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Methylation status and expression of human telomerase reverse transcriptase in ovarian and cervical cancer

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Abstract

Objectives. Activation of telomerase, the enzyme that synthesizes the telomere ends of linear chromosomes, has been implicated in human cell immortalization and cancer cell pathogenesis. The expression pattern of human telomerase reverse transcriptase (*hTERT*), the telomerase catalytic subunit gene, is correlated with telomerase activity. The promotor region of the *hTERT* gene has been located in a CpG island and may therefore be regulated, at least in part, by DNA methylation. The potential for methylation-mediated regulation of *hTERT* gene expression in ovarian and cervical cancer tissue has not been investigated up to now. The aim of this study was to investigate the expression and methylation pattern of *hTERT* in ovarian and cervical cancer tissue and their correlation with clinicopathological features and outcome of the disease.

Methods. A total of 223 tissues were analyzed for *hTERT* methylation using MethyLight: 65 patients with cervical cancer and 124 with ovarian cancer were studied. The control group consisted of 20 normal ovarian tissues and 14 normal cervical tissues. Quantitative *hTERT* expression analysis was carried out in a subgroup of patients using real time PCR.

Results. *hTERT* expression was statistically significantly higher in ovarian and cervical cancer tissue in comparison to normal tissue. While methylation of *hTERT* in cervical cancer was significantly more frequent in comparison to normal cervical tissue, the difference between ovarian cancer and normal ovarian tissue was not significant. No correlation was detected between hypermethylation of *hTERT* and *hTERT* mRNA expression. Both ovarian cancer and normal ovary showed an increase in *hTERT* methylation with increasing age. *hTERT* expression was not correlated with prognosis, whereas cervical and ovarian cancer patients with unmethylated *hTERT* had significantly better overall survival.

Conclusion. At least in some tumor entities, *hTERT* methylation is a function of age and is associated with a poorer outcome, irrespective of *hTERT* expression.
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Keywords: DNA methylation; Telomerase; Cervical cancer; Ovarian cancer

Introduction

Recent developments have implicated short telomere length and telomerase activation as critical elements in cellular immortalization and cancer. The end of the chromosome, the telomere, plays a critical role in chromosome structure and function, in that it affords chromosome genes protection from degradation or recombination as it is resistant to exonucleases and ligases [1–3]. A certain length of the telomere is important for cell division, and the telomere may serve as a “mitotic clock” for cell proliferation [4–6]. Normal human somatic cells express low or undetectable telomerase activity and progressively lose telomeric sequences with cell division [7–9]. Thus, in immortal eukaryotic cells as well as in cancer cells, augmentation of telomerase activity is apparently necessary to balance telomere loss with de novo synthesis of telomeric DNA so as to maintain

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sufficient length of the telomere to ensure proliferation [10,11]. The enzyme telomerase is a ribonucleoprotein, comprising an RNA template (hTR) [12] and protein components, the telomerase-associated protein (TP1) [13] and the catalytic subunit (*hTERT*) [14–16]. A close correlation between *hTERT* expression and telomerase activity has been demonstrated [14–16]. *hTERT* is presently considered to be the most important factor in the formation of functional telomerase [17–20]. Approximately, 85–95% of tumorigenic tissue expresses *hTERT* [21], making it a valuable tool in cancer diagnosis. Telomerase activity has been demonstrated in various types of gynecologic cancers [22,23]. Data on *hTERT* expression in cervical cancer has revealed that 0–33% of normal cervices exhibited *hTERT* mRNA expression, whereas 80–100% of the cervical cancers showed *hTERT* expression [24–27]. In ovarian cancer tissue, *hTERT* expression or telomerase activity was observed in 80–93% [28,29], in borderline tumors in 0–80% [28,29], in benign cysts in 0–36% [28,29], and in normal ovary in 0–30% [23,28].

Analysis of the 5′gene regulatory region revealed the presence of a CpG island and a high overall GC content [30–33]. This feature combined with the presence of binding sites for methylation-sensitive transcription factors within the *hTERT* core promoter [31,34] suggests a possible role for methylation in the regulation of *hTERT* gene expression. While a recent study demonstrated a positive correlation among hypermethylation of the *hTERT* promoter, *hTERT* mRNA expression, and telomerase activity in normal and tumor tissues from various organs [35], others found no or only a partial correlation [36,37]. Recently, an in vitro study has shown that demethylation of the *hTERT* gene promotor reduces *hTERT* expression and telomerase activity and shortens telomeres [38]. The potential for methylation-mediated control of *hTERT* gene expression in ovarian and cervical cancer tissue has not been investigated to date.

Until now, only two studies have described telomerase activity in association with clinicopathological data in cervical cancer [27,39]. Zhang et al. [39] more frequently detected high telomerase activity in advanced-stage disease than in early stage disease, while Wisman et al. [27] described no correlation between telomerase activity and tumor stage, differentiation grade or histological type. Activity of telomerase or its subunits was not related to survival.

