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Renal clear-cell carcinoma: an ultrastructural study on the junctional complexes

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Summary. Junctional complexes such as tight junctions, adherens junctions, and desmosomes play crucial roles in the structure and function of epithelial cells. These junctions are involved in increasing cell-cell contact and as well serve as signaling centers regulating multiple functions in epithelial cells. Carcinoma cell lines cultured in the laboratory generally lack junctional complexes. However, studies directed towards understanding the distribution of junctional complexes in human cancer tissues are lacking. In this study, we analyzed by electron microscopy the distribution of junctional complexes in patients diagnosed with renal clear-cell carcinoma. We found that both tight junctions and adherens junctions were drastically reduced in patients with cancer compared to normal tissues. Desmosomes were not detected in normal proximal tubules while distinctly present in cancer tissues. These results suggest that analysis of junctional complexes in human tumors should provide valuable information that might have prognostic and diagnostic value.

Keywords: Tight junction, Desmosome, Adherens junction, Renal clear-cell carcinoma

Introduction

Epithelia form a barrier between two biological compartments and regulate the molecular composition of, and exchange between the compartments they separate. In order to serve this function the plasma membrane of epithelial cells is divided into two functionally and biochemically distinct domains, the apical and basolateral (basal and lateral) plasma membranes (Rodriguez-Boulan and Nelson, 1989). Junctional complexes such as tight junctions, adherens junctions and desmosomes play crucial roles in the structure and function of epithelial cells. Tight junctions form a continuous belt at the boundary between the apical and lateral plasma membrane domains of neighboring epithelial cells and are structurally characterized by the close apposition of contiguous plasma membranes (Farquhar and Palade, 1963). They selectively regulate the passage of molecules across the paracellular space (*gate function*) and passively separate molecules into the apical and basolateral plasma membrane domains (*fence function*). A tight junction is crucial to maintain the polarized phenotype and the vectorial transport functions of epithelial cells. Recent studies indicate that tight junctions form a molecular platform involved in the regulation of cell polarity, vesicular trafficking and signal transduction (Mitic et al., 2000; Zahraoui et al., 2000; Tsukita et al., 2001). Adherens junctions are localized closely below the tight junction and are thought to function as signaling centers that may regulate the formation of other junctional complexes (Yap et al., 1997). Desmosomes are intercellular adhesive junctions that anchor intermediate filaments at membrane-associated plaques in adjacent cells to provide resilience and tensile strength to the epithelial monolayer and may also function as a signaling center (Green and Gaudry, 2000; Garrod et al., 2002).

In general, carcinoma (cancer derived from epithelial cells) lack junctional complexes. These cells also lack polarity and show more mesenchymal features with increased cell motility and invasive properties. Junctional complexes such as tight junctions, adherens junctions and desmosomes, increase the adhesive properties of epithelial cells. Loss of these junctions might contribute to the increased motility and invasiveness of carcinoma cells (Vleminckx et al., 1991; Rajasekaran et al., 2001b). Although it is well known that epithelial junctions are generally lost in cultured carcinoma cell lines, studies on the distribution of tight junctions, adherens junctions and desmosomes in solid tumors are lacking.

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Renal cell carcinoma is the most common cancer of the kidney, accounting for 80 to 85 percent of kidney tumors. Based on histopathological criteria, renal cell carcinoma (RCC) is classified into several subtypes among which clear-cell, papillary (chromophil) and chromophobe are the most prevalent. Clear-cell carcinoma is an aggressive form of RCC which arises from the proximal convoluted tubules and accounts for about 85% of RCC (Motzer et al., 1996).

In this study, using electron microscopy, we determined the junctional complexes in tumor tissues of patients diagnosed with renal clear-cell carcinoma. Our results suggest that tight junctions and adherens junctions are significantly reduced in tumors of grades I to III. However, we also found that the content of desmosomes significantly increased in tumors of all grades compared to normal tissues.

Materials and methods

Patient and tumor specimens

Tissues from a total of 26 consecutive patients diagnosed with RCC at UCLA were fixed for electron microscopy studies. Of these 16 patients diagnosed with renal clear-cell carcinoma were included in this study. Samples from six patients were not suitable for electron microscopy due to the presence of large amounts of necrotic tissue and were not included in the analysis. Age, sex, diagnosis, tumor grade and tumor size of patients used in this study is shown in Table 1. Two pathologists (GT and JS) blindly verified the diagnosis of the specimens. Tumors were graded according to Fuhrman grading system (Fuhrman et al., 1982). When available morphologically normal tissues from adjacent to tumor areas were also used in the analysis. In addition, kidney tissues from four transplantation biopsies were used as normal tissues to confirm the distribution of junctional complexes.

