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ORIGINAL ARTICLE

Expression of PinX1 and hTERT in basal cell carcinoma and their implications

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Abstract

Objective This study aimed to investigate the expression and significance of *PIN2/TERF1* interacting, telomerase inhibitor 1 (*PinX1*) and human telomerase reverse transcriptase (*hTERT*) in basal cell carcinoma (BCC).

Methods Real-time polymerase chain reaction and immunohistochemistry were performed to quantify the mRNA expressions and integrated optical density (IOD), respectively, of *PinX1* and *hTERT* in BCC specimens (n = 30), as well as in normal skin specimens (n = 15).

Results The mRNA expression level and IOD of *PinX1* in the BCC samples were both significantly lower than those in the control specimens ($P < 0.05$). Conversely, the mRNA expression level and IOD of *hTERT* in BCC were both significantly higher than that in the control samples ($P < 0.05$). The correlation between the expression levels of *PinX1* and *hTERT* showed no statistical significance ($P > 0.05$).

Conclusion Downregulation of *PinX1* and upregulation of *hTERT* expression may be associated with the activation and maintenance of telomerases in the induction of BCC.

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Key words: telomerase inhibitor 1 (*PinX1*); human telomerase reverse transcriptase (*hTERT*); basal cell carcinoma; real-time polymerase chain reaction; immunohistochemistry

Basal cell carcinoma (BCC) is a kind of epithelial malignant tumor derived from the epidermal basal cells or hair follicle outer root sheaths; however, its pathogenesis remains partly unclear. Telomeres are special structures in the eukaryotic chromosome ends, which play an important role in maintaining the stability of the chromosome. American scientist Elizabeth Blackburn first reported that telomerases were produced by the chromosome crown, and found that overexpression of these enzymes could trigger senescence and cancer; for these discoveries, she won the Nobel Prize in Medicine in 2009.

The endogenous telomerase inhibitor gene telomerase inhibitor 1 (*PinX1*) is a telomere regulating factor, which can directly interact with human telomerase reverse transcriptase (*hTERT*) in human cells, and which is thought to be a potential tumor suppressor gene [1]. Studies have shown that the activity of telomerases in skin tumors is higher than that in normal skin tissue [2].

In this study, we detected the mRNA expression level and integrated optical density (IOD) of *PIN2/TERF1* interacting, *PinX1* and *hTERT* in order to investigate the role of *PinX1* in the pathogenesis of BCC, and to provide a basis for future studies on telomere-targeted treatments.

Materials and methods

Tissue samples

A total of 30 skin specimens from patients with BCC (13 men and 17 women; age, 40–73 years; mean age, 57 years) were obtained from the Department of Dermatology, the Affiliated Hospital of Qingdao University, China, between 2011 and 2014. The BCC specimens were diagnosed by a clinician and by a professional pathologist. A total of 15 healthy control samples (8 men and 7 women; age, 35–74 years; mean age, 53 years) were obtained from individuals who underwent plastic surgery. The sex and age did not significantly differ between the two groups. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. All specimens were surgically resected and the specimens were embedded into paraffin and placed in a -80 ℃ refrigerator within 10 min.

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Table 1 Primer sequences of *PinX1*, *hTERT*, and *GAPDH*

Genes	Primer sequences (5'-3')	Products (bp)
PinX1	Positive: GGTGGTCTAAAGGAAAGGGTTT	154
	Reverse: ATGGGCAATCCAGTTGTCTT	
hTERT	Positive: CTCCCATTTCATCAGCAAGTTT	123
	Reverse: CTTGGCTTTCAGGATGGAGTA	
GAPDH	Positive: AGAAGGCTGGGGCTCATTTG	239
	Reverse: AGGGGCCATCCACAGTCTTC	

Materials

The reverse transcription reaction system kit and SYBR Green I chimeric fluorescence assay kit were purchased from Zhongshan Co. (China); the primers for *PinX1* and *hTERT* were purchased from Sangon Co. (China) (Table 1). Rabbit anti-human *PinX1* and *hTERT* polyclonal antibodies were procured from Bioss Co. (China). The general secondary biotinylated antibody (pv-6001), 3, 3'-diaminobenzidine (DAB) staining reagents, and immunohistochemical pen were purchased from Zhongshan Co. (China).

