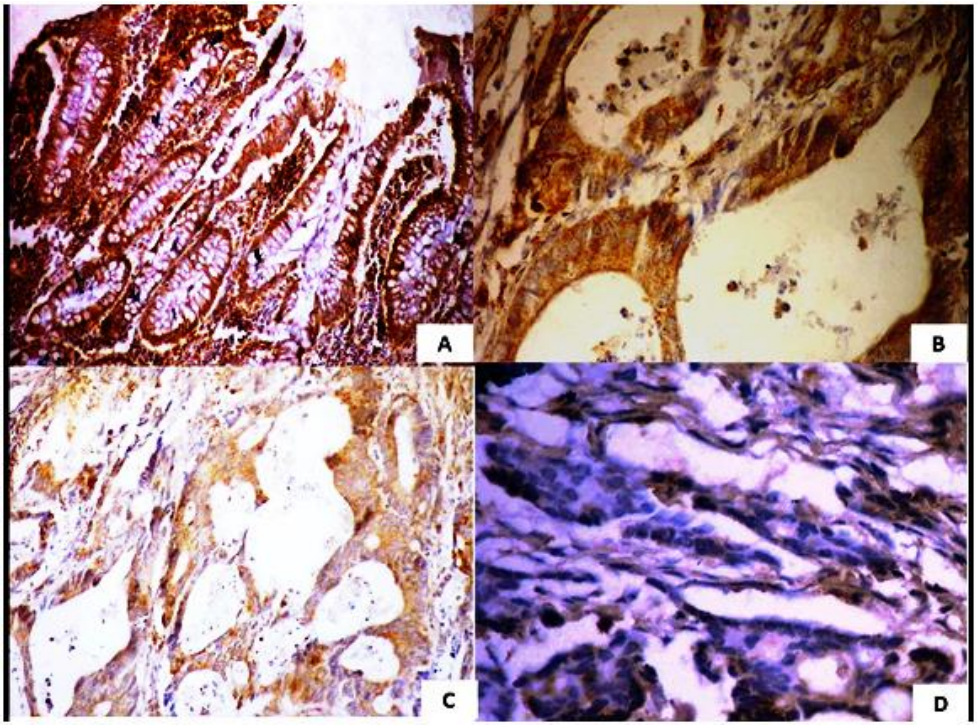

Fig. (**1**): Four representative samples of Survivin protein immunoreactivity, assessed by immunohistochemical staining in gastric carcinoma patients according to staining intensity. (A) Score 3, (B) Score 2, (C) Score 1, (D) Score 0.

Table (1): Correlation between Survivin expression and clinicopathological parameters of gastric cancer

	N	Survivin immunoreactivity								χ^2	^{MC}p
		Survivin Negative (n = 10)		Survivin positive (n=20)							
				Weak (n = 7)		Moderate (n = 8)		Marked (n = 5)			
		No.	%	No.	%	No.	%	No.	%		
Age											
<50	**9**	3	30.0	2	28.6	3	37.5	1	20.0	0.651	1.000
>50	**21**	7	70.0	5	71.4	5	62.5	4	80.0		
Sex											
Men	**18**	5	50.0	4	57.1	6	75.0	3	60.0	1.344	0.822
Women	**12**	5	50.0	3	42.9	2	25.0	2	40.0		
Tumor size											
≤5cms	**20**	8	80.0	6	85.7	6	75.0	0	0.0	10.729*	0.008*
>5cms	**10**	2	20.0	1	14.3	2	25.0	5	100.0		
Histopathological grade											
Differentiated	**18**	8	80.0	5	71.4	5	62.5	0	0.0	9.030*	0.025*
Undifferentiated	**12**	2	20.0	2	28.6	3	37.5	5	100.0		
Lauren classification											
Diffuse	**11**	3	30.0	2	28.6	3	37.5	3	60.0		
Intestinal	**9**	3	30.0	1	14.3	4	50.0	1	20.0	5.063	0.585
Mixed	**10**	4	40.0	4	57.1	1	12.5	1	20.0		
Depth of tumor											
T1	**4**	1	10.0	1	14.3	1	12.5	1	20.0		
T2	**8**	2	20.0	4	57.1	1	12.5	1	20.0	6.803	0.739
T3	**13**	4	40.0	2	28.6	5	62.5	2	40.0		
T4	**5**	3	30.0	0	0.0	1	12.5	1	20.0		
LN											
N0	**4**	1	10.0	1	14.3	2	25.0	0	0.0		
N1	**7**	3	30.0	2	28.6	1	12.5	1	20.0	3.642	0.992
N2	**7**	2	20.0	2	28.6	2	25.0	1	20.0		
N3	**12**	4	40.0	2	28.6	3	37.5	3	60.0		
Stage											
Stage II	**10**	3	30.0	4	57.1	2	25.0	1	20.0		
Stage III	**13**	4	40.0	2	28.6	6	75.0	1	20.0	8.374	0.187
Stage IV	**7**	3	30.0	1	14.3	0	0.0	3	60.0		