In ovarian cancer no significant correlation between telomerase activity and clinicopathological features was observed [40,41].

In several malignancies, for example, leukemias, breast cancer, and meningiomas, higher telomerase activity has been linked to a poorer prognosis [42–46]. No studies have yet been published investigating *hTERT* methylation and expression as prognostic factors concerning survival in ovarian and cervical cancer.

The aim of this study was to investigate the expression and methylation pattern of *hTERT* in ovarian and cervical cancer tissue and their correlation with clinicopathological features and outcome of the disease.

Materials and methods

Patients and samples

Tumor samples were retrieved from the tissue bank of the Department of Obstetrics and Gynecology, Innsbruck University Hospital (Innsbruck, Austria). In this tissue bank, tumor specimens are prospectively collected from patients consecutively operated for gynecological cancers at the Department of Obstetrics and Gynecology, Innsbruck University Hospital. Clinical, pathological, and follow-up data are stored in a database in accordance with hospital privacy rules. Tumor samples and clinical data are collected with the consent of patients.

Specimens were brought to the pathologist immediately after resection, and part of the tissue was placed in liquid nitrogen and stored at −80°C until lyophilization. A total of 124 patients with ovarian cancer (age, 24–88 years; mean, 61 years) and 65 patients with cervical cancer (age, 22–81 years; mean, 49 years), all treated at the Department of Obstetrics and Gynecology, University of Innsbruck, between 1989 and 2000, were included in this study. The control group consisted of 20 normal ovarian tissues (age, 30–81years; mean, 63 years) and 14 normal cervical tissues (age, 34–69; mean, 47 years).

All patients were staged according to the International Federation of Gynecology and Obstetrics (FIGO) staging system.

Cervical cancer treatment—according to international standards—consisted in stage IA of conization or simple hysterectomy. Patients with FIGO stage IB or IIA underwent radical hysterectomy according to Wertheim (type Piver III) with pelvic lymphadenectomy. Postoperatively, all patients with positive lymph nodes were given external pelvic radiation at 40 Gy in 20 fractions. Patients not eligible for surgery and patients with cervical cancer stage IIB or IIIA were given both intracavitary and external radiotherapy. Patients with tumors in stage IIIB or IVA received either combined intracavitary and external radiation or external radiation alone. Additionally, eight patients received chemotherapy.

With the exception of FIGO stage Ia (grading I or II), all ovarian cancer patients received a platinum-based chemotherapy following surgery.

All patients were followed-up after primary treatment, at our department, at intervals increasing from 3 months to 1 year until death or the end of the study, respectively. Follow-up information was available from all patients. The follow-up period ranged from 1 month to 13.2 years (median 4.2 years) for cervical cancer patients and 1 month to 13.9 years (median 3.5 years) for ovarian cancer patients. Routine examinations including systemic review, tumor marker

testing (SCC antigen and CA 125), pelvic examination, chest X-ray, pelvic computer tomography, or magnetic resonance imaging were performed to evaluate the outcome of the disease, which was classified as disease-free, relapse, death—according to the WHO criteria for clinical response. In total, 30 (46.2%) cervical cancer patients and 68 (54.8%) ovarian cancer patients had died, and 36 (55.4%) and 63 (50.8%) patients had relapsed.

RNA extraction and RT reaction

Because not enough sufficient tissue from all patients for RNA extraction was available, we investigated *hTERT* mRNA expression from patients with unmethylated *hTERT* promoter and from patients with the highest methylation (PMR values) of the *hTERT* promoter. Total cellular RNA was extracted from the tumor specimens using the acid guanidium thiocyanate–phenol–chlorophorm method [47]. Integrity was evaluated by assessing the 18S- and 28S-ribosomal RNA bands in 2% ethidium bromide-stained agarose gel. RNA concentration was measured by spectrophotometric analysis.

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Reverse Transcription of RNA was performed in a final volume of 20 μl containing 1× RT-Buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 5 mM $MgCl_2$,), 40 units of rRNasin® RNase Inhibitor (Promega, Madison, WI), 10 mM dithiothreitol, 200 units of M-MLV Reverse Transcriptase (Gibco BRL, Gaithersburg, MD), 5 μM random hexamers (Applied Biosystems, Foster City, CA), and 400 ng of total RNA. The samples were first incubated at 65°C for 5 min and then quick-chilled on ice. After addition of the M-MLV enzyme, the samples were incubated at 25°C for 10 min and 37°C for 50 min, followed by heating at 70°C for 15 min to inactivate the reverse transcriptase enzyme.