Methods

Real-time polymerase chain reaction (PCR)

Extraction of total RNA and synthesis of cDNA: we extracted the total RNA from 100 mg tissue using the Trizol one-step method. Using the total RNA, we measured the content of A260/A280 and determined its purity with an ultraviolet spectrophotometer. A 1.5% formaldehyde denaturing agarose gel electrophoresis was used to detect the integrity of the RNA. A260/A280 in the samples was detected in a ratio of 1.8–2, and no degradation was observed.

A total of 2 μg total RNA was used to make cDNA by reverse transcription reaction using a cDNA synthesis kit. Subsequently, the cDNA was stored in a -20 ℃ refrigerator.

Real-time fluorescent quantitative PCR: using the cDNA as a template, we detected the mRNA expressions of *PinX1* and *hTERT* in the BCC and normal skin tissues with the SYBR Green I chimeric fluorescence assay kit and BioRad fluorescence quantitative PCR instrument. The amplification reaction conditions were as follows: a) *PinX1* PCR: 10 s pre-degeneration at 95 ℃, 1 cycle; 10 s degeneration at 95 ℃, 30 s annealing (renaturation) at 55 ℃, 40 cycles; b) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) PCR: 10 s pre-degeneration at 95 ℃, 1 cycle; 10 s degeneration at 95 ℃, 30 s annealing at 60 ℃, 40 cycles; c) *hTERT* PCR: 10 s pre-degeneration at 95 ℃, 1 cycles; 10 S degeneration at 95 ℃, 30 s annealing at 60 ℃, 40 cycles. The objective gene specific amplification was relatively quantified by the $2^{-\triangle\triangle Ct}$ method. We analyzed the PCR amplification and melting curves after the reactions, and the specificity of the amplification was evaluated using 2% agarose gel electrophoresis.

Immunohistochemistry

Paraffin-embedded tissues were sliced into 3-μm sections and heated for 1 h at 64 ℃. Subsequently, these sections were deparaffinized in xylene and rehydrated with an alcohol gradient. PinX1 and hTERT sections were repaired at a high pressure for 2 min by using citrate buffer at pH 6.0. Endogenous peroxidase was inactivated with 3% hydrogen peroxide for 10 min. Next, the sections were incubated with the primary antibodies of PinX1 and hTERT (1:70 concentration) and placed in an incubator for 1 h at 37° ℃. Afterwards, the general secondary biotinylated antibody (pv-6001) was added, and the sections were incubated in a working liquid wet box for 30 min at 37°C. The sections were subsequently visualized using DAB for 90 s, stained with hematoxylin, dehydrated, and mounted. Between each step, the samples were soaked thrice in phosphate-buffered saline (PBS) solution. For the negative control, PBS was used instead of the first antibody.

Statistical analyses

The results were analyzed using SPSS version 17.0. Comparisons between the BCC and control samples were analyzed by using the independent *t*-test. The correlations between the gene and protein expressions of the two groups were analyzed by using the Pearson linear correlation. $P < 0.05$ was considered statistically significant.

Results

Real-time PCR

Total RNA purity and integrity

The sample ratio of A260/A280 of 1.8–2 suggested that the purity of the total RNA was good; three visible strips of 28S, 18S, and 5S, with suitable sizes and proportions, were observed under ultraviolet light, indicating that the total RNA extraction was achieved without degradation.

mRNA expression levels of PinX1 and hTERT

Electrophoresis of the PCR amplification products of *PinX1*, *hTERT*, and GAPDH showed obvious electrophoresis bands at 154, 123, and 239 bp, respectively (Fig. 1). The relative mRNA expression of *PinX1* in the BCC group (2.75 ± 1.07) was significantly lower than that in the controls (4.33 ± 2.92; $t = 4.128$, $P < 0.05$). The relative mRNA

