UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
DOCTORAL SCHOOL

PhD THESIS

THE ROLE OF EPITHELIAL-MESENCHYMAL TRANSITION IN THE PROGRESSION OF CLEAR CELL RENAL CELL CARCINOMA

PhD COORDINATOR:
Prof. univ. dr. CRISTIANA EUGENIA SIMIONESCU

PhD STUDENT:
BIANCA CĂTĂLINA NAIDIN (ANDREIANA)

CRAIOVA 2019

SUMMARY

INTRODUCTION

Epithelial-mesenchymal transition (EMT) has received great attention lately, with numerous scientific reports on the gene alterations, the expression of ARNm, a protein that is compatible with EMT and their potential correlation to tumor invasion, prognosis and metastasis.

EMT is a biologic process through which epithelial cells lose polarity and cell-cell contact and develop a mesenchymal phenotype [340]. The process is characterized by the loss of epithelial markers and the overexpression of mesenchymal markers, which gives it a key role in the process of invasion and metastasis [133, 316]. This process implies the molecular reprogramming of the cell through lower E-cadherin expression, which maintains cell-cell adhesion and the reorganization of the cytoskeleton, with an increase in N-cadherin expression caused by transcription factors like SNAIL, SLUG, TWIST1 and ZEB1.

Keywords: ccRCC, AE1/AE3, Vimentin, E-cadherin, N-cadherin, P-cadherin, SNAIL, SLUG, TWIST1, ZEB1, Fibronectin.

RESEARCH SYNTHESIS

CHAPTER I. Epidemiology and risk factors for clear cell renal cell carcinoma

Renal cell carcinoma (RCC) represents 4% of adult malignancies [369] and is the third most frequent type of tumor with urologic localization, after prostate and bladder carcinoma. Worldwide, RCC incidence, incidence rate and mortality vary substantially, being higher in more developed countries compared to developing countries [210].

RCC causes include three major risk factors: smoking, obesity and hypertension. While epidemiology studies have determined that each of these factors has an etiological connection to RCC, the underlying mechanisms and the interactions between them have been only partially determined [257].

Chapter II. Clear cell renal cell carcinoma carcinogenesis

Renal cell carcinogenesis includes the involvement of numerous signaling pathways. Each of the 7 genes known for the involvement in the pathogenesis of RCC: VHL(von Hippel-Lindau, MET(gene that codes the tyrosine-kinase protein), FLCN (folliculin), TSC1(tuberous sclerosis complex 1), TSC2 (tuberous sclerosis complex 2), FH (hydrate fumarate) and SDHB(succinate dehydrogenase B) are involved in signaling pathways that react to metabolic stress and/or nutritive stimulation [181, 355].

CHAPTER III. The role of epithelial-mesenchymal transition in the prognosis of clear cell renal cell carcinoma

EMT is a complex process during which cells lose their epithelial characteristics and develop a mesenchymal phenotype [183]. During EMT, cancer cells escape from the primary site and invade distant tissues through blood and lymph vessels. Moreover, EMT phenotype is often followed by a decrease of the level of epithelial markers E-cadherin and zonuela occludens 1 (ZO-1) and an increase in the level of mesenchymal and transcription markers like N-cadherin, Twist1 and Twist2, which results in an increased motility [42, 357]. Even more than that, studies have reported an association between the expression of E-cadherin or vimentin and tumor progression and survival rate (OS) in various types of cancer, including lung cancer and nasopharyngeal carcinoma [149]. It was also determined that SLUG and SNAIL decrease the level of E-cadherin by bonding to the E-cadherin promotor [223].

PERSONAL RESEARCH

STUDY SCOPE AND OBJECTIVES

This study aims to evaluate the involvement of EMT in the carcinogenesis of clear cell renal cell carcinoma (ccRCC) to try and identify possible prognosis factors and other targets for therapy. The research is important because determining a statistically significant correlation between the studied markers and prognosis factors supports the involvement of EMT in ccRCC carcinogenesis, as well as their use for the prognosis and therapy of ccRCC patients.

CHAPTER IV. Material and methods

We have performed an analytical, retrospective and prospective study on 50 cases of ccRCC diagnosed between 2015-2017.

The clinical and epidemiological study has provided data on year, patients' sex and age and the size of the tumor.

The histopathological study tracked the main histological parameters of the tumor: Fuhrman grade, vascular invasion, fat tissue invasion, invasion and tumor capsule disruption, the presence/absence of metastasis adenopathy, presence/absence of distant metastasis, tumor stage, necrosis, sarcomatoid transformation and growth pattern.