Electron microscopy and quantification of junctional complexes

Fresh tissue was immediately fixed in 2.5% glutaraldehyde and 2% paraformaldehyde, post fixed with 1% osmium tetroxide, dehydrated in graded acetone and embedded in epoxy resin. Semi-thin sections were stained with a polychromatic stain (modified Mallory Heidennoin stain) for selection. Ultra-thin sections were stained with uranyl acetate and triple lead. Electronmicrographs were prepared on a Philips EM208S electron microscope using an AMT digital camera.

For each normal and tumor tissue 20-25 electron micrographs at low (3000x) and high (30000x) magnification were taken. The junctions were visualized as electron dense region in the cell-cell contact area at low magnification and identified by standard features (Rajasekaran et al., 2001a,b). These areas were then photographed at higher magnification to further validate the type of junction. Junctions located at the apico-lateral region beneath the microvilli in which the intercellular space was almost not visible at high magnification were interpreted as tight junctions. In tumor tissues where microvilli at the apical plasma membrane were absent the electron dense intercellular region where the intercellular space could not be detected at high magnification was interpreted as tight junction. In normal tissues adherens junctions were visualized as electron dense regions located immediately below the tight junction where the intercellular space was clearly discernable. In tumor tissues without tight junctions adherens junctions were visualized by their electron dense nature and by the lack of intermediate filaments at the junction area. Desmosomes were visualized by the presence of intermediate filaments associated with the electron dense region at the intercellular contact sites. Desmosome-like junctions were categorized by their small size and the presence of intermediate filament associated with them.

Quantification of the number of junctions per cell-cell contact was essentially done as described earlier (Rajasekaran et al., 2001a). The number of junctions in randomly selected low magnification images was manually counted and the number of cell-cell contacts visualized in respective electron micrographs was determined. The number of junctions observed was normalized to the number of cell-cell contacts to obtain the number of junctions per cell-cell contact.

Satistical analysis

Each subject's specimen was categorized as being from a grade 1, grade 2, or grade 3 tumor or normal. We also recorded patient age, sex, tumor size, and evidence for metastatic disease. The mean number of junctions per cell-cell contact was compared among the tumor and normal groups by analysis of variance. Various mixed-effect models were also assessed to take into account any differences in intrasubject variability due to the number of observed cell-cell contacts. These alternate analyses were similar to those using standard analysis of variance, and only the latter results are reported. The Kruskal-

Table 1. Pathology of tumor specimens analyzed.

PATIENT	AGE/SEX	DIAGNOSIS	GRADE	TUMOR SIZE
1	45/F	Clear-cell	I	6.3 cm
2	52/M	Clear-cell	I	8.2 cm
3	51/F	Clear-cell	I	7.4 cm
4	64/M	Clear-cell	II	6.2 cm
5	53/F	Clear-cell	II	6 cm
6	47/M	Clear-cell	II	4 cm
7	59/F	Clear-cell	II	8 cm
8	73/F	Clear-cell	II	4.1 cm
9	72/M	Clear-cell	III	6.1 cm
10	61/M	Clear-cell	III	9.2 cm

Wallis test was used for the analyses of desmosomes because of unequal variances among the groups. Summary statistics for the number of junctional complexes per cell-cell contact are presented as mean ± standard error.

Results

Normal Kidney

Figure 1 shows the electron micrograph of proximal tubules of a morphological normal kidney tissue. The apical surface of the proximal tubule cells showed numerous microvilli (Fig. 1, MV) that collectively were recognized as brush border in the light microscope. The apical cytoplasm contained vesicles (Fig. 1, V) and lysosomes (Fig. 1, LY). Numerous longitudinally oriented mitochondria (Fig. 1, M) were seen within the interdigitating processes and directly adjacent to the infolded plasma membrane. At the apico-lateral lateral region junctional complexes (Fig. 1A, arrows) were readily detected at low magnification. At higher magnification these junctional complexes were identified as tight junction (Fig. 1B, TJ) and adherens junction (Fig. 1B, AJ). Adherens junctions were observed as elongated electron dense region immediately below the tight junction. Although tight junctions and adherens junctions were distinctly present, desmosomes were not detected in morphologically normal kidney samples obtained from cancer patients' kidney tissues. Further analysis of normal kidney tissues obtained from transplantation biopsy specimens confirmed the absence of desmosomes (our unpublished results) indicating that this is not a feature associated with tumor adjacent tissues. Quantification of the electron microscopic data (Fig. 5) revealed 0.869±0.076 and 1.05±0.12 tight junctions and adherens junctions per cell-cell contact in proximal tubules, respectively. As expected this result indicates that one tight junction and one adherens junction is present per cell-cell contact in morphological normal proximal tubule cells. These electron microscopic features observed in normal kidney tissue were similar to published reports (Brenner and Rector, 2000).

