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ORIGINAL ARTICLE

TCF-4 Microsatellite Instability Mutation and Expression of Splicing Forms in Human Bladder Cancer

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T cell factor (TCF)-4 and β-catenin are well recognized as key regulators in many developmental processes. TCF-4 binding with β-catenin can activate the transcriptional activity of downstream target genes (e.g. *c-myc* and *cyclin-D1*). Upregulation of TCF/β-catenin activity can promote carcinogenesis in many tissues. However, its precise role in bladder cancer is still unclear. Since typical activating mutations have not been previously reported in the bladder, we examined whether *TCF-4* mutations occur in human bladder carcinoma cell lines. In the present study, interestingly, *TCF-4* gene mutations were found in human bladder carcinoma cell lines as shown by reverse transcription polymerase chain reaction and a sequencing method. A *TCF-4* microsatellite instability (MSI) phenotype was identified to be an (A)8 repeat arising from the deletion of an A in the (A)9 coding repeat. Moreover, immunofluorescence analysis showed that the frameshift mutant of TCF-4 was exclusively localized in the nucleus of bladder cancer cells. Collectively, our data indicate that *TCF-4* MSI+ and the expression of spliced forms appear in human bladder cancer cells, and suggest a role of the TCF-4-mediated signal pathway in progression of bladder cancer.

Key Words: alternative splicing; bladder carcinoma; microsatellite instability; *TCF-4*

Introduction

Bladder cancer was the eighth most common malignancy in Taiwanese men in 2005 and the 12th leading cause of cancer mortality.[1] Recent research has identified that the Wingless/Wnt signaling pathway is involved in altering cell fate in several developmental processes as well as in morphogenesis, mitogenesis and tumorigenesis.[2,3] The Wnt signaling pathway plays a critical role in development and growth regulatory mechanisms.[4–6] A critical downstream factor in this pathway, β-catenin, is associated with a cytoplasmic protein complex comprised glycogen synthase kinase-3β (GSK-3β), adenomatous polyposis coli (APC), and axin.[7–9] Phosphorylation of β-catenin by GSK-3β leads to proteosomal degradation of β-catenin. Mutations in genes encoding *APC* or β-*catenin* that enhance the stability of β-catenin have been

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found in colon carcinoma and melanoma.[10,11] Transcriptional targets of this signaling pathway include cancer-associated genes, such as *cyclin D1* and *c-myc*.[12,13] Since the APC/β-catenin/T cell factor (TCF) pathway plays a vital role in cancer development, it is important to understand the role of TCF in pathogenesis of tumors.

Recently, genes with mononucleotide runs in their coding microsatellite sequences have come to be considered to be candidates for mutational inactivation in microsatellite instability (MSI)+ tumors.[14–16] MSI tumors are characterized by defective mismatch repair and accumulation of frameshift mutations in numerous genes harboring repeats in their coding sequences. Mutations in coding monotonic runs within *TGF-βRII*, *IGFIIR* and *BAX* genes are detected in cancers with MSI.[17–20] Mutations targeting monotonic runs within two mismatch repair genes, *hMSH3* and *hMSH6,* have also been identified in MSI+ cancers.[21,22] These findings suggest that a repetitive sequence in the coding region is a target of MSI and is associated with tumorigenesis.

To determine the role of TCF-4 in development and in bladder cancer, analysis of TCF-4 variants was conducted in human bladder cancer cell lines with varying degrees of differentiation for associations with clinicopathological markers. Our data showed that the *TCF-4* MSI mutation and expression of spliced forms occur in bladder carcinoma cell lines.

Materials and Methods

Cell culture

TSGH8301, BFTC905, and T24 bladder carcinoma cell lines were obtained from the Bioresource Collection and Research Center (BCRC, Taiwan). Cell lines were maintained in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and incubated in 5% CO_2 at 37°C.

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from cultured TSGH8301, BFTC905, and T24 bladder carcinoma cell lines using TRIzol reagent (Life Technologies, Inc., Carlsbad, CA, USA). Reverse transcribed cDNA was performed using Superscript II RT RNase H-reverse transcriptase and random primers (Life Technologies, Inc.). Quantity and quality of mRNA from all samples were verified by RT-PCR amplification of the *β-actin* cDNA. Amplification of *TCF-4* transcripts was performed using the Perkin-Elmer GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, CT, USA) and oligonucleotide primers (TCF-4F: 5′-ATGCCGCAG CTGAACGGCGGT-3′; TCF-4R: 5′-CTATTCTAAAGACTT GGTGAC-3′). Thermal conditions of the system were as follows: 94°C for 2 minutes; 30 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 2 minutes; one cycle at 72°C for 10 minutes. The resulting PCR products (20 μL) were resolved in a 2% agarose gel and visualized under UV light after ethidium bromide staining.

