The detection of SAS1B in serum provides clues for early diagnosis of thyroid cancer

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Abstract. – OBJECTIVE: The incidence of thyroid cancer is rising globally. Most patients progress slowly, but some patients develop lymph node and distant metastasis earlier, and their prognosis is poor. Therefore, early diagnosis and warning of malignancy are very meaningful for such patients. SAS1B gene is a newly discovered protein expressed on the surface of mature egg cells and has metalloendopeptidase activity. We aimed at exploring whether SAS1B is involved in the occurrence of thyroid cancer, and at providing evidence for early diagnosis and targeted therapy of thyroid cancer.

PATIENTS AND METHODS: In this study, a rabbit anti-human SAS1B polyclonal antibody was prepared by gene recombination technology. The indirect ELISA method was used to detect the SAS1B protein expression in the serum of 69 patients with thyroid cancer and 55 normal controls, and the relevant pathological factors were analyzed. Immunohistochemistry and PCR technology were used to investigate the expression levels of SAS1B protein and mRNA in 30 thyroid cancer tissues and 23 control thyroid tissues.

RESULTS: The titer of SAS1B recombinant antibody was 1:51200. The expression of SAS1B in the serum of patients with thyroid cancer was higher than that in the normal control group ($p<0.01$). The antibody had a good sensitivity in serum detection of cancer patients ($p=0.008<0.01$), the linear regression analysis result was that the expression of SAS1B gene was related to tumor envelope invasion and lymph node metastasis ($p=0.003<0.01$, $p=0.003<0.01$), and it was irrelevant to the patient's gender, age, tumor mass size, number of cancer foci, pathological stage, etc. ($p>0.05$). The results of immunohistochemistry showed that SAS1B protein was mainly located in the cytoplasm and membrane of thyroid cancer cells. The expression intensity in thyroid cancer tissues was higher than that in control tissues ($p<0.05$), but it was not expressed in normal thyroid tissues. Antibodies showed a good sensitivity that was used to detect thyroid cancer tissues ($p=0.000<0.01$). The results of ordinary PCR detection using thyroid cancer tissue and control thyroid tissue showed that the amplification products of the three domains (N-terminal, C-terminal and catalytic domain) of the SAS1B gene showed high expression in thyroid cancer tissue. q-PCR results showed that the expression of SAS1B gene in thyroid cancer and control thyroid tissue was higher than that in control group ($p<0.05$), and the genes of Aurora A and BARD1 related to centrosome replication and DNA replication forks protection during the proliferation were highly expressed in thyroid cancer tissue. The study results suggested that SAS1B was involved in the carcinogenesis of thyroid cancer. The Hum_mPLoc.2.0 software, PSORT II software and UniProt software were used to predict that SAS1B protein had secretory protein properties.

CONCLUSIONS: The above data indicate that the SAS1B gene is closely related to the process of thyroid cancer and can serve as a good tumor marker that can be used for early diagnosis and early warning of thyroid malignancy.

Key Words:
SAS1B gene, Thyroid cancer, Antibody, Tumor marker.

Introduction

Thyroid cancer is a common malignant tumor in the endocrine system and its incidence is increasing worldwide[1,2]. Germany predicts that from 2020 to 2030, thyroid cancer may leapfrog

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to the country's highest incidence of cancer[3]. In the UK there are nearly 3,000 new cases of thyroid cancer each year, and about 350 deaths from this disease each year, accounting for 10% of the total deaths from malignant tumors[4]. The incidence rate in China is also an upward trend year by year[5]. The pathological type of papillary thyroid cancer is the most common type of thyroid cancer, accounting for about 85%. Although most patients progress slowly and have a good prognosis, some patients have early lymph node and distant metastasis; their prognosis is not good, thus an early diagnosis is very important for thyroid cancer. At the same time, research on therapeutic targets for thyroid cancer is also very urgent[6]. Thyroid has endocrine functions, mainly composed of follicular cells and a small amount of parafollicular cells. The follicular epithelium is the origin cell of most thyroid malignant tumors, but the mechanism of canceration is still unclear. More and more clinical practice shows that a precise targeted therapy is the inevitable way of tumor treatment, and the search for specific targets is particularly important[6-9].

The SAS1B (Sperm Acrosomal SLLP1 Binding protein, SAS1B) gene was recently discovered on the surface of mature egg cell membranes[10-12]. The SAS1B protein of egg cells is located in the cortical granule by the 52DKDIPAIN64 amino acid motif[13]. After fertilization, SAS1B will exert metal endopeptidase activity. After cutting the zona pellucida protein 2 (ZP2), the sclerosis of the zona pellucida is triggered to ensure zygote development[14]. SAS1B protein is a protein with a zinc finger structure and has metalloendopeptidase activity, which is also called astaxin-like metallo-endopeptidase (ASTL). Recent studies have found that it is also found in cancers such as uterine cancer and pancreatic cancer, and it is expressed on the cell surface[15,16]. The thyroid is the largest endocrine gland in the human body, and the secreted thyroxine is related to many life activities[17]. Whether SAS1b is involved in the occurrence of thyroid cancer, which maintains increasing incidence, is the problem to be urgently solved. In this study, a rabbit anti-human SAS1B polyclonal antibody was prepared by gene recombination technology. The expression of SAS1B protein in serum and tissues of patients with thyroid cancer was detected by ELISA method and immunohistochemistry method, and SAS1B mRNA and cell proliferation-related gene Aurora A[18] and BARD1[19] expression changes in thyroid cancer and control tissues were detected by polymerase chain reaction to explore whether SAS1B is involved in the occurrence of thyroid cancer and to provide evidence for early diagnosis and targeted therapy of thyroid cancer.

Patients and Methods

Sample Collection

The histological classification of thyroid cancer was based on World Health Organization's criteria for primary epithelial tumors (2018 edition), and the TNM staging of thyroid cancer was based on the American Joint Committee on Cancer staging standards (8th edition). The sera of 69 newly treated patients with thyroid cancer were collected from the Oncology Department of the People's Hospital of Inner Mongolia Autonomous Region, General Surgery of the Affiliated Hospital of Inner Mongolia Medical University and Baotou Cancer Hospital, from January 2013 to December 2016. The average age was 49.6 years (39 to 69 years old). None of the patients received surgery, radiation, chemotherapy, or targeted biological therapy prior to sample collection. The serum samples from 55 healthy volunteers, with an average age of 45.6 years (35 to 64 years), collected during the same period, served as the control. All enrolled patients and healthy volunteers filled out the informed consent form. Thyroid cancer tissue specimens from 30 patients, with an average age of 47.4 years (33 to 68 years), undergoing surgery were collected during the same period. Thyroid tissue specimens from 23 cases of thyroid adenoma, with an average age of 42.3 years (36 to 63 years), served as the control. A part of the thyroid tissue specimen was stored in liquid nitrogen for experimental use, while the other part was fixed in 10% neutral formaldehyde and stained with hematoxylin-eosin, which were read by the senior pathologists for further diagnosis.

