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Role of survivin in the pathogenesis of papillary thyroid carcinoma

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ABSTRACT. The purpose of this study was to assess the correlation between the survivin gene and the occurrence and pathogenesis of papillary thyroid carcinoma (PTC). Sixty patients with PTC and no preoperative chemotherapy were recruited for the study and 30 thyrophyma patients receiving operative treatment in Drum Tower Hospital (Nanjing, China) were included as the control group. The protein expression levels of survivin were assessed by immunoblotting and immunohistochemical analysis of tissues from both patient groups. For survivin gene knockdown experiments, two target sequences were selected based on the mRNA sequence of survivin and two pairs of siRNA interference sequences were designed and synthesized accordingly. The siRNAs were shown to be successfully transfected into SW579 carcinoma cells and the resulting survivin knockdown was assessed by RT-PCR and immunofluorescence. Survivin was shown by immunohistochemistry to be distributed in the cytoplasm of PTC and thyrophyma cells, with the signal being significantly stronger in PTC cells than in thyrophyma cells and statistical analysis of immunostaining data further showed survivin to be more highly expressed

(P < 0.05) in the PTC tissue than in the thyrophyma tissue. Transfection of SW579 cells with siRNA was found to be effective in knocking down the expression levels of survivin: 87.3 and 76.2% knockdown was achieved with sh-Survivin-1 and sh-Survivin-2, respectively. The findings reported here show that survivin is highly expressed in PTC and may therefore play a role in the occurrence, lymph node metastasis, and clinical staging of PTC.

Key words: Survivin; Papillary thyroid carcinoma; Pathogenesis; Signaling pathway

INTRODUCTION

Thyroid cancer is the most common endocrine tumor and 5-10% of clinically identified thyroid nodules are found to be thyroid cancer. In the last 30 years, thyroid cancer morbidity has increased significantly-from approximately 10/1,000,000 to approximately 30/1,000,000-40/1,000,000 in China and a 300% increase in the USA. These thyroid cancers are primarily papillary thyroid carcinoma (PTCs; (Yazdani et al., 2012), which is the most common thyroid cancer. PTC accounts for approximately 70-80% of thyroid cancers and is often found in young females with good prognoses (Min et al., 2013). PTC grows slowly and can be restricted within the thyroid gland for many years. Lesions can spread from the primary sites to other sites and to regional lymph nodes; however, these can also remain restricted for many years, resulting in these tumors often being undetected (Singh et al., 2013). Pathologically, PTC patients have well-differentiated columnar epithelia exhibiting papillae as well as clear nuclei and eosinophilic cytoplasm. Concentric deposition of calcium salts is often observed in PTC (Wang et al., 2013d). Apart from nodules and local enlargement of lymph nodes, the clinical manifestations of PTC are few (Zhao et al., 2013). Cold nodules are observed through thyroid nuclein scanning and the deposition of dotted calcium salts can be observed in the tumor via X-ray examination of the neck (Lee et al., 2013). Papillary carcinoma growth is stimulated by thyroid-stimulating hormone and the carcinomas can be shrunk with thyroid hormones. Eighty percent of PTC patients survive approximately 10 years (Brahma et al., 2013).

Survivin is a new member of the anti-apoptosis protein (inhibitor of apoptosis, IAP) family and is the most potent apoptosis inhibitor. Survivin has complex functions, including inhibition of apoptosis, promotion of cell transformation, participation in cell mitosis and angiogenesis, and furthermore plays a role in the drug tolerance of tumor cells (Wang et al., 2013c). Survivin is tumor-specific and is only expressed in tumors and embryonic tissues. Survivin is furthermore closely associated with the differentiation, proliferation, infiltration, and metastasis processes in tumor cells. Survivin acts directly on caspase signaling, primarily by inhibiting the activity of caspase-3 and caspase-7. Caspase signaling may also be indirectly inhibited by survivin via P21 (Vandghanooni et al., 2011). Survivin is furthermore known to bind to the cell cycle regulatory factor CDK4 resulting in the activation of CDK2/cyclin-E and the subsequent phosphorylation of ribosomes (Hartgerink et al.). This can initiate cells into the cell cycle after phosphorylation of ribosomes to accelerate transformation at the G1/S stage (Aynaci et al., 2013; Liarmakopoulos et al., 2013).

