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## **Upregulation of WT1 in uterine sarcomas**

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### **Abstract**

**Aim:** Overexpression of Wilms' tumor gene (WT1) has been proven in several tumors. Previous research of our group on the cell cycle of uterine leiomyosarcoma (LMS) and carcinosarcoma (CS) suggested a possible role for WT1. We therefore intended to further explore the expression pattern of WT1 in uterine sarcomas.

**Methods:** 27 CS, 38 LMS, 15 endometrial stromal sarcomas (ESS) and seven undifferentiated sarcomas (US) were collected. WT1 expression was evaluated by immunohistochemistry (IHC) in 87 samples, by RT-PCR (m-RNA expression) in 23 random selected samples and by Western blotting in 12 samples, separating cytoplasmic and nuclear proteins. A pilot study to detect mutations (exons 7–10) was performed on eight samples.

**Results:** IHC showed WT1 positivity in 12/27 CS, 29/38 LMS, 7/15 ESS and 4/7 US. All-but-one sample had a positive RT-PCR. All Western blottings were positive with more cytoplasmic expression in 9/12 cases. No mutations were found.

**Conclusions:** WT1 is overexpressed in uterine sarcomas. Since increased levels of mRNA determine the biological role, WT1 might contribute to uterine sarcoma tumor biology.

## Introduction

Wilms' tumor gene 1 (WT1) is located on chromosome 11p13. Several studies have shown that WT1 is necessary for the development of the genito-urinary tract and of several other mesodermally derived tissues, including heart, spleen and adrenal glands. Thirty-six protein isoforms of WT1 are identified although there exist four main isoforms, WT1 (+/+) (+/-) (-/+ (-/-), depending on two alternative splice sites (exon 5 and KTS). WT1(+KTS) and WT1(-KTS) appear to be most determining in developing organs (1). The role of WT1 seems to be multifunctional. WT1 is thought to play a role in transcriptional regulation, RNA metabolism (possibly splicing) and possibly as well in translation (1).

Mutations in WT1 contribute to paediatric renal cancer (Wilm's tumor), glomerular nephropathy and gonadal dysgenesis. During the last decade, its role in carcinogenetic pathways was further extended to breast cancer, oesophageal cancer, primary astrocytic tumors, pancreatic ductal adenocarcinoma, lung cancer, head and neck squamous cell carcinoma, colorectal adenocarcinoma, primary thyroid cancer, desmoid tumors, human bone and soft-tissue sarcoma, children's rhabdomyosarcoma and the haematological malignancies (2-12).

Uterine sarcomas are relatively uncommon but encompass leiomyosarcoma (LMS), carcinosarcoma (CS), endometrial stromal sarcoma (ESS) and undifferentiated sarcomas (US) according to traditional classification systems (13,14). Recent textbooks classify CS as a subtype of endometrial carcinoma rather than a uterine sarcoma (15). However, the sarcoma component is believed to be less sensitive to systemic treatment modalities and therefore might be likely to determine prognosis (16,17).

The oncogenic role of WT1 in pelvic gynaecologic tumors was only recently subject to investigation. Whereas overexpression as determined by immunohistochemistry in serous ovarian carcinomas is as high as 91.1%, conflicting results in endometrial cancer were observed and a potential (18,19) genetic difference between serous ovarian and uterine carcinomas was proposed (18-25). Using a panel of seven microsatellites, we encountered loss of heterozygosity for D11S904 in 4/21 (19%) CS and 4/22 (18%) LMS (26). Furthermore, we encountered a low incidence of PTEN mutations in both LMS and CS (27). Given these similarities in CS and LMS and given the proximity of D11S904, we hypothesised that a similar oncogenic pathway including WT1 might be involved in both entities (28). Recent data support our hypothesis since 7/10 (70%) of CS were immunohistochemically positive for WT1 (22). Similarly, immunohistochemical positivity for WT1 was observed in 9/10 (90%) (29) and in 13/14 (93%) ESS (30). A recent study also looked for expression of WT1-mRNA in 5 LMS. They all showed WT1 expression, though the measured amount was rather low (31).

This study was designed to further explore our hypothesis that WT1 contributes to the carcinogenetic pathway of uterine sarcomas.

## Materials and methods

### *Study population*

After approval of the protocol by the local ethical committee tumor samples were collected thanks to a national multicentric approach. The distribution of uterine sarcoma subtypes after central pathological review (Ph M), is presented in **Table 3.1**. Mitotic index, cellular atypia and coagulative tumor cell necrosis were pathological criteria that were used to diagnose leiomyosarcoma according to recent guidelines (13).