Grade I Tumors

Figure 2 shows representative electron micrographs of renal clear-cell carcinoma tissues from patients diagnosed with grade I tumors. The cells of the tumor tissues did not show the elongated shape with interdigitating membranes which is seen in normal proximal tubule cells. However, the cells of the tumor tissue appeared to adhere to one another (Fig. 2A). The cytoplasm showed abundant lipid (Fig. 2A, asterisk) and glycogen, a characteristic feature of clear-cell carcinoma (Krishnan and Truong, 2002). A drastic reduction of mitochondria was also seen in all these tumor tissues. In grade I tumor samples, microvilli (Fig. 2, MV) were sparsely present. Although less frequent compared to normal proximal tubules, tight junctions (Fig. 2A, insert, and Fig. 2B) and adherens junctions (Fig. 2B) were observed in some cells of the tumor tissues. Quantification of the EM data revealed the mean number of tight junctions and adherens junctions in grade I tumors were 0.228±0.103 and 0.275±0.109, respectively (Fig. 5). Interestingly, desmosome-like structures (0.582±0.210/cell-cell contact) were observed in these tumor tissues.

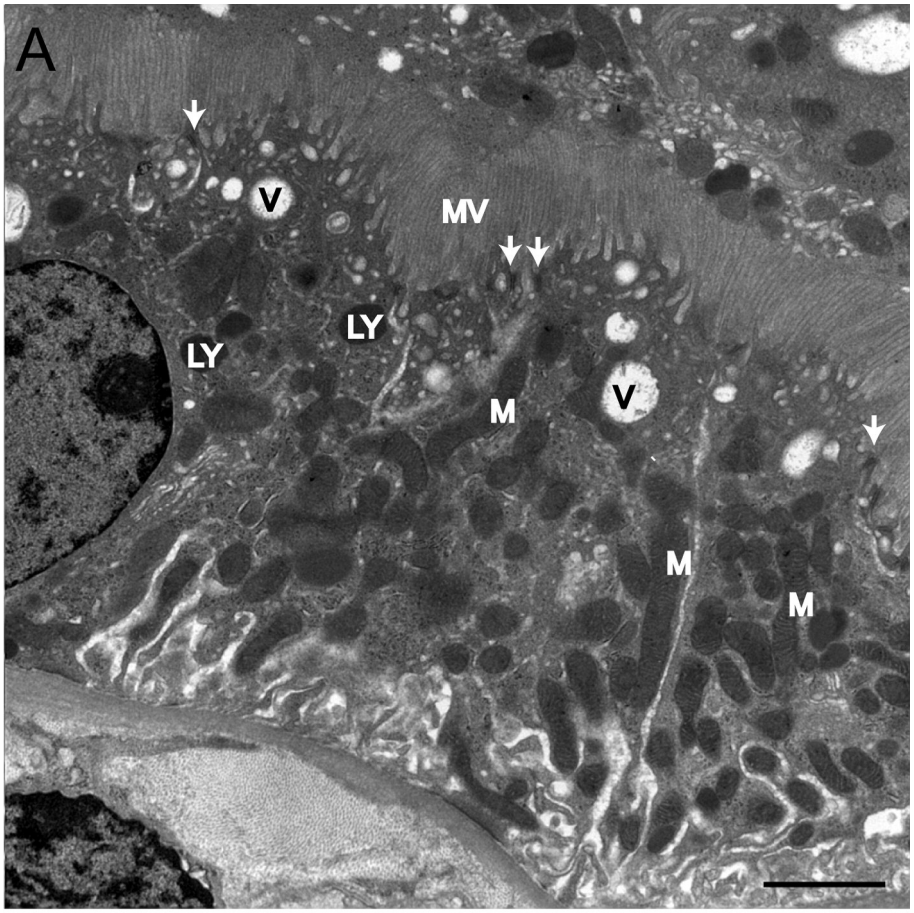
Grade II Tumors

In grade II tumors (Fig. 3), cells showed abundant glycogen granules, lipids, and reduced mitochondria and were adherent to each other like cells of grade I tumors. Microvilli and tight junctions were rarely observed. However, adherens junction-like structures were distinctly seen in these tumor tissues (Fig. 3B). Quantification of the EM data revealed the mean number of tight junctions and adherens junctions in grade II tumors with 0.148±0.065 and 0.369±0.164, respectively (Fig. 5). Desmosome-like structures were seen in cells of grade II tumors (0.704±0.289/cell-cell contact).

Grade III Tumors

Cells of tumors of grade III showed abundant glycogen granules, lipid, and reduced mitochondrial content and the cells were adherent to each other (Fig. 4). As in grade II tumors, microvilli and tight junctions were rarely observed. Adherens junctions were distinctly observed (Fig. 4B). Quantification of the EM data revealed the mean number of tight junctions and adherens junctions in grade III tumors were 0.198±0.177 and 0.245±0.130, respectively (Fig. 5). One of the striking observations in these tumors is the increased number of desmosomes (0.964±0.089/cell-cell contact). While some of these desmosomes were large and well developed (Fig. 4B, insert) several of these desmosomes were small and poorly developed.

