Wilms Tumor Gene 1 (WT1) is a Prognostic Marker in High-Grade Uterine Sarcoma

An Coosemans, MD,†‡ Ben Van Calster, PhD,§ Godelieve Verbist,* Philippe Moerman, MD, PhD,|| Ignace Vergote, MD, PhD,*‡ Stefaan W. Van Gool, MD, PhD,†‡ and Frédéric Amant, MD, PhD*‡*

Introduction: *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 [n = 24], undifferentiated sarcoma [n = 9]), and carcinosarcoma (n = 38) was performed. Patients were followed up for at least 12 months. *Wilms tumor gene 1* expression was determined by immunohistochemistry. Data on recurrence (progression-free survival) and overall survival (OS) were available for all patients. Univariate and multivariate analyses 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. Wilms tumor gene 1 was a predictor of survival in the univariate analysis: the hazard ratio of WT1 positivity was 2.44 (95% confidence interval, 1.34–4.71) for progression-free survival and 2.48 (95% confidence interval, 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.

Key Words: *Wilms tumor gene 1, WT1*, Uterine sarcoma, Prognosis

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Wilms tumor gene 1 (WT1) is located on chromosome 11p13. It has many molecular functions, which are partially explained by different splicings of WT1 RNA.[1] The 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] whereas its overexpression in several malignancies suggests a role in tumorigenesis.[2]

Uterine sarcomas are relatively uncommon but encompass leiomyosarcoma (LMS), carcinosarcoma (CS), endometrial stromal sarcoma (ESS), and undifferentiated sarcomas (US), according to traditional classification systems.[3] Recent textbooks classify CS as a subtype of endometrial carcinoma rather than as 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 5-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 overexpression in the different subtypes of uterine sarcoma.[7] Wilms tumor gene 1 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. Endometrial stromal sarcomas were excluded because they are low-grade sarcomas with an indolent behavior. Carcinosarcomas were included because of their poor prognosis, which seems to be related to the sarcoma component. We hope to highlight the potential clinical use of WT1 as a target for immunotherapy.

*Laboratory of Experimental Gynecology, †Laboratory of Experimental Immunology, Departments of ‡Child and Women, §Electrical Engineering (ESAT), and ||Human Pathology, UZ Gasthuisberg, Katholieke Universiteit Leuven, Leuven, Belgium.
Address correspondence and reprint requests to Frédéric Amant, MD, PhD, Department of Gynaecologic Oncology, University Hospital Leuven, Herestraat 49, 3000 Leuven, Belgium.
E-mail: frederic.amant@uzleuven.ac.be.

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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. Progression-free survival was calculated from the day of diagnosis until the day of recurrence or death (whichever was sooner). Overall survival was calculated from the day of diagnosis until the day of death or the day of the last follow-up. The use of patients in this study was approved by the local ethical committee.

Patients' characteristics are presented in Table 1.

Immunohistochemistry

Wilms tumor gene 1 staining was performed and interpreted semiquantitatively as described previously.[7] In brief,

TABLE 1. Overview of patients' characteristics

Variable	Statistical Test	Results
Age, yrs	Median (P_{25}, P_{75})	65 (57–70)
Sarcoma Subtype	n (%)	
LMS		24 (33.8)
CS		38 (53.5)
US		9 (12.7)
Stage (see Appendix 1)	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)
MI*		8.6 (5.2–12.2)
Follow-up Time, mo	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 mo (95% CI, 7–13)
OS	Median survival time	22 mo (95% CI, 15–30)
	Five-year survival	32%

P_{25}, P_{75}: 25th and 75th percentile, ie, the lower and upper quartile.
*The missing values (n = 21 [30%]) were imputed using MI: 100 imputations were created using predictive mean matching regression with $k = 5$ and age, sarcoma subtype, stage (I vs rest), WT1 expression, length of follow-up, censoring, and the interaction between follow-up and censoring as predictors. This results in 100 complete data sets. Analyses were performed on each complete data set, and the results were combined to obtain the final results according to standard MI procedure. The MI of size was done separately for the PFS and OS analyses (thus, each time, 100 complete data sets were produced).
LMS, leiomyosarcoma; CS, carcinosarcoma; US, undifferentiated sarcoma; PFS, progression free survival; OS, overall survival; MI, multiple imputation.

