Alternative Splicing-Related Factor YT521

An Independent Prognostic Factor in Endometrial Cancer

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Background: YT521 is a splicing factor involved in alternative splicing regulation of several tumor biological important genes. Two messenger RNA (mRNA) isoforms due to YT521 exon6 alternative splicing exist, with so far unknown functional consequences. Further evidence exists for a direct influence of YT521 expression in tumorigenesis because its mRNA level is changed in tumors compared with physiological tissue. We investigated the potential impact of YT521 expression on tumor biological parameters in endometrial cancer (EC).

Methods: Real-time reverse transcription–polymerase chain reaction specifically detecting YT521 exon6-retention and exon6-skipping mRNA isoforms and immunohistochemistry were performed in a cohort of 130 EC tissue samples.

Results: Whereas YT521 exon6-retention mRNA was detectable in 86 (66.2%), the exon6-skipping isoform mRNA was expressed in only 8 (6.2%) of all EC samples. On the protein level, 104 (80%) of EC samples showed nuclear expression. The mRNA levels of exon6-skipping isoform were not correlated to any of the clinicopathological parameters of EC. In contrast, YT521 exon6-retention mRNA expression was positively correlated to metastasis ($R = 0.196$, $P = 0.026$) and inversely correlated to the protein expression levels ($R = -0.205$, $P = 0.019$). In univariate analyses, higher levels of YT521 exon6-retention mRNA were correlated to a poorer progression-free survival ($P = 0.003$), and this is confirmed by multivariate analyses ($P = 0.019$). The negative YT521 protein expression was correlated to poorer overall and disease-specific survival ($P = 0.036$ and $P = 0.034$), respectively, in univariate analyses. They are also confirmed by multivariate analyses ($P = 0.021$ and $P = 0.010$, respectively).

Conclusions: We characterized for the first time in a clinical setting a new but rare exon6-skipping mRNA splicing isoform of YT521. Furthermore, we identified YT521 as a potential new independent prognostic factor for patients with EC: the lack of YT521 protein in tumor cells was highly predictive for a poor overall and disease-specific survival and independent from the histological subtypes.

Key Words: YT521, Alternative splicing, Endometrial cancer, Prognostic factor

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Alternative pre–messenger RNA (mRNA) splicing is an important mechanism for genetic diversity. Approximately 70% to 80% of human genes undergo alternative splicing, which generates different mRNA and consecutively protein isoforms out of 1 single gene.[1,2] Owing to the addition or deletion of functional domains, changing affinities, and altering mRNA stability, this mechanism has vast effects on cellular functions. Furthermore, specific alterations in splicing patterns have been found in association with cancer, with biological consequences in cell transformation, motility, and metastasis.[3–6] Splicing factors that play a crucial role through concentration changes or alterations of their expression patterns have significant impact on mRNA alternative splicing.[7,8] As a result, changes of splicing pattern in cancer are often accompanied by alterations in expression pattern of splicing factors.

YT521 is a ubiquitously expressed nuclear splicing factor, which was discovered through an association of its glutamic acid/arginine–rich domain with splicing factor hTRa2beta1.[9,10] YT521 itself is alternatively spliced and 2 isoforms of YT521 mRNA exist (NM_001031732 and NM_133370, respectively) due to the inclusion or skipping of its alternative exon6. However, at present, the functional consequences of these 2 mRNA isoforms and their corresponding proteins have not been reported. YT521 is involved in splice site selection in a concentration-dependent manner,[11] either by sequestering splicing factors via protein-protein interaction or by binding to nucleic acids via its YTH domain.[10,12,13] In recent studies, YT521 regulated CD44 splicing in a concentration-dependent manner by reducing inclusion of alternative CD44 exon v5.[12] Because CD44 is known to be a tumor biological important adhesion molecule and regulated by hTRa2beta1,[14,15] these findings drew our interest toward YT521.

Furthermore, it became evident that YT521 might be involved in the tumorigenesis of hormone-dependent cancers. Moreover, there is accumulating evidence that YT521 nuclear localization and its subnuclear organization are regulated during the cell cycle and strongly influenced by tamoxifen.[11] These findings indicate that YT521 and its biological impact might be endocrine-dependent. Because endometrial cancer (EC) represents the most common hormone-dependent gynecological malignancy, we were interested in the potential clinical impact of YT521 expression in this tumor entity.

