CLINICAL STUDY

Cyclin E correlates with malignancy and adverse prognosis in adrenocortical tumors

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Abstract

Objective: In many cases, the prognosis of an adrenocortical tumor cannot be determined from pathologic findings alone. We investigated cyclin E levels as a potential marker.

Methods: We studied 57 tumors by immunohistochemical staining with an anticyclin E antibody. We also evaluated clinical and pathologic factors (McFarlane staging and Weiss score) and previously validated genetic markers (17p13 loss of heterozygosity, 11p15 uniparental disomy, and overexpression of the IGF-II gene) for these tumors. Disease-free survival was estimated in 49 patients who underwent curative surgery.

Results: Cyclin E overproduction ($\geq 5\%$) was associated with the malignant phenotype and was strongly correlated with tumor size ($P < 0.0001$), Weiss score ($P < 0.0001$) and the presence of genetic abnormalities in tumors ($P < 0.001$) (nonparametric Wilcoxon test and Fisher's exact test). Within a median follow-up of 44.1 months, seven patients exhibited a recurrence and two patients died from other causes. Cyclin E overproduction was significantly associated with shorter disease-free survival in univariate analysis ($P = 0.016$; RR: 7.6), as were histologic grade (Weiss score ≥ 4 ; $P = 0.0006$; RR: 18), 17p13 LOH ($P = 0.014$, RR: 14.9), 11p15 UPD ($P = 0.003$, RR: 11.8) and overexpression of the IGF-II gene ($P = 0.015$, RR: 13.8).

Conclusion: This study shows that cyclin E overproduction is of adverse prognostic significance in adrenocortical tumors.

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Introduction

Adrenocortical carcinomas are tumors with a poor prognosis (1). In cases of localized adrenocortical tumors (McFarlane stages I and II) (2), pathologic examination with determination of Weiss score has been shown to be useful for distinguishing between benign and malignant tumors (3). However, the results of this examination are ambiguous in some cases (4). Recent studies of these tumors have shown that genetic markers are associated with malignant phenotype (4–14). These genetic abnormalities consist of strong overexpression of the IGF-II gene and maternal 11p15 uniparental disomy (UPD) with loss of the maternal allele and duplication of the active IGF-II paternal allele (6, 7, 9), 17p13 loss of heterozygosity (LOH) (4, 5, 8), 2p16 LOH (10) and 11q13 LOH (10–13). In malignant tumors, we previously showed that maternal 11p15 UPD is responsible for the loss of expression of the CDKN1C (also known as p57KIP2)

gene (15). This gene encodes a cyclin-dependent kinase inhibitor (CKI) involved in the G1/S phase of the cell cycle. We also showed that the production of G1 cyclins and G1 cyclin-dependent kinases (CDK), including cyclin E and CDK2, is upregulated in tumors overexpressing the IGF-II gene (15). Cyclins, particularly G1 cyclins, play an important role in the transformation and progression of many cancers (16, 17). Cyclin E production is increased in a number of human cancers including esophageal, gastrointestinal, liver, genitourinary, hematologic, lung, skin, breast and smooth tissue cancers (17–35). Cyclin E levels may also provide prognostic information, and cyclin E could even be a potential target for anticancer treatment (17). Cell-cycle components have not been extensively investigated in adrenocortical tumors and the prognostic value of cyclin E levels remains to be evaluated.

The aim of this study was to evaluate cyclin E levels by immunohistochemical means in a series

of 57 well-documented sporadic adrenocortical tumors and to assess the prognostic value of this marker. We found that cyclin E overproduction was associated with malignancy and was a good predictor of shorter disease-free survival in adrenocortical tumors.

Patients and methods

Patients

Between 1987 and 1998, sporadic adrenocortical tumor (other than Conn's adenoma) was diagnosed in 57 adult patients attending the Endocrinology Department of Cochin Hospital (Paris, France). This group of 57 patients was from among the patients reported in a previous study (4). None of these patients presented features of any tumor-predisposing syndrome (Beckwith–Wiedemann, McCune–Albright, multiple endocrine neoplasia type 1 or Li–Fraumeni syndromes). Clinical data and hormonal status were evaluated as previously described (1). Tumor stage was assessed according to the McFarlane classification (2). Informed consent for the analysis of leukocyte and tumor DNA and for access to the data collected was obtained from all the patients, and the study was approved by an institutional review board (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Cochin Hospital, Paris). After surgery, patients were examined twice a year for 2 years and annually thereafter. Hormonal evaluation, chest radiograph, and computerized tomography (CT) scans of the abdomen and thorax were carried out at each evaluation. The patients were followed until their date of death, their last examination or the end of the follow-up period. The minimal follow-up period was 12 months.