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paraffin-embedded tissue slices were stained with monoclonal mouse antihuman Wilms tumor 1 (Clone 6F-H2; Dakocytomation, Carpinteria, Calif) at a dilution of 1:400. Horseradish peroxidase–labeled dextran polymer coupled to antimouse secondary antibody (EnVision+ System Labeled Polymer-HRP antimouse, Dakocytomation) 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 categorized 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. and P.M.).

Depending on their availability, some samples were simultaneously stained for estrogen (ER) and progesterone receptor (PR) positivity (CS [n = 29], LMS [n = 17], and US [n = 6]). The staining protocol for ER and PR was as follows. Endogenous peroxidase activity was blocked by 0.5% hydrogen peroxide in methanol. Antigen retrieval treatment was carried out for 2 hours at 90°C in a Tris-HCl buffer at pH 9 with 1 mmol/L EDTA (for ER) and in a citrate buffer at pH 6 (for PR). Slides were then cooled in warm and cold water baths. Nonspecific binding was blocked before monoclonal mouse antihuman ERα clone 1D5 (dilution, 1:50; Dakocytomation) and monoclonal mouse antihuman PR clone 16 (dilution, 1:40; Novocastra Reagents, Zaventem, Belgium) were used as antibody against ER and PR, respectively. Slides were incubated with the antibodies overnight at 4°C. After washing, slides were incubated for another 30 minutes with peroxidase- conjugated goat antimouse (Jackson ImmunoResearch Laboratories, Suffolk, UK). Staining was performed using 3,3'-diaminobenzidine as chromogen, followed by Mayer counterstaining. Scoring was based on a recent publication by O'Cearbhaill.[8] Tumors with less than 10% ER and PR 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 WT1-negative tumors were described using the same statistics and were compared using the Mann-Whitney *U* test,[9] the Fisher exact test,* or the log-rank test[10] as appropriate. The median follow-up time in the study was computed using the reverse Kaplan-Meier estimator.[11,12] Univariate analysis was performed using median survival time, Kaplan-Meier curve with the log-rank test,[10] and hazard ratios (HRs). 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 HR estimates the change in hazard of recurrence/death of disease for a unit increase in the predictor. Multivariable analysis was performed using the Cox proportional hazards regression model.[10] The following prognostic markers were evaluated: stage (I–IV), age (years), size of the tumor (centimeter), and sarcoma subtype (CS vs LMS/US). The strength of the effects was 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.[13]

Tumor size was missing for 21 patients (30%). Using multiple imputation (MI),[14,15] 100 imputations were created using predictive mean matching regression[16] with age, sarcoma subtype, stage, WT1 expression, length of follow-up, censoring (ie, 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 rules). The MI of size was done separately for the PFS and OS analyses.

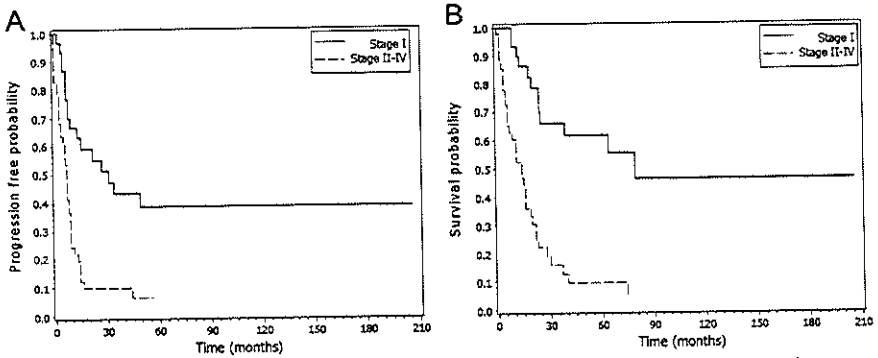


FIGURE 1. Survival curves of high-grade uterine sarcoma according to stage. A, Progression-free survival. B, Overall survival. (Stage I [n = 30], stages II–IV [n = 41]).

