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Immunopathology of Adrenal and Renal Cortical Tumors

Coordinated Change in Antigen Expression Is Associated with Neoplastic Conversion in the Adrenal Cortex

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A series of adrenal cortical adenomas (ACA) and carcinomas (ACC), as well as normal adrenal cortex have been studied by a panel of 11 antibodies to characterize antigenic changes that may distinguish these morphologically similar entities. Normal adrenal cortex and ACA express low-molecular weight cytokeratin intermediate filaments. However, none of the six primary or seven metastatic ACCs were found to express detectable levels of cytokeratins. In contrast, vimentin was seen in all ACCs studied and was heterogeneously expressed by ACAs. However, its expression was usually confined to stromal elements of the normal adrenal cortex. We conclude that adrenal cortical cells undergo characteristic changes in intermediate filament expression during the process of neoplastic conversion and malignant transformation. Undetectable expression of cytokeratins and strong expression of vimentin is associated with malignant adrenal cortical lesions. In addition, we examined the antigenic phenotype of a series of primary renal cell carcinomas (RCC). Renal cell carcinomas express cytokeratins, while ACCs do not. The majority of primary RCCs express Lewis blood group isoantigens (most commonly Lewis X), while ACAs and ACCs do not. The panel of antibodies described here may help to distinguish morphologically similar lesions of like histogenesis (ACAs vs. ACCs) and lesions of different histogenesis (adrenal vs. renal) on the basis of their composite antigenic phenotypes. (Am J Pathol 1990, 136:1077–1084)

The morphologic distinction of adrenal cortical adenomas (ACA) from adrenal cortical carcinomas (ACC) can be difficult. A number of investigators have attempted to establish criteria to distinguish these lesions. Histologic parameters that have been found most useful are presence of necrosis, nuclear atypia, mitotic rate, and vascular/capsular invasion.[1–5] Weight is generally believed to be the best predictor of behavior, although tumors weighing less than 50 g have metastasized and tumors weighing more than 100 g may not recur after surgical removal.[6–8] Tumor aneupolidy, as determined by DNA content analysis, can further substratify tumors.[9,10] However, none of the criteria predicts the ultimate behavior of all tumors.

Another diagnostic problem is the distinction between ACCs and renal cell carcinoma (RCC). The histogenesis of lesions involving the superior pole of the kidney and the adrenal gland can be difficult to determine because of location and similarities in histologic appearance. The differential diagnosis of metastatic tumors with clear or oncocytic cytoplasm often includes ACCs and RCCs.

We tested a panel of monoclonal antibodies on ACA, ACC, and RCC to determine their respective antigenic phenotypes and found that these lesions can be distinguished antigenically. In addition, we report changes in the antigen expression of adrenal cortical neoplasms that characterize progression from benign to malignant lesions.

Materials and Methods

Cases of ACA, ACC, and RCC reviewed at Memorial Sloan-Kettering Cancer Center (MSKCC) were studied.

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Table 1. *Immunohistochemical Reagents*

Antibody	Pretreatment/digestion	Source	Antibody dilution
AE-1	0.1% pepsin, 30 minutes	Sig	$1/10^3$
CAM 5.2	0.1% pepsin, 30 minutes	BD	$1/10^2$
Vimentin	0.025% trypsin, 7 minutes	Biog	$1/10^3$
Chromogranin	0.05% saponin, 30 minutes	Hyb	$1/10^4$
pS-100	0.05% saponin, 30 minutes 0.0025% pronase, 4 minutes	Dako	$1/10^3$
Blood group antigens†	0.05% saponin, 30 minutes	Sig	1/8

* All reagents are mouse monoclonal antibodies except that to pS-100, which is a polyclonal rabbit antisera, and the *Ulex europaeus* lectin.
† Antibodies to blood group antigens A, B and Lewis a, b, Y, and X. *Ulex europaeus* lectin used to identify H.
Antibody Sources: Sig: Signet Labs, Dedham, MA; BD: Becton-Dickenson, San Jose, CA; Biog: Biogenex, San Ramon, CA; Hybritech, San Diego, CA; Dako, Carpinteria, CA.