Primers and probes

Primers and probes for *hTERT* and for the TATA box-binding protein (TBP; a component of the DNA-binding protein complex TFIID as endogenous RNA control) were used according to Bieche et al. [48,49]. BLASTN searches were conducted to confirm the total gene specificity of the nucleotide sequences chosen for the primers and probes. To avoid amplification of contaminating genomic DNA, the probe was placed at the junction between two exons. The nucleotide sequences of the oligonucleotides used for TBP were 5′-CAC GAA CCA CGG CAC TGA TT-3′ (forward primer), 5′-TTT TCT TGC TGC CAG TCT GGA C-3′ (reverse primer), and 5′FAM-TGT GCA CAG GAG CCA AGA GTG AAG A-3′TAM (probe), and for *hTERT* 5′-TGA CAC CTC ACC TCA CCC AC-3′ (forward primer), 5′-CAC TGT CTT CCG CAA GTT CAC-3′ (reverse primer) and 5′FAM-ACC CTG GTC CGA GGT GTC CCT GAG-3′TAM (probe).

Real-time PCR amplification

PCR reactions were performed using an ABI Prism 7700 Detection System (Applied Biosystems) with a total volume of 25 μl reaction mixture containing 5 μl of each appropriately diluted RT sample (standard curve points and patient samples), 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of each primer, and 250 nM of the probe. The thermal cycling conditions comprised initial incubation at 50°C for 2 min, a denaturing step at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 65°C for 1 min. Each experiment included a standard curve with five cDNA concentrations, a control sample (T47D cell-line), 25 patients, and a no-template control. The standard curves were generated using serially diluted solutions of standard cDNA derived from the MCF-7 carcinoma cell line. Real-time PCR assays were conducted in triplicate for each sample, and mean value was used for calculation.

Determination of transcription level

The amount of the target and endogenous reference was determined from the standard curve. For value normalization, the target amount was divided by the endogenous reference amount. To verify the reproducibility of each assay, the normalized value of the control sample was always determined. Only experiments in which the control value was within a range ±2 SD were accepted.

DNA isolation and sodium bisulfite conversion

Genomic DNA from ovarian and cervical cancer specimens was isolated using a QIAmp tissue kit (Qiagen, Hilden, Germany). Sodium bisulfite conversion of genomic DNA was performed as described previously [50]. The beads were incubated for 15 h at 50°C to ensure complete conversion.

hTERT methylation analysis

Sodium bisulfite-treated genomic DNA was analyzed by the MethyLight technique as described [50,51]. As reference genes, *ACTB* and *COL2A1* were used. For *hTERT* methylation, two separate PMR values (separately calculated for *ACTB* and *COL2A1*) were calculated. The average was used as the final percentage of fully methylated reference (PMR) value. The following primers and probes were used for the MethyLight reactions: *hTERT*: 5′-GGATTCGCGGGTATAGACGTT-3′ (forward primer), 5′-CGAAATCCGCGCGAAA-3′ (reverse primer), 5′-6FAM-CCCAATCCCTCCGCCACGTAAAA-BHQ-1-3′ (probe);

ACTB: 5′-TGGTGATGGAGGAGGTTTAGTAAGT-3′ (forward primer), 5′-AACCAATAAAACCTACTCCTCCCTTAA-3′ (reverse primer), 5′-6FAM-ACCACCACCCAACACACAATAACAAACACA-BHQ-1-3′ (probe); *COL2A1*: 5′-TCTAACAATTATAAACTCCAACCACCAA-3′ (forward primer), 5′-GGGAAGATGGGATAGAAGGGAATAT-3′ (reverse primer), 5′-6FAM-CCTTCATTCTAACCCAATACCTATCCCACCTCTAAA-BHQ-1-3′ (probe).

Statistical analysis

Associations between categorical variables were tested with the Pearson's chi square test and the Fisher's Exact Test. Differences in median of age were examined with the Mann–Whitney *U* test. The Kaplan–Meier method was used for univariate survival analysis, and the log rank test was used to assess the difference between survival curves. Cox's proportional hazards analysis was used to estimate the prognostic effects of various variables. A *P* of less than 0.05 was considered statistically significant. These statistical calculations were performed using SPSS, version 11.0, for Windows.

