

RESEARCH ARTICLE

MEKK3 and Survivin Expression in Cervical Cancer: Association with Clinicopathological Factors and PrognosisXue-Quan Cao¹, Hong-Sheng Lu^{1*}, Ling Zhang², Li-Li Chen¹, Mei-Fu Gan³**Abstract**

Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 3 (MEKK3) is an important protein kinase and a member of the MAPK family, which regulates cellular responses to environmental stress and serves as key integration points along the signal transduction cascade that not only link diverse extracellular stimuli to subsequent signaling molecules but also amplify the initiating signals to ultimately activate effector molecules and induce cell proliferation, differentiation and survival. To explore the relationship between MEKK3 and cell apoptosis, clinicopathology and prognosis, we characterize the expression of MEKK3 and survivin in cervical cancer. MEKK3 and survivin expression was measured by RT-PCR and Western blotting of fresh surgical resections from 30 cases of cervical cancer and 25 cases of chronic cervicitis. Protein expression was detected by tissue microarray and immunohistochemistry (En Vision) in 107 cases of cervical cancer, 86 cases of cervical intraepithelial neoplasia (CIN), and 35 cases of chronic cervicitis. Expression patterns were analyzed for their association with clinicopathological factors and prognosis in cervical cancer. Expression of MEKK3 and survivin mRNA was significantly higher in cervical cancer than in the controls ($p < 0.05$). MEKK3 and survivin expression differed significantly between cervical carcinoma, CIN, and cervicitis ($p < 0.05$) and correlated with clinical stage, infiltration depth, and lymph node metastasis ($p < 0.05$). MEKK3 expression was positively correlated with survivin ($p < 0.05$). Kaplan-Meier survival analysis showed that MEKK3 and survivin expression, lymph node metastasis, depth of invasion, and FIGO stage reduce cumulative survival. Cox multivariate regression analysis showed that MEKK3, survivin, and clinical staging are independent prognostic factors in cervical cancer ($p < 0.05$). Expression of MEKK3 and survivin are significantly increased in cervical cancer, their overexpression participating in the occurrence and development of cervical cancer, with protein expression and clinical staging acting as independent prognostic factors for patients with cervical cancer.

Keywords: MEKK3 - survivin - cervical cancer - prognosis

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Introduction

Cervical cancer is the second common malignancy in women, implicated in 30 million deaths each year; it is one of the leading causes of death in women in developing countries (Karimi et al., 2009; Caffarel et al., 2014). The recurrence rate in patients with advanced cervical cancer is high and 60% of patients develop chemotherapeutic resistance (Cadron et al., 2007), thus reducing survival. There is important clinical significance and prognosis for early diagnosis of cervical cancer, targeted therapy, and prognostic evaluation.

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 that regulate early embryonic development of the cardiovascular system, a variety of inflammatory and immune responses, and

cytokines via the growth factor-induced signaling pathway (Yang et al., 2001; Xu et al., 2004). Protein kinases activate mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF- κ B) signaling pathways, and are thus closely associated with the occurrence and development of a variety of tumors including breast (Samanta et al., 2004) and esophageal cancer (Kumar et al., 2007). High expression of MEKK3 is accompanied by increased activity of IKK and NF- κ B and of chemotherapy drug resistance factors, including Bcl-2, Bcl-X, survivin, and XIAP. Survivin is the smallest member of the inhibitor of apoptosis protein family with cell periodicity and is expressed only in G2/M phase (Kitamura et al., 2006; Srivastava et al., 2012). Survivin BIR function by binding with mitotic microtubules influences the activity of Caspase-3 of most downstream effector molecule during apoptosis and inhibition of apoptosis (Wand et al., 2004; Karami et al., 2013). Increased expression of survivin

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is associated with the occurrence and development of cervical cancer and is expected to become a molecular marker for early diagnosis and prognosis in cervical cancer (Lu et al., 2010; Wu et al., 2012).

It is unclear whether there is abnormal expression of MEKK3 in cervical cancer or if there is a relationship between MEKK3 expression and the occurrence or development of cervical cancer. The relationship between MEKK3 and survivin in cervical cancer is also unclear. We analyzed the expression of MEKK3 and survivin in cervical cancer and chronic cervicitis tissues by RT-PCR and western blotting. Expression in cervical cancer, CIN, and chronic cervicitis was detected by immunohistochemistry and analyzed in the context of, analyzing the expression of MEKK3 and survivin in cervical cancer and their relationship with clinicopathological factors and prognosis and correlation between the expression of MEKK3 and survivin, the aim is to explore the molecular pathogenesis of cervical cancer.