Formalin-fixed (10% buffered formalin) paraffin-embedded tissues were used. Paraffin-embedded tissue blocks were obtained in those cases processed at other hospitals. The avidin-biotin peroxidase conjugate method was used with minor modifications.[11] Briefly, 5-μm thick tissue sections were cut onto slides coated with 0.005% poly-l-lysine (Sigma Chemical Co., St. Louis, MO) and incubated at 60°C for 2 hours. After rehydration the endogenous peroxidase activity was quenched by incubation in 1.0% H_2O_2 (diluted in distilled water) for 10 minutes. The slides were washed in phosphate-buffered saline and exposed to protease digestion and/or detergent,[12] as indicated in Table 1. After washing in phosphate buffered saline (PBS), sections were incubated in 5% horse serum/PBS or 5% rabbit serum (in the case of antibody to pS-100) for 1 hour at room temperature. This was aspirated, replaced with appropriately diluted primary antibody (Table 1), and incubated in sealed moisture chambers for 12 hours at 4°C. The sections were then washed in PBS and biotinylated horse anti-mouse immunoglobulin (Vector Labs, Burlingame, CA) (or sheep anti-rabbit immunoglobulin (Vector Labs) in the case of pS-100) was incubated for 1 hour at room temperature. After washing in PBS, the sections were incubated with avidin-biotin complex (1:100 dilution in PBS; DAKO, Santa Barbara, CA) for 1 hour at room temperature. After a final rinse with PBS, the sections were incubated in 3,3'-diaminobenzidine (0.06% in PBS) (Sigma) with 0.003% H_2O_2 for 10 to 15 minutes. After counterstaining with modified Harris hematoxylin (Fisher, Orangeburg, NY) the sections were dehydrated and coverslipped with Permount. The primary antibodies along with their working dilutions and commercial sources are listed in Table 1. Dilutions were in 2% bovine serum albumin (BSA)/PBS. Also listed in Table 1 are the enzymatic/detergent pretreatments used.

In the case of reactivity with the lectin *Ulex europaeus*, endogenous peroxidase in tissue sections was quenched as described. Sections were then incubated with *Ulex europaeus* lectin (1:500 dilution in PBS, Vector Labs) for 2.5 hours at room temperature. The slides were rinsed in PBS and sections were incubated in 5% rabbit serum for 1 hour at room temperature, aspirated, and incubated with goat anti-lectin immunoglobins (Vector Labs) for 12 hours at 4°C. The remainder of the procedure is as described, with the use of biotinylated rabbit anti-goat immunoglobulins (1:1500 in PBS/BSA; Vector Labs).

Results

Clinical and Pathologic Findings

The clinical/pathologic features of the ACA and ACC (primary and metastatic) are given in Tables 2, 3, and 4. None of the ACAs (Table 2) has recurred, with a follow-up time ranging from 2 to 30 months (mean, 17 months). Primary ACCs (Table 3) were considered malignant either on the basis of metastases (at the time of or after diagnosis) (cases 7 to 10), direct invasion of contiguous structures (case 12), or based on size and histologic findings, particularly necrosis (case 11). The metastatic ACCs (Table 4) had primary tumors of the adrenal cortex documented at MSKCC or outside institutions, and in the latter cases microscopic material was reviewed at MSKCC.

All cases classified as renal cell carcinoma were primary tumors and all had clear or tubular cell features.

Immunohistochemical Results (Table 5)

Cytokeratins

Two widely available antibodies that recognize low-molecular weight cytokeratins were used (AE-1, Cam

Table 2. *Adrenal Cortical Adenomas*

Case	Age	Sex	Tumor weight (g)	Clinical presentation
1	79	F	60	Sarcoma*
2	39	F	18	↑ 17 ketosteroids
3	71	F	19	NS
4	40	M	66	Mass
5	57	M	24	Mass
6	50	M	73	Mass

NS, not stated.
* Preoperative diagnosis based on fine-needle aspiration.

Table 3. *Primary Adrenal Cortical Carcinomas*

Case	Age	Sex	Tumor weight (g)	Clinical	Comments
7	63	M	2400	↑ 17-ketosteroids	Mets present at primary resection
8	41	F	960	NS	Mets at 2 years
9	17	M	410	Cushings	Liver and lung mets
10	49	M	1100	NS	Mets present at primary resection
11	63	M	450	↑ 17 ketosteroids	Necrosis, complete replacement adrenal gland
12	30	F	900	NS	Invasion of liver

NS, not stated; Mets, metastasis.