Therefore, survivin plays important role in the cell cycle and apoptosis as a new member of the anti-apoptosis protein. To assess the correlation between the survivin gene and the occurrence and pathogenesis of papillary thyroid carcinoma (PTC), we evaluated protein expression levels of survivinby different methods. For survivin gene knockdown experiments, two pairs of siRNA were successfully transfected into SW579 carcinoma cells.

MATERIAL AND METHODS

Materials

The rabbit anti-survivin antibody and rabbit anti-β-actin monoclonal antibody were purchased from Sigma (St. Louis, MO, USA); the TaqMan microRNA reverse transcription kit was purchased from invitrogen (Carlsbad, CA, USA).; PVDF membrane was purchased from Shanghai Baoman Biotechnology Co. Ltd. (Shanghai, China); the HRP marked goat anti rabbit IgG was purchased from Sigma (St. Louis, MO, USA); SW579 cells were purchased from Bio-Rad (Beijing, China); siRNA knockdown and control fragments were purchased from Amresco (Solon, OH, USA).

Collection of clinical data

Sixty patients with PTC who underwent surgical excision from January 2012 to March 2013 were selected for this study and none of the study participants had received preoperative chemotherapy. Thirty thyrophyma patients receiving operative treatment in Drum Tower Hospital were selected as the control group. Diagnoses of all cases were based on the clinical data of patients and pathologies were confirmed postoperatively. Tumor tissue was obtained at the tumor sites within 20 minutes after tumor excision. Part of the tissue was embedded with paraffin for pathological diagnosis and immunohistochemical detection and the remaining specimen was cryopreserved in two tubes containing liquid ammonia for use in subsequent experiments. Based on the data of cases and pathological diagnoses of the patients, the following clinical data were recorded: name, gender, age, tumor size, lymphatic metastasis, and clinical stages of gall. The AJCC staging system was used for clinical staging of tumors.

Immunohistochemistry

Dimethylbenzene solution or gradient alcohol was routinely used for dewaxing of tissue samples. H_2O_2 (3%) was freshly prepared with distilled water and samples were washed with distilled water three times for 2 min each after inactivation of endogenous peroxidase for 10 min at room temperature. The sections were immersed in 0.01 M citrate buffer solution (pH 6.0), heated in a microwave oven for 5 min at a high temperature, cooled for 10 minutes, and then washed three times with 0.1 M PBS for 5 min each. The samples were blocked for 20 min at room temperature with nonimmune serum blocking solution (added to samples dropwise), after which surplus liquid was removed, primary antibodies (or PBS for negative control) were added dropwise to samples, and samples were then stored overnight at 4°C. After incubation with primary antibodies, the samples were washed three times with 0.1 M PBS (5 min each time) and then at 37°C for 20 min with biotinylated goat anti-rabbit IgG (secondary antibody). After incubation with secondary antibodies the samples were washed with 0.1 M PBS (three times, 3 min each) and then incubated with horseradish peroxidase-labeled streptavidin (diluted with PBS) at 37°C for 20 min. The samples were washed with 0.1 M PBS (three times, 3 min each), after which freshly prepared DAB color developing agent was added dropwise. The samples were washed with sufficient water to terminate the reaction. Slight counterstaining with hematoxylin was carried out, after which the samples were dehydrated, clarified, and mounted with neutral resin. IgG with the same antibody species was used for substitution and the remaining steps were the same as the above. Five fields were photographed microscopically to allow for immunohistochemical analysis to be carried out.

