Endocrine, *vol. 22, no. 3, 193–201, December 2003* 0969–711X/03/22:193–201/$25.00

HSP90 Is a Key for Telomerase Activation and Malignant Transition in Pheochromocytoma

Carsten Boltze,[1] Hendrik Lehnert,[2] Regine Schneider-Stock,[1] Brigitte Peters,[3] Cuong Hoang-Vu,[4] and Albert Roessner[1]

Departments of [1]Pathology, [2]Endocrinology and Metabolism, and [3]Biometrics, Otto-von-Guericke-University Magdeburg, Magdeburg, Germany; and [4]Clinic of General Surgery, Martin-Luther-University Halle-Wittenberg, Halle, Germany

Recent studies on a limited number of pheochromocytomas (PCs) revealed a potential role of telomerase in the malignant transition of these tumors. Telomerase is a ribonucleoprotein complex that includes the telomerase RNA component (hTR), the telomerase-associated protein (TP1), the telomerase catalytic subunit (hTERT), and the heat-shock protein 90 (HSP90). The interactions between these subunits and the activation machinery of telomerase are still unclear. To test whether the expression and regulation of telomerase subunits are reflected in the malignant transition of PCs, we determined their mRNA and/or protein expression in 28 benign and 9 malignant PCs and compared the results with telomerase activity. Reverse transcriptase polymerase chain reaction analysis revealed that TP1 was ubiquitously expressed. hTR was found in all malignant (100%) and in 13/28 (46%) benign PCs. By contrast, hTERT was clearly associated with aggressive biologic behavior. All the malignant (100%) but only 2/28 benign (7%) PCs expressed hTERT. HSP90 was increased in malignant PCs but was also expressed at a lower level in benign tumors. High telomerase activity was measurable in only hTERT-positive tissues. Our data indicate that hTERT, HSP90, and telomerase activity are upregulated in malignant cells of the adrenal medulla. Overexpression of HSP90 is an important factor in the activation of telomerase via hTERT. The common expression of hTERT and telomerase activity thus represents an additional prognostic marker that may identify more aggressive tumors.

Key Words: HSP90; telomerase; pheochromocytoma; hTERT.

Introduction

Complex mechanisms regulating cellular life-span have evolved in mammalian cells. Normal cells show a strictly limited growth potential and senescence after a defined number of cell divisions. By contrast, tumor cells often exhibit an apparently unlimited proliferation potential and are termed *immortalized*. It has been proposed that the progressive shortening of the ends of the eukaryotic chromosomes, the telomeres, is an important component of senescence and is involved in the control of the cell cycle. The conventional DNA replication machinery cannot copy extreme terminal sequences of the lagging strand during replication of linear chromosomes; thus, 50–200 bp of telomeric DNA will be lost during each cell division. This problem is remedied by production of telomerase, a ribonucleoprotein enzyme-complex that synthesizes and maintains telomeric DNA by adding the hexanucleotide repeat units on the 3' ends of the single-stranded DNA *(1)*.

Telomerases consist of several protein components and an RNA subunit. The intrinsic RNA component of telomerase complex (hTR) displays low sequence homology but shares a conserved, predicted secondary structure consisting of a stem, a pseudoknot, and a set of stem-loop structures. Expression of hTR is no predictor of telomerase activity, because hTR is expressed in all the cells *(2)*. The catalytic subunit of the telomerase complex is the human homolog of the yeast *Saccharomyces cerevisiae* gene EST2, a specialized reverse transcriptase (RT) that relies on an associated RNA to provide a template for the synthesis of DNA repeats. Accordingly, it is designated hTERT (human telomerase reverse transcriptase), also known as hTRT, hTCS1, TP2, and hEST2 *(3)*. The heat-shock protein (HSP) 90 has been demonstrated to bind to hTERT and is considered a telomerase subunit *(4)*. Geldanamycin, an HSP90 inhibitor, has been found to reduce the activity of reconstituted telomerase in cell extracts, demonstrating the role of HSP90 in the holoenzyme complex *(5)*. Besides hTERT and hTR, which are apparently both necessary and sufficient to form the catalytic core of telomerase, several other proteins that form a complex with the core enzyme have been identified *(6)*. One telomerase-associated protein, TP1, which is presumably the human homolog of the *Tetrahymena* telomerase

Received July 1, 2003; Revised August 25, 2003; Accepted August 25, 2003.

Author to whom all correspondence and reprint requests should be addressed: Carsten Boltze, Department of Pathology, Otto-von-Guericke-University, Leipziger Strasse 44, D-39120 Magdeburg, Germany. E-mail: carsten.boltze@medizin.uni-magdeburg.de

p80 gene, is a large, ubiquitously expressed protein (230–240 kDa) that binds to hTR *(7)*. Telomerase is expressed by most malignant cells and is normally inactive in most somatic cells, with the exception of proliferative stem cells, male germ cells, and activated lymphocytes *(8)*. The activation of telomerase in malignant neoplasms seems to be an important step in tumorigenesis in order to gain the ability to proliferate indefinitely and to become immortal.