5.2). Cytokeratin expression was seen in all examples (four of four) of normal adrenal cortex (Figure 1A). The staining was observed to be focal and generally restricted to the zona glomerulosa. All examples (six of six) of ACAs showed expression of cytokeratins as well. However, a range of expression was noted. While some tumors demonstrated a strong staining pattern (Figure 1B), others showed weak staining, with only rare cells positive (Figure 1C). While all ACAs studied had focal reactivity with Cam 5.2, only two of six cases exhibited detectable expression of the epitopes recognized by antibody AE-1. In contrast, none of the 13 ACCs had detectable staining with either AE-1 or Cam 5.2 (Figure 1D). All examples of the primary renal cortical carcinomas (17 of 17) demonstrated expression of cytokeratins, in most cases staining with both AE-1 (12 of 17) and Cam 5.2 (17 of 17) (Figure 1E). Protease digestion was used in all cases to enhance immunoreactivity, as outlined in Materials and Methods.

Vimentin

Vimentin expression in the normal adrenal cortex was primarily confined to stromal elements, with only questionable reactivity seen in adrenal cortical cells (Figure 2A). Adrenal cortical adenomas exhibited a range of expression, with some tumors showing staining mainly in stromal elements (Figure 2B), while others had strong expression of vimentin in the neoplastic cells (Figure 2C). Vimentin expression was observed in all (13 of 13) ACCs studied, including all primary and metastatic lesions (Figure 2D). The staining was generally strong and fairly uniform. In the case of renal cell carcinomas, 5 of 17 primary lesions exhibited focal expression of vimentin.

Table 4. *Metastatic Adrenal Cortical Carcinomas*

Case	Age	Sex	No. of metastatic sites studied
7*	63	M	1
8*	44	F	1
10*	49	M	2†
13	63	M	4†
14	51	F	2†
15	45	M	1
16	52	F	2†

* Primary site immunophenotyped.
† All metastatic sites were immunophenotypically similar.

pS-100

Expression of S-100 protein was observed in normal adrenal cortex (3 of 4), ACAs (2 of 3), and ACCs (8 of 12), as well as in the majority of renal cell carcinomas (16 of 17).

Chromogranin

Chromogranin expression was not observed in any of the adrenal cortical lesions or examples of normal adrenal cortex studied. However, the adrenal medulla showed strong expression of choromogranin in the normal adrenal glands studied.

Blood Group Antigens

Monoclonal antibodies directed against the major blood group antigens A and B, as well as those against Lewis a, b, X, and Y were studied. In addition, the lectin *Ulex europaeus* was used to define blood group H expression. Cells derived from adrenal cortex (normal adrenal cortex, ACAs, and ACCs) were found to lack detectable expression of all blood group antigens studied (Figures 3A and B). None of the RCCs studied showed detectable expression of the major blood group antigens (A, B, and H), but 13 of 17 RCCs exhibited expression of Lewis-type antigens: Lewis X (12 of 17), Lewis a (3 of 17), and Lewis b (1 of 17) (Figure 3C). None showed expression of Lewis Y.

Expression of Cytokeratin and Vimentin in ACA

As previously noted, ACAs showed a range of staining for both cytokeratins and vimentin. Table 6 shows that the

Table 5. *Antigen Expression in Adrenal Cortical Neoplasms and Renal Cell Carcinomas*

	AE-1	Cam 5.2	Vimentin	Chromogranin	S-100	BGA*
Normal adrenal cortex	1/4	4/4†	0/4‡	0/4	3/4	0/4
ACA	2/6	6/6†	4/6‡	0/6	2/3	0/3
ACC primary	0/6	0/6	6/6	0/6	5/6	0/6
metastases	0/7	0/7	7/7	0/7	3/6	0/6
RCC	12/17	17/17	5/17	ND	16/17	13/17§

* BGA: Blood group antigens A, B, H, and Lewis a, b, X, and Y.
† Focal expression in all cases.
‡ Stromal elements stained in all cases.
§ Lewis a, b or X positive; A, B, H, and Lewis Y negative.
ND, not done.

expression of these two classes of intermediate filament proteins appears to be inversely related in individual lesions. In particular, cases 3 and 5 had strong focal expression of cytokeratins (Figure 1B), while the expression of vimentin in each of these cases was confined primarily to stromal elements (Figure 2B). Conversely, case 1 showed weak expression of cytokeratins, with only a few cells reactive, while the expression of vimentin in this case was strong and fairly uniform (Figures 1C and 2C). We did not observe any consistent morphologic differences between these antigenically distinct ACAs. However, the tumor that exhibited the weakest expression for cytokeratin (and strong expression for vimentin) was one of the larger ACAs (case 1, 60 g).