Microscopy

The tumors were fixed in formalin and embedded in paraffin, and 4 μ m sections were cut and stained with hematoxylin and eosin. The sections were examined (A.L.) to assess Weiss score (0–9) (3) on the basis of the presence or absence of the following nine histologic features: high mitotic rate, atypical mitoses, high nuclear grade, low percentage of clear cells, necrosis, diffuse tumor architecture, capsular invasion, sinusoidal invasion and venous invasion.

Genetic analysis

The allelic status at 17p13 and 11p15 loci and the evaluation of IGF-II messenger content in tumors were performed as previously described (9).

Immunohistochemical staining

Sections of 4 μ m from formalin-fixed tissue embedded in paraffin were mounted on Superfrost/Plus glass

slides. The paraffin was eliminated by incubating the sections in xylene and then rehydrating them. For antigen retrieval, sections were heated in a microwave oven for a total of 20 min in 10 mmol sodium citrate buffer at pH 6.0. The slides were incubated with monoclonal anticyclin E antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:200 for 60 min at room temperature. Sections were then incubated with the streptavidin-biotin-peroxidase complex, and the marker was detected by the enzymatic precipitation of 3,3'-diaminobenzidine tetrahydrochloride in 0.5 mmol Tris. The slides were counterstained with Mayer's hematoxylin.

Immunostaining was assessed at a multihead microscope by two independent pathologists (E.T. and A.L.) blinded to McFarlane stage, Weiss score and outcome. For the few discrepant cases, a consensus was reached by a new joint examination of the slides. Cells with nuclear staining were scored as stained cells (36). The cyclin E-stained sections were all examined at high magnification, and a labeling index (LI; percentage of stained cells) was attributed to each case. The intensity of staining was not scored. Cyclin E staining was considered negative if the cyclin E LI was < 5% and positive if the cyclin E LI was $\geq$ 5%. We chose to use 5% as the cutoff value on the basis of previous studies (36).

Statistical analysis

Disease-free survival was estimated in patients with curative surgery by the method of Kaplan–Meier. Disease-free survival was the time in months between the initial operation and documented recurrence or the end of the study. Survival was censored if the patient was still alive or had died from other causes. Survival curves were compared and tested for statistical significance by log rank tests (univariate analysis).

Cox proportional hazard model was used to study the independent prognostic value of cyclin E expression on disease-free survival, adjusted for 17p13 LOH and Weiss score in patients who had these data available (bivariate analysis).

The chi-square and Wilcoxon tests were used to compare groups for noncensored qualitative and quantitative variables respectively. The threshold for significance was $P \leq 0.05$. Calculations were performed with the SAS package (SAS Institute, Inc, Cary, NC, USA).

Results

Features at presentation

The characteristics of the patients are summarized in Table 1. The patients were 23–79 years old (44.9 ± 15.1 years). The ratio of women to men was 51/6. Forty-seven patients (82%) were referred for endocrine symptoms, and 51 patients (90%) presented

Table 1 Features at presentation in 57 patients with sporadic adrenocortical tumors.

	All patients (n = 57)	McFarlane stage I (≤ 5 cm) (localized) (n = 31)	McFarlane stage II (> 5 cm) (localized) (n = 14)	McFarlane stage III–IV (disseminated) (n = 12)
Age (mean±s.d.)	44.9±15.1	44.4±15.5	45.2±13.4	45.8±16.9
Sex (F/M)	51/6	30/1	13/1	8/4
Clinical presentation n (%)				
Endocrine symptoms	47 (82)	26 (84)	10 (71)	11 (92)
Cushing's syndrome	31 (54)	24 (78)	3 (21)	4 (34)
Cushing's syndrome and virilization	6 (10)	0 (0)	2 (14)	4 (34)
Virilization	9 (16)	1 (3)	5 (36)	3 (24)
Endocrine hypertension	1 (2)	1 (3)	0 (0)	0 (0)
Incidentaloma	10 (18)	5 (16)	4 (29)	1 (8)
Functional status n (%)				
Glucocorticoid only	33 (58)	27 (87)	3 (21)	3 (25)
Sex steroids only	5 (9)	0 (0)	5 (37)	0 (0)
Mixed	13 (23)	1 (3)	3 (21)	9 (75)
Nonfunctional	6 (10)	3 (10)	3 (21)	0 (0)
Curative surgery n (%)	49 (86)	31 (100)	14 (100)	4 (33)

an abnormal hormonal profile. Forty-nine patients (86%) underwent curative surgery.