Table 3.1. Distribution of sarcoma sub-types (n)

|       | Total | Primary tumor | Recurrent/metastatic tumor |
|-------|-------|---------------|----------------------------|
| CS    | 27    | 25            | 2                          |
| LMS   | 38    | 30            | 8                          |
| ESS   | 15    | 10            | 5                          |
| US    | 7     | 6             | 1                          |
| Total | 87    | 71            | 16                         |

CS: carcinosarcoma; LMS: leiomyosarcoma; ESS: endometrial stromal sarcoma; US: undifferentiated sarcoma

#### *Immunohistochemistry (IHC)*

Immunohistological staining was performed on paraffin-embedded tissue slices of all 87 samples. They were deparaffinized and rehydrated with ethanol. Endogenous peroxidase activity was blocked by 0.5 % H<sub>2</sub>O<sub>2</sub> in methanol. We then used a heat-induced epitope retrieval system. We used incubation by hand in water with citrate at pH 6 for 45 minutes at 90°. The slides were cooled in warm and cold waterbath. Monoclonal mouse anti-human Wilms' Tumor 1 clone 6F-H2 (Dakocytomation code M3561, Carpinteria, California) was used as antibody (dilution 1:400) against WT1 in this study. Slides were incubated with the antibody at room temperature for 2 hours. Slides were then washed with a buffer and incubated for another 30 minutes with horseradish peroxidase labelled dextran polymer coupled to antimouse secondary antibody (Envision+System Labelled Polymer- HRP anti-mouse, Dakocytomation, Carpinteria, California). Positive staining was identified by the presence of a brown reaction product (DAB). Counterstaining with Mayer's Haematoxylin was performed. Fetal kidney served as a positive control, whereas omission of the primary antibody served as negative control. Immunohistochemical staining was interpreted semiquantitatively. Of each patient sample, one tissue slide was evaluated. We assessed the distribution pattern (focal, multifocal or diffuse), the percentage and the intensity of the positive cells, being 0 (negative), 1 (weak), 2 (moderately) or 3 (strong). A scoring system was based on the multiplication of percentage and intensity of positive cells, being negative (0-20), weak (21-80), moderately (81-180) and strong (181-300). The cut off point in this study was 20. This point was chosen after judging all the samples in combination with RT-PCR results. Examination of immunohistochemical staining was performed by 2 investigators (A C en Ph M).

#### *RNA reverse transcriptase*

RNA was isolated from snap sarcoma tissue (23 samples, 100mg each : 6 CS, 6 LMS, 6 ESS, 5 US) using a monophasic solution of phenol and guanidine thiocyanate (TriPure Isolation Reagent, Roche molecular Biochemicals), according to the firm's guidelines. The RNA was dissolved in DEPC treated water. Spectrophotometry (260nm/280nm) was used to determine the concentration. RNA was converted into cDNA using Taqman Reverse Transcriptase Reagents (Applied Biosystems). Reverse transcription was performed during 46 minutes (25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes by ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

#### *Real time polymerase chain reaction (RT-PCR)*

After making cDNA, a dilution of 1:16 seemed most fitting for cDNA duplication. To each sample (5µl), 1,25µl of primers and probes for both WT1 (FAM-dye) and 18S (VIC-dye) (housekeeping gene) were added, together with 12,5µl MasterMix (Applied Biosystems). For all the samples we used doubles. Genomic contamination was checked by adding DEPC treated water to the mix. RT-PCR was performed twice as follows: 50° for 2 minutes, 95° for 10 minutes, followed by 50 cycles of 95° for 15 seconds and 60° for 1 minute.

WT1 expression (CT value) was measured by ABI PRISM 7000 Sequence Detection System. Value was compared to 18S expression, in order to obtain the normalized expression of WT1. The index delta-CT was calculated for each sample. The smaller delta-CT shows higher expression of target gene in each sample. Delta-CT values were inserted in a logarithmic function, resulting in a value, indicating the relative WT1 expression (meaning in comparison to a non-WT1-expressing sarcoma sample). The higher the value, the higher the relative WT1 expression.