Main Reagents and Instruments

cDNA Synthesis Kit (K1621) and ArktikThermalCycler were obtained from Thermo Fisher Scientific (Waltham, MA, USA); Tissue Total RNA Extraction Kit (DP431) and High-fidelity PCR MasterMix (KT201) were purchased by Tiangen Company (Beijing, China); Fluorescent Quantitative PCR Reagent was from TOYO (Osaka, Japan); Peroxidase color developing solution and S-P kit (9710) were from Maixin Company (Fuzhou, China); TMD Kit was obtained from Sigma-Aldrich (St. Louis, MO, USA).

SAS1B Antibody Preparation

pET-22b-SAS1B prokaryotic expression plasmid was constructed using gene cloning technology, BL21 (DE3) cells were transformed and expanding culture was adopted to prepare SAS1B protein, which was then purified using ÄKTA pure 25 protein purifier and Millipore 10 kDa ultrafiltration technology, and Japanese big ear white rabbit was immunized. The resulting rabbit anti-human SAS1B polyclonal antibody was detected by indirect ELISA.

ELISA Method was Used to Detect the Expression of SAS1B Protein in the Serum

The experiment was conducted in 3 multiple wells at least, that were coated with the rabbit antiserum antibody (1:400 dilution) by incubating at 37°C overnight. The next day, the wells were washed with PBST solution, dried and then incubated with blocking solution on a warm bath at 37°C for 2 hours. After washing with PBST solution again, 100 µL of diluted serum (1:100) samples were added to each well and incubated at 37°C for 2 h. The wells were then washed and treated with HRP-labeled secondary antibody (1:8000 dilution) and incubated at 37°C for 1 h. After wash, the Tetramethylbenzidine (3, 3', 5, 5'-Tetramethylbenzidine, TMB) chromogenic working solution was added and allowed to react for 10-15 min in the dark at room temperature, after which the reaction was stopped by adding the stop-solution and the OD was measured at 450 nm wavelength.

Preparation of Tissue Sections

The thyroid tissue, fixed with 10% neutral formaldehyde for 24 hours, was washed with running water for 12 hours before subjecting to gradient dehydration. The tissues were then embedded in paraffin and sectioned using a microtome at 4 µm thickness, and the slices were spread in distilled water at 45°C. They were then attached to the glass slide after spreading, and the water was let evaporate over the tissue on the baking sheet.

Hematoxylin-Eosin Staining

Tissue sections were dewaxed in xylene and rehydrated under decreasing concentrations of ethanol. The sections were then dipped in hematoxylin staining solution for 5 minutes after which the unbound hematoxylin dye on the surface of the tissue was washed off with distilled water. The slide was then immersed in 1% hydrochloric acid solution for 10 s and soaked in distilled water for 10 min, followed by eosin staining for 10 s. The slices were then dehydrated using 70% to 100% ethanol, immersed in xylene for 5 minutes and sealed with neutral gum, before observing under a microscope.

Immunohistochemical Staining

The dewaxed and rehydrated thyroid tissue sections were subject to antigen retrieval by microwaving the slides under citric acid buffer solution at medium and low fire power for 12 minutes, after which they were allowed to cool naturally. After washing twice with PBS buffer, peroxidase blocker was added dropwise to the tissue section and it was washed off with PBS after 10 minutes. A 50 µL of serum was then added dropwise and allowed to stand for 10 minutes before being absorbed with absorbent paper, followed by overnight incubation with 50 µL of SAS1B IgG (diluted 1:500) at 4°C. After washing 3 times with PBS buffer on the next day, the tissue sections were incubated with 50 µL of biotin-labeled secondary antibody for 20 minutes at room temperature and washed off with PBS buffer. Later, the tissue section was allowed to react with a 50 µL of anti-biotin peroxidase for 10 minutes, followed by peroxidase (DAB) for color development. After the brown-yellow reaction was observed under the microscope, the reagents were washed off with distilled water, counterstained with hematoxylin dye solution for 2 minutes, washed thoroughly and dehydrated by dipping in gradient ethanol for 5 minutes. The tissue was made transparent by immersing in xylene for 10 minutes and a coverslip was applied before observing under the microscope.

Western Blot Detection

About 0.1 g of frozen thyroid tissue sample was prepared for Western blot analysis by grinding in a grinder with liquid nitrogen. The finely ground tissue samples were collected into a 1.5 mL centrifuge tube and lysed in lysis buffer. The insoluble materials were separated by centrifuging the lysate at 12000 rpm at 4°C for 30 min, and the protein supernatant was aspirated and stored at -40°C. After mixing with protein loading buffer, the protein supernatant was subject to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which was carried out at 200 V constant pressure for 45 min. The membranes were then blocked by adding 5% skimmed milk for 1 hour at room temperature. After washing with TBST for at least 30 min, the membrane was incubated with

SAS1B rabbit polyclonal antibody (1:1000 dilution) for 2 hours at room temperature. After washing with TBST solution again, the membrane was incubated with HRP-labeled goat anti-rabbit secondary antibody (1:10000) at room temperature for 40 min. The membrane was then washed again with TBST solution before adding ECL color developing solution, after which the membrane was placed on the detection instrument to image the protein band.

Extraction of Total RNA from Tissues

Finely ground thyroid tissue samples were lysed by adding 300 μL of lysis buffer, followed by incubation with proteinase K (1:60 dilution in RNase-Free ddH2O) in a 56°C-water bath for 20 min. The solution was then centrifuged for 5 min and the supernatant was aspirated to which absolute cold ethanol was added, yielding white flocculent precipitate. All the liquids, including flocs, were transferred into the adsorption column CR3, installed on to the collection tube provided in the kit. The column was centrifuged for 1 min, and then was added with 350 μL of Transfer protein solution RW1 and again centrifuged for 1 min. DNase I working solution was added to the column and placed on an ice bath for 15 min, followed by addition of 350 μL of deproteinization solution RW1 and centrifuged for 1 min. The column then received 500 μL rinse solution RW and allowed to stand at room temperature for 2 min, followed by centrifuge for 1 min. The above steps were repeated once again and the residual liquid of the adsorption column CR3 was dried in ultra-clean Taichung for 3 min. About 50 μL of RNase-Free ddH2O was then added into the center of the filter membrane and allowed to stand for 2 minutes, after which the RNA was obtained by centrifuging the contents for 2 minutes into a new RNase-Free centrifuge tube, which was stored at -80°C.

Reverse Transcription

Reverse transcription was performed to obtain cDNA, by incubating 3 μL of total RNA, 1 μL of Oligo (dT) and 8 μL nuclease-free water, added in sequence into a sterile nuclease-free PCR tube, at 65°C for 5 min. The mixture was then cooled on an ice bath, centrifuged and treated with reaction buffer Solution (5×) 4 μL, ribonuclease inhibitor 1 μL, dNTP mixture (10 mM) 2 μL and M-MuLV reverse transcriptase 1 μL. The mixture was centrifuged and allowed to react in a PCR machine at 42°C for 60 minutes, to obtain the cDNA which was stored at -40°C.