Results

hTERT expression and clinicopathological characteristics

Quantitative RT-PCR was performed on RNA retrieved from 14 normal cervices, 12 normal ovaries, 45 cervical cancers, and 40 ovarian cancers. *hTERT* expression was categorized in low and high expression according to the median. In all 14 normal cervical tissues, *hTERT* expression was low, whereas *hTERT* expression was high in 64% of cervical cancer tissues (29 out of 45). The difference in

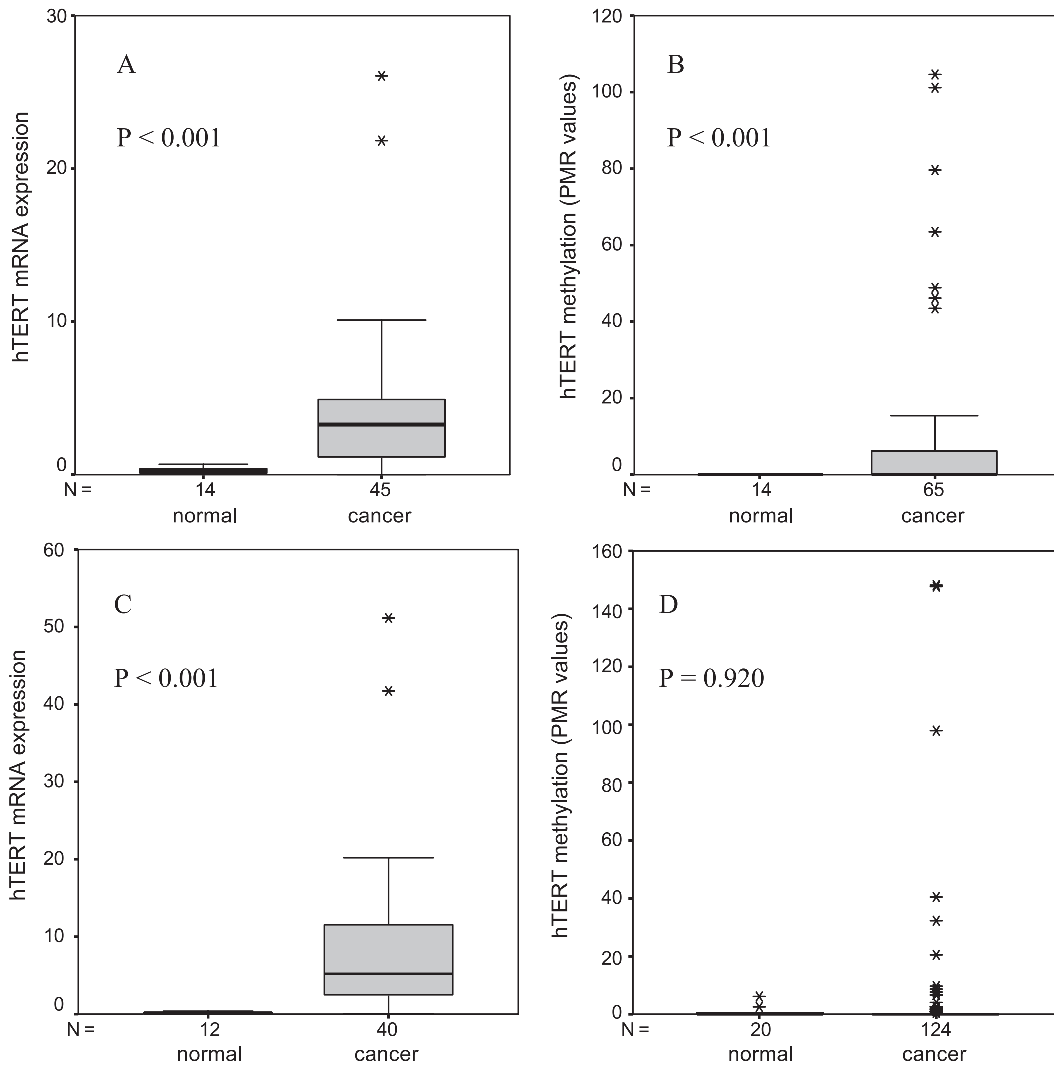

Fig. 1. *hTERT* mRNA expression in normal/cancer tissue of the uterine cervix (A) and normal/cancer tissue of the ovary (C). *hTERT* methylation in normal/cancer tissue of the uterine cervix (B) and normal/cancer tissue of the ovary (D).

Table 1A
hTERT methylation and expression: association with clinicopathological features in cervical cancer patients

Characteristics	*hTERT* methylation, no. of patients (n = 65)		P^a	*hTERT* expression, no. of patients (n = 45)		P^a
	Negative	Positive		Low	High	
Stage			0.077			0.438
FIGO I	21	16		10	19	
FIGO II	2	7		2	5	
FIGO III	2	8		2	1	
FIGO IV	2	6		1	4	
Tumor grade			0.507			0.530
1	2	7		3	3	
2	16	22		8	18	
3	7	8		3	7	
Histology			0.601			0.330
Squamous	22	31		11	23	
Adeno	3	4		4	2	
Adenosquamous	1	3		1	3	
Carcinofibrom	1	0		0	1	
Age (mean ± SD)	47.2 ± 14.9	50.7 ± 13.5	0.252[b]	50.2 ± 14.5	47.2 ± 14.4	0.522[b]

Tumor grade was unknown in three cases.
FIGO stage was unknown in one case.
[a] Based on the χ^2 Pearson test.
[b] Based on the Mann–Whitney U test.

hTERT expression levels between normal and cervical cancer tissues was statistically highly significant (Fig. 1A). The distribution of low and high *hTERT* expression within the clinicopathological characteristics revealed no statistical difference (Table 1A).

