

Variation in Sp1 binding sites correlates with expression of survivin in breast cancer

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Abstract. Survivin is the smallest member of the inhibitor of apoptosis (IAP) family and is deregulated in breast cancer, where it is associated with a poor overall prognosis. It is well established that survivin overexpression predominately occurs at the transcriptional level. Numerous transcription factors bind to specific sequences in the promoter regions of genes and are involved in transcriptional regulation. Specificity protein (Sp) 1 binding sites have been found in the promoter region of the survivin gene. The present study aimed to investigate whether variations in Sp1 binding sites affect survivin expression. Nested polymerase chain reaction followed by DNA sequencing were performed to analyze the survivin gene promoter region in 42 breast cancer tissue samples. Furthermore, survivin expression was assessed using immunohistochemistry. High survivin protein expression was found in 66.7% (28/42) of breast cancer tissue samples. In addition, 15 variations in seven Sp1 binding sites were detected in 12 samples and Sp1 binding site variation was found to be associated with low survivin expression in the 42 samples. These findings suggested that variations in Sp1 binding sites may be associated with survivin expression.

Introduction

Survivin, encoded by the human baculoviral IAP repeat containing 5 (BIRC5) gene, is the smallest member of the inhibitor of apoptosis (IAP) family and is involved in several

distinct molecular processes, including cell division, intracellular signaling and apoptosis (1). Survivin is rarely expressed in normal human tissues, but is widely expressed in the majority of cancer types, including neuroblastoma, melanoma and hepatocellular carcinoma, as well as brain, esophageal, laryngeal, gastric, colorectal, bladder, renal, uterine, ovarian, lung, breast and pancreatic cancer. Moreover, survivin is associated with increased tumor aggression and poorer clinical outcome (2,3). Due to the high global expression of survivin in cancer, survivin has been proposed to be a molecular target for anticancer therapy (4-7).

The expression of survivin in breast cancer has attracted much research attention. Over the past year, several studies have investigated survivin mRNA and protein expression in breast cancer, as well as the correlation between survivin expression and clinicopathological factors and prognosis in patients with breast cancer. Survivin has been reported to be expressed in numerous types of breast cancer and overexpression of survivin has been associated with a poor overall prognosis (8-14).

The mechanisms underlying survivin overexpression in cancer have yet to be elucidated; however, survivin expression has been proposed to be regulated primarily at the transcriptional level (15). Genetic variation in the survivin promoter region may be responsible for survivin expression, particularly the -31G/C single-nucleotide polymorphism (SNP), which has been shown to affect survivin expression, thereby influencing overall susceptibility to cancer (16,17). The -31G/C SNP is located at the cell cycle-dependent element/cell cycle homology (CDE/CHR) repressor region and mediates survivin expression through functional disruption of binding at the CDE/CHR repressor motif (18). However, -31 G/C polymorphism has not been found to be associated with survivin expression in breast cancer (19). Analysis of the putative promoter region of BIRC5 has revealed that the ubiquitous transcription factor specificity protein (Sp) 1 may be responsible for the constitutive survivin expression in tumors (20,21). Furthermore, numerous other transcription factors, including retinoblastoma protein, p53, signal transducer and activator of transcription 3, c-Myc, E2F, Krüppel-like factor (KLF) 5 and KLF4, may be involved in the regulation of survivin expression (22,23). Among these transcription factors, Sp1 has attracted the most research attention. Sp1 has been proposed to be the predominant reason

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Abbreviations: IAP, inhibitor of apoptosis; BIRC5, human baculoviral IAP repeat containing 5; Sp1, specificity protein 1

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for constitutive survivin expression in tumors (20,21). There are at least seven potential Sp1 binding sites in the core promoter region of survivin, according to the canonical or close to canonical sequence (G/T)(G/A)GGCG(G/T)(G/A)(G/A)(C/T), with at least three of the Sp1 binding sites reported to be functionally active (20-22). Furthermore, other transcription factors, including KLF4, have been found to regulate survivin expression through direct interaction with Sp1 (23,24).

Thus, Sp1 may be the key to understanding survivin expression in cancer. *In vitro* assays have revealed that Sp1 mutation may decrease survivin promoter activity, as well as survivin expression (22). The present study aimed to investigate whether Sp1 binding sites vary in breast cancer tissues and to analyze the correlation between Sp1 binding site variations and survivin expression.