Macroscopic findings and Weiss score

Macroscopic findings and Weiss scores are summarized in Table 2. Forty-five patients (79%) had localized tumors: 31 patients (54%) had tumors of ≤ 5 cm in size (McFarlane stage I), and 14 patients (25%) had tumors of > 5 cm in size (McFarlane stage II). Twelve patients (21%) had disseminated tumors at diagnosis (McFarlane stages III and IV): four patients had tumors invading adjacent organs (kidney: one, veins: three), and eight patients had distant metastases; all of these tumors were > 5 cm in size.

All patients with obviously malignant tumors (stages III and IV) had a Weiss score of 4 or more, and 42 patients (93%) with localized tumors (stage I and II) had a Weiss score of 3 or less.

Genetic abnormalities

Most patients (98%; n = 56) were informative for at least one 11p15 marker, and 81% (n = 46) were informative for at least one of the three 17p13 markers. The prevalence of 17p13 LOH and 11p15 UPD and overexpression of the IGF-II gene are shown in Table 2. The presence of 17p13 LOH and 11p15 UPD and overexpression of the IGF-II gene were not associated with adrenal hyperfunction. Genetic abnormalities were frequent in stage III–IV tumors, which were obviously malignant, and were less common in localized tumors (stages I and II).

Analysis of cyclin E levels

Figure 1 shows cyclin E LI as a function of McFarlane stage (Fig. 1A), Weiss score (Fig. 1B) and presence of genetic abnormalities (Fig. 1C).

Table 2 Macroscopic, microscopic, genetic and immunohistochemical features in 57 patients with sporadic adrenocortical tumors.

	All patients (n = 57)	McFarlane stage I (localized) (n = 31)	McFarlane stage II (localized) (n = 14)	McFarlane stage III–IV (disseminated) (n = 12)	P (disseminated vs localized)
Tumor size (cm, mean±s.d.)	7.4±5.5	3.5±0.7	9.8±4.7	14.7±4.1	< 0.0001 ^a
Weiss score (mean±s.d.)	2.2±2.7	0.4±0.6	2.4±1.8	6.5±1.3	< 0.0001 ^a
Genetic abnormalities					
17p13 LOH n (%)	17/46 (37)	2/25 (8)	7/11 (64)	8/10 (80)	< 0.001 ^b
11p15 UPD n (%)	18/56 (32)	1/30 (3)	7/14 (50)	10/12 (83)	< 0.001 ^b
IGF-II overexpression n (%)	21/56 (37)	1/30 (3)	9/14 (64)	11/12 (92)	< 0.001 ^b
Cyclin E LI					
Median (range)	1 (0–80)	0 (0–10)	5 (0–70)	17.5 (0–80)	< 0.001 ^a

^aNonparametric Wilcoxon test.

^bFisher's exact test or chi-square test.

LOH: loss of heterozygosity; UPD: uniparental disomy; LI: labeling index.

Figure 1 Cyclin E levels (% labeled cells; LI: labeling index) as a function of McFarlane staging (A), Weiss score (B) and the presence or absence of genetic abnormalities (17p13 LOH, 11p15 UPD and overexpression of the IGF-II gene) (C). The cutoff value of 5% is indicated by a dotted line.

In the 31 patients with stage I tumors, cyclin E LI was 0–10% (median: 0%) (Fig. 1A). Three patients (10%) had cyclin E LI of $\geq 5\%$, with associated genetic abnormalities in two of these patients, one of whom had a Weiss score of 2. In the 14 patients with stage II tumors, cyclin E LI was 0–70% (median: 5%). Seven patients (50%) had cyclin E LI of $\geq 5\%$. In the 12 patients with stage III–IV tumors, cyclin E LI was 0–80% (median: 17.5%). Ten patients (83%) had cyclin E LI of $\geq 5\%$.

In the 42 patients that had tumors with a Weiss score of 3 or less (Fig. 1B and Fig. 2A), cyclin E LI was 0–70% (median: 0%) (Fig. 1B and Fig. 2B). Seven patients (17%) had cyclin E LI of $\geq 5\%$, and six of these seven patients had associated genetic

abnormalities. In the 15 patients with tumors with a Weiss score of 4 or more (Fig. 1B and Fig. 2C), cyclin E LI was 0–80% (median: 15%) (Fig. 1B; Fig. 2D and E). Thirteen patients (87%) had cyclin E LI of $\geq 5\%$. Cyclin E overproduction was not associated with a specific Weiss score criterion.

