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Clear cell renal cell carcinomas – epithelial and mesenchymal immunophenotype

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Abstract

Clear cell renal cell carcinomas (CCRCCs) are the most common kidney tumors that despite current advances in diagnosis continue to have high rates of metastasis and mortality. In this study, we analyzed the cytokeratin (CK) AE1/AE3 and vimentin immunoexpression in 26 CCRCCs in relation to histopathological prognostic parameters. Immunoreactions were positive and heterogeneous in all analyzed cases. CK AE1/AE3 immunoexpression was associated with low grade and early stage lesions, while vimentin immunoexpression was associated with high grade and advanced lesions. The aspect may be used to determine the tumor heterogeneity and a better patients' stratification for therapy.

Keywords: clear cell renal cell carcinoma, immunophenotype, cytokeratin, vimentin.

Introduction

Clear cell renal cell carcinomas (CCRCCs) represent approximately 70–80% of malignant kidney tumors, being the most aggressive carcinomas with this location, due to the high rate of metastasis and mortality [1, 2]. The main risk factors involved in the occurrence of renal carcinomas are age between 50–70 years old, male gender, toxic exposure, and genetic and epigenetic changes [1, 3, 4].

Although in recent years the rate of diagnosis of renal tumors, especially in the initial stages increased significantly, mainly due to diagnostic imaging methods, the mortality rate of lesions remained high, over 90% of renal tumor metastases belonging to a CCRCC [5, 6]. Although the biomolecular mechanisms involved in the development of renal carcinomas are partly characterized, some studies highlight the need for routine markers with diagnostic and prognostic potential [1]. In the case of CCRCC is signaled the usefulness of some histopathological parameters, such as tumor grade and stage, in order to appreciate the expression of these markers [7].

CCRCCs are particular tumors, with a mixed, epithelial and mesenchymal profile, the lesions expressing both cytokeratins (CKs) and vimentin. In the literature, there are numerous studies that have analyzed the usefulness of these markers in the CCRCC positive and differential diagnosis, but relatively few data are related to the expression of the markers in relation to the histopathological prognostic parameters of the lesions [8, 9]. The frequency of CK AE1/AE3 and vimentin positivity, as well as CCRCC specific tumor heterogeneity, can provide information on aggressive lesions. Vimentin is a mesenchymal marker, but its overexpression has been described in gastrointestinal, lung, breast, and prostatic carcinomas [10]. Furthermore, by implication in the epithelial–mesenchymal transition, vimentin is an attractive therapeutic target for carcinomas [10, 11].

In this study, we analyzed the epithelial and mesenchymal immunophenotype of CCRCCs in relation to the histopathological prognostic parameters of the lesions.

Materials and Methods

In this study, we analyzed 26 cases of CCRCCs diagnosed during 2013–2017, in the Department of Pathology, Emergency County Hospital, Craiova, Romania, the patients being hospitalized and investigated in the Department of Urology of the same Hospital. The biological material was represented by radical nephrectomy specimens, fixed in 10% buffered neutral formalin, processed for paraffin embedding and Hematoxylin–Eosin (HE) staining. The histopathological assessment of the tumors was done according to the latest literature data [12]. The study included conventional cases of CCRCC without any other previous oncological therapy.

We investigated clinicopathological parameters (age, gender, tumor size, tumor grade and stage) in relation to the epithelial (CK AE1/AE3) and mesenchymal (vimentin) markers (Table 1).

In order to immunostaining, the sections were prepared for incubation with primary antibodies (dewaxing in xylene, rehydrating in alcohols, endogenous enzyme and unspecific blocking, microwaving for antibody retrieval), at 4°C, overnight. The working system was represented by Labelled Streptavidin-Biotin 2 (LSAB2) system (Dako, Redox, Romania, code K0675), and we used 3,3'-diaminobenzidine tetrahydrochloride (Dako, Redox, Romania,

code K3468) as chromogen. External positive controls, external negative controls and internal positive controls were used for the immunostaining reactions. The assessment of reactions was done by using the positivity index (PI) resulting by reporting the number of labeled cells to the total number of tumor cells counted at 40× microscope objective. For each case were counted at least 5000 cells from areas with the most immunosignals. Also, the reactions were analyzed descriptively in relation to the intensity and distribution of the signals. Because some markers were present at the level of stromal elements, the reactions were quantified by two pathologists who agreed on the number of labeled cells.