Ordinary PCR Detection of SAS1B Gene Expression

It was found in the NCBI database that the human SAS1B gene has three important functional domains, so the research team designed the primer sequences for the N-terminal, C-terminal and catalytic subunit domains of the SAS1B gene. RT-PCR primers used are as follows: GAPDH forward 5'TGCACCACCAACTGCTTAGC3', GAPDH reverse 5'GGCATGGACTGTGGTCATGAG3' (87 bp); SAS1B N-terminus forward 5'GCGCCCCTGGCCTCCAGCTGCACA3', SAS1B N-terminus reverse 5'CACGACACCACTACCACCACCCATGGG3' (237 bp); SAS1B C-terminus forward 5'GGCTGCAGCCCAAGTGGCCCCAGG3', SAS1B C-terminus reverse 5'AGCAA CACCGGGGGCACCTGCTCC3' (309 bp); SAS1B Catalytic domain forward 5'GAGGTCCCCTTCCTGCTCTCCAGC3', SAS1B Catalytic domain reverse 5'GGCATGGGACCCTCTCCCACGGGG3' (579 bp). The volume of RT-PCR amplification system was 25 μL, Template 2 μL, Primer 2 μL, 2×Taq PCR Mix 12.5 μL, and RNase-free water was 8.5 μL. PCR reaction conditions were: 94°C pre-denaturation 3 min, 30 cycles (94°C denaturation 30 s, 55°C annealing 30 s, 72°C extension 1 min), and 72°C extension 5 min.

Fluorescence Quantitative PCR Detection of SAS1B Gene Expression

Real-time fluorescent quantitative PCR technology was used to analyze the changes in SAS1B gene expression in thyroid cancer and control tissues. SAS1B gene primers redesigned according to the design principles of real-time fluorescent quantitative PCR primers were as follows: SAS1B Forward 5'CTCCTTGCCAGGTGTGATCC3', SAS1B Reverse 5'TGCAGGAATGTCCTTGTCCC3' (127 bp). At the same time, the Bard1 and Aurora A genes related to proliferation were amplified, with the following primers sequences: Aurora A Forward 5'TGGAATATGCACCACTTGGA3', Aurora A Reverse 5'ACTGACCACCC A AAATCTGCT3'(208 bp); BARD1 Forward 5'GTTAAGCAAACGGTGCCCTC3', BARD1 Reverse 5'GGGACTAGACATCACTCGCC3' (192 bp). Bard1 annealing temperature was 59.6°C, SAS1B annealing temperature was 60.0°C, and Aurora-A annealing temperature was 56.0°C. The volume of the amplification system was 20 μL, SYBR labeled real-time PCR reaction solution 10 μL, Primer 1 μL, template 2 μL, and nuclease-free water was 7 μL. PCR reaction program was: 95°C 30 s, 40 cycle (95°C 5 s, variable temperature 10 s, 72°C 20 s), and 65°C 5 s.

Statistical Analysis

The amplification parameters of the template in the Real-time quantitative PCR instrument were used to analyze the expression intensity of the gene. The CT value is the number of the amplifications of the PCR instrument when the intensity of the fluorescent signal in the PCR tube reaches the set threshold. The ΔCT value is the difference between the CT value of the target gene and the internal reference gene. The ΔΔCT value is the average CT value of the experimental group minus the average CT value of the control group. Since the ΔΔCT value is small, the multiple difference is commonly used, namely $2^{-\Delta\Delta CT}$, representing the relative expression level of genes. We used a light microscope to take pictures with the same pixels and magnification. The immunohistochemical images of each sample in the Image J analysis system were randomly collected in 5 fields of view and used for semi-quantitative analysis of the images. From the Western blot experiments, the average value of the optical density (OD) measured by the system was used as the measurement value of the SAS1B expression level of the sample. The Western blot electrophoresis image was converted into an 8-bit grayscale image, and then the image was inverted in black and white to correct the optical density. The obtained value was the measured value of the protein expression level. The measured values of immunohistochemistry and Western blot were then statistically processed using the *t*-test of SPSS19.0 software (SPSS Inc., Chicago, IL, USA).

Results

SAS1B Recombinant Protein Purification Results and SAS1B Antibody Titer

The SAS1B recombinant protein was expressed at an induction concentration of 1.25 mg/L IPTG, and the SAS1B recombinant protein was eluted on the ÄKTA pure 25 protein purifier with pH 8.1 buffer. The SAS1B recombinant protein band in the No. 11 collection tube was single (Figure 1). Japanese white rabbit was immunized using the purified protein. The ELISA method detected the absorbance of antibody serum and the serum before immunization, and the antibody titer reached at least 1:51200 (Figure 2).

The Expression of Serum SAS1B in Patients with Thyroid Cancer and its Relationship with Clinicopathological Factors

The expression level of serum SAS1B protein in patients with thyroid cancer (0.7845±0.01358) was higher than that of normal controls (0.7001±0.02297), and the difference was statistically significant ($p<0.01$) (Table I and Figure 3).

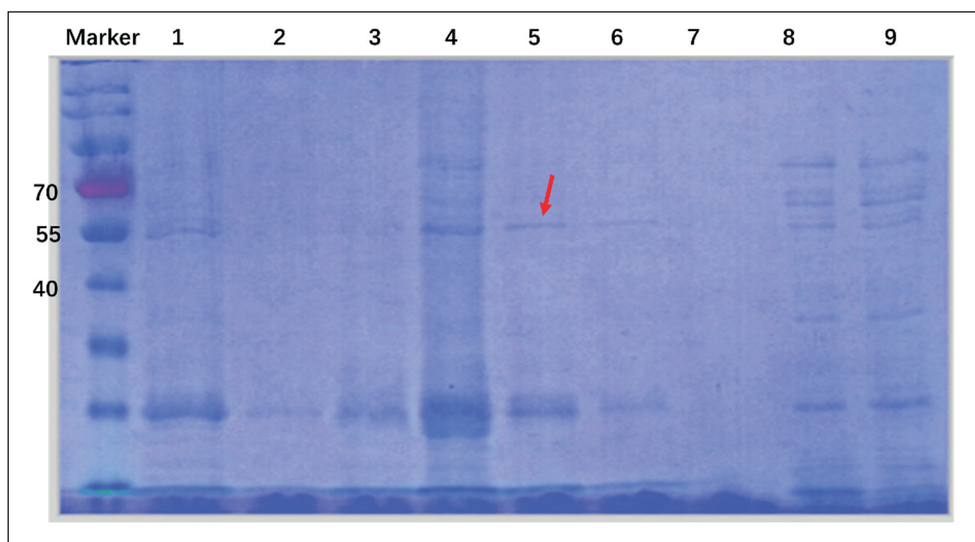

Figure 1. Electrophoresis of SAS1B recombinant protein after purification. The left is the standard molecular weight of the protein of Thermo Fisher Company (26616), 1st-3rd, 5th-9th are the proteins of different collection tubes after protein purification, and 4th is the result of electrophoresis of the 9th lane protein which purified with 10 KD ultrafiltration tube. The collection tube protein corresponding to lane 5 is used for subsequent experiments.