Materials and Methods

Patients

We collected specimens from 193 cases of cervical intraepithelial lesions including 86 cases of CIN (28 cases of CIN I, 26 cases of CIN II, and 32 cases of CIN III) and 107 cases of cervical cancer (81 cases of squamous cancer and 26 cases of adenocarcinoma) from Taizhou Hospital from February 2003 to May 2007. For controls, we collected specimens from 35 cases of chronic cervicitis. Being confirmed by pathological diagnosis, of 107 cervical cancer cases, the mean age was 43.5 years old (27-71 years old). The cervical cancer patients had not received chemotherapy, radiotherapy, or immunotherapy prior to surgery. Pathological differentiation of squamous cancer revealed cervical cancer grades as follows: high differentiation (22 cases), middle differentiation (43 cases) and low differentiation (16 cases). Clinical pathological staging (FIGO, 2000) revealed 37 cases of stage I, 44 cases of stage II, and 26 cases of stage III or IV. Stromal infiltration depths were as follows: 28 cases had no infiltration beyond the superficial myometrium and 79 cases had infiltration beyond the myometrium. Lymph node metastasis had occurred in 26 cases; 81 cases had no metastasis. Follow-up was performed through telephone calls or letters. Survival time was defined as the time from diagnosis to death or to the final examination. Postoperative follow-up lasted for over three years: 72 cases were followed-up for more than one year and 44 cases for more than three years, including 28 cases of survival and 16 cases of death due to tumor recurrence and/or metastasis.

Thirty cases of cervical cancer, including 28 cases of squamous cancer and 2 cases of adenocarcinoma, were obtained from Taizhou Central Hospital and Taizhou Hospital from August 2012 to December 2013. Mean patient age was 45.7 years old (35-80 years old). In the control group, the mean age was 40.1 years old (32-45 years old). All samples were cervical specimens obtained during total resection hysterectomy for uterine leiomyoma or adenomyosis, and chronic cervicitis was confirmed

by pathological diagnosis. All specimens were quickly frozen at -80°C for 30 min prior to use for RT-PCR and western blotting. The study was approved by Taizhou Central Hospital and Taizhou Hospital Ethics Committee and prior informed consent was obtained from all patients and family members.

Tissue microarray

We collected 107 cases of cervical cancer, 86 cases of CIN, and 35 cases of chronic cervicitis for tissue microarrays. We reviewed all hematoxylin- and eosin- (H&E) stained slides and selected the most representative areas of cervical cancer, CIN and chronic cervicitis samples. 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 from the TMA using a Leica RM2165 fully motorized rotary microtome prepared for subsequent and H&E and immunohistochemical staining.

Quantitative PCR

Preserved specimens from 30 cases of cervical cancer and 25 cases of chronic cervicitis were used; each specimen weighed about 50 to 100 mg. For quantitative RT-PCR, total RNA was isolated using the RNeasy Mini Kit, cDNA was synthesized using random primer and SuperScript II. The genes were amplified with the Power SybrGreen PCR Master (Mix Becton Dickinson, USA) according to the manufacturer's instructions. Gene expression was quantified by the comparative cT-Method, normalizing cT-values to a housekeeping gene (GAPDH) and calculating the relative expression values. The sequences of the primers were as follow: MEKK3: 5'-AATGTGCCAACCAAGTCTCC-3' (forward) and 5'-TCCAGAGCACTCACCTCCTT-3' (reverse); survivin: 5'-GTGAACGGATACCTCTCTATATGCTG-3' (forward) and 5'-CTGACTATCACCGTTACCAGAAC TG-3' (reverse); and GAPDH: 5'-GCTCACCATGGATG ATGATATC-3' (forward) and 5'-GCCAGATTTTCTCCAT GTCGTC-3' (reverse) (all from Jierui Biological Engineering, Shanghai). Independent experiments were repeated three times and results were determined by the relative quantitation method ($\Delta\Delta Ct$).

