**Complex Cadherin Expression in Renal Cell Carcinoma**

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**Abstract**

E-cadherin is an intercellular adhesion protein expressed by most epithelia. Decreased expression of E-cadherin correlates with tumor aggressiveness in most carcinomas. In renal cell carcinoma (RCC), however, this correlation is not well established and the prevalence of negative tumors is higher than in other carcinomas. Our immunofluorescence study of α-catenin expression in 20 RCC cell lines revealed a typical honeycomb staining pattern in all of the lines, whereas only six expressed E-cadherin. This suggested that other cadherins are expressed in RCC lines. Indeed, immunoprecipitation with an anti-α-catenin antibody resulted in coprecipitation of proteins of M, 125,000–135,000. Using Western blot, these proteins react with a pan-cadherin antibody. To identify these cadherin related proteins, RT-PCR using degenerated primers and sequence comparisons was carried out. We then assessed the expression of the identified cadherins. N-cadherin mRNA was present in all cell lines; and cadherin 6 mRNA was detected in 16 lines. Cadherin 11 (mRNA) and E-cadherin (protein) were expressed in five and six lines, respectively. A cadherin 4 transcript was observed in only one line, whereas no P-cadherin protein could be detected. Expression of the four main cadherins was also found in normal kidney (two samples tested) and RCC specimens (four samples). Thus, RCC and normal kidney express a complex set of cadherins.

**Introduction**

Cadherins constitute a large family of transmembrane glycoproteins. The so-called classical cadherins share a common structure, including a highly conserved cytoplasmic region, a transmembrane region, and an extracellular domain in which homologies define type I and type II cadherins. The most extensively characterized members of this family, E-, P-, and N-cadherins, belong to the type I subgroup and mediate Ca⁺⁺-dependent cell-cell adhesion in a variety of cell types. Their carboxyl terminus is bound to either β- or γ-catenin (plakoglobin), which in turn interacts with α-catenin that bridges the complex to the actin-based cytoskeleton. The importance of the correct assembly of this complex is exemplified in the fact that cadherins deleted of the 70-carboxyl terminal amino acids are unable to mediate cell adhesion.

In most carcinomas, E-cadherin expression decreases with tumor grade and stage (1). In RCC,2 however, E-cadherin expression is found infrequently and even low-grade tumors do not express E-cadherin (2). A possible explanation is that the renal proximal tubule epithelium, from which RCCs presumably originate, does not express E-cadherin. Indeed, in normal kidney, E-cadherin is expressed in Bowman’s capsule and in all tubular segments except the proximal convoluted and straight tubules (3). However, using immunohistochemistry we have been able to show α-catenin expression at the intercellular borders in all tubules.3 Thus, it is likely that functional α-catenin-cadherin complexes are present in all segments of the nephron. We hypothesized that other classical cadherins form complexes with α-catenin and mediate cell-cell adhesion in RCC. In this article, we used RCC cell lines to characterize RCC for expression of classical cadherins.

**Materials and Methods**

**RCC Cell Lines.** RCC cell lines (a kind gift from Dr. L. J. Old, New York, NY) were cultured in RPMI 1640 with 10% FCS and 1% glutamine.

**Immunohistochemistry.** Cells were grown on glass slides, fixed with 3% paraformaldehyde, and then permeabilized using 0.2% Triton X-100 in PBS. Monoclonal antibody HEC1D (Takara) was diluted 1:20 and A-CAM (clone G4C; Sigma) 1:30. Serum of a mouse immunized with a glutathione S-transferase/α-catenin fusion protein (nucleotides 454–1981 of human α-catenin, kindly provided by Dr. W. Isaacs, Baltimore, MD) was diluted 1:1000. On Western blot, this serum recognized a single band comigrating with α-catenin.

