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Diagnostic and Prognostic Value of Fas and Telomeric-Repeat Binding Factor-1 Genes in Adrenal Tumors

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It is often difficult to distinguish histologically between an adrenal cortical cancer and a benign adenoma, or to predict the prognosis of patients with adrenal cortical cancers. In this investigation, we examined whether apoptosis-regulating genes, bcl-xL and fas, and a telomere-related gene, telomeric-repeat binding factor-1 (TRF-1), differ between adrenal cortical cancers and benign adrenal tumors. Tissues from 4 adrenal cortical cancers were compared with 7 normal adrenal tissues, 17 cortical adenomas, 4 cortical hyperplasias, and 20 pheochromocytomas for expressions of bcl-xL and fas by RT-PCR, and for expressions of TRF-1 by real-time quantitative RT-PCR. All benign adrenal tissues expressed both the antiapoptosis gene, bcl-xL, and proapoptosis gene, fas, but the adrenal cortical cancers expressed only bcl-xL and not fas. TRF-1 increased by more than 30-fold in the adrenal cortical cancers, compared with benign adrenal tissues, and inversely correlated with the prognosis of patients with the adrenal cortical cancers. This lack of expression of fas in adrenal cortical cancer may help to distinguish it from benign adrenal tumors. The level of TRF-1 expression may be helpful prognostically for patients with adrenal cortical cancers. (*J Clin Endocrinol Metab* 88: 3690–3693, 2003)

ADRENAL CORTICAL CANCER is an aggressive cancer that is often resistant to surgical and medical treatment. Patients with adrenal cortical cancer often have a poor prognosis (1). It is sometimes difficult to determine whether a small (<4 cm) adrenal cortical neoplasm is benign or malignant histologically (2). The prognosis of patients with adrenal cortical cancer depends mainly on the stage of cancer at diagnosis (3). Several studies have shown genetic abnormalities, including overexpression of IGF-2, loss of heterozygosity at several sites, and abnormalities of several oncogenes in adrenal cortical carcinoma (4, 5).

In a previous investigation, we reported that benign adrenal tissues express both the antiapoptosis gene bcl-2 and the proapoptosis gene bax. Adrenal cortical cancers, in contrast, express only bcl-2 but not bax. We also found that there was increased expression of the telomere-related gene, human telomerase reverse transcriptase (hTERT), in adrenal cortical cancers when compared with benign adrenal tumors. hTERT expression also seemed to correlate with the prognosis of patients with adrenal cortical cancer (6).

To further confirm whether the difference in expression of apoptosis-regulating genes and telomere-related genes would help to distinguish between benign and malignant adrenal cortical tumors or to predict the prognosis of patients with adrenal cortical cancers, we examined the expression of other apoptosis-regulating genes, bcl-xL and fas, and a telomere-related gene, telomeric-repeat binding factor-1 (TRF-1).

Abbreviations: hTERT, Human telomerase reverse transcriptase; TRF-1, telomeric-repeat binding factor-1.

Materials and Methods

Materials

We analyzed 52 adrenal surgical specimens from patients who underwent adrenalectomy for primary adrenal tumors at the University of California San Francisco between 1995 and 2000. After resection, the specimens were frozen and stored at −80 C until RNA extraction. These adrenal specimens include 7 normal tissues adjacent to adrenal tumors (from 2 patients with aldosterone-secreting adenomas, 3 patients with nonfunctioning adrenal adenomas, and 2 patients with benign pheochromocytomas), 17 cortical adenomas (from 10 patients with aldosterone-secreting tumors and 7 patients with cortisol-secreting tumors), 4 cortical hyperplasias (from 3 patients with Cushing's disease and 1 patient with ectopic ACTH syndrome), 20 benign pheochromocytomas, and 4 cortical cancers. The 20 pheochromocytomas were presumed to be benign, because long-term follow-up has not detected any metastasis. All 4 adrenal cortical cancers secreted cortisol (Table 1). This investigation was approved by the Committee on Human Research, University of California San Francisco. We measured bcl-xL and fas gene expressions by RT-PCR, and TRF-1 gene expression by real-time quantitative RT-PCR.

Total RNA extraction and cDNA synthesis

RNA was extracted from adrenal specimens by the acid-phenol-guanidinium method. The quality of RNA samples was measured by the intensity of the 18S and 28S RNA bands, under UV light, after electrophoresis and staining with ethidium bromide. RNA was transcribed into first-strand cDNA using oligo dt primer for RT-PCR or using hexamers for real-time quantitative RT-PCR.