MATERIALS AND METHODS

Tissue Arrays

Tissue microarrays (TMAs) originating from 130 endometrial carcinomas of hysterectomy specimens were obtained from the Institute of Pathology of the University Medical Center Freiburg, and the corresponding patient data were extracted from an automated database. All patients included in the study were diagnosed with EC between October 1, 1993, and December 6, 2005, and were treated at the Department of Obstetrics and Gynecology of the University Medical Center Freiburg. All patients received hysterectomy, bilateral salpingo-oophorectomy, whereas in selected cases according to the national guidelines at the time, a selective pelvic lymphadenectomy, with or without para-aortic lymphadenectomy, was performed. In detail, lymph node sampling or dissection was generally performed in patients with advanced tumor stages, deep myometrial invasion, high-grade tumors, or aggressive histological subtypes. Lymphadenectomy was not performed in patients who had either high surgical risk, gross intraperitoneal disease, or minimal risk owing to early-stage (pT1a or pT1b) or low-grade differentiation at the time of intraoperative frozen section analysis. All hematoxylin-eosin–stained slides were reviewed by 2 experienced gynecopathologists (A.z.H. and M.O.-V.) with respect to diagnosis, histological subtype, histological grade, myometrial invasion depth, and lymph node involvement. Few divergent evaluations were discussed together, and common consent was found by the 2 pathologists. Histological classification was according to the World Health Organization (WHO) system as well differentiated, moderately differentiated, and poorly differentiated, respectively. Patients without lymphadenectomy and with no gross intraperitoneal disease were staged by the classic pathological parameters. In the case of recurrences or disease progression, diagnosis was confirmed even by imaging evidence or histology.

RNA Extraction and Real-Time Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from formalin-fixed paraffin-embedded blocks as described recently.[16] RNA quality check was performed by photometry accepting an $A_{260/280}$ ratio higher than 1.7. Two micrograms of total RNA was used for complementary DNA synthesis using M-MLV reverse transcriptase (Promega, Mannheim, Germany) and 10 pmol/L random hexamer primers (New England Biolabs GmbH, Frankfurt, Germany) in a total reaction volume of 50 μL. The assay was performed under standard conditions: 42°C for 90 minutes and at 94°C for 5 minutes.

For real-time polymerase chain reaction (PCR) amplification of the YT521 exon6-retention isoform (primer forward 5′-GAAGTGGAAGCTCTGCATCA-3′ and reverse 5′-GACAGCACGAACGGAAGATG-3′), YT521 exon6-skipping isoform (primer forward 5′-GCATCAGAGTCA TATGCAGATC-3′ and reverse 5′- GCTTTGGCAAGAGA CACATTC-3′), and ribosomal protein S18 (RPS18, primer forward 5′-TACTCAACACCAACATCGATGGGC-3′ and reverse 5′-GCTTTCCTCAACACCACATGAGCA-3′) as internal controls, respectively, the Quantitect qPCR Mastermix (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol. Each reaction mix contained 20 ng of complementary DNA, 5 μmol/L primer, 10 μL of qPCR Mastermix. Each sample had 2 replicates, and expression levels of the target genes were evaluated with the average C_t values of the real-time PCR. The PCR conditions are as follows: 40 cycles at 95°C for 20 seconds, at 60°C for 20 seconds, and at 72°C for 20 seconds, respectively. Because increasing copy numbers of the target gene leads consecutively to lower C_t values, the relative quantification of YT521 was calculated according to the following formula: mRNA level = $2^{(\text{mean } Ct \text{ HKG} - \text{mean } Ct \text{ GOI})}$, where HKG is the housekeeping gene and GOI is the gene of interest.[17]