In ovarian cancer, high *hTERT* expression was observed in 62.5% (25 out of 40) of tissues, whereas in all normal ovarian tissues, *hTERT* expression was low. *hTERT* expression levels in ovarian cancer were statistically significantly different from expression levels in normal ovarian tissue (Fig. 1C). FIGO stage, histology, tumor grade, and age showed no correlation with *hTERT* expression (Table 1B and Fig. 2B).

hTERT methylation and clinicopathological characteristics

hTERT methylation was detectable by quantitative real-time PCR in none of the 14 normal cervical tissues, in 38 of 65 (58.5%) cervical cancer tissues, in 6 of 20 (30%) normal ovarian tissues, and in 37 of 124 (29.8%) ovarian cancer tissues. While methylation of *hTERT* in cervical cancer was detected significantly more frequently in comparison to normal cervical tissue (P < 0.001; Fisher's Exact Test; Fig. 1B), the difference between ovarian cancer tissues and normal ovarian tissues was not significant (Fig. 1D).

Tables 1A and B show associations between various clinicopathological data and *hTERT* methylation in cervical

Table 1B
hTERT methylation and expression: association with clinicopathological features in ovarian cancer patients

Characteristics	*hTERT* methylation, no. of patients (n = 124)		P^a	*hTERT* expression, no. of patients (n = 40)		P^a
	Negative	Positive		Low	High	
Stage			0.262			0.161
FIGO I	16	10		6	5	
FIGO II	3	3		2	1	
FIGO III	57	18		7	15	
FIGO IV	9	6		0	4	
Tumor grade			0.077			0.376
1	4	0		0	1	
2	59	20		12	15	
3	24	17		3	9	
Histology			0.074			0.639
Serous cystadeno	38	18		8	12	
Mucinous cystadeno	37	8		6	8	
Endometrioid	8	8		1	3	
Clear cell	4	3		0	2	
Age (mean ± SD)	59.5 ± 14.2	66.0 ± 10.0	0.025[b]	61.0 ± 18.1	63.9 ± 13.6	0.679[b]

hTERT methylation: FIGO stage was unknown in two cases.
[a] Based on the χ^2 Pearson test.
[b] Based on the Mann–Whitney U test.

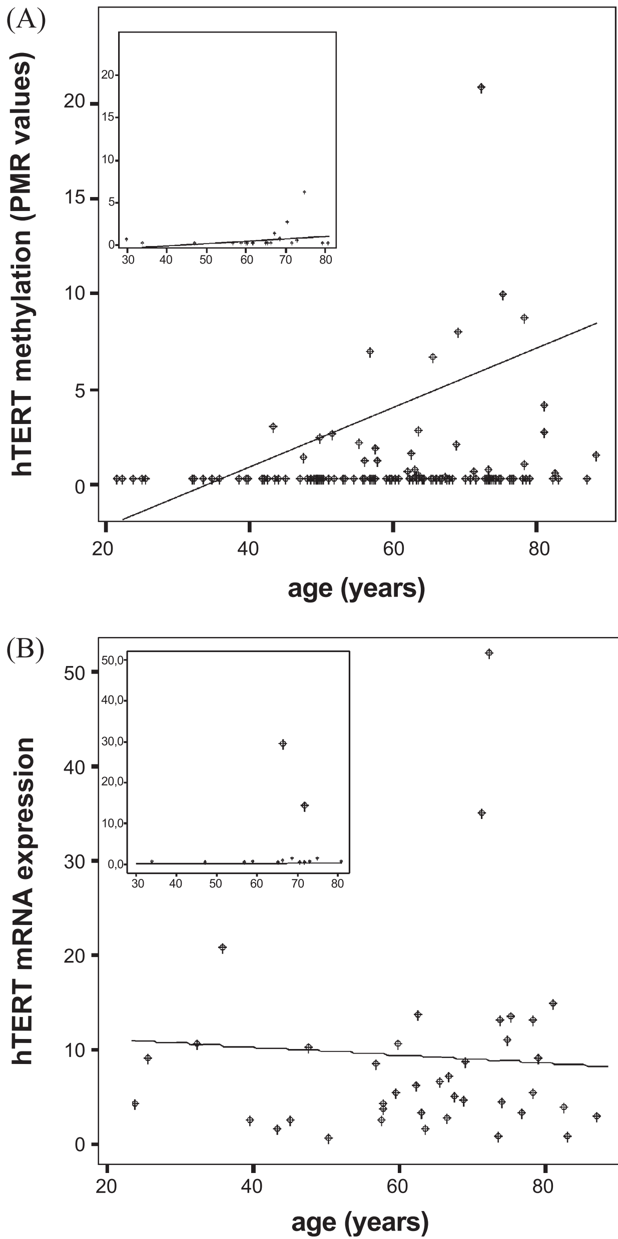

Fig. 2. Age-dependent *hTERT* methylation (A) and expression (B) in normal ovarian tissue (insert) and ovarian cancer tissue. Five PMR values above 25 are not shown. All five patients were older than 55 years.

