EXPERIMENTAL STUDY

Discerning malignancy in adrenocortical tumors: are molecular markers useful?

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Abstract

Objective: Adrenocortical carcinoma (ACC) is a rare neoplasm with poor prognosis. Discerning ACCs from benign adenomas histologically may be difficult if invasion into surrounding tissues or metastases are missing.

Design: In order to establish molecular markers for malignancy, we analyzed seven normal adrenals, three massive macronodular ACTH-independent adrenocortical hyperplasias (MMAHs), 30 adrenocortical adenomas (ACAs) and ten ACCs.

Methods: All tissues were studied for the presence of alterations in the p53 tumor suppressor gene using the PAb 1801 antibody, which detects mutant p53 protein and the pYNZ22 microsatellite marker to show loss of heterozygosity (LOH) at 17p, for expression of the proliferation-associated antigen Ki67 using the MIB1 antibody, for the rate of apoptotic tumor cells with the TdT-mediated dUTP biotin nick end labeling (TUNEL) method, and for LOH of 11q13 (menin gene locus) with the D11S956 microsatellite marker.

Results: 0/3 MMAH, 1/28 ACA and 3/10 ACC revealed immunopositive staining for p53. LOH for pYNZ22 was observed in 1/3 MMAH, 1/23 informative ACA and 6/6 informative ACC. The rate of apoptotic cells was significantly higher in ACC ($P < 0.0001$ by ANOVA) than in ACA but there was some overlap between groups. The Ki67 index (% immunopositive cells) was $1.9 \pm 1.30\%$ (mean $\pm$ s.d.) in normal adrenals, $3.47 \pm 1.37\%$ in MMAH, and $2.11 \pm 1.01\%$ in ACA. ACC had the highest Ki67 index of $11.94 \pm 7.58\%$ distinguishing all ACC from the ACA and MMAH studied with a cut-off level of 5%. LOH for 11q13 was detected in 2/3 MMAH, 5/26 ACA and 6/8 ACC.

Conclusions: We conclude that a Ki67 index above 5% is a sensitive and specific indicator of ACC and may be useful in the differentiation of adenomas from carcinomas.

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Introduction

Between 1.4 and 8.7% of all autopsies and roughly 1% of all abdominal CT scans reveal clinically non-recognized adrenal tumors. The majority of these incidentally discovered adrenal masses has little or no impact on the patient's health. Although adrenocortical carcinoma (ACC) is rare – the incidence is approximately 1 per 1.7 million inhabitants per year in the USA – its highly aggressive behavior and 2 years survival rate of only 50% makes exclusion of malignancy desirable in all adrenal tumors (1, 2). Assessing the malignant potential of adrenal tumors *in vivo* with non-invasive diagnostic techniques is difficult. Tumor size, endocrine activity and differences of signal intensity in T_1 and T_2 -weighted magnetic resonance imaging scans are helpful (3). Because of the high

number of benign clinically silent adrenal adenomas, surgical removal is only recommended in larger tumors, since in small tumors the benefits do not outweigh the risks of surgery (4, 5).

Discerning malignancy in adrenal tumors is often difficult even in resected tumors. In adrenocortical tumors, Hough *et al.*, Weiss *et al.* and van Slooten *et al.* established algorithms of histopathological features to diagnose malignancy (6–9). However, these algorithms are of limited value under routine conditions, especially since some of the criteria require clinical information, which may not be available in every case. Reliable molecular or immunohistochemical markers hold the potential to make assessment of adrenal malignancy much easier, as it has been shown for other endocrine and non-endocrine tumors. For example, loss of expression of the retinoblastoma gene product has

