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Biomarkers of epithelial-mesenchymal transition in localized, surgically treated clear-cell renal cell carcinoma

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Abstract

Introduction. It has been suggested that the metastatic potential of neoplastic cells can be predicted on the basis of their biological features, including expression of proteins involved in the epithelial to mesenchymal transition (EMT). Therefore, the purpose of this work was to (1) evaluate the expression of EMT markers: ZEB2, vimentin, N-cadherin, TWIST, PTEN, survivin, E-cadherin, Ki-67 and GLUT-1, (2) assess mutation status of two genes: *PIK3CA* and *KRAS*, and (3) investigate the potential relationships between the studied biomarkers and clinicopathological factors in clear-cell renal cell carcinoma (ccRCC).

Material and methods. Tumor tissue samples (embedded in paraffin blocks) from 159 patients undergoing radical nephrectomy were analyzed. Proteins expression was evaluated immunohistochemically. DNA mutations were analyzed on DNA isolated from tumor tissue and amplified by real-time PCR detection using suitable fluorescent labeled TaqMan assays.

Results. One hundred and seven men and 52 women of mean age of 63.1years were enrolled. Fifty four cancers at pTNM stage I–II and 98 at pTNM III–IV stage were diagnosed. There were 30 Fuhrman grade G1, 61 Fuhrman G2, 49 Fuhrman G3 and 19 Fuhrman G4 tumors. A negative correlation between ZEB2 ($p = 0.047$, $r = –0.172$) or E-cadherin expression ($p = 0.027$, $r = –0.191$) and TNM was observed. Positive association between grade and Ki-67 ($p < 0.001$), survivin ($p < 0.001$), vimentin ($p < 0.001$) immunoreactivity and negative association between TWIST expression ($p = 0.029$) or PTEN expression ($p = 0.013$) were found. Ki-67 expression was positively correlated with survivin ($p < 0.001$, $r = 0.617$), vimentin ($p = 0.001$, $r = 0.251$) and N-cadherin ($p = 0{,}009$, $r = 0.207$) immunoreactivity which can suggest tumor aggressiveness. TWIST was negatively correlated with E-cadherin ($p < 0.001$, $r = –0.284$), vimentin ($p < 0.001$, $r = –0.297$) and N-cadherin ($p < 0.002$, $r = –0.241$). ZEB2 was not associated with ccRCC grade but was negatively correlated with E-cadherin ($p = 0.055$, $r= –0.153$) and PTEN ($p = 0.006$). GLUT-1 expression was inversely linked to E-cadherin expression ($p = 0.022$, $r= –0.182$). Mutations in *PIK3CA* and *KRAS* genes were not found in any of the studied ccRCC tumors.

Conclusions. Low-grade tumors showed higher expression of ZEB2 and TWIST proteins than high-grade tumors, which can suggest that EMT in ccRCC begins at early stages of tumor development and, therefore, evaluation of these proteins, together with other biomarkers, may be useful for assessment of the tumor metastatic potential.
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Key words: ccRCC, EMT; surviving; vimentin; Ki-67; GLUT-1; TWIST; PTEN; ZEB2; *PIK3CA*; *KRAS*; IHC

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Introduction

Clear-cell renal cell carcinoma (ccRCC) has the highest incidence and accounts for 70% to 80% of all histological types of renal cell cancers (RCCs) [1]. This is a heterogeneous disease and its natural course is still unpredictable, *i.e*. the prognosis of patients at a similar stage of locally advanced ccRCC varies widely. Morbidity and mortality in advanced ccRCC is high. Up to a quarter of patients display distant metastases or advanced regional disease at the time of presentation and diagnosis [2].

In many tumors, biological markers can identify prognostic subgroups for individual treatment; however, at present, clinical use of biomarkers to predict outcome in RCC is not validated [3] and urine biomarkers as well as liquid biopsy analysis of circulating tumor DNA remain the area of active investigation [4]. The prognosis of patients with localized ccRCC and tumor metastatic potential cannot be accurately predicted by standard variables such as clinicopathological characteristics. Therefore, after surgical treatment of advanced cancer and the occurrence of single metastatic focus, the physician is facing the problem of choosing the right therapy — surgery/radiation or systemic treatment. It has been shown that the limited/high metastatic potential can be predicted on the basis of biological features of tumor cells: expression of proteins involved in the transformation from epithelial to mesenchymal phenotype [5, 6] or genetic mutations responsible for acquisition of the ability to metastasize of cancer cells [7]. Some of them become a therapeutic target for the treatment of RCC. *PTEN* (phosphatase and tensin homolog detected on chromosome 10) is a tumor suppressor gene involved in the homeostatic maintenance of the phosphatidylinositol 3 kinase (*PI3K)/AKT* cascade [8]. Inactivation of PTEN causes elevated *AKT* activation. The most important gene mutations for the development and progression of metastatic disease are: *PIK3CA* — playing a role in tumor cell invasion, and *KRAS* — an oncogene involved in signal transduction in hypoxia-inducible pathway activated by epidermal growth factor receptor (EGFR), which become targets for drug intervention in RCC [1, 6, 9].

