Int. J. Cancer: **114,** 202–208 (2005)

Upregulation of Wilms' tumor gene 1 (*WT1*) in desmoid tumors

Saeid Amini Nik[1], Peter Hohenstein[2], Ali Jadidizadeh[1], Kim Van Dam[1], Adriana Bastidas[1], Rachel L. Berry[3], Charles E. Patek[3], Bernadette Van der Schueren[1], Jean-Jacques Cassiman[1] and Sabine Tejpar[1]*

[1]*Center for Human Genetics, University of Leuven, Leuven, Belgium*
[2]*Comparative and Developmental Genetics, MRC Human Genetics Unit, Western General Hospital, Edinburgh, United Kingdom*
[3]*Sir Alastair Currie, Cancer Research UK Laboratories, Molecular Medicine Centre, Western General Hospital, Edinburgh, United Kingdom*

Desmoid tumors (aggressive fibromatosis) are locally invasive soft tissue tumors in which β-catenin/TCF3 mediated Wnt signaling is activated. More than 80% of desmoid tumors contain activating mutations in β-catenin. It has been shown that the Wnt signaling pathway interacts with Wilms' tumor gene 1 (*WT1*) in normal kidney development and plays a role in the genesis of some Wilms' tumors. About 15% of Wilms' tumors contain *WT1* mutations and of these, about 50% contain β-catenin mutations. This overlap in mutation pattern of *WT1* and β-catenin in Wilms' tumor suggests that these 2 genes may collaborate in the genesis of a subset of Wilms' tumors. To investigate whether this hypothesis could be extended to other Wnt-dependent tumor types, we searched for *WT1* mutations and studied *WT1* expression in β-catenin mutant desmoid tumors. We investigated the expression of *WT1* mRNA and protein in desmoid tumors. Medium to high abundant levels of *WT1* mRNA were detected by TaqMan quantitative PCR in all tested desmoids cells, whereas adjacent normal fibroblasts showed less expression of *WT1*. Western blot analysis and immunohistochemistry confirmed this overexpression at the protein level. A mutational screen of the *WT1* zinc-finger region by sequence analysis did not identify any mutations. Finally, we investigated a possible role of β-catenin on *WT1* regulation and vice versa. Overexpression of different β-catenin mutants in the HEK293T cell line did not modulate *WT1* promoter activity and *WT1* did not affect β-catenin /TCF transcriptional activity in this cell line. These results show that the wild-type *WT1* gene is strongly overexpressed in β-catenin mutant desmoid tumors and may play a role in tumorigenesis of desmoid tumors, similar to what has been suggested in some epithelial malignancies.
© *2004 Wiley-Liss, Inc.*

Key words: *WT1*, desmoid tumor, Wnt pathway, β-catenin, aggressive fibromatosis

Desmoid tumors, also called aggressive fibromatosis, are benign soft tissue tumors, composed of a clonal proliferation of fibroblast-like cells. They presumably arise from fascia or musculo-aponeurotic structures and show a locally invasive and aggressive behavior. Although they do not metastasize, their locally infiltrative growth leads to important morbidity and mortality. Little is known about the pathogenesis of these tumors. Desmoids can occur as sporadic lesions, or as a part of familial adenomatous polyposis (FAP), which is caused by germ-line mutations in the adenomatous polyposis coli (APC) gene. Our group and others[1,2] have shown previously that sporadic desmoid tumors all contain elevated levels of β-catenin and that this is due to somatic mutations in either the APC gene or in the β-catenin gene occurring in the tumor cells.

Using transgenic mice expressing conditional stabilized β-catenin, it was demonstrated recently that β-catenin stabilization in fibroblasts is sufficient to cause aggressive fibromatosis.[3] β-catenin is a key component of the Wnt signaling pathway. Upon Wnt signaling via the canonical pathway or through oncogenic mutations, the β-catenin protein is stabilized, accumulates, and translocates to the nucleus, where it interacts with members of the TCF/Lef family of transcription factors to modulate the transcription of target genes.[4] Four different members of the TCF family have been detected in humans: TCF-1, Lef-1, TCF-3 and TCF-4.[5] In colorectal cancer, nuclear β-catenin forms a complex with TCF-4 and activates target genes, such as c-myc, cyclin D1, MMP7, fra1, c-jun and PPAR delta.[6] In desmoids similar transcriptional activity is found by a β-catenin /TCF3 complex,[7] but for yet unknown reasons the genes regulated *in vivo* by this complex are different than those in colorectal cancer.[8]

Two independent studies have suggested that deregulation of the Wnt signaling pathway plays a role in the genesis of some Wilms' tumors. Maiti *et al.*[9] reported β-catenin mutations in 21 of 153 (14%) of Wilms' tumors confirming an earlier observation showing β-catenin mutations in 6 of 40 (15%) Wilms' tumors.[10] In both studies, the mutations involved phosphorylation sites that are critical determinants of β-catenin stability. Interestingly, the study by Maiti *et al.*[9] showed a highly significant correlation between β-catenin mutations and Wilms' tumor gene 1 (*WT1*) mutations; 19 of 20 β-catenin-mutant tumors had also sustained *WT1* mutations and 50% of the tumors with *WT1* mutations had a β-catenin mutation. This overlap in mutation pattern suggests that these 2 genes may collaborate in the genesis of a subset of Wilms' tumors. Additionally a positive regulation of Wnt4 by *WT1* has been identified in kidney development.[11,12] This possible relation between *WT1* and Wnt pathway encouraged us to study *WT1* expression in desmoid tumors.

To understand the possible interaction of *WT1* and the Wnt pathway in desmoids we first established the expression profile of *WT1* in desmoid tumors and compared it to that of adjacent normal tissue (fascia) of the same patient. Mutation analysis of *WT1* was carried out on desmoid tumors. Finally, using a transient transfection system in HEK293T, we looked for a possible interaction between *WT1* and β-catenin at the gene expression and protein level. The results will show that, although wild-type *WT1* is overexpressed at the mRNA and protein level in desmoids, no evidence for a direct genetic interaction with the canonical Wnt pathway (β-catenin) could be found.