The immunohistochemical study evaluated the expression of certain markers involved in EMT in ccRCC, such as AE1/AE3, Vimentin, E-cadherin, N-cadherin, P-cadherin, SNAIL, SLUG, TWIST1, ZEB, Fibronectin.

The results were analyzed statistically, and statistical tests were used to evaluate the differences between the immunoreaction scores of each case, with a significant level difference of $p<0.05$.

CHAPTER V. RESULTS

During the clinical epidemiological study, we determined that most patients were in the 60-69 age category, over half of them were males (64%) and most cases were located in the right kidney (60%).

Histological analysis showed that most cases (17 cases, 34%) were in the category >4cm and ≤7 cm, most cases were low Fuhrman grade (37 cases, 74%) and half of the cases showed a mixed pattern (25 cases, 50%). Necrosis was present in 26 cases (52%), fat tissue invasion was present in 32 cases (64%) and vascular invasion was present in only 11 cases (22%). According to pTNM classification, we determined that most tumors were tumor stage III, 30 cases (60%).

The immunoreaction for AE1/AE3 was identified in all 50 cases of ccRCC in the immunohistochemical analysis, with cytoplasm localization, with superior staining in low Fuhrman grade and incipient stage cases.

Vimentin immunoreaction was identified in all 50 cases of ccRCC in the immunohistochemical analysis, with cytoplasm localization, with superior staining in high Fuhrman grade and advanced stage cases.

The immunoreaction for E-cadherin was identified in 70% of the 50 cases of ccRCC in the immunohistochemical analysis, with cytoplasm localization and superior staining in low Fuhrman grade and incipient stage cases.

N-cadherin immunoreaction was identified in 72% of the 50 cases of ccRCC in the immunohistochemical analysis, with cytoplasm and membrane localization and a higher CS in high Fuhrman grade cases.

P-cadherin immunoreaction was identified in 92% of the 50 cases of ccRCC in the immunohistochemical analysis, with cytoplasm, membrane and nuclear localization and a higher SC in high Fuhrman grade cases.

Snail immunoreaction was present in 92% of the cases, with cytoplasm localization, low Fuhrman grade ccRCC showed an average percentage of marked cells 19.05±11.77, with low and moderate reaction intensity.

Slug immunoreaction was identified in 96% of the cases with a higher average percentage of positive tumor cells in incipient stages I and II (54.58±25.97; 57±33.27) and a predominantly moderate reaction intensity.

Twist1 immunoreaction was identified in 90% of the cases and the average percentage of positive tumor cells was higher in advanced stages III/IV (45.64±23; 65±21.79), with moderate and high reaction intensity.

ZEB1 immunoreaction was identified in 70% of the cases, ccRCC with low Fuhrman grade had an average percentage of marked tumor cells of 35.51±22.14, reaction intensity was predominantly low, with an average CS of 2.35.

Fibronectin immunoreaction was identified in 78% of the cases, with cytoplasm and nuclear pattern. The staining varied according to Fuhrman grade in both percentage of marked cells and intensity.

CHAPTER VI. Discussions

ccRCC represents 65-75% of all renal carcinoma [210]. Data from the literature shows that this malignant neoplasia has a maximum level of incidence in the 6-7th decade of life and it appears more often in men [210]. Initial tumor size is reflected in TNM staging and it has a prognostic role in RCC [58, 270]. Fuhrman grade and tumor stage are considered the most important prognostic factors in ccRCC, multiple studies indicating the Fuhrman classification system as a prognostic

factor for survival independent from tumor stage [88, 239]. Reports from the literature confirm that the increased incidence of vascular invasion is associated with late tumor stages [117]. Also, data from the literature confirm our result, with multiple studies correlating the tumor stage and tumor size, vascular invasion, necrosis and 5 years survival rate [377].

CcRCC is a tumor with a mixed pattern, epithelial and mesenchymal, these lesions expressing both cytokeratin (CK) and Vimentin. There are studies that reported a 100% specificity and 88% sensitivity of CH AE1/AE3 for ccRCC metastases [348]. Vimentin overexpression is correlated with bad prognosis and tumor growth, being intensively studied as EMT marker, but its role in cancer progression remains uncertain [271].

Classic cadherins are cell adhesion transmembrane glycoproteins and they include E-cadherin, N-cadherin and P-cadherin [101]. An important feature of EMT is the loss of E-cadherin expression [271, 133]. In recent years, there was a higher interest in the cadherin switch, which is increasingly used to monitor the EMT process [372].