Discrimination between benign pheochromocytoma (BP) and malignant pheochromocytoma (MP) and, in particular, prediction of malignancy remain highly difficult tasks. With the exception of the demonstration of distant metastases, there are no clear-cut criteria that distinguish between benign and malignant tumors in the adrenal medulla. Both capsular invasion and focal vascular invasion are insufficient criteria since both may be observed in BPs as well. According to the long-term follow-up studies of Linnoila et al. *(9)*, Goldstein et al. *(10)*, and John et al. *(11)*, the following features are more frequent in malignant tumors: male predominance, extraadrenal location, greater tumor weight (<80 g), confluent tumor necrosis, and the presence of vascular invasion and/or extensive local invasion. Hyaline cytoplasmic globules were found in 59% of benign and in 32% of malignant tumors. Extraadrenal location, coarse nodularity of the primary tumor, confluent tumor necrosis, and the absence of cytoplasmic hyaline globules were most predictive of malignancy. Similarly, employing immunohistochemical or molecular markers still remains an inconclusive means in predicting malignant behavior. Thus far, only the MIB-1-labeling index (<3%) has been proven important *(12)*. Thus, the diagnostic dilemma of determining the nature of pheochromocytoma (PC) still needs to be resolved. This is particularly important, because approx 10–15% of PCs are probably malignant *(13)*.

There is a steadily growing interest in the potential application of telomerase as a diagnostic and prognostic marker. For this reason, the aim of the present investigation was to analyze the dynamics of various genes (TP1, hTR, hTERT, and HSP90) of the telomerase ribonucleoprotein enzyme-complex in PCs and to study whether the expression of these genes and telomerase activity could serve as a diagnostic marker distinguishing benign from malignant neoplasms. Furthermore, the mechanism of activation and regulation between the telomerase subunits was investigated.

Results

Clinicopathological Data

Comparing demographic with clinical data, we found the following pattern: the mean age of the BP group was 54.3. The patients of the MP group with poorer clinical prognosis were, on average the older (mean age: 60.4 yr; $p = 0.067$; Table 2). The MP group consisted of 20 males and 8 females, whereas 6 males and 3 females constituted the BP group ($p = 0.786$; Table 2). Of 28 patients with BP, 24 were still alive and 4 died of another disease after a 44.8-mo follow-up (range: 24–72 mo). Neither recurrences nor metastases were detectable during this time. In the MP group, eight of nine patients had tumor metastasis after a tumor-free time of 17.8 mo (ranging from 0 to 42 mo). Only one patient (no. 34; Table 1) was tumor free after 37 mo. Two patients (nos. 36 and 37) had metastases (lymph nodes and liver) at the time of surgery. Four of nine MP patients died of disease.

Proliferation Study

The mean tumor weight in BPs (80.5 g) and MPs (94.5 g) was not statistically different ($p = 0.12$). By contrast, MPs had a substantially higher proliferation rate than BPs (10.8 vs 1.3%; $p < 0.001$). Considering MPs and BPs as one group, there is no significant correlation between proliferation rate and tumor weight ($p = 0.09$). In MPs alone, however, proliferation rate correlates significantly with tumor weight ($p = 0.02$).

Telomerase Genes and Activity in PCs

TP1

The mRNA coding for telomerase-associated protein was ubiquitously and equally expressed in all BP and MP (Fig. 1, Table 1).

hTR

The intrinsic RNA component of telomerase complex showed a different expression pattern. RT-polymerase chain reaction (PCR) revealed that hTR was expressed in 13 of 28 BPs (46%) and in all 9 MPs (100%; Fig. 1 and Table 1). No statistically significant correlation was observed between hTR expression and age ($p = 0.54$), sex ($p = 0.78$), tumor size ($p = 0.67$), or proliferation index ($p = 0.11$) in BPs.

hTERT

The expression of mRNA for the catalytic subunit hTERT was associated with malignancy. hTERT was expressed in only 2 of 28 BPs and in all of the 9 MP ($p < 0.01$; Fig. 1 and Table 2). In the two hTERT-positive BPs (nos. 27 and 28; Table 1), there was a strong infiltration of lymphocytes, suggesting chronic inflammation. These two tumors were also found among the largest PCs in BPs and derived from the oldest patients. In addition, the proliferation index was elevated. Complete agreement was found between mRNA and protein expression. Protein expression was only detectable in mRNA-positive cases. In these cases, the share of stained nuclei was 28.1% for BPs and 72.1% for MPs ($p < 0.001$) (Fig. 2 and Table 1).

HSP90

All of the BPs expressed the HSP90 protein in the cytoplasm and, sporadically, in the nuclei at a similarly low level. The Remmele-Score ranged between 3 and 6 (mean: 4.50). Patients 27 and 28 with positive hTERT expression also showed a low HSP90 expression (Remmele-Score: 4;

Table 1
Clinical and Tumor-Specific Data,
Expression of Telomerase-Related Genes (TP1, hTR, hTERT, HSP90), and Telomerase Activity in BP and MP