Material and methods

Cell lines and materials

The cell line HEK293T was obtained from the American Type Tissue Culture Collection and was cultured in DMEM (Invitrogen, Merelbeke, Belgium), supplemented with 10% FCS (HyClone, Erebodegem-Aalst, Belgium), penicillin and streptomycin (Invitrogen, San Diego, CA) at 37°C with 5% CO_2. The Anti-*WT1* antibody (Ab-2 clone MWT12) was from NeoMarkers (Fremont, CA) for Desmoid Western blot and the anti-*WT1* antibody (clone 6F-H2, Dako, Carpinteria, CA) was used in the other experiments.

Grant sponsor: Iranian Ministry of Health and Medical Education; Grant sponsor: European Union Marie Curie; Grant sponsor: 'Kom op Tegen Kanker', Fund for Scientific Research; Grant number: G.033.02; Grant sponsor: Belgian Federation Against Cancer; Grant number: I.32.2000.F-13.

***Correspondence to:** Center for Human Genetics, University of Leuven, Campus Gasthuisberg, Herestraat 49, Leuven B-3000, Belgium. Fax: +32-16-345997. E-mail: sabine.tejpar@med.kuleuven.ac.be

Received 5 May 2004; Accepted after revision 30 August 2004
DOI 10.1002/ijc.20717
Published online 11 November 2004 in Wiley InterScience (www.interscience.wiley.com).

Samples and cell cultures

Surgical resection specimens were obtained from patients undergoing surgery for desmoid tumor at University Hospital of Leuven, Belgium. After resection of the tumor, part of the material was immediately snap-frozen in liquid nitrogen and stored at −80°C. Normal fascia was also collected from the resection boundary. Formalin-fixed and paraffin-embedded samples of all tumors were stained with hematoxyline/eosine and examined histologically for the presence of tumor cells by a pathologist at our University Hospital.

Primary cell cultures of 5 desmoid tumours were derived by collagenase treatment of tissue biopsies and grown in DMEM supplemented with 10% FCS. Normal fascia tissue at a safe margin from the resection sides was also obtained and processed in an identical manner. The cultures were split when confluent and all studies were carried out using cultures in passage 2. To confirm the tumoral origin of the cultured cells, all primary tumour cultures were examined for the presence of nuclear β-catenin by immunohistochemistry and for transcriptional TCF-dependent activation by TOP/FOP transfection experiments. Only tumor cultures demonstrating clear nuclear β-catenin and a significant high TOP/FOP ratio were used. Two of five tumours with elevated β-catenin that we used in our experiments contained no β-catenin but APC mutations. Desmoid tumors used in our study were assessed for nuclear β-catenin and had an increased TOP/FOP ratio. In parallel, fascia primary cultures were checked for the absence of nuclear β-catenin and transcriptional TCF-dependent activation.

RNA extraction

Total RNA was extracted from primary desmoid and fascia cultures using the RNeasy kit (Qiagen), following the manufacturer's instructions.

RT-PCR (TaqMan)

Quantitative PCR was carried out by ABI PRISM 7000 Sequence Detection Systems (Applied Biosystems, Foster City, CA). After RNA extraction, as described above, cDNA was synthesized using random primers (Amersham Pharmacia, Buckinghamshire, UK) and SuperScript II (Life Technologies, Bethesda, MD). Probes and primers were designed by Primer Express 1.0 (Applied Biosystems). Sequences of the primers and probes are available upon request. Using the TaqMan PCR kit (Eurogentec, Seraing, Belgium), The PCR protocol was carried out as recommended by Applied Biosystems. Basically, quantitative values are obtained from the threshold cycle number at which the increase in the signal associated with an exponential growth of PCR products starts to be detected. Standard curves for targets and the housekeeping control gene PBGD (Porphobilinogen Deaminase) were drawn by Excel (Microsoft, Redmond, WA) upon the Ct (threshold cycle) values, and the relative concentrations of the standards and the relative concentrations for desmoid and fascia samples were calculated from the detected Ct values and the equation of the curves. Values obtained for targets were divided by the values of PBGD to normalize for differences between samples.

Genomic contamination of the samples was checked by NAC (no amplification control) samples, which did not contain reverse transcriptase enzyme during the cDNA preparation.

Northern blot

For Northern blotting, 25 μg of total RNA was denatured in a MOPS/formaldehyde/formamide buffer and run on a 1% agarose gel. The RNA was transferred onto Hybond-N nylon membranes (Amersham Pharmacia) overnight by capillary force. Specific cDNA sequences of *WT1* and PBGD were amplified by RT-PCR, isolated and used as probes. After prehybridization, hybridization was carried out overnight at 68°C in an ExpressHyb hybridization solution (BD Biosciences, Palo Alto, CA) with a ^{32}P-labeled probe. Membranes were washed during 1 hr at 42°C with a 2xSSC, 0.1% SDS solution and during 1 hr at 62°C with a 0.1 × SSC, 0.1% SDS solution. After autoradiography, all Northern blots were stripped and hybridized with a cDNA probe for PBGD to control for RNA loading and transfer efficiency.

Plasmids

The delta N89 β-catenin plasmid was a gift from Polakis (South San Francisco, CA). This CMV-neo-bam vector lacks the first 89 codons of the β-catenin gene crucial for protein degradation. Expression vector for mutant β-catenin, S33-β-catenin and S45-β-catenin (codon 33 and 45 substitution of tyrosine for serine) were gift from Vogelstein (The Johns Hopkins University Medical Institutions, Baltimore, MD). The *WT1* promoter construct, containing the 800 nt human Wilms' tumour gene 5′-flanking region (The *WT1* promoter region spanning −291 nt to +509 nt), was a kind gift from Dr. H. Soejima (Saga, Japan).