RT-PCR

Table 2 shows the primers for bcl-xL and fas (7). The PCR condition for bcl-xL and fas was as follows: one cycle of 94 C (2 min) and 92 C (2 min), 40 cycles of 55 C (30 sec), 72 C (90 sec), and 92 C (45 sec). PCR was conducted with 50 μl reaction volumes of 1× PCR buffer II (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 200 nM each primer, 200 μM

TABLE 1. Characteristics of adrenal tumors

	Normal tissue adjacent to tumor n = 7	Adenoma n = 17	Hyperplasia n = 4	Pheochromocytoma n = 20	Cortical cancer n = 4
Aldosterone-secreting	2	10			
Cortisol-secreting		7	4		4
Cushing disease			3		
Ectopic ACTH-secreting			1		
Benign pheochromocytoma	2			20	
Nonfunctioning	3				

TABLE 2. Primer and probe sequences used in RT-PCR and real-time quantitative RT-PCR

Gene and oligonucleotide	Sequence 5′–3′
bcl-xL	
Forward primer	GGAGCTGGTGGTTGACTTTCT
Reverse primer	CCGGAAGAGTTCATTCACTAC
fas	
Forward primer	AGACTGCGTGCCCTGCCAAGA
Reverse primer	CAGGATTTAAGGTTGGAGATT
TRF-1	
Forward primer	GCCTCCCAAAGTGCTGAGATT
Reverse primer	AAGGCCACAAACCAAGTCCTT
Probe	TGTGAGCCACTGCGTCCTGCCTAAA

of each deoxynucleotide triphosphate, and 0.025 U/μl Taq polymerase with 2 μg cDNA. Fifteen microliters of the RT-PCR products were electrophoresed through 1.5% agarose gels and stained with ethidium bromide and examined under UV light. The following PCR products were detected: bcl-xL (379 bp) and fas (413 bp).

Real-time quantitative RT-PCR

Primers, a probe, and PCR conditions to measure the human β-glucuronidase gene (used as an endogenous RNA control) expression have been previously described (6). The sequences of the oligonucleotide primers and the hybridization fluorescent probe for TRF-1 gene were designed and synthesized by Biosearch Technologies, Inc. (Novato, CA) (Table 2). PCR for TRF-1 was conducted, in triplicate, with 50 μl reaction volumes of 1× PCR buffer A (Applied Biosystems), 6.5 mM $MgCl_2$, 900 nM each of primers, 200 nM each of deoxynucleotide triphosphates, 100 nM probe, 0.025 U/μl Taq Gold (Applied Biosystems), and 50 ng cDNA, using ABI 7700 Prism (Applied Biosystems). The cycling conditions for TRF-1 were as follows: 1 cycle of 95 C (12 min), 45 cycles of 95 C (15 sec) and 60 C (1 min). Relative TRF-1 gene expression was determined by the δ-δ threshold cycle method (6, 8). Sequence Detection (version 1.7, Applied Biosystems) was used for analysis. The threshold cycle values for each set of three reactions were averaged for all subsequent calculations (6, 9).

Statistical analysis

Relative TRF-1 gene expression among the various adrenal tissues was compared using the Mann-Whitney *U* test. Differences were considered significant at $P < 0.05$.

Results

RT-PCR

Figure 1 shows representative bcl-xL (379 bp) and fas (413 bp) gene expression in various adrenal tissues. All adrenal tissues expressed bcl-xL gene. Normal adrenal tissues (7 of 7), cortical adenomas (17 of 17), cortical hyperplasias (4 of 4) and pheochromocytomas (20 of 20) expressed the fas gene, but cortical cancers (0 of 4) did not. Thus, all adrenal tissues, benign or malignant, expressed the antiapoptosis gene, bcl-xL. In contrast, all the benign adrenal tumors, but none of the cortical cancers, expressed the proapoptosis gene, fas.

