

Analysis of *WT1* in Granulosa Cell and Other Sex Cord-Stromal Tumors¹

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Abstract

The molecular genetic events involved in the etiology of granulosa cell, Sertoli cell, and Leydig cell tumors are unknown. The expression of the Wilms' tumor suppressor gene *WT1* in granulosa and Sertoli cells prompted us to analyze this gene for mutations in 11 granulosa cell tumors, three Leydig cell tumors, and one Sertoli/Leydig cell tumor. Although most of these tumors express *WT1* mRNA, none harbors a *WT1* mutation in the zinc finger domains where >90% of *WT1* mutations in sporadic Wilms' tumors have been found. In addition we were able to exclude tumor-specific loss of heterozygosity in 13 of 15 cases. Taken together these results suggest that the *WT1* gene is unlikely to play an important role in the development of sex cord-stromal tumors.

Introduction

The term SCS⁴ tumor refers to neoplasms of specialized gonadal stroma tumors containing cells resembling Sertoli cells, Leydig cells, and granulosa cells, in varying combinations and degrees of differentiation. Little is known about the etiology and pathogenesis of these tumors. For instance it is not known whether Sertoli and granulosa cells originate from the sex cords that develop from the surface germinal epithelium of the gonadal ridge (1) or from the gonadal mesenchyme, from which the Leydig cells originate (2). The occurrence of mixed tumors of both well differentiated and incompletely differentiated components would seem to favor the common progenitor hypothesis and justifies, at present, the utilization of one term for morphologically diverse tumors.

WT1, the recently characterized Wilms' tumor suppressor gene located at chromosome 11p13 (3-5), has been shown to be involved in the development of several hereditary and sporadic Wilms' tumors (for a review see Ref. 6). Normal tissue expression of *WT1* is restricted to a small number of organs: kidneys; gonads; uterus; and spleen (3, 7). Within the human kidney, expression is restricted to the condensing blastemal cells, renal vesicles, and glomerular epithelium (8). In the mouse, gonadal expression is restricted to the granulosa cells of the ovaries and the Sertoli cells of the testis (9). In these cell types *WT1* mRNA levels increase during fetal development and remain elevated during adult life (9).

The recent description of a homozygous *WT1* exon 9 mutation within the DNA binding domain of *WT1* in the juvenile granulosa cell tumor of a patient with the Denys-Drash syndrome suggested the possibility that *WT1* function might be abrogated in such and possibly other SCS tumors (10). These results prompted us to analyze *WT1* in 15 SCS tumors. The objectives of this study were to determine

whether *WT1* is expressed in granulosa and other SCS tumors, to determine the frequency of tumor loss of heterozygosity for chromosome 11p13 markers in SCS tumors, and to determine whether *WT1* zinc finger mutations contribute to the development of these neoplasms.

Materials and Methods

Tumor Samples. Tumor samples from 15 patients with an SCS tumor were derived from the tumor banks of the Tissue Procurement Facility of the University of Alabama Cooperative Human Tissue Network, Birmingham, AL (SCS-1 and SCS-2), the Pediatric Tissue Procurement of the National Cancer Institute Cooperative Human Tissue Network, Columbus, OH (SCS-3 to SCS-6), and the Department of Pathology of The Cleveland Clinic Foundation, Cleveland, OH (SCS-7 to SCS-15). All tumors were obtained at the time of surgery and were snap-frozen in dry ice and stored at -80°C. SCS-1, SCS-2, SCS-3, SCS-9, SCS-10, SCS-11, and SCS-14 were diagnosed as granulosa cell tumors; SCS-4 and SCS-5 were diagnosed as juvenile granulosa cell tumors; SCS-7 was diagnosed as a granulosa-theca cell tumor; and SCS-15 was diagnosed as a mixed sex-cord stromal tumor, predominantly granulosa cell tumor with minor components of Sertoli-Leydig cell tumor. SCS-12 and SCS-13 were diagnosed as a Leydig cell tumor (SCS-13 arose within an ovary), and finally SCS-6 was diagnosed as a Sertoli/Leydig cell tumor.

