Expression of telomerase (hTERT) in aldosterone-producing adrenocortical tumors

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Abstract. Telomerase was found in cancers and immortalized cell lines, but only occasionally in normal tissues, thus suggesting that measurement of its hTERT subunit might help distinguishing benign from malignant tumors. Data on hTERT expression in adrenocortical tumors are scant and mostly confined to non-functioning tumors. Therefore, we investigated whether hTERT expression may predict malignancy in aldosterone producing adrenocortical tumors. We measured hTERT mRNA with a real-time one-step reverse transcription (RT) polymerase chain reaction (PCR) method, based on the use of hTERT-specific fluorescence resonance energy transfer (FRET) probes, in 19 adrenalectomized patients with aldosterone-producing adenomas (APAs), in whom long-term follow-up (median 7 years, range 5-14 years) data were available. We also studied two rare aldosterone-producing carcinomas (APCs), eight adrenocortical carcinomas (ACs), twelve normal adrenal cortexes, and two malignant cell lines (NCI-295H and SW-13). Telomerase activity and hTERT immunoreactivity were also investigated. Of interest, we detected hTERT mRNA in 58% of APAs at levels similar to those of malignant tumors, which all consistently showed hTERT expression. In hTERT expressing tumors, immunocytochemistry showed the nuclear expression of hTERT. No hTERT expression was found in the normal adrenocortical tissue. No histopathology differences were observed between hTERT-positive and -negative APAs; however, a patient originally held to have an hTERT-positive APA was retrospectively classified as APC because of metastatic spread. In conclusion, RT-PCR measurement of hTERT mRNA is a hallmark of malignant adrenocortical tumors, but identifies also a subset of hTERT-expressing APAs that might show metastatic spread at long-term follow-up.

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Introduction

Telomeres, the repeated sequences at the chromosome ends, undergo shortening on continuous cell proliferation (1). Since they stabilize chromosomes and prevent DNA degradation, their progressive shortening with life is thought to provide a signal for cellular senescence. Telomerase (hTERT) is a ribonucleoprotein complex that catalyzes the addition of telomeric repeats to the 3'-end of chromosome DNA, thereby preventing the loss of telomeric sequences at each cell division (2). Albeit variously distributed in adult somatic cells, telomerase activity was found usually low in normal differentiated tissues (3). By contrast, hTERT activity is clearly detectable in cancer cell lines (5), and in most human cancer tissues (4), suggesting its usefulness as a marker of human cancers and index of poor prognosis (5-12).

Adrenocortical tumors are highly prevalent in the general population (13,14); nonetheless, the differentiation of malignant from benign neoplasms is often difficult (15), not only on clinical but even on histopathology ground. Several criteria have been claimed to allow discrimination between these tumors (16,17), including some molecular markers as the DNA index (18,19) the expression of the proliferating cell antigen (20), the p53 protein (21), the adrenal 4 binding protein (22), the c-Myc protein (18), or the insulin-like growth factor II gene (23). Unfortunately, to date none of these markers has gained wide acceptance because of poor accuracy.

The hTERT activity has been demonstrated in adrenal tumors, including few adrenocortical carcinomas (ACs) (24-27), but data are limited (27,28). Investigators used either the traditional semiquantitative TRAP (telomerase repeat amplification protocol) assay, or a quantitative determination of hTERT activity assay. These methods do not lend themselves to routine use on the tiny amount of tissue available through fine-needle biopsy, which is occasionally used to differentiate benign from malignant adrenocortical tumors.

The technological development in molecular analysis techniques have allowed to investigate the expression of hTERT with unprecedented sensitivity and accuracy. In the present study we, therefore, exploited the use of a novel commercially available quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR) method based on fluorescence resonance energy transfer (FRET) technology

to measure hTERT in a relatively large collection of benign adrenocortical aldosterone-producing adenoma (APA) patients, in whom long-term follow-up data were available, some very rare aldosterone-producing carcinomas (APC) patients and few ACs.

Patients and methods

Patients and follow-up. Consecutive patients referred to Department of Clinical and Experimental Medicine for arterial hypertension and found to have primary aldosteronism caused by an aldosterone-producing tumor, according to published criteria (29), were enrolled for this study. In these cases, tumor tissue was obtained at adrenalectomy and used for the molecular and immunocytochemical studies. After adrenalectomy, the patients were regularly seen at follow-up visits at our outpatient clinic. Telephone interviews with patients or, if unavailable, with close relatives or family doctors, were used to collect information on the few not attending regular follow-up visits. In addition, cases of AC were retrospectively identified from our tumor bank and their tissue was used for the molecular analysis as described below.