Western blotting

Proteins were extracted from tissues of 30 cases of cervical cancer and 25 cases of chronic cervicitis. Proteins were electrophoresed under reducing conditions on 4-12% acrylamide gels and then transferred to a nitrocellulose membrane. To block nonspecific binding, the membrane was incubated with 2% nonfat dry milk in Tris-buffered saline at room temperature for 1 h. Subsequently, the membrane was either incubated with primary antibody to MEKK3 (1:1000, Ab40756, abcam, Cambridge, UK), Survivin (1:1000, NB 500-201, Novus Biologicals, Acris Antibodies, Hiddenhausen, Germany) and GAPDH (1:1000, 10494-1-AP, protein tech, USA) in the blocking buffer overnight at 4°C. Then, the membrane was incubated in horseradish peroxidase-conjugated goat anti-mouse IgG at room temperature for 1.5 h. The protein was visualized using the ECL detection kit.

Immunohistochemical method

Baking slices for two hours, dewaxed to water conventionally. Hydrogen peroxide (3%) was added to inactivate endogenous peroxidase at room temperature for 10 min, 0.1 mmol/L citrate buffer for antigen retrieval, 3% BSA closed for 30 min, primary antibodies were added (MEKK3 and survivin antibodies were diluted 1:100) at 4°C overnight. The secondary antibody was added with conventional DAB color and hematoxylin, and samples were mounted and observed under a light microscope. PBS was substituted for primary antibody as a negative control, and known positive slips were used as positive controls. Brown cytoplasmic particles represented MEKK3-positive staining. Survivin was mainly located in the cytoplasm, and occasionally the nucleus. At least five high-power fields were analyzed and ≥ 1000 cells were counted; $< 10\%$ positive cells was defined as negative and $\geq 10\%$ was defined as positive.

Statistical Analysis

The SPSS 13.0 statistical software package was used to analyze the data by independent sample t-test; count data were analyzed by chi-square test, correlations between MEKK3 and survivin protein expression were determined by Spearman's rank correlation analysis. 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

Real-time PCR determination of MEKK3 and survivin expression

Amplification and melting curve analysis of MEKK3, survivin, and the GAPDH reference yielded threshold cycle numbers (Ct) between 18 and 30. Amplification curve was smooth, fluorescence absorption spectrum of the s-shaped curve shaped well, and had reached a plateau. The melting curves were unimodal and specific, with peaks was at 79.87°C, 81.35°C, and 80.67°C,

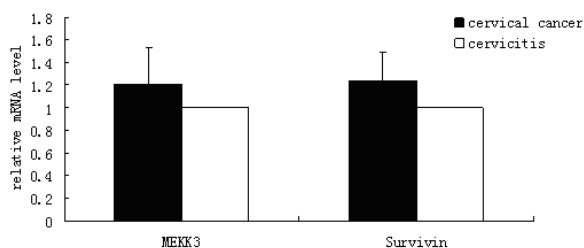


Figure 1. Relative Expression of MEKK3 and Survivin mRNA in Cervical Cancer and Chronic Cervicitis

respectively, indicating that primer specificity is sufficient for quantitative detection. Therefore, this system can be used for quantitative analysis of MEKK3 and survivin expression.

Transcript expression of MEKK3 and survivin in cervical cancer and chronic cervicitis

There was the expression level of MEKK3, survivin mRNA in cervical cancer and chronic cervicitis, the expression in cervical cancer was significantly increased. Relative expression of MEKK3 and survivin in cervical cancer was 1.2083 ± 0.3215 and 1.2395 ± 0.2571 , respectively, significantly higher than in the controls ($t=3.229, p < 0.05$; $t=4.638, p < 0.05$; Figure 1).

MEKK3 and survivin protein expression in cervical cancer and chronic cervicitis western blotting

Expression levels of MEKK3 and survivin in cervical cancer were 1.015 ± 0.148 and 1.047 ± 0.382 ; in chronic cervicitis, the levels were 0.881 ± 0.040 and 0.563 ± 0.036 . Expression of MEKK3 and survivin was significantly higher in cervical cancer than in controls ($t=4.389, p < 0.05$; $t=6.302, p < 0.05$; Figure 2).