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TABLE 2. Characteristics of WT1-positive and WT1-negative tumors

Variable	Statistical Test	WT1-Positive Tumors n = 49 (69%)	WT1-Negative Tumors n = 22 (31%)	P
Age, yrs	Median (P_{25}, P_{75})	65 (57, 73)	65 (57, 69)	0.642*
Sarcoma Subtype	n (%)			0.343†
LMS		19 (39)	5 (23)	
CS		25 (51)	13 (59)	
US		5 (10)	4 (18)	
Stage	n (%)			0.174†
I		18 (37)	13 (59)	
II–IV		31 (63)	9 (41)	
Size of Tumor	Median (P_{25}, P_{75})			0.715*
Complete cases		9.0 (5.5, 12.1)	8.5 (5.0, 12.0)	
MI		8.9 (5.7, 12.2)	8.2 (4.5, 12.2)	
PFS	Median survival time	7 mo	29 mo	0.004‡
OS	Median survival time	16 mo	64 mo	0.007‡
	Five-year survival	18%	55%	

P_{25}, P_{27}: the 25th and 75th percentiles, ie, the lower and upper quartiles.
*Mann-Whitney *U* test.
†Fisher's exact test.
‡Log rank test for Kaplan-Meier curves.
LMS, leiomyosarcoma; CS, carcinosarcoma; US, undifferentiated sarcoma; PFS, progression free survival; OS, overall survival; MI, multiple imputation.

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

RESULTS

Descriptive statistics of the patients are presented in Table 1. The median survival time was 9 months (95% CI, 7–13) for PFS and 22 months (95% CI, 15–30) for OS . Using life-table analysis, the 5-year OS was 32%. The 5-year OS was 61% in patients with a stage I sarcoma in contrast to only 10% in patients with higher-stage sarcomas (Fig. 1, Kaplan-Meier curves).

The characteristics of WT1-positive versus WT1-negative tumors are presented in Table 2. The patients' age and tumor size were similar in both groups. The WT1-positive patients had higher stages and worse prognosis for

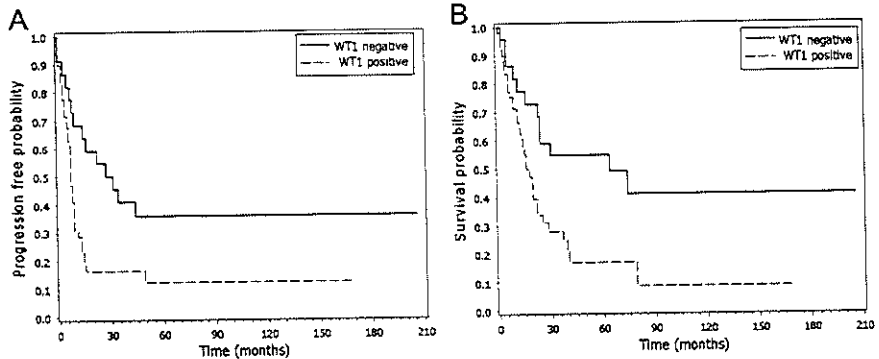


FIGURE 2. Survival curves of WT1-positive versus WT1-negative high-grade uterine sarcoma. A, Progression-free survival. B, Overall survival. (WT1-positive tumors [n = 49], WT1-negative tumors [n = 22]).

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PFS and OS than the WT1-negative patients (Fig. 2, Kaplan-Meier curves). The HR 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. Patients without WT1 in the tumor had a 5-year OS of 55% in contrast to 18% for patients with a WT1-positive tumor.

Results of the multivariate Cox regression analysis are shown in Table 3. 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, 1.11–4.25; $P = 0.023$) and 3.13 (95% CI, 1.58–6.19; $P = 0.001$), respectively. For death from the disease, the HRs of WT1 and stage were 2.47 (95% CI, 1.13–5.41; $P = 0.024$) and 3.50 (95% CI, 1.62–7.59; $P = 0.002$), respectively. No strong evidence for violations of the linearity and proportional hazards assumptions were found. Inasmuch as CSs are histologically classified as endometrial carcinomas,[4] we checked the interaction between WT1 status and tumor subtype. The interaction between WT1 status and CS was weak ($P = 0.486$). 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. Because of the weak strength of these interactions, these observations are merely trends.