Case no./ biologic behavior	Age (yr)/ sex	Tumor weight (g)	Proliferation index (%)	Expression of					Telomerase activity	Tumor-free survival (mo)[b]
				TP1	hTR	hTERT		HSP90[c]		
						RT-PCR	IHC[a]			
1 BP	50 f	12	0.1	+	–	–	0	6	–	48 (AL)
2 BP	27 f	13	0.1	+	–	–	0	4	–	56 (AL)
3 BP	38 m	27	0.3	+	–	–	0	4	–	24 (AL)
4 BP	65 m	28	1.1	+	–	–	0	4	–	37 (AL)
5 BP	64 f	32	0.5	+	+	–	0	3	–	60 (AL)
6 BP	58 m	38	0.3	+	+	–	0	4	–	71 (AL)
7 BP	67 m	44	1.2	+	+	–	0	6	–	28 (DAD)
8 BP	63 m	50	1.0	+	–	–	0	6	–	54 (AL)
9 BP	48 m	51	2.2	+	+	–	0	4	–	62 (DAD)
10 BP	62 f	54	0.3	+	+	–	0	6	–	38 (AL)
11 BP	35 m	58	0.7	+	–	–	0	4	–	34 (AL)
12 BP	37 f	62	0.4	+	–	–	0	4	–	46 (AL)
13 BP	48 m	68	0.2	+	–	–	0	4	–	28 (AL)
14 BP	51 m	72	1.1	+	–	–	0	6	–	39 (AL)
15 BP	64 m	78	1.4	+	+	–	0	3	–	50 (AL)
16 BP	59 m	81	0.1	+	+	–	0	4	–	72 (AL)
17 BP	61 m	88	0.5	+	–	–	0	4	–	29 (DAD)
18 BP	64 m	92	0.7	+	+	–	0	6	–	48 (AL)
19 BP	53 m	93	2.3	+	+	–	0	4	–	42 (DAD)
20 BP	61 f	94	1.2	+	+	–	0	6		38 (AL)
21 BP	33 m	108	2.2	+	–	–	0	6	–	44 (AL)
22 BP	37 f	117	1.4	+	–	–	0	3	–	46 (AL)
23 BP	48 m	134	1.1	+	–	–	0	3	–	28 (AL)
24 BP	65 m	138	2.5	+	–	–	0	4	–	37 (AL)
25 BP	64 f	152	1.8	+	+	–	0	4	–	60 (AL)
26 BP	58 m	163	2.7	+	–	–	0	6	–	71 (AL)
27 BP	68 m	132	5.2	+	+	+	30.2	4	–	28 (AL)
28 BP	72 m	177	3.3	+	+	+	25.9	4	–	35 (AL)
29 MP	63 m	43	3.6	+	+	+	30.9	12	+	42 (MET/AL)
30 MP	61 f	54	2.4	+	+	+	88.3	9	+	18 (MET/AL)
31 MP	53 m	62	6.2	+	+	+	63.1	9	+	14 (MET/AL)
32 MP	47 f	63	7.2	+	+	+	44.0	12	+	36 (MET/AL)
33 MP	67 m	70	24.3	+	+	+	59.0	12	+	8 (MET/DOD)
34 MP	66 m	98	11.1	+	+	+	90.8	12	+	37 (AL)
35 MP	62 f	122	3.8	+	+	+	95.3	9	+	5 (MET/DOD)
36 MP	59 m	147	12.9	+	+	+	94.1	9	+	0 (MET/DOD)
37 MP	66 m	192	25.8	+	+	+	83.2	8	+	0 (MET/DOD)

[a]IHC, immunohistochemistry. Numbers under IHC represent percentage of immunopositively stained nuclei.
[b]AL, alive; DAD, died of another disease; DOD, died of disease; MET, metastasis.
[c]HSP data = Remmele-Score.

Fig. 2 and Table 1). By contrast, MPs were very strongly immunopositive for HSP90 (Remmele-Score: 8–12; mean: 10.22; $p < 0.01$; Fig. 2, Table 2).

Telomerase Activity

All 28 BPs were negative for telomerase activity. By contrast, T-activity was detectable in all MPs ($p < 0.001$) (Fig. 3, Tables 1 and 2). Telomerase activity was only measurable in tissues that expressed the catalytic subunit hTERT ($p < 0.001$). In BPs, expression of hTERT does not lead to activation of telomerase.

Discussion

In the last few years, telomerase has attracted considerable interest as a discrimination marker and a possible target for therapeutic intervention of malignant tumors. In colorectal and gastric carcinomas *(14,15)*, breast tumors

Table 2
Uni- and Multivariate Analyses
of the Demographic, Tumor-Specific, and Experimental Data[a]

Factor	BP (n = 28) (mean ± SD)	MP (n = 9) (mean ± SD)	p Value
Age (yr)	54.29 ± 12.36	60.44 ± 6.64	0.067
Tumor weight (g)	80.57 ± 45.79	94.56 ± 49.97	0.441
Proliferation (%)	1.28 ± 1.172	10.81 ± 8.784	<0.01
HSP90 (score)	4.50 ± 1.380	10.22 ± 2.710	<0.01
Positive for	n (%)	n (%)	
TP1	28 (100)	9 (100)	n.i.
hTR	13 (46)	9 (100)	<0.01
hTERT	2 (7)	9 (100)	<0.01
Telomerase	0 (0)	9 (100)	<0.01
Sex/male	20 (71)	6 (67)	
Sex/female	8 (29)	3 (33)	0.786
Factor	Confidence interval	Risk	p Value
hTERT	1.085–39.012	6.506	0.040

[a]Multivariate analysis (logistic regression) with biologic behavior as dependent factor, and age, sex, tumor weight, proliferation index, HSP90 expression, and hTR and hTERT alterations as independent factors. Telomerase and TP1 were excluded. After statistical reduction, only hTERT was identified as an independent prognostic marker. n.i., noninformative because no differences were found.

