EXPERIMENTAL STUDY

Molecular analysis of *CDKN1C* and *TP53* in sporadic adrenal tumors

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Abstract

Objective: To evaluate the roles of the *CDKN1C* (*P57KIP2*) gene, which encodes for the cyclin-dependent kinase inhibitor CDNC, and the *TP53* tumor suppressor gene in adrenal tumorigenesis, as a means of investigating the molecular basis of sporadic adrenal tumors, which is unknown.
Design: Screening for the presence *CDKN1C* and *TP53* mutations and analyzing the expression pattern of CDNC, P53 and its downstream effector CDN1 (P21*WAF1/CIP1*) in a series of 79 sporadic adrenal tumors.
Methods: Single-strand conformation polymorphism and sequencing were used for mutation analysis of *CDKN1C* and *TP53* in blood and adrenal tissue samples. In a subgroup of 48 tissues, *CDKN1C* expression was evaluated by RT-PCR and immunohistochemistry. Immunohistochemical analysis of P53 and CDN1 was performed.
Results: No somatic mutations of *CDKN1C* were found in the tumors analyzed, in spite of low/absent CDNC expression in adrenocortical adenomas and carcinomas. Mutations in the *TP53* gene were present in 70% of adrenocortical carcinomas, associated with abnormal P53 and CDN1 expression, but not in benign neoplasms. In the normal adrenal cortex, CDNC expression was strictly nuclear and confined to the cortical zone (i.e. zona glomerulosa and reticularis), with no staining in the medulla.
Conclusions: Mutations in the *TP53* gene are frequent in adrenocortical carcinomas and might be used as a marker of malignancy. In the normal adrenal cortex, the zone-specific pattern of expression of CDNC suggests a role in adrenal differentiation.

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Introduction

Management of adrenocortical masses still raises much concern about the diagnosis of malignancy. Indeed, clinical and morphological data cannot reliably distinguish between benign and malignant adrenal masses, and often the nature of a localized tumor cannot be assessed even at histology. Identification of molecular markers of malignancy has been demonstrated to be helpful in the diagnosis and characterization of several haematologic and solid neoplasms. However, the molecular events leading to malignancy are largely unknown for adrenal tumors, even though a multi-step model with a sequence of genetic events, involving overexpression of proto-oncogenes or inactivation of tumor suppressor genes, or both, have been hypothesized (1). Rearrangements, loss of heterozygosity and abnormal imprinting at the 11p15.5 chromosomal region, associated with low *CDKN1C*

(*P57KIP2*) and *H19* RNA expression and increased *IGF2* mRNA levels, have been reported in adrenocortical carcinomas (1). Mutations within candidate genes, such as *TP53*, have also been demonstrated in adrenocortical carcinomas (1). A putative role for these alterations in adrenal tumorigenesis is suggested also by the association, although rare, of adrenocortical carcinomas with familial tumor syndromes, such as the Beckwith–Wiedemann syndrome and the Li–Fraumeni syndrome. The molecular basis of these syndromes is represented by abnormalities within 11p15.5, including *CDKN1C* mutations, and germline *TP53* mutations respectively (1).

It is against this background that the *CDKN1C* and the *TP53* genes have been considered as candidate genes for adrenal tumorigenesis. In the present study we screened a large series of sporadic adrenal tumors for mutations in the *CDKN1C* gene and for expression of *CDKN1C* mRNA and the

Table 1 Clinical and pathological data of adrenal tumors ($n = 79$). Values in parentheses are ranges.