and ovarian cancer tissues of patients with undetectable (negative) and detectable (positive) *hTERT* methylation. No statistically significant differences were found, except that *hTERT* methylation in ovarian cancer was detected more frequently in older patients. *hTERT* methylation was never observed in ovarian cancer or in normal ovaries in patients younger than 40 or 60, respectively. In both ovarian cancer and normal ovary, an increase in frequency of *hTERT* methylation with increasing age was observed, while *hTERT* mRNA expression was seen to be independent of age (Fig. 2).

hTERT expression and methylation

All normal cervical tissues showed low *hTERT* expression and were negative for *hTERT* methylation. *hTERT* expression was low in all 12 normal ovarian tissue samples and in four of these *hTERT*, methylation was observed. In cervical cancer, 8 out of 16 (50%) tissue samples with low *hTERT* expression were negative for *hTERT* methylation, and 13 out of 29 (44.8%) tissue samples with high *hTERT* expression were negative for *hTERT* methylation (P = 0.765; Fisher's Exact Test). In ovarian cancer tissues, 40% (6 out of 15) with low *hTERT* expression and 52% (13 out of

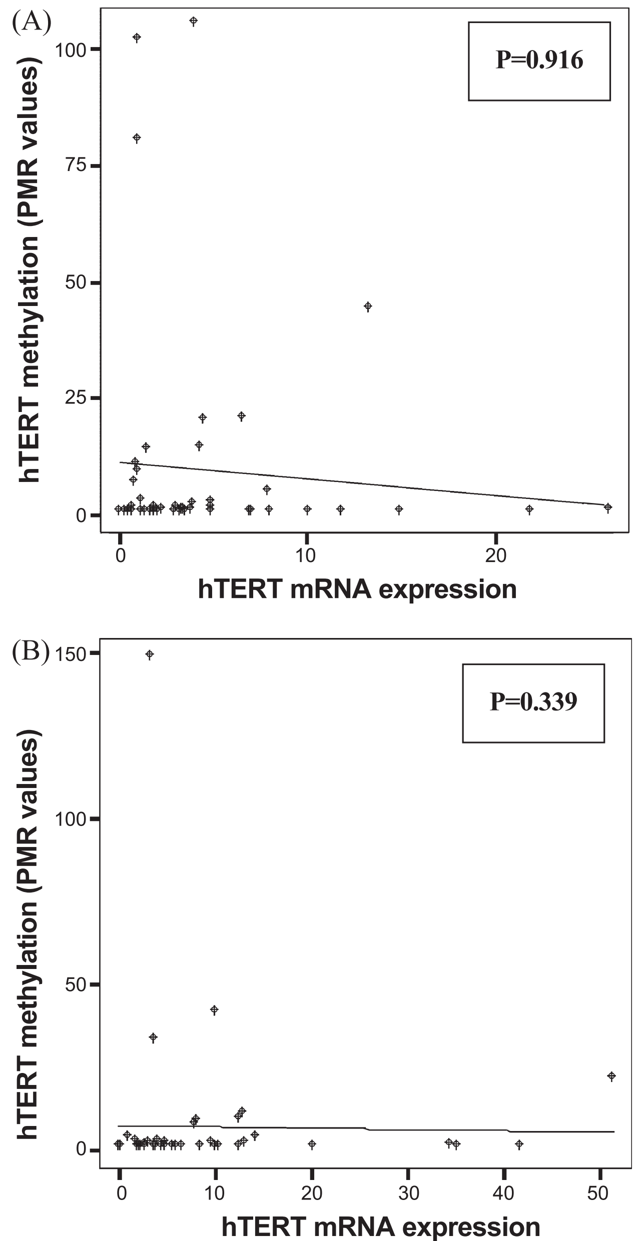

Fig. 3. *hTERT* mRNA expression and methylation in cervical (A) and ovarian (B) cancer.

25) with high *hTERT* expression were methylated (P = 0.527; Fisher's Exact Test). Even using nonparametric correlation (Spearman-Rho), no association could be observed between PMR values for *hTERT* and expression levels (Fig. 3).