χ^2: Chi square test
MC: Monte Carlo test
*: Statistically significant at $p \leq 0.05$

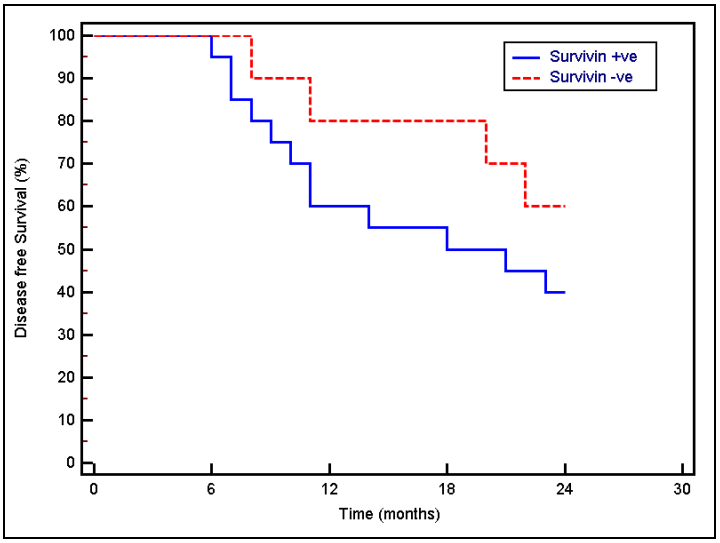

Fig. (2): Kaplan-Meier survival curve for disease free survival

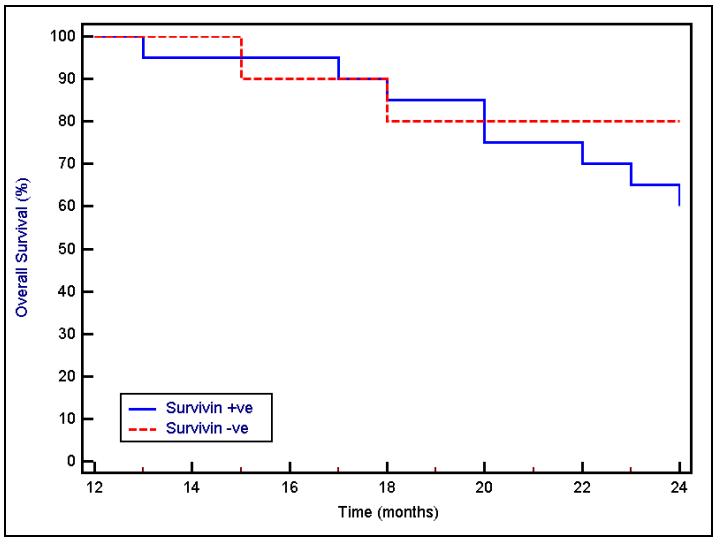

Fig. (3): Kaplan-Meier survival curve for overall survival

DISCUSSION

Development of gastric cancer, like many other malignancies, is a multi-step process involving the accumulation of mutations and changes in cell cycle regulatory mechanisms. The detection of these alterations in the early stage of cancer development may shed new light into the gastric carcinogenesis process.