Materials and methods

Tissue sample collection and immunohistochemistry. Breast cancer tissue samples were obtained from Loudi Central Hospital (Loudi, China). All samples were confirmed by pathologists. Paraffin-embedded samples were cut into 4-mm sections and immunohistochemistry was performed using a standard streptavidin peroxidase (SP) method. Slides were dewaxed using xylene and rehydrated with serial dilutions of ethanol. Antigen retrieval was then performed and endogenous peroxidase activity was blocked using 3% hydrogen peroxide and nonspecific staining was blocked using 10% normal goat serum (Vector Laboratories Inc., Burlingame, CA, USA). Slides were incubated with anti-survivin antibodies diluted 1:100 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C in a humidified chamber overnight, followed by incubation with biotin-conjugated secondary antibodies (ZSGB-BIO, Beijing, China) for 30 min and incubation with streptavidin peroxidase for 15 min. 3,3'-diaminobenzidine tetrachloride (DAB; ZSGB-BIO) was added to the slides to detect peroxidase activity. Sections were then dehydrated using alcohol and cleared using xylene. The slides were assessed by two individual investigators. Survivin immunoreactivity was scored between 0 and 4 based on the color and percentage of cells showing distinct nuclear and/or diffuse cytoplasmic immunohistochemical staining. Informed consent was obtained from all patients prior to sample collection and the protocol was approved by the Institutional Review Board of Loudi Central Hospital (Loudi, China).

DNA extraction, nested polymerase chain reaction (PCR) and DNA sequencing. Genomic DNA was extracted from three paraffin-embedded sections representative of breast cancer tissues. The DNA was then isolated using a DNA Extraction from Paraffin-Embedded Tissue kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. The concentration and purity of the extracted DNA was assessed using spectroscopy with a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). Nested PCR analysis was performed to detect Sp1 binding sites in the survivin gene promoter region. The primer sequences were as follows: External primers: SurO1, forward 5'-CGCGTTCTTTGAAAGCAGTCGAG-3' and SurO2, reverse 5'-GAAGGCCAGTTCTTGAATGTAGAG-3', which were used for the first round of PCR; and internal primers:

SurI1, forward 5'-GCTAGGTGTGGGCAGGGACGAGCTG-3' and SurI2, reverse 5'-GATTCAAATCTGGCGGTTAATGG-3', which were used to amplify a 207 bp fragment in a final volume of 50 µl. A total of 5 µl PCR product was separated on a 1.5% agarose gel and stained with ethidium bromide, followed by analysis under ultraviolet (UV) light. Amplified products which were observed as clear bands were sequenced in duplicate in the forward and reverse directions using a BigDye® Terminator sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA) and an ABI Prism 3730XL DNA Analyzer (Applied Biosystems, Inc.) according to the manufacturer's instructions. Sequences were compared against the archived sequence of the human survivin gene in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>).

Statistical analysis. All statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) for windows. The χ^2 test was performed to analyze the correlation between Sp1 binding site mutations in the survivin gene promoter region and survivin expression in breast cancer tissues. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Survivin expression in 42 breast cancer tissues detected using immunohistochemistry. Survivin expression was analyzed in 42 patients with breast cancer using immunohistochemistry. Samples were scored between 0 and 4 based on survivin expression. The numbers of samples exhibiting survivin expression with a score of 0, 1, 2, 3 and 4 were 7 (16.7%), 7 (16.7%), 12 (28.6%), 12 (28.6%) and 4 (9.5%), respectively. Scores of 0 and 1 were defined as 'low expression' and scores of 2-4 were defined as 'high expression'. Among the 42 tissue samples, 14 tissues exhibited low survivin expression, while 28 samples had high survivin expression.

Analysis of Sp1 binding site variation in the survivin gene promoter region. In order to compare nucleotide sequences, survivin gene promoter fragments were amplified from the genomic DNA isolated from the 42 breast cancer tissue samples using nested PCR. The intended sequence was 207 bp long, containing seven Sp1 binding sites from -34 to -240 upstream of the translation initiation site of the survivin gene (Fig. 1A). The PCR products were separated on a 1.5% agarose gel. PCR fragments which exhibited single clear bands under UV light were sequenced (samples 1, 3 and 4; Fig. 1B). In total, 15 variations on seven Sp1 binding sites were observed in 12 samples. No Sp1 binding site variations were found in the other 30 samples. Among these variations, Sp1 binding site E, which is located at the 'Sp1-complex' (21), was observed to have the greatest mutation frequency, while the Sp1 binding site A exhibited no variation (Table I).

Correlation between variation in Sp1 binding sites in the survivin gene promoter region and survivin expression. In order to analyze the correlation between variation in Sp1 binding sites in the survivin gene promoter region and survivin expression, χ^2 tests were performed and a significant correlation was observed ($P < 0.05$, Table II). As shown in Fig. 2, case 1 was a sample which exhibited no Sp1 binding

Table I. Variations in Sp1 binding sites in the survivin gene promoter in 42 patients with breast cancer.