Diagnosis	No.	Sex (F/M)	Median age (yr)	Median size (cm)
Adrenocortical carcinoma†				
Non-functioning	3	2/1	45 (43–65)	8 (5–12)
Functioning‡	7	6/1	46 (27–69)	9.5 (8–20)
Suspect adrenocortical carcinoma				
Non-functioning	3	2/1	52 (35–68)	4.5 (4–6)
Androgen-secreting	1	1	44	8
Adrenocortical adenoma				
Non-functioning	17	11/6	58 (46–70)	3.5 (2.5–5)
Aldosterone-producing	16	10/6	36 (28–57)	2 (0.5–3)
Cortisol-producing	8	7/1	47 (19–58)	3 (2.5–5)
ACTH-dependent adrenal hyperplasia	6	5/1	36 (17–48)	
Pheochromocytoma	17	7/10	20 (12–52)	5 (3–8)
Malignant pheochromocytoma	1	0/1	35	8

†Stage I–II = three patients; stage III–IV = six patients.
‡Local recurrences of adrenocortical carcinoma in two patients.

encoded protein CDNC. In the same tumor samples, we performed mutational analysis of *TP53* and immunohistochemical evaluation of the expression of P53 and of its downstream effector CDN1 (P21*WAF1/CIP1*).

Materials and methods

Patients and tissues

A total of 79 patients with sporadic adrenal tumors were investigated. Clinical and pathologic data are summarized in Table 1. Normal adult adrenals were obtained from 10 patients who underwent nephrectomy for kidney cancer. Patients underwent clinical, radiological, and hormonal evaluation as previously described (2). They were considered to have functioning tumors on the basis of the clinical picture and of abnormal hormone levels. Staging of adrenocortical carcinomas was preformed according to the MacFarlane criteria (3). Diagnosis of benign, suspect and malignant adrenocortical tumors was based on widely accepted criteria, including tumor mass, presence of metastasis or recurrence, mitotic rate, nuclear grade, necrosis and capsula and/or vascular invasion (4). Tissue specimens, removed at surgery, were snap-frozen in liquid nitrogen and stored at $-80\,^{\circ}\text{C}$ until required for DNA extraction. To avoid contamination with surrounding tissue, non-neoplastic areas were removed macroscopically, and only a central portion of each tissue specimen was used. Blood samples from all patients were available. Blood and tissues were collected after informed consent had been given by the patients and with the approval of the local ethics committee.

PCR-single-strand conformation polymorphism analysis and sequencing

DNA was isolated from leukocytes and tissue specimens by standard procedures using proteinase k digestion and the phenol–chloroform method. All samples were screened for mutations in the coding sequence of the *CDKN1C* and the *TP53* genes by single-strand conformation polymorphism (SSCP) analysis, direct sequencing, or both. Oligonucleotide primer sequences used for PCR and sequencing are available on request. Sequencing was performed on an ABI PRISM 310 DNA sequencer (Applied Biosystem, Foster City, CA, USA) using ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit with AmpliTaq DNA Polymerase FS (Applied Biosystem).

RT-PCR and CDKN1C mRNA analysis

RT-PCR was performed in a subgroup of 19 tissues, including normal adult adrenal (five samples), ACTH-dependent adrenocortical hyperplasia (three samples), functioning and non-functioning adrenocortical adenoma (five samples) and functioning and non-functioning adrenocortical carcinoma (six samples). Total RNA was isolated from tissues after a single step acid guanidium–phenol–chlorophorm extraction procedure using RNAzol (Biotech Laboratories, Inc., Huston, TX, USA). Random-primed cDNAs were generated from total RNA using MuLV reverse transcriptase. PCR of the *CDKN1C* cDNA was performed using oligonucleotide primers that span the intron between exons 3 and 4 and yield a 255 bp product. As the internal control, a 100 bp fragment of β-*actin* was also amplified by RT-PCR. PCR products, when amplified for 30 cycles, remained within the exponential phase of the reaction on the linear range for both *CDKN1C* and β-*actin*. RT-PCR products were run on agarose gels and the images digitized and subjected to Image Master 1D software analysis (Amersham Pharmacia Biotech, Uppsala, Sweden).