The *WT1* gene constructs, composed of 4 different *WT1* isoforms *WT1* (−/−), *WT1* (+/−), *WT1* (−/+) and *WT1* (+/+), were provided by J. Pelletier (McGill University, Canada). The first alternative splice site (exon 5) consists of 17 amino acids (VAAGSSSSVKWTEGDSN), and the second alternative splice site consists of 3 amino acids (KTS).

Transfections

For transient transfections, HEK293T cells were plated into 6-well plates (1.5×10^5 cells/well) in DMEM supplemented with 10% FCS and grown to 60% confluence. Next, cells were transiently transfected using Fugene (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions. Total DNA concentrations were kept constant with empty vector DNA (PcDNA). At 30 hr after transfection, the cells were harvested and luciferase activity was determined using the Promega luciferase assay kit. All luciferase activity values were normalized to β-galactosidase values, which served as internal controls for the transfections. Transfections were carried out in triplicate and the means ± SD of at least 3 independent experiments are presented.

Western blot

Proteins were extracted from HEK293T cells and from desmoid and fascia primary cultures to perform Western analysis with anti-*WT1* and anti-β-catenin antibody. As a positive control, HEK293T cells were transiently transfected with 500 ng of different human *WT1* expression vectors.

Proteins were extracted from all cell types using NE-PCR: nucleic and cytoplasmic extraction buffers (Perbio Science, Erembodegem, Belgium) following the manufacturer's instructions to divide nuclear and cytoplasmic protein extracts.

Protein samples were heated at 95°C for 10 min before they were applied to gel electrophoresis on a 10% BisTris gel (Invitrogen). The size-fractionated proteins were electroblotted on Hybond-C-super nitrocellulose membranes (Amersham Biosciences) for 90 min at 170 mA. The membranes were blocked with PBS-Tween 20 (0.1%), with 5% milk powder for 2 hr at room temperature and incubated with a different commercially available anti-*WT1* antibody. Western blot on desmoids samples were carried out by using Wilm's Tumor Protein (*WT1*) Ab-2 (Clone MWT12, NeoMarkers) (1/1,500 dilution, 4°C overnight). Western blot on HEK293T cells was carried out by using the N-terminal monoclonal mouse anti-human *WT1* antibody, clone 6F-H2 (Dako) (1/2,500 dilution, 4°C overnight); 6F-H2 recognizes a region in the amino-terminal 84 aa of *WT1*. Mouse monoclonal (6C5) to GAPDH (loading control; Abcam, Cambridge, UK) was used to check the loading amount of each lane (1/10,000, dilution, 4°C overnight).

After washing in PBS-Tween 20, the membranes were incubated with a second anti-mouse antibody (Goat anti-mouse) (1:3,000 dilution) coupled to horseradish peroxidase (Prosan) and washed again. Finally, immunoreactive bands were detected by ECL according to the manufacturer's instructions (Amersham Biosciences).

Immunohistochemistry

The antigens for *WT1* were retrieved by microwave treatment of the dewaxed sections in urea buffer (0.8 M, pH 6.4) for 2 × 10 min. The sections were subsequently washed for 5 min with PBS, incubated for 20 min in blocking serum and incubated overnight with the *WT1* antibody (C19, Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:3,000). As negative control, tissue sections were incubated with 0.1% BSA/PBS. The next day, the sections were washed for 2 × 5 min with PBS, incubated with Biotinylated secondary antibody for 30 min in blocking serum. After quenching for 5 min with 3% H_2O_2, they were washed for 10 min in PBS, and subsequently stained with ABC Vectastain reagent (Vector Labs, Burlingame, CA; PK-6101) and DAB (Vector Labs, SK-4100) according to the manufacturer's instructions. The sections were counterstained with Methyl Green.

Results

WT1 *expression in Desmoid tumors*

WT1 mRNA levels were measured quantitatively in primary cultures of desmoid tumor cells and compared to those of non-tumoral fibroblasts of the same patient. The results were normalized to the levels of porphobilinogen deaminase (PBGD), a housekeeping gene. In 5 tumors analyzed the *WT1* expression was 2–35 fold higher than in the respective fascia cells (Fig. 1*a*). The expression of *WT1* was also assessed and confirmed in the primary tumors (snap frozen material) using TaqMan analysis. *WT1* expression in primary tumor samples was even found to be higher (1.18–6.47-fold, 3 independent experiments) than in the corresponding cultures.

Northern blot analysis was carried out to validate the TaqMan results. A major band of 3 Kb was detected in the desmoid samples, whereas the fascia samples showed a weaker expression signal (Fig 1*b*). RNA of the HTB182 lung carcinoma cell line was used as positive control.

To confirm at the protein level the observed overexpression of *WT1* at the mRNA level, Western blot analysis was carried out with desmoid and fascia protein extracts. Using an anti-*WT1* antibody, an approximately 54-KDa band was seen in all desmoid samples. To better define the sublocalisation of *WT1* in desmoids tumor, the total protein extract was fractionated in a cytoplasmic and a nuclear as mentioned in materials and methods and western blot was carried out on both fractions. (Fig 1*c*). The results showed that *WT1* was mainly expressed in the nuclear fraction. The size corresponded to the + exon 5 *WT1* isoform. Quantitative PCR showed that the majority (55–91%) of the *WT1* mRNA, which is expressed in desmoid tumors is the one with exon 5 (data not shown).

Further analysis of *WT1* expression at the protein level in desmoid tumors was done by immunohistochemistry. The pattern of *WT1* expression by immunohistochemistry is mainly nuclear, very little cytoplasmic staining was observed. Between 62–92% of cells, depending on the sample analyzed and the area within the samples, are positive for *WT1* at detectable levels. No specific staining pattern within the samples could be identified (Fig. 2*a*).

Mutation analysis of WT1 *in desmoid tumors*

Analogous to the mutations of *WT1* found in Wilms' tumors, direct sequencing of genomic DNA was carried out on the last 4 exons of the *WT1* gene where its 4 zinc fingers are encoded. Each exon was sequenced in 4 desmoids expressing high levels of *WT1*. No mutations or deletions were detected.

