Overexpression of the *h*Biot2 gene is associated with development of human cervical cancer

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Abstract. The novel gene human Biot2 (*h*Biot2) was first reported by our laboratory. Previously, we indicated its function of proliferation and carcinogenesis in human endometrial cancer. In the present study, we aimed to investigate whether *h*Biot2 played a similar role in human cervical cancer. We tested *h*Biot2 expression profile in cervical cancer, the corresponding adjacent normal tissues, normal cervix and the cervical cancer cell lines by RT-PCR and compared the mean value of *h*Biot2 expression between cervical cancer and normal cervix, and cervical cancer with or without lymphatic metastasis by real-time PCR. The location of *h*Biot2 in normal cervix and cancer tissues together with the corresponding adjacent normal tissues was determined by RNA-RNA *in situ* hybridization (ISH). *h*Biot2 expression in the cervical cancer (20/25), the corresponding adjacent normal tissues (3/12), normal cervix (17/18) and the cervical cell lines (2/3) was shown by RT-PCR. The mean value of *h*Biot2 expression was higher in the cervical cancer than in the normal cervix (0.478±1.612 vs. 0.091±0.107, P=0.0004), higher in the lymphatic metastasis than in the non-lymphatic metastasis in the cervical cancer (1.117±2.483 vs. 0.052±0.071, P=0.014). *h*Biot2 expression location was mainly in the parenchymal cells of the cervical cancer and normal cervix rather than in the stromal cells. Overexpression of *h*Biot2 is associated with early and interim development of human cervical cancer.

Introduction

The Human Genome Project (HGP) was launched in 1990 and a draft of the sequences of the human genome was reported in February 2001. The euchromatic sequence of the human genome with high accuracy and nearly complete coverage was reported in 2004 (International Human Genome Sequencing Consortium, 2004). Following the publication of this project, much importance was focused on finding, cloning and analyzing novel genes to determine their sequences, as well as their expression locations and functions (1). As altered gene expression is a common feature of neoplastic cells, researchers had sought to identify genes which are abundantly or specifically expressed in tumor tissues when contrasted with corresponding adjacent normal tissue (2,3). This comparison is potentially a very useful tool in the understanding of carcinogenesis and tumor progression.

Rat Biot2 is a novel gene first reported and named by our laboratory (GenBank accession no. AY845219). A portion of this novel gene was identified using the rabbit serum immunized with human mammary cancer and ovarian cancer cells to screen the rat testis cDNA expression library using the serological analysis of the recombinant cDNA expression library (SEREX) approach (4). The full length cDNA of Rat Biot2 was next obtained by the Rapid Amplification of cDNA Ends (RACE) technique and the Expressed Sequence Tags (EST) technique. The previous study and the bioinformatics analysis revealed that Rat Biot2 may correlate with sperm development in rat and have potential function to stimulate proliferation of cells (5,6). *h*Biot2 (human Biot2), as the human homologous gene of Rat Biot2, was obtained using homology analysis in NCBI (National Center for Biotechnology Information) (GenBank accession no. AK057324). The full-length cDNA sequence of *h*Biot2 is located in chromosome 10 (10p11.22) with 1819 bp and composed of 18 exons and 17 introns. The open reading frame (ORF) is 804 bp and encodes the protein of 267 amino acids. The ORF is composed of 9 exons and 8 introns. In a previous study, it was indicated that *h*Biot2 played an important role in carcinogenesis in human endometrial cancer (7). Invasive cervical cancer is a leading cause of cancer death in women, however, clinical outcomes vary significantly and are difficult to predict (8). Therefore, it is necessary and urgent to search for an effective tumor marker to predict and diagnose cervical cancer in its early stage. In the current study, we studied the expression profile of *h*Biot2 in cervical cancer and the relationships between expression value and clinicopathological variables.

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Table I. Primer and probe sequences used in RT-PCR and real-time PCR (5'-3').