Discussion

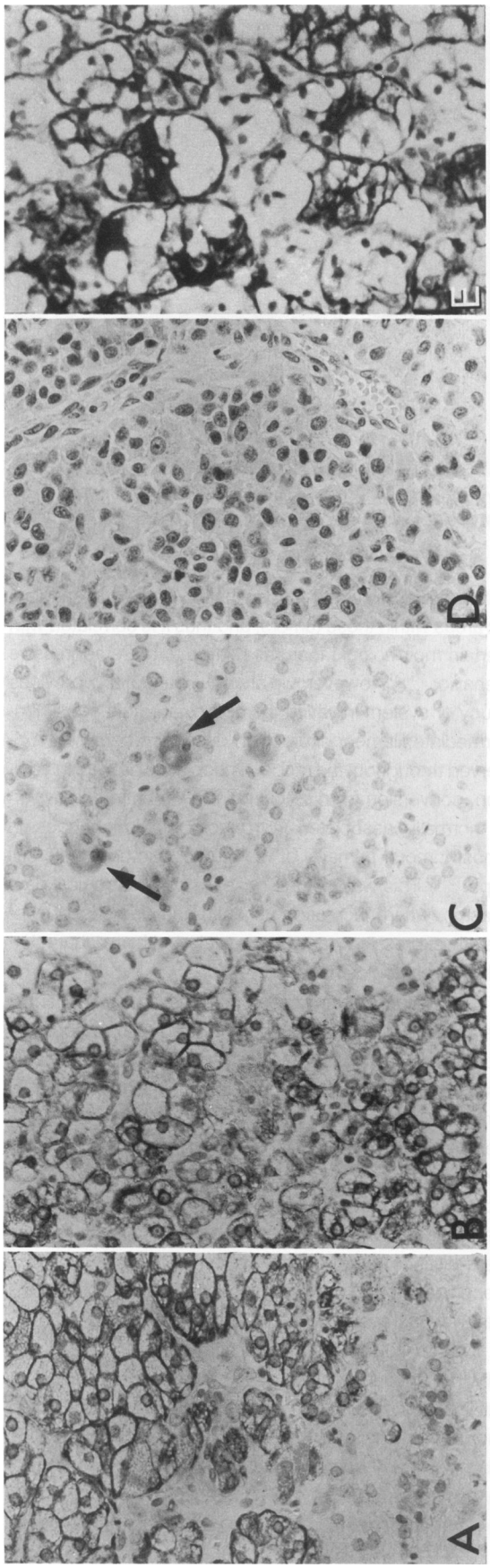
When faced with a moderately large but apparently well-circumscribed adrenal cortical tumor, the distinction between adenoma and carcinoma can be difficult. A series of well-defined histologic characteristics, including vascular/capsular invasion, necrosis, nuclear pleomorphism, and architectural features have been developed to aid in this distinction.[1-5] More recently the DNA content of the neoplastic cells comprising the tumor has been shown to separate lesions with more aggressive behavior.[9,10] However, none of these criteria can predict outcome in all cases. In this study we have used a panel of commonly available monoclonal antibodies to define the antigenic phenotype of ACAs and ACCs.

Previous reports have indicated that no clear distinctions could be made based on the antibodies used.[13,14] Miettinen et al[13] found that tumors arising in the adrenal cortex could be distinguished from those arising in the medulla on the basis of intermediate filament expression, but observed no consistent distinctions between ACAs and ACCs. Wick et al,[14] using a more extended panel of antibodies, also concluded that there were no clear differences in the antigenic phenotypes of ACAs and ACCs.

In contrast to the results obtained in the current study, these investigators found that a proportion of ACCs express cytokeratins.[13,14] In the study of Miettinen et al,[13] both frozen and paraffin-embedded tissues were used and there were no obvious differences between these types of tissue preservation. Three different monoclonal antibodies to cytokeratins, developed by that group, were used. Wick et al[14] used paraffin-embedded tissues in their study and found that, even with protease treatment, few ACCs expressed detectable levels of cytokeratins. A common feature of both these studies was that relatively few ACCs expressed cytokeratins, usually at decreased levels. In addition, Miettinen et al[13] reported that increased expression of vimentin was seen in ACC, compared with ACA or normal adrenal cortex, which is consistent with our findings.