Epithelial-mesenchymal transition (EMT) is related to proliferation, invasion and metastasis of tumor [5, 9, 10]. Increased cell proliferation can be detected by the assessment of expression of the Ki-67 antigen, which is present during all active phases of the cell cycle. EMT is typified by a common spectrum of changes in cell morphology, gene expression, and signaling pathways, which can confer resistance to therapy [10–12]. During EMT many cancer cells lose epithelial cell features and acquire mesenchymal cell features including repression of E-cadherin and overexpression of N-cadherin, and cytoskeletal protein — vimentin [10–12]. Transcriptional downregulation of E-cadherin appears to be an important event in the progression of various epithelial tumors [11, 13]. However, recently, it has been shown on a mouse model that rather than being a binary process, EMT develops from epithelial to completely mesenchymal states through an intermediate state [14]. Hypoxia and EMT have been identified as key events in tumor invasion and metastasis [15]. Stabilization of hypoxia-inducible factor 1α (HIF-1α) (constantly expressed in RCC [16]) has been shown to control, directly or indirectly, the expression of EMT-related transcription factors, array of genes that encode proteins essential for cancer cell functioning under hypoxic conditions, such as ZEB2 (zinc finger E-box-binding homeobox also known as Smad interacting protein-1 (SIP1)) that shows specific DNA binding activity to the E-cadherin promoter and inhibits E-cadherin transcription [10, 17], TWIST [10, 18] and glucose transporter 1 (GLUT-1) [19].

It has been found that hypoxia promotes EMT in vitro by regulating TWIST/E-cadherin pathway [11]. Recent studies have demonstrated that TWIST is remarkably upregulated in a large number of malignant tumors, plays a crucial role in the onset of metastatic behavior by promoting cell migration and motility [20], and inhibits apoptosis [21]. Tumor hypoxia may contribute to the conversion of differentiated tumor cells into cancer stem cells [22]. This may be correlated with overexpression of survivin, a protein that inhibits apoptosis, regulates cell division and enhances angiogenesis [23]. In ccRCC it has been shown that overexpression of survivin leads to high metastatic capacity of cells and poor patients' outcome [24].

EMT and markers of recurrence in ccRCC have not yet been established [6]. Therefore, the purpose of the study was to: (1) evaluate the expression of mesenchymal cell markers: ZEB2, vimentin, N-cadherin, TWIST, and survivin, E-cadherin, PTEN, Ki-67, and GLUT-1 (2) assess mutations in two genes: *PIK3CA* (p.E542K and p.H1047R) and *KRAS* (p.G12D and p.G13D) and (3) investigate the potential relationships between the expression of biomarkers and clinical-pathological variables as well as the possibility to predict metastatic potential of ccRCC.

Material and methods

Patients and samples. The study protocol was approved by the local Ethical Committee at the Regional Medical Chamber in Cracow. All procedures performed in the study

Table 1. Procedures for immunohistochemical reactions

Antigen	Antibody Clone	Manufacturer	Antigen retrieval	Dilution	Incubation
Ki-67	Mouse monoclonal MIB-1	DAKOCytomation Denmark A/S Cat. No. M7240, Glostrup, Denmark	TRS (Tris Retrieval Solution), (pH = 6.0), water bath, 96° C, 50 min	1 : 100	4°C overnight
E-cadherin	Mouse monoclonal NCH-38	DAKOCytomation Denmark A/S Cat. No. M3612, Glostrup, Denmark	TRS, (pH = 6.0), water bath, 96° C, 40 min	1 : 50	4°C overnight
N-cadherin	Mouse monoclonal 6G11	DAKOCytomation Denmark A/S Cat. No. M3613, Glostrup, Denmark	TRS, (pH = 6.0), water bath, 96° C, 40 min	1 : 75	4°C overnight
PTEN	Rabbit monoclonal D4.3	Cell Signaling Cat. No. 9188 Danvers, MA, USA	TRS, (pH = 6.0), water bath, 96° C, 50 min	1 : 50	37°C 60 min
TWIST	Rabbit polyclonal	Ab-cam Cat. No. Ab49254 Cambridge, MA, USA	TRS, (pH = 6.0), water bath, 96° C, 50 min	1 : 75	4°C overnight
Vimentin	Mouse monoclonal V9	Bio Genex Cat. No. MU074-UC Fremont, CA, USA	none	1 : 200	4°C overnight
Survivin	Mouse monoclonal EP2880	Bio Genex Cat. No. NU503-UC, Fremont, CA, USA	TRS, (pH = 6.0), water bath, 96° C, 40 min	1 : 30	4°C overnight
ZEB2	Rabbit polyclonal	Sigma-Aldrich Cat. No. HPA003456, St. Louis, MO, USA	TRS, (pH = 6.0), water bath, 96° C, 40 min	1 : 300	4°C overnight
GLUT-1	Rabbit polyclonal	EMD Millipore Corporation Cat.No. 07-1401 Temecula, CA, USA	TRS, (pH = 6.0), water bath, 96° C, 40 min	1 : 200	4°C overnight

involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from all individuals included in the study.

Between November 2013 and January 2016, a group of 159 patients with locally advanced ccRCC (clinical stage I–IV) were included into the study in which a tumor biopsy was taken during curative surgery.

Tumors were classified according to the WHO classification of renal neoplasms [25], clinical (cTNM) and pathological (pTNM) stages according to the AJCC TNM 2010 classification [26] as well as Fuhrman grade [27] were assessed.