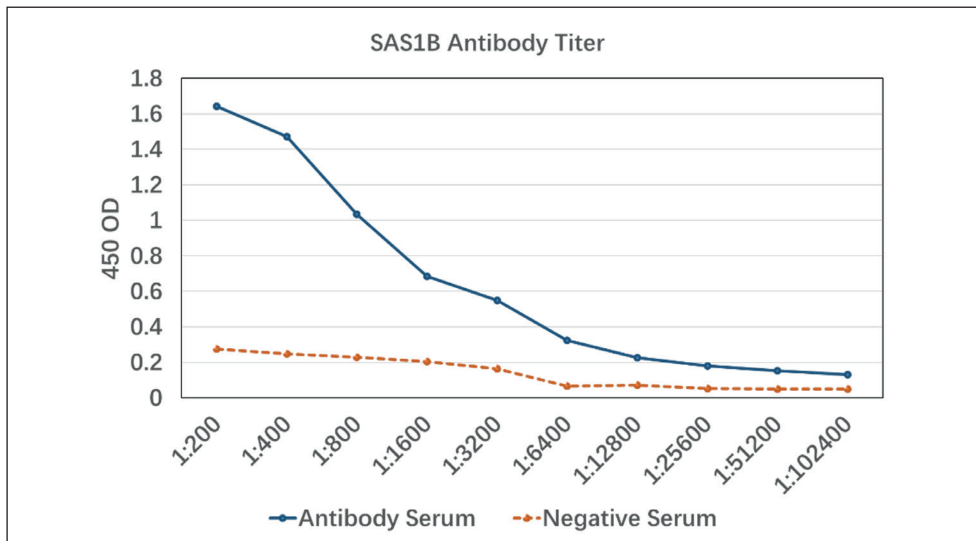

Figure 2. Detection of serum titer of SAS1B antibody by indirect ELISA method. The upper curve is the OD value of antibody serum, and the lower curve is the OD value of rabbit serum before immunization.

The area under the receiver operating characteristic (ROC) curve representing the sensitivity of the serological test results was 0.815, p=0.008<0.01, which was very significant (p<0.01) (Figure 4). The ROC curve indicates that the prepared rabbit anti-human SAS1B antibody has very good sensitivity and can be used for clinical auxiliary diagnosis of thyroid cancer. Further analysis of clinicopathological factors in patients with thyroid cancer confirmed that the expression level of SAS1B was not associated with age (p=0.575), sex (p=0.645), tumor diameter (p =0.807), number of cancer foci (p=0.451) and TNM staging (p =0.831). However, the expression of SAS1B was closely related to envelop invasion (p =0.003) and lymph node metastasis (p =0.001) (Table II).

Localization and Degree of Expression Analysis of SAS1B Protein in Thyroid Cancer Tissue HE Staining Results

Round or elliptical follicles could be seen in normal thyroid tissue under high magnification, and the follicular wall of the gland was surrounded by a single cuboidal epithelium and the follicular cavity was filled with red jelly-like substance (Figure 5). However, the follicles in thyroid cancer tissue were small or even disappeared with no obvious boundaries and reduced jelly-like substance. The thyroid cancer cells appeared large with rich cytoplasm and large/irregular nucleus. Further, the cells were irregularly arranged with disordered polarity. Within the thyroid cancer tissues, the papillary branches and fibrovascular interstitium were evident under the microscope, and interstitial sclerosis had sand granules (Figure 5).

Table I. SAS1B protein expression in serum of thyroid cancer.

Group	n	OD value ($\bar{x}$±s)	p
Thyroid cancer	69	0.7845±0.01358	p=0.00097
Normal	55	0.7001±0.02297	

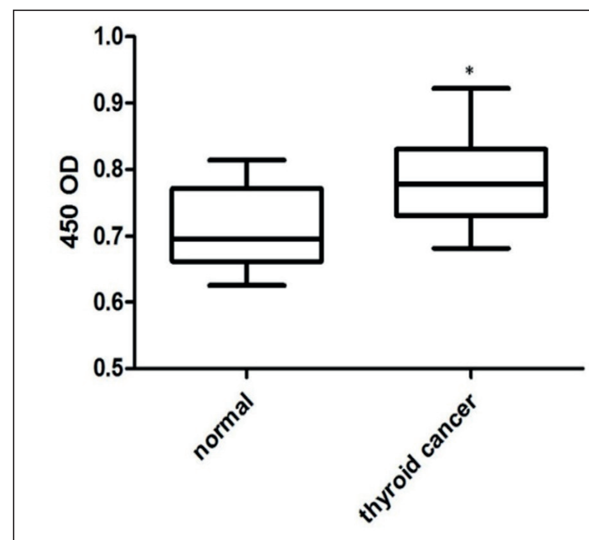

Figure 3. SAS1B protein expression in serum of patients with thyroid cancer. The level of SAS1B protein in serum of patients with thyroid cancer is higher than that of control group. *p<0.05.

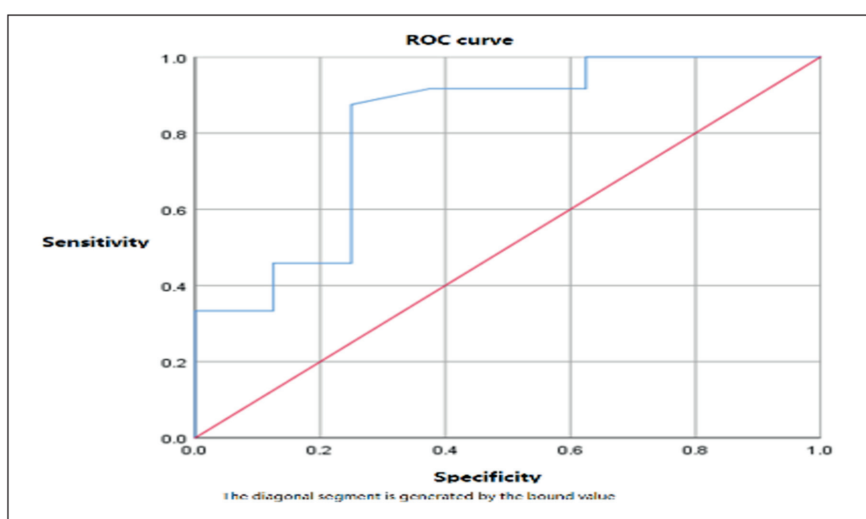

Figure 4. ROC curve of serum SAS1B in the diagnosis of thyroid cancer. The area under the curve (AUC) is 0.815, p=0.008. It shows that the SAS1B antibody is very sensitive in the diagnosis of thyroid cancer.