Effect of β-catenin on WT1 *expression*

Stabilized β-catenin together with TCF transcription factors can activate target genes containing TCF binding sites. In HEK293T cells transient transfection of activated β-catenin constructs (S33, S45, Delta.N.89 β-catenin) can effectively activate TCF/β-catenin dependent reporter constructs. These Top/Fop reporter constructs contain wild-type (WT) and mutated TCF-4 consensus binding sites, respectively. Depending on the β-catenin construct used, activation ratios between 35–55-fold were found (Fig. 3*a*).

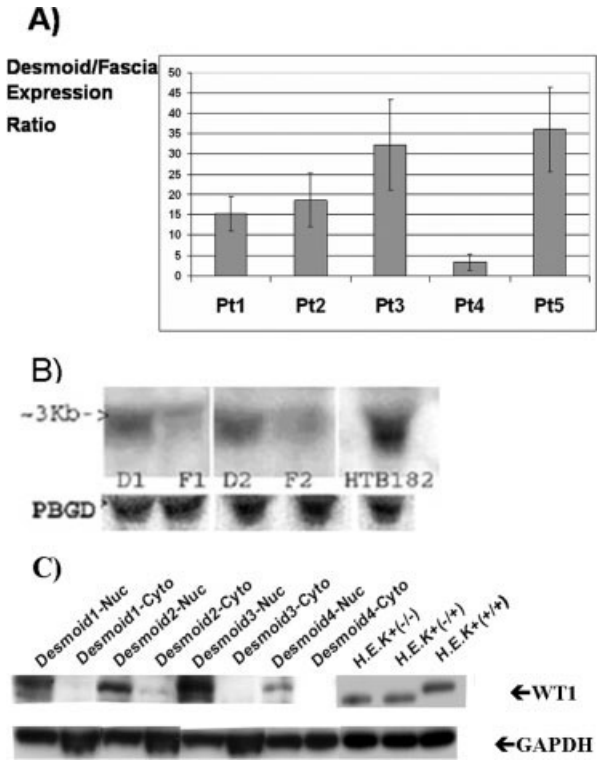


FIGURE 1 – WT1 expression in Desmoid tumors. (*a*) Comparison of *WT1* expression between desmoid samples with respective fascia of the same patient. Note that *WT1* is upregulated in all 5 tested patients (Pt). Each bar is the mean of 3 independent experiments. The SD is indicated by a vertical line. (*b*) Northern Blot analysis of *WT1* RNA in 2 desmoid samples (D) compared to control fascia (F) from the same patients. Total RNA (25 μg) from the cells was subjected to electrophoresis, transferred to nitrocellulose membranes, and hybridized with a human *WT1* probe. To confirm equal loading of total RNA, the blot was stripped and hybridized with a probe from the human PBGD gene, which showed equal expression in all samples. (*c*) Western blot analysis of *WT1* expression by desmoid cells. Twenty micrograms of a cytoplasmic and a nuclear protein fraction of 4 desmoid samples were collected and subjected to immunoblot analysis with an anti-*WT1* monoclonal antibody. To show the same amount of loading, the blot was stripped and hybridized with a loading control antibody (GAPDH) as described in material and methods. HEK293T cells were transfected with 3 different *WT1* construct (*WT1*−/−, *WT1*−/+, *WT1*+/+) as positive controls and to visualize the *WT1* isoform detected in desmoid cells. The HEK293T blot was developed after 30 min of ECL reaction to avoid a too strong messy band.

To determine whether the expression of the *WT1* promoter could be modulated by β-catenin, the *WT1* promoter was co-transfected into HEK293T cells with different β-catenin mutant constructs. Figure 3*b* shows that the *WT1* luciferase reporter activity did not change significantly in response to β-catenin overexpression.

To analyze a potential interaction between β-catenin and *WT1*, we overexpressed β-catenin in HEK293T cells. Using transient transfection, overexpression of different β-catenin constructs did not change the expression of *WT1* at the RNA (data not shown) and the protein level (Fig. 3*c*).

Effect of WT1 *on β-catenin activity*

Having shown that activated β-catenin does not influence the expression of *WT1*, we next examined if *WT1* expression could have a modulating effect on β-catenin activity. Different *WT1*

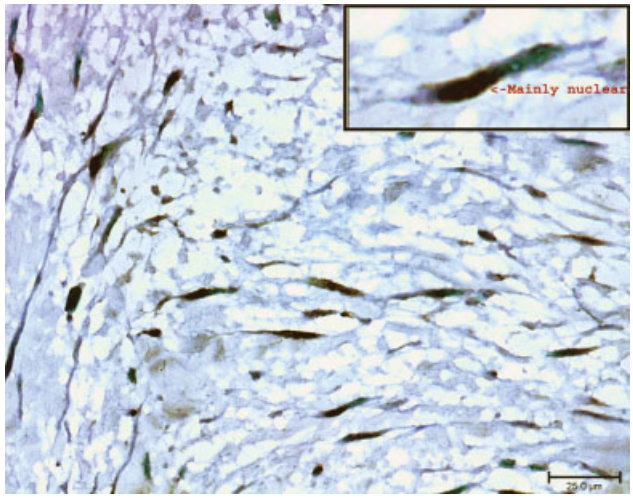


FIGURE 2 – *WT1* protein localization in the desmoid cells. Immunohistochemistry showed that *WT1* is expressed in desmoid cells, mainly as a nuclear protein. Note that many desmoid cells reacted positively.

isoforms were overexpressed in the HEK293T cell line together with TOP or FOP reporter constructs. Figure 4*a* shows that the TOP/FOP reporter ratio did not change significantly in response to *WT1* overexpression ($p > 0.05$).

Using transient transfection, over-expression of different *WT1* constructs did not change β-catenin expression at the protein level (Fig. 4*b*). This does not exclude an effect of *WT1* on β-catenin at the transcription level because the β-catenin could be degraded due to an intact phosphorylation machinery in HEK293T cells. It suggests that no connection exists between the degradation of β-catenin and *WT1*.