Immunoblotting

For immunoblotting, the protein was extracted from collected tissue samples and separated with 10% SDS-PAGE as previous described (Kamiya et al., 1998). The gel was transferred to a PVDF membrane (Solvay Chemicals, Belgium) and blocked for one hour. The rabbit anti-survivin and goat anti-rabbit IgG antibody were used as primary and secondary antibody at dilution of 1:1000 and 1:10000, respectively. Signal was visualized with the ECL kit (Amersham International, Amersham, UK). Image J software (NIH, Bethesda, MD, USA) was used to compare the gray values between the proteins of interest and the internal control protein, as well as between the phosphorylated protein and the total protein.

siRNA transfection

The SW579 thyroid cancer cell line was cultured and transfected with target siRNA. The experimental groups were a control group (transfection with the control interference fragment), the survivin interference group 1 (transfection with the survivin interference fragment 1), and the survivin interference group 2 (transfection with the survivin interference fragment 2). A fluorescence microscope was used to assess the transfection results, allowing the transfection efficiency to be calculated.

Blank and recombinant plasmids (1 µg each) were subjected to double digestion by *Eco*RI and *Hin*dIII at 37°C for 2 h as those used to linearize the blank plasmid. The plasmids (0.5 µg) were then subjected to PCR amplification with the following primers: survivin interference group 1, 5'-TACGATACAAGGCTGTTAGAGAG-3' (sense primer) and 5'-TAGAAGGCACAGTCGAGG-3' (anti-sense primer); survivin interference group 2, 5'-TACGACACTAGGTTGTTAGAGAG-3' (sense primer) and 5'-TAGAACGCAGAGTGGACG-3' (anti-sense primer). Reactions (total volume of 25 µL) consisted of plasmid (0.5 µg), 10X PCR buffer (2.5 µL), 25 mmol/L $MgCl_2$ (2 µL), 10 mmol/L dNTP (0.5 µl), 5 U/µl TaqDNA polymerase (0.5 µL), 10 mmol/L primers (0.5 µL each), and distilled water. The recombinant plasmids (5 µg) were also labeled to allow for the transfection outcomes to be assessed by fluorescence microscopy in terms of transfection efficiency.

Quantification of survivin gene expression by RT-PCR

Differences in the expression levels of survivin in the three groups of successfully transfected cells were assessed by RT-PCR. Total RNA was extracted from cells using the Trizol method, after which cDNA was synthesized from the RNA by reverse transcription. Using specific primers, PCR amplification was performed to allow for fluorescence-based quantitation of the gene expression. Sequences of the primers were 5'-CCACAGGAAACCACCCAATA-3' (survivin upstream primer), 5'-GCTGATGAGGCATTGGGAAT-3' (survivin downstream primer), 5'-AACCGGTGAAGGACTGGTG-3' (β-actin upstream primer), 5'-GGTGGGTAGAGTGTGGTTT-3' (β-actin downstream primer). PCR reaction volumes were 10 µL and were composed of cDNA (1 µL), survivin (or β-actin) upstream and downstream primers (0.2 µL each), 2X Premix Ex Taq (5 µL), and H_2O (3.6 µL) (Antonaci et al., 2008).

Statistical analysis

Statistical analysis of the data was carried out using SPSS 15.0 statistical software. A variance analysis and an independent *t*-test were performed for means and data were expressed

as mean ± standard deviation (± s). Transfection efficiencies were compared with the X^2 test. Differences with $P < 0.05$ were considered significant.

RESULTS

Patient data

The group of study participants (N = 60 PTC patients) consisted of 22 males and 38 females aged 20-60 years (average 38.8 ± 19.7 years). All participants were inpatients and were pathologically diagnosed definitely post operation. Based on the AJCC staging system, the cases consisted of 27 Stage I cases, 12 Stage II cases, 14 Stage III cases, and 7 Stage IV cases (Table 1). The group of control patients (N = 30 thyrophyma patients during the same period) consisted of 9 males and 21 females aged 20-60 years (average 37.6 ± 18.4 years). All cases were inpatients and pathologically diagnosed definitely post operation.

Table 1. General clinical data of papillary thyroid carcinoma.