Immunohistochemistry

All immunohistochemistry (IHC) stains were performed on formalin-fixed paraffin-embedded human EC TMAs. The rabbit polyclonal antibody of YT521 was raised against a mixture of 2 YT521 protein peptides (P1: RSARSVILIFSVRE SGKFQCG; P2: KDGELNVLDDILTEVPEQDDECG) fused to keyhole-limpet hemocyanin.[11] Sections were deparaffinized in xylene and were rehydrated. Epitope retrieval was achieved by heating the sections in DAKO target retrieval solution (high pH) for 12 minutes.[18] Endogenous peroxidase was blocked with 3% H_2O_2 for half an hour. After 2 washes in phosphate-buffered saline, slides were incubated with DAKO protein block serum-free for 1 hour to block unspecific staining. The sections were incubated with the polyclonal antibody (dilution, 1:1000) overnight. Then, slides were washed in phosphate-buffered saline and incubated with peroxidase-labeled antirabbit immunoglobulins at room temperature for 2 hours. Staining was achieved by 3,3' Diaminobenzidine (VECTASTAIN; Vector Laboratories, Inc, Linaris GmbH, Wertheim-Bettingen, Germany). All slides were counterstained with hemalaun and, after dehydration, were mounted with Entellan (Merck, Darmstadt, Germany).

Immunostained arrays were independently evaluated by 2 experienced gynecopathologists (A.z.H. and M.O.V.) blinded to the clinical data. Results were divided into negatively and positively stained subgroups, respectively.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences software (SPSS Version 15.0; SPSS Inc., Chicago, IL). χ^2 and Fisher exact tests were performed to analyze real-time PCR and IHC results with clinicopathological parameters, and Spearman test was applied to identify relevant correlations. Univariate analyses of overall survival (OS; patients who died at follow-up were considered as uncensored, whereas patients alive at follow-up were considered as censored), disease-specific survival (DSS; patients who died of EC were considered as uncensored, whereas patients who died of other reasons or alive at follow-up were considered as censored), and progression-free survival (PFS; patients who died of EC or alive with recurrence were considered as uncensored, whereas patients who died of other reasons or alive without recurrence at follow-up were considered as censored) were performed with Kaplan-Meier survival curves[19] and were compared by the log-rank test. Prognostic models used Cox regression for multivariate analyses of survival, and the variables were entered in an enter method.[20] Data were adjusted for age, International Federation of Gynecology and Obstetrics (FIGO) stage (stage I and II vs stage III and IV), grade (G1 and G2 vs G3), histological subtypes (endometrioid vs nonendometrioid), myometrial invasion depth (<1/2 vs ≥1/2), postoperative therapy (without postoperative therapy vs radiotherapy, chemotherapy, and combined chemoradiotherapy), mRNA level (low vs high), and YT521 IHC status (negative vs positive), respectively (the first category in bracket was reference category). Significant differences were set as $P < 0.05$ at the 2-sided test (SPSS version 15.0).

RESULTS

Patients

The clinicopathological characteristics of the 130 patients diagnosed with EC included in the study are summarized in Table 1. The median age was 66 years (range, 36–94 years), with 55 patients (42.3%) younger than 65 years

TABLE 1. Clinicopathological parameters of the analyzed EC

	No. Patients	%
Age, yr		
<65	55	42.3
≥65	75	57.7
Histological subtype		
Endometrioid	96	73.8
Nonendometrioid	34	26.2
Grade		
G1	31	23.8
G2	65	50.0
G3	34	26.2
FIGO stage		
I	84	64.6
II	14	10.8
III	21	16.1
IV	11	8.5
Myometrial invasion depth		
<1/2	55	42.3
>1/2	75	57.7
Lymph node involvement		
Negative	77	59.2
Positive	22	16.9
Unknown	31	23.9
Metastases		
Negative	121	93.1
Positive	9	6.9
Postoperative therapy		
Without postoperative therapy	40	30.7
Radiotherapy	74	57
Chemotherapy	5	3.8
Combined chemoradiotherapy	11	8.5
Recurrence during follow-up		
No	111	85.4
Yes	19	14.6
Outcome		
Alive	113	86.9
Died of cancer	12	9.2
Died of other causes	5	3.9
Total (n = 130)		