Immunohistochemistry

Brown cytoplasmic particles represented MEKK3 positivity (Figure 3). Survivin was mainly located in the cytoplasm and occasionally in the nucleus, appearing as brown particles in Figure 4. Expression rates of MEKK3 protein in 81 cases of cervical squamous cancer, 86 cases of CIN, and 35 cases of chronic cervicitis were 75.3% (61/81), 47.7% (41/86), and 11.4% (4/35), respectively. Survivin protein expression rates were 88.9% (72/81), 48.8% (42/86), and 0.0% (0/35) in the same groups. The differences between groups were significant ($p < 0.05$; Table 1).

MEKK3, survivin, and clinicopathological factors in cervical cancer

The expression of MEKK3 was significantly associated with FIGO staging, invasive depth, and lymph node metastasis ($p < 0.05$); MEKK3 expression varied with age, histological type, and classification, although

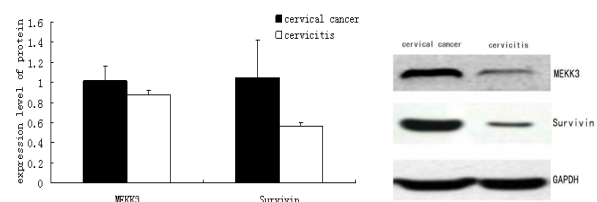


Figure 2. Expression of MEKK3 and Survivin Protein was Detected in Cervical Cancer and Chronic Cervicitis by Western Blotting (means \pm SD)

Table 1. Expression of Mekk3 and Survivin in Cervical Cancer, Cin, and Chronic Cervicitis

Cervical lesions	n	MEKK3		χ^2	P	Survivin		χ^2	P
		Positive cases	Positive rate(%)			Positive cases	Positive rate(%)		
Cervicitis	35	4	11.4			0	0		
CIN	86	41	47.7			42	48.8		
Squamous cancer	81	61	75.3	41.374	0	72	88.9	82.059	0

Table 2. MEKK3, Survivin, and Clinicopathological Factors in Cervical Cancer

Clinicopathological factors		n	MEKK3		χ^2	P	Survivin		χ^2	P
			Positive cases	Positive rate(%)			Positive cases	Positive rate(%)		
Age(years)	≤35	27	18	66.7	0.502	0.479	23	85.2	0.031	0.86
	>35	80	59	73.7			67	83.8		
Histological type	Squamous cancer	81	61	75.3	1.85	0.174	71	87.7	3.13	0.077
	adenocarcinoma	26	16	61.5			19	73.1		
Classification (squamous cancer)	I	22	14	63.6	2.243	0.326	18	81.8	1.639	0.441
	II	43	34	79.1			39	84.8		
	III	16	13	81.3			15	93.8		
FIGO staging	I	37	21	56.8	11.831	0.003	26	70.3	8.8	0.012
	II	44	31	70.5			39	88.6		
	III+IV	26	25	96.2			25	96.2		
Invasive depth	≤1/2	28	15	53.6	6.358	0.012	19	67.9	7.498	0.006
	>1/2	79	62	78.5			71	89.9		
Lymph node metastasis	yes	24	22	91.7	5.954	0.015	24	79.5	5.844	0.016
	no	83	55	66.3			66	100		

Table 3. Multivariate Analysis of Factors Influencing Prognosis in Cervical Cancer

Variables	Wald	Exp (B)	95%CI	P
Invasive depth	0.011	0.894	0.114~7.042	0.915
Lymph node metastasis	0.497	0.055	0.390~7.392	0.497
FIGO staging	6.088	0.046	0.004~0.531	0.014
Expression of MEKK3	4.876	0.044	0.03~0.703	0.027
Expression of survivin	5.799	0.016	0.005~0.582	0.016

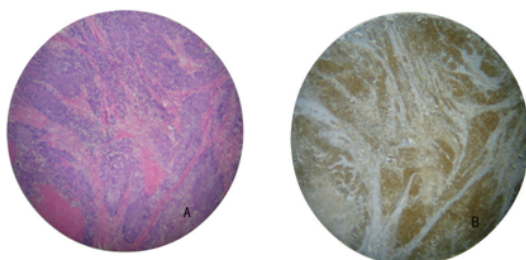


Figure 3. Tissue Microarray Site Map (HE x50)

the relationship was not significant ($p < 0.05$). Survivin expression correlated with clinical staging, invasive depth, and lymph node metastasis in cervical cancer ($p < 0.05$), but was not associated with age, histological type, or classification ($p > 0.05$; Table 2).