	Gene	Forward primer	Reverse primer	Probe
RT-PCR	*h*Biot2	CGGGATCCTGGAAGCCAAGTCAGTAACAAAATG	CCGCTCGAGCTGCATCTGTTTGTATTCAGCATCT	
	GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC	
Real-time PCR	*h*Biot2	CCTAAGATCTCCACCAACAG	CTGATCGCAATGTCCATA	ATCCATTTTACGGTATGCTTTGCCC
	GAPDH	GACTCATGACCACAGTCCATGC	AGAGGCAGGGATGATGTTCTG	CATCACTGCCACCCAGAAGACTGTG

Materials and methods

Tissues. For scientific purposes, all samples were collected with the permission of the ethics committee of Second West China Hospital of Sichuan University and with the consent of the patients or the relatives. All tissues were from patients who underwent an operation between September 2005 to May 2006 in the Department of Gynecology and Obstetrics (The Second West China Hospital of Sichuan University, Chengdu, Sichuan, China), which included normal cervix (n=18), cervical cancer (n=25) and the corresponding adjacent normal tissues (n=12). The corresponding adjacent normal tissues were obtained 5 cm beyond the boundary of the cancerous tissue, which were myometrium in essence. Each samples were cut into two pieces, one was harvested and embedded in Tissue-Tek OCT compound (Sakura, Tokyo, Japan) within 10 min after resection from the patients and snap-frozen in liquid nitrogen before storage at -80˚C (9). The other piece was fixed in fresh 10% neutral-buffered formalin for 24 h at room temperature.

Cell lines. The cervical cancer cell line HeLa was obtained from the American Type Culture Collection (ATCC, Manassas, USA). Another two cervical carcinoma cell lines Siha, Caski were kindly provided by Gynecological Cancer Laboratory, Second West China Hospital of Sichuan University (The Gynecological Cancer Laboratory obtained the cell lines from ATCC). Cell lines were cultured in DMEM and RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (10-14). Cells were incubated at 37˚C in humidified atmosphere of 5% CO_2. Cells ($1x10^7$) were collected and washed with PBS twice before they were treated with 1 ml of TRIzol reagent for RNA isolation.

Reverse transcription PCR (RT-PCR). Total RNA from fresh, rapidly frozen tissues and cultured cells samples were isolated with single-step isolation method using TRIzol reagent (15) (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Total RNA were used at once or stored in -80˚C until used.

The primers for *h*Biot2 were designed with the Jellyfish Software. The primer sequences are shown in Table I. RT-PCR was performed with the One-step Reverse transcription PCR reagent (Takara) according to the manufacturer's instructions. The glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. Reverse transcription was carried at 42˚C for 50 min using 1 μl RNA in 25 μl reaction system. Amplification reactions were 40 cycles (94˚C 30 sec, 55˚C 30 sec, 72˚C 30 sec). The RT-PCR products were separated on a 1% agarose (GeneTech, Co. Limited) gel and visualized with ethidium bromide.

Quantitative real-time reverse transcription-PCR. Quantitative RT-PCR analysis was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems). Total RNA was completely the same as the RNA used in RT-PCR. Primers and TaqMan-probe for *h*Biot2 were designed with the assistance of the Invitrogen Co. The primers and probe sequences are listed in Table I.

Real-time PCR were performed in two steps according to the manufacturer's instructions (code nos.: DRR035S and DRR039S, Takara). The first step was reverse transcription using 2 μl RNA in 20 μl reaction system. Samples were incubated for 15 min at 42˚C and for 2 min at 95˚C. The second step was amplification reactions. The reaction conditions were as follows: 95˚C for 2 min, followed by 95˚C (10 sec), 50˚C (10 sec) and 72˚C (34 sec) for 45 cycles (16-18). To obtain the relative quantitation of gene expression, a validation experiment was done to test that the target (*h*Biot2) and reference (GAPDH) efficiencies of amplification were equal at different template dilutions (19). Target and reference probe sets were amplified in triplicates in separate wells of 96-well plates and data were averaged from the values obtained in each reaction (20,21). Results were analyzed using ABI PRISM sequence detection system software (version 2.1 Applied Biosystems), which was the calculation of ΔCt (ΔCt = average Ct_{hBiot2} - average Ct_{GAPDH}). The expression quantities of *h*Biot2 was normalized to that of GAPDH with a ratio using $2^{-\Delta Ct}$. The procedure used has been described elsewhere (22,23). Real-time PCR was performed to analyze *h*Biot2 expression in normal cervix and cervical cancer.