TABLE 2. Spearman test results

	Metastasis (No vs Yes)	Exon6-Retention mRNA Level (Low vs High)	IHC (Negative vs Positive)
Exon6-retention mRNA level (low vs high) correlation coefficient	0.196	—	−0.205
P	0.026*	—	0.019*
IHC (negative vs positive) correlation coefficient	−0.015	−0.205	—
P	0.864	0.019*	—

Spearman test showed that YT521 exon6-retention mRNA levels were correlated with metastasis and inversely correlated with YT521 protein expression levels (*$P < 0.05$).

at the initial diagnosis. Of these patients, 84 (64.6%), 14 (10.8%), 21 (16.2%), and 11 (8.5%) patients were diagnosed to have FIGO stages I, II, III, and IV, respectively. Moreover, conditions of 31 (23.8%), 65 (50.0%), and 34 (26.2%) patients were diagnosed as G1, G2, and G3, respectively. The most common histological diagnosis was the endometrioid type with 96 (73.8%) of the patients, whereas 34 (26.2%) presented with a serous/clear cell or undifferentiated type. Twenty-two patients (16.9%) were found to have lymph node involvement, 77 patients (59.2%) were found to be lymph node–negative, whereas those of 31 patients (23.9%) were unknown. At the initial diagnosis, metastases were found in 9 patients (6.9%), whereas 121 (93.1%) patients had no evidence of metastases. Forty patients (30.7%) did not have postoperative therapies, whereas 74 (57%), 5 (3.8%), and 11 patients (8.5%) undertook radiotherapy, chemotherapy, and combined chemoradiotherapy, respectively, after surgery. The median follow-up time was 17 months (mean, 26 months; range, 1–110 months). Seventeen patients died: 12 due to EC and 5 due to other causes. There were 19 patients (14.6%) observed with tumor recurrence after therapy during the follow-up period.

YT521 mRNA Expression in Endometrial Cancer

Real-time reverse transcription–PCR results of each sample were normalized against RPS18 gene expression to partially quantify mRNA copies of YT521. Interestingly, we identified for the first time in a clinical setting the exon6-skipping isoform of YT521 mRNA, which was amplified in 8 samples (6.2%), whereas the exon6-retention isoforms were detected in 86 samples (66.2%). Owing to the small number, no statistical analyses were performed for the rare-expressed exon6-skipping YT521 isoform. A further investigation focused on the exon6-retention mRNA isoform. The relative quantification of the 75th percentile or higher of all analyzed samples was defined as high level, whereas that of less than the 75th percentile of all analyzed samples was defined as low level. χ^2 Test demonstrated that YT521 exon6-retention mRNA levels were associated with metastasis ($P = 0.040$), which was confirmed by an additional Spearman test, revealing a significant positive correlation with metastasis (correlation coefficient = 0.196, $P = 0.026$; Table 2). Interestingly, the YT521 exon6-retention mRNA level was inversely correlated to YT521 protein expression levels (correlation coefficient = −0.205, $P = 0.019$; Table 2). However, no further correlations to the clinicopathological parameters such as age, histological type, grade, FIGO stage, myometrial invasion depth, or lymph node involvement were detectable.

YT521 Protein Expression in Endometrial Cancer

Immunohistochemistry revealed that YT521 expression was restricted to the nucleus with a diffuse staining pattern (Fig. 1). One hundred four samples (80%) displayed positive nuclear staining and 26 samples (20%) showed

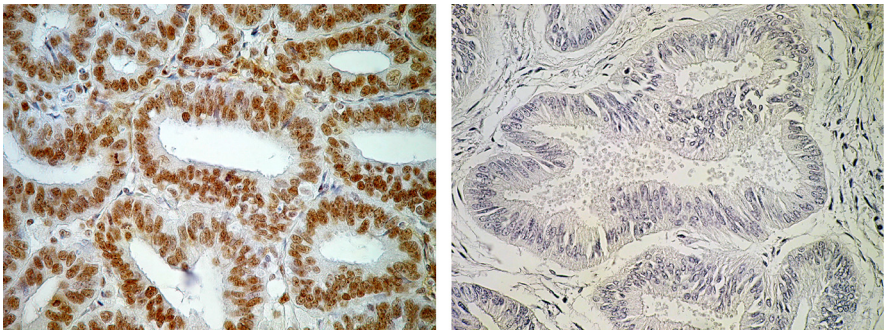

FIGURE 1. YT521 expression is restricted to the nucleus without cytoplasmic staining. Staining pattern is diffuse within the nucleus: positive (left) and negative (right).