Immunohistochemical analysis and scoring. Protein expression was evaluated immunohistochemically on histological specimens using a suitable antibody and BrightVision visualization system (Bright Vision detection system, cat. no. DPVB-110HRP, Immunologic, Duiven, Netherlands). Following rehydration, and antigen retrieval (TRS — Target Retrieval Solution (10 ×), cat. No. S1699, DakoCytomation Denmark A/S, Glostrup, Denmark) 5 μm sections were incubated with proper antibody according to the procedure given in Table 1. Next, the sections were stained with diaminobenzidine (DAB — Peroxidase substrate KIT, cat.no. SK-4100, Vector Laboratorie, Inc., Burlingame, CA, USA), counterstained with hematoxylin, dehydrated and mounted. Negative control slides (omitting the primary antibody) and positive controls (with known protein expression) were included in each staining run. Additionally, for GLUT-1 red blood cells, and for PTEN, ZEB2 stromal and inflammatory cells served as internal positive control. Immunoreactivity was scored as the number of positive tumor cells over total (500–1000) tumor cells — labeling index (LI): several (5–7) microscopic fields of each tumor tissue sections were examined. PTEN protein expression was considered positive when > 30% of tumor cells exhibited nuclear staining and ZEB2 was assessed at the invasive front (hot spot) of the tumors. Slides were evaluated by two investigators unaware of the clinicopathological variables.

qPCR analysis of *KRAS* and *PIK3CA* mutation status. DNA was isolated from 5 μm thick formalin-fixed, paraffin-embedded tumor tissue sections. ReliaPrep FFPE gDNAMiniprep System (Promega Corp Madison, WI, USA) was used for DNA extraction according to the manufacturer's protocol.

qPCR reactions were performed using ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Two mutations in *KRAS* and two in *PIK3CA* genes were analyzed in each patient.

For establishing *PIK3CA* mutation status, three different assays on the same plate were run: (i) *PIK3CA* 775 TaqMan® Mutation Detection Assay (Assay ID Hs00000831_mu) to detect H1047R mutation, (ii) *PIK3CA* 760 TaqMan® Mutation Detection Assay (Assay ID Hs00000822_mu) for E542K mutation, (iii) *PIK3CA* TaqMan Mutation Detection Reference Assay (Assay ID Hs00001025_rf) to detect conservative fragment of *PIK3CA* gene which serves as quality control of isolated DNA. Similarly, to establish *KRAS* mutation status, three different assays on the same plate were run: (i) *KRAS* 521 TaqMan® Mutation Detection Assay (Assay ID Hs00000121_mu) for G12D mutation detection, (ii) *KRAS* 532 TaqMan® Mutation Detection Assay (Assay ID Hs00000131_mu) for G13D mutation, (iii) *KRAS* TaqMan Mutation Detection Reference Assay (Assay ID Hs00000174_rf) to detect conservative fragment of *KRAS* gene. All TaqMan Mutation Detection Assays were supplied by Applied Biosystems.

The final volume of qPCR reaction was 20 μl per well. Each well contained the following reagents: 2 μl of proper TaqMan® Mutation Detection Assays, 4 μl (20 ng) of isolated DNA, 10 μl of TaqMan Genotyping Master Mix (Applied Biosystems), 0.4 μl of Exogenous IPC Template DNA (internal positive control), 2 μl of Exogenous IPC Mix (Applied Biosystems) and 1.6 μl of nuclease-free water suitable for PCR (Ambion, Austin, TX, USA). The following thermocycling conditions were applied: initial denaturation — 95°C, 10 minutes; 5 cycles: 92°C, 15 seconds, 58°C for 1 minute; 40 cycles: 92°C for 15 seconds, 60°C for 1 minute. Data obtained from qPCR reactions were analyzed using Mutation Detector Software to determine the presence or absence of mutations.

Statistical analysis. Statistical analysis was performed with STATISTICA v. 12 (Statsoft, Krakow, Poland). For determination of mean values of variables and standard errors of means (SE) the descriptive statistics were used. Intergroup differences in the mean values were tested with one-way ANOVA test (grade) or Student's t-test. Associations between investigated categorical parameters and clinicopathological variables were evaluated by Pearson's Chi^2 test. The relationship between proteins expression (continuous variables) was tested with Pearson correlation and between proteins expression and TNM and pTNM (categorical variables) using Spearman correlation. Differences were considered significant at p value of < 0.05.

Table 2. Gender-related clinicopathological characteristics of ccRCC patients

Variable	N	Sex		*p* value*
		Women	Men	
Age (years) mean ± SD	159	63.4 ± 10.4 (N = 52)	62.9 ± 9.5 (N = 107)	0.768
cTNM (N = 154)				
1 2 3 4	74 30 18 32	28 10 4 8	46 20 14 24	0.447
pTNM (N = 152)				
1 2 3 4	48 6 73 25	19 2 18 9	29 4 55 16	0.351
G (according to Fuhrman) (N = 159)				
1 2 3 4	30 61 49 19	15 21 9 7	15 40 40 12	0.031

Results

Demographic and clinicopathological characteristics of the ccRCC patients

The analyzed group of ccRCC patients consisted of 107 men and 52 women of median age of 64.0 (30–85) years which did not differ between male and female patients (Table 2). The patients' clinicopathological characteristics are listed in Table 2. The majority (104) of patients were diagnosed with cTNM stage 1–2 and pTNM stage 3–4 (98) and there was no statistically significant difference in stage between both sexes (Table 2). In five (3.1%) patients cTNM and in seven (4.4 %) patients pTNM was not assessed (nephron sparing surgery). Ninety one patients had low-grade tumors (30 cases — G1, according to the Fuhrman system, and 61 G2, while 68 patients — high grade tumors (49 G3 and 19 G4 cases). This variables differed ($p = = 0.031$) between male and female subgroups (Table 2).