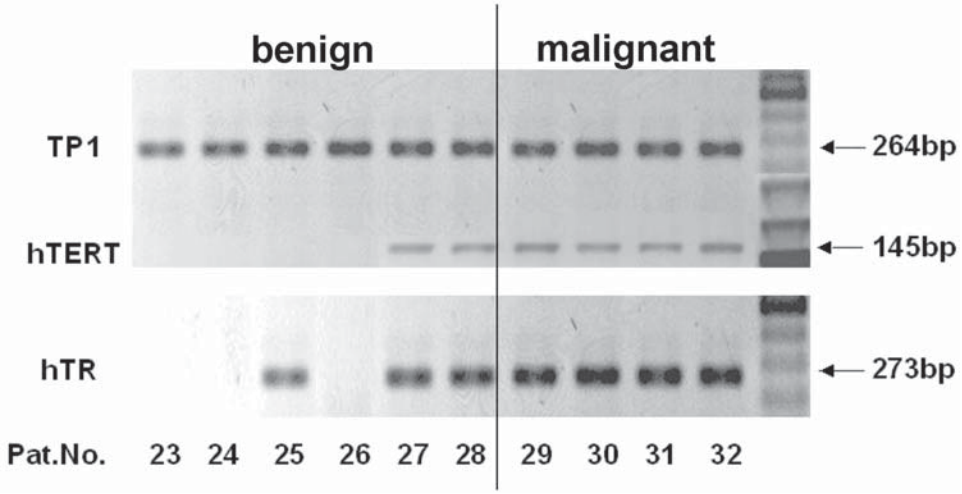

Fig. 1. Examples for expression of telomerase-related genes in PC tissue of 10 patients. Patient's 23–28 had BP; patient's 29–32 had MP. For further clinical data see Table 1.

(16), thyroid carcinomas *(17)*, and liposarcomas *(18)*, telomerase activity has been described as a marker that correlated significantly with malignancy and progression of the clinical tumor stages. However, recent studies reported that telomerase activity was also expressed in normal nonneoplastic tissues and nonneoplastic hyperproliferative lesions *(16,19)*. Analysis of the components of the human telomerase complex indicates that their expression often does not correlate with telomerase activity. This applies to the RNA component hTR, which is expressed irrespective of the immortalization status *(20)*, and to TP1, which is ubiquitously expressed *(7)*. However, the newly cloned putative telomerase subunit hTERT may indeed be a marker for immortalization *(21,22)*. hTERT was shown to be expressed in tumor cell lines and in a number of tumors. By contrast, no expression was detected in normal fibroblast-derived