Group		Number of cases	Percentage
Gender	Male	22	36.67
	Female	38	63.33
Age	≤40	26	43.33
	>40	34	56.67
Size	≤2cm	32	53.33
	>2cm	28	46.67
Lymph Node Metastasis	Yes	35	58.33
	No	25	41.67
Clinical Stages	Stage I and II	39	65.00
	Stage III and IV	21	35.00

IHC analysis of survivin expression in PTC tissue

H&E staining showed PTC cells to be arranged in an island-shaped pattern and interstitial blood sinuses to be rich: size and morphology of the cancer cells were relatively consistent (Figure 1A-a). Thyrophyma cells were found to vary in size and were round, oval, or irregular in shape. Follicles covered the simple cuboidal epithelial cells and the follicular cavity was filled with a large amount of red colloid (1A-b).

Immunoblotting shows an increased expression of survivin in PTC

The expression of survivin in PTC and thyrophyma tissues was assessed immunohistochemically and the results of this assessment showed survivin to be distributed in the cytoplasm of PTC and thyrophyma cells, with the signal in PTC cells being significantly stronger than that in the thyrophyma tissue (Table 2, Figure 1B, and Figure 1C). To exclude increases in signal due to non-specific immunohistochemical staining, immunoblotting was used to assess survivin expression. The immunoblotting analysis demonstrated good specificity for the survivin protein, since the signal appeared as a single band at the expected size. The expression levels of surviving (normalized to the internal control β-actin) were found to be significantly higher in PTC tissue than in thyophyma tissue (Figure2A, Figure 2B, and Table 3).

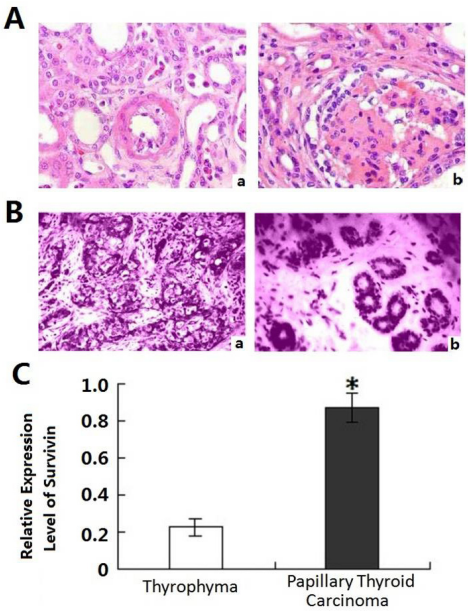

Figure 1. A. H&E staining of papillary thyroid carcinoma (a) and thyrophyma (b) tissue specimens. **B.** Immunostaining of survivin in tissue specimens of papillary thyroid carcinoma (a) and thyrophyma (b). **C.** Statistical result of immunostaining of survivin in tissue specimens of papillary thyroid carcinoma and thyrophyma. *P < 0.05 compared with the thyrophyma group.

Table 2. Comparison of survivin expression in two groups as quantified by immunohistochemistry.

Group	Relative expression level of survivin
Thyrophyma	0.227 ± 0.096
Papillary thyroid carcinoma	0.874 ± 0.113*

Compared with the thyrophyma group, *P < 0.05.

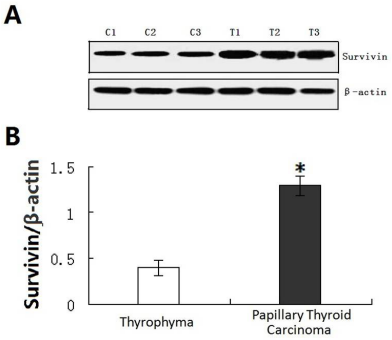

Figure 2. A. Comparison of the protein expression levels of survivin in tissue specimens of papillary thyroid carcinoma and thyrophyma, where C1, C2, and C3 represent three control thyrophyma tissues and T1, T2, and T3 represent three papillary thyroid carcinoma tissues. β-Actin served as an internal control. **B.** Statistical results of immunoblot analysis of survivin in tissue specimens of papillary thyroid carcinoma and thyrophyma. *P < 0.05 compared with the thyrophyma tissue.