been proven to be useful in the diagnosis of parathyroid carcinoma (10, 11) unless some overlap between benign and malignant lesion has to be considered (12). Several groups have tried to establish markers for malignancy in adrenocortical tumors. Overexpression of insulin-like growth factor-II (IGF-II, ref. 13), IGF-I receptor (14) and high levels of IGF-binding protein-2 (15) have been demonstrated in a high percentage of malignant adrenocortical tumors, but has not been detected in benign adrenal tumors. Marx *et al.* (16) found that abrogation of MHC class II expression was a feature of ACC but may be found in some adenomas as well. After investigating cellular DNA content by flow cytometry and immunohistochemical distribution of *c-myc*, vimentin, proliferating cell nuclear antigen (PCNA) and epidermal growth factor receptor, Suzuki *et al.* concluded that only DNA ploidy and distribution of immunohistologically detectable *c-myc* oncoprotein has some diagnostic value and that morphologic and clinical studies are the best available methods in discerning malignancy in adrenocortical neoplasms (17). Cote *et al.* (18) found expression of low-molecular weight cytokeratin intermediate filaments in ACA (adrenocortical adenoma) and normal adrenal but not in seven ACCs. In contrast, vimentin was seen in all ACCs studied and was heterogeneously expressed by ACAs. They conclude that undetectable expression of cytokeratins and strong expression of vimentin is associated with malignant adrenal cortical lesions (18). Goldblum *et al.* showed that PCNA expression does not correlate with histological diagnosis and clinical outcome in adrenal tumors, however, Ki67 expression significantly does (19).

In order to test the diagnostic potential of molecular markers in adrenocortical neoplasms we studied 43 adrenocortical masses, evaluating the use of p53 tumor suppressor gene alterations (20, 21), Ki67 expression, the rate of apoptosis, and allelic loss at the 11q13 locus near the multiple endocrine neoplasia type-1 (MEN-1) gene (22, 23) in differentiating benign from malignant lesions.

Patients and methods

Patients and DNA isolation

We studied 43 patients with a variety of adrenal diseases (clinical data see Table 1). Neoplastic tissue

was obtained at adrenalectomy. The tissue was snap-frozen immediately and stored at -80 °C for a maximum of 11 years. Diagnosis was made by clinical and histological data following established criteria (8, 9), together with a follow-up of the patients for a minimum of 12 month (24). Endocrine work up included measurement of urinary free cortisol, serum renin and aldosterone, urinary tetrahydroaldosterone, urinary catecholamine and performing of low dose dexamethasone suppression test where applicable. Blood was simultaneously collected for leukocyte DNA extraction. DNA was isolated from frozen tissue and leukocytes using the Qiagen Blood & Cell Culture DNA Kit and protocol (Qiagen, Hilden, Germany). Seven human adrenal glands were obtained from brain-dead patients during organ removal for transplantation and studied as reference tissue.

p53 Immunohistochemistry and loss of heterozygosity of 17p

Immunohistochemistry was performed on frozen tumor sections using the monoclonal IgG mouse antibody PAb 1801 (Ab 2, Oncogene Science, Manhasset, NY, USA) according to the instructions of the manufacturer and described in detail previously (20). Loss of heterozygosity (LOH) indicating allelic loss was analyzed with the pYNZ22 microsatellite, a variable number of tandem repeat (VNTR) marker at 17p near the p53 gene. The rate of heterozygosity in examined populations is approximately 80% (25). DNA from frozen tissue or leukocyte DNA was amplified by PCR and analyzed by ethidium bromide stained agarose gel electrophoresis. Primers, protocol and PCR conditions were similar to those reported recently (20). Data of p53 immunohistochemistry from 25 out of the 50 samples of the present series have been published previously (20).

TdT-mediated dUTP biotin 3'OH nick end labeling (TUNEL)

The rate of apoptotic cells was assessed by the TUNEL method (26). In brief, 10 µm tissue cryosections were fixed and incubated with the TdT enzyme at 37 °C as primary antibody; a peroxidase coupled digoxigenin antibody was used as secondary antibody and

Table 1 Clinical data of the patients studied.

Diagnosis	n	Mean age in years (range)	Mean tumor size (cm)	Range of tumor size (cm)	Sex (F/M)
Human adrenal glands*	7	42 (15-68)	-	-	4/3
MMAH	3	38 (32-45)	4.0	3.0-5.0	2/1
ACA	30	50 (30-69)	3.0	0.7-7.0	23/7
ACC	10	37 (1-69)	10.7	7.0-16.5	5/5

*Human adrenal glands were obtained from brain-dead patients during organ removal for transplantation. F, female; M, male.

Table 2 p53 alterations, apoptosis, Ki67 index and LOH of 11q13 in sporadic adrenal tumors. The number of non-informative cases is shown in parentheses. *P* value for significance of difference: Carcinoma vs adenoma **P* < 0.0001.