Adrenocortical specimen. Twenty-nine adrenocortical tumors, including APAs (n=19) and ACs (n=10), of which 2 were aldosterone-producing carcinomas (APCs), were studied. Histologically normal adrenocortical tissue obtained at surgery from 12 patients with renal cancer undergoing unilateral nephrectomy and ipsilateral adrenalectomy served as controls. Tissue specimens were obtained at surgery under sterile conditions, immediately frozen in liquid nitrogen, and stored at -80˚C until extraction, as previously described (30). An AC cell line (NCI-295H) and a small cell carcinoma of the adrenal cortex line (SW13) from a metastatic lesion to the adrenal (both from the American Type Culture Collection, Rockville, MD) were also investigated.

Histopathology examination. The following parameters, derived from the Weiss criteria (31) (tumor extension, nuclear atypia, necrosis, fibrosis, capsule, pushing edges, multiples nodules, zona fasciculata-like cells, zona glomerulosa-like cells, compact reticular cells, hybrid cells, oncocytes and balloon cells), were determined in a blinded manner to the hTERT mRNA and telomerase activity results in the 19 APAs.

Measurement of hTERT mRNA. Total RNA was isolated using the guanidine isothiocyanate method (Omnizol Kit™, Euro Clone, Milan, Italy) from frozen tissue or with the proteinase K method (High Pure RNA Paraffin Kit, Roche, Milan, Italy) from fixed tissue. The method used to measure hTERT mRNA entails a novel commercially available real-time RT-PCR method, the LightCycler Telo*TAGGG* hTERT Quantification Kit™ (Roche), that is specifically adapted for PCR in glass capillaries using the LightCycler Instrument™ (Roche), and was applied for the first time to adrenal tumors. Since for this approach it is crucial that the RNA preparation is free of contaminating DNA, which can lead to overestimation of the amount of RNA, the quality of the RNA was systematically checked with use of the lab-on-chip technology in an Agilent Bioanalyzer 2100 with the RNA6000 Nano Assay (Agilent

Technologies, Cernusco, Italy). hTERT-encoding mRNA was reverse transcribed to a 198 bp cDNA, which was then amplified with specific primers in a one-step RT-PCR reaction. The amplicon was detected by fluorescence using a specific pair of FRET probes that hybridize to an internal sequence of the amplicon during the annealing phase. One probe was labeled at the 5'-end with LC-Red 640, and to avoid extension, modified at the 3'-end by phosphorylation. The other probe was labeled at the 3'-end with fluorescein. Hybridization to the template DNA allows the two probes to come in close proximity, resulting in FRET between the fluorophores. Primers and probes are furnished with the Telo*TAGGG* hTERT Quantification Kit™. The mRNA encoding for porphobilinogen deaminase (PBGD) was similarly processed for use as housekeeping gene in a separate one-step RT-PCR. This served as both a control for RT-PCR performance and as a reference for relative quantification. A control RNA was also used to allow for ensuring attainment of reliable and reproducible data. The primers used were selected to exclusively reverse transcribe and amplify the mRNA encoding the functionally active hTERT protein and PBGD. They were chosen to span exon-intron boundaries, to prevent occurrence of co-amplification of genomic DNA. hTERT primer sequence is as follows: forward, 5'-CTGAGGAATGCATGTATGCT-3'; and reverse, 5'-ATGCTATCTGAGCCGTCT-3' (GeneBank NM# 000190). In pilot experiments we found that hTERT levels could be specifically and quantitatively detected down to a 10-100 copy sensitivity in <45 min. However, optimal results were obtained by using 100-200 ng total RNA per reaction. Results were analyzed as absolute number of hTERT mRNA copies and also as cycle threshold (Ct) of hTERT relative to PBGD.

