Aberrant activity of Wnt/Frizzled signaling pathway in renal cancer cell lines

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Abstract The expression of Wnt, Wnt receptor-Frizzled, and several other key components in Wnt pathway in renal cancer cell lines was studied. The result of semi-quantitative RT-PCR has shown that the expression level of Wnt5A and hFz5 mRNA were higher in renal cancer cell line (GRC-1) than in normal renal cell line (HK-2). This result has been confirmed by in situ hybridization. The expression of β-catenin protein was obviously higher in GRC-1 than in HK-2 (P<0.01), but there were no different expressions of its mRNA between 3 lines. The reasons of the overexpression of β-catenin has been investigated by means of immunocytochemistry, SSCP and so on, no mutation of β-catenin gene and APC were found. That means that the overexpression of Wnt5A/hFz5 might be the reason of overexpression of β-catenin. It was concluded that the aberrant activity of Wnt pathway might play an important role in renal cell carcinoma.

Keywords: Wnt/Frizzled, β-catenin, signaling pathway, renal cell carcinoma.

The Wnt gene belongs to an ever-expanding family of proto-oncogenes (there are now at least 17 known family members) that are expressed in species ranging from Drosophila to human beings[1]. Wnt5A is a member of this family, which encodes the conserved proteins with characteristics of secreted growth factor and plays a pivotal role in the formation of many kinds of tumors and biological development[2]. Following the isolation of Wnt receptor-Frizzled, more and more attention has been paid to the role of Wnt pathway in occurrences of tumors. It has been found that Wnt interacts with its receptor-Frizzled which activates a cytoplasmic protein-dishevelled (DSH), the activated DSH suppresses the activity of the complex which consists of GSK3, APC, Axin and β-catenin. The inactivitied complex induces the accumulation of free β-catenin in cytoplasm. β-catenin, an onco-protein, is a key protein in Wnt signaling pathway, which can translocate into nucleus after binding with T cell factor (TCF) or lymphocyte enhance factor (LEF), transcription factor and subsequently regulates the expression of specific target gene (for example, c-myc) resulting in the normal cell to malignant turnover[3]. At present, the aberrant activity of this signaling pathway in colorectal cancer, breast cancer and some other kinds of malignant tumors has been reported[4], but the expression of Wnt pathway in renal cell carcinoma (RCC) is not clear yet. For investigation of the role of Wnt pathway in RCC, we studied the expression of several key components in Wnt pathway in three cell
lines (GRC-1, RCC-949, HK-2) by means of immunocytochemistry, Western blot, RT-PCR and in situ hybridization.

1 Material and methods

(i) Cell culture. Six urinary malignant tumor cell lines, namely renal cell carcinoma: GRC-1 and RCC-949, bladder cancer: T24, EJ and BIU-87, prostatic cancer: PC-3M, and normal renal tubular epithelial cell: HK-2, primary cultured normal renal fibroblasts: PRF, were obtained from the Immunological Lab of the Institute. HK-2 are from human no-death cell of renal tubular. The generation and characteristics of these cell lines have been described elsewhere [9, 10]. All cell lines were maintained in tissue culture with RPMI-1640 containing 16% fetal calf serum (FCS), 10 mmol/L Hepes and 2 mmol/L L-glutamine in 37°C, 5% CO2. The cells were studied within 4 weeks after resuscitation.

(ii) Methods. Single-step method [7] was used to extract total RNA from cells for RT-PCR. AMV reverse Kit and Taq polymerase were from Promega Company (USA). Each RNA sample was simultaneously reverse transcribed into cDNA in a total volume of 25 μL containing oligo-dT 0.1 μg as an antisense primer, dNTP 1 μL (10 mmol/L each), RNasin 100 U, AMV 10 U and no-RNAase water. 2 μL of the reverse-transcribed mixture was taken as template, and PCR were performed in 50 μL of reaction mixture containing 25 mmol/L MgCl2, 4 μL, dNTP (10 mmol/L each) 1 μL, each sense (50 pmmol/L) and anti-sense (50 pmol/L) primers separately 0.5 μL, and Taq polymerase 1.5 U. Parallel control reaction was carried out with water replacing cDNA mixture. Amplification was performed after 5 min of pre-denaturation at 94°C according to the following varied reaction conditions: Wnt5A and hFz5, 35 cycles of 94°C, 40 s, 62°C, 1 min, 72°C, 1 min and a final elongation of 10 min at 72°C. β-catenin, 40 cycles of 94°C, 1 min 30 s, 56°C, 1 min, 72°C, 1 min 30 s and a final elongation 10 min at 72°C. The same volume of cDNA mixture was used as template for parallel PCR reaction performed in the presence of β-actin primers. The varied sequences of primers in PCR were Wnt5A: F(5′-GACCTGGTCTACATCGACCCTCC-3′), R(5′-GCAGCACCAGTGGAATTGCA-3′), hFz5: F(5′-CCGCACCAAGACGAGGAGC-3′), R(5′-AAACGCCGGCCAGCCTCCAC-3′), β-catenin: F(5′-ATGGAAACCAGAACAAAAGC-3′), R(5′-GCTACTGTGTTCTGATGAG3′), β-actin: F(5′-GTGGGAAAAATACACCCCTT-3′), R(5′-GTGGCCATCTCTTGGCTGAGTC-3′). The PCR products were analyzed by electrophoresis on 1% agarose gel (Sigma, USA). Scanning and quantitation of ethidium bromide stained PCR products were performed using the Gel Doc 1000 system controlled by IBM 586 personal computer. The integrated volumes of each band of products were compared separately with the counterpart of β-actin.

Immunocytochemistry was used to detect the expression of β-catenin and APC protein. The primary antibody was rabbit polyclonal anti-β-catenin and rabbit polyclonal anti-APC (1 : 50). The second antibody was goat anti-rabbit monoclonal antibody (1 : 100). The control was performed with PBS as primary antibody. The cells which were brown-yellow stained were taken as the positive. To confirm the results of immunocytochemistry, Western blot method was used. The ratio of primary antibody to secondary antibody were 1 : 100. The procedure was discrised in ref. [8] and developed according to the enhanced chemiluminescence protocol (Amershain International).

In situ hybridization was used to confirm the results of RT-PCR. The plasmids pRK5-hFz5 were from Dr. He Xi (Harvard University, USA) and became the probe of hFz5 after being amplified, extracted and endonuclease digested. The probe of Wnt5A was from RT-PCR products. The probes were labeled with Digoxin according to the protocol (BM, Germany) and stored at -20°C. For the procedure of hybridization see ref. [8]. The cells which show black-blue or purple-blue stained were taken as the positive.

The SSCP method was used to detect the mutation of β-catenin. The PCR products of β-catenin were denatured at 65°C for 10 min and immediately cooled. Electrophoresis was performed on polyacrylamide/bis-acrylamide gel and the gels were silver stained.

All experiments were repeated at least three times. Data were represented with the means ±SE and data sets were compared using the student’s t test.