Relationship between clinicopathological variables and proteins expression

The immunoreactivity of the studied proteins was assessed in all tumors. In Figure 1, nuclear positivity for Ki-67 (Fig. 1A), survivin (Fig. 1B), PTEN (Fig. 1F), TWIST (Fig. 1G), ZEB2 (Fig. 1I), membranous for E-cadherin (Fig. 1C), N-cadherin (Fig. 1D),

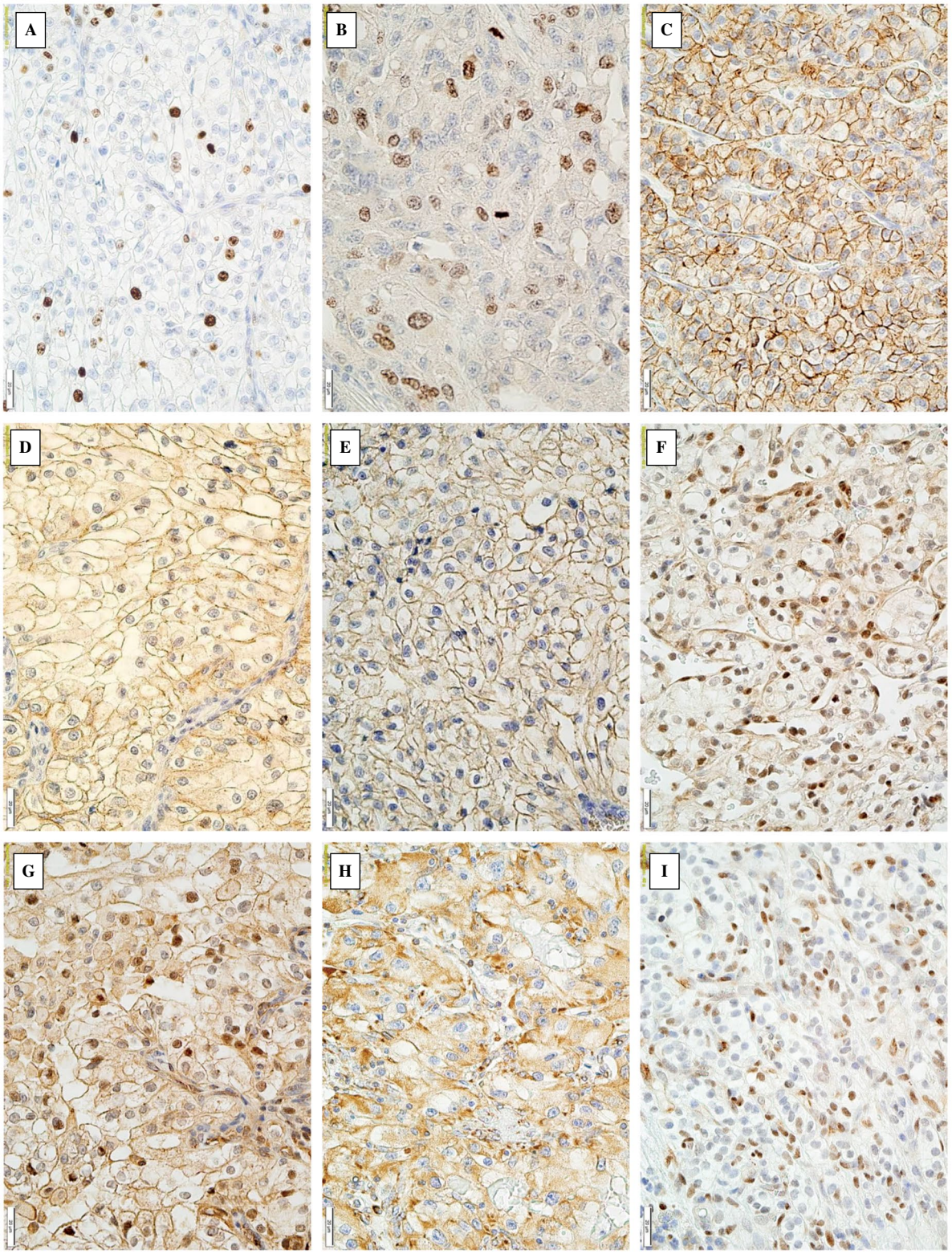

Figure 1. Immunoreactivity of the studied proteins in the tumor tissue of 159 ccRCC patients. Nuclear immunopositivity of: (**A**) Ki-67 (grade [G] 2, male patient; (**B**) survivin (G4, male patient); (**F**) PTEN (G2, male patient); (**G**) TWIST (G3, male patient); (**I**) ZEB2 (G1, female patient). Membranous staining of: (**C**) E-cadherin (G1, female patient); (**D**) N-cadherin (G3, male patient). Membranous/cytoplasmic expression of (**E**) GLUT-1 (G3, male patient), and cytoplasmic staining of (**H**) vimentin (G4, male patient) in primary ccRCC. The slides were stained by immunohistochemistry as described in Methods. Scale bars = 20 μm.