Table 3. Comparison of survivin expression in two groups as quantified by immunoblotting.

Group	Survivin/β-actin
Thyrophyma	0.392 ± 0.126
Papillary thyroid carcinoma	1.287 ± 0.272*

Compared with the thyrophyma tissue, *P < 0.05.

Transfection of SW579 cells with survivin siRNA

To verify that siRNA transfections were effective, the synthesized siRNA was used to transfect SW579 thyroid cancer cells. After optimization of the transfection conditions, a transfection efficiency of 93.6% was achieved. Fluorescent labeling of the siRNA allowed for successfully transfected cells to be identified by fluorescence microscopy (Figure 3A). The siRNA transfection was considered effective based on these results and transfection with the siRNAs was therefore deemed suitable for survivin knockdown.

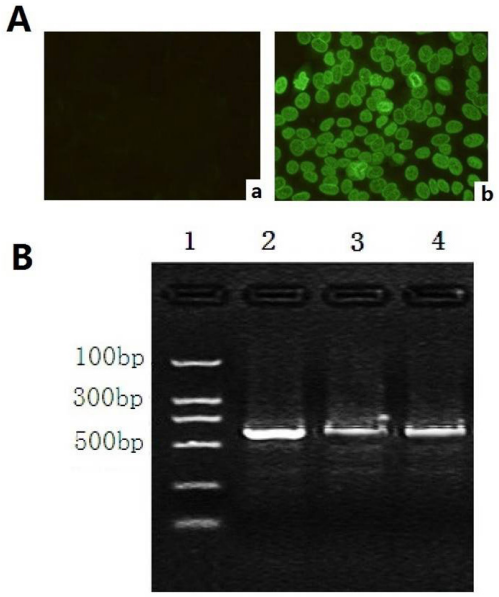

Figure 3. A. Fluorescence microscopy images of untransfected (a) and siRNA-transfected (b) SW579 cells. **B.** RT-PCR quantification of survivin expression in SW579 cells. 1: marker; 2: transfection with control siRNA; 3: transfection with sh-Survivin-1 siRNA; 4: transfection with sh-Survivin-2 siRNA.

siRNA knockdown of survivin expression

Reverse transcription was performed with the RNA extracted from the siRNA-transfected cells, after which the survivin gene was amplified with RT-PCR for relative quantification. The RT-PCR results showed that siRNA knockdown of survivin was successful: the level of survivin mRNA was significantly reduced by both sh-Survivin-1 siRNA (87.3% knockdown) and sh-Survivin-2 (76.2% knockdown) as shown in Figure 3B and Table 4.

Table 4. Comparison of survivin mRNA levels in three groups after siRNA knockdown.

Group	Survivin/actin
Control Interference Group	1.000 ± 0.000
Survivin Interference Group 1	0.237 ± 0.062*
Survivin Interference Group 1	0.375 ± 0.074*

Compared with the control group, * P < 0.01.

DISCUSSION

The morbidity of PTC accounts for approximately 1-2% of systemic malignant tumor morbidities and exhibits an increasing trend in consecutive years, which significantly increases particularly in females and poses a great risk to human health (Zhang et al., 2013). The specific pathogenesis of thyroid cancer still remains unclear. Based on clinical research and epidemiological investigations; however, the onset of thyroid cancer has been associated with various factors including ray radiation, hormonal effects, and iodine intake (Pyo et al., 2013). A number of oncogenes and cancer suppressor genes participate in thyroid cancer occurrence and progression and the main genes among these are survivin, TRK, BRAF, RAS, and cancer suppressor genes such as p53 (Wang et al., 2013b).