Immunohistochemistry Staining Results

Under the microscope, brown or tan positive particles (positive stain) could be seen on the cell membrane and in the cytoplasm of thyroid cancer cells. A small amount of SAS1B protein expression could also be seen in the thyroid tissue adjacent to cancer, but no SAS1B expression was seen in normal thyroid tissue, and in the negative control samples that used PBS instead of the primary antibody. A t-test analysis comparing SAS1B expression between thyroid cancer tissues and control tissues (Table III and Figure 6) revealed a statistically significant difference (Figure 7A), and the area under the ROC curve for SAS1B expression in thyroid cancer tissue was 0.940, p=0.000<0.001, indicating a highly significant difference (p<0.001, Figure 7B).

Table II. Correlation Between SAS1B Expression and Clinicopathologic Characteristics in thyroid cancer.

Clinicopathological Variable	No.	*t*-value	*p*-value
Gender			
Female	26	0.463	0.645
Male	43		
Age			
<45	37	0.563	0.575
≥45	32		
Tumor size			
<4 cm	38	- 0.245	0.807
≥4 cm	31		
Tumor number			
Solitary	29	- 0.759	0.451
Multiple	40		
Envelope violation			
No	27	3.064	0.003
Yes	42		
TNM staging			
I+II	39	- 0.214	- 0.813
III+IV	30		
Lymph node metastasis			
No	28	3.463	0.001
Yes	41		

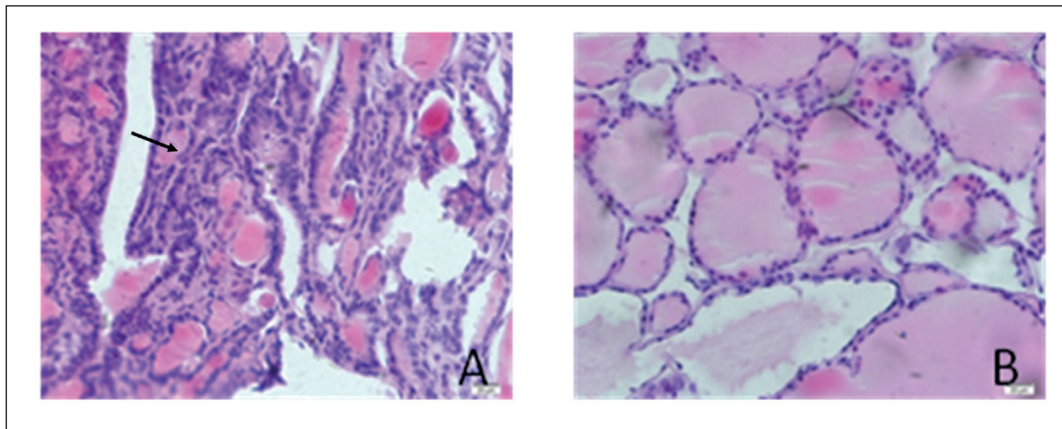

Figure 5. HE staining of thyroid cancer (×200). **A,** Thyroid cancer tissue. **B,** Normal thyroid tissue. Papillary branches and fibrovascular interstitium can be seen in thyroid cancer cells, and there are sand granules in interstitial sclerosis (→).

Table III. Comparison of SAS1B expression between in thyroid cancer tissues and control tissues.

Group	n	OD value ($\bar{x}$±s)	p
Thyroid cancer	30	128.37±23.93	$p < 0.05$
Control	29	73.82±18.11	

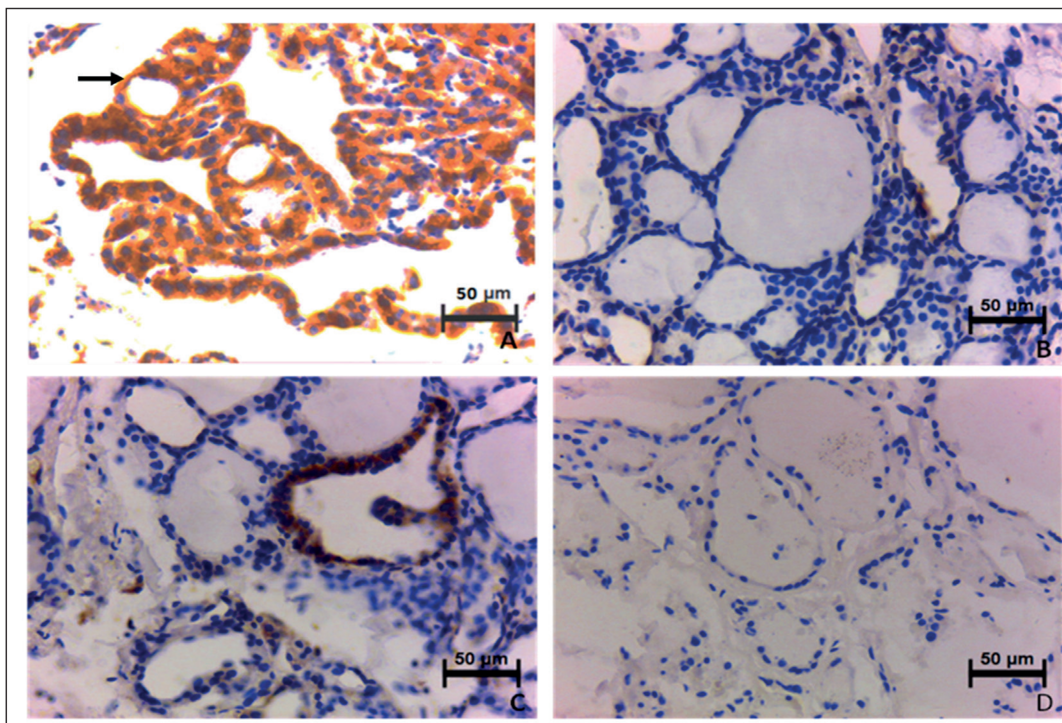

Figure 6. SAS1B protein expression in thyroid cancer and control tissues (×200). **A,** Thyroid cancer. **B,** Thyroid control. **C,** Next to thyroid cancer. **D,** Negative control. Positive expression indicated by arrow (→).