Table 3. The association between protein expression and clinicopathological variables in ccRCC patients

Variable	Labeling index (%) x ± SE								
TNM	**Ki-67**	**Survivin**	**E-cadherin**	**GLUT-1**	**Vimentin**	**TWIST**	**N-cadherin**	**ZEB2**	**PTEN[1]**
1	22.9 ± 1.4	5.4 ± 0.6	34.7 ± 2.6[2]	62.8 ± 2.7	42.8 ± 2.7	25.7 ± 1.8	59.8 ± 3.3	20.8 ± 1.4[3]	50.0
2	24.5 ± 2.2	5.4 ± 1.1	27.4 ± 4.4	66.1 ± 3.6	37.4± 3.7	24.5 ± 3.0	53.8 ±5.0	16.9 ± 2.0	33.3
3	25.6 ± 2.8	7.3 ± 1.7	22.5 ± 3.7	73.8 ± 5.5	43.3 ± 5.7	27.2 ± 4.5	60.4 ± 7.8	20.6 ± 3.0	26.7
4	26.0 ± 2.3	7.8 ± 1.1	26.6 ± 3.3	66.2 ± 4.0	38.7 ± 4.8	24.4 ± 3.9	60.0 ± 4.3	13.8 ± 2.6	26.9
pTNM									
1	22.1 ± 1.5	4.8 ± 0.5[4]	35.8 ± 3.3[5]	60.3 ± 3.5	43.1 ± 3.2	26.8 ± 2.3	59.0 ± 4.1	21.3 ± 1.5	53.7
2	20.0 ± 3.1	3.3 ± 1.1	31.0 ± 10.4	65.5 ±3.0	35.0 ± 9.1	26.3 ± 7.1	51.0 ± 7.0	16.5 ± 3.4	16.7
3	25.5 ± 1.3	6.6 ± 0.7	27.9 ± 2.4	69.8 ± 2.3	39.2 ± 2.8	23.0 ± 1.9	58.6 ± 3.3	17.7 ± 1.6	34.7
4	27.7 ± 2.7	9.0 ± 1.4	22.2 ± 3.8	63.7 ± 5.4	43.6 ± 5.9	28.2 ± 4.6	56.4 ± 5.3	15.7 ± 2.8	36.4

[1]percentage of PTEN positive tumors, [2]$p = 0.078$, [3]$p = 0.044$, [4]$p = 0.014$, [5]$p = 0.064$. Statistically significant differences were analyzed by one-way ANOVA test.

membranous/cytoplasmic for GLUT-1 (Fig. 1E) and cytoplasmic for vimentin (Fig. 1H) are presented. The mean labeling index values for each protein and the correlation between proteins expression and TNM and pTNM are presented in Table 3. With the increase of clinical tumor stage (cTNM) expression of E-cadherin ($p = 0.027$, $r = –0.167$) and ZEB2 ($p = 0.046$, $r = –0.207$) significantly decreased. Weak positive correlations between pTNM and expression of survivin ($p = 0.028$, $r = 0.178$) and weak negative association with E-cadherin ($p = 0.038$, $r = –0.186$) and ZEB2 ($p = 0.031$, $r = –0.175$) were observed. However, there was a statistically significant increase in the rate of tumor cell proliferation (Ki-67 LI, $p < 0.001$), expression of survivin ($p < 0.001$), vimentin ($p < 0.001$), decrease of TWIST expression ($p = 0.029$) and percentage of PTEN- positive tumors ($p = 0.013$) with tumor grade (Fig. 2). Grade 4 tumors showed the highest immunoreactivity for Ki-67, survivin and vimentin, and the lowest expression of TWIST and PTEN (Fig. 2). A statistically significant relationship between tumor grade and biomarker expression has not been demonstrated for GLUT-1 ($p = 0.344$), E-cadherin ($p = 0.354$), N-cadherin ($p = 0.648$) and ZEB2 ($p = 0.216$).

Correlation between immunoexpression of the studied proteins

Analysis of the correlation between protein expressions showed that the expression of vimentin ($p = 0.001$, $r = 0.252$), N-cadherin ($p = 0.009$, $r = 0.207$) and survivin ($p < 0.001$, $r = 0.617$) was positively correlated with Ki-67. TWIST expression was negatively correlated with the expression of vimentin ($p < 0.001$, $r = –0.297$), E-cadherin ($p < 0.001$, $r = –0.284$) and N-cadherin ($p = 0.002$, $r = –0.241$). Survivin was positively associated with vimentin ($p < 0.001$, $r = 0.345$) whereas GLUT-1 showed negative correlation with E-cadherin ($p = 0.022$, $r = –0.182$). ZEB2 was inversely linked to E-cadherin ($p = 0.055$, $r = –0.153$) and PTEN ($p = 0.006$).

Gene mutations

None of the patients showed any mutations in the *PI3KCA* and *KRAS* genes in ccRCC tumor tissue.