Taken together, these results indicate that in renal clear cell carcinoma the number of tight junctions and adherens junctions was drastically reduced compared to normal tissues ($P<0.01$ and $P=0.01$, respectively). In cancer tissues desmosomes were distinctly present compared to normal tissues ($P=0.03$). The mean number of desmosomes per cell-cell contact in the three patients with known metastases was 1.17±0.33 compared to 0.48±0.15 for the five patients with no known metastases ($P=0.07$). Unfortunately, archival information about metastases was missing on two other patients, and these results are at best only suggestive of a true association between desmosome frequency and metatstatic potential. No further statistical distinction could be made relating the frequency of junctional complexes to the tumor grade, tumor size, reported evidence of metastatic disease or age or sex of patient.



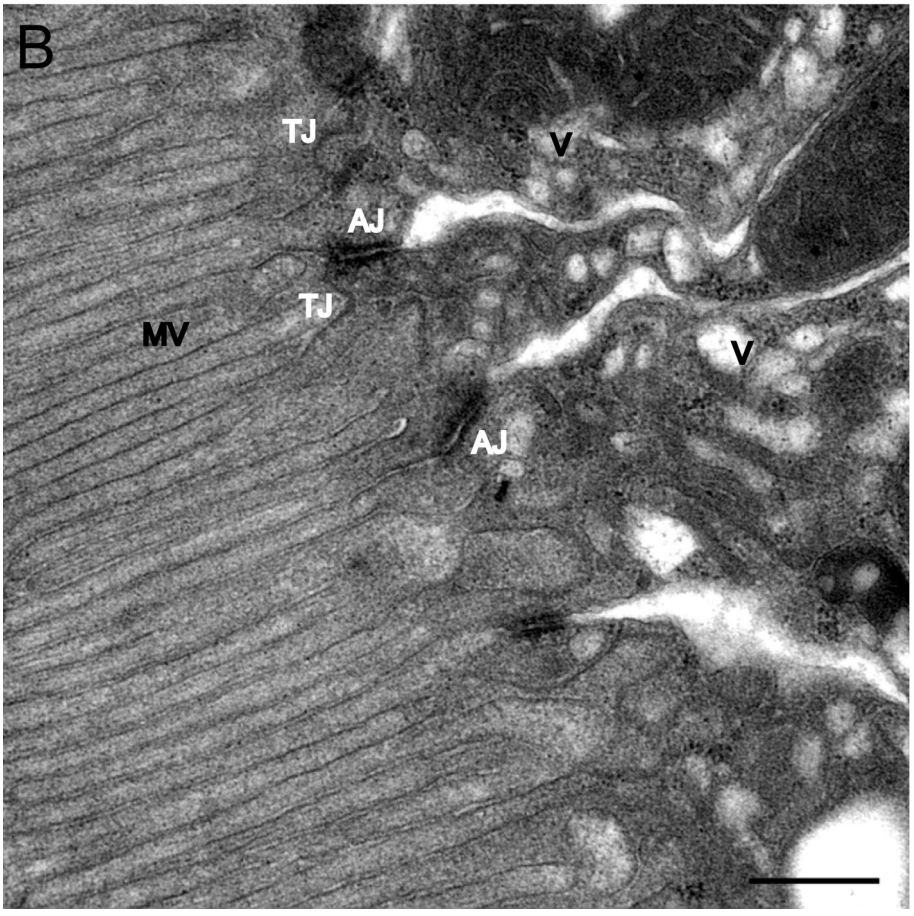


Fig. 1. Electron micrograph of morphologically normal kidney samples showing proximal tubules. **A.** Low magnification electron micrograph showing proximal tubule cells. LY: lysosome; V: vesicle; MV: microvilli; M: mitochondria; arrows demonstrate apical junctional complexes. **B.** High magnification EM showing tight junctions (TJ) and adherens junctions (AJ). Bars: A, 2μm; B, 0.5 μm.