Recent studies have shown the involvement of many transcription factors with key roles in EMT including SNAIL, SLUG, Twist1 and ZEB1 [203]. Reports from the literature confirm the results of our research on the overexpression of SNAIL in ccRCC with high Fuhrman grade [206]. Other studies have determined that the increased expression of SLUG was significantly associated with improved survival in contrast with Snail expression, which was had a negative association with survival, while E-cadherin association was insignificant [206]. Other studies reported that Twist1 has a crucial role in tumor aggressiveness [107, 233], but even though Twist1 expression was associated with bad prognosis in ccRCC [107, 233], its pathological significance was not fully understood [233].

For Fibronectin, Kondisetty's study is one of the few that show that the expression of this marker in RCC cytoplasm is associated with a higher rate of mortality caused by disease, indicating a possible role in the progression of these carcinoma [148].

CHAPTER VII. Conclusions

The study performed on 50 cases of ccRCC allowed the following conclusions:

- The *clinical epidemiological* analysis for the investigated period of time (2015-2017) indicated an average diagnosis age of 60.18 ±10, most patients being males (64%), with an average tumor size of 6.7±2.9 cm, most of them falling in the >4 and ≤7 cm category;
- The *histopathological* study of the analyzed cases revealed that over half of the ccRCCs had a low Fuhrman grade (74%) and 26% had high Fuhrman grade;
- The mixed histological pattern was present in half of the cases (50%), tumor necrosis in 52%, vascular invasion in 22% and fat tissue invasion in 64% of the cases;
- PTNM staging indicated: 24% of the cases were stage I, 10% were stage II, 60% were stage III and 6% were stage IV;
- The *immunohistochemical* study covered all 50 cases of ccRCC, using markers involved in EMT: epithelial, mesenchymal and transcription markers;
- The immunostaining for AE1/AE3 was identified in all 50 cases, statistical analysis showed significant associations between the expression of AE1/AE3 an Fuhrman grade ($p=0.008$);
- The immunostaining for Vimentin was identified in all cases, with significant associations between the expression of Vimentin and Fuhrman nuclear grade ($p=0.000$) and vascular invasion ($p=0.047$);
- The immunostaining for E-cadherin was identified in 70% of the cases, with significant associations between high CS for E-cadherin and low Fuhrman nuclear grade ($p=0.000$), the absence of vascular invasion ($p=0.031$); moreover, E-cadherin showed a linear negative correlation with Vimentin ($p<0.05$, Pearson);
- The immunostaining for N-cadherin was identified in 72% of the cases, with significant associations between N-cadherin expression and Fuhrman grade ($p=0.048$);
- The immunostaining for P-cadherin was identified in 92% of the cases, with significant associations between P-cadherin expression and Fuhrman grade ($p=0.049$); even more, there is a linear negative correlation between P-cadherin and E-cadherin ($p<0.05$, Pearson), and a linear positive correlation between P-cadherin and N-cadherin ($p<0.05$, Pearson);
- SNAIL immunoreaction was identified in 92% of the cases, with significant associations between SNAIL expression and Fuhrman nuclear grade ($p=0.007$), fat tissue invasion ($p=0.03$) and tumor stage ($p=0,007$); we also determined a positive linear correlation between N-cadherin and SNAIL ($p<0.05$, Pearson);

- SLUG immunostaining was identified in 96% of the cases, with significant associations between the expression of SLUG and Fuhrman grade ($p=0.008$), fat tissue invasion ($p=0.001$), tumor extension ($p=0.006$), vascular invasion ($p=0.011$), necrosis ($p=0.014$) and tumor stage ($p=0.006$); there was also a positive linear correlation between SLUG and E-cadherin ($p<0.05$, Pearson) and a negative correlation between SLUG and Vimentin ($p<0.05$, Pearson);
- The immunostaining for Twist1 was identified in 90% of the cases, with significant associations between Twist1 expression and Fuhrman nuclear grade ($p=0.003$), tumor extension ($p=0.05$), fat tissue invasion ($p=0.001$) and tumor stage ($p=0.03$);
- Immunostaining for ZEB1 was identified in 70% of the cases, with significant associations between ZEB1 expression and Fuhrman grade ($p=0.008$);
- Fibronectin staining was identified in 78% of the cases, with higher staining for high Fuhrman grade tumors; statistical analysis showed no significant associations with the studied histopathological factors ($p>0.05$).

Selective bibliography

42. Chang JW, Gwak SY, Shim GA, et al. EZH2 is associated with poor prognosis in head-and-neck squamous cell carcinoma via regulating the epithelial-to-mesenchymal transition and chemosensitivity. Oral Oncol. 2016; 52: 66-74.