#### *Western blotting*

Proteins were extracted from snap frozen sarcoma tissue, stored at -80°C (12 samples, 40mg each: 4 CS, 3 LMS, 4 ESS, 1 US) using NE-PCR protein extraction kit (Perbio Science, Erembodegem, Belgium), according to the firm's guidelines. Consequently, nuclear and cytoplasmic proteins were separated. Protein samples were heated at 95°C for 7 minutes before applying to gel electrophoresis (10% BisTris gel, Invitrogen). During 90 minutes, proteins were electroblotted on Hybond-C-super nitrocellulose membranes (Amersham Biosciences) at 170mA. The membranes were blocked with PBS-Tween 20 (0.1%) and 5% milk powder during one hour. Afterwards, they were incubated with WT1 antibody. Monoclonal mouse anti-human Wilms' Tumor 1 clone 6F-H2 (Dakocytomation code M3561, Carpinteria, California) was used as antibody (dilution 1:2500, 4°C overnight). After washing in PBS-Tween 20, the membranes were incubated during one hour with a second anti-mouse antibody (Goat anti-mouse) (dilution 1:3000), coupled to horseradish peroxidase and washed again. Finally, immunoreactive bands were detected by developing in film.

#### *Mutation Analysis*

DNA extraction:

DNA was isolated from snap frozen sarcoma tissue, stored at  $-80^{\circ}\text{C}$  (8 samples, approximately 40 mg each: 3 CS, 2 LMS, 2 ESS, 1 US) using High Pure PCR Product Purification Kit (Roche Diagnostics Corporation). The last 4 exons were amplified in five PCRs (PCR primer sequences available on request). DNA amplification of exon 7, 8 and 9 was performed in a total volume of 50  $\mu\text{l}$  containing 1 $\times$  PCR buffer (Roche), 2 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  dNTPs (Amersham Pharmacia), 10 pmol of each primer (forward and reverse), and 3U of AmpliTaq Gold DNA polymerase (Roche) and 2 $\mu\text{l}$  genomic DNA (concentration ranging from 31.5 ng/ $\mu\text{l}$  till 87.1 ng/ $\mu\text{l}$ ). The Expand Long Template PCR system kit (Roche) was used for DNA amplification of exon 10. It was performed in a total volume of 50 $\mu\text{l}$  volume containing 1 $\times$  Expand Long Template Taq polymerase buffer1, 1.75 mM  $\text{MgCl}_2$ , 3.75 U Expand Long Template enzyme mix (containing Taq and Tgo DNA polymerases), 350  $\mu\text{M}$  dNTP, 300 nM of each primer (forward and reverse) and 2 $\mu\text{l}$  genomic DNA (concentration ranging from 31.5 ng/ $\mu\text{l}$  till 87.1 ng/ $\mu\text{l}$ ). Amplification of exon 7, 8 and 9 was performed using the following temperature profile: 1 cycle of 5 minutes at  $95^{\circ}$ , 35 cycles of 30 seconds at  $95^{\circ}$ , 30 seconds at  $58^{\circ}$ , 1 minute at  $72^{\circ}$  and 1 cycle of 10 minutes at  $72^{\circ}$ . Amplification of exon 10 was performed using the following temperature profile: 1 cycle of 5 minutes at  $95^{\circ}$ , 35 cycles of 30 seconds at  $95^{\circ}$ , 30 seconds at  $60^{\circ}$ , 1 minute at  $78^{\circ}$  and 1 cycle of 10 minutes at  $72^{\circ}$ . PCR amplification was checked by 1% agarose gel electrophoresis in 1 $\times$  TBE.

#### Sequencing analysis :

For sequence analysis of the PCR products, the amplicons were purified using QIAquick PCR Purification kit (qiagen) according to the manufacturer's guidelines. Cycle sequencing was performed directly on the purified PCR product by BigDye terminator chemistry according to the manufacturer's recommendations (Applied Biosystems; Model 3100 genetic analyzer, BigDye Terminator v3.0 Cycle Sequencing) (sequencing primers available on request). All sequence changes were confirmed on both strands.

#### Statistical analysis

Analysis was carried out using the Microsoft Excel 2003. Statistical significance was ascribed to  $p < 0.05$ . The means and standard deviations (SD) of WT1 expression were determined for each subset of patients, and compared using two-way  $t$ -test. The qualitative variables were analyzed by two-way tables.