Table 1 – ***The antibodies and immunostaining protocol***

Antibody	Clone / Manufacturer	Dilution	Pretreatment	External positive control
CK AE1/AE3	AE1/AE3 / Dako	1:100	Microwaving in citrate buffer, pH 6	Skin
Vimentin	SP20 / Thermo Scientific	1:150	Microwaving in citrate buffer, pH 6	Colon

CK: Cytokeratin.

For the statistical analysis, we used the one-way analysis of variance (ANOVA) and Pearson's tests within Statistical Package for the Social Sciences (SPSS) 10 software. The results were considered significant for values of $p<0.05$.

For the images acquisition was used the Nikon Eclipse E600 microscope equipped with Lucia 5 software.

Ethical aspects have been respected in this study.

Results

The study of clinicopathological data indicated the higher frequency of CCRCC in male patients (69.2%), over 50 years old (80.7%), the mean age at diagnosis being 60.1±10.4 years. Tumor dimensions ranged between 2.5–19 cm, with an average of 7.3±3.5 cm. Most cases were classified as Fuhrman 1 tumors (46.1%), extension pT1 tumor (53.9%) and without lymph node (92.3%) or distant (96.1%) metastasis (Table 2).

Table 2 – ***Clinicopathological parameters***

Clinicopathological parameters	No. of cases
Age [years]	<50: 5, >50: 21
Gender	Males: 18, Females: 8
Tumor size [cm]	<7: 13, >7: 13
Degree of differentiation	F1: 12, F2: 8, F3: 4, F4: 2
Tumoral extension (pT)	T1: 14, T2: 8, T3: 3, T4: 1
Lymph node status (pN)	N0: 24, N1: 2
Distant metastasis (pM)	M0: 25, M1: 1
Tumor stage	I: 14, II: 8, III: 3, IV: 1

F: Fuhrman grade.

Most cases (53.9%) belonged to stage I (pT1N0M0), followed by stage II (pT2N0M0 – 30.7%), stage III (pT3N0M0 – 7.7%, pT3N1M0 – 3.8%) and stage IV (pT4N0M1 – 3.8%) (Table 2). In this study, we found the same number of cases for tumor extension (pT) and tumor stage.

The immunohistochemical (IHC) analysis indicated for both markers, cytoplasmic reactions in tumor cells in all analyzed cases. The quantification of the performed IHC reactions indicated differences in the expression of CK AE1/AE3 and vimentin in relation to the histopathological parameters analyzed.

Thus, the CK AE1/AE3 reactions had the highest mean PI values for low grade carcinomas, respectively for Fuhrman 1 lesions (50.8±12), compared to CCRCC Fuhrman 2 (34.2±6.2), Fuhrman 3 (32.5±6.4) or Fuhrman 4 (24.5%) grades, the aspects being statistically significant ($p=0.001$, one-way ANOVA) (Figure 1, A–E). Depending on the tumor extension (pT) and tumor stage, the mean PI CK AE1/AE3 values were higher in the case of stage I lesions (44.2±12.4) compared to tumor stage II (39.3± 15.2), stage III (35±8.6) and stage IV (30%), but the aspects were statistically insignificant ($p=0.526$, one-way ANOVA) (Figure 1F).

In the case of vimentin, the immunoreactions indicated superior mean PI values in the case of high-grade carcinomas, respectively Fuhrman 3 (61.2±4.7) and Fuhrman 4 (62.5%) grades, compared to Fuhrman 1 (36.2±5.2) and Fuhrman 2 (46.8±7) grades, aspects that were statistically significant ($p<0.001$, one-way ANOVA) (Figure 2, A–E). Depending on tumor extension (pT) and tumor stage, the vimentin mean PI values were superior for stage II CCRCC (48.1±13.6), stage III (51.6±15.2) and stage IV (60%), compared to lesions in stage I (41.4±8.6), but the aspects were statistically insignificant ($p=0.526$, one-way ANOVA) (Figure 2F).

On the analyzed sections, we found different degrees of tumor heterogeneity, the CK AE1/AE3 immunosignals being superior in low-grade tumor areas, while the vimentin reactions were more frequent in the case of high-grade lesions (Figure 3). The immunoreactions have moderate intensity in case of CK AE1/AE3 and moderate/strong intensity in case of vimentin immunostaining.