Cloning and sequences

PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands) and cloned into the pGEM-T easy vector (Promega Co., Fitchburg, MI, USA). The nucleotide sequence was determined using an ABI PRISM 310 DNA Sequencer (Perkin-Elmer).

Plasmid construction

The TCF-4 MSI+ expression clone was constructed by PCR with appropriate primers providing an initiation codon and a stop codon and was cloned into the pCDNA3-HA (Invitrogen, Carlsbad, CA, USA) and pEGFP-C1 (Clontech Laboratories, Inc., San Francisco, CA, USA) vectors. The constructs were confirmed by restriction endonuclease digestion and sequencing. Detailed procedures for plasmid construction are available on request.

Transfection and fluorescent microscopy

Subcellular localization of MSI+ TCF-4 was examined by transfection and fluorescent microscopy. Transfection was performed in duplicate on cell plates at a density of 3×10^5 cells/well in six-well plates, using 1 mL of Opti-MEM reduced serum medium (Life Technologies, Inc.), 5 μL of lipofectamine 2000 reagent, and 1 μg of each plasmid (pEGFP-C1-TCF-4). After 24 hours, images were obtained by fluorescent microscopy (Olympus Co., Tokyo, Japan).

Results

Bladder carcinoma cell lines express TCF-4 splicing forms

TCF families are well known as key activators/repressors in many developmental processes. TCF-4 binding with β-catenin can activate transcriptional activity and activate downstream target genes (e.g. *c-myc* and *cyclin-D1*). Mutations in *β-catenin* contribute to elevation of β-catenin/TCF transactivation in colorectal cell lines.[23] Since the APC/catenin/TCF pathway plays an important role in colorectal

cancer development, mutational analysis of these genes is critical for determining their functions. Therefore, mutational analysis of the *TCF-4* gene in bladder carcinoma cell lines was performed by RT-PCR (Figure 1). Figure 1 shows PCR amplification of TCF-4 cDNA fragments with primers designed from the positions of 1291 to 1791. The amplified TCF-4 cDNA fragments were 300–500 bp. Four new TCF-4 splicing forms were designated as TCF-4E*, TCF-4E*(d17), TCF-4E(d17), and TCF-4B(I17) (Figure 2). They differ in the Groucho-binding domain and the region immediately downstream of the High-Mobility-Group box of *TCF-4*.

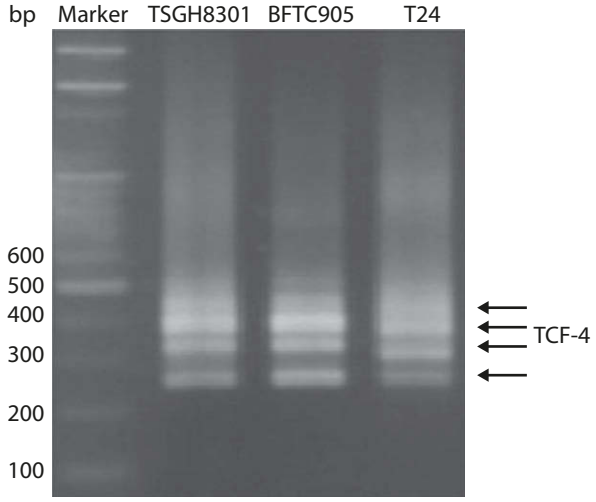

Figure 1 Mutational analysis of T cell factor-4 cDNA in bladder cancer cells by reverse transcription polymerase chain reaction. Total cellular RNA was isolated from cultured TSGH8301, BFTC905, and T24 bladder carcinoma cell lines.

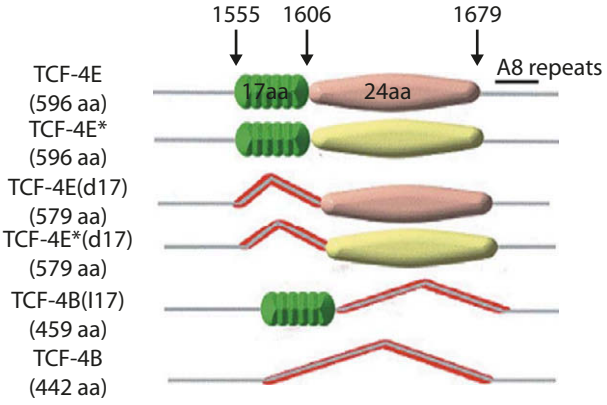

Figure 2 Four new T cell factor (TCF)-4 splicing forms. These new splicing forms were designated as TCF-4E*, TCF-4E*(d17), TCF-4E(d17), and TCF-4B(I17). Splicing sites and the number of amino acids are indicated. The amino acids sequence DLSAPKKCRARFGLDQQNNWCGPR is depicted by pink box and the amino acids sequence DSNTPKKCRALFGLDRQTLWCKPR is depicted by yellow box.