## Results

### *IHC, RT-PCR and Western blotting*

A summary of the IHC, RT-PCR and Western blotting results is presented in **Table 3.2**. Immunohistochemical staining was positive with more prominent cytoplasmic expression. Only 2 samples had a nuclear staining (**Figure 3.1**). When we compared primary versus recurrent disease in the same patient, we noticed the same or reduced staining pattern. In LMS, we had 4 patients with IHC results for primary and recurrent disease in recurrent ones, compared to primary tumors. The staining was the same in 2 cases and reduced in the 2 other recurrent cases. In US, only one patient could be matched. The staining was identical. In ESS, 2 patients were found, one had the same staining, the other one had a reduced staining pattern. No patients could be matched for CS. WT1-mRNA expression could be measured in all-but-one sample. Almost half of the samples had a moderate expression, 35% had a strong expression. Of the 23 samples, 3 had to be omitted because of inconclusive results. Western blotting results showed presence of WT1 protein in all 12 samples (**Figure 3.2**). The majority (75%) of samples had more cytoplasmic expression of WT1.

### *Mutation analysis*

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 8 samples expressing high levels of *WT1*. No mutations or deletions were detected.

### *Clinical correlation*

Out of 87 tested patients, 20 patients (4 CS, 5 ESS and 11 LMS/US) have been chosen randomly in order to evaluate WT1 expression at RNA and study their clinical outcome. Parameters that were studied include stage (stage I-II vs III-IV) and recurrence/dying of disease (poor versus good prognosis group). Although, WT1 expression in LMS/US and CS was much higher in cases with a higher stage and poor prognosis, a statistical analysis did not show any significant correlation (p-value >0.05). This pattern was not observed in ESS.

|   | LMS         | CS           | ESS        | US        |
|---|-------------|--------------|------------|-----------|
| <b>IMMUNOHISTOCHEMISTRY</b>                             |             |              |            |           |
| <b>Overall staining</b>                                 | 29/38 (76%) | 12/27 (44%)* | 7/15 (47%) | 4/7 (57%) |
| <b>Weak</b>   | 15/29       | 6/12         | 7/7        | 1/7       |
| <b>Moderate</b>   | 7/29        | 1/12         | 0/7        | 1/7       |
| <b>Strong</b>   | 7/29        | 5/12         | 0/7        | 2/7       |
| <b>Distribution pattern</b>                             |             |              |            |           |
| <b>Focal</b>  | 1/29        | -            | 0/7        | -         |
| <b>Multifocal</b>                                       | 14/29       | 5/12         | 3/7        | 1/4       |
| <b>Diffuse</b>  | 13/29       | 4/12         | 4/7        | 3/4       |
| <b>Multifocal-diffuse</b>                               | 1/29        | 3/12         | -          | -         |
| <b>Subcellular localisation</b>                         |             |              |            |           |
| <b>Cytoplasmic</b>                                      | 29/29       | 11/12        | 6/7        | 3/3       |
| <b>Nuclear and cytoplasmic</b>                          | -           | 1/12         | 1/7        | -         |
| <b>RT-PCR</b>   |             |              |            |           |
| <b>Overall relative WT1-mRNA expression<sup>°</sup></b> | 6/6 (100%)  | 4/4 (100%)   | 6/6 (100%) | 3/4 (66%) |
| <b>Negative</b>   | 0/6         | 0/4          | 0/6        | 1/4       |
| <b>Weak</b>   | 2/6         | 1/4          | 0/6        | 0/4       |
| <b>Moderate</b>   | 3/6         | 2/4          | 1/6        | 3/4       |
| <b>Strong</b>   | 1/6         | 1/4          | 5/6        | 0/4       |
| <b>WESTERN BLOTTING</b>                                 |             |              |            |           |
| <b>Overall WT1-protein expression</b>                   | 3/3         | 4/4          | 4/4        | 1/1       |
| <b>More cytoplasmic expression</b>                      | 3/3         | 4/4          | 2/4        | -         |
| <b>More nuclear expression</b>                          | -           | -            | 2/4        | -         |
| <b>Comparabel expression</b>                            | -           | -            | -          | 1/1       |

CS:  
carci

nosarcoma; LMS: leiomyosarcoma; ESS: endometrial stromal sarcoma; US: undifferentiated sarcoma

\* The carcinoma and sarcoma component were not assessed separately

<sup>°</sup> Results are in comparison to the only non-WT1-expressing sarcoma sample. Two samples of CS and one sample of ESS are not registered in this table, because of inconclusive results.

