RESEARCH ARTICLE

Expression and Prognostic Role of MEKK3 and pERK in Patients with Renal Clear Cell Carcinoma

Qi Chen[1], Hong-sheng Lu[2]*, Mei-fu Gan[3], Lan-xi Chen[2], Kai He[2], Guang-min Fan[2], Xue-quan Cao[2]

Abstract

Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 3 (MEKK3) is an important serine/threonine protein kinase and a member of the MAPK family. MEKK3 can effectively activate the MEK/ERK signaling pathway and promote an autocrine growth loop critical for tumor genesis, cell proliferation, terminal differentiation, apoptosis and survival. To explore the relationship between MEKK3 and cell apoptosis, clinicopathology and prognosis, we characterize the expression of MEKK3, pERK and FoxP3 in the renal clear cell carcinoma (RCCC). Protein expression was detected by tissue microarray and immunochemistry in 46 cases of RCCC and 28 control cases. Expression levels of CD3+ ,CD3+CD4+,CD3+CD8+,CD4+CD25+, CD4+CD25+ FoxP3+ were assessed by flow cytometry and analyzed for their association with pathological factors, correlation and prognosis in RCCC. Expression of MEKK3, pERK and FoxP3 was significantly up-regulated in RCCC as compared to control levels (p<0.01), associated with pathological grade (p<0.05)and clinical stage (p<0.05). CD4+CD25+ Foxp3+ Treg cells were also significantly increased in RCCC patients (p<0.05). Cox multivariate regression analysis showed that MEKK3, pERK expression and patholigical stage were independent prognostic factors in patients with RCCC (p<0.05). MEKK3 can be used as an important marker of early diagnosis and prognostic evaluation in RCCC. It may be associated with imbalance of anti-tumor immunity and overexpression of pERK. Expression of MEKK3 and pERK are significantly increased in RCCC, with protein expression and clinical stage acting as independent prognostic factors.

Keywords: MEKK3 - pERK - FoxP3 - renal clear cell carcinoma - prognosis

Introduction

Renal carcinoma is the most common cancer of the kidney and became a great threat to human's health. It accounts for roughly 80% of malignant kidney tumors. Among renal carcinomas, approximately 80% are classified as Renal clear cell carcinoma (RCCC) (Yoshimura et al., 2013). However, chemotherapy and radiotherapy treatment have limited utility for those patients with metastatic RCCC (Escudier et al., 2007). If caught early, tumors can be effectively removed surgically. So there is important clinical significance and prognosis for early diagnosis of RCCC, targeted therapy, and prognostic evaluation. But today, very little is known about the molecular and cellular mechanisms involved in the development of RCCC (Lu et al., 2014).

Genetic and epigenetic events are implicated in the development of human cancer and result in aberrantly activated signals pathways. Among these pathways, activation of the Ras/Raf /mitogen-activated and extracellular signal-regulated kinase kinase (MEK)/ extracellular signal-regulated kinase (ERK) signaling

cascade promotes an autocrine growth loop critical for tumor genesis, cell proliferation, terminal differentiation, apoptosis and survival (Young, 2013). Mitogen -activated protein kinase/extracellular signal-regulated kinase kinase kinase 3 (MEKK3) is a mitogen-activated protein kinase kinase kinase (MAP3K) family of serine/threonine protein kinases, and MEKK3 can effectively activate the MEK/ERK signaling pathway. Phosphorylated ERK1/2 (*p* ERK1/2) activates transcription factors that regulate gene expression involved in proliferation and survival. (CHEN, 2011).

The MEK/ ERK pathway is one of the best-characterize kinase cascades in cancer cell biology. It is triggered by either growth factors or activating mutations of major oncogenic proteins (Cindy Neuzillet, 2014),and it is closely associated with the occurrence and development of breast cancer (Kamal et al., 2013). Forkhead Box P3 (FoxP3) is a key transcription factor in regulatory T cells (Tregs) and relates to the tumor immune escape, High FOXP3 regulatory T-cell density in the sentinel lymph node is associated with downstream non-sentinel lymph-node metastasis in gastric cancer (Lee et al., 2011). The

*[1]Department of Clinical Laboratory, [2]Department of Pathology, Taizhou Central Hospital, Taizhou, [3]Department of Pathology, Taizhou Hospital, Linhai, Zhejiang, China *For correspondence: luhongsheng00@163.com*

expression of Foxp3 associated with the progression and metastasis of tumor (Wang et a1., 2012; Zeng et a1., 2013). The relationship between MEKK3 and pERK in RCCC is still unclear. These reports clearly emphasize the need for in-depth investigations of the relationship between MEK/ERK pathway and MEK/ ERK pathway in human cancers.