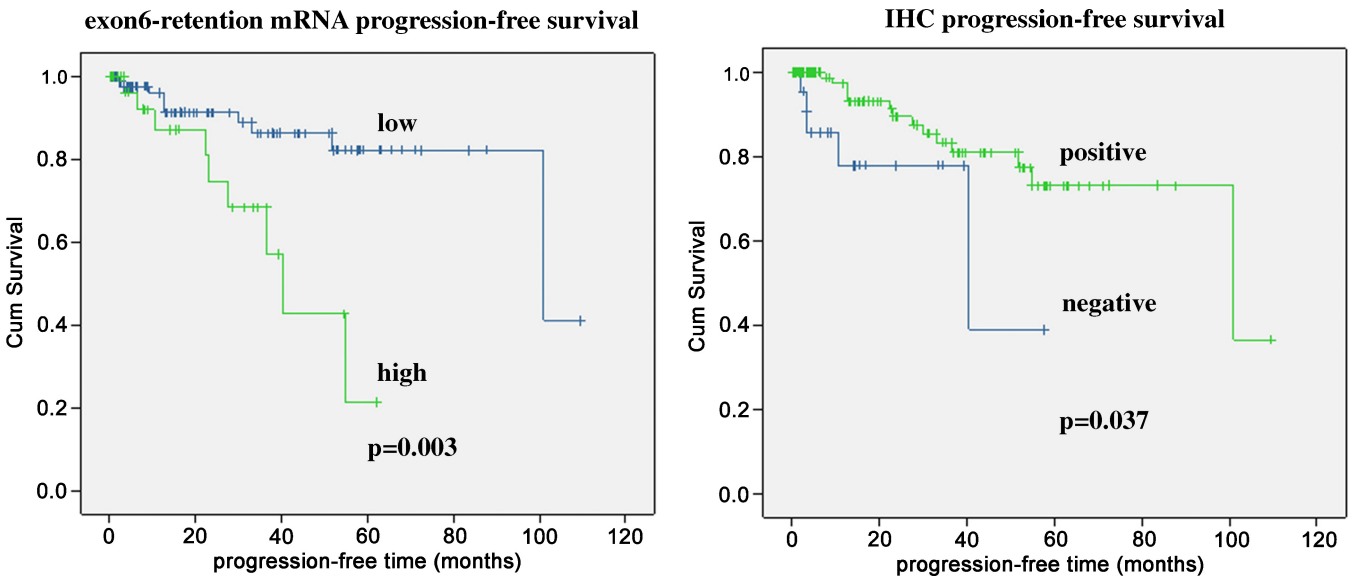

FIGURE 2. Kaplan-Meier survival curves of PFS for exon6-retention isoform mRNA level and YT521 IHC (log-rank: $P = 0.003$ and $P = 0.037$, respectively).

negative staining. χ^2 analysis did not reveal any significant association between YT521 IHC and age, grade, histological subtype, FIGO stage, myometrial invasion depth, postoperative therapy, lymph node involvement, or metastasis status.

Survival Analyses

As for the exon6-retention mRNA, univariate survival analysis did not demonstrate any significant differences in OS ($P = 0.269$) or DSS ($P = 0.450$). However, patients with a lower level of YT521 exon6-retention mRNA showed a highly significantly better PFS ($P = 0.003$, log-rank test; Fig. 2). On the other hand, patients with positive IHC staining had a significantly better survival in comparison to those with negative staining in PFS, DSS, and OS analyses ($P = 0.037$, $P = 0.034$, and $P = 0.036$, respectively, log-rank test; Figs. 2, 3, and 4).