Discussion

We have shown previously that desmoid tumors contain β-catenin mutations in 75% of cases, APC mutations in 15% and yet unknown mutations in the rest. All desmoid tumors, including the mutation negative cases, have nuclear β-catenin and elevated TCF transcriptional activity, indicative of Wnt signaling activation ratio.[1,2]

Although β-catenin is identified as a key molecule in desmoids,[1,2] the molecular fingerprint of desmoids still remains largely unclear. It is not known which other signaling pathways could be involved in these tumors. The Wilms' tumor gene was originally identified in Wilms' tumor and plays a central role in embryonic kidney development. *WT1* is expressed at high levels not only in most types of leukemia but also in various types of solid tumors, including lung and breast cancer.[13,14] Some reports suggested that deregulation of the Wnt signaling pathway plays a role in the genesis of some Wilms' tumors. One group reported β-catenin mutations in 21 of 153 (14%) Wilms' tumors[9] confirming an earlier observation showing β-catenin mutations in 6 of 40 (15%) Wilms' tumors.[10] In these studies, the mutations involved phosphorylation sites that are critical determinants of β-catenin stability. Interestingly, the study by Maiti *et al.*[9] showed a highly significant correlation between β-catenin mutations and *WT1* mutations; 19 of 20 β-catenin-mutant tumors had also sustained *WT1* mutations and 50% of the tumors with *WT1* mutations had a β-catenin mutation. This overlap in mutation pattern suggests that these 2 genes operate in distinct pathways and may collaborate in the genesis of a subset of Wilms' tumors.

A positive regulation of Wnt4 by *WT1* was suggested during kidney development.[11,12] It has been shown that mice with a homozygous *Wnt-4* null mutation (*Wnt-4*$^{-/-}$) died within 24 hr of birth due to kidney failure.[15] All neonatal and 18.5 days *post-coitum* (dpc) *Wnt-4*$^{-/-}$ embryos have agenic kidneys showing a small amount of undifferentiated mesenchyme interspersed with branches of collecting duct epithelium.[15] Despite some degree of ureteric epithelium branching, the early mesenchymal condensates failed to form pretubular aggregates. This absence of further development of any mesenchyme-derived structures suggested that expression of *Wnt-4* was important for the process of mesenchymal-epithelial transition (MET) during metanephric nephrogenesis. In these mice, early MET markers, including *WT1*, *Pax2*, and *N-myc*, remained unchanged until 14 dpc suggesting that *Wnt-4* is downstream of these genes. *WT1* is upregulated in expression at the point of the mesenchyme to epithelial transition.[16] The frequent observation of persistent blastema and unsuccessful attempts at epithelialization in Wilms' tumors implicate *WT1* in the same pathway. This was confirmed recently by the use of siRNA-mediated knockdown of gene expression in kidney organ culture.[12] It was found that a decrease in *WT1* expression resulted in disturbance of nephrogenesis as observed in *Wnt4* knockout mice. In addition, Wnt4 siRNA treatment resulted in the same phenotype, without a change in *WT1* expression, suggesting *WT1* is upstream of Wnt4 but not *vice versa*. Moreover, it has been shown that *Wnt-4* expression levels decrease in the presence of a dominant-negative *WT1* protein, implying that *WT1* may positively regulate Wnt4 during kidney development.[11] Data on the pathways activated by Wnt4 (canonical, non-canonical or both) is at present conflicting, but at least in chicken chondrogenesis Wnt4 seems to be signaling via the canonical β-catenin mediated pathway.[17] If this is universally applicable, it might provide a link between *WT1* and β-catenin deregulation in Wilms' tumors.

Recently, it has been shown that in the Wilms' tumor with *WT1* mutations, the nuclear accumulation of β-catenin is associated with rhabdomyogenesis.[18] These findings prompted us to search for mutations of *WT1* and study the expression profile of *WT1* in desmoid tumor as well as possible interactions of this protein with β-catenin. Direct sequencing of genomic DNA of the *WT1* gene was carried out to evaluate any possible mutation in this gene that might lead to its overexpression. No mutation or deletion was detected.

To determine the expression profile of *WT1* in desmoids, we analyzed *WT1* mRNA in cultured desmoid tumors and in the respective fascia of the same patient. *WT1* was overexpressed in the desmoid cells compared to the respective fascia. Northern blot analysis confirmed the overexpression; this expression was about 70% compared to the HTB1382 lung cancer cell line.

The *WT1* protein was detected in desmoids cells, mainly in the nuclear fraction as indicated both by Western blot and immunohistochemistry.