We analyzed the expression of MEKK3, pERK and FoxP3 by immunohisto- chemistry. Expression level of CD3+, CD3+CD4+,CD3+CD8+,CD4+CD25+, CD4+CD25+ FoxP3+ were detected by Flow cytometry. Analyzing protein of MEKK3, pERK and FoxP3 associated with pathological factors, correlation and prognosis. The aim is to explore the molecular pathogenesis of RCCC.

Materials and Methods

Patients were selected from Taizhou central Hospital between May 2007 and June 2014. The patient population included 28 men and 18 women, with an average age of 53.22±12.41 years (range 29 to 81 years). Cases were graded based on the 2004 Worlds Health Organization (WHO) pathological Fuhrman nuclear grading standards. In all, 21 cases were classified as grade I, 16 cases as grade II, 7 cases as grade III, and 2 cases as grade IV. According to 2004 WHO clinical staging standards, 23 RCCC patients were stage I, 15 were stage II, 5 were stage III, and 3 were stage IV. 28 healthy volunteers included 18 men and 10 women, in the corresponding period were set as the control group, with an average age of 55.31±10.30 years (range 27 to 75 years). Histopathological examination and immunohistochemistry staining were performed using cancer tissues from the 46 RCCC patients enrolled in the study. Paraffin-embedded RCCC tissues (46 cases) and normal renal tissues (28 cases) were retrieved and tissue micro- array slides were constructed according to a previously published method (Lu et al., 2010). The microarray contained 126 cases specimen in total including RCCC and CG.

Tissue microarray

Histopathological examination and immunohistochemistry staining were performed using cancer tissues from the 46 RCCC patients enrolled in the study. A manual tissue arrayer was used to punch 2.0-mm-diameter cylinders from each donor block and transfer them to the recipient paraffin block. 4μm-thick multiple sections were cut using a Leica RM 2165 fully motorized rotary microtome prepared for subsequent and immunohisto-chemical staining. Paraffin-embedded RCCC tissues (46 cases) and normal renal tissues (46 cases) were retrieved and tissue microarray slides were constructed according to a previously published method. The microarray contained 92 cases specimen in total including RCCC and control group (CG).

Monoclonal antibodies

Fluorochrome-conjugated mAb used were FITC-CD4 clone SK3, APC-CD25 clone 2A3, PE-FoxP3 clone 259D/C7, FITC- Mouse-IgG1 clone X40, PE- Mouse-IgG1 clone MOPC-21, APC-Mouse-IgG1 clone SJ25C1,CD3+-FITC/CD8+-PE/CD45+-PerCP/ CD4+-APC (SK3, 2D1,

SK1, SK7) (BD Biosciences, San Jose, CA).

Flow-cytometric analysis

Peripheral blood mononuclear cells in PBS with 5% heatinactivated fetal calf serum were then stained for the surface markers CD4 (FITC), CD25 (APC) and followed by intracellular staining for FoxP3 (clones 259D/C7) using a permeabilization kit (BD Biosciences), according to the manufacturer's instructions. another tube was added with CD3+-FITC/CD8+-PE/CD45+-PerCP/ CD4+ -APC (SK3, 2D1, SK1, SK7). Cells were washed twice in staining and analyzed immediately using a FACSCalibur cytometer and FACS software (BD Biosciences). Quadrants and box gates were set in reference to isotype controls, and percentages of the parent CD4+, CD25+, FoxP3+population were calculated.

Statistical analysis

The SPSS 17.0 statistical software package was used to analyze the data by independent sample t-test. Count data were analyzed by chi-square test. Survival rates were determined by the Kaplan-Meier method. Survival rates were compared between groups by using the Log-rank method, and the COX proportional hazard model was used for multivariate analysis. $p < 0.05$ was considered statistically significant.
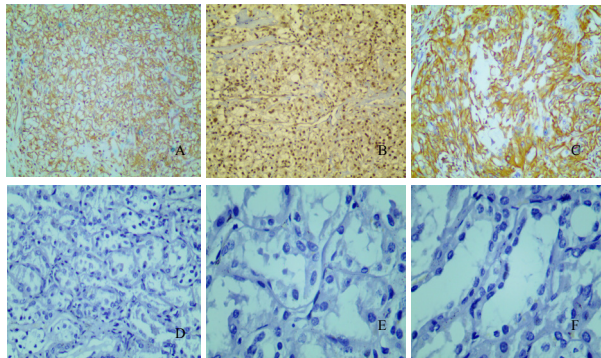
Results