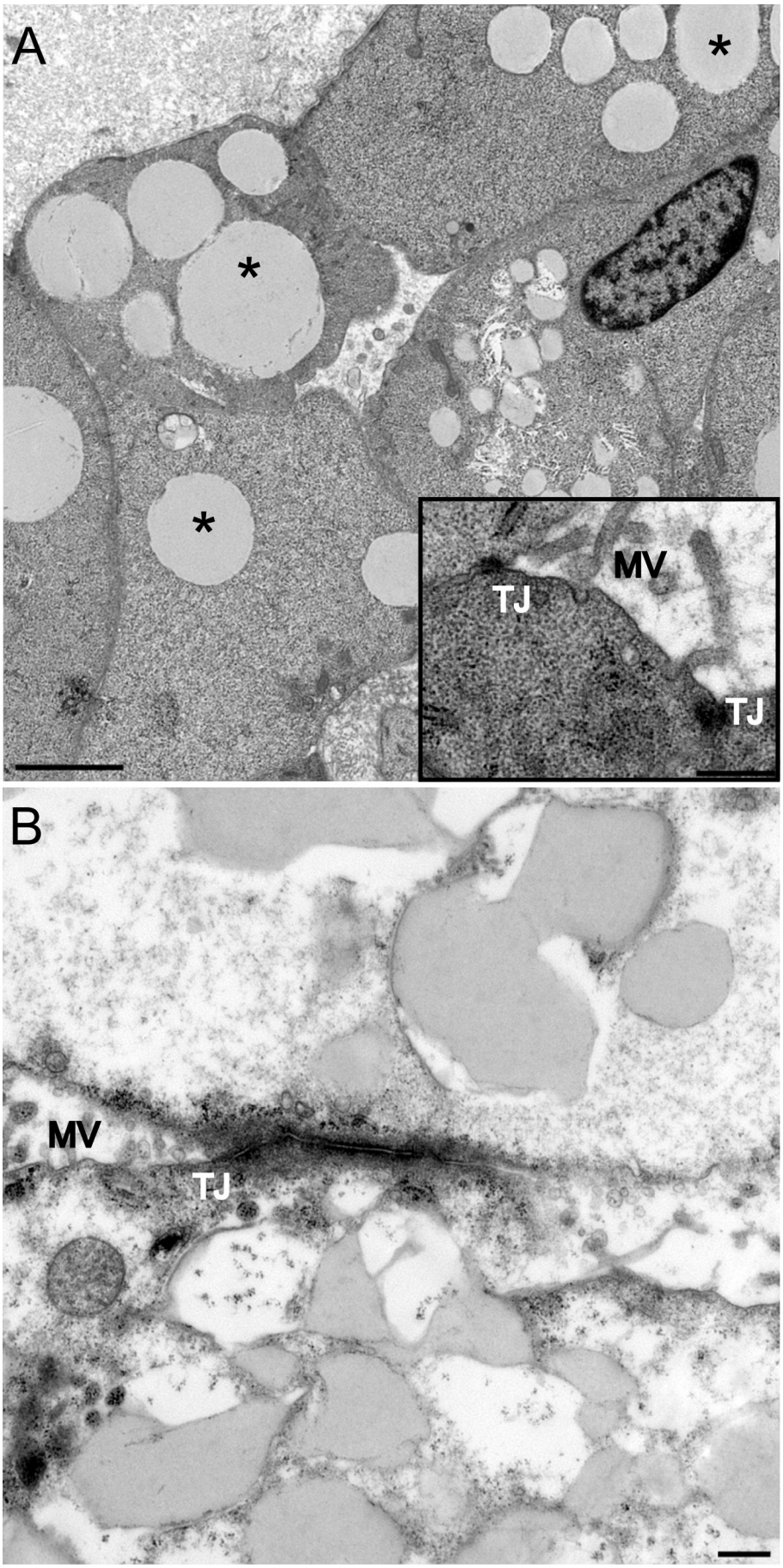


Fig. 2. Electron micrograph of a grade I renal cell carcinoma tumor. **A.** Low magnification electron micrograph showing cell-cell contacts of the tumor cells. *: lipid vesicle. Insert demonstrates tight junctions (TJ) and microvilli (MV). **B.** High magnification electron micrograph showing tight junction (TJ) in the tumor cell. Bars: A, 2 µm; B, Insert in A, 0.5 µm.