Discussion

We studied expressions of proteins known to be involved in the EMT process, and *PI3KCA* and *KRAS* genes mutations in order to assess their association with clinicopathological variables in ccRCC. The results of our study might indicate that the ability of ccRCC to undergo EMT may not be directly associated with histological grade, suggesting the need for biological markers assessment. We have shown the change from epithelial to mesenchymal phenotype based on higher expression of vimentin, N-cadherin, Ki-67, survivin and also repression of E-cadherin and PTEN which may demonstrate an increased potential for metastasis. ZEB2 was inversely linked to E-cadherin and PTEN which suggests its role in the EMT process. However, ZEB2 and TWIST expression was negatively associated with tumor grade. Additionally, TWIST expression was negatively correlated with the expression of vimentin, E-cadherin and N-cadherin. This may indicate two functions of the TWIST protein, depending on the tumor grade: (i) higher expression of this protein in differentiated tumors may be indicative of the acquisition of tumor stem cell traits by neoplastic cells, which promotes tumor growth, and (ii) negative correlation with E-cadherin

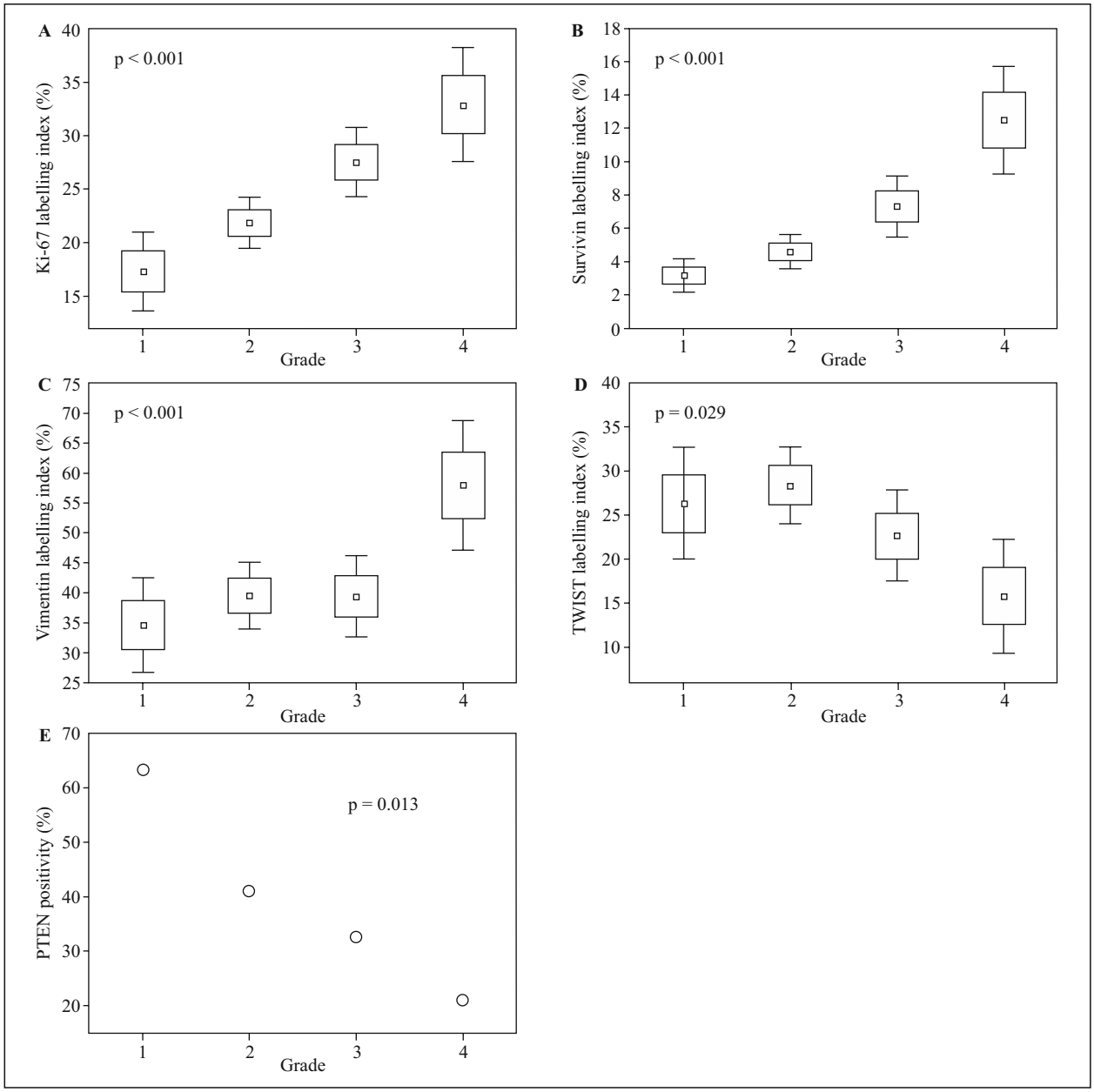

Figure 2. Relationships between histological grade of ccRCC (Fuhrman scale) and immunoexpression of Ki-67 (**A**), Survivin (**B**), Vimentin (**C**), TWIST (**D**) and PTEN (**E**). One-way ANOVA test (**A–D**) and Pearson's Chi^2 test (**E**) were used to analyses the differences. Symbols represent mean values ± SE.

may be indicative of EMT induction. Both functions are important in the EMT process. We showed high expression of GLUT-1, even in well differentiated tumors, and its negative correlation with E-cadherin proves that hypoxia favors the EMT process, what may support general statement [10, 15]. In our study, values of mean labeling index of the analyzed proteins are within the ranges found in renal cell carcinomas [19, 24, 28–37]. However, it is difficult to compare the results because the authors often did not provide raw data but the information on cut-off value for positive or negative expression important for patients' survival. Furthermore, they analyzed not only ccRCC but all histological types of RCC [29–32, 34–35], and also often used tissue microarrays (0.6 mm tissue blocks) with much lower number of cells counted (risk of exclusion of defective cores) [19].