Immunohistochemistry

Tissue chip staining was good and arranged in neat rows, and organizational structure to all of the sites maintained well. There was no obvious necrosis tissue (Figure 1).
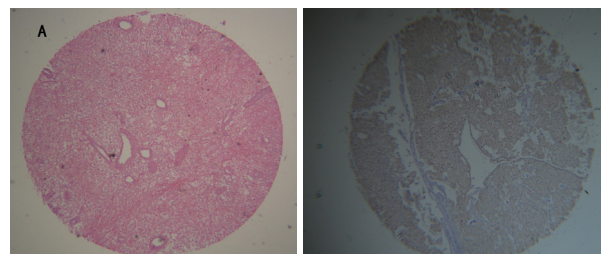
Figure 1. Tissue Microarray Site Map(HE×50)

Figure 2. The Expression of MEKK3 , pERK and FoxP3 in RCCC and CG (En Vision 400×). A) Positive expression of MEKK3 in RCCC; B) Positive expression of pERK in RCCC; C) Positive expression of FoxP3 in RCCC; D) Negative expression of MEKK3 in CG; E) Negative expression of pERK in CG; F) Negative expression of FoxP3 in CG

Brown cytoplasmic particles represented MEKK3 positivity (Figure 2A). pERK was located in the nucleus appearing as brown particles in Figure 2B. FoxP3 was located in the cytoplasm (Figure 2C). MEKK3, pERK and FoxP3 appeared negative expression in negative CG (Figure 2D, E, F).

In RCCC, the positive expression of MEKK3, pERK and FoxP3 were 69.6% (32/46), 73.9% (34/46) and 80.4% (37/46), respectively. In CG, the positive expression of MEKK3, survivin and stat3 were 7.1% (2/28), 3.6% (1/28) and 7.1% (2/28), respectively. Compared with RCCC, MEKK3, pERK and FoxP3 in CG showed a significantly lower percentage of positive staining (χ^2=37.11, $p<0.01$; χ^2=43.96, $p<0.01$; χ^2=10.27, $p<0.01$) (Table 1). These findings suggested that MEKK3, pERK and FoxP3 expression are significantly up-regulated in RCCC.

*MEKK3, pERK, FoxP3 and Clinicopathological Factors in Renal Clear Cell Carcinoma*

Positive association between MEKK3, pERK and FoxP3 expression with pathological grade (χ^2=19.28, $p<0.05$; χ^2=14.15, $p<0.05$; χ^2=8.68, $p<0.05$) and clinical stage (χ^2=14.16, $p<0.05$; χ^2=9.80, $p<0.05$; χ^2=7.71, $p<0.05$) were evident (Table 2).

Percentages of T cells, T cell subsets and Treg in the peripheral blood

We analyzed the levels of CD3+ T cells, CD3+CD4+ T cells and CD3+CD8+ T cells. The cells were analyzed by flow cytometry and the quantitative results were shown in Figure 3. Foxp3 was expressed on CD4+CD25+ Treg cells. The expression of a typical scatterplot of Foxp3 gated on CD4+ CD25+ T cells in representative patients and controls was shown in Figure 4. As shown in Table 3, No statistical difference of percentage of CD3+ T cells, CD3+CD4+ T cells and CD3+CD8+ T cells was found between RCCC and CG patients ($p>0.05$). CD4+CD25+ Treg cells were significantly increased in RCCC patients (10.9±1.7%) compared to CG (9.3±1.3%) ($p<0.05$). CD4+CD25+ Foxp3+ Treg cells were also significantly increased in RCCC patients (4.5±1.2%) compared to CG (3.2±0.5%)($p<0.05$).
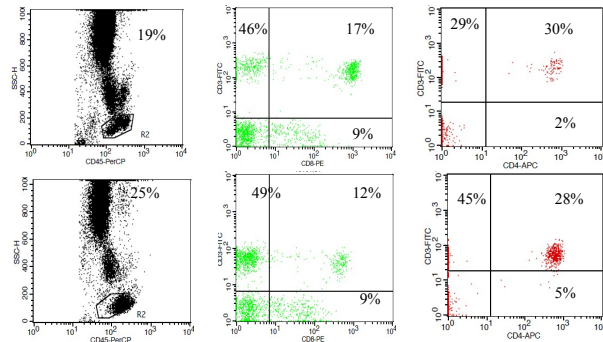
Figure 3. The Scatterplot of T Cells, T Cell Subsets. A) Percentages of Lymphocyte in RCCC; B) Percentages of CD3+ and CD4+ T cells in RCCC;C) Percentages of CD3+ and CD8+ T cells in RCCC; D) Percentages of Lymphocyte in CG;E) Percentages of CD3+ and CD4+ T cells in CG;F) Percentages of CD3+ and CD8+ T cells in CG
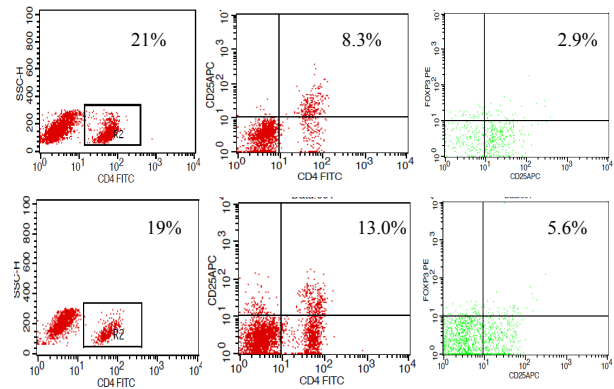
Figure 4. the scatterplot of Treg. A) Percentages of CD4+ T cells in RCCC; B) Percentages of CD4+ and CD25+ Treg in RCCC; C) Percentages of CD4+ CD25+ FoxP3+Treg in RCCC; D) Percentages of CD4+ T cells in CG; E) Percentages of CD4+ and CD25+ Treg in CG; F) Percentages of CD4+ CD25+ FoxP3+Treg in CG