DNA Extraction and Analyses. Tumor DNA was extracted as previously described (11). Tumor LOH was determined by using two intragenic *WT1* polymorphic markers: a 50-base pair CA repeat in the 3' untranslated sequence of 31E1 (12, 13); and a restriction fragment length polymorphism in exon 7 (see "Results"). Both polymorphisms were detected by PCR amplification of tumor DNA only, since genomic DNA from the patients was not available for analysis. We evaluated tumor LOH based upon the presence or absence of two alleles in the PCR-amplified tumor DNA. SSCP was performed to determine if any of these tumors contained *WT1* deletions, insertions, or point mutations. Exons 6-10 of the *WT1* gene (including the 4 exons that encode for the 4 *WT1* zinc fingers) from all 15 SCS tumors were amplified by PCR and subjected to SSCP analyses. The primers and conditions used for PCR and the SSCP assay have been described before (13-15).

Sequencing. The mechanisms used for direct sequencing of biotinylated PCR amplified tumor DNAs have recently been described (13, 15).

RNA Extraction and Analysis. Total cellular RNA was prepared from fresh frozen tumor tissue by the guanidine isothiocyanate/phenol/chloroform extraction method (16). Reverse transcriptional PCR was used to generate complementary DNA. In brief, 1 to 5 µg of total RNA were reversed transcribed using oligodeoxythymidylate and Moloney-murine leukemia virus reverse transcriptase (BRL, Bethesda, MD) (17, 18). Following inactivation, one-tenth (2 µl) of the reaction was amplified by PCR with Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) and primers OSCAR-2 (5'-TTCTCCCG-GTCCGACCACCT-3') and OSCAR-3 (5'-TGACACGACGGACCCTGTG) which encompass the 4 zinc fingers of the *WT1* gene, and, as a control, P538SQ15 (5'-TTTGATGCTGTCCCCGGACG-3') and P538Q21 (5'-GGAAACCGTAGCTGCCTGG-3') which encompass codons 42-103 the p53 gene (18), using a Perkin Elmer Thermocycler (Perkin Elmer).

Results

To determine whether *WT1* is expressed in SCS tumors, we performed reverse transcriptase PCR of RNA isolated from 10 granulosa cell and 3 Leydig cell tumors. The results (Fig. 1) show *WT1* expression in seven of the granulosa cell tumors and in two of the Leydig cell

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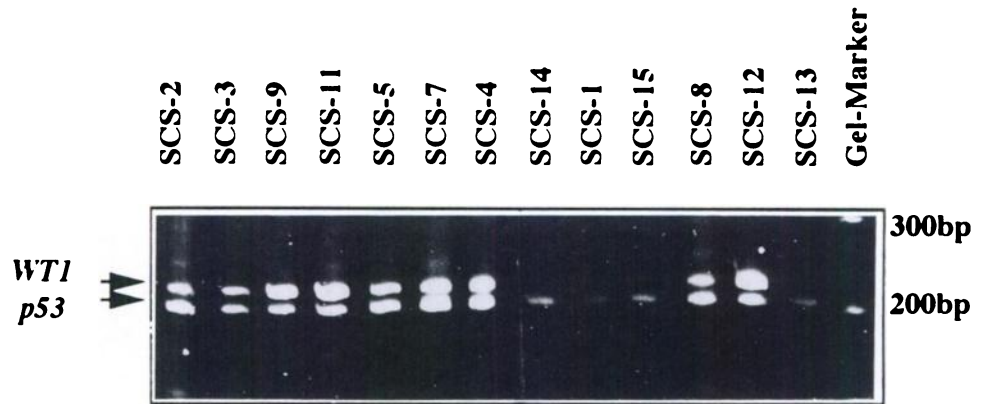
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⁴ The abbreviations used are: SCS, sex cord-stromal; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism; LOH, loss of heterozygosity.