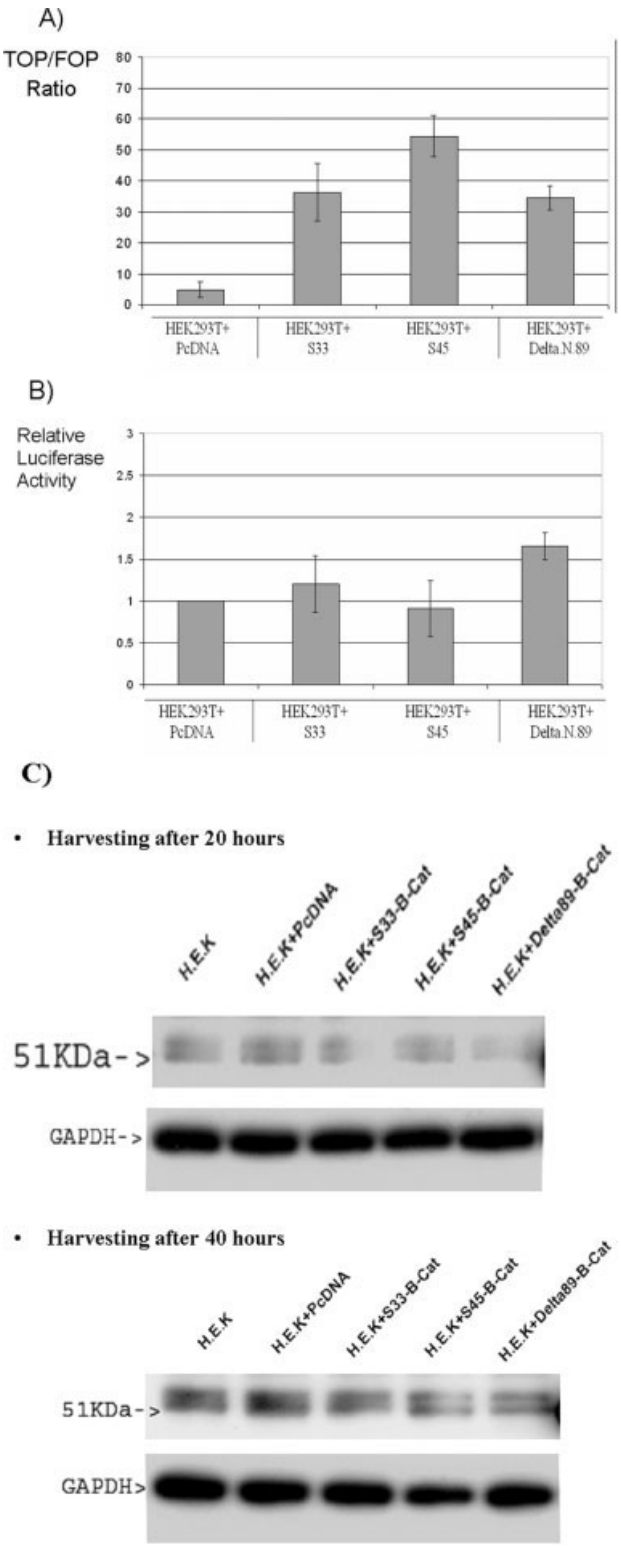
The results of the mutation analysis suggest that *WT1* somatic mutations do not play a major role in the overexpression of *WT1* found in desmoid tumors and also are in keeping with the current hypothesis that overexpression of the wild-type *WT1* gene plays an important role in the tumorigenesis of some primary cancers.

Quantitative PCR, using 2 sets of primers and probes, one with the sequence of exon 5 showed that the majority (55–91%) of *WT1*, which is expressed in desmoid cells, is the one with exon 5. It is known that there are 4 major isoforms of *WT1* represented by 2 alternative splice sites.[19–21] The first alternative splice site either includes or excludes 17 amino acids encoded by exon 5 and is only expressed in mammals.

The function of this domain is not clear because mice engineered to express *WT1* isoforms without exon 5 develop normally and are fertile.[22] The second alternative splice site occurs in intron 9 and includes or excludes 3 amino acids, KTS. *In vitro*, *WT1* isoforms without KTS (*WT1* [−KTS]) bind DNA and regulate transcription whereas those with the KTS (*WT1* [+KTS]) insertion primarily regulate RNA processing but not gene activity.

To explain the overexpression of *WT1* in desmoids, we studied potential genetic interactions between *WT1* and β-cate-

nin. Both are overexpressed in these tumors and potential interactions between *WT1* and Wnt signaling (of which β-catenin is the downstream effector) have been described during embryonic development of the kidney. β-catenin is a transcriptional co-activator, known to regulate target gene expression together with the TCF/Lef family of transcription factors. We evaluated if the WT promoter was sensitive to β-catenin regulation. Promoter reporter assays of the *WT1* promoter showed that different mutant or deleted β-catenin constructs did not



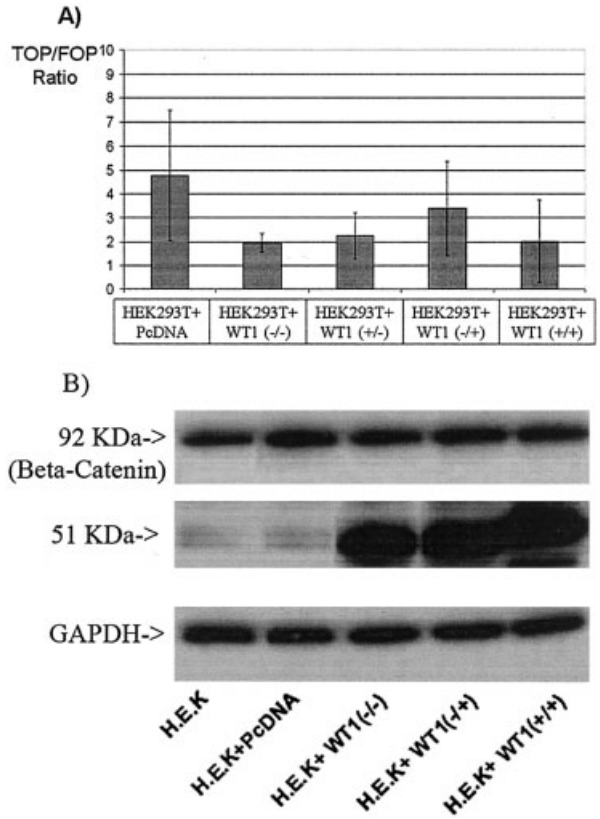


FIGURE 4 – Effect of *WT1* on β-catenin activity. (*a*) TCF transcriptional activity in transfected HEK293T cells. The ratio is the ratio of pTOPFLASH/pFOPFLASH luminescence normalized for transfection efficiency by using β-galactosidase expression vector, which served as internal controls for the transfections. Each bar gives the mean and SD of at least 3 independent experiments. The overexpression of *WT1* did not modulate the TOP/FOP ratio significantly. (*b*) Western blot analysis of *WT1* and β-catenin expression in transfected HEK293T cells. Ten micrograms each of nuclear protein extract of transfected and non-transfected HEK293T cells were subjected to immunoblot analysis, by probing with an anti-*WT1* monoclonal antibody. The blot was stripped and hybridized with an anti-β-catenin and a loading control antibody (GAPDH) as described in material and methods. HEK293T cells were transfected with 3 different *WT1* constructs (*WT1* (−/−), *WT1* (−/+) and *WT1* (+/+) and harvested after 20 hr. Harvesting the transfected cells after 40 hr did not affect the results (data not shown). *WT1* did not change the level of β-catenin protein. Similar experiments were carried out on cytoplasmic extracts with the same results (data not shown).