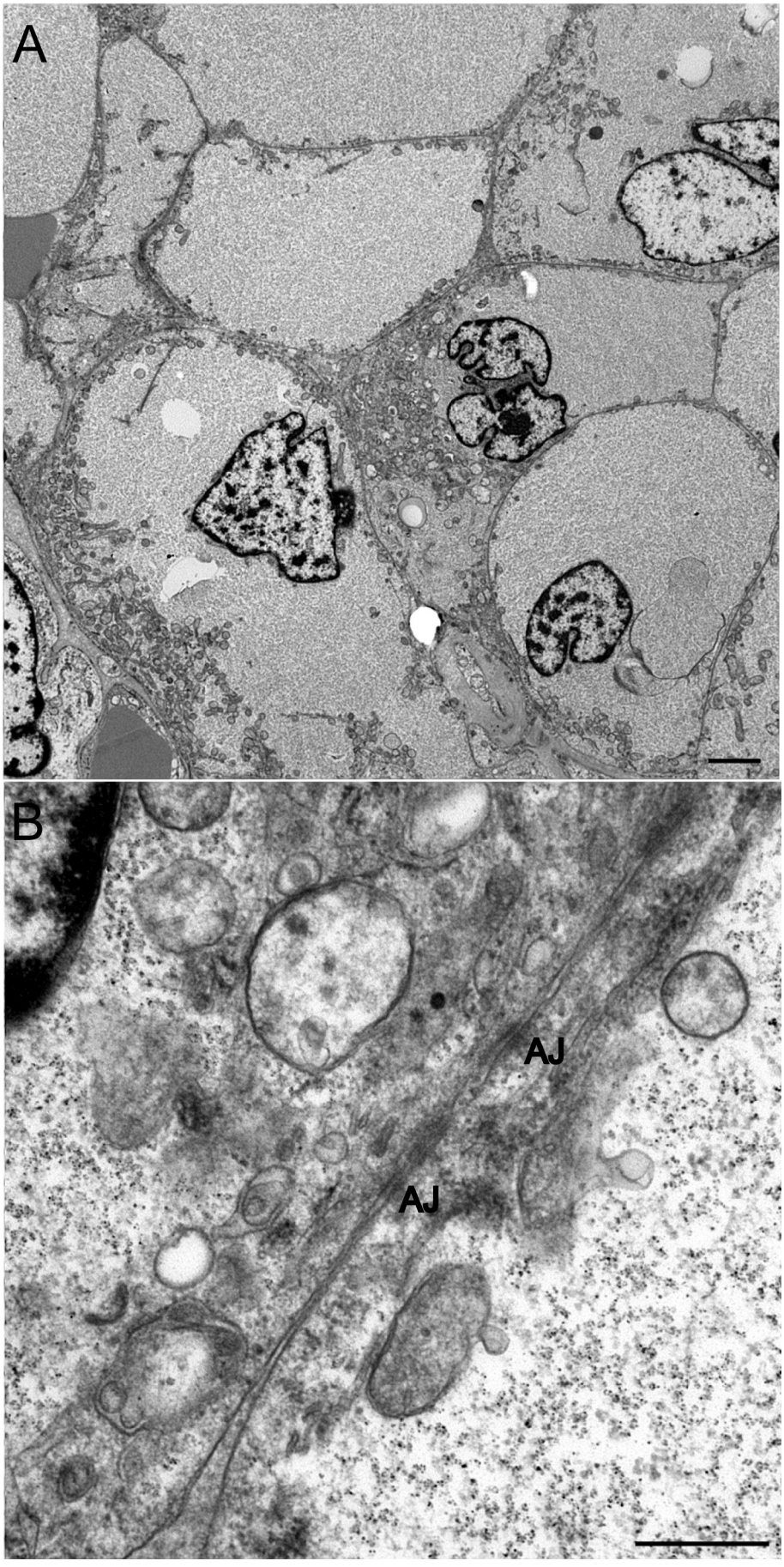


Fig. 3. Electron micrograph of a grade II renal cell carcinoma tumor. **A.** Low magnification electron micrograph showing cell-cell contacts of the tumor cells. **B.** High magnification electron micrograph showing the presence of adherens junctions (AJ). Bars: A, 2 µm; B, 0.5 µm.