**Immunoprecipitation.** Cells were metabolically labeled with [35S]methionine (250 μCi/ml) overnight. Proteins were extracted in 2% Triton X-100, 20 mM Tris (pH 7.4), 150 mM NaCl, 2 mM CaCl₂, 50 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein extracts containing 10⁷ cpm were incubated with 1 μl α-catenin mouse antiserum. Complexes were collected with protein G-agarose (Oncogene Science) and resolved on a 7% SDS-polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane. After overnight autoradiography, the membrane was processed for immunoblotting with a pan-cadherin polyclonal antibody diluted 1:1600 (Sigma).

**Western Blot.** Twenty-five μg of proteins were separated using a 7% SDS-PAGE and transferred onto nitrocellulose filters. Immunoblots were analyzed using ECL (Amersham) and HEC1D or P-cadherin (Transduction Laboratories), both diluted 1:500.

**RT-PCR.** Total RNA was extracted from the cell lines with TRIzol (Life Technologies, Inc.) and treated with DNase. The cDNA was synthesized using superscript reverse transcriptase (Life Technologies, Inc.). PCR was carried out using primers described previously (4). PCR products were subclassed into a PGEM vector and sequenced using sequencing 2.0 (United States Biochemical). A computer-assisted homology search was performed with the EMBL (release 44) and Genbank (release 90) nucleotide sequences data bases.

**Northern Blotting.** Ten μg RNA were denatured with glyoxal, separated on 1% agarose, and transferred to Hybond N+ membranes. Probes corresponding to nucleotides 579–1643 for N-cadherin, 522–2186 for cadherin 4, 25–465 for cadherin 6, and 2759–3256 for cadherin 11 were generated by RT-PCR and labeled with [32P]dATP by random priming. Blots were hybridized overnight in 250 mM Na₂HPO₄ (pH 7.2), 1 mM EDTA, 1% BSA, and 7% SDS at 65°C and then washed at 65°C successively in 250 mM Na₂HPO₄ (pH 7.2), 1 mM EDTA, and 1% SDS; in 125 mM Na₂HPO₄ (pH 7.2), 1 mM EDTA, and 1% SDS; and in 50 mM Na₂HPO₄ (pH 7.2), 1 mM EDTA, and 1% SDS. To ensure that the expressions we evaluated were not in vitro artifacts, we also included two normal kidneys and four RCC samples.

**Results and Discussion**

Using immunohistochemistry, we found that all of the cell lines expressed α-catenin at the cell-cell borders. In contrast, E-cadherin was expressed in only six cell lines (Table 1 and Fig. 1). Since in L cells, which do not express any cadherin, α-catenin is unstable, the staining pattern observed here probably reflects the involvement of α-catenin in a protein complex, possibly with a cadherin (5).

To investigate which protein stabilized α-catenin, we immunoprecipitated α-catenin from seven cell lines; one of them (SKRC 59)
expresses E-cadherin. Autoradiography after SDS-PAGE revealed several coprecipitating proteins. One of them was present in all of the cell lines and comigrated with β-catenin. A weak band with the same electrophoretic mobility as γ-catenin was found in some cell lines. Interestingly, a band coinciding with E-cadherin (M₀ 120,000) was present only in SKRC 59, but bands corresponding to $M_0$ 125,000–135,000 were observed in all of the lines (Fig. 2A). Western blot analysis of these immunoprecipitates with an anti-pan-cadherin antibody showed that the proteins constituting these bands were immuno logically related to cadherins (Fig. 2B).

To characterize further these putative cadherins, we performed RT-PCR using degenerated primers corresponding to the cytoplasmic region of classical cadherins. Indeed, the pan-cadherin antibody used in the Western blot analysis was raised against this part of the molecule. Because slight differences in the electrophoretic mobility of the cadherin-related bands were noticed, possibly reflecting the presence of different proteins, we chose four cell lines representative of the various mobilities but not expressing E-cadherin at the protein level (SKRC-7, -10, -24, and -52). Sequence analysis of the RT-PCR products revealed that several cadherins are expressed. N-cadherin was the most frequently cloned and was present in the four cell lines (five of five clones in SKRC-24, six of eight in SKRC-52, four of eight in SKRC-7, and only one of eight in SKRC-10). Cadherin 6 was found in two cell lines (five of eight clones in SKRC-10 and three of eight in SKRC-7). Other cadherins were found in only one line: cadherin 11 (two of eight clones in SKRC-10), cadherin 4 (one of eight clones in SKRC-7), P-cadherin (one of eight clones in SKRC 52), and E-cadherin (one of eight clones in SKRC-52).