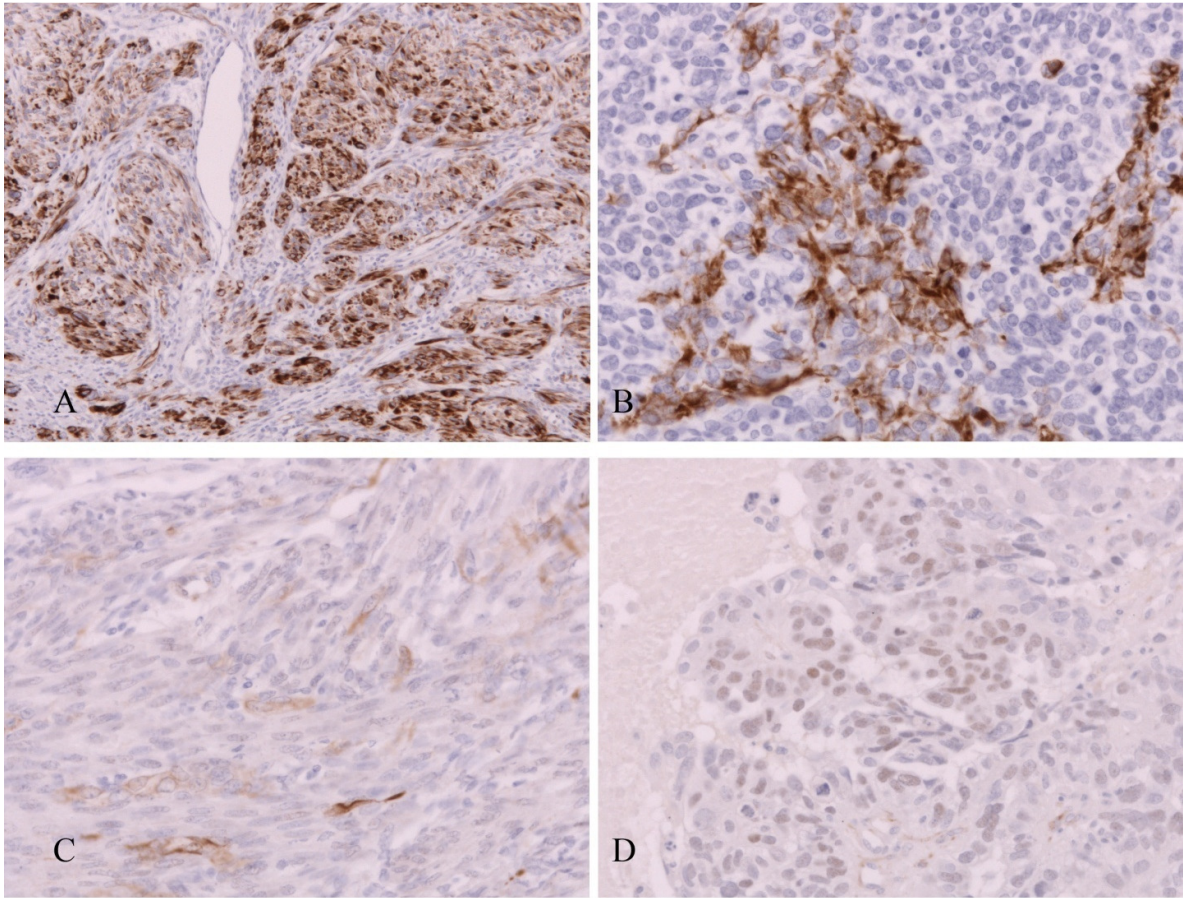


Figure 3.1. Immunohistochemical staining in uterine sarcomas. (A) Leiomyosarcoma. Cytoplasmic staining. Strong intensity. (x11,6, Linear, Res. 1292x968). (B) Endometrial Stromal Sarcoma. Cytoplasmic staining. Strong intensity. (x40, Linear, Res. 1292x968). (C) Carcinosarcoma. Cytoplasmic staining. Weak intensity. (x40, Linear, Res. 1292x968). (D) Nuclear staining. Weak intensity. (x40, Linear, Res. 1292x968).