Most of the investigated samples were negative for ER and PR. For PR, 10 (59%) of 17 LMSs, 6 (100%) of 6 USs, and 19 (65.5%) of 29 CSs were negative. For ER, 6 (40%) of 15 LMSs, 5 (83%) of 6 USs, and 16 (55%) of 29 CSs were negative. This contrasts to the 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/PR. For CS, the percentages were even higher: 66% and 69% of samples were positive for WT1 and negative for ER and PR.

TABLE 3. Multivariate Cox regression analysis, after MI (n = 71)

Progression-Free Survival	Hazard Ratio	95% CI	*P*
Variable			
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 yrs)	1.15	0.86–1.55	0.337
Size, cm	1.00	0.93–1.07	0.966
Overall survival			
Variable			
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 yrs)	1.27	0.91–1.77	0.156
Size, cm	1.04	0.96–1.12	0.316

CS, carcinosarcoma.
"Other" includes leiomyosarcoma and undifferentiated sarcoma.

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, revealed that 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,[17] leukemia,[18] and hepatocellular carcinoma.[19] No studies have investigated possible molecular mechanisms that might explain the correlation between WT1 and prognosis, but some hypotheses have been raised. For hepatocellular carcinoma, Sera et al[19] speculated that WT1 overexpression 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 insulinlike growth factor receptor. Inoue et al[18] 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 seems 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 et al[20] 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 et al[21] 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, LMSs are believed to have a worse prognosis than CS. However, Rovirosa et al[22] reviewed this and concluded, based on 3 studies, together including 303 patients, that the prognosis of LMS and CS were equivalent. In contrast, 2 large studies of 820 patients altogether attributed a worse prognosis to LMS. In our smaller study, no clear differences in prognosis were found between LMS and CS. However, because 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, although 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 work of Bell et al[23] 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 because all our patients were Caucasian. Adjuvant therapy was also excluded as a prognostic factor in our multivariate analysis. Recent work from the European Organisation for Research and Treatment of Cancer group revealed no effect of postoperative radiotherapy on PFS and OS in patients with LMS or CS.[24] Previously, it has been shown that adjuvant chemotherapy does not result in a survival benefit.[25] We also decided not to include lymph node status as a separate prognostic factor. In LMS, surgical exploration does not include lymphadenectomy,[26] 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 vs II–IV), subtype (CS vs LMS/US), and size in our multivariate analysis.

Despite 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/PR expression stresses the negative influence of WT1 on prognosis because ER and PR are associated with better differentiation and, overall, a more indolent behavior. However, this is in contrast to the findings of Carvalho et al.[27] They assessed 30 uterine LMS and found that only 23% were positive for WT1 and most were positive for ER and PR. 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 a true expression.

High-grade uterine sarcoma are resistant to standard treatment,[26] therefore, new treatment options need to be explored. Immunohistochemical studies on the presence of platelet-derived growth factor receptor α (PDGFRα), human epidermal growth factor receptor 2 (HER-2/NEU), ER, and PR have been conducted recently (for overview, see Amant et al[26]). 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 inasmuch as WT1 has presented itself as a highly immunogenic target.[28] Immunotherapy in general has proven benefit. Wilms tumor gene 1 peptide immunotherapy has been effective in malignant glioma and lung carcinoma.[29] 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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Appendix 1: FIGO classification with corresponding TNM classification

FIGO[a]	TNM	T	Tumor
I	T1 N0 M0	1	Tumor confined to corpus uteri
II	T2 N0 M0	2	Tumor invades cervix but does not extend beyond uterus (for CS) *or* the tumor extends to the pelvis (for LMS and US)
III	T3 N0 M0		
	T3 N1 M0		
	T1 N1 M0	3	Tumor spreads locally (serosa, adnexa, ascites, and vagina) (for CS) *or* the tumor invades the abdomen with exclusion of bladder and rectum (for LMS and US)
	T2 N1 M0		
IV	T4 N0 M0		
	T4 N1 M0		
	T4 N0 M1	4	Tumor invades bladder mucosa and/or bowel mucosa
	T4 N1 M1		
	T1 N0 M1	N	Lymph nodes
	T1 N1 M1	0	No regional lymph node metastasis
	T2 N0 M1	1	Metastasis in pelvic and/or para-aortic lymph nodes
	T2 N1 M1		
	T3 N0 M1	M	Metastasis
	T3 N1 M1	0	No distant metastasis
		1	Distant metastasis

FIGO, International Federation of Gynecology and Obstetrics; TNM (tumor_nodes_metastasis), classification of malignant tumors.