Currently, using clinical parameters alone we cannot accurately predict the clinical outcome of patients after surgery. The discovery of molecular biological prognostic factors may aid in a more accurate prediction of clinical outcome and may also reveal novel predictive factors and therapeutic targets.[16]

Survivin, the anti-apoptotic gene has been extensively studied as a possible new biomarker in gastric cancer. It functions as a key regulator of mitosis and programmed cell death. The regulation of Survivin expression and function can occur at various levels including transcription, differential splicing, protein degradation and

intracellular sequestration via different ligands.[17] The expression of Survivin is upregulated at a transcriptional level by the nuclear factor-kB.[18] Survivin degradation occurs via the ubiquitin-proteasome pathway in the G1 phase of the cell cycle and is stabilized when bound to heat shock protein 90.[19-21]

The current study demonstrated that there was no statistical significant association between Survivin expression and patient demographic data including age and sex. The same result has been reported by other authors as in the study of Yu et al [22], Kyo et al [23] and others who demonstrated that there was no association between positive expression of Survivin and patient's age or sex.

For gastric cancer, previous studies showed the Survivin expression rate between 34.5% and 82.0% of cancerous tissues.[24,25] In gastric carcinoma or other human carcinomas, the overall expression of Survivin as reported in different studies is difficult to compare due to different score systems and different antibodies used. However, in the present work 66.7% of gastric carcinomas showed positive Survivin expression, so our findings were in line with those previously studied.

Our results regarding tumor size demonstrated that increased Survivin expression occurred more frequently in tumors of larger size than in their smaller-sized counterparts as indicated by the statistical significant association between Survivin expression and tumor size. In accordance with our findings Kyo et al [23], Lee et al [26] and others reported a significant association between Survivin expression and tumor size.

According to our findings, a significant association was found between Survivin expression and tumor grading. Positive Survivin expression was significantly higher in undifferentiated adenocarcinomas than in differentiated tumors. Also Survivin expression was higher in diffuse type than in intestinal or mixed carcinomas although the difference was not statistically significant. The above findings were in accordance with those reported by other authors Sun et al [27], Chen et al [28] and Zhang et al.[29] But in contrast to our findings Yu et al, Kyo et al [22,23] reported that there was no significant association between Survivin expression and tumor grade.

In the current study, Survivin expression showed no association with the invasion depth of the tumor, lymph node involvement and tumor stage confirming the findings reported by previous studies.(22,23) Contrary to our obtained data, an immunohistochemical study of Survivin expression in gastric cancer by Lee et al demonstrated that expression of Survivin was significantly associated with depth of invasion, lymph node metastasis, tumor stage and poor survival.[26]

In the present study, based on the Kaplan-Meier curves we found that increased Survivin expression was associated with poor disease free and overall survival although the difference was not statistically significant. Several reports showed that Survivin expression was correlated with poor survival of patients and contributed to chemo-resistance in gastric cancer.[30] In the literature, Survivin expression was shown to predict poor prognosis and shorter survival in various human cancers including laryngeal carcinoma [31], colorectal carcinoma [32], hepatocellular carcinoma [33], lymphoma [34], urothelial carcinoma [35], small adenocarcinoma of the lung [36], and esophageal carcinoma.[37] Previous studies demonstrated that increased Survivin expression was also associated with increased risk of recurrence, loco-regional lymph node invasion and distant metastasis.[38,39] Our results were in agreement with those previously studied. On the other hand, and in contrast to our obtained data Survivin expression was linked to good prognosis in different tumors including transitional cell carcinoma of bladder [40], and osteosarcoma[41] where its expression was linked with prolonged survival and good response to neoadjuvant chemotherapy.

Since recent experimental and clinical evidence showed that the anti-apoptotic gene, Survivin, regulates cancer cell proliferation as well as tumor-associated angiogenesis and is correlated with patient survival, these findings suggest that it may be used as prognostic factor and target for gastric cancer treatment. Hence targeting Survivin, at least by drugs that interfere with its expression or inhibit its function might have promising effects in reducing aggressiveness of the disease and may be expected to prevent cancer cells from using alternative salvage pathways during treatment and consequently increasing the disease free and overall survival in gastric cancer patients.[42]

In vitro and in vivo studies showed that inhibiting Survivin reduces tumor growth potential and sensitizes tumor cells to chemo-therapeutic agents, irradiation and immunotherapy.[43]

Finally, we conclude that the anti-apoptotic gene, Survivin, may be a useful biomarker of prognosis in patients with gastric cancer, consequently increased expression would be expected to predict a poor prognosis. Survivin may aid in a more accurate prediction of clinical outcome of gastric cancer and may also be a novel therapeutic target.

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