Immunohistochemistry

CDNC expression was investigated *in situ* in a subgroup of 48 adrenal tissues, including normal adult (10

samples) and fetal (one sample) adrenals, ACTH-dependent adrenocortical hyperplasia (four samples), functioning and non-functioning adrenocortical adenomas (five samples), functioning and non-functioning adrenocortical tumors (suspect malignant, four samples; malignant, seven samples) and pheochromocytomas (17 samples).

The immunohistochemical analysis for CDNC was performed on formalin-fixed paraffin-embedded sections using microwave antigen retrieval and a streptavidin–biotin peroxidase technique (LSAB Dako, Carpinteria, CA, USA), as described previously (5). A goat affinity-purified polyclonal antibody raised against a peptide corresponding to amino acids 286–305 mapping at the carboxy terminus of human CDNC was used (C-20 cat. No. sc-1040 Santa Cruz Biotechnology, Santa Cruz, CA, USA).

The immunohistochemical analysis for P53 and CDN1 was performed as previously described (6) using DO-1 MoAb (Dako, Glostrup, Denmark) specific for P53 and EA10 MoAb (Oncogene Science, Uniondale, NY, USA) recognizing CDN1.

Results

Mutations of the CDKN1C gene in sporadic adrenal tumors

SSCP analysis and sequencing of *CDKN1C* revealed several neutral or conservative polymorphisms in 18 of 79 adrenal tumors (three adrenocortical carcinomas, six non-functioning adrenocortical adenomas, four aldosterone-producing adenomas, one cortisol-producing adenoma, four pheochromocytomas) and in one normal adrenal sample. Polymorphisms were found also in the corresponding blood samples. These included the PAPA-repeat length polymorphism in exon 2 (1715–1727del) in six cases, $1776C \rightarrow T$ transition in exon 2 (A183A) in nine cases, 2773–2774insG in intron 3 in 12 cases (which have been reported also by others (7), and three novel polymorphisms: $1407C \rightarrow T$ (R60R) in exon 2 in one case, $2708C \rightarrow G$ (G305G) and $2726G \rightarrow A$ (P311P) in exon 3 in the other two. At variance with the benign tumors, the three adrenocortical carcinomas showed $1776C \rightarrow T$ and 2773–2774insG heterozygous polymorphisms in blood DNA, but were hemizygous in tumor DNA, suggesting loss of heterozygosity. No relationships between DNA polymorphism and phenotype (including age, sex, functioning status, size, histological diagnosis) were apparent from our data. All samples showed both $2079G \rightarrow C$ transversion and 2600–2601insC in intron 2, confirming previous reports (7). None of the adrenal tumors contained *CDKN1C* somatic coding or splice-signal mutations.

CDKN1C expression in normal adrenal tissues and sporadic adrenal tumors

In normal adrenal gland (10 samples) a number of CDNC expressing cells were observed at immunohistochemistry, confined to the cortical zone, with completely negative results in the medulla. The immunostaining was strictly nuclear, and variable in intensity. The proportion of CDNC expressing cells was highly variable and related to the location within the cortex. In the *zona glomerulosa* 5–10% CDNC positive cells were present. The *zona fasciculata* was devoid of positive cells in all samples, with the exception of few scattered nuclei observed in a minority of samples. The proportion of positive cells reached the greatest values in the *zona reticularis*, with 10–20% cells expressing CDNC (Fig. 1). Similar levels of CDNC expression were observed in a single sample of fetal adrenal (12 weeks old). In all samples of cortical hyperplasia, a pattern of CDNC expression similar to that observed in normal adrenal tissue was observed, with variable levels of expression in different zones. In contrast, most samples of adenoma (four of five) showed very few positive cells, with a single case expressing a proportion of positive cells akin to normal adrenal cortex. A low level of CDNC expression was documented in most adrenocortical tumors (six of seven) clearly defined as malignant on the basis of pathological and clinical criteria, and in most adrenal tumors characterized by atypical morphology, but lacking consistent clinico-pathological features of malignancy (Table 2). Nevertheless, a focal immunostaining was observed in two out of four of these cases. No particular features could be documented (including sex, age, morphology, tumor dimension, or functioning status) to account for this phenotypic heterogeneity. Most cells in pheochromocytomas were devoid of CDNC expression, with the exception of focal collections of strongly staining cells in four of 17 investigated cases.