Table 1. Expression of MEKK3, pERK and FoxP3 in RCCC and CG

Group	n	MEKK3		χ^2	P	pERK		χ^2	P	FoxP3		χ^2	P
		Positive cases	Positive rate(%)			Positive cases	Positive rate(%)			Positive cases	Positive rate(%)		
RCCC	46	32	69.6	37.11	0	34	73.9	43.96	0	37	80.4	10.27	0
CG	28	2	7.1		1	2	3.6			2	7.1		

Table 2. MEKK3, pERK, FoxP3 and Clinicopathological Factors in RCCC and CG

Cases	n	MEKK3		χ^2	P	pERK		χ^2	P	FoxP3		χ^2	P
		Positive cases	Positive rate(%)			Positive cases	Positive rate(%)			Positive cases	Positive rate(%)		
Pathological grade													
1	23	8	34.8	19.28	0	10	43.4	14.15	0	13	56.5	8.68	0.01
2	15	14	93.3			14	93.3			14	93.3		
3	5	5	100			5	100			5	100		
4	3	3	100			3	100			3	100		
Clinical stage													
1	21	9	42.9	14.16	0	11	52.4	9.8	0	13	61.9	7.71	0.01
2	16	14	87.5			14	87.5			15	93.8		
3	7	7	100			7	100			2	100		
4	2	2	100			2	100			2	100		

Table 3. Percentages of T cells ,T cell subsets and Treg in the peripheral blood in RCCC and CG

Group	CD3+	CD3+CD4+	CD3+CD8+	CD4+CD25+	CD4+ D25+FoxP3+
RCCC	61.7±6.6	34.5±6.1	25.3±5.5	10.9±1.7	4.5±1.2
CG	59.2±5.4	33.9±4.2	22.8±5.9	9.3±1.3	3.2±0.5
χ^2	0.83	3.05	0.38	4.8	29.62
P	0.37	0.09	0.54	0.03	0

Table 4. Multivariate Analysis of Factors Influencing Prognosis in RCCC

Variables	Wald	Exp(B)	95%CI	*P*
MEKK3	2.265	1.811	0.836~3.925	0.032
pERK	5.926	5.349	1.387~20.638	0.015
FoxP3	0.539	0.612	0.165~2.268	0.463
Patholigical grade	6.193	3.286	1.288~8.387	0.013
Clinical grade	1.23	1.497	0.734~3.054	0.267
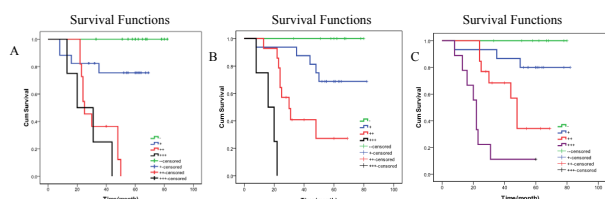
Figure 5. A) MEKK3 Expression in RCCC and Kaplan-Meier Survival Analysis; B) pERK Expression in RCCC and Kaplan-Meier Survival Analysis; C) FoxP3 Expression in RCCC and Kaplan-Meier Survival Analysis

Survival analysis

Univariate analysis showed that MEKK3, pERK and FoxP3 expression, pathological grade and clinical stage were associated with prognosis ($p<0.05$), In univariate, the prognostic factors of $p<0.05$ was put into the COX proportional hazard model analysis. MEKK3, pERK expression and patholigical stage were independent prognostic factors of patients with RCCC (Table 4, Figure 5).