Survival analysis

To determine whether any prognostic significance was attached to differences in *hTERT* expression and *hTERT* methylation, we compared the clinical outcome of cervical and ovarian cancer patients with *hTERT* expression and methylation status. A trend to better overall survival for patients with low *hTERT* expression was observed (Figs. 4A, C). Comparison of mean levels of *hTERT* mRNA expression in patients who died of disease and patients that did not revealed no significant differences in ovarian cancer (P = 0.359) and cervical cancer (P = 0.765). Both cervical and ovarian cancer patients with methylated *hTERT* had a significantly poorer overall survival in comparison to patients with unmethylated *hTERT* (Figs. 4B, D). To assess for independent prognostic significance, a Cox proportional hazard model analysis was performed. The logistic regression model included tumor stage, histology, grade of differentiation, age, and *hTERT* methylation status. In ovarian cancer, left tumor mass after surgery (no tumor left, less than

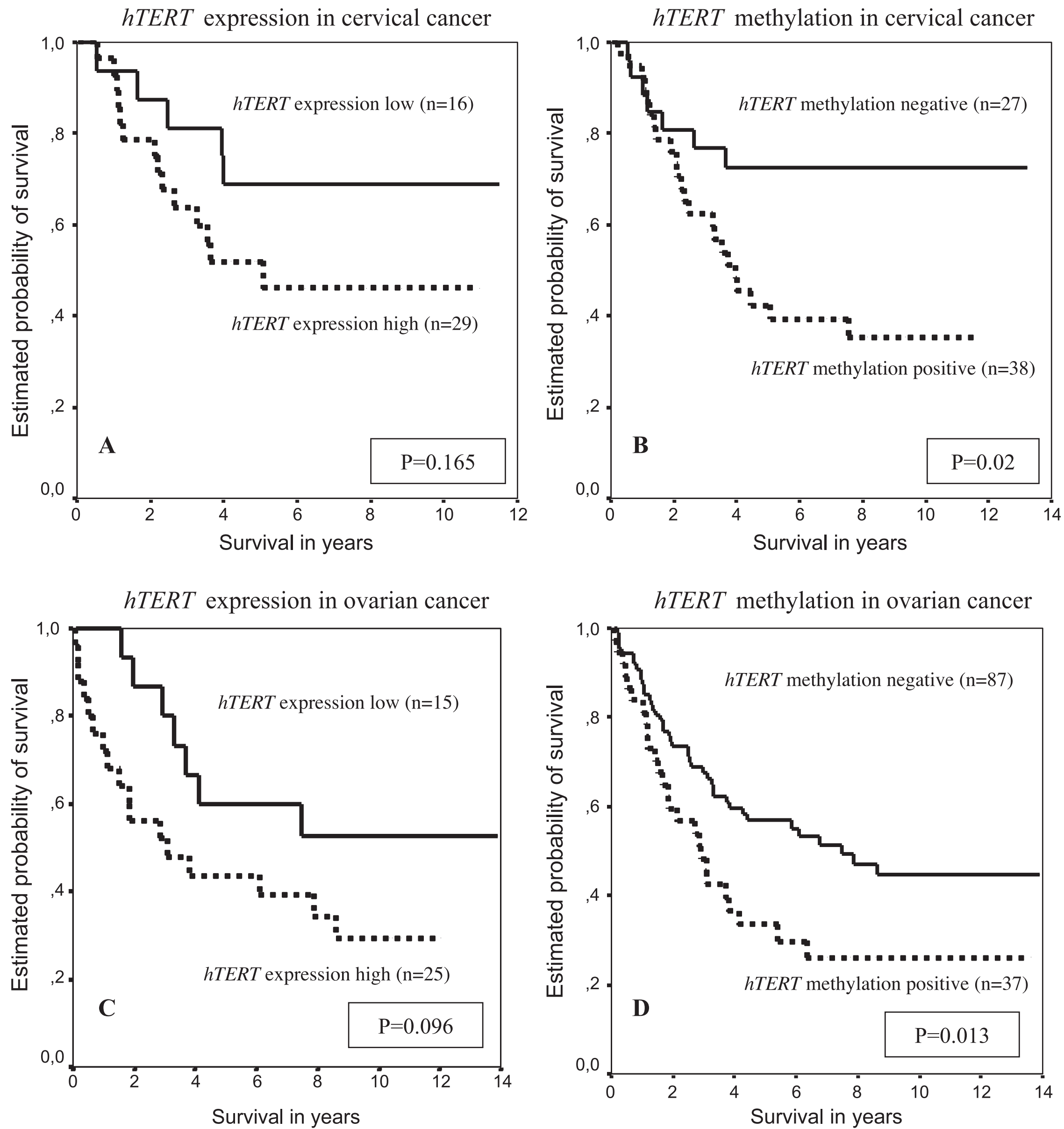

Fig. 4. Survival of cervical and ovarian cancer patients according to their *hTERT* expression level (low vs. high) and *hTERT* methylation status (positive vs. negative) in tumor tissues. In parentheses, the number of patients per group. The P of significance for differences in the survival function of the two groups was determined by the log-rank test.