The correlation between MEKK3 and survivin expression

In 107 cases of cervical cancer, 70 were positive for expression of MEKK3 and survivin in 70 cases; 10 patients were negative for both; there was a positive correlation between MEKK3 and survivin expression ($r_s = 0.298, p = 0.002$).

Survival analysis

Univariate analysis showed that MEKK3 and survivin expression, lymph node metastasis, depth of invasion, and clinical stage were associated with prognosis ($p > 0.05$); the three-year cumulative survival rate was 48.27% for patients with positive MEKK3 expression and 93.33% for MEKK3-negative patients (Log-rank test, $\chi^2 = 8.40, p = 0.046$; Figure 5). The three-year cumulative survival rate for patients with positive expression of survivin was 46.67%, versus survivin-negative patients, whose survival

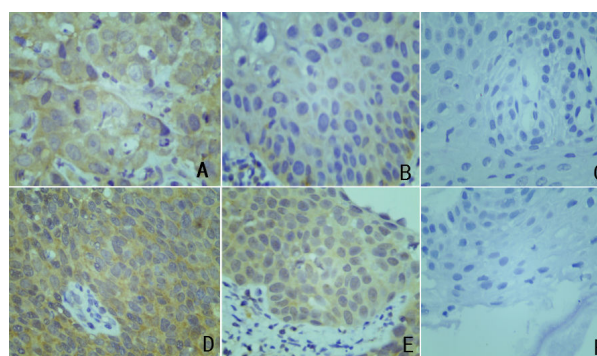


Figure 4. The Expression of MEKK3 and Survivin in Cervical Cancer, CIN, and Chronic Cervicitis (EnVision 400x). A) Positive expression of MEKK3 in cervical cancer; B) Positive expression of MEKK3 in CIN; C) Negative expression of MEKK3 in chronic cervicitis; D) Positive expression of survivin in cervical cancer; E) Positive expression of survivin in CIN; F) Negative expression of survivin in cervical cancer

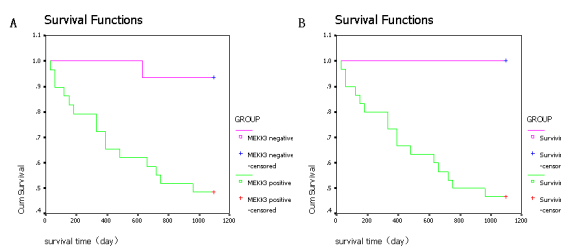


Figure 5. A MEKK3 Expression in Cervical Cancer and Kaplan-Meier Survival Analysis; 5B Survivin Expression in Cervical Cancer and Kaplan-Meier Survival Analysis

rate was 100% (Log-rank test, $\chi^2 = 10.36, p = 0.001$). Figure 6. In univariate, the prognostic factors of $p < 0.05$ was put into the COX proportional hazard model analysis, MEKK3 and survivin expression and FIGO stage were independent prognostic factors of patients with cervical cancer. Table 3.

Discussion

MAPKs and stress-activated protein kinases function regulate cellular responses to environmental stress. They serve as key integration points along the signal

transduction cascade that not only link diverse extracellular stimuli to subsequent signaling molecules but also amplify the initiating signals to ultimately activate the effector molecules and induce cell proliferation, differentiation and survival (Chang et al., 2001; Johnson et al., 2002). MEKK3 is an important protein kinase and a member of the MAPK family. It is a cytoplasmic protein expressed in many mammalian tissues. It is 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- κ B pathway. MEFs deficient for MEKK3 exhibit inhibition of LPA IKK-induced activation of NF- κ B and express MIP-2 and IL-6 (Sun et al., 2009; Kazuhiro et al., 2010). Deng et al (Deng et al., 2007) found that MEKK3 participates in the Ang1/Tie2 signaling pathways, which regulate epithelial cell proliferation and survival. We showed that MEKK3 transcript and protein were significantly higher in cervical cancer than in chronic cervicitis. Immunohistochemistry showed that MEKK3 expression was significantly higher in cervical cancer than in CIN and chronic cervicitis. MEKK3 may activate MAPKs and NF- κ B signaling including JNK, p38, and ERK5 (Johnson et al., 2002; Craig et al., 2008), thus participating in cervical epithelial carcinogenesis. Expression of MEKK3 was associated with clinical staging, invasive depth, and lymph node metastasis. *In vitro* experiments confirmed that using siRNA technology to silence MEKK3 can make expression levels of MEKK3 decline by 5% in mice embryonic cells with osteosarcoma, at the same time observing ability of invasion and metastasis of osteosarcoma cell to decrease markedly (Yu et al., 2006).