We have found that in the cases studied here there is consistent and distinctive pattern of antigen expression in comparing normal adrenal cortex, ACAs, and ACCs (Table 5). All examples of normal cortex studied showed cytokeratin expression, but only minimal (or equivocal) expression of vimentin, generally confined to stromal elements (Figures 1A and 2A). The majority of ACAs studied expressed cytokeratin; a range of expression was noted, with some tumors showing strong staining and others weak and focal staining (Figures 1B and C). Adrenal cortical adenomas also showed a range of expression of vimentin, with some tumors having weak/minimal expression, while others exhibit strong reactivity (Figures 2B and C). Adrenal cortical adenomas tend to show expression of both cytokeratins and vimentin (Tables 5 and 6). None of the 13 ACCs studied, including examples of primary and metastatic lesions, showed detectable expression of cytokeratins. However, all showed strong, fairly uniform reactivity for vimentin (Figures 1D and 2D).

There appears to be a coordinated change in intermediate filament expression in comparing normal adrenal cortex, ACA, and ACC. Normal cortex expresses cytokeratins, primarily in the zona glomerulosa, but only minimally expresses vimentin. Adrenal cortical carcinomas show the reverse; none showed reactivity for cytokeratins, but



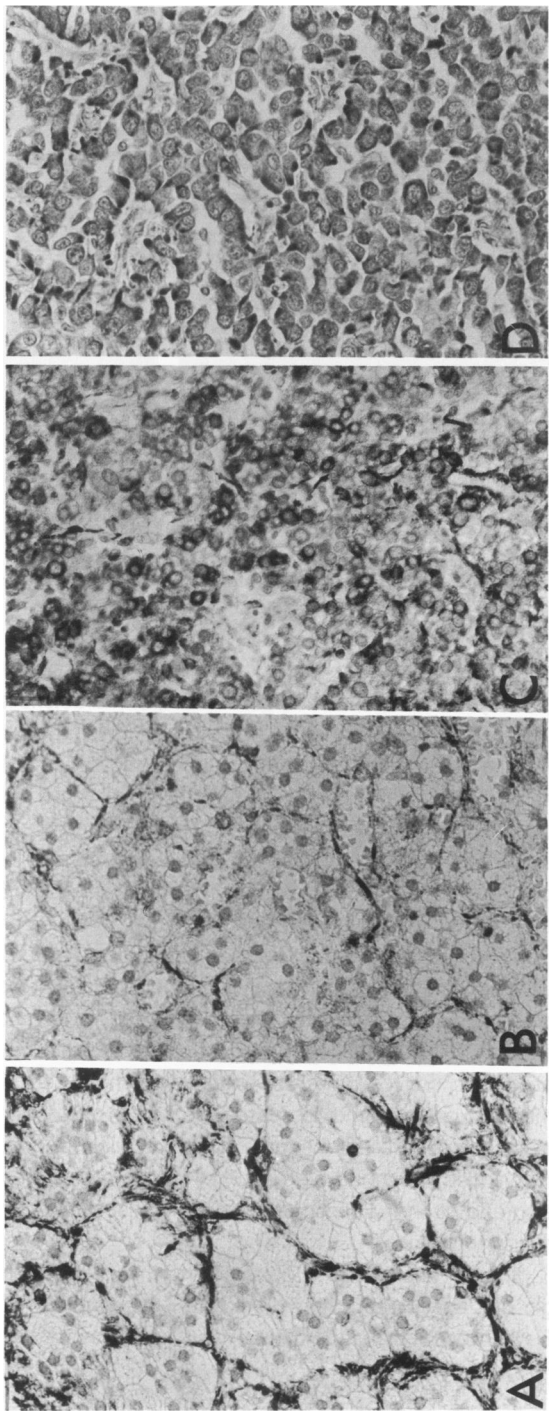


Figure 1. *Reactivity with antibody to cytokeratins (Cam 5.2) (×250).* **A:** *Normal adrenal cortex, positive reactivity;* **B:** *Adrenal cortical adenoma (case 3). Note strong focal reactivity;* **C:** *Adrenal cortical adenoma (case 1). Note weak staining of few cells.* **D:** *Adrenal cortical carcinoma (case 12). No reactivity seen.* **E:** *Renal cell carcinoma, positive reactivity.* **Figure 2.** *Reactivity with antibody to vimentin (×250).* **A:** *Normal adrenal cortex, staining confined to stromal elements;* **B:** *Adrenal cortical adenoma (case 3). Staining primarily confined to stromal elements:* **C:** *Adrenal cortical adenoma (case 1). Note strong reactivity;* **D:** *Adrenal cortical carcinoma (case 12), positive reactivity.*