Multivariate analysis was applied to identify the independent prognostic impact on the outcome parameters in our cohort of 130 patients. Age, histological subtypes, grading, FIGO stage, myometrial invasion depth, postoperative therapy, lymph node involvement, metastasis, exon6-retention mRNA levels, and IHC status were included and analyzed in

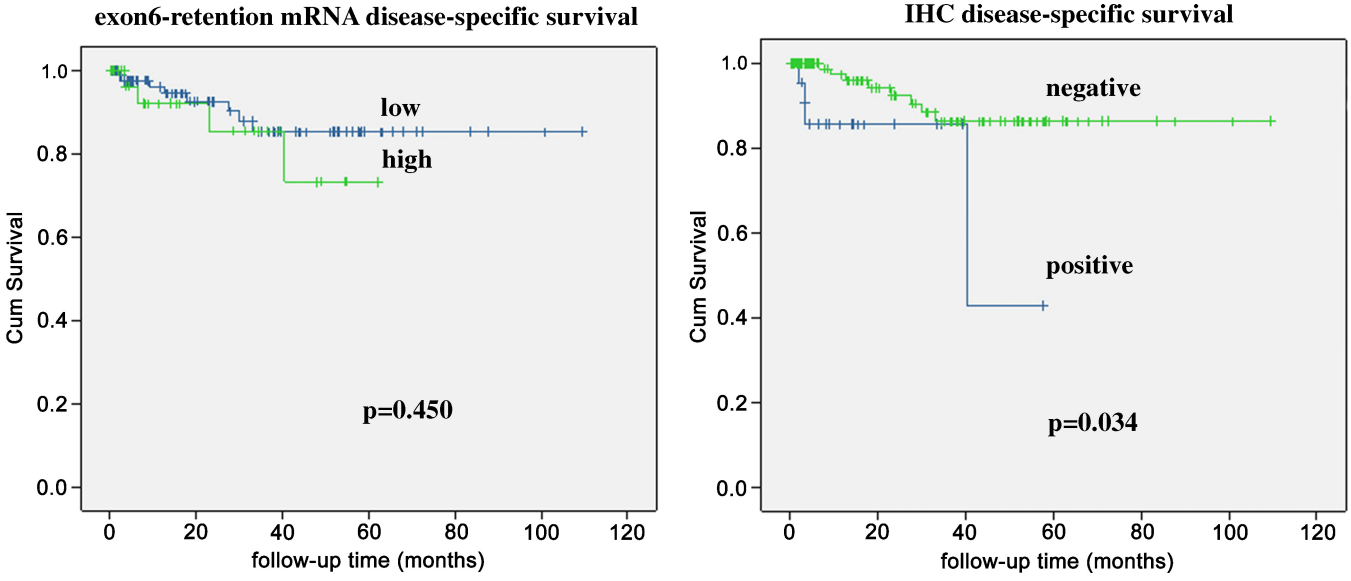

FIGURE 3. Kaplan-Meier survival curves of DSS for exon6-retention isoform mRNA level and YT521 IHC (log-rank: $P = 0.450$ and $P = 0.034$, respectively).