We systematically investigated three genetic abnormalities in all tumors: 17p13 LOH, 11p15 UPD, and IGF-II mRNA overproduction. Tumors were assigned to one of two groups: no genetic abnormality (none of these markers present) and with genetic abnormalities (at least one of these markers present). In the 32 tumors with no genetic abnormality (Fig. 1C), cyclin E LI was 0–5% (median: 0%). In the 25 tumors with at least one genetic abnormality, cyclin

E LI was 0–80% (median: 10%), and 19 patients (76%) had cyclin E LI of $\geq 5\%$. Cyclin E overproduction was not associated with a specific genetic abnormality.

Disease-free survival analysis

We carried out a prognostic study for the 49 patients that underwent curative surgery. In this group, the median duration of follow-up was 44.1 months (range: 6.7 months–8.7 years). The Kaplan–Meier estimate of disease-free survival was 86% (95% CI: 74.7–96.4) at 5 years. During the follow-up period, two patients died of unrelated causes: ovarian cancer (one case) and amyloidosis (one case). Seven patients (14%) displayed recurrence 6.7 months to 7.9 years after initial surgery (median: 29 months).

In univariate analysis, large tumor size, high histologic grade, 17p13 LOH, 11p15 UPD and overexpression of the IGF-II gene were found to be strong predictors of shorter disease-free survival (Table 3 and Fig. 3A–D). Thirty-six patients showed fewer than two genetic abnormalities and 18 patients showed at least two genetic abnormalities. The presence of two or more genetic abnormalities was highly predictive of shorter disease-free survival, with a relative risk of 40 (95% CI: 5.1–313.6). The disease-free survival curve shown in Fig. 3E indicated that cyclin E overproduction was also highly predictive of shorter disease-free survival, with a relative risk of 7.6 (95% CI: 1.5–39.1; $P = 0.016$; Table 3). Indeed, the Kaplan–Meier estimate of disease-free survival was 97% at 5 years (95% CI: 90.6–100) in the group of patients with a cyclin E LI of $<5\%$ and 59% at 5 years (95% CI: 31.6–86.9) in the group of patients with a cyclin E LI of $\geq 5\%$.

We then investigated whether the combination of cyclin E labeling with other known prognostic factors, such as Weiss score or 17p13 LOH, would improve the prognostic evaluation. Cyclin E was not found to have an independent prognostic value when compared with Weiss score (RR = 3.6, 95% CI: 0.5–25.4; $P = 0.20$) or 17p13 LOH (RR = 0.7, 95% CI: 0.1–5.9; $P = 0.73$), but the multivariate analysis was impaired by the size of the series and the low frequency of events (recurrence in seven cases).

Discussion

Benign and malignant adrenocortical tumors are usually distinguished by clinical, hormonal and

Figure 2 Hematoxylin-eosin (HE) and immunohistochemical (IHC) staining for cyclin E of two different adrenocortical tumors. (A and B) Adrenocortical tumor with a Weiss score of 0 (A: HE $\times 400$; B: IHC staining of cyclin E $\times 400$). (C–E) Adrenocortical tumor with a Weiss score of 8 (C: HE $\times 400$; D: IHC staining of cyclin E $\times 25$; E: IHC staining of cyclin E $\times 400$).

Table 3 Univariate analysis comparing disease-free survival for various prognostic factors (log rank tests).

Variable	Relative risk	95% confidence interval	P value
Tumor size (cm) (≤ 5 vs > 5)	*	*	0.0001
Histologic grade (Weiss score) (≤ 3 vs ≥ 4)	18	3.5–94.1	0.0006
17p13 LOH (normal vs LOH)	14.9	1.7–127.3	0.014
11p15.5 UPD (normal vs UPD)	11.8	2.3–60.8	0.003
IGF-II gene expression (normal vs high)	13.8	1.7–114.8	0.015
Cyclin E LI (< 5 vs $\geq 5\%$)	7.6	1.5–39.1	0.016

LOH: loss of heterozygosity; UPD: uniparental disomy; LI: labeling index.