TCF-4 MSI phenotype in bladder carcinoma cell lines

Human TCF-4 is a member of the TCF-4/lymphoid enhancing factor family, and is an important transcription factor. Deregulation of TCF/β-catenin activity can promote carcinogenesis in many tissues. Alteration of the components in the Wnt signaling pathway in colorectal cancer with MSI has been demonstrated. However, one group could not confirm an (A)10 or more sequence in colorectal cancer by sequence analysis.[24] The *TCF-4* MSI+ phenotype has been found in leukemia cell lines, but not in colon cancer cell lines.[25] Moreover, these cell lines exhibit deletion and insertion of 1–2 As in an (A)9 repeat resulting in (A)7, (A)8, (A)10 and (A)11 repeats. We examined whether *TCF-4* mutations occur in human bladder carcinoma cell lines with different degrees of differentiation. We found that *TCF-4* gene mutations occurred in bladder carcinoma cell lines as shown by RT-PCR and sequencing. We describe, for the first time, a *TCF-4* MSI+ phenotype in bladder cancer cell lines with deletion of an A in the (A)9 coding repeat resulting in an (A)8 repeat. Figure 3 shows that the *TCF-4* MSI+ phenotype had an (A)8 repeat resulting from deletion of an A in the [A] 9 coding repeat in bladder cancer cells. β-catenin interacts with transcription factors of the TCF family and subsequently activates genes that are

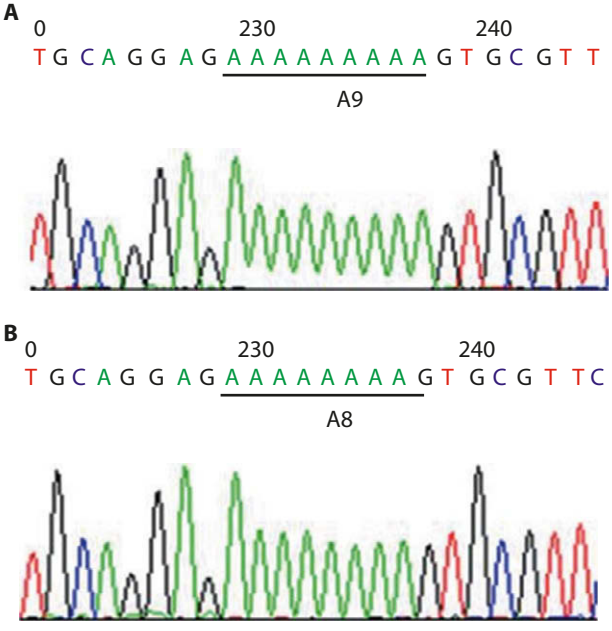

Figure 3 Nucleotides sequence analysis of T cell factor (TCF)-4. (A) Wildtype TCF-4 sequence, (A)9 in normal bladder cells. (B) Mutated TCF-4 sequence (A)8 in TSGH8301 bladder cancer cells.

