WT1 is a prognostic marker in high grade uterine sarcoma

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Abstract

Objective: Wilms' tumor gene 1 (WT1) contributes to uterine sarcoma tumor biology. In this study, we aimed to clarify the prognostic value of WT1.

Methods: A retrospective clinical and histopathological review of 71 women with high grade uterine sarcoma (leiomyosarcoma (LMS) (n = 24), undifferentiated sarcoma (US) (n = 9)) and carcinosarcoma (CS) (n = 38) was performed. Patients were followed up for at least 12 months. WT1 expression was determined by immunohistochemistry. Data on recurrence (progression free survival (PFS)) and overall survival (OS) were available for all patients. Univariate and multivariate analysis of WT1 expression were carried out using Kaplan-Meier curves and Cox regression, respectively.

Results: Forty-nine (69%) tumors were WT1 positive. Forty-seven (66%) patients died of the disease, with a median OS time of 22 months. WT1 was a predictor of survival in the univariate analysis: the hazard ratio of WT1 positivity was 2.44 (95% CI 1.34-4.71) for PFS and 2.48 (95% CI 1.26-4.90) for OS. Multivariate analysis including stage, age, tumor size and sarcoma subtype, identified only stage and WT1 positivity as independent prognostic markers for survival.

Conclusions: The identification of WT1 as a prognostic marker confirms its role in high grade uterine sarcoma and carcinosarcoma tumor biology.

Introduction

Wilms' tumor gene 1 (WT1) is located on chromosome 11p13. It has many molecular functions, which are partially explained by different splicing of WT1 RNA (1). WT1 is thought to have a role in the regulation of transcription, RNA metabolism and translation. It has a central role in embryonic development (1) while its over expression in several malignancies suggests a role in tumorigenesis (2).

Uterine sarcomas are relatively uncommon but encompass leiomyosarcoma (LMS), carcinosarcoma (CS), endometrial stromal sarcoma and undifferentiated sarcomas (US), according to traditional classification systems (3). Recent textbooks classify CS as a subtype of endometrial carcinoma rather than a uterine sarcoma (4). However, the sarcoma component is believed to be less sensitive to systemic treatment modalities and therefore might be likely to determine prognosis (5). Current treatment options for uterine sarcoma are limited and its prognosis is poor with a five year survival as low as 50% for stage I, and only 10% in more advanced stages, stage (6) being the most important prognostic factor.

Recently, we demonstrated WT1 over expression in the different subtypes of uterine sarcoma (7). WT1 positivity, as determined by immunohistochemistry (IHC), was observed in 76% of all LMS, 44% of CS, 47% of ESS and 57% of US.

The aim of this study was to further explore the role of WT1 in uterine sarcoma tumor biology and to correlate WT1 expression with clinical outcome in a larger series. ESS were excluded because they are low grade sarcomas with an indolent behaviour. CS were included because of their poor prognosis which appears to be related to the sarcoma component. We hope to highlight the potential clinical use of WT1 as a target for immunotherapy.

Materials and methods

Study population

A retrospective clinical and histopathological review of 71 women with a diagnosis of high grade uterine sarcoma or carcinosarcoma and a follow up of at least 12 months for living patients was performed. Progression free survival (PFS) and overall survival (OS) were calculated for all patients. PFS was calculated from the day of diagnosis until the day of recurrence or death (whichever was sooner). OS was calculated from the day of diagnosis until the day of death or the day of the last follow up. The use of patient material was approved by the local ethical committee.

Patient characteristics are presented in **Table 3.4**.

Table 3.4. Overview of patient characteristics.