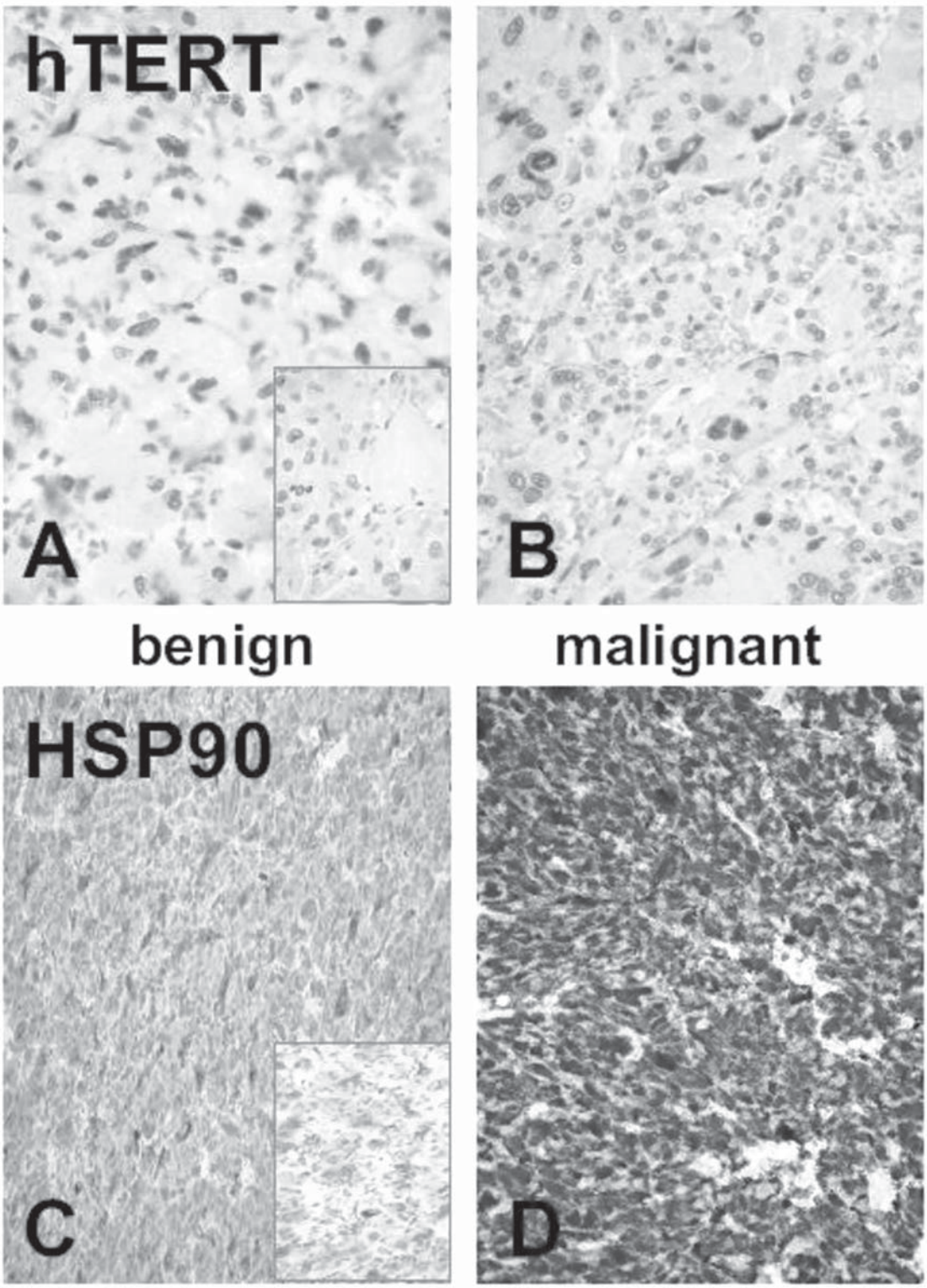

Fig. 2. Detection of hTERT and HSP90 protein expression in PCs. (**A**) Ninety-three percent of BPs were immunonegative; only two cases showed positivity (inset = patient no. 28). (**B**) By contrast, all MPs were immunopositive for hTERT with strongly stained nuclei. (**C**) All the BPs expressed HSP90 at a similarly low level. In addition, patient 28 with positive hTERT expression showed a low HSP90 expression (inset, Remmele-Score = 4). (**D**) MPs had a very strong immunopositivity for HSP90 (Remmele-Score = 9–12).

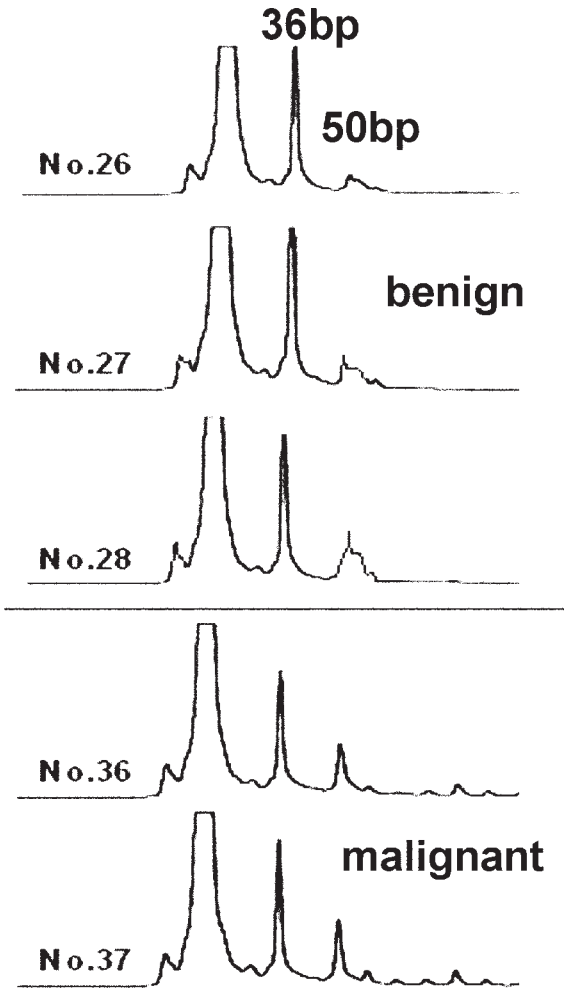

Fig. 3. Nonradioactive TRAP assay in BPs and MPs. BPs (cases 26–28) without telomerase activity only showed the 50-bp increment and the 36-bp internal control, whereas the high-positive MPs (cases 36 and 37) showed a ladder-like pattern of PCR products. BPs 27 and 28 were immunopositive for hTERT, but telomerase negative.

mortal cell lines or in normal tissues, such as heart, brain, placenta, liver, skeletal muscle, breast, ovary, and prostate *(2)*.