In situ hybridization (ISH). Before and during the processing of hybridization, all equipment and materials were treated with 0.1% DEPC. The frozen samples embedded in OCT compound were cut in 5 μm thick sections and mounted on α-aminopropyl triethoxysilane (APES)-coated slides. They were stored at -80˚C until needed (15).

The gene *h*Biot2 fragment was subcloned into the pGEM-T vector (Promega). The recombinant PGEM®-T Easy Vector/*h*Biot2 plasmids were isolated according to the instruction of Invitrogen PureLink™ Quick Plasmid Miniprep kit (Invitrogen Corp., Carlsbad, USA) and then enzymatic cut by *Bam*HI and *Xoh*I, respectively. The enzymatic cutting products were purified according to the instruction of EZ-10 Spin Column DNA Gel Extraction kit (Imperial Bio-Medic Lit Corp., Madhyamarg, India) for the temple DNA of the anti-sense RNA probe and sense RNA probe, respectively. Digoxigenin-labeled sense and anti-sense riboprobes were prepared by *in vitro* transcription using a DIG RNA Labeling kit (SP6/T7; Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions (20,24). The probes were stored at -80˚C and used within two months.

Prior to the procedure of ISH, the sections stored at -80˚C were recovered to room temperature and heated the sections in a stove for 2 h at 60˚C to fix the RNA in the tissues. The process of ISH was performed under the direction of protocols (15,25,26). The negative controls were determined by the substitution of sense for the anti-sense probe. The cells stained blue were identified as positive cells that could express *h*Biot2.

Histological analysis. The other piece of the tissue samples fixed in fresh 10% neutral-buffered formalin were cut into 5 μm sections and stained with hematoxylin and eosin (H&E). These sections were examined by experienced pathologists under a light microscopy to confirm histology.

Statistical analysis. Signed test was used to analyze the difference between the cervical cancer and the normal cervix,

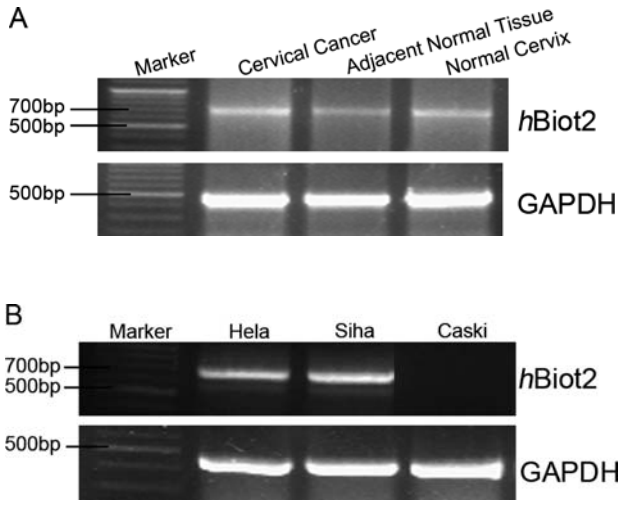

Figure 1. Using RT-PCR to detect the *h*Biot2 (700 bp) expression in cervical cancer tissues and cell lines. (A) *h*Biot2 was detected in cervical cancer (20/25), the corresponding adjacent normal tissues (3/12) and normal cervix (17/18). (B) *h*Biot2 was detected in the cervical cancer cell lines Hela, and Siha, but not in Caski.

Man-Whitney U test was used to analyze the cervical cancer patients with or without lymphatic metastasis. Statistical analysis was performed using the SPSS 16.0 statistical software (SPSS Inc., Chicago, USA) and statistical significance was set at $P<0.05$.