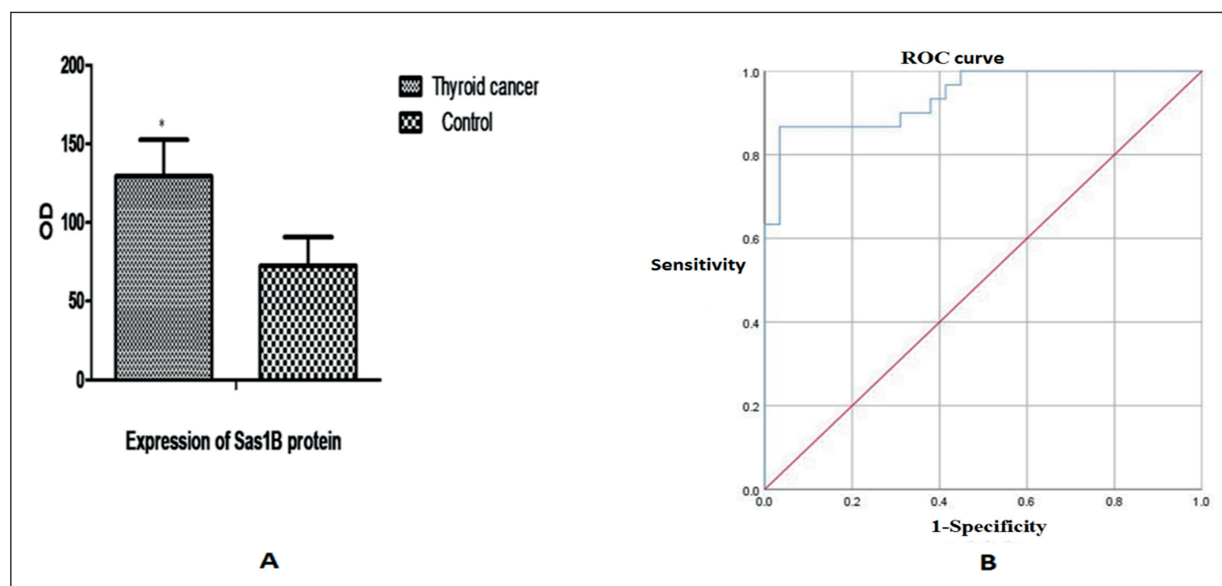

Figure 7. Protein expression of SAS1B in thyroid cancer tissues and its sensitivity analysis.**A**, The expression of SAS1B protein in thyroid cancer tissue is higher than that in control tissue. **B**, ROC curve analysis of SAS1B between thyroid cancer and control tissues. *$p<0.01$. ROC curve shows that the area underneath is 0.940, $p=0.000<0.01$. The SAS1B antibody has significant sensitivity in the diagnosis of thyroid cancer.

Western Blot Results of SAS1B Expression in Thyroid Cancer Tissue

The expression of SAS1B protein in thyroid cancer tissue was significantly higher than that in the adjacent control group ($p<0.05$) (Figure 8).

SAS1B mRNA Expression Results in Thyroid Cancer Tissue SAS1B RT-PCR Amplification Results

The thyroid tissue samples were subjected to RNA extraction, reverse transcription, and PCR amplification. The electropherogram results of the amplified products of SAS1B N-terminus domain, SAS1B C-terminus domain and SAS1B catalytic domain (Catalytic domain), are consistent with the size of the amplified fragments designed by the primers, confirming the expression of SAS1B mRNA in thyroid cancer tissues (Figure 9).

SAS1B Real-Time Fluorescent Quantitative PCR Results

The SAS1B melting curve showed a single peak, and there was a significant difference in SAS1B mRNA expression between thyroid cancer and control tissues ($p<0.05$) (Table IV, Figure 10).

Bard1 and Aurora A Real-Time Fluorescence Quantitative PCR Results

The melting curves of Aurora A and Bard1 showed a single peak, and there were significant differences in the expression of Aurora A mRNA and Bard1 mRNA between thyroid cancer and control tissues ($p<0.05$) (Table V, Figure 11).

Discussion

Thyroid cancer has increased by 300% in the last 40 years. There are 54,000 newly diagnosed cases in the United States each year[20,21]. In Asian population the incidence rate of thyroid cancer is even higher, with China reporting 201,000 newly diagnosed cases in 2019[22]. Among the four types of thyroid cancer, the pathological type of papillary thyroid cancer (PTC) is the most common type of thyroid cancer, accounting for about 85%. Although most patients progress slowly and have a good prognosis, some have a poor prognosis due to the early occurrence of lymph node and distant metastasis[20,23]. With the increased understanding of the molecular mechanism of thyroid cancer increases, molecular marker research for diagnosis, prognosis, monitoring and treatment has become more and more important[24]. The latest guidelines of the American Association of Endocrine Surgeons and the American Thyroid Association (ATA) guidelines have further recognized the increasing importance of molecular markers in the diagnosis and treatment of thyroid cancer[25,26]. The research on circulating biomarkers of thyroid

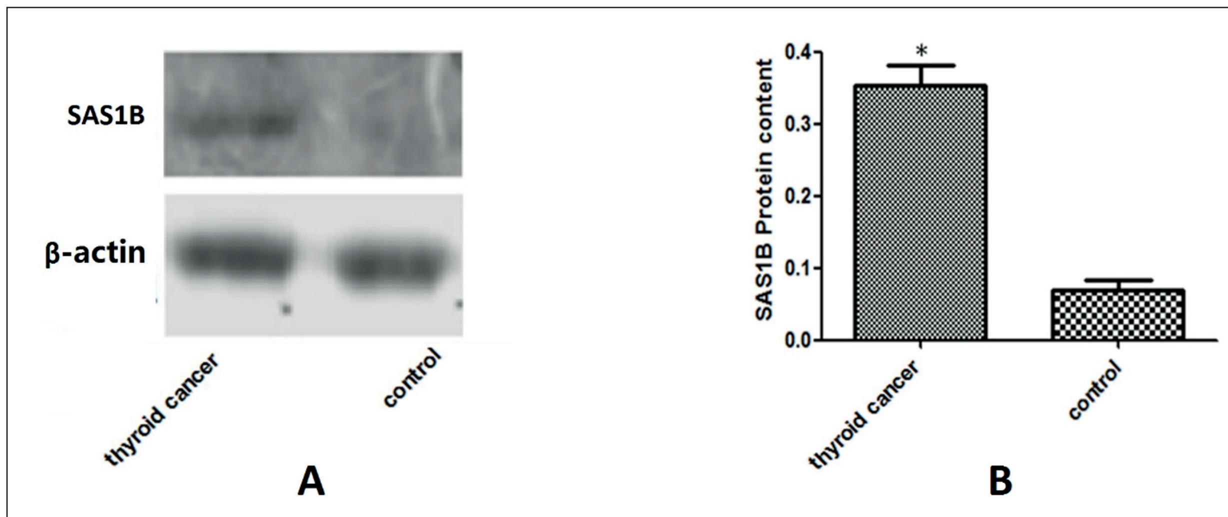

Figure 8. Expression of SAS1B protein in thyroid cancer. **A,** Western Blot results of SAS1B expression in thyroid cancer tissues. **B,** Bar graph shows that SAS1B protein expression in thyroid cancer tissues is higher than that in control tissues (p<0.05).

cancer is in its infancy because differentiated thyroid cancer plasma samples and tissue samples have low consistency. The diagnostic application of circulating tumor DNA (ctDNA) is questionable[27,28]; circulating miRNAs in biological fluids have high stability and tissue specificity, but miRNA requires multiple combined detection to have a certain meaning. miRNA alone cannot be used as a malignant tumor marker, and miRNA research results cannot be achieved for precise targeted therapy[23,29,30]. Some scholars conducted genome and transcriptome studies on 123 cases of PTC patients, including 36 cases of metastatic cancer, but no obvious molecular markers were found[31]. Many scholars tend to write molecular testing into the diagnosis and treatment of thyroid cancer, so the research work of screening specific tumor biomarkers is very important.