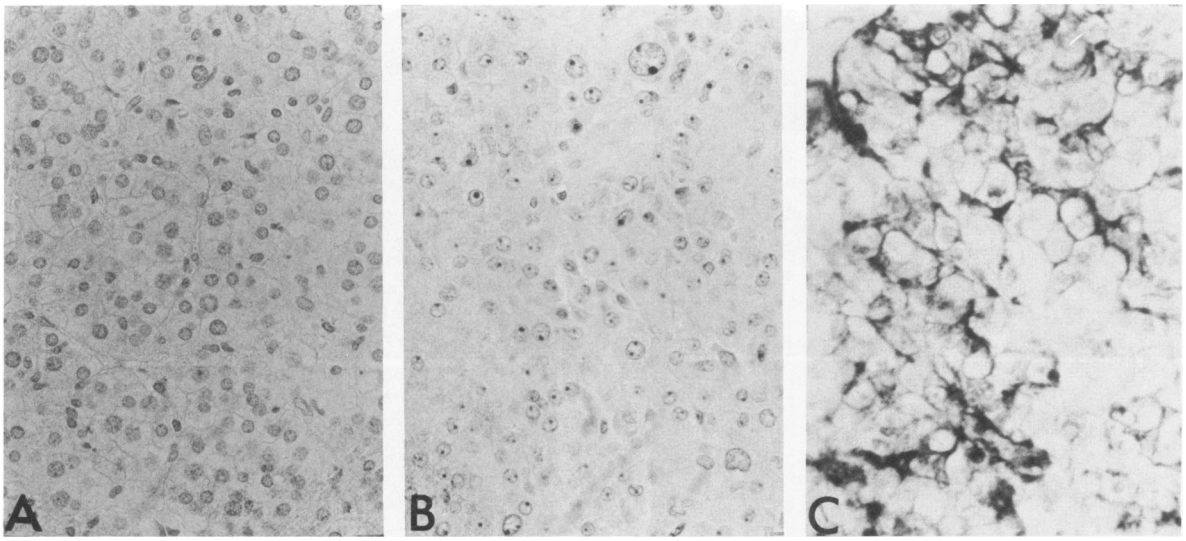


Figure 3. *Reactivity with antibodies to blood group antigens (Lewis X) (×250).* A: *Adrenal cortical adenoma, no reactivity seen;* B: *adrenal cortical carcinoma, no reactivity seen;* C: *renal cell carcinoma, positive reactivity.*

all expressed vimentin. There is a range of expression of both cytokeratins and vimentin in ACA, a characteristic not seen in normal cortex or ACC. In ACA, this range of intermediate filament expression appears to be coordinated in individual lesions. Adrenal cortical adenomas that show strong reactivity for cytokeratins only weakly express vimentin, while those that are weakly reactive for cytokeratins show strong expression of vimentin (Table 6 and Figures 1B, 2B and 1C, 2C).

These results suggest that adrenal cortical cells undergo characteristic changes in intermediate filament expression during the process of neoplastic conversion and malignant transformation. Our data would indicate that neoplastic conversion is associated with increased vimentin expression and decreased cytokeratin expression at the cellular level. Furthermore, it appears that using the techniques described here, lack of cytokeratin expression and strong vimentin expression is associated with malignant lesions.

It is well known that malignant tumors of epithelial origin can coexpress both cytokeratins and vimentin.[15-20] Furthermore, increased vimentin expression is associated with more highly anaplastic epithelial tumors and anaplastic areas within these tumors, and thus it is correlated with certain morphologic features related to more aggressive behavior.[15-20] However, we are not aware of any previous study or system in which the coordinated changes in intermediate filament expression described here were observed throughout the range of neoplastic growth. In addition, conversion from cytokeratin to vimentin expression is normally associated with morphologic changes in most tumors, primarily of the metaplastic or spindle cell type.[15-20] This was not the case for adrenal cortical neoplasms, where increased vimentin and decreased cytokeratin expression was not associated with any characteristic morphologic changes.