Densitometric analysis of RT-PCR for *CDKN1C* mRNA showed greatest expression in normal adrenal gland and adrenocortical hyperplasia. At variance with this, *CDKN1C* mRNA levels were lower or absent in functioning and non-functioning adenomas (5–70% and 0–30% of that of normal adrenal respectively) and in adrenocortical carcinomas (0–15% of that of normal adrenal) (Fig. 2).

Mutations of the TP53 gene in sporadic adrenal tumors

PCR-SSCP analysis and sequencing of *TP53* showed mutations in tumor DNA from six of seven (86%) functioning adrenocortical carcinomas, in one of three (33%) non-functioning adrenocortical carcinomas, and in one of four (25%) suspect non-functioning adrenocortical carcinomas. Sequencing of paired leukocyte
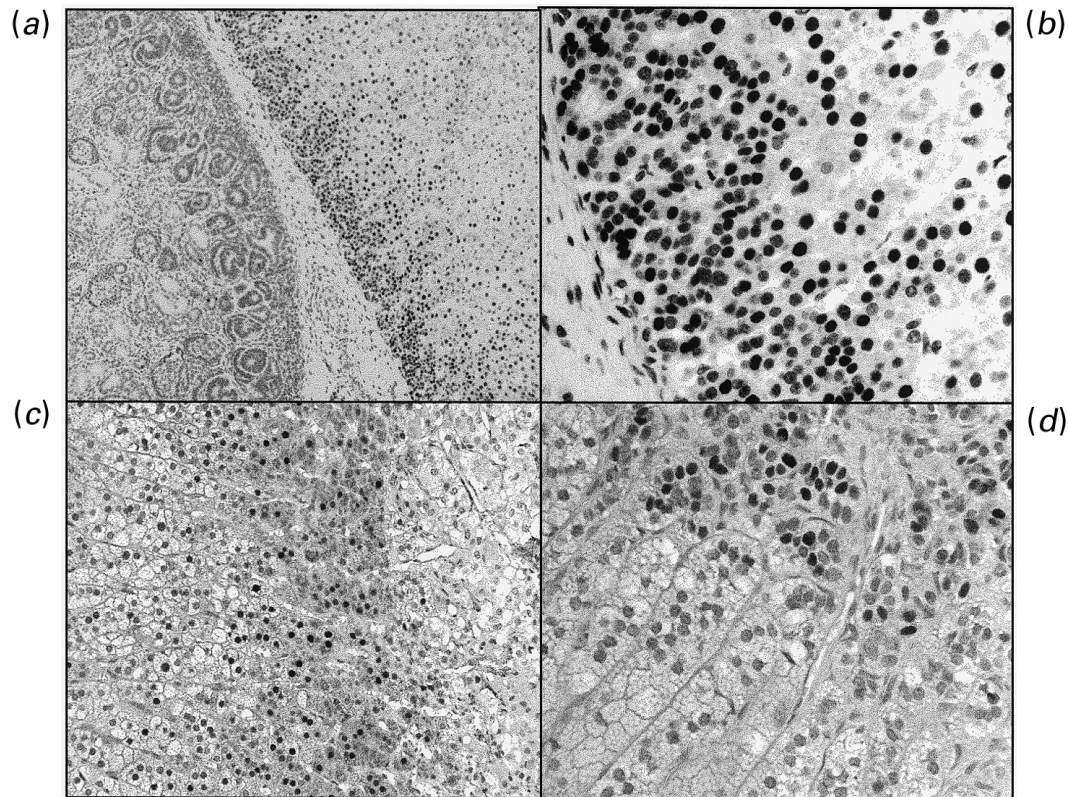
Figure 1 Expression of CDNC in normal adrenal tissues. (*a*) and (*b*) Fetal adrenal gland; (*c*) and (*d*) normal adrenal gland (*fasciculata, reticularis, glomerulosa*).