Measurement of telomerase activity. The activity of telomerase was determined with the TRAPeze Elisa Detection Kit™ (Chemicon Europe, Chandler Ford, UK) in 14 adrenocortical tumors (12 APAs, 1 AC and 1 APC). As this assay requires freshly frozen tissue, all specimens were obtained at surgery, immediately frozen in liquid nitrogen, and stored at -80˚C until used. Each sample was assayed for telomerase in duplicate, starting from protein extracts of the tissue. In each extract the protein concentration was measured by the Pierce BCA method. Heat-inactivated controls were obtained after incubation of each sample extract at 85˚C for 10 min. As positive and negative controls, protein extracts of a telomerase positive cells (10^6 cells) and normal adrenal glands (n=7), were evaluated in parallel. The presence of telomerase activity was tested using the modified TRAP assay with biotinylated primer b-TS and dinitrophenyl (DNP) labelled dCTP. In this assay protocol, telomerase reaction and amplification utilizes a reverse primer (RP) with modified sequence, instead of the CX primer described originally. For labeling of PCR products a deoxynucleotide mixture containing dCTP labelled with DNP were added to the reaction mixture. The labeled products were immobilized onto streptavidin-coated microtiter plates via biotin-streptavidin interaction and detected by anti-DNP antibody conjugated to horseradish peroxidase (HRP). The amount of TRAP products was determined by means of the HRP activity using 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. Colour development

Table I. Anthropometric and biochemical features of the patients with APA and APC.

Parameter	APA (n=19)	APC (n=2)
Age (year)	54±12	60±11
Body mass index	25.5±2.9	22.0±2.8
Systolic blood pressure (mmHg)	178±26	212±25
Diastolic blood pressure (mmHg)	102±16	112±11
Serum K^+ concentration (mmol/l)	3.3±0.4	3.2±0.3
Aldosterone plasma concentration (pmol/l)	413±334	843±11
PRA (ng Ang-I/ml/h)	0.48	0.81

Number of patients is shown in brackets.

was measured at 450 nm with a reference wavelength of 690 nm. Mean absorbance of the negative control was subtracted from the sample absorbance. When the difference in absorbance $A_{450 nm}$ to $A_{690 nm}$ was ≥0.150 units, the samples were regarded as having telomerase activity.

hTERT immunocytochemistry. Paraffin-embedded sections were incubated with the polyclonal antibody NCL-hTERT (clone 44F12; Novocastra Laboratories, Newcastle upon Tyne, UK) at 1:100 dilution, for 2 h at room temperature. After washing in phosphate-buffered saline (PBS, pH 7.4) for 5 min, the samples were incubated with HRP/Fab polymer conjugate (EnVision+Dual Link System-HRP [DAB+]™, DakoCytomation, Glostrup, Denmark) for 30 min at room temperature, followed by washing in PBS. The detection of the bound HRP was accomplished with the avidin-biotin complex method using 3',3'-diaminobenzidine (DAB) as a chromogen. The specificity of the immunostaining was checked by running negative controls with the replacement of the primary antibody with preimmune serum.

Statistics. Statistical comparison between groups was performed using the Wilcoxon's signed rank test. Correlation was assessed by scatter plot and Pearson's correlation coefficient.

Results

Baseline clinical features and follow-up of the patients. The baseline anthropometric and biochemical data of the patients with APA and APC are summarized in Table I. As anticipated by the study design, elevated plasma aldosterone and suppressed plasma renin activity (PRA) were seen in both groups. No differences between groups achieved statistical significance likely because of the limited power imposed by the quite small sample size of the APC group and to the wide variation of aldosterone values. The median follow-up of the APA patients after adrenalectomy was 7 years (range 5-14 years). All patients were alive and free from recurrence except one, who was initially diagnosed as APA, but thereafter developed recurrence and widespread metastatic disease (32), and therefore was classified as APC.
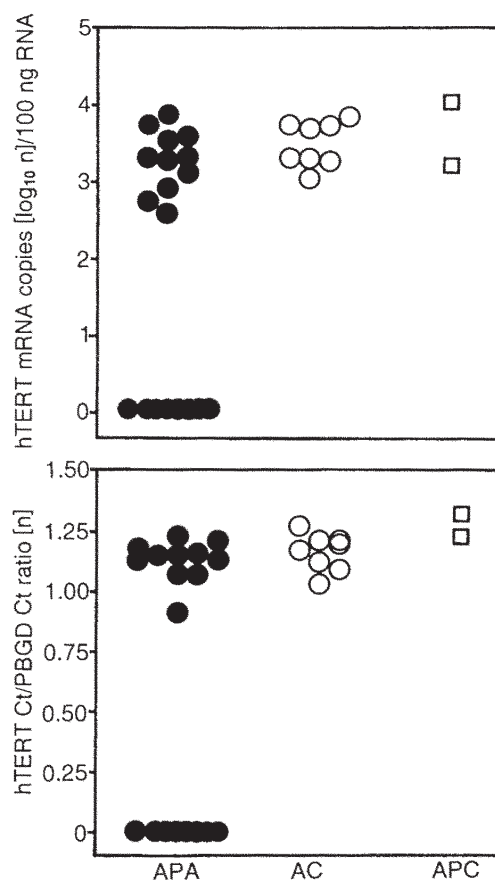