Results

hBiot2 expression in normal cervix, cervical cancer and the corresponding adjacent normal tissues. Twenty-five cases of cervical cancer together with 12 cases of the corresponding adjacent normal tissues, as well as 18 cases of normal cervix were involved in this study. The cervical cancers were squamous cell cancer and the FIGO (International Federation of Gynecological and Obstetrics) stage was Ia-IIIc. The expression of *h*Biot2 (700 bp) was detected in cervical cancer (20/25), the corresponding adjacent normal tissues (3/12) and normal cervix (17/18) by RT-PCR (Fig. 1A).

hBiot2 expression in cervical cancer cell lines. The expression of *h*Biot2 was detected in the cervical cancer cell lines Hela, and Siha, but not in Caski by RT-PCR (Fig. 1B).

The mean expression of hBiot2 both in the cervical cancer and the normal cervix in real-time RT-PCR. Since the expression frequency of *h*Biot2 in cervical cancer (20/25) and in normal cervix (17/18) was similar, we further performed real-time RT-PCR in order to investigate expression value differences of *h*Biot2 between them. The mean value of *h*Biot2 expression was higher in the cervical cancer than in the normal cervix (0.478±1.612 vs. 0.091±0.107, P=0.0004) (Fig. 2A).

In cervical cancer, 2 groups were separated based on with or without lymphatic metastasis. Higher expression was seen in lymphatic metastasis compared with non-lymphatic metastasis (1.117±2.483 vs. 0.052±0.071, P=0.014) (Fig. 2B).

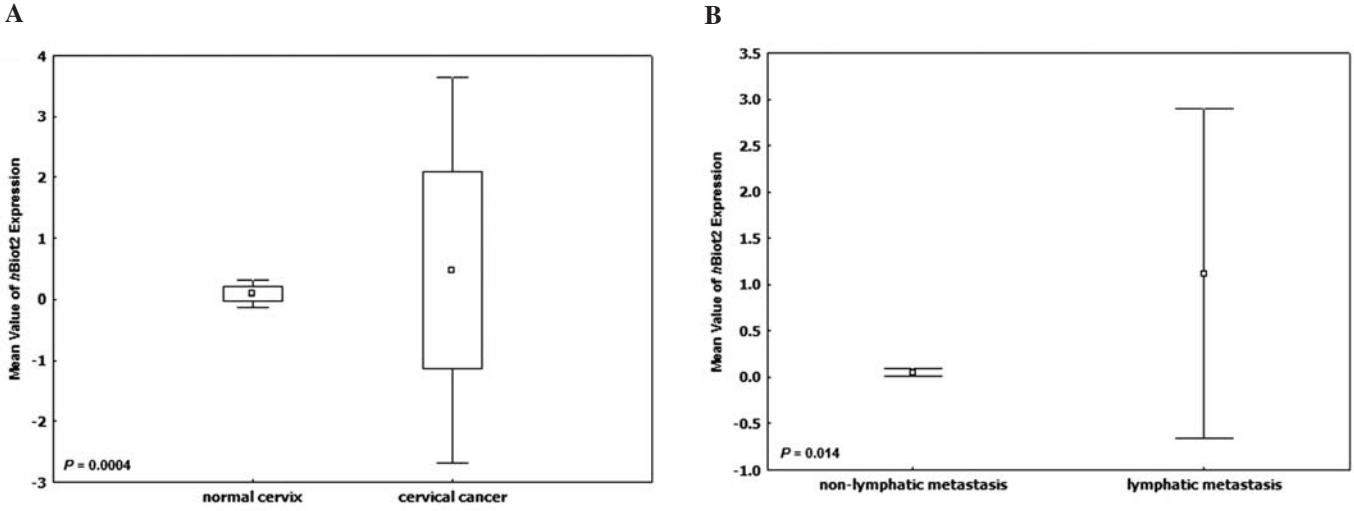

Figure 2. Using real-time PCR to detect the mean value of *h*Biot2 expression in cervical cancer and normal cervix. (A) The mean value of *h*Biot2 expression was higher in the cervical cancer than in normal cervix (0.478±1.612 vs. 0.091±0.107, P=0.0004). (B) Higher expression in lymphatic metastasis compared with non-lymphatic metastasis in cervical cancer (1.117±2.483 vs. 0.052±0.071, P=0.014).