Variable	Statistical test	Results
Age, years	Median (P ₂₅ , P ₇₅)	65 (57-70)
Sarcoma subtype	N (%)	
LMS		24 (33.8)
CS		38 (53.5)
US		9 (12.7)
Stage <small>(see Appendix 1)</small>	N (%)	
I		30 (42.2)
II		8 (11.3)
III		13 (18.3)
IV		20 (28.2)
Tumor size (cm)	Median (P ₂₅ , P ₇₅)	
50 completes cases		8.5 (5.0-12.0)
multiple imputation **		8.6 (5.2-12.2)
Follow up time, months	Median (range)	66 (0.5-289)
Recurrent disease	N (%)	
No		16 (22.5)
Yes		55 (77.5)
Death	N (%)	
No		24 (33.8)
Yes		47 (66.2)
PFS	Median survival time	9 months (95% CI, 7-13)
OS	Median survival time	22 months (95% CI, 15-30)
	Five-year survival	32%

P₂₅, P₂₇: the 25th and 75th percentile, i.e. the lower and upper quartile

N: number, LMS: leiomyosarcoma, CS: carcinosarcoma, US: undifferentiated sarcoma, PFS: progression free survival, OS: overall survival, MI: multiple imputation

Appendix 1 : FIGO classification with corresponding TNM classification

FIGO ⁸	TNM	T	Tumor
I	T1N0M0	1	Tumor confined to corpus uteri
II	T2N0M0	2	Tumor invades cervix but does not extend beyond uterus [for CS] <i>or</i> the tumor extends to the pelvis [for LMS and US]
III	T3N0M0 T3N1M0 T1N1M0 T2N1M0	3	Tumor spreads locally (serosa, adnexa, ascites, vagina) [for CS] <i>or</i> the tumor invades the abdomen with exclusion of bladder and rectum [for LMS and US]
IV	T4N0M0 T4N1M0 T4N0M1 T4N1M1 T1N0M1 T1N1M1 T2N0M1 T2N1M1 T3N0M1 T3N1M1	4	Tumor invades bladder mucosa and/or bowel mucosa
		N	Lymph nodes
		0	No regional lymph node metastasis
		1	Metastasis in pelvic and/or para-aortic lymph nodes
		M	Metastasis
		0	No distant metastasis
		1	Distant metastasis

FIGO : International Federation of Gynecology and Obstetrics

TNM : TNM (tumor_nodes_metastasis) classification of malignant tumors

Immunohistochemistry

WT1 staining was performed and interpreted semiquantitatively as described previously (7). In brief, paraffin-embedded tissue slices were stained with monoclonal mouse anti-human Wilms' Tumor 1 [Clone 6F-H2 (Dakocytomation, Carpinteria, California)] at a dilution of 1:400. Horseradish peroxidase labelled dextran polymer coupled to anti-mouse secondary antibody (Envion+System Labelled Polymer- HRP anti-mouse, Dakocytomation, Carpinteria, California) was used as a secondary antibody. One tissue slide was evaluated for each patient. A score for each slide was calculated by multiplying the percentage and intensity of positive cells and then categorised as negative (0 - 20), weak (21 - 80), moderate (81 - 180) and strong (181 - 300). Examination of immunohistochemical staining was performed by 2 investigators (A C en Ph M).

Depending on their availability, some samples were simultaneously stained for estrogen (ER) and progesterone (PgR) receptor positivity (CS (n = 29), LMS (n = 17) and US (n = 6)). Staining protocol for ER and PgR was as follows. Endogenous peroxidase activity was blocked by 0.5% H₂O₂ in methanol. Antigen retrieval treatment was carried out for 2 hours at 90°C in a Tris-HCL buffer at pH 9 with 1 mM EDTA (for ER) and in a citrate buffer at pH 6 (for PgR). Slides were then cooled in warm and cold water baths. Non specific binding was blocked before monoclonal mouse anti-human ERαclone 1D5 (Dakocytomation, Carpinteria, California – dilution 1:50) and monoclonal mouse anti-human PgR clone 16 (Novocastra Reagents, Zaventem, Belgium – dilution 1:40) were used as antibody against ER and PgR respectively.

Slides were incubated with the antibodies overnight at 4°C. After washing, slides were incubated for another 30 minutes with Peroxidase- conjugate Goat anti-Mouse (Jackson Immuno Research Laboratories, Suffolk, UK). Staining was performed using DAB as chromogen, followed by Maeyercounterstaining. Scoring was based on a recent publication by O’Cearbhaill(9). Tumors with less than 10% ER and PgR nuclear positivity were scored as negative. Tumors with more than 10% staining were scored as positive.