Figure 1. Scatter plot of hTERT mRNA, expressed either as absolute (number of copies; upper panel) or relative (ratio of hTERT to PBGD Ct; lower panel) expression in APA (solid circles), AC (empty circles), and APC (empty squares). The carcinoma tissues consistently showed hTERT mRNA, while the APA tissues showed a quite heterogeneous expression with some tumors showing no detectable hTERT mRNA and some exhibiting an expression similar to that of the cancer tissues.

hTERT mRNA. Fig. 1 shows the mRNA level found in the APA, APC and AC tissues. Data are shown either as absolute number of mRNA copies (upper panel) per 100 ng of total RNA or as expression relative to the housekeeping gene PBGD (lower panel). Of much interest, 58% of the APAs evidenced levels of hTERT mRNA similar to those seen in APCs and ACs, while no expression could be detected in the normal adrenal gland (Table II). The lack of hTERT expression was confirmed in all these cases by repeating the assay after re-extraction of the RNA from a different aliquot of the specimens and also by showing: i) the expression of PBGD and several other genes and ii) the hTERT expression in positive samples run in parallel with the same master mixture and under identical experimental conditions. Thus, while the hTERT was consistently expressed in the malignant tumors, its expression was quite heterogeneous in APA.

hTERT mRNA and tumor size. Since at the time of the diagnosis APCs and ACs usually display a size larger than APAs, we wonder whether any relationship between size of the tumor and the amount of hTERT mRNA existed. With use of correlation analysis we could find no significant relation between the hTERT mRNA and size or surface area of the tumor (Fig. 2).

Table II. Percent of positive samples at the hTERT mRNA, telomerase activity (TRAP) and hTERT immunocytochemical assays.

Percent of positive samples	NAC	APA	APC	AC
hTERT mRNA	0 (12)	58 (19)	100 (2)	100 (8)
Telomerase activity	0 (7)	33 (12)	0 (1)	100 (1)
hTERT-ir	0 (2)	67 (3)	0 (1)	50 (4)

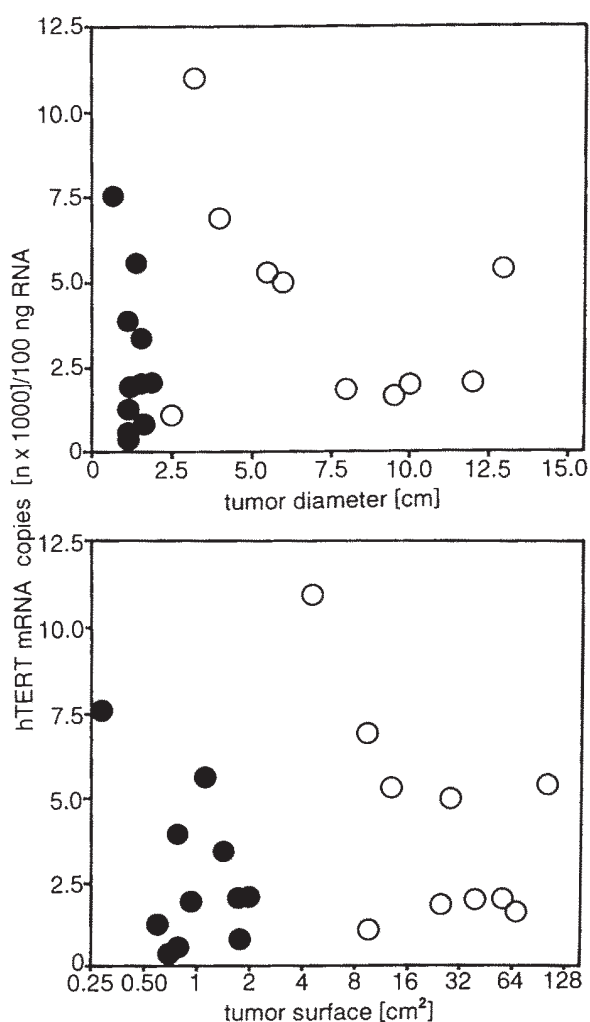NAC, normal adrenal cortex. Number of samples is shown in brackets.