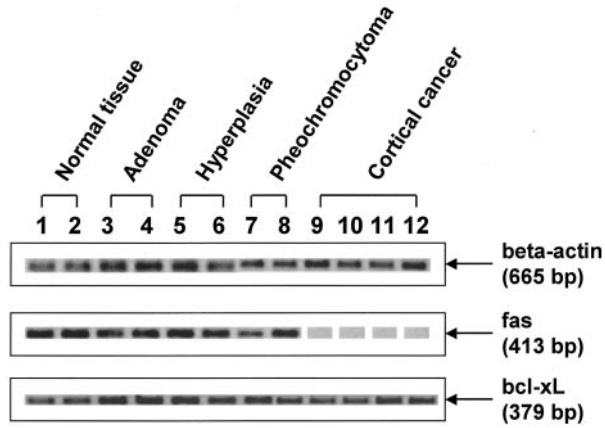

FIG. 1. Representative expressions of bcl-xL and fas genes in adrenal tumors. RT-PCR products from adrenal tissues were electrophoresed on 1.5% agarose gel stained with ethidium bromide. Expression of β-actin gene was used as an internal control. Normal tissue, Normal adrenocortical tissues adjacent to adrenal tumors (lanes 1 and 2); Adenoma, aldosterone-secreting (lane 3) and cortisol-secreting (lane 4) adrenal cortical adenomas, Hyperplasia, Cushing's disease (lane 5) and ectopic ACTH-syndrome (lane 6); Pheochromocytoma, lanes 7 and 8; Cortical cancer, adrenal cortical cancer (lanes 9–12).

Real-time quantitative RT-PCR

Figure 2 shows, and Table 3 summarizes, the amount of relative TRF-1 gene expression in the adrenal tumors studied. TRF-1 gene expression was significantly higher in adrenal cortical cancers than in normal adrenal tissues, cortical adenomas, cortical hyperplasias, and pheochromocytomas. The three patients (cases 1–3) who had a very high TRF-1 gene expression died 5–24 months after adrenalectomy. The one patient (case 4) who did not have an increased TRF-1 gene expression is still alive 5 yr, after adrenalectomy, with distant metastasis.

Discussion

In this investigation, we documented that all benign and malignant adrenal tissues that were evaluated expressed the antiapoptosis gene, bcl-xL, but only adrenal cortical cancers failed to express the proapoptosis gene, fas. bcl-xL inhibits apoptosis, whereas fas induces apoptosis (10, 11). Increased bcl-xL and decreased fas expression have been observed in other cancers (12, 13). The decreased expression of fas in adrenal cortical cancers may help them bypass the control of programmed cell death. Determination of antiapoptosis and proapoptosis gene expression may also help to differentiate between benign and malignant adrenal tumors, because none of our four adrenal cortical cancers expressed fas.

bcl-xL is one of the bcl gene family members, and it inhibits

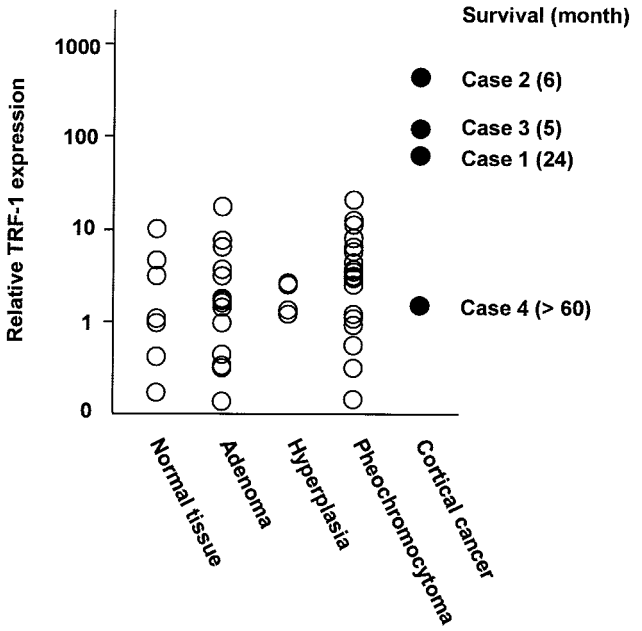

FIG. 2. Relationship between relative expression of TRF-1 gene and prognosis of adrenal cortical cancer. Relative gene expressions of adrenal tissues were measured by real-time quantitative RT-PCR. Normal tissue, Normal tissues adjacent to adrenal tumors; Adenoma, adrenal cortical adenoma; Hyperplasia, adrenal cortical hyperplasia; Cortical cancer, adrenal cortical cancer; Survival, survival period after adrenalectomy.