Discussion

The MEK/ERK pathway plays a role in all known biological functions acquired during the multistep development of human tumors (Hanahan andWeinberg, 2011). Deregulation of the MEK /ERK pathway is observed in several cancers and yields multiple changes in the expression of numerous genes involved in tumor cell differentiation, proliferation, survival, migration, and angiogenesis (McCubrey et al., 2007; Cao et al., 2014). MEKK3 is an important protein kinase and a member of the MEK family, it is a cytoplasmic protein expressed in many mammalian tissues. It encoded on human chromosome 17q24, and is a 71-kDa protein (Blank et al., 1996). MEKK3 could specifically combine basic structure in the front with PB1 acidic cluster domain of tail, connecting the tumor necrosis factor receptor associated factor 6 (TRAF6), composed of TRAF6-p62-MEKK3 complexes, participating in IKK complex ubiquitin process and activate the NF-ϰB pathway. (Craig et al., 2008). ERK1/2 is an important member in the mitogen-activated protein kinase (MAPK) family. One mechanism by which the ERK1/2 pathway increases global protein translation is through phosphorylation (Christophe et al., 2010). Phosphorylated ERK1/2 (pERK1/2) is the activated form of ERK1/2, and take part in the regulation of various physiological and pathological pathways such as cell differentiation, proliferation, and apoptosis.

Compared with RCCC, MEKK3, pERK and FoxP3 in CG showed a significantly lower percentage of positive staining ($\chi^2=37.11$, $p<0.01$; $\chi^2=43.96$, $p<0.01$; $\chi^2=10.27$, $p<0.01$). Immunohistochemistry showed that MEKK3, pERK and FoxP3 expression was significantly higher in RCCC than in CG, which is consistent with previous findings (Hasan et al., 2014). We also found that expression of MEKK3, pERK and FoxP3 was associated with pathological grade and clinical stage, in which positive association between MEKK3, pERK and FoxP3 expression with pathological grade ($\chi^2=19.28$, $p<0.05$; $\chi^2=14.15$, $p<0.05$; $\chi^2=8.68$, $p<0.05$) and clinical stage ($\chi^2=14.16$, $p<0.05$; $\chi^2=9.80$, $p<0.05$; $\chi^2=7.71$, $p<0.05$) were evident.

The percentages of T cells and T cell subsets in the peripheral blood indicate the functional status of the immune system. Treg play significant roles in the development and maintenance of immune responses. Shinohara et al.(2009) found when MEKK3 was knocked out the quantity of peripheral T cells were reduced. MEKK3 regulated T cell development via IKK-induced activation of NF-ϰB . MEKK3 is essential for lymphopenia induced T cell proliferation and survival, The exact underlying mechanism for this is not fully understood (wang et al.2009). We found T cells and T cell subsets were no statistical difference between RCCC and CG patients ($p>0.05$). However, we showed CD4+CD25+ Treg cells were significantly increased in RCCC patients (10.9±1.7%) compared to CG (9.3±1.3%) ($p<0.05$). CD4+CD25+ Foxp3+ Treg cells were also significantly increased in RCCC patients (4.5±1.2%) compared to CG (3.2±0.5%) ($p<0.05$). Tregs are important mediators of peripheral immune tolerance, acting via multiple mechanisms to suppress cellular immunity including antitumor responses. We suspect RCCC patients have an imbalance of anti-tumor immunity, and it may be associated with the over- expression of MEKK3.

Univariate analysis showed that MEKK3, pERK and FoxP3 expression, pathological grade and clinical stage were associated with prognosis ($p<0.05$), In univariate, the prognostic factors of $p<0.05$ was put into the COX

proportional hazard model analysis. MEKK3 and pERK expression and patholigical stage were independent prognostic factors of patients with RCCC.

In summary, MEKK3 and pERK expression was closely associated with the occur- rence, development, and prognosis of RCCC, and can be used as an important marker of early diagnosis and prognostic evaluation. It may be associated with imbalance of anti- tumor immunity.Targeted therapy of MEKK3 combined with apoptosis-promoting therapy may provide a new strategy for treatment of chemotherapeutic-resistant tumors. Our study may help to clarify the molecular mechanisms involved in the pathogenesis of RCCC.

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