FIGURE 3 – Effect of β-catenin on *WT1* expression. (*a*) TCF transcriptional activity in transfected HEK293T cell line. Ratio of luminescence is the ratio of pTOPFLASH/pFOPFLASH when normalized for transfection efficiency. Each bar gives the mean and SD of at least 3 independent experiments. Note that overexpression of β-catenin can increase TOP/FOP ratio from 4.8- to 35–55-fold. (*b*) *WT1* promoter luciferase assays. Luciferase transfection assays with *WT1* promoter were carried out in HEK293T cells as described in Material and Methods. The control was transfected with the empty PcDNA vector and was set arbitrarily to one. None of the β-catenin constructs significantly modulated the *WT1* promoter activity. (*c*) Western blot analysis of *WT1* expression in transfected HEK293T cells. Ten micrograms of nuclear protein extract of transfected and non-transfected HEK293T were subjected to immunoblot analysis by probing with an anti- *WT1* monoclonal antibody. To show the same amount of loading, the blot was stripped and hybridized with an antibody against GAPDH as described in material and methods. HEK293T cells were transfected with 3 different β-catenin constructs (S33-β-catenin, S45-β-catenin and DeltaN-89-β-catenin). β-Catenin did not change the expression of *WT1*. Similar experiments were done on cytoplasmic extracts with similar results (data not shown).

modulate *WT1* promoter activity in HEK293T cells, while they stimulated TCF/β-catenin reporter constructs in these cells (Fig. 3). Thus a direct effect of β-catenin on *WT1* expression seems unlikely. Little information is available regarding the molecular mechanisms responsible for regulating the expression of the *WT1* gene. Transcription factor Sp1 fulfills an important role in controlling *WT1* gene expression.[23] Additional transcription factors involved in activation of the *WT1* gene are nuclear factor-κB, PAX2, and PAX8.[24–26] Furthermore, *WT1* is capable of autoregulating its own expression in a negative manner.[27] Finally, phosphorylation of *WT1* interferes with its nuclear translocation, as well as with DNA binding, thus providing an additional level of regulation of *WT1* action.[28] To detect any indirect effect of β-catenin on *WT1*, we overexpressed β-catenin constructs in HEK293T cell line. Western blot analysis on harvested cells after 20 and 40 hr of transfection demonstrated that β-catenin did not change the expression of *WT1* at the protein level (Fig. 3). Taken together, these results suggest that the β-catenin/TCF complex might not be directly involved in *WT1* expression or influence the stability of the protein.

The reverse hypothesis was examined to see whether *WT1* could modulate β-catenin. Different *WT1* isoforms did not change the TOP/FOP ratio in HEK293T cells (Fig. 4), suggesting that no direct effect of *WT1* on the β-catenin -TCF complex occurred at the transcriptional level. A similar negative effect was observed at the protein level (Fig. 4). It is known that a GC-rich DNA sequence, GCGGGGGCG, is recognized by *WT1*,[29] and the promoters of genes containing this sequence such as EGR1,[30] c-myc,[31] *WT1*,[27] and PAX2[32] have been shown to be regulated by *WT1 in vitro*. Data on *in vivo* relevance of these genes as downstream targets of *WT1* is surprisingly scarce.

There is not enough evidence to show a direct oncogenic contribution of *WT1* to the pathogenesis of desmoid tumors. More efforts are in progress to determine other possible interactions of *WT1* and the Wnt pathway.

In conclusion, expression profiles of desmoid cells exhibited that wild-type *WT1* is upregulated in this tumor. The mechanism of this upregulation is as yet unclear, as is the case for other tumors in the literature shown to contain elevated levels of *WT1*.[13,14]

Our findings of *WT1* overexpression in tumoral fibroblasts add to the growing spectrum of mainly epithelial tumors containing high levels of *WT1*. The exact role of *WT1* in tumorigenesis remains to be elucidated. In addition, our findings in desmoid tumors provide an additional example of coexistence of *WT1* and Wnt/β-catenin signaling in a single model, such as already described during kidney development. Although our study could not show any mechanism of direct interaction between these pathways, this might be due to limitations of our *in vitro* analysis. Further functional analyses of these pathways in model systems are warranted.

Acknowledgements

We would like to thank Dr. N. Hastie for critical reading of the manuscript. The following investigators generously provided plasmids: Dr. B. Vogelstein (S33 and S45 β-catenin), Dr. P. Polakis (delta N89 β-catenin); Dr. H. Soejima (*WT1* promoter) and Dr. J. Pelletier (*WT1* constructs). We thank Professor De Wever, Professor R. Sciot and Professor D. Uyttendaele for providing samples. S. Amini Nik and A. Jadidizadeh are supported by the Iranian Ministry of Health and Medical Education. Peter Hohenstein is a European Union Marie Curie research fellow.