58. Chan Y, Yu Y, Wang G, et al. Inhibition of MicroRNA-381 Promotes Tumor Cell Growth and Chemoresistance in Clear-Cell Renal Cell Carcinoma. Med Sci Monit. 2019;25:5181-5190.

340. Voulgari A, Pintzas A. Epithelial–mesenchymal transition in cancer metastasis: mechanisms, markers and strategies to overcome drug resistance in the clinic. Biochim Biophys Acta. 2009; 1796(2):75–90.

348. Weinbreck N, Marie B, Bressenot A, et al. Immunohistochemical markers to distinguish between hemangioblastoma and metastatic clear-cell renal cell carcinoma in the brain: utility of aquaporin1 combined with cytokeratin AE1/AE3 immunostaining. Am J Surg Pathol. 2008; 32(7):1051–1059.

355. Wong SHM, Fang CM, Chuah LH, et al. E-cadherin: Its dysregulation in carcinogenesis and clinical implications. Crit Rev Oncol Hematol. 2018;121:11-22.

369. Zacchia M, Vilasi A, Capasso A, et al. Genomic and proteomic approaches to renal cell carcinoma. J Nephrol. 2011; 24(2):155–164.

375. Zeng J, Zhan P, Wu G, et al. Prognostic value of Twist in lung cancer: Systematic review and meta-analysis. Transl Lung Cancer Res. 2015;4:236-41.

316. Thiery J.P. Acloque H., Huang R.Y, et al. Epithelial-mesenchymal transitions in development and disease. Cell. 2009; 139(5): 871–890

270. Sanjmyatav J, Matthes S, Muehr M, et al. Identification of high-risk patients with clear cell renal cell carcinoma based on interphase-FISH. Br J Cancer, 2014; 110(10): 2537–2543.

271. Satelli A, Li S. Vimentin in cancer and its potential as a molecular target for cancer therapy. Cell Mol Life Sci. 2011; 68(18):3033–3046.

272. Savagner P. Epithelial-mesenchymal transitions: from cell plasticity to concept elasticity, Curr. Top. Dev. Biol. 2015; 112: 273–300.

206. Mikami S, Katsube KI, Oya M, et al. Expression of Snail and Slug in renal cell carcinoma: E-cadherin repressor Snail is associated with cancer invasion and prognosis. Lab Invest. 2011; 91(10):1443–1458.

203. Meng FD, Li Y, Tian X, et al. Synergistic effects of snail and quercetin on renal cell carcinoma Caki-2 by altering AKT/mTOR/ERK1/2 signaling pathways. Int J Clin Exp Pathol. 2015; 8(6):6157–6168.

223. Naber HP, Drabsch Y, Snaar-Jagalska BE, et al. Snail and Slug, key regulators of TGF-β-induced EMT, are sufficient for the induction of single-cell invasion. Biochem Biophys Res Commun. 2013; 435: 58-63.

210. Moch H, Humphrey PA, Ulbright TM, Reuter VE. World Health Organization (WHO) Classification of tumours of the urinary system and male genital organs. International Agency for Research on Cancer (IARC) Press. Lyon, France, 2016.

257. Randall E Harris – Epidemiology of chronic disease. Global Perspectives. Jones & Bartlett Learning, 2013.

183. Liu Y, Zeng S, Jiang X, et al. SOX4 induces tumor invasion by targeting EMT-related pathway in prostate cancer. Tumour Biol. 2017; 39(5):1010428317694539.

181. Linehan WM, Srinivasan Ramaprasad, Schmidt Laura S. The genetic basis of kidney cancer: a metabolic disease. Nat Rev Urol. 2010; 7(5): 277–285.

149. Kong FF, Qu ZQ, Yuan HH, et al. Overexpression of FOXM1 is associated with EMT and is a predictor of poor prognosis in non-small cell lung cancer. Oncol Rep.2014; 31: 2660-2668.

133. Kalluri R, Weinberg R.A. The basics of epithelial-mesenchymal transition. J Clin. Invest. 2009; 119(6): 1420–1428

107. Harada KI, Miyake H, Kusuda Y, et al. Expression of epithelial–mesenchymal transition markers in renal cell carcinoma: impact on prognostic outcomes in patients undergoing radical nephrectomy. BJU Int. 2012; 110(11c):E1131–7.

48. Frank I, Blute ML, Cheville JC, et al. An outcome prediction model for patients with clear cell renal cell carcinoma treated with radical nephrectomy based on tumor stage, size, grade and necrosis: the SSIGN score. J Urol. 2002; 168:2395–400.