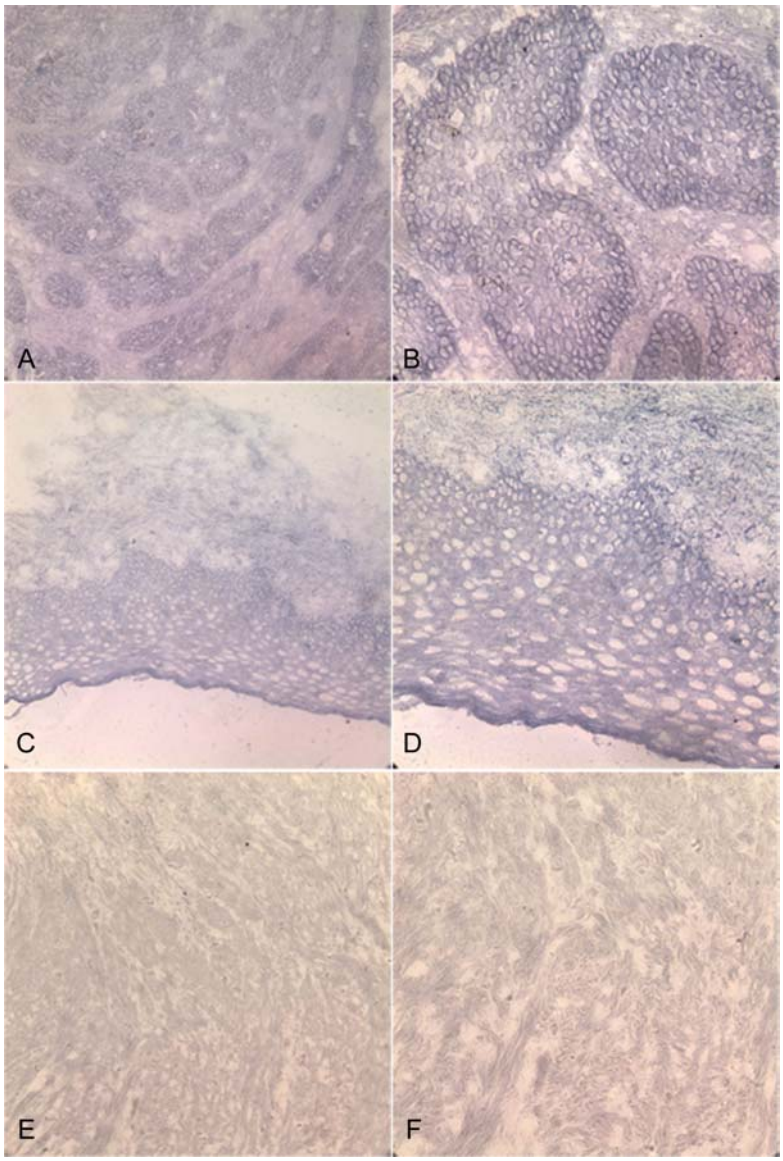

Figure 3. RNA expression of *h*Biot2 was determined by ISH on the frozen sections in cervical cancer, the corresponding adjacent normal tissues and normal cervix (x200 in the left, x400 in the right). *h*Biot2 expressed strongly in the cytoplasm of tumor cells (A and B) and normal cervix (C and D). Corresponding adjacent normal tissues (myometrium in essence) did not express *h*Biot2 (E and F).

RNA expression of hBiot2 determined by ISH in cervical cancer, the corresponding adjacent normal tissues and normal cervix. In order to reveal what type of cells expressing *h*Biot2, we performed ISH to localize *h*Biot2 expression in mRNA level. The cells expressing *h*Biot2 were identified by the blue stain in the cytoplasma. *h*Biot2 expression was mainly detected in the cervical cancer and normal cervix cells, which were detected only in the squamous cells in the malignancy of cervix and normal cervix, respectively. *h*Biot2 was only slightly expressed in normal myometrium (Fig. 3).