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Wilms' tumor gene 1 (WT1) is a prognostic marker in high grade uterine sarcoma

A. Coosemans,MD[1,2,3*], B. Van Calster,PhD[4**], G. Verbist[1], Ph. Moerman,MD,PhD[5], I. Vergote,MD,PhD[1,3], S.W. Van Gool,MD,PhD[2,3***], F. Amant,MD,PhD[1,3***]

[1] Laboratory of Experimental Gynecology, UZ Gasthuisberg, Katholieke Universiteit Leuven, Belgium

[2] Laboratory of Experimental Immunology, UZ Gasthuisberg, Katholieke Universiteit Leuven, Belgium

[3] Department of Child and Women, UZ Gasthuisberg, Katholieke Universiteit Leuven, Belgium

[4] Department of Electrical Engineering (ESAT), Katholieke Universiteit Leuven, Belgium

[5] Department of Human Pathology, UZ Gasthuisberg, Katholieke Universiteit Leuven, Belgium

* A Coosemans is Ph.D. fellow of the Research Foundation - Flanders (FWO)

** BVC is a postdoctoral researcher of the Research Foundation – Flanders (FWO)

*** SWVG and FA are Senior Clinical Investigators of the Research Foundation – Flanders (FWO)

Corresponding author

Prof. Dr. Frédéric Amant

UZ Gasthuisberg

Department of gynecologic oncology

Herestraat 49

3000 Leuven

Belgium

Phone : +32 16344252 - Fax : +32 16344629 - Email : frederic.amant@uz.kuleuven.ac.be

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) and 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: Fourty-nine (69%) tumors were WT1 positive. Fourty-seven (66%) patients died of the disease, with a median OS time of 22 months. WT1 was a significant predictor of survival in the univariate analyses: median PFS (OS) survival time was 7 (16) months for WT1 positive tumors and 29 (64) months for WT1 negative tumors ($p = 0.004$ and 0.007 respectively). Multivariate analysis including stage, age, tumor size and sarcoma subtype, identified only stage and WT1 positivity as strong 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 [2-5] while its overexpression in several malignancies suggests a role in tumorigenesis [6].

Uterine sarcomas are relatively uncommon but encompass leiomyosarcoma (LMS), carcinosarcoma (CS), endometrial stromal sarcoma (ESS) and undifferentiated sarcomas (US), according to traditional classification systems [7,8]. Recent textbooks classify CS as a subtype of endometrial carcinoma rather than a uterine sarcoma [9]. However, the sarcoma component is believed to be less sensitive to systemic treatment modalities and therefore might be likely to determine prognosis [10,11]. Current treatment options for uterine sarcoma are limited resulting in an impaired prognosis. Five year survival is as low as 50% for stage I, and only 10% in more advanced stages. Stage [12,13] is hitherto the most important prognostic factor.

Recently, we demonstrated WT1 overexpression in the different subtypes of uterine sarcoma [14]. 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 to the clinical outcome. ESS were excluded because they are low grade sarcomas with an indolent behavior [15]. CS were included because of the determined role of the sarcoma component.

Materials and methods

Study population

A retrospective clinical and histopathological review of 71 women with the diagnosis of high grade uterine sarcoma or carcinosarcoma and a follow up of at least 12 months was performed. Of all patients progression free survival (PFS) and overall survival (OS) were calculated. PFS was calculated from the day of diagnosis till the day of recurrence or death (whichever occurred first). OS was calculated from the day of diagnosis till the day of death or the day of the last follow up.

Patient characteristics are presented in Table 1.

Immunohistochemistry

WT1 staining was performed and interpreted semiquantitatively as described earlier [14]. 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 secondary antibody. Of each patient sample, one tissue slide was evaluated. A scoring system was based on the multiplication of percentage and intensity of positive cells, being 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).

Part of the samples, based on their availability, were simultaneously stained for estrogen (ER) and progesterone (PR) receptor positivity (CS (n=29), LMS (n=17) and US (n=6)).
Protocol nog te opmaken : bij lieve langsgaan
The scoring was based on a recent publication of O'Cearbhaill [17]. Tumors with up to 10% of ER and PR nuclear positivity were scored as negative. Tumors with more than 10% staining were scored as positive.