Because an accurate method for diagnosing malignant PC does not exist, telomerase activity, which indicates the presence of immortal cells, and the catalytic subunit of telomerase, might represent useful diagnostic tools. The present study is the first for which a sufficiently large number of samples were available. It demonstrates that hTERT was clearly and highly significantly associated with differentiation, whereas the telomerase RNA component (hTR) was found in all malignant and in almost half of BPs. In addition, telomerase activity was measurable only in the hTERT-positive tissues, suggesting that these markers represent an important addition to prognostic markers in PC. Our data now clearly demonstrate that telomerase is not expressed in benign PC cells. To date, only telomerase activity has been investigated in a few studies, and the results achieved were equivocal. In one study, low mean telomerase activity was found in eight of eight BPs and in two of three MPs. Telomerase activity was high in only one MP *(23)*. Two other studies yielded divergent results, showing that, based on a highly sensitive PCR detection method, telomerase activity was detected in 1/13 adrenal cortical tumors and in 2/7 PCs; these telomerase-positive tumors were found to exhibit pathologic features suggesting a malignant potential *(24)*. Similarly, in another study, telomerase activity was detected neither in normal adrenal medulla nor in BPs, whereas all three MPs showed elevated telomerase activity *(25)*. Thus, our data clearly exceed the data, reported in previous studies, demonstrating the importance of both telomerase activity and hTERT expression. From these data, it now appears to be clear that the study of multiple components of the telomeric complex is necessary to discriminate between these two types of PCs.

Our data confirm that TP1 is ubiquitously expressed in benign and malignant tumors *(7)*, as well as in BP and MP. The expression of hTR showed a very different picture. hTR expression does not correlate with telomerase activity. Interestingly, telomerase activity is also measurable without hTR expression, and hTR is also expressed in telomerase-negative cells, an observation that has already been described in other cell systems *(2,19)*. In these cases, the restoration of telomerase activity through ectopic expression of hTERT leads to telomere lengthening with a significant extension of life-span. This definitively excludes TP1 and hTR as markers distinguishing between BP and MP.

The expression of hTERT was associated with malignancy. Only two benign neoplasms (7%) showed positivity. However, these cases had no telomerase activity in the telomerase repeat amplification protocol (TRAP) assay. At first, we could not explain this phenomenon. The critical reanalysis of these positive cases revealed that they contained extensive lymphoid infiltrates with germinal centers, which was also observed in other studies *(26,27)*. Recent studies investigated other binding proteins (i.e., molecular chaperone p23, HSP90) of the telomerase complex *(28)*. HSP90 has been demonstrated to bind to hTERT and contribute to telomerase activity *(4)*. To investigate this fact in PCs, we conducted an HSP90 protein expression study. Although HSP90 was detectable in all tissues, significant up-regulation was found in the telomerase-positive MPs. The two hTERT-positive BPs and all the other BPs showed only low protein expression. In a p23 study (data not shown), no differences were found between MP and BP.

HSP90 and p23 have been identified as a component of progesterone and glucocorticoid receptor complexes. Subsequently, it was found that the presence of both molecules is required to maintain these receptors in a ligand-binding state *(29)*. These observations led to the concept of a molec-

ular chaperone machinery or foldosome that mediates the assembly of a biologically active protein complex. Similarly, HSP90 and p23 in the telomerase complex may also serve this foldosome function in assembling the active holoenzyme. This mechanistic model and our results indicate that overexpression of not only hTERT but also HSP90 is strongly associated with the activation of telomerase. Only the nine malignant PCs were telomerase positive in the TRAP assay. These tumors expressed hTERT and overexpressed HSP90. In addition, hTR and TP1 were detectable in the MPs.

In summary, our data, obtained from a representative number of BPs and MPs, show that telomerase activity is upregulated in neoplastic cells. The data also show that the concomitant expression of hTERT and telomerase activity and overexpression of HSP90 are potential prognostic markers for the identification of invasive tumors but may also be used as additional diagnostic markers for the classification of PCs.

Materials and Methods

Ethical Approval

The studies involving the use of human tissues described in this article were approved by the local ethical committees, and all patients gave their written consent.

PC Tissues

PC tissue was obtained from 37 patients undergoing surgery between 1994 and 2000. All tumors showed the "classic" histology and the typical immunohistochemical pattern (positive immunoreactivity for vimentin, chromogranin, synaptophysin, and S100). To predict the malignant potential of these tumors, we used four validated criteria from long-term follow-up-studies: metastasis, vascular invasion, MIB-1 labeling >3%, and tumor weight >80 g. A PC was considered to be malignant if metastases were found or all of the other three criteria were applied. According to these criteria, there were two clinically defined subject groups: 28 benign and 9 malignant intramedullar PCs. The benign group consisted of tumor samples obtained from 8 females and 20 males (median: 52.2 yr; range: minimum of 27 yr, maximum of 72 yr; follow-up: median of 44.8 mo; range: minimum of 24 mo, maximum of 72 mo). The MP group consisted of three females and six males (median: 60.4 yr; range: minimum of 47 yr, maximum of 67 yr; tumor-free survival time: median: 17.8 mo; range: minimum of 0 mo, maximum of 42 mo) (Tables 1 and 2).

RNA Isolation and RT-PCR

RNA was isolated from frozen PC tissues using the Trizol reagent (Gibco, Munich, Germany) according to the manufacturer's recommendations. cDNA was synthesized from total RNAs using a Superscript II-Kit (Gibco).