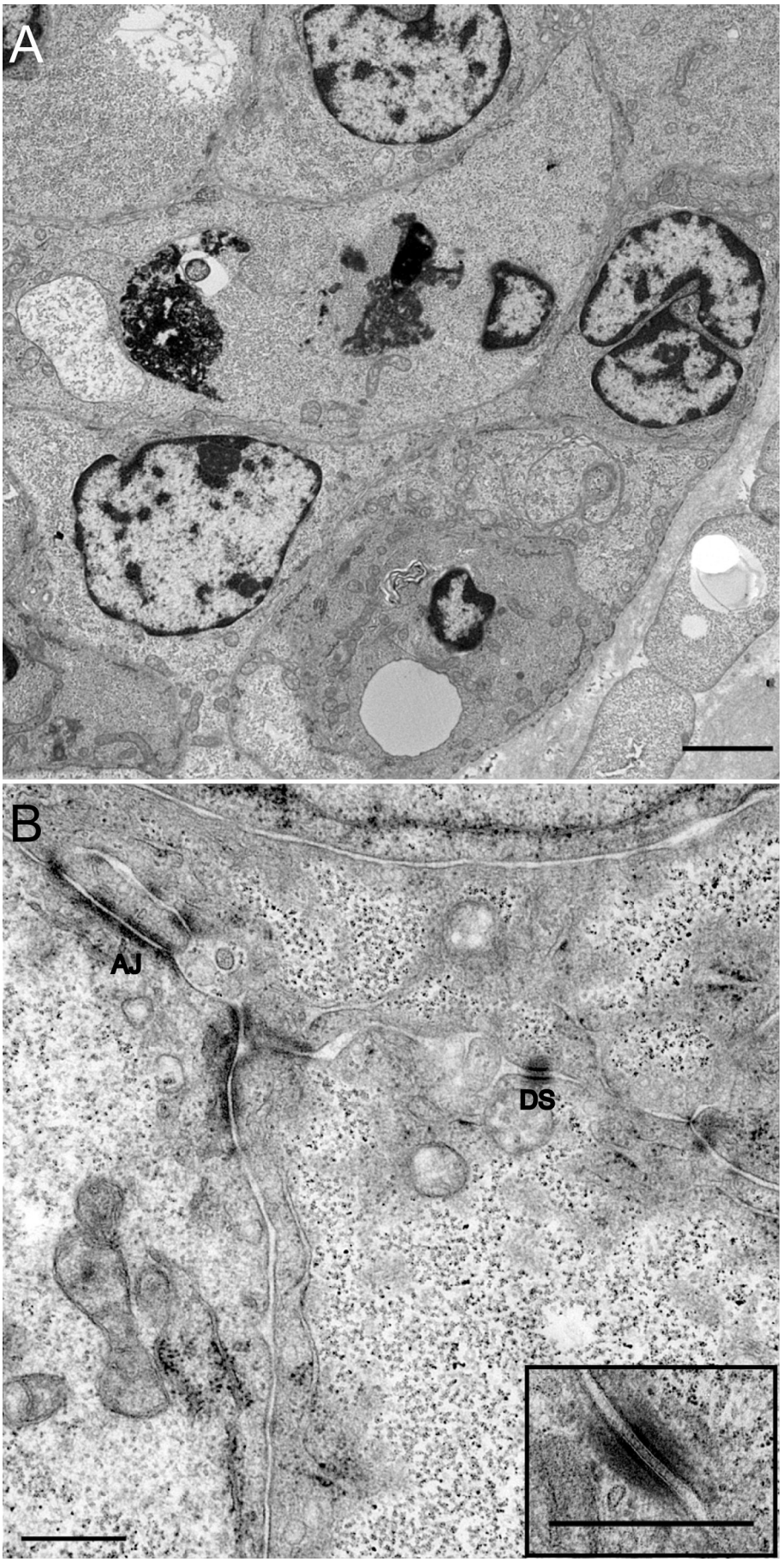


Fig. 4. Electron micrograph of a grade III renal cell carcinoma tumor. **A.** Low magnification electron micrograph showing cell-cell contacts of the tumor cells. **B.** High magnification electron micrograph showing the presence of adherens junctions (AJ) and desmosomes (DS). Insert demonstrates a higher magnification of a well-defined desmosome. Bars: A, 2 μm; B, Insert in A, 0.5 μm.

Discussion

This study provides evidence that the numbers of tight junctions and adherens junctions are drastically reduced in renal clear-cell carcinoma tumor tissues compared to normal tissues. While tight junctions and adherens junctions were reduced there was an increase in the number of desmosomes in these tumor tissues. To our knowledge this is the first study describing the distribution of junctional complexes in human solid tumors derived from renal clear-cell carcinoma. Although limited number of cases representing each grade of tumors was used in this study, this initial study provides evidence that the tight junctions, adherens junctions and desmosomes are differentially regulated in human kidney tumors. A further study with a larger number of patients should provide more insights into whether changes in the junctional complexes in renal clear-cell carcinoma have diagnostic or prognostic value.

Tight junctions constitute a crucial organelle in epithelial cells. They are made up of several membrane spanning proteins (occludin, claudins, junctional adhesion molecule) and cytoplasmic plaque proteins (ZO-1, ZO2, ZO-3, cingulin, 7E6, and symplekin) (Tsukita and Furuse, 2000). It is possible that loss of any of these tight junction proteins might be associated with the reduced number of tight junctions in renal cancers. In salivary gland tumors loss of occludin expression is associated with the lack of tight junctions and polarity of these cells (Li and Mrsny, 2000). Alternatively, proteins involved in the regulation of tight junctions such as E-cadherin (Gumbiner et al., 1988) and Na,K-ATPase (Rajasekaran and Rajasekaran, 2003) might be affected leading to the loss of tight junctions in carcinoma. We have shown earlier that Na,K-ATPase ß-subunit expression and enzyme activity are highly reduced in clear-cell RCC (Rajasekaran et al., 1999). Subsequently, we have shown that Na,K-ATPase ß-subunit (Rajasekaran et al., 2001b) and enzyme activity are essential for the assembly (Rajasekaran et al., 2001a; Rajasekaran and Rajasekaran, 2003) and maintenance (Rajasekaran et al., 2003) of tight junctions in epithelial cells. Therefore, it is possible that reduced Na,K-ATPase function might be associated with the reduced number of tight junctions in clear-cell RCC.