2 cm left inside, more than 2 cm left inside) was included instead of tumor stage. In cervical cancer patients, only tumor stage ($P = 0.007$), and in ovarian cancer, only age ($P < 0.0001$) and left tumor mass ($P < 0.0001$) were of independent prognostic significance.

Discussion

This study examined *hTERT* expression and methylation pattern and the correlation with clinicopathological characteristics and survival in ovarian and cervical cancer.

It was shown that expression of *hTERT* correlates with telomerase activity during cellular differentiation and neoplastic transformation [15,16]. A strong correlation has been observed between *hTERT* mRNA expression and telomerase activity in a variety of epithelial cancers including cervical and ovarian carcinomas [16,26,52].

In our study, high *hTERT* expression was observed in ovarian and cervical cancer tissue in 62.5% and 64%, respectively. Normal ovarian and cervical tissue revealed only low *hTERT* expression. The median expression level of *hTERT* in cervical and ovarian cancer tissue was 18 and 50 times higher, respectively, as compared to normal tissue (Figs. 1A, C and 2B). These findings are in agreement with studies reporting *hTERT* expression predominantly in ovarian and cervical cancer but only rarely in benign tumors or normal tissue [24–26,28,29,53]. So far, several studies have reported the presence of telomerase activity or *hTERT* in association with clinicopathological characteristics in cervical or ovarian cancer [27,39–41]. It has been shown that high telomerase activity is related to advanced stage in cervical cancer, while no correlation was detected in ovarian cancer. Our study did not observe a relation between *hTERT* mRNA expression and FIGO stage, histology, grade of differentiation, or age in cervical or ovarian cancer.

A number of transcription factors, tumor suppressors, cell cycle inhibitors, cell fate-determining molecules, hormone receptors, and viral proteins have been implicated in the control of *hTERT* expression [34,54]. Recently, a role for regulation of *hTERT* expression and telomerase activity by methylation was suggested by the presence of a dense CpG-rich region in the promotor of *hTERT* [31]. The studies investigating methylation in the transcriptional control of *hTERT* are conflicting. While some authors describe a strong correlation between *hTERT* methylation and telomerase activity [35,55,56], others found no or only a partial correlation [36,37]. Our results revealed no relation between *hTERT* expression and methylation status, neither in ovarian nor in cervical cancer tissue. An explanation for these conflicting results may be the possibility that *hTERT* expression is differently regulated in various tissues. As mentioned above, *hTERT* gene regulation is influenced by several control mechanisms. One can speculate that these control mechanisms mask the regulatory influence of methylation in the expression of *hTERT* in several cancer tissues.

Methylation changes are known to be associated with aging in the normal epithelium and have been described, for instance, for the *estrogen receptor*, *p16* exon 1 and *MYOD* [57]. Age-dependent methylation in cancer tissue has not been observed up to now. In our study, we observed a trend to increasing *hTERT* methylation with increasing age in both ovarian cancer and normal ovarian tissue. Several reasons have been supposed for age-related methylation in normal epithelium. It has been suggested that some CpG islands progressively lose protection from methylation, because repetitive transcription of a gene weakens the putative DNA–protein interactions that may be protective [58]. Another potential cause of methylation to be considered is damage by reactive oxygen species that could trigger or accelerate CpG island methylation [59,60].

It has been reported that high *hTERT* expression or telomerase activity is associated with poor prognosis in several malignancies [48,61,62]. In cervical cancer, no correlation between telomerase activity and survival was observed [27,39]; no data for ovarian cancer exist concerning this correlation. Our own data revealed a trend to better overall survival for patients with low *hTERT* expression in both ovarian and cervical cancer patients.

There is increasing evidence that the methylation status of certain genes in various tumor entities might provide independent prognostic information [63–65]. To date, no studies have been published investigating *hTERT* methylation as a prognostic factor concerning survival in ovarian and cervical cancer. Our study demonstrates for the first time that *hTERT* methylation is of prognostic significance in ovarian and cervical cancer. Although univariate analysis revealed significant results, multivariate analysis including FIGO stage, histology, grade of differentiation, and *hTERT* methylation showed no independent prognostic value for *hTERT* methylation. The lack of independent prognostic significance in cervical cancer patients may be due to the smaller number of investigated patients. In ovarian cancer, a higher age was observed among those patients with *hTERT*-methylated tumor tissue. This effect could contribute to the poorer prognosis of these patients.

It remains to be seen whether age-determined *hTERT* methylation of tumor cells selects for resistant clones, which are responsible for relapse and subsequently lead to death.

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