In this study, we did not find statistical associations of CK AE1/AE3 and vimentin immunoexpression with other investigated parameters. The analysis of the mean PI values of the investigated markers indicated a significant negative linear correlation between the expression of CK AE1/AE3 and vimentin ($p=0.004$, Pearson's test) (Figure 4).

Discussions

Currently, is known the Von Hippel–Lindau pathway involvement and overexpression of hypoxia-inducible factor 1-alpha (HIF-1α) in CCRCC initiation and progression [9]. There are also described CCRCC prognostic markers, such as cluster of differentiation 44 (CD44), carbonic anhydrase 9 (CA9), p53, Ki67, proliferating cell nuclear antigen (PCNA), chemokine receptors CXCR3 and CXCR4, epithelial cell adhesion molecules (EpCAMs) [9, 13].

Also, among the most widely used markers of positive and differential diagnosis of CCRCC are CKs, vimentin, paired box gene (PAX) 2, PAX8, renal cell carcinoma marker (RCC Ma), CD10, CA9 [8]. CCRCC expresses more commonly low molecular weight CKs as well as vimentin [14].

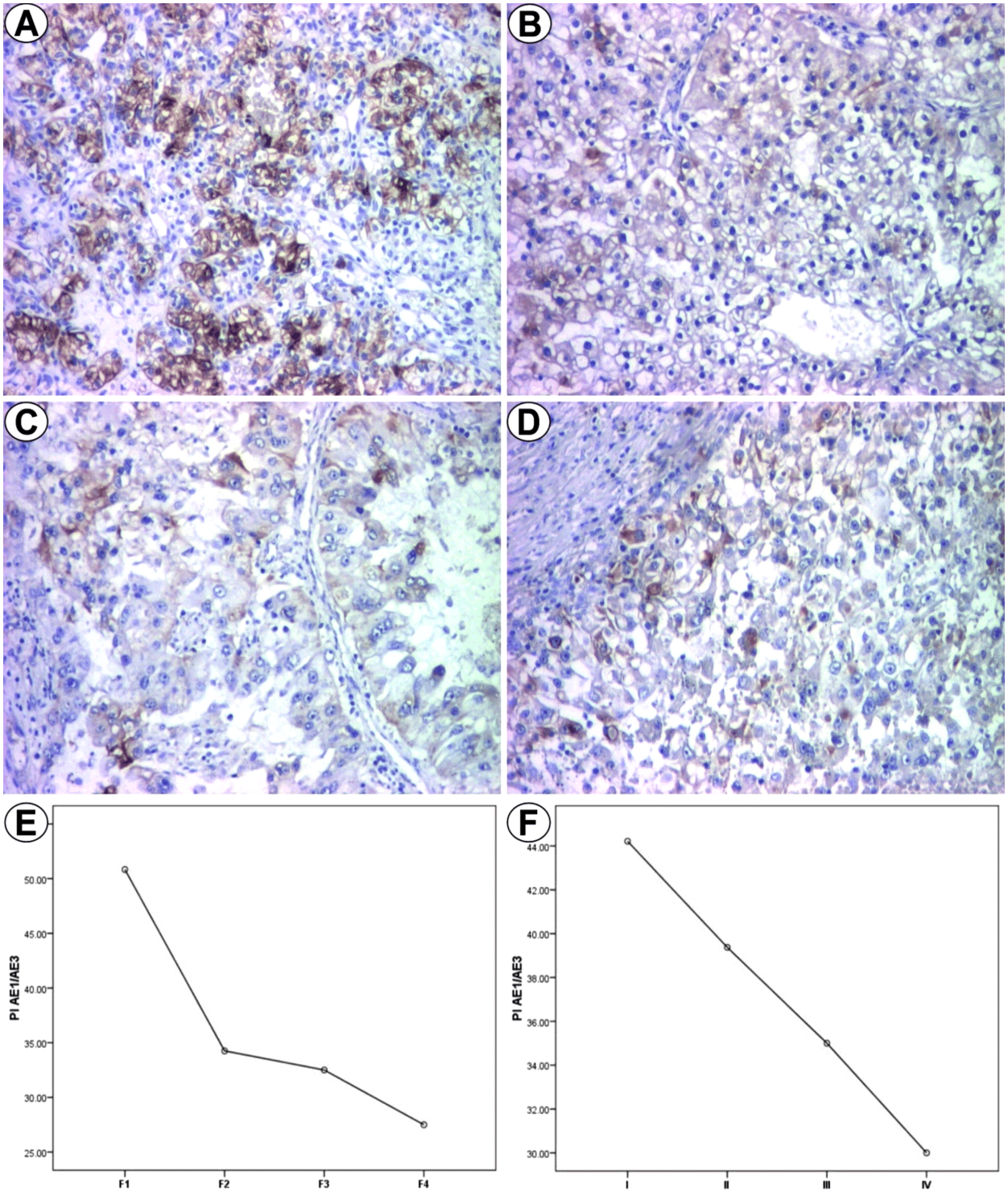

Figure 1 – ***CCRCC: (A) Fuhrman 1; (B) Fuhrman 2; (C) Fuhrman 3; (D) Fuhrman 4; (E) PI values in relation to tumor grade; (F) PI values in relation to tumor stage. Anti-CK AE1/AE3 antibody immunostaining: (A–D) ×100. CCRCC: Clear cell renal cell carcinoma; PI: Positivity index; CK: Cytokeratin.***