References

1. Alman BA, Li C, Pajerski ME, Diaz-Cano S, Wolfe HJ. Increased β-catenin protein and somatic APC mutations in sporadic aggressive fibromatoses (desmoid tumors). Am J Pathol 1997;151:329–34.
2. Tejpar S, Nollet F, Li C, Wunder JS, Michils G, dal Cin P, Van Cutsem E, Bapat B, van Roy F, Cassiman JJ, Alman BA. Predominance of β-catenin mutations and β-catenin dysregulation in sporadic aggressive fibromatosis (desmoid tumor). Oncogene 1999;18:6615–20.
3. Cheon SS, Cheah AY, Turley S, Nadesan P, Poon R, Clevers H, Alman BA. beta-Catenin stabilization dysregulates mesenchymal cell proliferation, motility, and invasiveness and causes aggressive fibromatosis and hyperplastic cutaneous wounds. Proc Natl Acad Sci USA 2002;99:6973–8.
4. Morin PJ. Beta-catenin signaling and cancer. Bioessays 1999;21: 1021–30.
5. Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. Genes Dev 1997;11:3286–305.
6. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509–12.
7. Tejpar S, Li C, Yu C, Poon R, Denys H, Sciot R, Van Cutsem E, Cassiman JJ, Alman BA. Tcf-3 expression and beta-catenin mediated transcriptional activation in aggressive fibromatosis (desmoid tumour). Br J Cancer 2001;85:98–101.
8. Denys H, Jadidizadeh A, Amini Nik S, Van Dam K, Aerts S, Alman BA, Cassiman JJ, Tejpar S. Identification of IGFBP-6 as a significantly downregulated gene by beta-catenin in desmoid tumors. Oncogene 2004;23:654–64.
9. Maiti S, Alam R, Amos CI, Huff V. Frequent association of beta-catenin and WT1 mutations in Wilms' tumors. Cancer Res 2000;60: 6288–92.
10. Koesters R, Ridder R, Kopp-Schneider A, Betts D, Adams V, Niggli F, Briner J, von Knebel Doeberitz M. Mutational activation of the beta-catenin proto-oncogene is a common event in the development of Wilms' tumors. Cancer Res 1999;59:3880–2.
11. Sim EU, Smith A, Szilagi E, Rae F, Ioannou P, Lindsay MH, Little MH. Wnt-4 regulation by the Wilms' tumour suppressor gene, WT1. Oncogene 2002;21:2948–60.
12. Davies JA, Ladomery M, Hohenstein P, Michael L, Shafe A, Spraggon L, Hastie N. Development of an siRNA-based method for repressing specific genes in renal organ culture and its use to show that the Wt1 tumour suppressor is required for nephron differentiation. Hum Mol Genet 2004;13:235–46.
13. Oji Y, Miyoshi S, Maeda H, Hayashi S, Tamaki H, Nakatsuka S, Yao M, Takahashi E, Nakano Y, Hirabayashi H, Shintani Y, Oka Y, et al. Overexpression of the Wilms' tumor gene WT1 in *de novo* lung cancers. Int J Cancer 2002;100:297–303.
14. Koesters R, Linnebacher M, Coy JF, Germann A, Schwitalle Y, Findeisen P, von Knebel Doeberitz M. WT1 is a tumor-associated antigen in colon cancer that can be recognized by *in vitro* stimulated cytotoxic T cells. Int J Cancer 2004;109:385–92.
15. Stark K, Vainio S, Vassileva G, McMahon AP. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature 1994;372:679–83.
16. Pritchard-Jones K, Fleming S, Davidson D, Bickmore W, Porteous D, Gosden C, Bard J, Buckler A, Pelletier J, Housman D, Van Heyningen V, Hastie ND. The candidate Wilms' tumour gene is involved in genitourinary development. Nature 1990;346:194–7.
17. Hartmann C, Tabin CJ. Dual roles of Wnt signaling during chondrogenesis in the chicken limb. Development 2000;127:3141–59.
18. Fukuzawa R, Heathcott RW, Sano M, Morison IM, Yun K, Reeve AE. Myogenesis in Wilms' tumors is associated with mutations of the WT1 gene and activation of Bcl-2 and the Wnt signaling pathway. Pediatr Dev Pathol 2004;7:125–37.
19. Hastie ND. Life, sex, and WT1 isoforms: three amino acids can make all the difference. Cell 2001;106:391–4.
20. Haber DA, Sohn, RL, Buckler AJ, Pelletier J, Call KM, Housman DE. Alternative splicing and genomic structure of the Wilms' tumor gene WT1. Proc Natl Acad Sci USA 1991;88:9618–22.
21. Lee SB, Haber DA. Wilms' tumor and the WT1 gene. Exp Cell Res 2001;264:74–99.
22. Natoli TA, McDonald A, Alberta JA, Taglienti ME, Housman DE, Kreidberg JA. A mammal-specific exon of WT1 is not required for development or fertility. Mol Cell Biol 2002;22:4433–8.
23. Cohen HT, Bossone SA, Zhu G, McDonald GA, Sukhatme VP. Sp1 is a critical regulator of the Wilms' tumor-1 gene. J Biol Chem 1997;272:2901–13.
24. Dehbi M, Hiscott J, Pelletier J. Activation of the wt1 Wilms' tumor suppressor gene by NF-κB. Oncogene 1998;16:2033–9.
25. McConnell MJ, Cunliffe HE, Chua LJ, Ward TA, Eccles MR. Differential regulation of the human Wilms' tumour suppressor gene (WT1) promoter by two isoforms of PAX2. Oncogene 1997;14:2689–700.

26. Fraizer GC, Shimamura R, Zhang X, Saunders GF. PAX 8 regulates human WT1 transcription through a novel DNA binding site. J Biol Chem 1997;272:30678–87.
27. Rupprecht HD, Drummond IA, Madden SL, Rauscher FJ 3rd, Sukhatme VP. The Wilms' tumor suppressor gene WT1 is negatively autoregulated. J Biol Chem 1994;269:6198–206.
28. Ye Y, Raychaudhuri B, Gurney A, Campbell CE, Williams BR. Regulation of WT1 by phosphorylation: inhibition of DNA binding, alteration of transcriptional activity and cellular translocation. EMBO J 1996;15:5606–15.
29. Rauscher FJ 3rd, Morris JF, Tournay OE, Cook DM, Curran T. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science 1990;250:1259–62.
30. Morris JF, Madden SL, Tournay OE, Cook DM, Sukhatme VP, Rauscher FJ 3rd. Characterization of the zinc finger protein encoded by the WT1 Wilms' tumor locus. Oncogene 1991;6:2339–48.
31. Hewitt SM, Hamada S, McDonnell TJ, Rauscher FJ 3rd, Saunders GF. Regulation of the proto-oncogenes bcl-2 and c-myc by the Wilms' tumor suppressor gene WT1. Cancer Res 1995;55:5386–9.
32. Ryan G, Steele-Perkins V, Morris JF, Rauscher FJ 3rd, Dressler GR. Repression of Pax-2 by WT1 during normal kidney development. Development 1995;121:867–75.