Diagnosis	<i>n</i>	p53 alterations		Apoptosis: % TUNEL-positive cells±s.d.	Proliferation: % Ki67-positive cells±s.d.	MEN-1 locus LOH at D11S956
		p53 over- expression	LOH at pYNZ22			
Human adrenal glands	7	—	—	0.19±0.06	1.89±1.30	—
MMAH	3	0/3 (0)	1/3 (0)	0.50±0.31	3.47±1.37	2 ^a /3 (0)
Adrenal adenoma	30	1/28 (2)	1/23 (7)	0.44±0.29	2.11±1.01	5/26 (4)
Adrenal carcinoma	10	3/10 (0)	5/5 (2)	4.57±4.43*	11.94±7.58*	6/8 (2)

^aLOH was present in some but not all noduli.

positive cells were visualized with diaminobenzidine as chromogen. Specimens were counterstained with methyl green. All reagents and protocols were supplied by the manufacturer (Oncor, Gaithersburg, MD, USA).

Ki67 Immunohistochemistry

On 10 µm tissue cryosections, pretreated with blocking serum, the monoclonal antibody MIB1 (Dianova, Hamburg, Germany) was used to detect specifically Ki67 antigen. Bound MIB1 antibody was conjugated with a biotinylated IgG. Ki67 positive cells were finally visualized by the biotin–avidin–peroxidase complex system revealing a brown nuclear signal. Specimens were counterstained with hematoxylin.

Analysis of immunohistological data

At a magnification of 500× the number of positive cells were counted and divided by the number of total cells per high power field (HPF). For each tumor the mean of five HPFs was calculated and expressed as percentage (%) positive cells. p53 overexpression was defined as nuclear p53 immunoreactivity in more than 1% of tumor cells.

11q13 LOH

D11S956 is a VNTR marker on 11q13 at the centromeric side of the MEN-1 gene locus. Its rate of heterozygosity is 88% (27). PCR was used to amplify the alleles according to the following protocol: initial denaturation at 94 °C for 3 min, followed by 27 cycles (denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and primer extension at 72 °C for 1 min), terminated by a final step of primer extension at 72 °C for 6 min. The primers used were: 5'-GATCAGTAAT-TAGCCAGACTCTAGG-3' and 5'-GGTTTTGGAGCTTA-AGGAGG-3' (27).

Statistics

All values are expressed as mean±s.d. Significance of differences was assessed by ANOVA using the StatView

5.0 (SAS Institute, Cary, NC, USA). A *P* < 0.05 was considered statistically significant. Correlation between parameters was determined by linear regression analysis.

Results

p53 alterations

Overexpression of p53 was detected in none of massive macronodular ACTH-independent adrenocortical hyperplasias (MMAH), was rarely present in ACA (1/28), but was found in approximately 30% of ACC (Table 2). LOH of p53 was rarely found in benign lesions (1/23), but in all ACC informative for pYNZ22 (*n* = 6).

Apoptosis

The rate of apoptotic cells was significantly higher (*P* < 0.0001) in ACC than in the other adrenocortical tissues (Fig. 1), but there was some overlap between groups, limiting its prognostic significance. Two out of

Figure 1 Mean (±s.d.) rate of apoptotic cells (gray bars) and Ki67-positive cells (white bars) of the different tissues. **P* < 0.0001, ACC vs ACA. NA=normal adrenal.

Figure 2 Apoptosis index: distribution in normal adrenal (NA), MMAH, ACA and ACC.

Figure 3 Ki67 index: distribution in normal adrenal (NA), MMAH, ACA and ACC. The line represents the cut-off between ACA and ACC of 5%.

Figure 4 LOH studies: LOH indicating allelic loss was analyzed with the pYNZ22 microsatellite marker near the p53 gene (patients 1–3) and D11S956 marker on 11q13 at the centromeric side of the MEN-1 gene locus (patients 4–6). LOH of tumor DNA in patients 1 (ACC) and 4 (ACA), no LOH of tumor DNA in patients 2 (ACA) and 5 (ACC), non-informative patient 3 (pheochromocytoma) and 6 (adrenocortical adenoma). M=marker, L=leucocyte DNA, T=tumor DNA.

30 (6.6%) ACA had an apoptotic index higher than 1% and 1/10 (10.0%) ACC had one lower than 1% (Fig. 2).

