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Original Article

NEDD9 expression is correlated with epithelial-to-mesenchymal transition markers in colorectal cancer

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Abstract: The epithelial-to-mesenchymal transition (EMT) is a critical step in tumor metastasis. NEDD9 has been shown to be an oncogene in colorectal cancer. However, little is known about the relationship between NEDD9 and EMT in colorectal cancer metastasis. A total of 63 pairs of freshly frozen colorectal cancer tissues and adjacent noncancerous tissues were evaluated for NEDD9 gene expression using quantitative real-time PCR. The expression of NEDD9 and three epithelial-mesenchymal transition (EMT)-related proteins (E-cadherin, β-catenin and vimentin) was examined in 122 colorectal cancers by immunohistochemistry. The expression of NEDD9 was markedly increased in colorectal cancer tissues compared with adjacent noncancerous tissues. The expression level of NEDD9 was positively correlated and TNM stage but not with other clinicopathological features of colorectal tumors. Furthermore, the expression of NEDD9 was strongly associated with the loss of epithelial marker E-cadherin and acquired expression of the mesenchymal markers nuclear β-catenin and vimentin. These findings suggested that NEDD9 might promote EMT and the progression of colorectal cancer, and thus may be a potential therapeutic target of colorectal cancers.

Keywords: NEDD9, EMT, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and second in women globally, and causes the fourth most cancer-related deaths [1]. Despite the substantial progress achieved in diagnosis and treatment for CRC in recent years, the overall 5-year survival rate remains unsatisfactory due to metastasis [2]. Therefore, it is imperative to identify novel molecular markers or factors for the early detection before distant metastasis appears and for prognosis of colorectal cancer.

NEDD9 (neural precursor cell expressed developmentally down-regulated 9, also known as HEF1, human enhancer of filamentation 1 and Cas-L, Crk-associated substrate lymphocyte type) was initially identified by its developmentally regulated expression pattern in early embryonic, but not adult [3-5]. NEDD9 plays important roles in cell cycle regulation and cell survival [6]. Overexpression of NEDD9 has also been identified in several human malignancies including breast cancer, melanoma, glioblastoma, head and neck squamous cell carcinoma (HNSCC), lung cancer, liver cancer, cervical cancer, gastric cancer, and pancreatic cancer [7-19]. Overexpression of NEDD9 has been found to promote tumor progression [16-19]. NEDD9 was recently reported to play a role in the progression and may serve as a novel prognostic marker for colorectal cancer [20]. It is believed that NEDD9 regulates cell adhesion, migration, invasion, and epithelial-mesenchymal transition (EMT) [21-24].

Although it has been demonstrated that NEDD9 plays an important role in the development of

Table 1. Relationship between NEDD9 expression levels and clinicopathological parameters of colorectal cancer samples

Variable	Numbers	NEDD9 relative expression (mean ± SEM*)	*P* value
Age (years)			0.62
≤ 65	30	0.53 ± 0.21	
> 65	33	0.59 ± 0.25	
Gender			0.55
Male	34	0.48 ± 0.19	
Female	29	0.52 ± 0.27	
Tumour site			0.47
Rectum	28	0.54 ± 0.19	
Colon	35	0.55 ± 0.26	
Tumour histology			0.16
Adenocarcinoma	44	0.47 ± 0.28	
Mucinous adenocarcinoma	19	0.58 ± 0.23	
Tumour differentiation			0.012
Well/Moderate	38	0.21 ± 0.17	
Poor	25	0.85 ± 0.36	
TNM stage			0.004
II	37	0.18 ± 0.09	
III	26	0.93 ± 0.34	
Lymph vascular invasion			0.07
Absence	33	0.36 ± 0.22	
Presence	30	0.62 ± 0.28	

*SEM: Standard error of mean.

colorectal cancer [20], its association with EMT in colorectal cancer has yet to be clarified. This study analyzed the expression of NEDD9 in archived colorectal cancer tissue samples using quantitative polymerase chain reaction or immunohistochemistry as well as the relationship between NEDD9 expression and levels of three EMT markers in colorectal cancer samples.

Materials and methods

Patients and tissues samples

This study was approved by the Research Ethics Committee of The Third Hospital Affiliated to Nantong University. Written informed consent was obtained from all of the patients. All specimens were made anonymous and handled according to the ethical and legal standards.