DNA revealed a wild-type sequence. In particular, missense mutations were identified in five stage II-III adrenocortical carcinomas (A161V, I162M, E180A, D208G, G245S) and in the suspect adrenocortical carcinoma (S185R). These included a female patient who, after 1 year of follow-up, exhibited an increase in tumor mass size from 3.5 to 4.5 cm, associated with subclinical hypercortisolism (8). Deletions were demonstrated in two recurrences from two aggressive stage IV adrenocortical carcinomas: the first tumor had a heterozygous 3 bp in-frame deletion (P191del); the second had two distinct hemizygous deletions in exon 8 (14571delC; 14575delG), leading to a frame-shift that modified the three amino acids downstream and resulted in a premature translation termination. Germline genetic polymorphisms (13342T → C (L194L) in exon 6 and 12139G → C (R72P) in exon 4) were also observed in an adrenocortical carcinoma and in four adrenocortical adenomas (one androgen-secreting and three non-functioning) respectively.

Table 2 CDNC, P53 and CDN1 expression in sporadic adrenal tumors.

Sample	No.	CDNC expression†			P53 expression†			CDN1 expression†		
		++	+	−	++	+	−	++	+	−
Normal adrenocortical tissue	10	10								
Adrenocortical hyperplasia	4	1	2	1						
Adrenocortical adenoma	5	1		4						
Suspect adrenocortical carcinoma	4			4‡			4	2	1	1
Adrenocortical carcinoma	7		1	6	1		6	2	1	4
Pheochromocytoma	16		4	12			16		1	15
Malignant pheochromocytoma	1			1			1	1		

†Immunohistochemical semi-quantitative evaluation of CDNC, P53 and CDN1 positive cells: −, negative; +, 5−10%; ++, 10−20% for CDNC, 10−15% for P53 and CDN1.
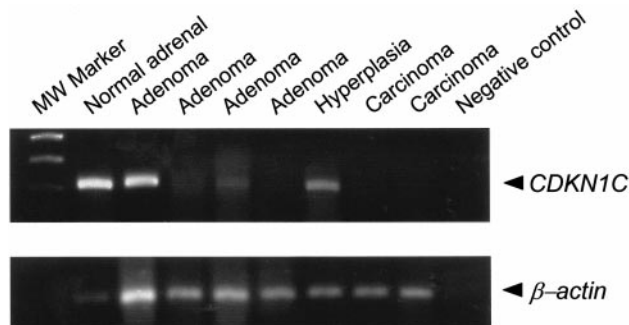‡Focal expression of CDNC in two cases.

Figure 2 Representative RT-PCR for *CDKN1C* and *β*-*actin* mRNA in normal adrenal tissue and adrenocortical tumors. Negative control is RT-PCR without reverse transcriptase.

P53 and CDN1 expression in sporadic adrenal tumors

Immunohistochemical analysis of P53 and CDN1 proteins, performed in 11 malignant/atypical adrenocortical tumors, revealed P53 overexpression (strong nuclear immunostaining in more than 50% cells) only in an adrenocortical carcinoma bearing an A161V missense mutation (Table 2). This tumor was characterized by complete lack of CDN1, suggesting that non-fuctional P53 protein was accumulated in neoplastic nuclei. All other investigated malignant cases did not contain a significant number of P53 positive nuclei (<5%), whereas CDN1 was variably expressed regardless of molecular abnormalities. All four investigated suspect adrenocortical tumors were P53-negative; overexpression of CDN1 was demonstrated in two. All 17 samples of pheochromocytoma were negative for P53, whereas CDN1 was overexpressed in a single malignant pheochromocytoma (Table 2).