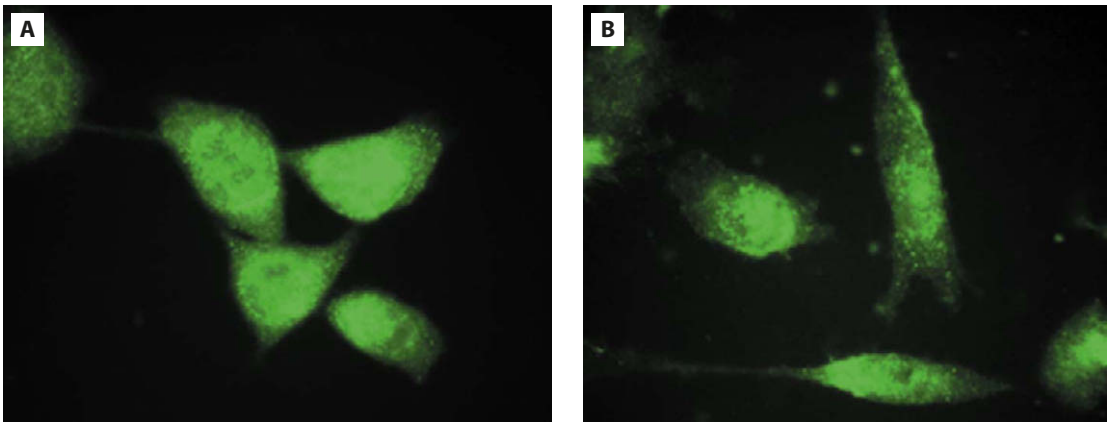

Figure 4 Immunolocalization of T cell factor (TCF)-4 microsatellite instability (MSI) in the human bladder cancer cell line TSGH8301 by immunofluorescence microscopy. (A) Transfection with the green fluorescent protein–tagged TCF-4 MSI+frameshift product (pEGFP-C1-TCF4A8). (B) Transfection with an empty vector (pEGFP-C1) in the human bladder cancer cell line TSGH8301.

responsive to TCF family members.[26] Thus defects in the β-catenin/TCF pathway may play a role in MSI+ *TCF4* in bladder cancer. To determine the role played by MSI+ *TCF4* in bladder cancer, we need to design a transfection assay with pTOPFLASH or pFOPFLASH, which directly assays β-catenin-TCF activity.

Localization of TCF-4 MSI+ frameshift gene products

We found that mutations of the *TCF-4* (A)9 repeat resulted in an (A)8 repeat of *TCF-4* in bladder carcinoma cell lines. We considered whether mutations in the (A)9 repeat of *TCF-4* influenced the expression and localization of TCF-4 in cells leading to changes in the interaction between β-catenin and TCF-4 complexes. To resolve this issue, transient transfection and fluorescent microscopy were performed. We found that GFP-tagged TCF-4 (A)8 was localized in the nuclei of bladder cancer cells (Figure 4). Control green fluorescence was observed with an empty vector. Based on these results, we believe that the localization of TCF-4 MSI+ remains unchanged. The cellular localization of TCF-4 MSI+ may perturb the protein–protein interaction between *TCF-4* and β-catenin. Further experiments are required to consolidate this putative role of MSI+ *TCF-4* with β-catenin in cancer progression.

Discussion

The multistep development of malignant tumors with increasing accumulation of genetic alterations from preneoplastic lesions to invasive carcinoma is an accepted model of carcinogenesis.[27] Molecular research on bladder cancer is beneficial for its prevention, diagnostics and therapy. Urothelial carcinoma of the bladder and upper urinary tract is a useful mode to study tumor development and progression.[28,29] Either genome-wide screens or candidate gene analysis can be conducted for elucidating molecular mechanisms that elicit bladder cancer. Human TCF-4 is a member of the TCF-4 or lymphoid enhancing factor family and is as an important transcription factor.[5] Recent studies have found that TCF-4 regulates a certain set of genes related to growth and differentiation of epithelial cells.[30] Studies on papillary bladder cancers for progression-related genetic alterations have shown that mutations in the Wnt pathway genes including APC and beta-catenin do not play an important role in urothelial carcinogenesis.[31] However, mutations of *TCF-4* have been found in two of four MSI upper urinary tract urothelial cell carcinomas.[29] Alternatively, tumors of the ureter and renal pelvis develop through a different genetic pathway in 30% of cases.[32]

Microsatellite repeats are widely distributed throughout the genome. Frameshift mutations in genes containing mononucleotide repeats are often observed in cancers exhibiting a high frequency of MSI.[14–16] *TGFβRII* was reported as the first target gene for instability in human colorectal MSI-H tumors.[17] The loss of mismatch repair proteins (hMSH2, hMLH1 or hMSH6) leads to a mutator phenotype with accumulation of genetic alterations in multiple repetitive sequences.[21,22] The transcription factor TCF-4 is one of the TCF-family proteins, which forms a transcription complex with β-catenin and plays an important role in maintaining normal epithelial growth and development of colorectal tumors.[33] Our data further suggest a fascinating possibility of the association of TCF-4 mutations with bladder cancer. The *TCF4* MSI+ phenotype has a deletion of the 1A nucleotide in (A)9 repeats, resulting in (A)8 repeats. Transfection with frameshift mutations showed that

the frameshift mutant is localized in nuclei. These data suggest that TCF mutations are involved in the development of bladder tumor. Currently, bladder tumor tissues and paired normal tissues from the same patients are being collected in the Department of Surgery and Division of Urology, Zouying Armed Forces General Hospital, Kaohsiung, Taiwan. The MSI status and mutations of TCF-4 in bladder tumor tissues will be evaluated in future studies.

Acknowledgments

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