Fig. 1. *WT1* and *p53* RNA expression of 13 SCS tumors. The reverse transcriptase PCR was performed as described in "Material and Methods." Reverse transcriptase PCR using two intronic *p53* primers shows that all SCS specimens (Lanes 1–13) contained RNA. Reverse transcriptase PCR with the two *WT1* intronic primers OSCAR-2 and OSCAR-3 shows the absence of a *WT1* PCR product for SCS-14, SCS-1, SCS-15, and SCS-13 (Lanes 8–10 and 13), indicating that *WT1* is not expressed in these 4 SCS tumors. bp, base pairs.



tumors. There was no *WT1* expression detected from the tumors in lanes 8 (SCS-14), 9 (SCS-1), 10 (SCS-15), and 14 (SCS-13). To determine if the lack of *WT1* expression was specific or related to low abundance of RNA, we also performed PCR amplification using the same first strand complementary DNA synthesis reaction with primers within exon 4 of the *p53* gene. *p53* is expressed in virtually all tissues, including kidney and Wilms' tumor. Tumors in Lanes 8, 9, 10, and 14 were shown to express *p53* mRNA (Fig. 1) indicating that the non-expression of *WT1* in these tumor samples was indeed specific. Sufficient material was not available to extract RNA from tumor specimens SCS-10 and SCS-6.

PCR-SSCP analysis used to search for deletions, insertions, or base pair mutations within the *WT1* exons 6 to 10 demonstrated a mobility shift in exon 7 for 7 of the 15 tumors analyzed (Fig. 2). Subsequent sequence analysis revealed a nucleotide change at position 5034 (CGA-CGG), a silent substitution which does not change the amino acid (Arg). This single nucleotide change introduces a second Fnu4H1 site (GCNGC) in the 244-base pair PCR product of *WT1* exon 7. Evaluation of 35 unrelated individuals revealed the following allelic frequency: 90% A1 (CGA); 10% A2 (CGG). Seven of 35 individuals are heterozygous and 8% are homozygous for A1, but none for A2. However, our previous study of Wilms' tumor (13) has revealed 3

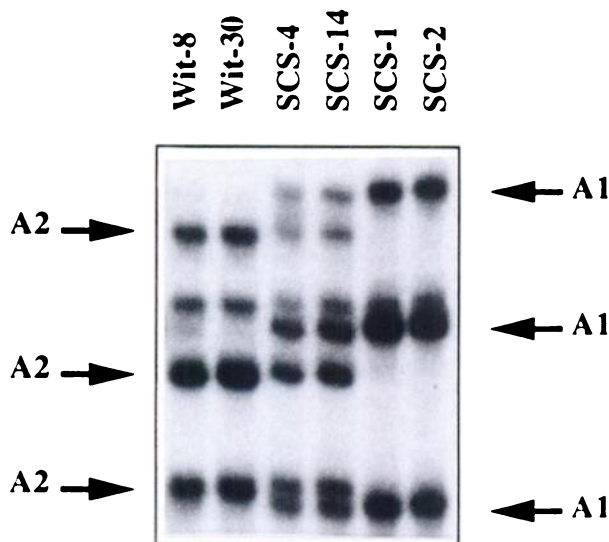


Fig. 2. PCR/SSCP analysis of *WT1*. The PCR/SSCP analysis was performed as described in "Material and Methods"; the primer set used for amplification of exon 7 has been described elsewhere (13). Lanes 1 and 2, the SSCP pattern of two sporadic Wilms' tumors, Wit-8 and Wit-30. These two tumors are homozygous for allele A2 (⁵⁰³⁴CGG). Lanes 3–6, the SSCP pattern of 4 SCS tumors. SCS-4 and SCS-14 demonstrate a heterozygous pattern, and SCS-1 and SCS-2 show a homozygous pattern for A1 (⁵⁰³⁴CGA). The heterozygous pattern was found in 7 of the 15 SCS tumors analyzed. Arrows, different conformers of both alleles A1 and A2.

cases homozygous for A2 (see Fig. 2). Codominant inheritance was determined by analyzing the segregation of the polymorphic allele in three informative families.

The unavailability of constitutional DNA from any of the patients restricted our ability to determine the presence of tumor LOH for chromosome 11p13 markers. However, demonstration of two alleles of both intragenic *WT1* polymorphic markers in 13 of the 15 tumors excludes LOH in these cases. The remaining 2 tumors (SCS-6 and SCS-7) may or may not have lost heterozygosity.