In our study there was no relationship between cTNM or pTNM and proteins' expression, apart from E-cadherin and ZEB2, where significant decrease

in expression levels was observed with cTNM which supports the earlier published study [30]. However, there was a statistically significant increase in the rate of tumor cell proliferation (Ki-67 LI), expression of survivin, vimentin, and a decrease in the percentage of PTEN- positive tumors with grade which is in agreement with other authors' results [28, 29, 31, 35]. The expression of GLUT-1, ZEB2, E-cadherin and N-cadherin was not associated with histological grade of ccRCC. In our material high hypoxia (GLUT-1 LI > 60%) was found even in differentiated tumors, and well-differentiated tumors showed higher expression of ZEB2 and TWIST proteins than high-grade tumors, which can suggest that EMT in ccRCC began early in tumor development.

It was reported that malignant carcinoma cells are characterized by the loss of both cell-cell adhesion and cellular differentiation, which correlate with E-cadherin downregulation [10]. During the EMT process epithelial cadherin (E-cadherin) is downregulated, while neural cadherin (N-cadherin) is upregulated, a phenomenon referred to as 'cadherin switch' that occurs in cancer cells of mesenchymal phenotype [10]. The results of our study suggests that downregulation of E-cadherin was not completed since the expression of E-cadherin did not decrease and expression of N-cadherin did not increase significantly with pTNM and tumor grade as shown in another study of renal cell carcinomas [30]. Furthermore, the expression of these proteins was positively correlated. This may imply the association between phenotypic plasticity of carcinoma cells and their metastatic potential and may be in agreement with study of Armstrong *et al.* [36] showing that circulating tumor cells from metastatic solid tumors (prostate and breast cancers) co-express E-cadherin and N-cadherin. Recently, it has been also shown that breast carcinoma cells with hybrid epithelial and mesenchymal phenotypes are more capable of entering the circulation and forming metastases [14].

In our study the expression of E-cadherin, even in low-grade tumors, was relatively low (mean 30%) in comparison to other cancer types [37, 38]. This may be connected with (1) expression of TWIST and ZEB2, repressors of E-cadherin, the expression of which was higher in low-grade tumors. Therefore, in ccRCC, the switch from E-cadherin to N-cadherin does not seem to promote EMT as in other tumor types [39]. Low expression of E-cadherin might also be caused by (2) hypermethylation of E-cadherin [10] or (3) controlling EMT by miR-200 family which targets E-cadherin repressor — ZEB2 [40, 41]. In our study, the immunoexpression of ZEB2 was inversely linked to E-cadherin immunoexpression which is in accordance with the results of other studies [17, 34]. In breast tumor cell lines it has been shown that ZEB2 may directly activate/upregulate genes typical for mesenchymal cells (such as vimentin) and cell migration [42]. However, we did not demonstrate correlation between ZEB2 and vimentin, although other authors [34] showed positive correlation between expressions of these proteins (they analyzed not only ccRCC but all histological types of RCC). Therefore, it seems that clinicopathological significance of ZEB2 expression is unclear or may be not important in mesenchymal transition of ccRCC cells (not fully expressed mesenchymal phenotype) that affects only a small fraction of the tumor cell population at any given time [13] or may show a transient process occurring during EMT [14]. In our study, vimentin expression was associated with increasing tumor grade. Other authors [33] observed significant increase in vimentin expression also with clinical stage which we cannot confirm. Our results have shown that hypoxia is high (mean GLUT-1 LI > 60%) even in differentiated ccRCC tumors, which may point to the inducible role of hypoxia in RCC etiology suggested by other authors [16, 43, 44]. This indicates that malignant progression is based on dynamic processes which cannot be explained solely by irreversible genetic alterations, but must be additionally regulated by the tumor environment. In our study, GLUT-1 expression was not associated with tumor stage and grade which was also reported by other authors [19, 32]. We found a weak negative correlation between GLUT-1 and E-cadherin expression which can suggest that more hypoxic areas favor EMT and repression of E-cadherin [10] and prove that these processes are linked in the pathogenesis of ccRCC.

Hypoxia is necessary for the induction of HIF-1, which has been described to induce tumor cell dedifferentiation towards an immature phenotype and similarly to maintain tumor cells with stem-cell properties [22]. HIF-1 induces genes encoding glycolytic enzymes and proteins that facilitate transport of glucose into cells. Similarly to other authors [6, 10, 44] we found negative correlation between GLUT-1 and E-cadherin immunoexpressions.