Discussion

Previous reports have suggested a role for genes located in the 11p15.5 region, including *CDNK1C*, in adrenal tumorigenesis (1, 9–11) To our knowledge, the present report is the first on mutational analysis of *CDKN1C* and immunohistochemical evaluation of its expression in normal and neoplastic adrenal tissues.

Our results indicate that mutations within the *CDKN1C* gene are rare events and do not account for reduced *CDKN1C* mRNA and protein levels observed in adrenocortical tumors. However, we cannot exclude the presence of mutations in the non-coding region of the gene, influencing expression or altering the start site, or hemizygous deletions of one allele, or other mutations missed by SSCP analysis. In accordance with others (9–11), our data showed a pattern of expression of *CDKN1C* mRNA and CDNC in hyperplastic adrenal that paralleled that of normal adrenal tissue, whereas it was very low in most adrenocortical adenomas and

carcinomas. As the majority of these tumors are known to be monoclonal in origin (12), low expression of *CDKN1C* may indeed reflect a growth advantage of a particular clone. Decrease in both RNA and protein expression in adrenal tumors suggests that post-translational modifications by the ubiquitin–proteasome system may not be important in regulating *CDKN1C* expression (13). At variance with this, loss of heterozygosity, imprinting abnormalities, or both, reported in most adrenocortical carcinomas (10, 11), could explain low *CDKN1C* expression. In our patients, immunohistochemical analysis demonstrated that CDNC is expressed in the normal adrenal cortex, but not in the medulla. Immunostaining of CDNC was strictly nuclear and, interestingly, was zone-specific – that is, negative in the adrenal *zona fasciculata*. This pattern of expression seems to reflect the differential regulation of cell proliferation, maturation and apoptosis in the normal adrenal gland, with cell proliferation predominant in the *zona fasciculata* and programmed cell death predominant in the *zona reticularis* and in the *zona glomerulosa* (14, 15). These data suggests that CDNC might play a part in the differentiation of the normal adrenal cortex (16).

TP53 somatic mutations were observed in 70% adrenocortical carcinomas, but not in benign lesions, suggesting that it may be possible to use their presence as a marker of malignancy. Although our analysis was performed in a relatively small series of adrenocortical carcinomas, the prevalence of mutations was greater than had been observed in other studies reporting *TP53* mutations in 20–30% of sporadic adrenocortical carcinomas, but rarely in adenomas (1, 17–20). Environmental factors or methodological aspects such as the scanning of the entire rather than the partial sequence of the gene may account for this difference. At variance with this, but in agreement with previous findings (20, 21), no *TP53* gene mutations were observed in pheochromocytomas, suggesting that *TP53* mutations are not important in the pathogenesis of these tumors, although a role in the development of malignant pheochromocytomas cannot be excluded (20). Immunohistochemical analysis of P53 and of its downstream effector CDN1 in adrenal cortical and medullary tumors showed a poor concordance with *TP53* sequence data. Only one adrenocortical carcinoma with a *TP53* mutation had the typical immunohistochemical pattern of P53 overexpression and decreased CDN1 expression. Discordant results between *TP53* mutations and immunohistochemical staining have been reported in other types of malignancy (22). In fact, deletion mutations, as in our two samples with negative staining, may not result in a P53 protein with increased stability, and, in the case of missense mutations, additional cellular events may be required for P53 overexpression (22). Thus immunohistochemical analysis alone (23, 24) does not detect all genetic alterations affecting *TP53* in adrenal tumors.

In conclusion, reduced *CDKN1C* expression occurs in the majority of adrenocortical adenomas and carcinomas, but is not due to genetic mutations. Mutations in the *TP53* gene are frequent in adrenocortical carcinomas and might be used as a marker of malignancy. Further investigations on the mechanisms of *CDKN1C* downregulation in adrenal tumors and on its role in the development of the normal adrenal cortex are required.

Acknowledgements

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