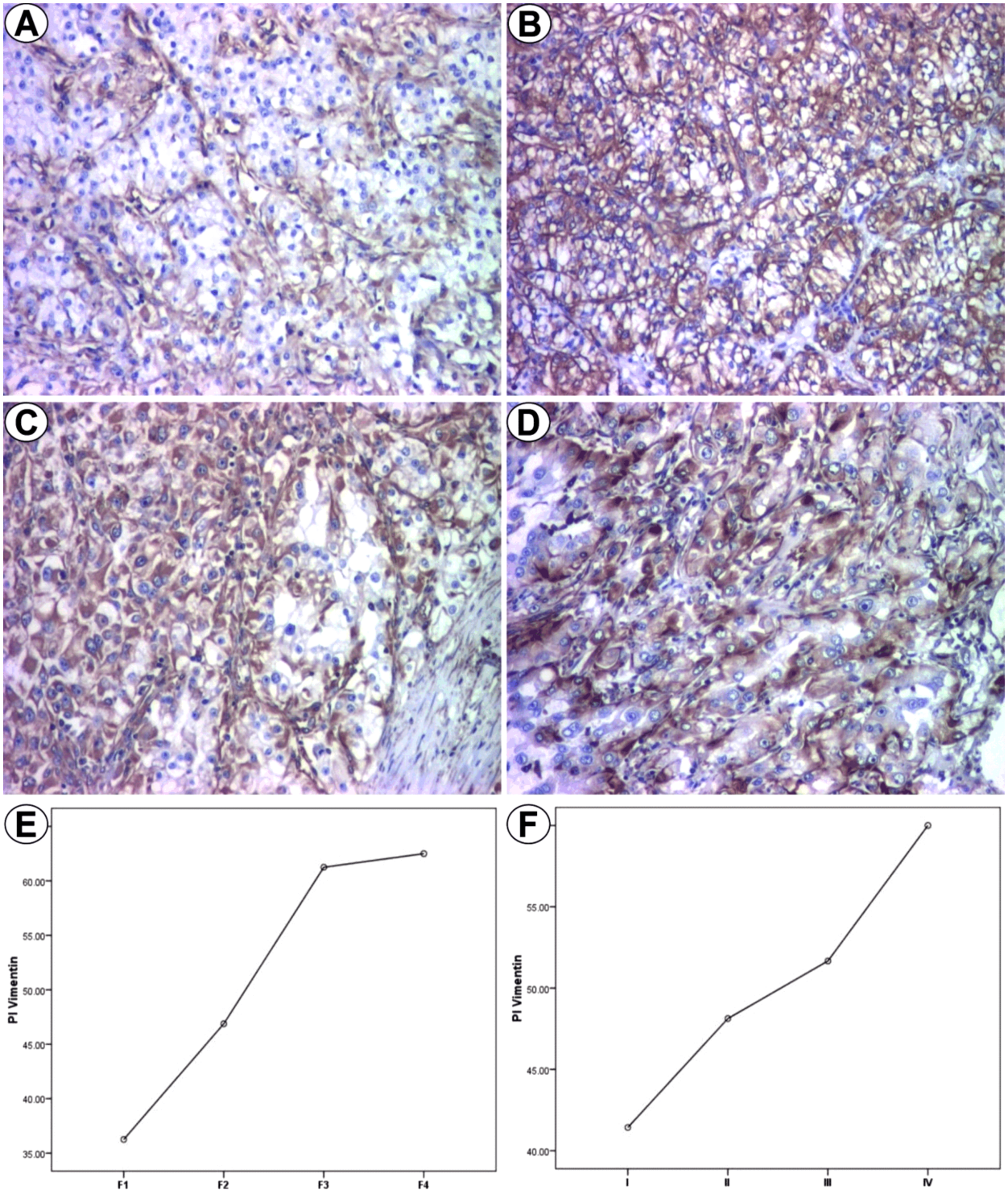

Figure 2 – ***CCRCC: (A) Fuhrman 1; (B) Fuhrman 2; (C) Fuhrman 3; (D) Fuhrman 4; (E) PI values in relation to tumor grade; (F) PI values in relation to tumor stage. Anti-vimentin antibody immunostaining: (A–D) ×100. CCRCC: Clear cell renal cell carcinoma; PI: Positivity index.***

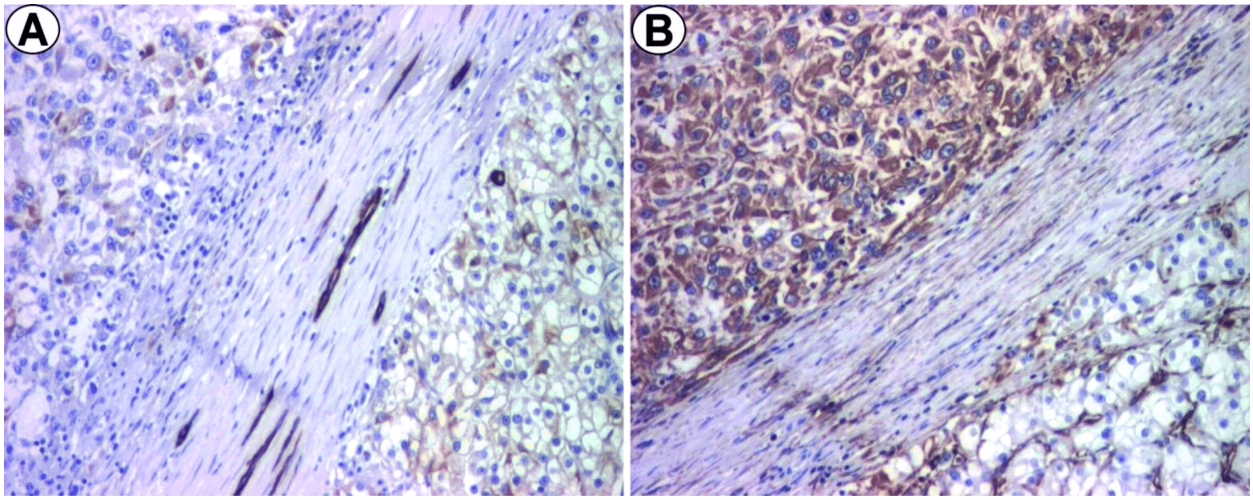

Figure 3 – ***CCRCC, heterogeneous tumor areas: (A) Anti-CK AE1/AE3 antibody immunostaining, ×100; (B) Anti-vimentin antibody immunostaining, ×100. CCRCC: Clear cell renal cell carcinoma; CK: Cytokeratin.***

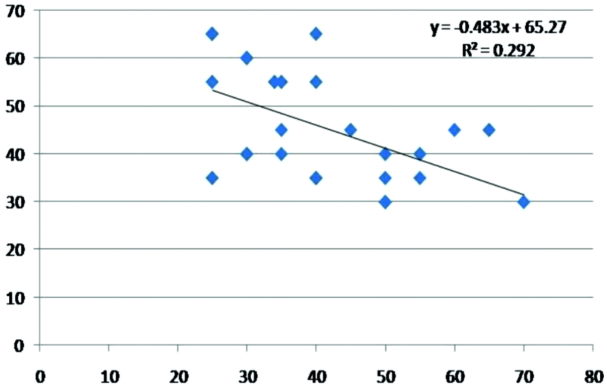

Figure 4 – ***CK AE1/AE2 and vimentin PI values distribution. CK: Cytokeratin; PI: Positivity index.***