Sp1 binding site	Variations				
	1	2	3	4	5
A	\	\	\	\	\
B	-184G/A	\	\	\	\
C	-171G/A	\	\	\	\
D	-150G/A	-151G/A	\	\	\
E	-129C/T	-130G/A	-132C/T	-140G/A	-141C/T
F	-94C/T	-95C/T	-101G/A	\	\
G	-82G/A	-84G/A	-87G/A	\	\

Sp, specificity protein.

Table II. Correlation between Sp1 binding site variation and survivin expression in 42 breast cancer samples.

Sp1 binding site	Low survivin expression, n (%)	High survivin expression, n (%)	Total n (%)	Value	
				χ^2	P-value
Mutated	8 (19.0)	4 (9.5)	12 (28.6)		
Not mutated	6 (14.3)	24 (57.1)	30 (71.4)		
Total	14 (33.3)	28 (66.7)	42 (100)	8.400	0.004

Sp, specificity protein.

A TCGAGGGGGCGCTAGGTGTGGGCAGGGACGAGCTGGC
GCGGCGTCGCTGGGTGCACCGCGACCACGGGCAGAG
CCACGCGGGCGGGAGGACTACAACCTCCCGGCACACCCC
GCGCCGCCCCGCCTCTACTCCAGAAGGCCGCGGGGG
GTGGACCGCCTAAGAGGGCGTGCCTCCCGACATGCC
CCGCGGCGCGCCATTAACCGCCAGATTTGAATCGCGGG
 ACCCGTTGGCAGAGGTGGCGGCGGGC**AT**GGGTGCC
 +1



Figure 1. Sequence analysis of the survivin gene promoter. (A) DNA sequence analysis of the 5' flanking region of the human survivin gene. Numbering is from the translation initiation site ATG. The 207 bp PCR sequence is shown in bold and the putative bindings sites for Sp1 are underlined and numbered A-G. (B) Nested PCR of the survivin promoter. PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and were observed under ultraviolet light. 1, 3 and 4 represent three samples which were subsequently sequenced. PCR, polymerase chain reaction; Sp, specificity protein.

a -150 G to A heterozygous variation and low expression of survivin (Fig. 2).

Discussion

Breast cancer is a heterogeneous group of tumors which differ in molecular characteristics, prognosis and response to therapy (25,26). Somatic mutations occurring at any stage of cancer development may affect the properties of the cancer cells. Certain recurrent somatic alterations in breast cancer have been reported, including human epidermal growth factor receptor 2 amplifications, which were the first successful therapeutic targets defined by a genomic aberration (27). Several studies have investigated mutations across different breast cancer subtypes (28-31) and recurrent somatic gene mutations, particularly in coding regions, including core-binding factor, beta subunit (28), TP53 (28) and GATA3 (32), have been identified in breast cancer. Genomic alterations which affect survivin expression in breast cancer cells have been reported and loss of heterozygosity in breast cancer has been associated with survivin expression (19). In addition, the survivin gene is located at 17q25, a hotspot for mutations in breast cancer and other tumors (33-35). Thus, the high mutation rate at the survivin gene promoter may be associated with genomic instability.

In addition to somatic gene mutations, SNPs have been a focus of cancer research. The -31 G/C SNP of the survivin gene promoter has been reported to be associated with gastric (36), colorectal (17), lung (37) and urothelial cancer (38) as well as

site variations in the survivin promoter and had high survivin expression. Case 2 had a -130 G to A homozygous variation and its survivin expression was low. Moreover, case 3 had