Statistical analysis

Univariate analysis was performed using median survival times, Kaplan-Meier curves with logrank tests, and hazard ratios. The median survival time represents the number of months within which 50% of the patients experienced the event of interest (i.e. disease progression or death). The HR estimates the change in hazard of recurrence/death of disease for a unit increase in the predictor. Next, a multivariate analysis was performed using Cox regression. The following prognostic markers were evaluated: stage (I to IV), age (years), size of the tumor (cm) and sarcoma subtype (LMS, CS, US). The strength of

the effects was described using the hazard ratio (HR) with its 95% confidence interval (CI) and likelihood ratio p-value. The proportional hazards assumption was investigated using Schoenfeld residual plots, and for continuous predictors the linearity assumption was investigated using Martingale residuals [18].

Tumor size was missing for 21 patients (30%), using multiple imputation (MI) [19,20]: 100 imputations were created using predictive mean matching (PMM) [21] regression with $k=5$ and age, sarcoma subtype, stage (I vs rest), WT1 expression, length of follow-up, censoring, and the interaction between follow-up and censoring (i.e. whether the event of interest, recurrence or death, has taken place or not) as predictors. This results in 100 complete data sets. Analyses were performed on each complete data set and the results are combined to obtain the final results, according to standard MI procedure. The MI of size was done separately for the PFS and OS analyses (thus, each time 100 complete data sets were produced).

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

Results

Descriptive statistics of the patients are presented in Table 1. The median survival time was 9 months (95% CI, 7-13) for PFS and 22 months (95% CI, 15-30) for OS . Using life-table analysis, the five-year OS was 32%. Five-year OS was 70% in patients with a

stage I sarcoma, in contrast to only 11% in patients with higher stage sarcomas (see Figure 1 for the Kaplan-Meier curves).

The characteristics of WT1 positive versus negative tumors are presented in Table 2. Patients age and tumor size were similar in both groups. Interestingly, WT1 positive patients had higher stages and worse prognosis for PFS (logrank p = 0.004) and OS (logrank p = 0.007) than WT1 negative patients (Figure 2). 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. Patients with absence of WT1 in the tumor had a five-year overall survival of 60%, while it was only 11% for patients with a WT1 positive tumor. PFS and OS were also negatively correlated with the WT1 score (negative vs weak vs strong), as shown in Figure 3.

Results of the multivariate Cox regression analysis are shown in Table 3. Based on the univariate analysis Kaplan-Meier curves, stage was modeled as I versus II-IV. The Cox regression model resulted in strong 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 = 1.1 to 4.25, p = 0.023) and 3.13 (95% CI = 1.58 to 6.19, p = 0.001) respectively. For death of 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 was found. Since CS are histologically classified as endometrial carcinomas [9] we checked interaction between WT1 status and tumor subtype. The interaction between WT1 status

and CS was weak (LR p-value = 0.486). If it is included in the multivariate model, for PFS the HR of WT1 for CS tumors is 1.73 (0.70-4.25) whereas for other tumors it is 2.74 (1.05-7.16), 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 is 1.79 (0.70-4.59) whereas for other tumors it is 3.98 (1.14-14.0), suggesting a weaker effect for CS tumors. Same comments as for the recurrence analysis. However, since sample sizes become smaller by making subgroups, it is not certain that the effect really is weaker.

The majority of investigated samples were negative for ER and PR : For PR, 10/17 (59%) of LMS, 6/6 (100%) of US and 19/29 (65.5%) of CS were negative, whereas this was the case in 6/15 (40%) of LMS, 5/6 (83%) of US and 16/29 (55%) of CS for ER. This in in contrast to the a 69% WT1 positivity in all investigated uterine sarcoma samples. Indeed, 62%/61% of high grade uterine sarcoma (LMS and US) showed an inverse result for WT1 and ER/PR. This was even more pronounced in CS : 66% for ER and 69% for PR.

Discussion

In this study, we investigated the prognostic significance of WT1 positivity in different types of high grade uterine sarcoma. Univariate analysis suggests worse prognosis if the tumor was positive for WT1. In a multivariate analysis, combining WT1 with age, sarcoma subtype, size and stage, WT1 is still an independent negative prognostic factor.