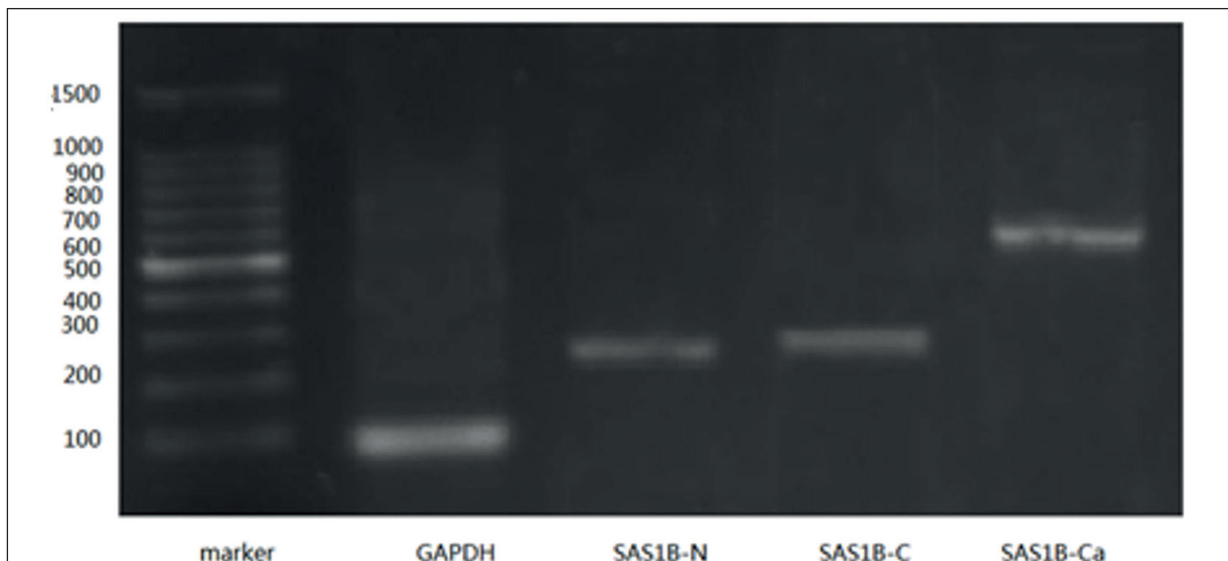

Figure 9. Electrophoresis of SAS1B gene different structure domain amplification products in thyroid cancer tissue. The size of internal reference control GAPDH amplified fragment is around the standard molecular weight of 100 bp, the SAS1B N-terminus structure domain amplified fragment is between the standard molecular weight of 200 bp~300 bp, the amplified fragment size of SAS1B C-terminus domain is nearly the standard molecular weight of 300 bp and the size of the amplified fragment of SAS1B's Catalytic domain is between 500 bp~600 bp of the standard molecular weight.

Table IV. Relative expression of SAS1B mRNA in thyroid cancer and control tissues.

Group	Relative quantification	*p*
Thyroid cancer tissue	0.621±0.163	
Control thyroid tissue	0.289±0.092	*$p<0.01$

*Comparison between the control group and thyroid cancer tissue, $p<0.01$, statistically significant difference.

Table V. Relative mRNA expression levels of Bard1 and Aurora A in thyroid cancer.

Group	Bard1 Relative quantification	*p*	Aurora-A Relative quantification	*p*
Thyroid cancer	0.893±0.119		1.102±0.285	
Control tissue	0.235±0.097*	$p<0.05$	0.359±0.067*	$p<0.05$

*Compared between control group and cancer tissue, $p<0.05$, statistically significant difference.

In their long research career, people can reprogram somatic cells to generate teratomas and even malignant tumors[32]. "Tumor-a developmental biology problem" was proposed in the 1970s[33]. From the fertilized egg to the adult, the organism has experienced the process of cell proliferation and differentiation. The reprogramming of the zygote development on the fertilized egg is a necessary condition to ensure embryonic development[34,35] and the expression of egg cell genes is closely related to fertilization. SAS1B is not expressed in primordial follicle cells. As the follicle matures, the expression becomes stronger. After fertilization, the N-terminus and enzyme active area of SAS1B protein on the surface of the egg cell will fall off from the egg membrane to the outer periphery of the cell[10]. In 2015, Pires et al[15] found that SAS1B gene was highly expressed in uterine cancer cells. In 2018, Knapp et al[16] found that SAS1B gene expression was increased in pancreatic cancer cells. None of the above studies reported that SAS1B was in expression characteristics in serum.

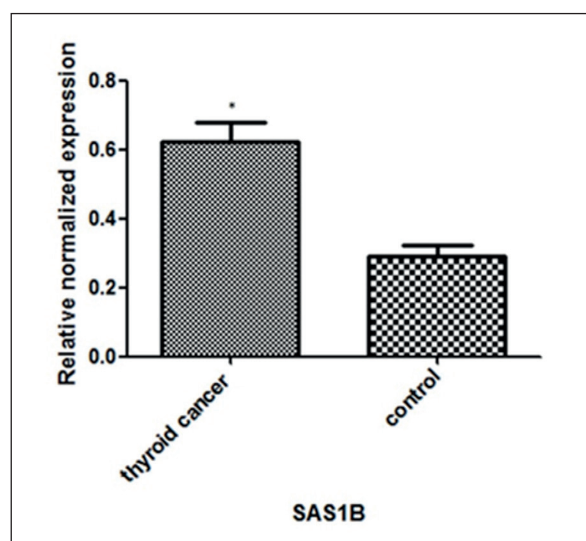

Figure 10. Different expression levels of SAS1B mRNA in thyroid cancer and control tissues. *Comparison of SAS1B mRNA expression between the control group and thyroid cancer tissues. $p<0.01$, the difference is statistically significant.

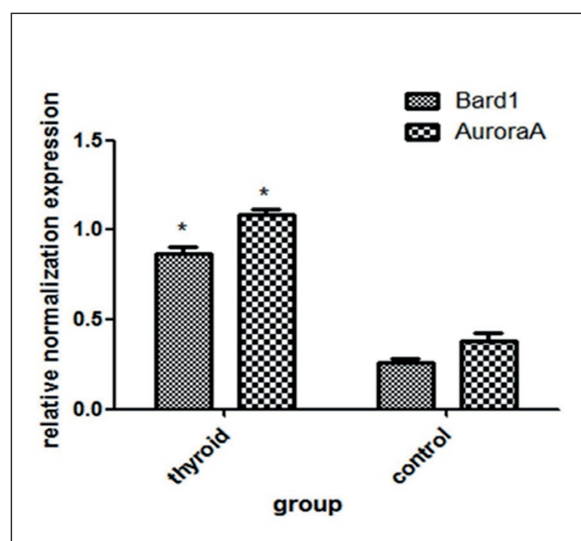

Figure 11. Relative expression levels of BARD1 mRNA and Aurora A mRNA in thyroid cancer and control tissues. *Compared between control group and cancer tissue, the expression level of BARD1mRNA and Aurora A mRNA in thyroid cancer tissue is higher than that in control thyroid tissue. $p<0.05$, the difference is statistically significant.