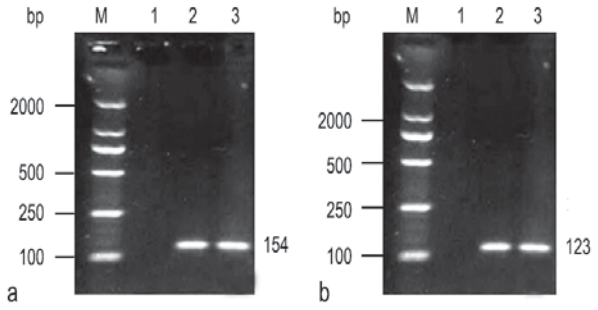

Fig. 1 Electrophoresis map of the PCR products. (a) Electrophoresis map of the PCR product of *PinX1*. M: *PinX1* PCR amplification product, 1: blank control, 2, 3: *PinX1* fragment (154 bp); (b) Electrophoresis map of the PCR product of *hTERT*. M: *hTERT* PCR amplification product, 1: blank control; 2, 3: *hTERT* fragment (123 bp)

expression of hTERT in the BCC samples (5.64 ± 2.37) was significantly higher than that in the controls (1.55 ± 0.58; $t = 5.413$, $P < 0.05$).

Correlation analysis of mRNA expression between hTERT and PinX1

No correlation was found between the mRNA expressions of hTERT and PinX1 by Pearson correlation analysis ($r = 0.032$, $P > 0.05$).

Immunohistochemistry

The positive cell staining of *PinX1* revealed yellow or brown staining in the nucleus. The positive cell staining of *hTERT* revealed yellow or brown staining in the nucleus and/or in the cytoplasm (Fig. 2). The image analysis system of Image-Pro Plus 6.0 was used to quantitatively analyze the staining in the BCC and control specimens, by measuring the IODs in order to determine the staining intensity. Five different high-power objective visual fields (× 400) were chosen randomly under an optical microscope, and their mean value was calculated.

Immunohistochemistry results: the expression of *PinX1* in normal skin tissues was positive. The IOD of *PinX1* in the BCC samples (65.30 ± 41.84) was significantly lower than that in the control group (137.97 ± 42.09; $t = 4.17$, $P < 0.05$). The expression of *hTERT* in normal skin tissues was negative or weakly positive, while the expression of *hTERT* in BCC was positive, and the IOD (156.58 ± 87.64) was significantly higher than that in normal tissues (32.45 ± 11.47; $t = 5.38$, $P < 0.05$; Fig. 2).

Correlation analysis of the IODs of *hTERT* and *PinX1*: no correlation was found between *hTERT* and *PinX1* by Pearson correlation analysis ($r = 0.17$, $P > 0.05$).

Discussion

BCC is a malignant epithelial tumor originating in the basal cell of the skin or its appendages, which is associated with slow development and a low metastasis rate;

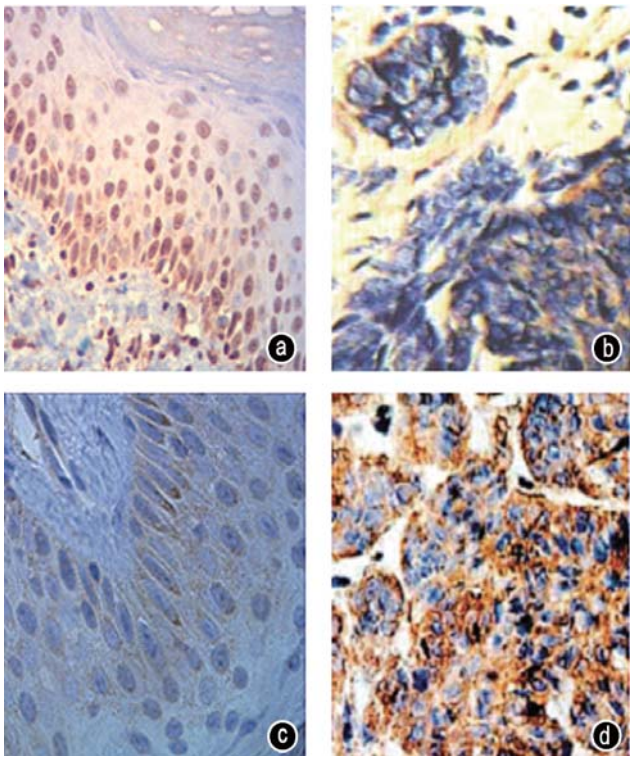

Fig. 2 Protein expression of PinX1 and hTERT in normal skin tissue or basal cell carcinoma. (a) Protein expression of PinX1 in normal skin tissue (× 400); (b) Protein expression of PinX1 in basal cell carcinoma (× 400); (c) Protein expression of hTERT in normal tissue (× 400); (d) Protein expression of hTERT in basal cell carcinoma (× 400)