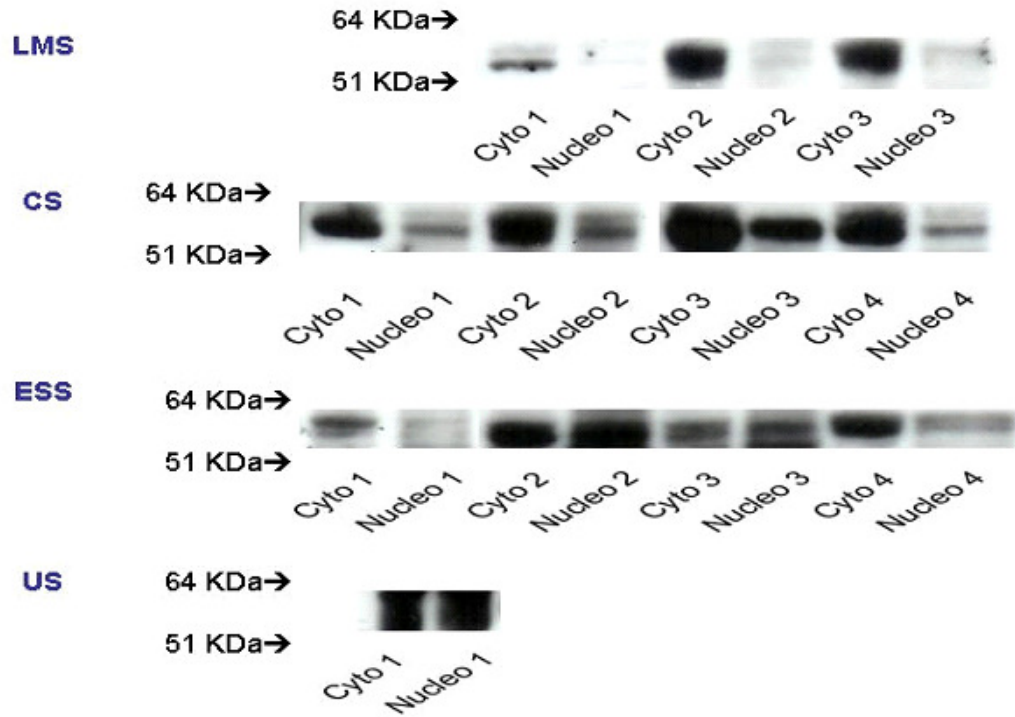


Figure 3.2. Immunoreactive WT1 bands as determined by Western blotting are presented. In 9 out of 12 samples, WT1 is more expressed in the cytoplasm. In US, there is no apparent difference between cytoplasmic and nuclear part. In 2 LMS, WT1 protein was more expressed in the nucleus. (LMS, leiomyosarcoma; CS, carcinosarcoma; ESS, endometrial stromal sarcoma; US, undifferentiated sarcoma).

## Discussion

In earlier work (28) we suggested a role for a gene localised on chromosome 11 in the carcinogenesis of uterine LMS and endometrial CS. The current study was designed to identify WT1 in uterine sarcomas. The results obtained both on RNA and protein level suggests that WT1 is present in all 4 investigated subtypes of uterine sarcomas. Previous studies in smaller series, detected WT1 expression immunohistochemically in 7/10 (70%) CS (22), whereas positivity of ESS was noted in 9/10 (90%) and 13/14 (93%) (29,30). So far, only one study was performed detecting low amounts WT1-mRNA in 5 LMS (31). In our study, we provide evidence of WT1 expression in all subtypes of uterine sarcoma. Furthermore, we provided the evidence of WT1 expression at RNA level in a large sample and detection of WT1 by immunoblotting in almost all tested samples. These data suggest that WT1 is present in a large fraction of uterine sarcomas, and since the level of expression determines the biological role, WT1 might contribute to uterine sarcoma tumor biology. In the majority of tested cases the results of the different techniques matched each other (Table 3). However, some data need clarification. Comparing IHC to RT-PCR in 87 tested samples for IHC, 10 samples (1 CS, 2 LMS, 2 US and 5 ESS) showed negative IHC but positive RT-PCR. We hypothesise tumor heterogeneity to contribute to this observation. Furthermore, the presence of a large amount of endothelial cells in ESS that contain a significant amount of WT1, might contribute to this discrepancy. Also technical considerations should be taken into account when results are interpreted. By making a homogenate of 100mg frozen tissue to extract RNA, tumoral and endothelial cells are mixed and both cell components will determine the results of RT-PCR, whereas with IHC the operator can visually eliminate WT1 staining in endothelial cells.