TABLE 3. Relative TRF-1 gene expressions in adrenal tumors

Tissue	No. of cases	Age (yr)	M/F	TRF-1 mRNA
Normal tissue	7	50.4 ± 9.0	3/4	2.97 ± 3.54[a]
Adenoma	17	47.8 ± 13.0	6/11	3.14 ± 4.52[a]
Hyperplasia	4	47.3 ± 5.5	1/3	1.96 ± 0.75[a]
Pheochromocytoma	20	48.3 ± 20.6	10/10	5.12 ± 5.30[a]
Cortical cancer	4	62.3 ± 8.6	2/2	162.65 ± 200.55

Normal tissue, Normal tissues adjacent to adrenal tumors; Adenoma, adrenal cortical adrenoma; Hyperplasia, adrenal cortical hyperplasia; Cortical cancer, adrenal cortical cancer; M, numbers of males; F, numbers of females. Relative gene expressions were represented in mean ± SD.

[a] $P < 0.05$ *vs.* adrenal cortical cancer.

apoptosis independent of bcl-2 (14). It has become increasingly clear that abnormality in the regulation of programmed cell death is a critical component in multistep tumorigenesis (15, 16). Increased expression of bcl-xL could be involved in malignant behavior, because it inhibits apoptosis and increases tumor growth (12). We previously reported that adrenal cortical cancers failed to express bax; bax and fas are both proapoptosis genes (6, 17). Bax-induced apoptosis is mitochondria-dependent, whereas fas-induced apoptosis is mitochondria-independent (11). Fas expression has been reported to be suppressed in thyroid cancers and also adrenal cancers (13, 18).

Telomeres, telomerase, and hTERT are related to tumor growth and proliferation (19–22). Telomeres are a repeating sequence of six bases, (TTAGGG)n at the ends of chromosomes. Telomeres shorten at each cell division. When telomere length is less than 4–6 kbp, normal cells can no longer proliferate. Telomerase can synthesize telomeric DNA repeats and compensate for such shortening. Many cancers have elevated telomerase activity that enables them to proliferate (23). hTERT elongates telomere length through activation of telomerase (24). TRF-1 binds to chromosome ends and limits telomere elongation (25).

Telomere length and telomerase activity are not always concordant. For example, thyroid cancer, in spite of having elevated telomerase activity, often has shorter telomeres than adjacent normal tissue (26, 27). We hypothesized that adrenal cortical cancers that had elevated hTERT expression also had increased TRF-1 expression. Telomere length is controlled by mechanisms that involve telomerase and TRF-1 (19, 28). TRF-1 binds to double-strand telomeric DNA and regulates telomere length without affecting the enzymatic activity of telomerase (25). Telomere length is shorter in some cancers, compared with adjacent normal tissue, despite higher telomerase activity in cancers (27, 29). This suggests a regulatory mechanism that limits telomere elongation by genes such as TRF-1, which is overexpressed in some cancers. hTERT gene expression examined in our previous investigation correlated with TRF-1 gene expression in adrenal cortical tumors ($r = 0.475$; $P < 0.05$, Pearson's correlation coefficient) but not in pheochromocytomas ($r = 0.009$) (6). This finding suggests that the feedback mechanism of TRF-1 to check telomere length is maintained in adrenal cortical tumors; thus TRF-1 expression increases with increased hTERT expression. Parallel to the level of expression of hTERT, the increased expression of TRF-1 may correlate with malignancy.

Increased hTERT gene expression activates cell proliferation in cancers. Prognosis of patients with breast cancer was reported to be inversely proportional to hTERT gene expression (30). Our data indicates that TRF-1 gene expression correlates directly with hTERT gene expression in adrenal cortical tumors. The one patient who expressed lowest TRF-1 expression was the only one who survived more than 5 yr after adrenalectomy and was alive with distant metastasis. In our previous study, this tumor also had the lowest expression of hTERT gene, compared with the other three adrenal cortical cancers (6). Thus, TRF-1 gene expression, in addition to hTERT gene expression, may also help to predict tumor behavior in patients with adrenal cortical cancer.

In conclusion, adrenal cortical cancers, in contrast with benign adrenal tumors, fail to express fas gene. In addition, the level of TRF-1 gene expression seems to correlate with adrenal cortical cancer aggressiveness. These observations should be confirmed by a larger study that includes more adrenal cortical cancers, but these findings may help to select patients for adrenalectomy and guide postsurgical treatment.

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