Ki67 expression

ACC had the highest Ki67 index ($12.98 \pm 7.11\%$) of all adrenocortical tumors (human adrenal glands $1.9 \pm 1.30\%$, MMAH $3.47 \pm 1.37\%$, ACA $2.11 \pm 1.01\%$) distinguishing significantly ($P < 0.0001$; Fig. 1) all carcinomas from benign adrenocortical tumors without any overlap (cut-off level 5%, Fig. 3). A weak but significant correlation between Ki67 expression and apoptotic index could be detected in the tissues studied ($R^2 = 0.15$, $P = 0.04$).

LOH at the MEN-1 locus

LOH of 11q13 was found in 19% (5/26) of ACA and 75% (6/8) of ACC. None of the ACA revealed LOH of the MEN-1 locus and of the p53 gene locus whereas six of ten ACC proved to have LOH of both loci (Table 3) (Fig. 4).

Discussion

The main finding of our study is that a Ki67 staining index over 5% separates benign from malignant adrenocortical tumors without overlap (Fig. 3). This indicates that a Ki67 index greater than 5% is a good marker for malignancy in adrenocortical neoplasms. Our data extend the recent findings of Goldblum *et al.* (18). Using archival material they showed that the Ki67 index was higher in 15 ACC compared with 19 ACA. However, in their series, 6 of 15 carcinomas had a Ki67 labeling index within the range of adenomas, limiting its diagnostic significance as a marker for malignancy. The discrepancy with our series may be due to technical differences. Whereas fresh frozen tumor tissue in the present series gave an intense nuclear Ki67 signal, Ki67 staining as reported by Goldblum *et al.* was shown to be discrete and sometimes cytoplasmic in localization. This is probably due to formalin fixation of the tissue with decreased protein antigenicity and/or reduced nuclear penetration of the antibody resulting in underestimation of the true number of Ki67-positive cells.

Table 3 Results for 43 adrenal lesions and seven human adrenal glands investigated for p53 alterations, apoptosis, proliferation and LOH of 11q13. NA = normal adrenal.

No.	Diagnosis	p53 alterations		Apoptosis: % TUNEL-positive cell	Proliferation: % Ki67-positive cells	MEN-1 locus LOH at D11S956
		p53 over-expression	LOH at pYNZ22			
1	NA	/	/	0.10	0.90	/
2	NA	/	/	0.16	3.80	/
3	NA	/	/	0.28	2.50	/
4	NA	/	/	0.16	2.40	/
5	NA	/	/	0.15	0.70	/
6	NA	/	/	0.20	2.70	/
7	NA	/	/	0.25	0.20	/
8	MMAH	—	+	0.85	4.10	+
9	MMAH	—	—	0.25	4.40	—
10	MMAH	—	—	0.4	1.9	+ ^a
11	ACA	—	—	n.i.	0.03	—
12	ACA	—	—	0.31	2.70	—
13	ACA	—	—	0.26	0.00	+
14	ACA	—	—	n.i.	4.00	+
15	ACA	n.i.	n.i.	0.43	2.20	+
16	ACA	—	—	0.31	1.70	n.i.
17	ACA	—	—	0.40	1.50	+
18	ACA	—	—	0.42	0.90	—
19	ACA	—	n.i.	0.34	1.20	—
20	ACA	—	n.i.	0.22	1.10	—
21	ACA	—	—	0.17	1.40	—
22	ACA	—	—	0.18	1.50	—
23	ACA	—	—	0.24	4.00	—
24	ACA	—	—	0.51	2.10	—
25	ACA	—	—	0.24	2.90	—
26	ACA	—	—	0.30	3.50	—
27	ACA	n.i.	—	0.39	2.00	n.i.
28	ACA	—	—	0.28	3.80	—
29	ACA	—	n.i.	0.36	0.70	—
30	ACA	—	n.i.	0.27	2.00	—
31	ACA	—	—	0.63	1.20	n.i.
32	ACA	—	n.i.	0.49	2.00	—
33	ACA	—	—	0.51	3.50	—
34	ACA	—	n.i.	0.30	2.40	—
35	ACA	—	—	0.12	4.00	—
36	ACA	—	—	0.71	1.90	+
37	ACA	—	—	0.48	2.30	—
38	ACA	—	—	1.28	2.30	—
39	ACA	+	+	0.70	1.80	n.i.
40	ACA	—	—	1.39	2.40	—
41	ACC	—	n.i.	3.95	7.60	—
42	ACC	—	+	1.16	9.30	+
43	ACC	+	n.i.	1.18	13.20	—
44	ACC	+	+	2.64	8.30	+
45	ACC	—	+	0.66	14.00	+
46	ACC	—	+	2.38	18.10	+
47	ACC	+	+	2.17	30.40	+
48	ACC	—	—	3.1	11.0	n.i.
49	ACC	—	—	9.3	5.5	+
50	ACC	—	—	9.3	12.4	n.i.