however, it can invade and destruct local tissue, and can cause infection and bleeding. The causes of BCC are complicated, mainly owing to abnormal expressions of a variety of oncogenes, tumor suppressors, and apoptosis-regulating genes. Recently, it has been reported that the avoidance of tumor immune surveillance may also play a role [3]. With the increasing numbers of chromosome splits and copies in most human cells along with aging, the telomere length becomes shorter and the cells show a more unstable karyotype, eventually resulting in cell death. Most malignant tumors can evade this physiological process through activation of telomerase in the tumor cells, which results in the telomeres recovering, extending, and essentially obtaining eternal life [4].

As a linear DNA, the terminal of the telomeres cannot be copied by DNA enzymes, resulting in the ends becoming gradually shortened upon the periodic replication, consequently leading to the cells gradually undergoing senescence or apoptosis [5]. Telomerases can use their own RNA template to synthesize telomeric DNA, and add it to the end of the telomeres to maintain stabilization of the telomere length and to cancel out the causes of the curtate and unstable telomeres [6].

Previous studies have confirmed that *PinX1* expression

is downregulated in lung, breast, bladder, and renal cell carcinomas, among other cancers, and *PinX1* has been speculated to play a role in the occurrence of cancer [7–8]. In this study, the experimental results showed that the expression level of *PinX1*, an endogenous telomerase inhibitor gene, in BCC tissues was significantly lower than in the normal tissues ($P < 0.05$), indicating that *PinX1* may participate in the downregulation of the telomerase activity in normal skin tissues, thereby maintaining cell apoptosis and inhibiting tumor growth. If *PinX1* is downregulated, the telomerase activity may not be inhibited effectively, thus resulting in the telomere length being maintained and in the growth of tumor cells. On the other hand, *hTERT* has been reported to show a high expression in tumor tissues and low or no expression in normal tissues [9]. As the catalytic subunit of telomerase, *hTERT* can use telomerase RNA as the template to synthesize telomeres through reverse transcription, and its level of expression has been shown to be a limiting factor of telomerase activity, and to reflect the telomerase activity in tissues [10]. Taken together, this suggests that telomerases may play a role in the process of epidermal cell proliferation and regeneration, suggesting the need for telomerase inhibitors in the treatment of malignant tumors.

The results of this study showed that as the integrant gene of expression of telomerase activity, the expression level of *hTERT* in BCC was significantly higher than that in normal tissue ($P < 0.05$), potentially prompting the telomerase activity in BCC tissues to be higher than that in normal tissues. However, the experimental results indicated no statistically significant correlation between *PinX1* and *hTERT*, and the specific mechanisms behind the downregulation and upregulation of these genes need to be addressed in future studies.

Currently, the occurrence mechanism of BCC is not completely understood. We speculate that it may be the result of a combined effect of abnormal expressions of many related genes. For instance, promoter methylation silencing of tumor suppressor genes plays an important role in a variety of malignant tumors, especially in BCC [11]. At the present, the treatment options for BCC include operation, radiotherapy, chemotherapy, and biological therapy; however, these can produce great damage. Whether telomerases may represent diagnostic markers and therapeutic targets in malignant tumors is a hot topic, and increasing evidence suggests that *PinX1* is a promising new cancer therapeutic target. Moreover, Lance Cowey found that the Hedgehog signaling pathway plays an important role in the pathogenesis of this disease, and this may also represent a potential therapeutic target, through the novel drug vismodegib, to inhibit the Hedgehog pathway. Recently, this drug has been approved by the USA Food and Drug Administration to treat BCC [12]. Therefore, we believe that PinX1 also shows good prospects for clinical application.

In summary, detecting the expression of *PinX1* and *hTERT* may have important clinical values for evaluating the development of BCC, and these genes may represent novel biomarkers for BCC. Further, regulating the expression of *PinX1* and *hTERT* may represent a new treatment method for BCC.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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