Statistical analysis

Descriptive statistics were based on medians, 25th and 75th percentiles, numbers and percentages. Patient, tumor and survival characteristics for patients with a WT1 positive and negative tumors were described using the same statistics, and were compared using Mann-Whitney (10), Fisher’s exact (10), and log rank tests (11), as appropriate. The median follow-up time in the study was computed using the reverse Kaplan-Meier estimator(12,13). Univariate analysis was performed using median survival times, Kaplan–Meier curves with log rank tests (11), and hazard ratios. The median survival time represents the number of months within which 50% of the patients experienced the event of interest (disease progression or death). The hazard ratio (HR) estimates the change in hazard of recurrence/death of disease for a unit increase in the predictor. Multivariable analysis was performed using Cox regression (11). The following prognostic markers were evaluated: stage (I to IV), age (years), size of the tumor (cm) and sarcoma subtype (CS versus LMS/US). The strength of the effects were described using the HR with 95% confidence interval (CI). The proportional hazards assumption was investigated using Schoenfeld residual plots, and for continuous predictors the linearity assumption was investigated using Martingale residuals (14).

Tumor size was missing for 21 patients (30%). Using multiple imputation (MI) (14,16): 100 imputations were created using predictive mean matching (PMM) regression (17) with age, sarcoma subtype, stage, WT1 expression, length of follow-up, censoring (i.e. whether the event of interest, recurrence or death, has taken place or not), and the interaction between follow-up and censoring as predictors. This resulted in 100 completed data sets. Analyses were performed on each completed data set and the results were combined to obtain the final results, according to standard MI procedure (Rubin’s rules). The MI of size was done separately for the PFS and OS analyses.

All statistical analyses were carried using SAS version 9.1.3 (SAS Institute, Cary, USA).

Results

Descriptive statistics of the patients are presented in **Table 3.4**. The median survival time was 9 months (95% CI, 7 to 13) for PFS and 22 months (95% CI, 15 to 30) for OS . Using life-table analysis, the five-year OS was 32%. Five-year OS was 61% in patients with a stage I sarcoma, in contrast to only 10% in patients with higher stage sarcomas (see **Figure 3.3**. for the Kaplan-Meier curves).

Figure 3.3. Survival curves of high grade uterine sarcoma according to stage. A. Progression free survival. B. Overall survival. [Stage I (n=30), stage II-IV (n=41)]

The characteristics of WT1 positive versus negative tumors are presented in Table 3.5.. Patients age and tumor size were similar in both groups. WT1 positive patients had higher stages and worse prognosis for PFS and OS than WT1 negative patients (see **Figure 3.4.** for Kaplan-Meier curves). The HR of WT1 positivity was 2.44 (95% CI, 1.34 to 4.71) for PFS and 2.48 (95% CI, 1.26 to 4.90) for OS. Patients with absence of WT1 in the tumor had a five-year overall survival of 55%, in contrast to 18% for patients with a WT1 positive tumor.

Table 3.5.Characteristics of WT1 positive and negative tumors.

Variable	Statistical test	WT1 positive tumors N=49 (69%)	WT1 negative tumors N=22 (31%)	p-value
Age, years	Median (P_{25} , P_{75})	65 (57, 73)	65 (57, 69)	0.642 ^a
Sarcoma subtype	N (%)			0.343 ^b
LMS		19 (39)	5 (23)	
CS		25 (51)	13 (59)	
US		5 (10)	4 (18)	
Stage	N (%)			0.174 ^b
I		18 (37)	13 (59)	
II-IV		31 (63)	9 (41)	
Size of tumor	Median (P_{25} , P_{75})			0.715 ^a
Complete cases		9.0 (5.5,12.1)	8.5 (5.0,12.0)	
Multiple imputation		8.9 (5.7,12.2)	8.2 (4.5,12.2)	
PFS	Median survival time	7 months	29 months	0.004 ^c
OS	Median survival time	16 months	64 months	0.007 ^c
	Five-year survival	18%	55%	

^a Mann-Whitney test; ^b Fisher's exact test; ^c logrank test for Kaplan-Meier curves

P_{25} , P_{75} : the 25th and 75th percentile, i.e. the lower and upper quartile

N: number, LMS: leiomyosarcoma, CS: carcinosarcoma, US: undifferentiated sarcoma, PFS: progression free survival, OS: overall survival, MI : multiple imputation