Figure 2. The scatter plots show the lack of correlation between hTERT mRNA absolute expression (number of copies) and either tumor diameter (upper panel) or tumor equatorial section surface (lower panel) in APA (solid circles) and AC (empty circles).

Telomerase activity. We measured the presence of telomerase activity by the TRAP assay. This assay requires freshly frozen tissue and therefore it was feasible in 12 APAs, one APC and AC, but not in the fixed tissue bank tumors. Detectable telomerase activity was found in 33% of all APAs, but only in 33% of those that had detectable

hTERT mRNA. Therefore, two thirds of hTERT mRNA expressing APAs were falsely negative at this assay and, conversely, one third of those not expressing hTERT mRNA were falsely positive. Detectable telomerase activity was found in the AC, but not in the only one of the two APCs that was available for this assay (Table II).

hTERT detection by immunocytochemistry. Immunocyto-chemistry allowed us to verify whether the expression of hTERT at the mRNA and enzyme-activity level was paralleled by that of immunocytochemically detectable amounts of the enzyme. These experiments, which were performed on a smaller number of specimen, showed staining almost exclusively at the nuclear level (Fig. 3), in keeping with results documenting an assembly of hTERT in the nucleoli (33). No staining was found in the negative controls (data not shown). The hTERT immunostaining was found in 67% of APAs, including two cases with expression of hTERT mRNA, and not in one APA showing no hTERT mRNA (Table II). Thus, there was marked heterogeneity of immunostaining across APA tissues that were consistent with the hTERT expression at the mRNA level. In malignant tumors, clear-cut staining was observed in two of four AC (Table II), but not in the APC case.

Histopathology examination. When the APAs were classified according to hTERT expression, we found no differences in any of the aforementioned Weiss parameters between those expressing or not expressing hTERT mRNA.

Discussion

Metastasizing or very large adrenocortical tumors are easily labeled as malignant, but for most adrenal tumors it is difficult to predict the malignant potential. To discriminate between metastasizing and non-metastasizing adrenal tumors a multi-factorial analysis has been proposed (17), but the method is cumbersome, and sometimes provides inaccurate results. Fine-needle biopsy is also occasionally used to this end, but the limited amount of tissue obtainable and the lack of molecular markers for accurately identifying malignancy have prevented its wide use. Many indexes have also been proposed (18-23, 34,35), but none of them was found to predict malignancy with accuracy in the single tumor.