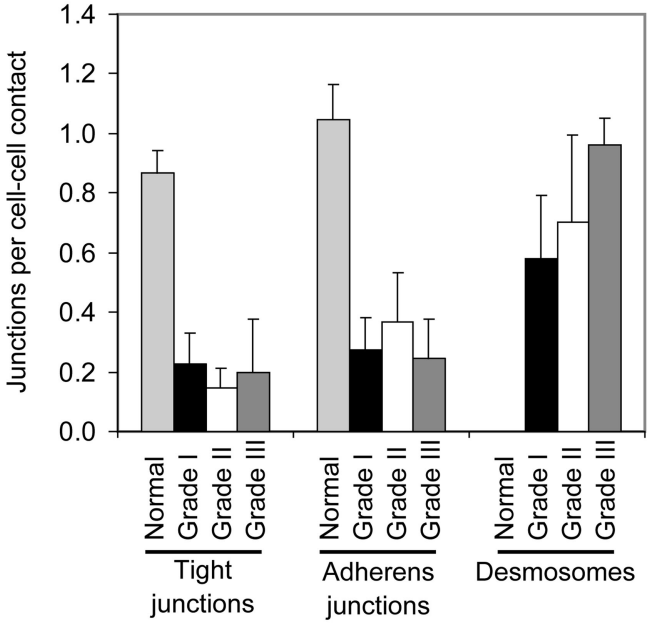


Fig. 5. Quantitative analysis of tight junctions, adherens junctions, and desmosomes in normal, grade I, grade II, and grade III renal cell carcinoma.

Tight junctions are crucial for maintaining the polarized phenotype of epithelial cells. Loss of tight junctions in RCC might facilitate the localization of basolaterally localized receptors, such as epidermal growth factor receptor (EGFR), to be aberrantly expressed at the apical plasma membrane. Apical expression of EGFR should allow its association with EGF present in the urine (Hirata and Orth, 1979; Espineda et al., 2003) and activation of EGF mediated signaling pathways. Alternatively, the lumenal EGF might seep through leaky tight junctions and activate EGFR localized to the basolateral domain (Mullin et al., 2000; Espineda et al., 2003). Thus loss of tight junctions in RCC might facilitate further development of the tumor. Future experiments should validate the consequence of loss of tight junctions in RCC.

Like tight junctions, adherens junctions were also drastically reduced in tumors of grade I through III. Adherens junctions are composed of cell adhesion molecules such as E-cadherin which mediates cell-cell contact in epithelial cells by homophilic interaction (Takeichi, 1990). It is well known that E-cadherin expression is important for adherens junction assembly in epithelial cells (Yap et al., 1997). While proximal tubule epithelial cells express predominantly N-cadherin (Tani et al., 1995), clear-cell RCC also expresses E-cadherin (Shimazui et al., 1996) and cadherin-6 (Paul et al., 1997). Whether altered expression of cadherins is involved in the disassembly and reduction of adherens junctions in clear-cell RCC remains to be elucidated.

Desmosomes were not detected in our samples of normal kidney proximal tubules. By contrast, the number of desmosomes markedly increased in tumor cells. Although the trend was statistically not significant it is interesting that grade III tumors showed the highest number of desmosomes. Several studies have described the presence of desmosomes in RCC (Tannenbaum, 1971; Dikshtein et al., 1978; Macke et al., 1985; Karthaus et al., 1987; Bird et al., 1991; Daneshmand et al., 2003). Interestingly, sarcomatoid RCC, which is the most aggressive form of RCC has desmosomes (Macke et al., 1985; Bird et al., 1991; Daneshmand et al., 2003). Since sarcomatoid RCC is not a distinct subtype, but rather an aggressive and dedifferentiated form of other major types of RCC including clear-cell type (Bertoni et

al., 1987), it appears that this form of RCC (usually grade 4) might exhibit increased numbers of desmosomes. The increased number of desmosomes in high grade and invasive tumors suggest that desmosomes might play a role in the aggressiveness and probably metastasis of RCC. In a study involving implantation of cells to developing chicken wing to test the invasive behavior of carcinoma cells, Tickle et al., (1978) showed carcinoma cells formed desmosomes and not tight junctions or adherens junctions when adhered to mesenchymal cells. This result indicates that desmosomes might participate in the attachment of carcinoma cells to the mesenchymal cells of the interstitium during invasion. Alternatively, desmosomes might facilitate establishment of cellular contact of carcinoma cells to other cell types at a metastatic site. In contrast, in breast cancer cell lines loss of desmosomal proteins is associated with increased invasion indicating that desmosomes have a protective function against tumor invasion (Tselepis et al., 1998). Future studies should determine whether desmosomes have a role in increasing the invasiveness of RCC cells and whether the number of desmosomes in RCC has any diagnostic or prognostic value.

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