In this study, SAS1B rabbit anti-human polyclonal antibody was prepared, and the indirect ELISA method was used to detect the presence of SAS1B protein in the serum of patients with thyroid cancer and the serum of normal control group. It was found that the expression of SAS1B in the serum of patients with thyroid cancer was higher than that of normal people. Linear regression analysis showed that the SAS1B is expressed in the thyroid cancer cell. The SAS1B expression pattern in thyroid cancer was related to the malignant clinicopathological characteristics, such as tumor envelope invasion (p=0.003), lymph node metastasis (p=0.001). However, the tumor mass size, TNM staging, age, gender, etc. were irrelevant with SAS1B expression. The ROC curve analysis of the test results found that it had a very good diagnostic sensitivity (the area under the curve was 0.780, p=0.000<0.01). Among the thyroid cancer serum samples selected in this study, 51 cases (74%) of papillary thyroid cancer, 16 cases (23%) of follicular thyroid cancer, and 2 cases (3%) of medullary cancer were included. Due to the extremely low incidence of undifferentiated thyroid cancer, serum samples could not be collected. The study found that thyroid cancer types between papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) had nothing to do with the expression intensity of SAS1B protein. The expression intensity of medullary carcinoma of SAS1B protein was higher than that of differentiated thyroid carcinoma (PTC and FTC), but due to the small number of cases, statistical analysis was not possible. Since differentiated thyroid cancer is currently the most common type in the world, the prognosis is generally very good, but individual cases owning a high degree of malignancy often metastasize early, even leading to patient death, causing patients and physicians to be caught off guard. This study surprisingly found that SAS1B expression in serum was related to the metastasis and aggressiveness of differentiated thyroid cancer, providing a basis for early judgment of clinical high-malignant thyroid cancer. Subsequent immunohistochemical staining of thyroid cancer tissue and control thyroid tissue showed that SAS1B protein was mainly located in the cytoplasm and membrane of thyroid cancer cells. The ROC curve of the absorbance value detected by immunohistochemistry showed that SAS1B protein exists in the thyroid cancer and the sensitivity of cancer diagnosis is high (the area under the curve was 0.940, p=0.000; <0.01). Western blot also confirmed that the expression of SAS1B protein in thyroid cancer tissues was higher than that in control tissues. All evidence suggests that SAS1B gene is involved in the occurrence and development of thyroid cancer. Aiming at the expression characteristics of SAS1B protein found in the serum of thyroid cancer, the research team used PSORT II software to predict the location of SAS1B protein and found that SAS1B protein can be located in the nucleus, mitochondria, cytoplasm, cell membrane, secretory vesicles, extracellular space, and Golgi bodies ***(Supplementary Figure 1)***. According to the UniProt database, the SAS1B protein is localized only in the cell membrane ***(Supplementary Figure 2)***. Hum-mPLoc.2.0 software was used to predict that the SAS1B subcellular localization only exists cell periphery. According to the results of immunohistochemical staining and bioinformatics, SAS1B protein should be located in the cytoplasm and membrane of thyroid cancer cells, and at the same time is secreted to the periphery of the cell and participates in the carcinogenesis of adjacent cells. The expression character of SAS1B in thyroid cancer is very significant for clinic work. Serological detection results could provide clues for early clinical diagnosis and warning of the malignant prognosis for differentiated thyroid cancer.

Polyclonal antibodies usually have high affinity, so they can form stable binding with a large number of antigens, and the production of polyclonal antibodies is more cost-effective[36]. Polyclonal antibodies have the ability to recognize multiple epitopes and have the possibility of cross-reaction. Setting up controls in experiments can improve the specificity of detection. In this study, a polyclonal antibody was prepared using the recombinant protein of SAS1B full-length amino acid sequence as the antigen. The titer of the antibody reached 1:51200. Benign thyroid disease of thyroid tissue was used as control for the test SAS1B expression in the thyroid cancer. The antibody was confirmed by immunohistochemical staining, Western Blotting and indirect ELISA method. SAS1B antibody not only can detect SAS1B protein in peripheral blood of thyroid cancer patients (p<0.05), but also investigates the SAS1B protein in the tissue of thyroid cancer. At the same time, RT-PCR and q-PCR were used to detect the expression level of SAS1B mRNA in thyroid cancer tissues and control thyroid tissues. The ordinary PCR results showed that the three important domains sequence of SAS1B N-terminal, carboxyl terminal and catalytic domain were checked out in thyroid cancer. It was confirmed by

q-PCR that SAS1B expression in thyroid cancer tissues was higher than that in control thyroid tissues ($p<0.01$). At the same time, q-PCR detected an abnormal increase in centrosome amplification kinase Aurora A (compared to the control group, $p<0.05$), and the expression of BARD1 also increased ($p<0.05$). Aurora A kinase is a marker enzyme of centrosome replication[37]. The amplification of Aurora A kinase leads to an increase in the number of centrosomes[38-40]. Overexpression of the Bard1 spliceosome containing the fourth exon can protect the replication fork promoting cells proliferation[41], while inhibiting Aurora kinase ubiquitination. The results of this study suggest that SAS1B upregulates the expression of Aurora A to cause the formation of multipolar spindle filaments, increases the number of aneuploid cells and promotes malignant cell cloning. Moreover, it upregulates the expression of BARD1 to protect DNA replication, inhibits Aurora A kinase degradation, and maintains malignant cell proliferation. SAS1B protein can also create a tumor metastasis microenvironment by secreting vesicles and promote envelope invasion and lymph node metastasis. We also used GENEMANIA software to predict SAS1B interaction proteins network, which suggests that SAS1B's ubiquitination is mainly mediated by VHL protein and the effects of proteases such as MEP1B, TLL1, TLL2, and BMP1, affect the extracellular matrix microenvironment. Furthermore, the transcription factor PCGF2 can regulate SAS1B activity and affects the formation of spindle filaments by theTUBB1 ***(Supplementary Figure 3)***. The SAS1B protein is involved in the formation of malignant cell proliferation and tumor metastasis microenvironment. SAS1B is a very potential new tumor marker in the occurrence of thyroid cancer; in particular, it provides a specific marker for the judgment of metastatic papillary thyroid cancer.

Conclusions

This study prepared SAS1B rabbit anti-human polyclonal antibody and investigated the expression characteristics of SAS1B in thyroid cancer cells and its relationship with clinicopathological characteristics. The study revealed for the first time the relationship between abnormal expression of SAS1B and thyroid cancer. In particular, SAS1B protein is found in the peripheral blood of thyroid cancer patients and thus can be a good biomarker for early diagnosis of thyroid cancer. Further, the close association of SAS1B expression with malignant aggressiveness of the disease can provide early warning for the poor prognosis of thyroid cancer.

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Conflict of Interests

The authors have no conflict of interest

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