Aldosterone-producing tumors are the most common cause of endocrine hypertension (36) and exhibit quite variable functional features and biological behavior. With a highly specific and sensitive real-time RT-PCR based on FRET probes we could quantify hTERT mRNA in a relatively large collection of specimens obtained from 29 patients with adrenocortical tumor. APAs responsive and non-responsive to RAS were described (37-39). More recently, with the use of bilateral simultaneous adrenal vein sampling, we documented the existence of ACTH-responsive and ACTH-non-responsive APAs. Of much interest and for the first time this study provides compelling evidence that the heterogeneity of APAs extends also to the expression of hTERT. We could identify two subsets of APAs: the larger one, comprising about 58% of them, that showed levels of hTERT expression comparable to those seen in malignant APCs and ACs; the
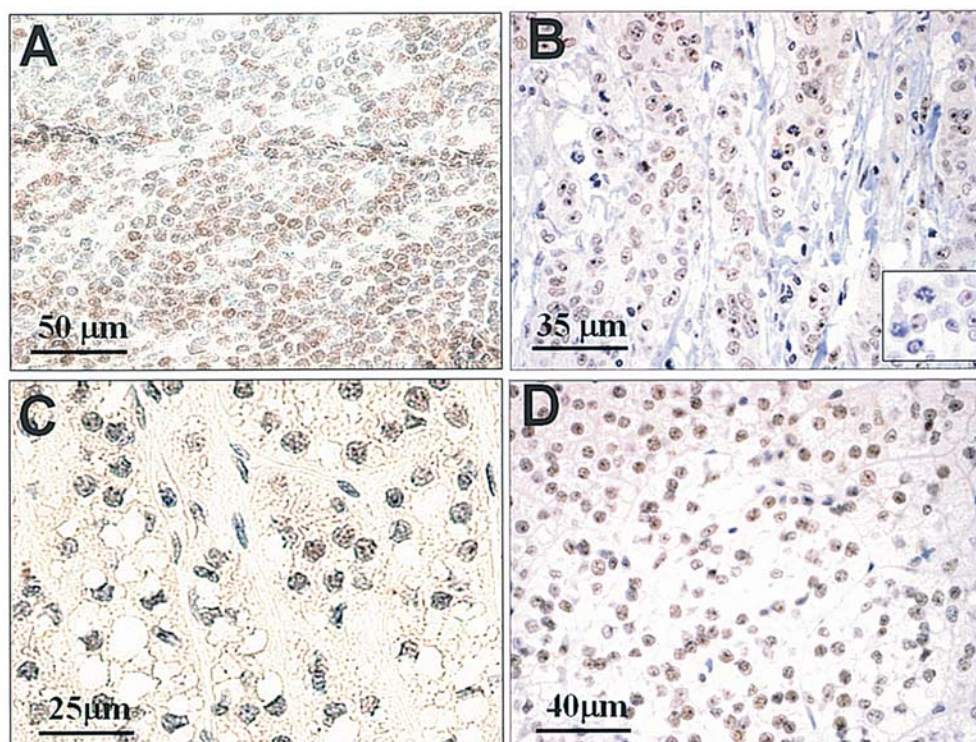
Figure 3. Immunocytochemical demonstration of the nuclear localization of hTERT staining in APA (A and B) and AC (C and D), that were hTERT mRNA and hTERT activity positive.

other, comprising 42% of the APAs, that resembled the normal adrenocortical tissue, because they showed neither hTERT expression nor immunocytochemically detectable hTERT. Obviously neither subgroup had any histological signs of malignancy. However, as the discrimination of APAs from APCs can be challenging on this ground, we gathered long-term follow-up data in these patients. In neither group could we find any evidence for recurrence of the tumor or development of metastasis with on important exception: a female initially held to have an APA who died after 18 months of metastatic APC (15,32). She showed level of hTERT expression similar to those of APC in the tumor tissue that had initially been considered to be an APA. Of further interest, in a male with disseminated APC, who showed hTERT expression in a fine-needle biopsy specimen from a lung metastatic node, hTERT mRNA could be detected with this RT-PCR method in the peripheral blood buffy coat, thus indicating the presence of APC cells expressing hTERT mRNA in the bloodstream. Thus, our data indicate that expression of hTERT was a consistent feature of APCs and ACs. Furthermore, they suggest that hTERT mRNA measurement, which in our hands was more sensitive than the TRAP assay, can occasionally help to identify APCs. We did observe some inconsistencies between the hTERT mRNA assay and immunocytochemistry on one hand, and the measurement of telomerase activity on the other. Whether these inconsistencies are intrinsic to the methods or due to some bias that might have occurred at handling the tissue for the more demanding TRAP assay assay remains to be investigated.

Given the rarity of APCs (15,32), and the ensuing limited number of specimen that could be examined, caution is mandatory and further studies are necessary before drawing conclusion. Moreover, in the majority of APAs the detection of hTERT mRNA with this highly sensitive RT-PCR assay did not predict a malignant clinical course, at least within the time frame of this study.

A heterogeneous biological behavior was described in the fortunately very rare APCs, but in these tumors the lack of accurate diagnostic markers usually translate in a late diagnosis and an ominous clinical course (32). A large tumor size was contended to be a good index of tumor growth capacity and, therefore, of malignancy. Accordingly, in a previous study of some ACs, telomerase activity significantly correlated with tumor size. In contrast with such contention, using a sensitive and accurate quantitative assay for hTERT expression we found no evidence for such correlation between tumor diameter or surface and hTERT mRNA level.

In conclusion, should our results be confirmed in larger series, the quantitative measurement of hTERT mRNA might become a useful index not only to distinguish normal adreno-cortical tissue from malignant adrenocortical tumors, but also for identifying a subset of 'benign' APAs that might deserve closer follow-up examination for careful watch on recurrence and/or metastatic spread.

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