For qRT-PCR, we collected 63 pairs of fresh colorectal cancer and matched adjacent normal tissue specimens from patients who underwent surgery between October 2011 and April 2012 in The Third Hospital Affiliated to Nantong University. In particular, adjacent normal tissues were taken 5-10 cm away from the tumor tissues. All specimens were immediately frozen in liquid nitrogen after surgery and stored at -80°C until RNA extraction. No patients received chemotherapy or radiotherapy prior to operation. Clinical information of these patients was summarized in **Table 1**.

For immunohistochemical assays, archived and paraffin-embedded colorectal cancer tissues and adjacent noncancerous tissues were collected from 122 patients who underwent surgery between 2001 and 2008 in The Third Hospital Affiliated to Nantong University. The cases selected were based on distinctive pathologic diagnosis of colorectal cancer, undergoing curative resection for tumor without preoperative chemotherapy or radiotherapy. The clinicopathological characteristics of these 122 patients were summarized in **Table 2**.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from colorectal cancer and adjacent noncancerous tissues using Qiazol reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The integrity of RNA was verified by electrophoresis on a 1% agarose gel stained with ethidium bromide. The quality and quantity of RNA were determined by ultraviolet spectroscopy. cDNA was synthesized using random hexamer primers and a Fermentas M-MLV reverse transcriptase (ThermoFisher, Waltham, MA, USA).

Quantitative real-time PCR

The expression level of NEDD9 gene was determined by quantitative real-time RT-PCR with β-actin as the internal control. The primers for the target gene were as follows: 5'-CGGGACCTTCTCGCTTTCATC-3' and 5'-GGTGGGTTGAGCCGTTTTCC-3' for NEDD9 and 5'-CGTCTTCCCCTCCATCGT-3' and 5'-GCCTCGTCGCCCACATAG-3' for β-actin. PCR was performed using Maxima™ SYBR Green/ROX qPCR Master MIX

Table 2. Correlation of NEDD9 expression with clinicopathological parameters

Variable	No. of patients	NEDD9 expression		*P* value
		Low	High	
Age (years)				
≤ 65	57	27	30	0.518
> 50	65	27	38	
Gender				
Male	48	23	25	0.513
Female	74	31	43	
Tumour site				
Rectum	49	25	24	0.218
Colon	73	29	44	
Tumour histology				
Adenocarcinoma	89	40	49	0.803
Mucinous adenocarcinoma	33	14	19	
Tumour differentiation				
Well/Moderate	76	40	36	0.017
Poor	46	14	32	
TNM stage				
II	67	37	30	0.007
III	55	17	38	
Lymph vascular invasion				
Absence	63	32	31	0.133
Presence	59	22	37	
E-cadherin				
Positive	56	31	25	0.023
Negative	66	23	43	
β-catenin				
Positive	51	17	34	0.039
Negative	71	37	34	
Vimentin				
Positive	48	15	33	0.002
Negative	74	39	35	

(ThermoFisher) on an Applied Biosystems StepOnePlus™ instrument. The PCR amplification conditions consisted of an initial denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 5 seconds, then extension for 15 seconds at 72°C. All samples were measured in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative levels of gene expression.

Immunohistochemistry

Immunohistochemistry was performed to detect the expression of NEDD9, E-cadherin, β-catenin and vimentin in formalin-fixed paraffin-embedded colorectal cancer specimens. Briefly, 4 μm thick sections were deparaffinized with xylene, then were incubated with 0.3% hydrogen peroxide for 30 min to block the endogenous peroxidases. After treatment with normal goat serum for 1 h to block nonspecific binding, primary antibodies against NEDD9 (1:100, ab75769, Abcam, Cambridge, MA, USA), E-cadherin (1:200, sc-8426, Santa Cruz, Santa Cruz, CA, USA), β-catenin (1:100, sc-7199, Santa Cruz) and vimentin (1:100, sc-32322, Santa Cruz) were added and incubated overnight at 4°C. Specified proteins were detected using a HRP Envision System kit (Dako, Gene Co. Ltd., Shanghai, China), and then were counterstained with hematoxylin. For the negative controls, the primary antibodies were replaced by mouse or rabbit IgG (Santa Cruz).