Discussion

One of the most important characteristics of cancer cells is the increased ability to proliferate and the decreased susceptibility to apoptosis (27). Moreover, studying the expression profile of a novel gene in tissues could help to determine the function of the gene and better understand the mechanism of carcinogenesis and cancer progression (28-30). In a previous study, we identified that *r*Biot2 gene could stimulate proliferation of cells in rat and mouse (5,6), and *h*Biot2 might play an important role in the development of human endometrial cancer (7). Expression of *h*Biot2 was higher in endometrial cancer than in the corresponding normal endometrium, and the expression was higher in poorly differentiated tumors compared with well differentiated ones. The results indicated that *h*Biot2 may have a potential function in carcinogenesis. In the current study, we investigated the expression of *h*Biot2 in human cervical cancer and cervical cancer cell lines, as the first study investigating the novel gene *h*Biot2 expression in human cervical cancer.

In the present study, it was shown that *h*Biot2 was expressed in cervical cancer, the corresponding adjacent normal tissues and normal cervix by RT-PCR. The expression frequency was quite different between in the cervical cancer, normal cervix and the corresponding adjacent normal tissues, which was lower in the corresponding adjacent normal tissues compared with cervical cancer and normal cervix, but the expression frequency was similar between the cervical cancer and normal cervix. As mentioned above, the corresponding adjacent normal tissues were myometrium in essence. Therefore, it seemed the difference among them was caused by epithelia cells instead of stromal cells, such as myometrium. We further investigated the expression amount of *h*Biot2 both in cervical cancer and normal cervix using real-time PCR. The results showed that mean value of *h*Biot2 expression was higher in cervical cancer than normal cervix, higher in lymphatic metastasis than non-lymphatic metastasis in cervical cancer. Thus, *h*Biot2 expression increased from normal cervix to cancer and also related to the metastasis potential, that is the higher the expression, the higher the metastasis potential. Taken together, *h*Biot2 may play an important role in the development of cervical cancer.

Moreover, we studied the cervical cancer cell lines, including Hela, Siha and Caski using RT-PCR. It was shown that *h*Biot2 was expressed both in Hela and Siha, no expression was seen in Caski. We speculated this could be because the Caski cell line was taken and originated from metastasis in the small intestine rather than the primary site, while the Hela and Siha cell lines were taken and originated from the cervix. The bioinformatics of tumor cells might change greatly once distant metastasis occur and expressed none or less *h*Biot2. However, combined with the results mentioned above, the trend was for higher expression of *h*Biot2, the the higher metastasis potential. We further suggest that *h*Biot2 was only related to local metastasis instead of distant metastasis, in other words, *h*Biot2 focused on the early and interim stage in the development of cervical cancer and played a lesser role in the late development. These results were consistent with other unpublished results, which found increasing *h*Biot2 expression during the early and interim development of colorectal cancer (CRC), and then decreased in the late period of CRC, while almost non-existation in liver metastasis. Therefore, overexpression of *h*Biot2 was associated with early and interim development of cervical cancer.

To rule out that the *h*Biot2 difference of expression was caused by the stromal cells, being a possible confounding variable in this study, we determined the localization of *h*Biot2 gene expressed in the cervical cancer, the corresponding adjacent normal tissues and normal cervix. The expression locations of *h*Biot2 on the frozen sections in cervical cancer, the corresponding adjacent normal tissues and normal cervix were performed by ISH of RNA level. Our results showed that *h*Biot2 expressed in the normal epithelial and tumor cells of the cervical cancer but not in the stromal cells and myometrium, which indicated that the value of *h*Biot2, determined by real-time PCR, in the both normal cervix and cervical cancer samples represented normal epithelial cells and cancer cells. This result agreed with the result that both cervical cancer and normal cervix expressed *h*Biot2, while the corresponding adjacent normal tissues (myometrium in essence) were only slightly expressed.

In conclusion, *h*Biot2 was expressed in epithelium of normal cervix and cervical cancer. Its overexpression was associated with early and interim development of cervical cancer and possibly could be a diagnostic factor in early stage of cervical cancer.

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