Discussion

The assumption that cells expressing *WT1* under physiological circumstances constitute targets for pathological changes following its inactivation predicts that *WT1* may play a role in the development of disorders of the kidney, spleen, uterus, and gonads, since *WT1* is expressed in these tissues. The description of *WT1* mutations in the Denys-Drash syndrome supports the assumption that a role for *WT1* is not limited to renal development (10, 15). Consequently, *WT1* expression in the granulosa cells of the ovaries and the Sertoli cells of the testes might predict that *WT1* plays a role in malignant processes originating from these cells. The recent description of a homozygous *WT1* mutation in a juvenile granulosa cell tumor (10) prompted us to analyze *WT1* in granulosa cell and other SCS tumors.

WT1 was, as expected, expressed in most granulosa cells tumors (Fig. 1; Table 1). Two exceptions (Table 1, patients 7 and 8) could reflect histopathological variation or result from inactivation of the promoter region of *WT1*, but this remains to be ascertained.

WT1 mRNA expression in two of the three Leydig cell tumors (Table 1) might not have been expected because these cells do not express this gene *in utero* (19). However, the origin of Leydig cell tumors might account for our finding, since these tumors originate from the gonadal mesenchyme, a mesodermal derivative. *WT1* expression has been demonstrated for several mesodermally derived tissues (19). Moreover, *WT1* mRNA expression has now been reported for several malignancies, including acute lymphoblastic leukemia, acute myelogenous leukemia (20), epithelial carcinomas of the ovary, endometrial tumors, and fallopian tube cancer (21).

Although this is a small series, tumor LOH in granulosa cell tumors was excluded in 10 of 11 specimens. The fact that most, if not all, granulosa cell tumors retain heterozygosity, as demonstrated by the presence of both alleles of either the 50-base pair CA repeat in the 3' untranslated sequence of *WT1*, or the *WT1* exon 7 polymorphism, suggests that homozygous inactivation of *WT1* is not a common event in the development of granulosa or Leydig cell tumors. This is supported by the fact that none of the analyzed SCS tumors demonstrated an SSCP shift for PCR amplicons 6–10, indicative of a *WT1* mutation. By contrast, 8 of 9 homozygous somatic point mutations thus far

Table 1 Features of 15 patients with a sex cord-stromal tumor

Patient	Tumor	Diagnosis	Organ	Sex	Age (yr)	mRNA	SSCP ^a	LOH
1	SCS-2	Granulosa	Left ovary	F	49	+	-	-
2	SCS-3	Granulosa	Left ovary	F	9	+	-	-
3	SCS-9	Granulosa	Right ovary	F	32	+	-	-
4	SCS-11	Granulosa	Left ovary	F	39	+	-	-
5	SCS-5	Granulosa	Ovary	F	13	+	-	-
6	SCS-7	Granulosa	Left ovary	F	83	+	-	NE ^b
7	SCS-4 ^c	Granulosa	Left ovary	F	12	+	-	-
8	SCS-14	Granulosa	Left ovary	F	38	-	-	-
9	SCS-1 ^d	Granulosa	Left ovary	F	28	-	-	-
10	SCS-15	Granulosa	Left ovary	F	54	-	-	-
11	SCS-10 ^e	Granulosa	Left ovary	F	67	No RNA	-	-
12	SCS-8	Leydig	Left testis	M	29	+	-	-
13	SCS-12	Leydig	Left testis	M	27	+	-	-
14	SCS-13	Leydig	Left ovary	F	73	-	-	-
15	SCS-6	Sertoli/Leydig	Left ovary	F	17	No RNA	-	NE

^a Single strand conformational polymorphism of *WT1* exons 6 to 10.

^b NE, not established.

^c Patient had Maffucci syndrome.

^d Also developed an endometrial carcinoma.

^e Also developed a renal cell carcinoma.

found in Wilms' tumor occur in the last five exons of the *WT1* gene (6). In a recent series of 41 Wilms' tumors, 3 were demonstrated to harbor a homozygous *WT1* mutation in the zinc finger region (7.3%) (13). From these data, we conclude that *WT1* mutations are unlikely to play an important role in the development of granulosa cell or other SCS tumors.

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