Figure 3.4. Survival curves of WT1 positive versus WT1 negative high grade uterine sarcoma. A. Progression free survival. B. Overall survival. [WT1 positive tumors (n=49), WT1 negative tumors (n=22)]

Results of the multivariate Cox regression analysis are shown in **Table 3.6.** Based on the Kaplan-Meier curves, stage was modeled as I versus II-IV. The Cox regression model showed

independent prognostic effects of stage and WT1 for both PFS and OS. For recurrence of disease (PFS), the HRs of WT1 and stage were 2.17 (95% CI = 1.11 to 4.25, $p = 0.023$) and 3.13 (95% CI = 1.58 to 6.19, $p = 0.001$) respectively. For death from the disease, the HRs of WT1 and stage were 2.47 (95% CI = 1.13 to 5.41, $p = 0.024$) and 3.50 (95% CI = 1.62 to 7.59, $p = 0.002$) respectively.

No strong evidence for violations of the linearity and proportional hazards assumptions were found. Since CS are histologically classified as endometrial carcinomas (4) we checked interaction between WT1 status and tumor subtype. The interaction between WT1 status and CS was weak (LR p -value = 0.486). If included in the multivariate model, for PFS, the HR of WT1 for CS tumors was 1.73, whereas for other tumors it was 2.74, suggesting a weaker effect for CS tumors. For OS, the same was true : the interaction between WT1 status and CS was weak ($p = 0.296$). If included in the multivariate model, the HR of WT1 for CS tumors was 1.79 whereas for other tumors it was 3.98, again suggesting a weaker effect for CS tumors. Due to the weak strength of these interactions, these observations are merely trends.

The majority of investigated samples were negative for ER and PgR. For PgR, 10/17 (59%) of LMS, 6/6 (100%) of US and 19/29 (65.5%) of CS were negative. For ER 6/15 (40%) of LMS, 5/6 (83%) of US and 16/29 (55%) of CS were negative. This contrasts to a 69% WT1 positivity in all investigated uterine sarcoma samples. Indeed, 62% and 61% of high grade uterine sarcoma (LMS and US) showed an inverse result for WT1 and ER/PgR. For CS, the percentages were even higher: 66% and 69% of samples were positive for WT1 and negative for ER and PgR, or vice versa.

Table 3.6. Multivariate Cox regression analysis, after multiple imputation (n=71)

A. Progression free survival.

Variable	Hazard ratio	95% CI	p-value
WT1 status (+ vs -)	2.17	1.11 – 4.25	0.023
Tumor (CS vs other)	0.47	0.22 – 1.00	0.050
Stage (I vs II-IV)	3.13	1.58 – 6.19	0.001
Age (per 10 years)	1.15	0.86 – 1.55	0.337
Size (cm)	1.00	0.93 – 1.07	0.966

B. Overall survival

Variable	Hazard ratio	95% CI	p-value
WT1 status (+ vs -)	2.47	1.13 – 5.41	0.024
Tumor (CS vs other)	0.69	0.29 – 1.63	0.398
Stage (I vs II-IV)	3.50	1.62 – 7.59	0.002
Age (per 10 years)	1.27	0.91 – 1.77	0.156
Size (cm)	1.04	0.96 – 1.12	0.316

CS :carcinosarcoma, other include leiomyosarcoma and undifferentiated sarcoma

CI : confidence interval

Discussion

In this study, we investigated the prognostic significance of WT1 positivity in different types of high grade uterine sarcoma. Univariate analysis suggests a worse prognosis if the tumor

was positive for WT1. Multivariate analysis, combining WT1 with age, sarcoma subtype, size and stage, WT1 is still an independent negative prognostic factor. These findings confirm the biological and clinical importance of WT1 in uterine sarcomas.

Similar observations for a role of WT1 in cancer biology have been made in breast cancer (18), leukemia (19) and hepatocellular carcinoma (20). No studies have investigated possible molecular mechanisms which might explain the correlation between WT1 and prognosis, but some hypotheses have been raised. For hepatocellular carcinoma Sera *et al.* (20) speculated that WT1 over expression might result from Hepatocyte Nuclear Factor 4 suppression, which has been proven to be associated with tumor progression. However, the authors also postulated a mechanism via p53 and Type I Insulin-like Growth Factor Receptor. Inoue *et al.* (19) reported that WT1 increase was the highest in rapidly proliferating immature hematopoietic cells from which leukemic cells are derived.