Table 6. *Comparison of Cytokeratin and Vimentin Expression in Adrenal Cortical Adenomas*

Case	Cam 5.2	Vimentin*
1	+ few cells	+++ many cells
2	++ focal	+−++ focal
3	+++ focal	− most cells (+ stroma)
4	++ focal	+ focal
5	++−+++ focal	− most cells (+ stroma)
6	++ focal	+ focal

Staining intensity: +, weak; ++, moderate; +++, strong; * stromal/vascular elements stained in all cases.

The antigenic phenotype of adrenal cortical hyperplasia appears to be distinct and unrelated to this neoplastic conversion because none of four hyperplastic nodules studied had detectable expression of either cytokeratin or vimentin (unpublished data). As previously noted, cytokeratin expression is principally seen in the zona glomerulosa, while vimentin is not seen well in any layers of the adrenal cortex. This may indicate that adrenal cortical hyperplasia (those studied here at any rate) may arise from cells distinct from those that give rise to adrenal cortical neoplasms, and that hyperplasia may not be part of the pathway of transformation in the adrenal cortex.

The second major issue investigated here was whether antibody panels could distinguish adrenal cortical lesions from RCC. These tumors can be quite similar morphologically, and they are included in the differential diagnosis of metastatic lesions that have clear or granular cytoplasm. In addition, the primary site of a tumor involving both the adrenal gland and upper pole of the kidney

can be difficult to determine, even after detailed microscopic examination.

Our results suggest that the most useful antibodies are those with specificity to cytokeratins and blood group antigens (Table 5). Adrenal cortical carcinomas did not express detectable levels of cytokeratins. Cytokeratins were demonstrated in all primary clear cell and tubular RCCs examined. The majority of primary RCCs express blood group antigens, but all ACAs and ACCs were negative (Table 5). Reactivity with antibodies to vimentin and protein S-100 do not contribute to the differential diagnosis. Although Wick et al[14] found that ACCs and RCCs did not express detectable levels of protein S-100, we found that many ACCs and the majority of RCCs showed positive reactivity to the antibody to protein S-100. Expression of protein S-100 by normal and malignant renal epithelium has been previously reported.[21,22]

Wick et al[14] also demonstrated that the majority of RCCs expressed cytokeratins as well. In addition, they found that RCCs expressed epithelial membrane antigen (EMA), while ACAs and ACCs were negative. They reported that RCCs reacted with antibodies to the major blood group isoantigens (A, B, and H),[14] but in our series none of the RCCs tested expressed detectable levels of these antigens. However, the majority of primary RCCs expressed Lewis-type blood group antigens, most commonly Lewis X, and occasionally Lewis a or b.[23] One possible reason for this discrepancy is that the antibodies to the major blood group antigens used in the previous study may have had unsuspected cross reactivity with Lewis-type antigens as well.

It is important to emphasize the fact that the antigenic phenotype for RCCs reported here pertains to primary renal cortical tumors with clear cell or granular cell features. We have found that metastatic RCC less commonly express Lewis blood group antigens.[23] In addition, some metastatic RCCs express low to undetectable levels of cytokeratins and high levels of vimentin (Cordon-Cardo et al, unpublished data), thus making their antigenic phenotype more similar to that of ACC. However, RCCs that lack expression of cytokeratins are generally poorly differentiated tumors, often with pronounced spindle cell features,[14] and thus can generally be separated from ACCs on morphologic grounds.

Finally, RCCs and adrenal cortical lesions can be distinguished by a series of antigens that are expressed by cells of proximal tubular origin.[24-26] These antigens are, however, only stable in frozen sections and were not used in the current study.

This study points out once again the importance of using antibody panels in evaluating lesions of uncertain histogenesis or malignant potential. While none of the antigens studied here are specific for adrenal cortical or renal cells, lesions of similar histogenesis (ACAs *vs.* ACCs) and lesions of different histogenesis (adrenal cortex *vs.* renal cortex) can be distinguished on the basis of their composite antigenic phenotypes.

An important future step will be to determine if those features that appear to distinguish ACAs from ACCs (primarily cytokeratin and vimentin expression) correlate with more established criteria (morphology, weight, and DNA content analysis), particularly for borderline lesions. We expect that, given more sensitive detection systems and a greater number of tumors studied, cytokeratin expression will be shown in at least a proportion of ACCs. However, the finding of strong vimentin expression associated with weak cytokeratin expression should raise the possibility of malignant potential in an adrenal cortical tumor.

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