Until recently, WT1 was believed to be localized mainly in the nucleus (2,4,6-8,11,12,18,19,20,22,25,32). However, published data on WT1 in solid tumors demonstrate its expression in the cytoplasm (children's rhabdomyosarcoma, primary astrocytic tumors, pancreatic ductal adenocarcinoma, colorectal adenocarcinoma, breast carcinoma in situ and lobular breast cancer) (3,5,9-11). Our results also highlight the expression pattern of WT1 mainly in cytoplasm in comparison to the nucleus. Immunohistochemistry showed positive cytoplasmic staining in 50 out of 87 samples. This was combined with a weak nuclear staining in 2 out of 50 samples (1 ESS and 1 CS). This expression pattern was confirmed by Western blotting. After separating the nuclear and cytoplasmic proteins, the amount of cytoplasmic WT1 protein was larger than the nuclear part in 9 out of 12 samples. The three other samples (2 ESS and 1 US) showed an identical expression pattern for nuclear and cytoplasmic proteins in two samples and a higher nuclear WT1 expression in only one sample (**Figure 3.2**). This sample had a negative immunohistochemical staining (**Table 3.3**).

Table 3.3. Comparison between the different techniques.

CS: carcinosarcoma; LMS: leiomyosarcoma; ESS: endometrial stromal sarcoma; US: undifferentiated sarcoma

| No | Subtype sarcoma | Recurrence | IHC | RT-PCR       | Western blotting         | Mutation analysis |
|----|-----------------|------------|-----|--------------|--------------------------|-------------------|
| 1  | LMS             | No         | Neg | +            | +, cytoplasmic           | Neg               |
| 2  | LMS             | No         | +++ | ++           | +, cytoplasmic           | Neg               |
| 3  | LMS             | No         | ++  | +++          | +, cytoplasmic           | NP                |
| 4  | LMS             | Yes        | +   | ++           | NP                       | NP                |
| 5  | LMS             | Yes        | +   | ++           | NP                       | NP                |
| 6  | LMS             | No         | Neg | +            | NP                       | NP                |
| 7  | CS              | No         | + * | +++          | +, cytoplasmic           | Neg               |
| 8  | CS              | No         | +   | ++           | +, cytoplasmic           | NP                |
| 9  | CS              | No         | Neg | +            | +, cytoplasmic           | Neg               |
| 10 | CS              | Yes        | Neg | Inconclusive | NP                       | NP                |
| 11 | CS              | No         | ++  | ++           | +, cytoplasmic           | Neg               |
| 12 | CS              | No         | Neg | Inconclusive | NP                       | NP                |
| 13 | US              |            | +++ | ++           | +, nuclear = cytoplasmic | Neg               |
| 14 | US              | No         | Neg | ++           | NP                       | NP                |
| 15 | US              | No         | +   | Neg          | NP                       | NP                |
| 16 | US              | No         | Neg | ++           | NP                       | NP                |
| 17 | US              | No         | +   | Inconclusive | NP                       | NP                |
| 18 | ESS             | Yes        | Neg | +++          | +, cytoplasmic           | NP                |
| 19 | ESS             | No         | Neg | +++          | +, nuclear = cytoplasmic | NP                |
| 20 | ESS             | No         | Neg | +++          | +, nuclear               | Neg               |
| 21 | ESS             | No         | Neg | +++          | NP                       | NP                |
| 22 | ESS             |            | Neg | ++           | +, cytoplasmic           | Neg               |
| 23 | ESS             | No         | Neg | +++          | NP                       | NP                |

IHC :neg (negative), + (weak), ++ (moderate), +++ (strong) – detection was in cytoplasm unless otherwise indicated (\* = nuclear and cytoplasmic expression)

RT-PCR :neg (negative), + (weak), ++ (moderate), +++ (strong)

Western blotting : + (positive), neg (negative), cytoplasmic (more cytoplasmic staining of WT1), nuclear (more nuclear staining of WT1)

Mutation analysis :neg (negative)

NP : not performed

Of course, most tested samples showed similar pattern, but there are some samples with apparent discrepancy between IHC and Western blotting as mentioned in Table 1, which can be explained by its technique. Western blotting fractionates the protein extracts based on their weight and exposes them to the antibody, whereas with IHC the total pool of proteins is exposed to the antibody. This makes Western blotting, in general, a more specific and sometimes more sensitive technique compared to IHC (33). However, for our results, the

abundance of WT1 protein in cytoplasm and not in the nucleus is the same for both the detection methods.