PCR primers were as follows: *TP1*: 5'-TCAAGCCAAA CCTGAATCTGAG-3' (sense) and 5'-CCCGAGTGAAT CTTTCTACGC-3' (antisense), amplicon 264 bp; *hTR*: 5'-CCTAACTGAGAAGGGCGTAGGC-3' (sense) and 5'-C TAGAATGAACGGTGGAAGGCG-3' (antisense), amplicon 273 bp; *hTERT*: 5'-CGGAAGAGTGTCTGGAGCAA-3' (sense) and 5'-GGATGAAGCGGAGTCTGGA-3' (antisense), amplicon 145 bp; *GAPDH*: 5'-CATCACCATCTT CCAGGAGCG-3' (sense) and 5'-TGACCTTGCCCACA GCCTTG-3' (antisense), amplicon 443 bp.

A negative control without cDNA was included in each experiment. PCR products were resolved on ultrathin native polyacrylamide gels crosslinked with piperazine diacrylamide, baked on GelBond Pag (FMC, Rockland, ME), and visualized by silver staining. Bands were directly quantified by laser densitometry VDS (Amersham Pharmacia Biotech, Denver, CO). Expression was evaluated by estimating the intensity ratio among the *TP1*, *hTR*, and *hTERT* signal, and the corresponding *GAPDH* signal. Normal placenta tissue was used as a negative control, and its expression level was defined as normal. In comparison with the placenta, threefold or more intensified signals were regarded as increased expression. The expression levels were classified as follows: –, no expression; +, low expression (3- to 10-fold); ++, high expression (more than 10-fold). Tissue from an expanded endometrioid ovarian carcinoma with a three- (*TP1*), three- (*hTR*), and fourfold (*hTERT*) increased expression was used as a positive control. To ensure consistency, each PCR reaction was carried out twice.

hTERT Immunohistochemistry

For immunohistochemical evaluation of *hTERT* on paraffin-embedded sections, a new staining procedure was developed, because the antibody used has only been applied to frozen tissue. Sections (3 µm) were pretreated in a microwave (5 min at 600 W and 15 min at 450 W) in Glycabuffer (pH 3.0) after deparaffinization and rehydratation. Endogenous peroxidase activity was inhibited with a 3% solution of H_2O_2 in pH 7.5 phosphate-buffered saline (PBS) for 15 min. Normal horse serum diluted 1:20 with PBS and containing 1% bovine serum albumin was incubated with the samples for 20 min to suppress nonspecific binding. Tissue sections were incubated with the polyclonal antibody TRT (H-231; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution for 1 h at 37°C. After washing in PBS, the samples were incubated with a 1:200 dilution of biotinylated antigoat secondary antibody (Vector) for 30 min at room temperature. The detection of the bound antibody was accomplished by the avidin-biotin complex method (Vectastain Elite ABC Kit; Vector). A 0.1% solution of 3,3'-diaminobenzidine (DAB) (5 min) (Sigma) was used as chromogen. Specificity of immunostaining was checked by omitting single steps in the immunohistochemical protocol and by replacing the primary antibody with nonimmune serum. Three sections of each tissue were examined by two independent reviewers blinded to their identity. In 10 high-

power fields of each section (six peripheral and four central high-power fields [HPF]), positively stained nuclei were counted and represented as a percentage.

HSP90 Immunohistochemistry

Immunohistochemical studies for *HSP90* also employed the avidin-biotin complex method. The 3-µm-thick paraffin-embedded sections were rehydrated step-by-step with descending concentrations of ethanol before the staining procedure. Endogenous peroxidase was blocked by incubation in methanol containing 0.3% H_2O_2 for 35 min. After washing in 0.01 *M* PBS, the slides were incubated with 10% normal goat serum for 60 min to block nonspecific binding sites. Normal goat serum was removed and followed by incubation with the specific polyclonal antibody *HSP90* (H-114; Santa Cruz Biotechnology) at a 1:100 dilution overnight at room temperature, with subsequent staining as described for *hTERT*. Immunostaining was repeated on at least one occasion, and the results were assessed by considering both the scoring intensity of staining and the proportion of maximally stained cells as recommended by Remmele et al. *(30)*. Maximally stained cells were proportionally distributed as follows: missing = 0, <10% = 1, 11–50% = 2, 51–80% = 3, >80% = 4. Staining intensity was classified as negative = 0, slight = 1, abundant = 2, or strong = 3. The product of staining intensity and percentage of positive cells describes the score (0–12). A score of 0–2 was regarded as negative or low, 3–6 as moderate, and >6 as strong.

Telomerase Assay

For telomerase activity analysis, a commercially available Telomerase PCR ELISA-Kit (Boehringer Mannheim, Germany) was used. This is a photometric enzyme immunoassay utilizing the TRAP assay with nonradioactive detection, which is an extension of the original method described by Kim and Wu *(31)*. The assay procedures were done as described in the kit manual. All determinations were performed in triplicate.

Proliferation Study

To detect the proliferation rate, an immunohistochemical study for the *Ki-67* antigen was done on representative paraffin sections with the use of appropriate positive and negative controls throughout. The monoclonal antibody anti-*Ki-67* (Mib-1, 1:50; Dianova, Hamburg, Germany) was used. The alkaline phosphatase antialkaline phosphatase method was carried out on 4-µm-thick tissue sections, previously deparaffinized with xylene for 15 min, and treated in a microwave oven using 0.01 mol/L of citrate buffer (pH 6.0) for 30 min. Incubation was done with the primary antibody at 37°C. After washing, the sections were incubated for 30 min at room temperature with a biotinylated horse antimouse antibody and an avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; Vector). The final reaction product was shown by incubation with DAB (0.1% solution, 5 min) (Sigma), and the nuclei were counterstained with Gui's hematoxylin. Positively stained nuclei (10 HPF/section) were counted and represented as a percentage.