Cytokeratins belong to the intermediate protein filament family, being involved in cytoskeleton maintenance, and which in the case of malignant tumors provide information about the origin, prognosis and/or response to the treatment of the lesions [15]. The diagnostic utility of CK AE1/AE3 is limited in case of CCRCC, and literature reports of this marker in relation to the histopathological parameters are rare. The aspect is due to the absence of CK18 in the CK AE1/AE3 cocktail, CK which is diffuse expressed in renal carcinomas, including CCRCC [8]. Some studies indicate for CCRCC a CK phenotype strictly restricted to CK18 and CK8 expression, others indicate the positivity of a small number of tumors for CK7, and other studies indicate the utility for the diagnosis of a large and complete panel of CKs [8, 16]. However, the aspect may be considered favorable for the study of the epithelial phenotype of CCRCC in relation to histopathological prognostic parameters, by excluding a diffuse expressed CK, such as CK18. In our study, CK AE1/AE3 immunoexpression was identified in all cases, being associated with low grade and early stage carcinomas. In the literature, are reported in CCRCC, CK AE1/AE3 positivity rates over 50% [17]. Also, data from the literature indicates a specificity and sensitivity of 100% and 88% of CK AE1/AE3 in CCRCC metastases [18].

Vimentin belongs to the intermediate family of proteins, being heavily expressed in mesenchymal structures [10]. CCRCC is one of the few carcinomas expressing vimentin, along with endometrial, thyroid, adrenal carcinomas, which is mainly used in the diagnosis of tumors. While some studies indicate vimentin expression in about 50% of CCRCCs, others indicate markers in over 80–100% of tumors [8, 9, 14]. Vimentin overexpression appears associated with a poor prognosis in renal carcinomas, independent of tumor stage and grade [9, 19]. In our study, the vimentin immunoexpression was identified in all cases, the immunostaining being associated with high grade and advanced stage lesions.

Immunoexpression of vimentin is generally associated with aggressive, poorly differentiated and high-risk metastatic forms of carcinoma. The aspect was also described in the case of epithelial–mesenchymal transition, a process involved in carcinomas progression, which consists in the loss of the epithelial phenotype and the acquisition of a mesenchymal, invasive and migratory tumor cell phenotype [11]. Thus, in invasive and metastatic carcinomas, some tumor cell groups, especially from the invasion front, express vimentin, which allows them to conformational change and migration, aspects that are regulated by complex biomolecular mechanisms in which the cadherinic switch, transcription and growth factors play a central role [11, 20]. The vimentin diffuse expression in CCRCCs can be associated under these conditions with an aggressive carcinoma status, with a reduced survival rate compared to other locations, despite the existing of some diagnosis and prognosis markers for these lesions. Preliminary studies indicate vimentin as the possible multifunctional therapeutic target in carcinomas, the inhibitors administered on experimental models being without toxicity and restricted effect on tumor epithelial cells [10].

Stage, size, degree and tumor necrosis are the most important prognostic factors for CCRCCs, being parameters used to stratify patients for therapy [7, 21]. Some studies indicate low inter-observatory concordance for the use of four-tiered Fuhrman grading in CCRCCs, while others indicate consistent similarity of this system with the simplified ones [21–23]. Although simplified grading systems may lead to lower overall costs for therapeutic lesion management, due to the varying degree of tumor heterogeneity present in the CCRCCs, the grouping of different degrees for the lesion assessment does not seem to be indicated [21]. Also, in this study we have found the presence of tumor heterogeneity, respectively the

presence of low or high-grade areas, aspects that have been observed by differences in the expression of investigated markers. This may support the utility of CK AE1/AE3 and vimentin to assess the degree of tumor heterogeneity and how it influences the prognosis of patients.

Conclusions

In this study, high-grade and advanced stages CCRCCs have associated high vimentin and reduced CK AE1/AE3 expression, suggesting the loss of epithelial phenotype and the acquisition of a mesenchymal one. This aspect may be used to determine the tumor heterogeneity and a better patients' stratification for therapy. Extensive studies are required to determine the opportunity of including CK AE1/AE3–vimentin immunophenotype in the biomarker panels used to identify aggressive CCRCCs.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Alex Emilian Stepan and Mioara-Desdemona Stepan contributed equally to this manuscript.

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