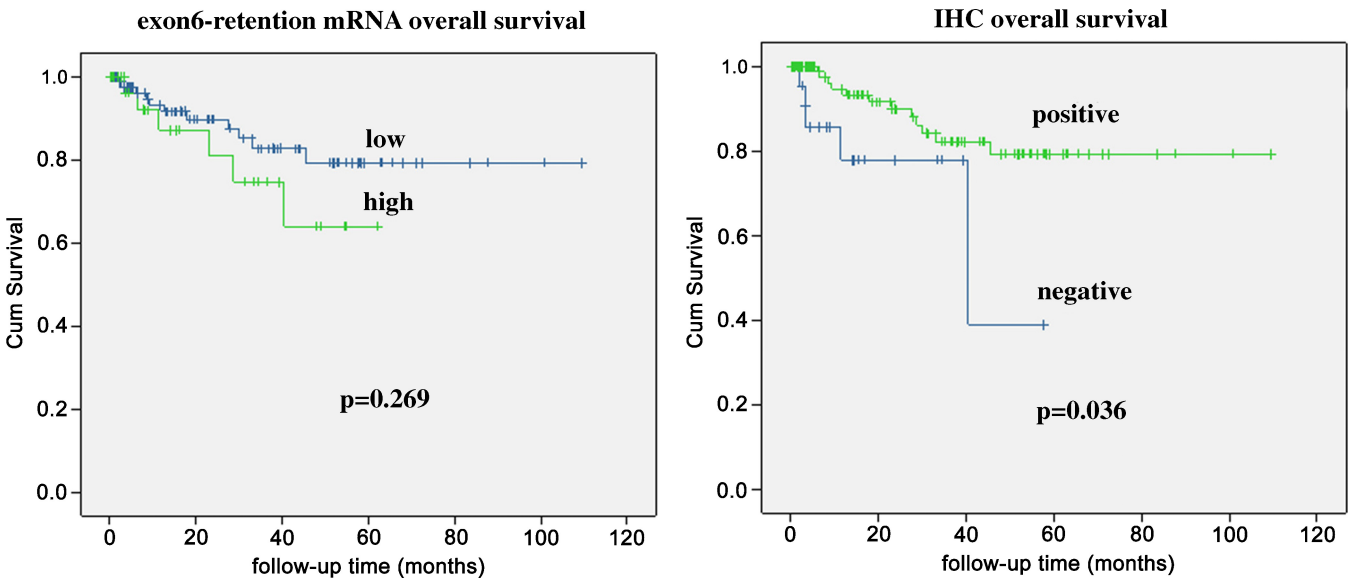

FIGURE 4. Kaplan-Meier survival curves of OS for exon6-retention isoform mRNA level and YT521 IHC (log-rank: $P = 0.269$ and $P = 0.036$, respectively).

the enter method of a Cox regression. We identified YT521 exon6-retention isoform mRNA level (HR = 5.196, $P = 0.019$) as an independent prognostic factor for PFS; YT521 IHC expression status (HR = 0.089, $P = 0.010$) as an independent prognostic factor for DSS. Furthermore, myometrial invasion depth (HR = 11.902, $P = 0.042$) and YT521 IHC expression status (HR = 0.193, $P = 0.021$) were identified as independent prognostic factors for OS.

One thing worth mentioning, neither YT521 exon6-retention mRNA nor IHC status could be correlated with FIGO stage. To elucidate the potential role of YT521 in early FIGO stages of EC, the patients with FIGO stage III and IV were excluded, and the survival analyses were redone accordingly. In univariate analyses, patients with a lower level of YT521 exon6-retention mRNA and positive IHC staining showed a significantly better PFS ($P = 0.001$, log-rank test) and OS ($P = 0.026$, log-rank test), respectively. Multivariate analysis failed to identify any of the previously mentioned parameters as an independent prognostic factor for PFS, DSS, or OS (Table 3). Although the multivariate analyses marginally failed to reach statistical significance, the P values of exon6-retention mRNA level in PFS ($P = 0.08$) and IHC status in OS ($P = 0.08$) and DSS ($P = 0.067$) indicate a statistical trend.

TABLE 3. Univariate and multivariate survival analyses results of early-stage patients (FIGO stage I and II) regarding YT521 exon6-retention mRNA level and IHC status

	Univariate Analysis, *P*			Multivariate Analysis, *P*		
	OS	DSS	PFS	OS	DSS	PFS
Exon6-retention mRNA	0.088	0.413	0.001*	0.605	0.976	0.084
IHC	0.026*	0.075	0.054	0.081	0.067	0.145

*$P < 0.05$.

DISCUSSION

Endometrial cancer is the most frequent gynecological malignancy, and in most cases, conditions of women are diagnosed at an early stage and are cured by surgery alone. Types 1 and 2 of EC exhibit distinct biological behaviors with consecutively different clinical courses. The type 1 carcinoma is related to hyperestrogenism, a younger age, and a good prognosis, whereas the more aggressive type 2 carcinoma is unrelated to estrogen and is found in older age. The prognostic significance of the morphological classification, however, is limited because 15% and 20% of patients diagnosed with type 1 EC will recur and 50% of type 2 will not.[21] Molecular data support the hypothesis of different genetic pathways for both tumor entities. Although loss of protein tyrosine phoshatase (Entrez gene: 5728) is found in up to 85% of type 1 EC, p53 mutations and Her2/neu overexpression are the major alterations in type 2 EC.[22] However, the prognostic value of these findings has not yet been established, and the identification of patients at high risk for recurrence is still an unsolved problem. In this study, we investigated the expression of splicing factor YT521 in EC as well as its potential impact as a prognostic factor in this common tumor type.