Statistical Analysis

Telomerase activity, expression of the different telomerase genes, and the clinical parameters including pathologic data were tested for statistical significance by Student's *t*-test and uni- and multivariate analysis. Values of $p < 0.05$ were considered to be statistically significant.

Acknowledgments

We gratefully acknowledge the technical assistance of Carola Kügler, Claudia Miethke, Nadine Wiest, and Kathrin Hammje.

References

1. Levy, M. Z., Allsopp, R. C., Futcher, A. B., Greider, C. W., and Harley, C. B. (1992). *J. Mol. Biol.* **225,** 951–960.
2. Avilion, A. A., Piatyszek, M. A., Gupta, J., Shay, J. W., Bacchetti, S., and Greider, C. W. (1996). *Cancer Res.* **56,** 645–650.
3. Nakamura, T. M., Morin, G. B., Chapman, K. B., et al. (1997). *Science* **277,** 955–959.
4. Holt, S. E., Aisner, D. L., Baur, J., et al. (1999). *Genes Dev.* **13,** 817–826.
5. Masutomi, K., Kaneko, S., Hayashi, N., et al. (2000). *J. Biol. Chem.* **275,** 22568–22573.
6. Lundblad, V. (1998). *Proc. Natl. Acad. Sci. USA* **95,** 8415–8416.
7. Harrington, L., McPhail, T., Mar, V., et al. (1997). *Science* **275,** 973–977.
8. Zhu, X., Kumar, R., Mandal, M., et al. (1996). *Proc. Natl. Acad. Sci. USA* **93,** 6091–6095.
9. Linnoila, R. I., Keiser, H. R., Steinberg, S. M., and Lack, E. E. (1990). *Hum. Pathol.* **21,** 1168–1180.
10. Goldstein, R. E., O´Neill, J. A. Jr., Holcomb, G. W. III, et al. (1999). *Ann. Surg.* **229,** 755–764.
11. John, H., Ziegler, W. H., Hauri, D., and Jaeger, P. (1999). *Urology* **53,** 679–683.
12. Clarke, M. R., Weyant, R. J., Watson, C. G., and Carty, S. E. (1998). *Hum. Pathol.* **28,** 522–526.
13. Mundschenk, J. and Lehnert, H. (1998). *Exp. Clin. Endocrinol. Diabetes* **106,** 373–376.
14. Okayasu, I., Mitomi, H., Yamashita, K., et al. (1998). *J. Cancer Res.* **124,** 444–449.
15. Katayama, S., Shiota, G., Oshimura, M., and Kawasaki, H. (1999). *J. Cancer Res.* **125,** 405–410.
16. Poremba, C., Böcker, W., Willenbring, H., et al. (1998). *Int. J. Oncol.* **12,** 641–648.
17. Hoang-Vu, C., Boltze, C., Gimm, O., et al. (2002). *Int. J. Oncol.* **21,** 265–272.
18. Schneider-Stock, R., Boltze, C., Jäger, V., et al. (2003). *J. Pathol.* **199,** 517–525.
19. Harley, C. B. (1991). *Mutat. Res.* **256,** 271–282.
20. Feng, J., Funk, W. D., Wang, S. S., et al. (1995). *Science* **269,** 1236–1241.
21. Ramakrishnan, S., Eppenberger, U., Mueller, H., Shinkai, Y., and Narayanan, R. (1998). *Cancer Res.* **58,** 622–625.
22. Nakayama, J., Tahara, H., Tahara, E., et al. (1998). *Nat. Genet.* **18,** 65–68.
23. Bamberger, C. M., Else, T., Bamberger, A. M., et al. (1999). *Exp. Clin. Endocrinol. Diabetes* **107,** 272–275.

24. Kinoshita, H., Ogawa, O., Mishina, M., et al. (1998). *Urol. Res.* **26,** 29–32.
25. Kubota, Y., Nakada, T., Sasagawa, I., Yanai, H., and Itoh, K. (1998). *Cancer* **82,** 176–179.
26. Aogi, K., Kitahara, K., Buley, M., et al. (1998). *Clin. Cancer Res.* **4,** 1965–1970.
27. Saji, M., Xydas, S., Westra, W. H., et al. (1999). *Clin. Cancer Res.* **5,** 1483–1489.
28. Chang, J. T. C., Chen, Y. L., Yang, H. A. T., Chen, C. Y., and Cheng, A. J. (2002). *Eur. J. Biochem.* **269,** 3442–3450.
29. Hutchinson, K. A., Stancato, L. F., Owens-Grillo, J. K., et al. (1995). *J. Biol. Chem.* **270,** 18841–18847.
30. Remmele, W., Hildebrand, U., and Hienz, H. (1986). *Virchows Arch.* **409,** 127–147.
31. Kim, N. W. and Wu, F. (1997). *Nucleic Acids Res.* **25,** 2595–2597.