* It was not possible to calculate relative risk because there were no recurrences in the group of patients with tumor size of ≤ 5 cm.

pathologic features (3, 37). However, in localized tumors (McFarlane stages I and II), it is sometimes difficult to distinguish between tumors with an adverse prognosis and those that are truly benign. Indeed, pathologic examination with determination of Weiss score is useful but subject to limitations. We therefore need to identify other prognostic factors for use on a routine basis. Tumorigenesis is tightly linked to abnormal regulation of the cell cycle, and there is growing recognition of the role of cyclins, CDK and CKI (17, 38). CKI downregulate the kinase activity of cyclin-CDK complexes, thereby negatively regulating cell-cycle progression (38). In a recent study, Stojadinovic *et al.* investigated in adrenocortical tumors important components of the p53 pathway by use of an immunohistochemical multimolecular profiling approach with Ki-67, p53, mdm-2, cyclin D1, Bcl-2, p21 and p27 antibodies (39). They demonstrated the molecular complexity and heterogeneity of adrenocortical carcinoma by showing 10 different phenotypes for 31 tumors, and they concluded that tumor staging and morphologic evaluation were essential in determining prognosis for patients with adrenocortical tumors. In a previous study, we have shown by a molecular approach that, in malignant adrenocortical tumors, 11p15 UPD is responsible for the loss of CDKN1C gene expression and is associated with increased expression of cyclin E, CDK2 and CDK4 and with high activity of G1 cyclin-CDK complexes (15). In a recent study based on transcriptome analysis, Giordano *et al.* (14) looked for genes specifically associated with malignant adrenocortical tumors. They clearly demonstrated frequent overexpression of IGF-II in malignant tumors, confirming previous data from several groups (6, 7, 9). More interesting, they also identified several other genes, including that encoding cyclin E, as being specifically associated with malignant adrenocortical tumors.

In this study, we used immunohistochemistry to investigate cyclin E levels and to assess their prognostic value in a series of 57 sporadic adrenocortical tumors. This series is interesting for two major reasons: it is a large series reporting well-documented clinical, hormonal, genetic and pathologic characteristics of

adrenocortical tumors in patients followed at a single center, and to our knowledge it is the first study investigating cyclin E levels in adrenocortical tumors by this method.

Cyclin E overproduction was associated with clearly clinically malignant tumors, and was correlated with larger tumor size, high Weiss score and the presence of genetic abnormalities. These immunochemical data confirm our previous Western immunoblot results that demonstrated an increase in cyclin E protein levels in malignant adrenocortical tumors (15). Our results are also consistent with those for other tumors, showing that cyclin E overproduction is associated with malignancy (23, 28, 40).

Cyclin E levels have been shown to be correlated with an adverse prognosis for breast (18, 19, 25, 27, 33, 35), stomach (20), lung (24, 30), liver (21), lymphoid tissue (41), ovarian (31, 40, 42) and urinary tract (22) cancers. This led us to evaluate the prognostic value of cyclin E in our series of tumors. The disease-free survival analysis demonstrated that cyclin E overproduction predicted shorter survival in adrenocortical tumors. This study provides the first demonstration that cyclin E levels have a predictive value for adrenocortical tumor recurrence. However, although this study is the largest available study for a relatively rare disease, the frequency of events (recurrences in seven cases) was low, and it was not possible to assess the prognostic value of cyclin E in multivariate analysis (43). Nevertheless, it may be possible to establish the independent prognostic value of cyclin E by extending this study to a larger series of patients with more outcome events.

Immunohistochemistry has been shown to be a useful method for assessing the prognostic value of cyclin E, particularly in breast ductal carcinoma (25), epithelial ovarian carcinoma (31, 42), non-small-cell lung carcinoma (30), colorectal carcinoma (44) and carcinoma of the ampulla of Vater (45). Our results indicate that this simple method is also useful in the prognostic assessment of adrenocortical tumors.

In conclusion, this study establishes that cyclin E overproduction is of adverse prognostic significance in adrenocortical tumors. We now need to extend this study to a larger series of patients with more outcome

Figure 3 Kaplan–Meier disease-free survival curves according to Weiss score (A) or comparing patients with tumors showing 17p13 LOH and normal heterozygous profiles (B), 11p15 uniparental disomy (UPD) and normal heterozygous profiles (C), high and normal IGF-II gene mRNA contents (D), and low or high cyclin E labeling index (E).

events to determine whether the evaluation of cyclin E levels could provide useful prognostic information beyond that provided by known genetic and pathologic prognostic factors.

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