We then evaluated the expression of these cadherins in our panel of cell lines at the mRNA (N-cadherin and cadherins 6, 11, and 4) or protein level (E- and P-cadherins).

A 3.4-kb N-cadherin transcript was present in all of the cell lines. Besides this major transcript, two minor transcripts (3.9 and 2.7 kb) can be seen in some lines. N-cadherin transcripts were found in the two normal kidneys and three of four RCCs showing that N-cadherin expression in cell lines is not an in vitro artifact (i.e., expression triggered by the culture conditions and not seen in vivo).

Transcripts of different sizes have been described for both mouse (6) and human N-cadherin (7). However, the transcripts found in human brain and muscle are bigger (5.2, 4.3, and 4 kb) than those observed in the RCC cell lines. Interestingly, in rat, it has been found that brain, muscle, and heart express a 5.2- and a 4.3-kb transcript, whereas lung, kidney, and liver express a 4.3- and a 3.5-kb transcript (8). It thus appears that the N-cadherin transcripts vary in a tissue-specific manner, with, in humans, a main 3.4-kb transcript in kidney and RCC. The significance of these size differences is still unknown; evidence for alternative polyadenylation has been found in human muscle (7). 

The widespread expression of N-cadherin in RCC cell lines was confirmed using immunofluorescence (Table 1). The fact that two lines (SKRC 18 and 28) with a clear mRNA expression are negative, as determined with immunohistochemistry, probably reveals posttran-

<table>
<thead>
<tr>
<th>Gene Selected series (%)</th>
<th>Reference series (%)</th>
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<tr>
<td>SKRC-1 (E, P) 1/45 (2)</td>
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</tr>
<tr>
<td>SKRC-6 (E, P) 26/45 (58)</td>
<td>Hahnet et al. (2)</td>
</tr>
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<td>SKRC-7 (E, P) 4/45 (9)</td>
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</tr>
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<td>SKRC-10 (E, P)</td>
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Table 1 Characteristics of the RCC cell lines and cadherin expressions

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<th>Cell line</th>
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* Expressions were assessed as follows: α-catenin, immunohistochemistry; E-cadherin, immunohistochemistry and Western blot; N-cadherin, Northern blot (first symbols) and immunohistochemistry (second symbols); cadherins 4, 6, and 11, Northern blot.

**W, weak expression.**

Fig. 1. E-cadherin (A and B) and α-catenin (C and D) immunofluorescence in RCC cell lines. SKRC-12 cells do not express E-cadherin (A) but they do express α-catenin (C) at their cell-cell contact sites, whereas SKRC-6 cells express both E-cadherin (B) and α-catenin (D) at the intercellular junctions. E-cadherin expression in SKRC-6 is generally weak as compared with α-catenin expression. Note that both cell lines have an epithelial phenotype.
posttranscriptional regulations. Posttranscriptional down-regulation of N-cadherin has indeed been described during retinal development (9).

Sixteen of the 20 cell lines as well as the 2 normal kidneys and 3 of 4 RCC samples expressed cadherin 6 (Fig. 3). Four major transcripts (9, 4, 3.4, and 2.7 kb) were observed in concordance with earlier studies (10). The 2.7- and 9-kb forms were not found in all cell lines. Moreover, we could clearly see in some cell lines a 4.3-kb transcript which was not described previously by Shimoyama et al. (10). Indeed, this transcript is not visible in the non-renal cell lines or in the normal organs they have tested, but it can be clearly seen in the KT12 RCC carcinoma they analyzed. Thus, this transcript may be kidney specific.