Survivin, a member of the inhibitors of apoptosis protein (IAP) family, regulates cell division (Li et al., 1998; LaCasse et al., 2008). Dysregulation of the survivin pathway may mediate initiation of malignant transformation and maintenance of the malignant phenotype of established tumors (Altieri et al., 2003). Survivin is encoded on chromosome 17q25 and is a 142-amino acid protein with a unique BIR domain. Survivin expression is low in normal adult tissue, with the exception of the thymus and embryonic organization (Gho et al., 2011), but is strongly expressed in several types of human cancer, including colon carcinoma (Hasan et al., 2014), breast carcinoma (Bongiovanni et al., 2014), and urothelial carcinoma (Chen et al., 2014). In this study, western blotting and real-time PCR analysis of fresh tissue showed significantly increased survivin expression in cancer versus chronic cervicitis, consistent with previous findings. Immunohistochemistry of 81 cases of cervical cancer, 86 cases of CIN, and 35 cases of chronic cervicitis tissues revealed 0.0%, 48.8%, and 88.9% survivin expression, suggesting that survivin specifically inhibits the activity of the most downstream effective molecule Caspase-3 and apoptosis (Wand et al., 2004); these differences may be used to predict the course of intraepithelial cervical lesions. Indarti et al.

(2013) found that determination of survivin expression by immunocytochemistry staining, along with other significant risk factors, can be used in a scoring system to predict the progressivity of CIN lesions. Application of this scoring system may be beneficial in determining the action of therapy towards the patient. We also found that survivin expression correlated closely with invasive depth, lymph node metastasis, and clinical progress. Overexpression of survivin may inhibit apoptosis of cervical cancer cells and enhance local invasion and distant metastasis of survivin-induced cancer cells by regulating MMP-7 expression (Gao et al., 2014).

Univariate analysis showed that MEKK3 and survivin expression, lymph node metastasis, depth of invasion, and clinical stage were associated with prognosis, in which three-year cumulative survival was 48.27% for patients expressing MEKK3 and 93.33% for those who were MEKK3-negative (Log-rank test, $\chi^2=8.40$, $p=0.046$). The three-year cumulative survival rate for patients positive for survivin expression 100% versus those who were survivin-negative (46.67%, log-rank test, $\chi^2=10.36$, $p=0.001$). Prognostic factors with $p<0.05$ were entered into a COX proportional hazard model; MEKK3 and survivin expression as well as FIGO stage were independent prognostic factors in patients with cervical cancer. This is consistent with a previous study in small cell lung cancer. Another study showed a combination of MEKK3 overexpression and node positivity is an important predictor of reduced disease-free survival and poor prognosis in ESCC (Hasan et al., 2014).

In the stable cell lines U373, Hep3B, and HEK293, MEKK3 is overexpression is associated with elevated NF- κ B binding activity; the cells were more responsive to cytokine stimulation and showed 2-4-fold higher basal expression of Bcl-2 and xIAP than the parental cells (Samanta et al., 2004). In this study, spearman rank correlation analysis revealed a positive correlation between MEKK3 and survivin expression. Thus, up-regulation of MEKK3 may activate NF- κ B and other signaling pathways, thus inducing antiapoptotic factors such as surviving (Samanta et al., 2009) and promoting the occurrence, infiltration, and metastasis of cervical cancer.

In summary, MEKK3 and survivin expression was closely associated with the occurrence, development, and prognosis of cervical cancer, and can be used as an important marker of early diagnosis and prognostic evaluation. Targeted therapy of MEKK3 combined with apoptosis-promoting therapy may provide a new strategy for treatment of chemotherapeutic-resistant tumors.

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