In our cohort of 130 patients diagnosed with predominantly type 1 EC, the exon6-retention YT521 mRNA isoform, encoding for the whole-length functional protein, was predominantly expressed (66%). Because the exon6-skipping mRNA isoform was only rarely detected (6.2%), we did not further investigate it statistically. No associations of YT521

exon6-retention mRNA expression with established clinicopathological parameters were found, especially histological type, grading, or FIGO stage. Interestingly, the statistical analyses revealed that expression levels of YT521 exon6-retention mRNA were directly linked to the patients' outcome because a lower level was significantly correlated with the absence of distant metastases as well as an improved PFS (Table 2 and Fig. 2). As for the protein expression patterns of YT521, positive nuclear IHC staining was dominant. In accordance with our mRNA, YT521 protein expression was not associated with any of the investigated clinicopathological parameters. However, the analyses regarding clinical outcome revealed that ECs with positive YT521 protein expression were characterized by a significantly improved clinical outcome regarding DSS and OS. This is in accordance with our findings that normal endometrial tissues express YT521 protein at high levels and might therefore play a crucial role in the homeostasis of cellular processes. Further survival analyses focusing on early FIGO stage EC patients confirmed that YT521 exon6-retention mRNA level and IHC status were significantly correlated with univariate PFS and OS, respectively (Table 3). Although the multivariate analyses did not show any significant results, the *P* values of exon6-retention mRNA level in PFS and IHC status in OS and DSS are near statistical significance. Therefore, there is a possibility that YT521 could be an independent prognostic factor in early stages (FIGO I and II) of EC. At a first glance, YT521 exon6-retention mRNA and protein expression levels were significantly inversely correlated (Table 2), and the opposite findings regarding the prognostic value of YT521 exon6-retention mRNA and protein levels seem to be contradictory. However, there are indeed some proteins existing that can autoregulate themselves from exhibiting a similar phenomenon, in which more protein will cause a degradation of its coding mRNA.[23] Meanwhile, the functional mechanism of YT521 exon6-skipping mRNA and its corresponding protein product is unknown, and it is of great interest to investigate further. This opens new perspectives of functional analyses on YT521 in the future.

The clinically important aspect of this study is the retrospective identification of a subgroup of approximately 20% (n = 26) of EC patients (in these 26 patients, only 5 were FIGO stage III or IV) with a negative YT521 protein expression. This group of patients exhibits, independent from other well-established clinicopathological features, a compromised clinical outcome with a dramatically increased risk for poorer DSS ($P = 0.010$) and OS ($P = 0.021$), respectively. This 20% of negative YT521 protein expressers might represent the 20% of patients with disease recurrence even with favorable classic prognostic markers. Our findings also underline the biological importance of the nuclear alternative splicing system. Because YT521 is involved in the splicing regulation of tumor biological important genes, expression changes might have eminent consequences on splicing decision of cancer-associated genes.[12] In our cohort of patients with EC, YT521 protein exerts a potential tumor suppressor activity, with influence on disease progression and metastasis.

In summary, our retrospective cohort analysis of YT521 expression in EC demonstrates a potential prognostic value of this splicing factor. Independent from the established prognostic parameters, this marker might be able to identify a subgroup of patients who are at high risk for disease recurrence and need additional intensified adjuvant treatment beyond surgery. Furthermore, these findings underline the putative functional significance of YT521 in EC, which is in addition supported by the known regulation. Although our results are promising in establishing a new potential prognostic factor by testing YT521 expression, it is yet too early to definitely draw a final conclusion. However, data on protein expression by immunohistochemistry seem to be more promising and feasible for the use in daily routine testing. Of course, larger cohort analyses and functional studies of YT521 are needed to confirm these results and to investigate the underlying molecular events.

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