Using Northern blot, the previously described 3.5-kb cadherin 11 transcript (4) was found in five cell lines (Fig. 3 and Table 1). Both normal kidneys and one of the RCC samples also expressed cadherin 11.

As previously stated, E-cadherin was expressed at the protein level in six cell lines (Table 1). This frequency is in agreement with other studies on RCC samples (19 and 35%). The frequency of negative cell lines (70%) is very high when compared to other cell lines of epithelial origin [e.g., 30% in colon (11), 33% in bladder (12), and 55% in breast (13) cancer cell lines], and is probably related to the fact that proximal epithelial cells, from which RCC originates, do not express E-cadherin.

A weak band, corresponding to a 6-kb cadherin 4 transcript, was seen in only one cell line (Table 1) and one of the RCC; using RT-PCR with specific primers, we could find low levels of cadherin 4 transcript in five more cell lines.
P-cadherin was not detectable using Western blot in any of the cell lines.

Altogether, the RCC cell lines investigated as well as normal kidney displayed a complex pattern of cadherin expression. It is already known that cadherin expression in kidney is highly structured, with differential expression in the various segments of the nephron. For instance, the proximal convoluted tubule and the proximal straight tubule express N-cadherin, the thick ascending limb, the distal convoluted tubule, and the collecting duct express E-cadherin, whereas the thin limb of the Henle loop coexpresses both molecules (3). Based on in situ hybridization studies of the developing kidney, it is likely that cadherins 6 and 11 also have a specific expression pattern in kidney. For cadherin 6, the signal obtained with the Northern blot of normal kidneys was weak when compared to those of the cell lines. This might indicate that in human adult kidney, cadherin 6 expression is also restricted to one particular compartment of the nephron. Indeed, in the rat developing kidney, cadherin 6 expression is restricted to the S-shaped body (the progenitor of, among others, the proximal tubule); no expression was found in the ureter or collecting duct (14). In the mouse developing kidney, a marked cadherin 11 expression was found in the metanephric mesenchyme surrounding the developing nephron but not in the epithelial nephron (15, 16). Based on the widespread expression of cadherin 11 in mesenchyme (15-17), one can expect that the signal obtained with normal kidney resulted from the expression by the stromal compartment.

It is tempting to relate the cadherin phenotype of RCC to the origin of these tumors along the nephron. The widespread expression of N-cadherin (also found by others in RCC samples) supports the generally admitted proximal tubular origin of RCC. However, the high proportion of tumors coexpressing N- and E-cadherins (up to 30% in our series) show that correlations between phenotype and tumor origin are not always straightforward. In these cases, E-cadherin is probably aberrantly expressed by tumors originating from the proximal tubule. Thus, E-cadherin expression would reflect perturbations of the differentiation process, similar to metastatic processes seen in other carcinomas.

Although almost all combinations of cadherins were found in the 20 lines studied here, no coexpression of E-cadherin and cadherin 11 was observed. This supports the current view that cadherin 11 is a mesenchymal marker, whereas E-cadherin is associated with epithelial differentiation. Indeed, five of six lines expressing E-cadherin have an epithelial phenotype (the sixth one having an intermediate phenotype), whereas four of the lines with a clear cadherin 11 expression are fibroblastic. Thus, expression of cadherin 11 may be related to reexpression of mesenchymal markers in RCC, similar to reexpression of vimentin (18).

Since N-cadherin is expressed by almost all of the cell lines, it seems unlikely that N-cadherin expression is an important factor of RCC aggressiveness. The same observation has been made in astrocytomas and glioblastomas (19). Recently, no correlation between N-cadherin expression and grade was found in a series of 34 RCCs (20). No insight into the role of cadherin 6 in kidney carcinogenesis can be inferred from our data. Furthermore, the accurate assessment of the role of all of these cadherins in the loss of epithelial integrity during kidney tumor development awaits studies at the protein level and experimental evidence such as transfection or use of blocking antibodies.

References
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