The occurrence and progression of thyroid cancer are also affected by various external environmental factors and the multi-step progression process involves a number of genes (Aframian et al., 2013). Molecular biological research has shown that normal human cells transform into malignant tumor cells based on molecular biological changes. As a result, the cell growth is not subject to the normal growth regulation and cell cycle, which in turn leads to the occurrence of malignant cellular changes. The occurrence and progression of thyroid cancer, just like other malignant tumors, also involves a number of oncogenes and cancer suppressor genes that play a role in enabling the transformation of normal cells into malignant cells with no growth control (Ito et al., 2003; Dong et al., 2006; Xiang et al., 2007; Nam et al., 2013; Wang et al., 2013a). Changes in the expression levels of these genes during different periods can be assessed to gain a better understanding of the pathogenesis of thyroid cancer. Based on current knowledge, the main molecular biological changes during the occurrence of thyroid cancer include mutations of a number of genes, changes in the expression levels of certain proteins, and changes in intracellular signaling pathways (Antonaci et al., 2008; Chen et al., 2012; González et al., 2013; Jariwala et al., 2013; Pannone et al., 2014).

The survivin gene, 15 kb in size, is located on chromosome 17q25 and contains four exons and three introns. The survivin gene product is a protein comprising 142 amino acids with a molecular weight of 16.2 kDa. The survivin protein has a human-mouse homology of 84.3% (Mostaan et al., 2013). Compared with other members of the IAP family, the structure of survivin is unique: the IAP family proteins generally contain 2-3 tandem baculovirus inhibitor of apoptosis protein repeats (baculovirus IAP repeat, BIR) molecules comprising 70 amino acids and containing a cysteine/histidine consensus sequence and a hydroxy terminal ring finger structure, of which the BIR molecules exert an anti-apoptotic effect (Yahya et al., 2012). Survivin, in contrast, contains only a single BIR functional domain and a three amino acid insertion sequence of Cys46-Pro47-Thr48 dimidiates the survivin. The hydroxyl terminal of survivin contains a coiled-coil structure instead of a ring finger structure, further distinguishing it from the other members of the IAP family (Taniguchi and Sasaki, 2012). By blocking the head and neck squamous cell carcinoma (HNSCC) cells at

different stages of cell proliferation (G_1, S, and G_2/M) using a cell division blocking agent, Khan and colleagues showed that survivin (in terms of endogenous mRNA levels) is hardly expressed at the G_1 stage and that the expression of survivin is increased 6-fold at the S stage and 40-fold at the G_2/M stage (relative to G_1 stage expression levels), indicating that survivin is primarily expressed at the G_2/M stage and is thus a regulator gene at the G_2/M stage of the cell cycle (Khan et al., 2012).

Cell apoptosis in the case of most apoptosis-inducing signals is known to occur via related protease (Varras et al., 2012). Survivin resists the induction of apoptosis at the G_2/M stage of the cell cycle through cell cycle-dependent expression. The overexpression of survivin in tumor cells is furthermore known to overcome the checkpoint of apoptosis and survivin moreover promotes abnormal proliferation of transformed cells through mitosis (Chen et al., 2012). The formation and progression of tumors is a multistep process with a complex mechanism (Zheng et al., 2011). Tumor formation and progression requires the activation of a number of factors, including oncogenes, cancer suppressor genes, reverse transcriptases, and anti-apoptotic genes as well as the inactivation of pro-apoptotic genes-an effect of simultaneous promoting and antagonistic effects (Shen et al., 2012).

Survivin is currently known to be expressed in all common malignant tumor types, including breast cancer, gastric carcinoma, renal carcinoma, melanocarcinoma, intestinal cancer, neuroblast cancer, and ovarian cancer; while not being expressed in normal tissues (Zahedi et al., 2012). This tumor specificity allows for survivin-targeted immunotherapy and genetic therapy to be investigated as a means of promoting apoptosis of tumor cells and inhibiting their proliferation without injuring and affecting healthy tissues (Lechler et al., 2011). Research findings indicate that survivin may be a potential target in possible therapeutic approaches for papillary thyroid carcinoma and screening of the survivin gene mutation may also be a good diagnostic criterion for papillary thyroid carcinoma.

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