Twenty-six studies, including at least 40 patients each have been carried out looking for prognostic factors in uterine sarcoma. From these studies it appears that stage, age, grade, menopausal status, subtype, size, mitotic index and adjuvant therapy are the most commonly studied prognostic factors. Two studies are highlighted here because of their large patient numbers. Kapp and colleagues (21) recently described the prognosis of 1396 patients with LMS. According to their multivariate analysis age, stage and grade were independent prognostic variables, together with race and primary surgery. Nemani and colleagues (22) described prognostic variables in 1855 patients with CS. They confirmed age and stage to be independent prognostic factors, together with race, lymphadenectomy and marital status.

Generally, LMS are believed to have a worse prognosis than CS. However, Rovirosa *et al.* (23) reviewed this and concluded, based on three studies, together including 303 patients, the prognosis of LMS and CS were equivalent. In contrast, two large studies of altogether 820 patients, attributed a worse prognosis to LMS. In our smaller study, no clear differences in prognosis were found between LMS and CS. However, since the histopathological origin of both sarcomas is different (4), we looked into a possible interaction between WT1 and sarcoma subtype. The prognostic effect of WT1 in CS seemed smaller than in the high grade mesenchymal tumors, though the small sample size of both subgroups may not be representative.

In the multivariate analysis, we have decided not to include mitotic index, based on the publication of Bell *et al.* (24) in 1994, proving that mitotic index is a poor predictor of prognosis when used as a univariate predictor. Grade was not included in the analysis because all sarcomas were high grade. Race could not be included in our study, since all our patients were Caucasian. Adjuvant therapy was also excluded as a prognostic factor in our multivariate analysis. Recent work from the EORTC group revealed no effect of post-operative radiotherapy on PFS and OS in patients with LMS or CS (25). Previously it has been shown that adjuvant chemotherapy does not result in a survival benefit (26). We also decided not to include lymph node status as a separate prognostic factor. In LMS, surgical exploration does not include lymphadenectomy (27), whereas for CS, a positive nodal status is reflected in tumor stage. Finally, we also excluded menopausal status as a separate prognostic factor, because we already included age as a continuous variable. Ultimately, we

included, besides WT1 expression, age, stage (I *versus* II-IV), subtype (CS *versus* LMS/US) and size in our multivariate analysis.

In spite of a relatively small number of patients, we were able to demonstrate that WT1 is an independent predictor of poor prognosis.

The inverse correlation between WT1 and ER/PgR expression stresses the negative influence of WT1 on prognosis, since ER and PgR are associated with better differentiation and overall a more indolent behavior. However, this is in contrast to the findings of Carvalho *et al.* (28). They assessed 30 uterine LMS and found only 23% were positive for WT1 and the majority was positive for ER and PgR. The discrepancy in WT1 positivity may in part be explained by the recording of WT1 nuclear staining, whereas it has been largely accepted now that WT1 cytoplasmic expression is also true expression.

High grade uterine sarcoma are resistant to standard treatment (27), therefore new treatment options need to be explored. Immunohistochemical studies on the presence of Platelet Derived Growth Factor Receptor α (PDGFR α), Human Epidermal Growth Factor Receptor 2 (HER-2/NEU), ER, PgR,... have been conducted recently (for overview see (27)). New treatment options which target these molecules have been suggested. Our data demonstrate the independent negative prognostic effect of WT1, suggesting the importance of WT1 in the tumor biology of high grade uterine sarcoma. Therefore, targeted treatment against WT1 might be a valuable option. Immunotherapy could offer a solution, since WT1 has presented itself as a highly immunogenic target (29). Immunotherapy in general has proven benefit. WT1 peptide immunotherapy has been effective in malignant glioma and lung (30). Dendritic cell-based immunotherapy has the potential to directly eliminate tumor cells or to influence their biological behavior. Our findings confirm the role of WT1 in uterine sarcoma tumor biology and support the ongoing investigation of WT1 immunotherapy in uterine sarcomas.

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