In a recent review on WT1, Peter Hohenstein (1) highlights this cytoplasmic expression of WT1 in tumors. The first molecular study demonstrating cytoplasmic sub localisation was published by Niksic and colleagues in 2004 (34). They show its shuttling capacity between the nucleus and the cytoplasm. Their results demonstrate that cytoplasmic WT1 protein is present in functional polysomal complexes, suggesting an involvement in translation, and that a significant proportion of WT1 is associated with ribonucleoprotein particles (RNPs), providing evidence for a role in the RNA metabolism of the cytoplasm.

In several tumors, an overexpression of the WT1 protein in the cytoplasm is noted (3,5,9-11). Based on these 2 cellular mechanisms (translation and RNA metabolism), Niksic and colleagues suggest that not only this overexpression but the cytoplasmic expression per se, may explain the oncogenic role of WT1 in tumors.

Nevertheless, several questions about its role in the sarcoma tumorigenesis remain to be answered. Currently, little is known about the pathobiology of uterine sarcomas. Several small studies have addressed the presence of oncogenes and tumor suppressor genes in uterine sarcomas. Valid data are merely available for *TP53* and these demonstrate its biological importance in uterine sarcomas. Interactions between WT1 and p53 mainly have been studied in mice. The presence or absence of WT1 in combination with the (non-)mutated condition of p53 appeared to influence the suppression of insulin-like growth factor-I receptor (IGF-IR) (35). Moreover, in the absence of p53, WT1 acts as a potent transcriptional activator of the early growth response gene 1 (EGR1) site (36). Since both WT1 and p53 are present, tumor growth in uterine sarcomas might be regulated via IGF-IR and EGR1. On the other hand, insulin-like growth factor 2 (IGF2) is a potentially physiological target gene for WT1 and proliferation of uterine sarcoma might also be regulated by insulin-like growth factor 2 (IGF2) (37). The notion that WT1 can stabilize p53, modulate its transactivational properties and inhibit its ability to induce apoptosis adds to a potential oncogenic role of WT1 (38). It would be interesting to validate these statements and to see if there is a correlation with for example the growth factor (IGF - EGR), in order to understand better its cytoplasmic sub localisation. Further research is necessary to establish this.

Our mutational study did not show any mutation in last 4 exons of tested samples. The occurrence of *WT1* mutations may become clinically apparent as a syndrome (WAGR – Wilm's tumor/Aniridia/Genitourinary abnormalities/Retardation and DDS – Denys-Drash Syndrome). Also, in a small proportion of other tumors mutation in *WT1* has been reported. They include sporadic cases of Wilms' tumor, acute myeloid and lymphoblastic leukaemia, mesothelioma and desmoplastic small round cell tumor (39). However, recent studies (5-7,12,32,40) were unable to show mutations in any of 10 exons of the *WT1 gene* in most cancers (eg primary breast cancer, de novo lung cancers, primary thyroid cancer, colorectal adenocarcinoma, desmoidtumors,...) (2,5,7,12,32,40). Our results therefore are in line with these observations.

As to the correlation between WT1 expression and clinical outcome, no significant pattern could be seen, though a tendency towards a worse prognosis with high WT1 expression

could be noticed. This was also proven for breast cancer (41). In a recently published study (31) a good correlation between WT1 expression and outcome was found. In their study they included 5 LMS. However, further prospective studies have to be set up to explore this more accurately.

It has been shown that WT1 protein is an attractive target for immunotherapy because of its tissue-restricted expression and high immunogenicity (1,42-44). In vitro and in vivo vaccination studies have been started and the first clinical evidence of WT1 peptide-based immunotherapy, merely in patients with haematological malignancies, recently has been reported (45-48). Absence of WT1 in normal uterine tissue and its moderate to high expression in uterine sarcoma without any mutation, imply an oncogenic related expression for WT1 and makes it a potential candidate for tumor specific immunotherapy in uterine sarcoma.

Uterine sarcomas are relatively uncommon, resulting in a paucity of information with regard to tumor biology. However, uterine sarcomas are refractory to systemic treatment modalities and the development of alternative treatment strategies deserves further exploration. The current report suggests the presence of WT1 in a large fraction of uterine sarcomas and increased mRNA levels might point toward a role of WT1 in uterine sarcoma tumor biology. Apart from insights in uterine sarcoma tumor biology, the current results address an attractive target for immunotherapy.

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