We reported that ZEB2 was negatively correlated not only with E-cadherin but also with PTEN. This may be in agreement with the study of Milella *et al.* [8] who showed that PTEN regulates a broad spectrum of biological functions, modulating the flow of information from membrane-bound growth factor receptors to nuclear transcription factors, in concert with other tumor supressors and oncogenic signaling pathways. In our study, expression of PTEN decreased with tumor grade, which suggests loss of inhibitory role of this protein on the PI3K/AKT/mTOR signaling pathway,

suggested by other authors [45]. However, we did not find mutations in *PIK3CA* and *KRAS* genes. Maybe it is because *PIK3CA* mutations are rare in ccRCC and are present only in 2–5% of tumors [46] or result from intratumoral molecular heterogeneity which should be studied by novel sampling strategies [47]. Similarly to other authors [47–50] we also did not detect mutations in *KRAS* gene. It seems that mutations in *PIK3CA* and *KRAS* are not significant events in the development of ccRCC. Lack of mutation in the *PIK3CA* gene responsible for the synthesis of the catalytic subunit of the phosphoinositide-3-kinase (PI3K) the key enzyme of this pathway, may be the cause of tumor resistance to everolimus — an inhibitor of the mTOR pathway. The finding of the absence of mutation in the *KRAS* gene in all studied patients, may help in qualification of patients for targeted treatment (lack of mutation in the *KRAS* gene may indicate tumor sensitivity to inhibitors of signal transduction for growth factors). Expression of mesenchymal proteins (vimentin, N-cadherin) was positively correlated with Ki-67 and survivin immunoexpression which was found also in another study [33] and indicates a higher potential for metastasis, because it was shown that survivin inhibits apoptosis and promotes cell proliferation [23].

In our study, TWIST expression was negatively associated with tumor grade, repression of vimentin, E-cadherin, and N-cadherin immunoreactivity. TWIST is known as the driver of EMT and is needed for tumor initiation and maintenance [20, 51]. It was shown that increase in the level of this protein during the EMT process is sufficient to suppress the death of cells initiated by a proapoptotic signal such as lack of cell adhesion [21]. Therefore, it has been suggested that TWIST can promote not only tumor invasion and metastasis but also is necessary for conferring cancer stem cell properties through EMT-independent mechanism [20, 52]. EMT and stem cell competence could be decisive for malignant tumor progression by allowing such tumor cells to detach, migrate and disseminate in the body and subsequently to form new colonies at the metastatic site [53].

In our study, TWIST was negatively correlated with E-cadherin, which confirms other authors` studies [17, 51]. TWIST expression was negatively associated with grade, contrarily to the results of Ohba and colleagues who analyzed 156 RCC including papillary and chromophobe tumors [54]. There may be two reasons for this discrepancy: (1) the authors considered nuclear and cytoplasmic staining of TWIST positivity, while we counted only nuclear expression, according to the data sheet from the manufacturer, because it was demonstrated that TWIST undergoes nuclear translocation under the effect of mechanotransduction (matrix stiffness) to induce EMT [55]; (2) it has been reported recently, that only cytoplasmic expression of TWIST was associated with higher tumor grade, and a significant difference between the nuclear expression of TWIST in different RCC subtypes was observed [56].

We have shown that TWIST expression was also negatively correlated with the expression of vimentin, N-cadherin and E-cadherin. This may indicate two functions of the TWIST protein, depending on the tumor grade. (1) Higher expression of this protein in differentiated tumors may be indicative of the acquisition of tumor stem cell traits by tumor cells, what is in agreement with the study of Friberg *et al.* [2], who had shown that even small RCCs have the capacity to generate metastases. (2) Negative correlation with E-cadherin may be indicative of EMT induction. Both functions are important in the EMT process.

If the transition or partial transition from epithelial to mesenchymal phenotype is molecularly linked to a dedifferentiation program, this could indicate that certain mesenchymal properties are related to a stemness phenotype. For example, TWIST expression in low-grade tumors has a role in inhibiting terminal differentiation by interfering with the ARF-p53 tumor suppressor pathway and apoptosis [21]. Beck and colleagues [20] showed that different levels of TWIST1 in mouse skin SCC regulate tumor initiation, stemness, and progression, but independently of its EMT function. Similarly as in our study, TWIST was expressed at early stages of skin tumorigenesis [20]. Also a direct link between the EMT, TWIST and stem-cell properties was described at the molecular level in mammary epithelial cells [57].

The EMT program and cancer stemness are mechanistically linked [52]. The EMT program predominantly operates during tumor metastasis, while cancer stem cell properties are also needed for tumor initiation. Therefore, it is unclear whether these two properties are independent or cooperate during different stages of tumor progression or represent an intermediate state between epithelial and mesenchymal phenotype [14].

Conclusions

The results of our study suggest that the ability of ccRCC to undergo EMT may not be directly linked to tumor malignancy. The change in epithelial-to-mesenchymal transition, based on higher expression of vimentin, N-cadherin, Ki-67, survivin and E-cadherin and PTEN repression may indicate an increased potential for metastasis. Although ZEB2 and TWIST were inversely linked to E-cadherin they were neg-

atively associated with ccRCC grade which suggests an early beginning of EMT program in ccRCC development, and points to the need for the biological markers assessment. The predictive value of all the biological markers will be revealed only after analysis of the correlation between biomarkers expression and clinical outcomes (relapse and metastasis, overall and progression free survival).

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' Contribution

AG and AS were involved in conception, design and data interpretation of the study. AS and JJ were engaged in data acquisition and patients' management. WW was responsible for histopathological analysis, while AC, AG, AA, AJW were involved in immunohistochemical analysis. AA and AJW performed mutation analysis. AG wrote the manuscript and all co-authors reviewed it.

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