^aLOH in one of two noduli analysed. n.i. = non-informative, / = not performed.

Data on the importance of the cell proliferation indices like Ki67 have been collected in other endocrine and non-endocrine tissues tumors. Vargas and colleagues found that more than 4% MIB1-positive cells in parathyroid tumors correlated strongly with malignancy (28). In pituitary adenomas, a Ki67 index higher than 1% predisposed to rapid tumor growth 2

years after initial surgery (29). To identify variables potentially predictive of malignancy among non-functioning pancreatic endocrine tumors, La-Rosa *et al.* found that vascular or perineural microinvasion and Ki67 proliferative index >2% were the most sensitive and specific variables correlated with malignancy (30). Basolo and colleagues (31) investigated 92

thyroid carcinomas of different histotypes for apoptotic and Ki67 indices. Whereas the apoptotic index was low in all subtypes of thyroid carcinoma, the Ki67 index was significantly different among the histotypes, increasing with tumor aggressiveness from 3.1% for papillary carcinoma to 5.6% for poorly differentiated carcinoma and 51.8% for undifferentiated carcinoma (31). Garzetti *et al.* reported that in comparison to cystadenomas and borderline tumors, the Ki67 antigen immunostaining was significantly higher in ovarian cystadenocarcinomas. Additionally, a significant relationship was observed between the Ki67 index and disease-free survival (32). Albers *et al.* reported in patients with clinical stage A non-seminomatous germ cell tumors who consecutively underwent retroperitoneal lymph node dissection that only those cases revealed metastases that had a MIB1 value higher than 52% (33).

In our series, p53 tumor suppressor gene alterations and LOH of 11q13 alone were not very useful markers. LOH of 17p and an elevated apoptotic index was predominantly seen in ACC. Those findings may provide a clue to malignancy in a given tumor, but also occur in ACA, limiting its prognostic significance. According to our series, combinations of these markers can give additional information if the malignant potential of a tumor is unknown: LOH of both 17p and 11q13 was present in none of the ACA, compared with 6/10 ACC. On the other hand, absence of p53 alterations and LOH of 11q13 together with a low apoptotic index makes malignancy unlikely in a given tumor (Table 3 and Fig. 2).

Our findings may have an impact on the approach to patients with incidentally detected adrenal masses. There is general agreement that adrenal tumors larger than 4–5 cm should be removed because of the risk of malignancy (4, 34). In these patients, careful analysis of the tumor tissue is required to identify those patients who harbor an ACC. In the past, identification of well differentiated ACC among benign ACA by means of histopathology was time-consuming and required specialist knowledge, as outlined recently (6, 8). Determination of Ki67 content, possibly in combination with other markers, could help to determine the malignant potential of a given tumor with more accuracy.

Surgical removal is not generally recommended in adrenal incidentalomas smaller than 5 cm in diameter. A frequently used method to evaluate a tumor's malignant potential in these cases is CT or ultrasound-guided fine needle aspiration biopsy or cutting biopsy. However, this approach is controversial, since the small amount of tissue yielded by the procedure makes the assessment of the pathological criteria (invasion of venous and sinusoids, necrosis, fibrous bands, tumor architecture, etc.) almost impossible. Adrenal biopsies are also associated with considerable morbidity and mortality due to pneumothorax, hemorrhage, incidental puncture of pheochromocytomas and

needle tract metastases outweighing the benefits for the patient. In this setting, reliable markers for malignancy could be a strong argument for biopsy, especially in the evaluation of small but suspicious silent adrenal masses.

In conclusion, in our series a Ki67 index above 5% was a valuable marker for malignancy in adrenocortical neoplasms. A high apoptotic index, especially in combination with LOH of 17p and 11q13, raises the probability of ACC.

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