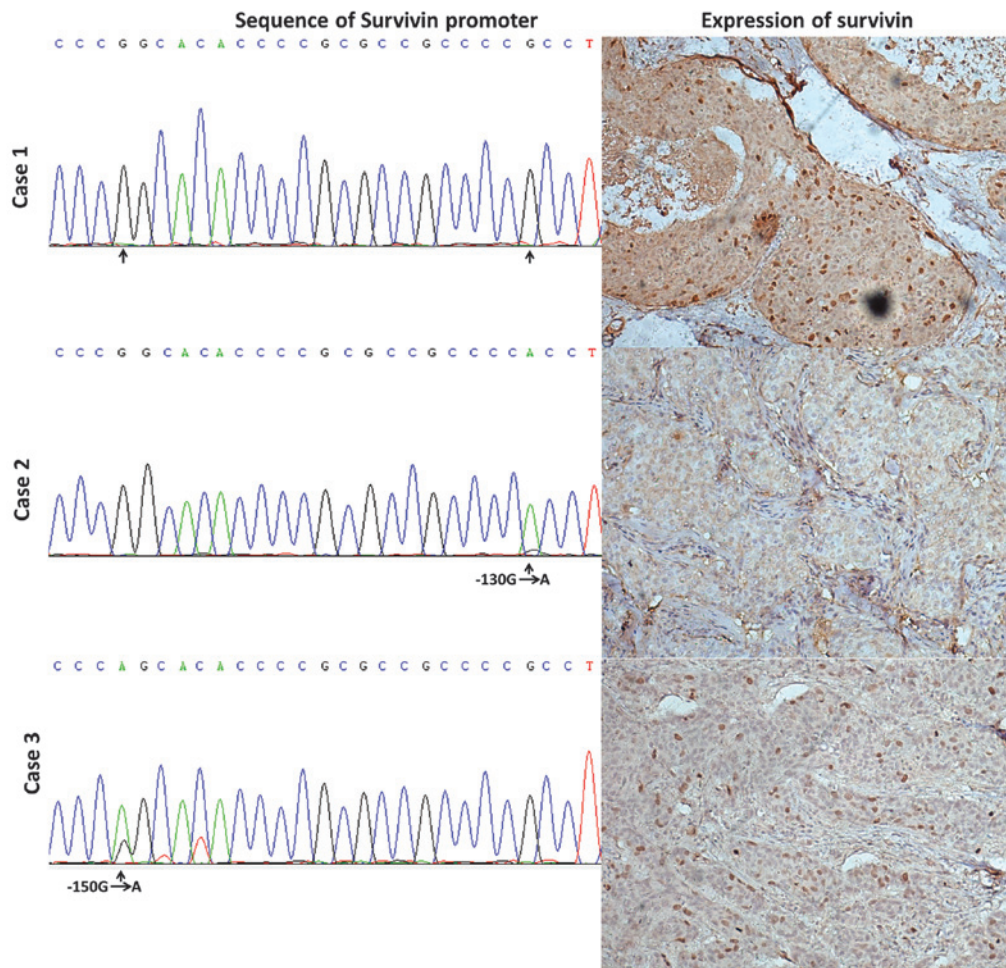


Figure 2. Sp1 binding site mutations in the survivin gene promoter and survivin protein expression in breast cancer tissues. Case 1 represents a case which had no putative Sp1 binding site mutations and high survivin expression. Case 2 represents a case which had a -130 G to A mutation and case 3 represents a case which had a -150 G to A mutation. Cases 2 and 3 had low survivin expression. Sp, specificity protein.

esophageal squamous cell carcinoma (39), and may be a risk factor for cancer development. However, the -31 G/C polymorphism has not been associated with cervical (40) or breast (32) cancer. Based on the central role of survivin in multiple cellular networks, it is important to elucidate the factors regulating and influencing survivin expression (6).

The transcription factor Sp1 is one of the most important regulators of survivin. Sp1 binds to GC-rich motifs in various promoters and is involved in numerous cellular processes, including cell differentiation, growth and apoptosis, as well as immune responses, DNA damage responses and chromatin remodeling (41). Polymorphisms of Sp1 binding sites which affect protein expression have been investigated in several studies (42-45), particularly in patients with cancer. For example, the -216 G/T polymorphism of the Sp1 binding site at the epidermal growth factor receptor promoter region has been reported to be associated with altered promoter activity and gene expression *in vitro* and *in vivo* (43). Furthermore, SNPs of the Sp1 binding site at the mouse double minute 2 homolog promoter have been associated with risk of breast and ovarian cancer, and Sp1 binding site polymorphisms at the matrix metalloproteinase-2 promoter have been correlated with risk of gastric (44) and lung cancer (45). Prior to the identification of Sp1 binding site polymorphisms at the survivin promoter,

survivin expression was shown to be regulated by Sp1 through several Sp1 binding sites in the survivin promoter, which were close to the translation initiation site, and mutations in these binding sites were found to decrease survivin expression *in vitro* (20-22,46,47). In the present study, low survivin expression was observed to correlate with Sp1 binding site variation in the survivin promoter *in vivo*. While the mechanism underlying the high mutation rate in the survivin gene promoter region and the functional significance of such mutations in breast cancer remain to be fully elucidated, the phenomenon suggests that survivin promoter genotypes may influence survivin expression, thus influencing individual susceptibility to the initiation and development of certain types of cancer.

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