Similar observations for a role of WT1 in cancer biology have been made in breast cancer [22], leukemia [23] and hepatocellular carcinoma [24]. Whereas we used IHC to determine WT1 in correlation to outcome, other authors determined WT1 expression at RNA level. None of the studies looked to molecular mechanisms explaining the correlation between WT1 and prognosis. However, some hypotheses were raised. For hepatocellular carcinoma Sera et al. [24] speculated that WT1 overexpression might result from HNF-4 suppression, which has been proven to be associated with tumor progression. On the other hand, the authors also postulated a mechanism via p53 and IGF-IR. Inoue et al. [22] noticed 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 conducted on 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 because of their large patient sample number. Kapp and colleagues [25] recently described prognosis in 1396 patients with LMS. According to their multivariate analysis age, stage and grade were independent prognostic variables, together with race and primary surgery. On the other hand, Nemani and colleagues [26] described prognostic variables in 1855 patients with CS. They confirmed age and stage to be independent risk factors in determining prognosis, together with race, lymphadenectomy and marital status.
Generally, LMS are believed to have worse prognosis than CS. Rovirosa et al [27] made an overview of studies, focusing on this parameter. Three studies, implying together 303

patients, concluded that the prognosis of LMS and CS was equal. In contrast, two large studies of together 820 patients, attributed a worse prognosis to LMS. In our study, no clear differences in prognosis were found between LMS and CS. However, since the histopathological origin of both sarcomas is different [9], we looked into a possible interaction between WT1 and the 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 leads to a possible distorted picture.

In the multivariate analysis, we have decided not to include mitotic index, based on the publication of Bell et al [28] in 1994, proving that MI has a poor performance in predicting 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 [29]. Previously it was shown that adjuvant chemotherapy did not result in a survival benefit [30]. We also decided to not include the status of the lymphadenectomy as a separate prognostic factor. It often is an unknown factor based on the surgical guidelines based on preoperatieve stage. This would result in incomplete and incorrect statistical analyses. Moreover, the status of the lymph nodes is most of the time covered by the stage, but of course, this is immediately a shortcoming since surgery is often based on preoperative staging. Finally, we also excluded menopausal status as a separate prognostic factor, because we already included age as a continuous. 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 relative small number of patients, which requires confirmation on larger scale, we were able to demonstrate that WT1 is an independent predictor of worse prognosis.

The inverse correlation between WT1 and ER/PR expression stresses the negative influence of WT1 on the prognosis, since ER and PR are associated with better differentiation and overall a more indolent behavior. However, this is in contrast to the findings of Carvalho et al [31]. In 30 uterine LMS, they found only 23% that was positive for WT1 and the majority was positive for ER and PR. The discrepancy for WT1 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.

It is well known that high grade uterine sarcoma are reluctant to standard treatment [32]. Therefore new treatment options are explored. Immunohistochemical studies on the presence of Platelet Derived Growth Factor Receptor alfa (PDGFRα) [33], Human Epidermal Growth Factor Receptor 2 (HER-2/NEU) [34], Estrogen Receptor (ER) [35], Progesterone Receptor (PR) [36],... have been conducted recently. New targeted treatment options towards 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 might offer the solution, since WT1 has

presented itself as a highly immunogenic target [37,38]. Immunotherapy in general has proven its benefit [38]. WT1 immunotherapy has been effective, including in high grade glioblastoma and lung cancer by injection of a modified WT1 peptide [39,40]. Also dendritic cell immunotherapy [41] offers possibilities as well to directly eliminate tumor cells or to influence at least their biological behavior.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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Figure and table legends

Table 1. Overview of patient characteristics.

Figure 1. 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)]

Table 2. Characteristics of WT1 positive and negative tumors.

Figure 2. 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)]

Figure 3. Survival curves of high grade uterine sarcoma based on the WT1 score. A. Progression free survival. B. Overall survival. [WT1 negative tumors (-) (n=22), WT1 weakly positive tumors (+) (n=16), WT1 strongly positive tumors (+++) (n=13). WT1 moderately positive tumors (++) (n=4) were excluded because of the small sample size.

Table 3. Multivariate Cox regression analysis after MI (n=71). A. Progression free survival. B. Overall survival.