Immunostaining was blindly evaluated by two independent pathologists. The percentage of NEDD9-positive cells was determined with a scale of 0 to 3: 0 for 0%, 1 for 1-33%, 2 for 34-66%, and 3 for 67-100%. The intensity of NEDD9 staining was scored from 0 to 3: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. NEDD9 immunostaining results were scored by multiplying the percentage of positive cells by their intensity and expression was classified as low (0-3) or high (4-9). For EMT makers (E-cadherin, β-catenin and vimentin), immunohistochemistry staining was evaluated using the method described earlier: positive staining of < 10% of tumor cells was deemed to be negative, whereas positive staining of ≥ 10% of tumor cells was considered to be positive [25].

Statistical analysis

All data were expressed as means ± standard error of mean (SEM) from at least three separate experiments. Statistical analyses were performed using SPSS version 16.0. Differences between groups were analyzed using a paired

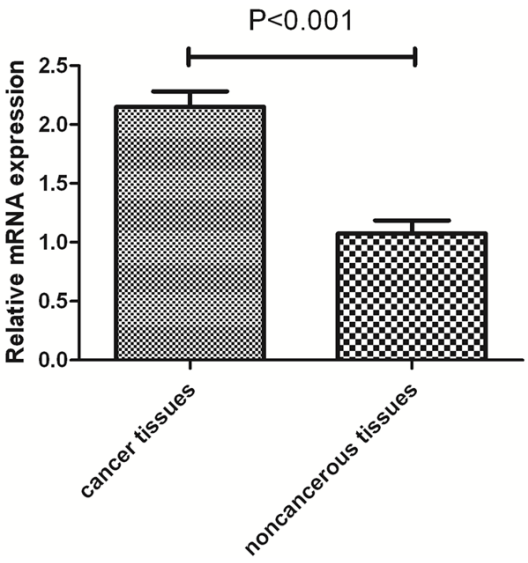

Figure 1. Relative expression of NEDD9 mRNA in colorectal cancer tissues compared to noncancerous tissues. The gene expression of NEDD9 in colorectal cancer tissues was significantly higher (2.35 ± 0.87) than in noncancerous tissues (1.18 ± 0.53; P < 0.001).

t-test or one-way ANOVA with post hoc multiple comparisons. Statistical significance was defined as $P \leq 0.05$.

Results

NEDD9 mRNA expression was elevated in colorectal cancer tissues

The mRNA level of NEDD9 was determined by real-time quantitative RT-PCR assays in 63 paired cancerous and matched adjacent noncancerous tissues. The expression levels of NEDD9 mRNA were significantly higher in colorectal cancer tissues than in adjacent noncancerous tissues (2.412 ± 0.498 vs. 1.128 ± 0.357; P < 0.001; **Figure 1**). Thus, NEDD9 may play important roles in the progression of colorectal cancer.

Correlation of NEDD9 expression with clinicopathological features in colorectal cancer

In order to examine the clinical importance of NEDD9 overexpression in colorectal cancer, the correlation between clinicopathological status of colorectoral tumors and levels of NEDD9 was evaluated. A significant positive association between the expression levels of NEDD9 and tumor differentiation or TNM stage was identified. A trend was also evident between lymph vascular invasion and NEDD9 levels although it did not reach statistical significance. No significant correlation was found between NEDD9 levels with other clinicopathological features of colorectal cancers (**Table 1**).

Immunohistochemical results for NEDD9 and EMT markers in colorectal cancer tissue samples

Representative immunohistochemical staining patterns observed for NEDD9 and three EMT marker proteins were shown in **Figure 2**. Different levels of positive staining for NEDD9 were observed mainly in the cytoplasm and membrane of cancer cells from colorectal cancer tissue samples. High NEDD9 expression was noted in 68/122 (55.7%) of colorectal cancer tissue samples and in 19/122 (15.6%) of adjacent matched noncancerous tissue samples (P < 0.001).

The levels of NEDD9 were inversely correlated with epithelial marker E-cadherin (P = 0.023) but positively correlated with both nuclear β-catenin (P = 0.039) and vimentin (P = 0.002) levels of colorectal cancer (**Table 2**).

Discussion

Colorectal cancer is one of the most common causes of cancer death worldwide [26]. Although several treatment modalities have been developed, the clinical outcome of prognosis continues to be poor in patients with advanced colorectal cancer [27]. Metastasis of colorectal cancer cells to vital organs is responsible for the majority of cancer deaths. Therefore, it is of great interest to identify valuable biomarkers for early diagnosis of patients with a high risk of metastasis, prognosis, and novel therapeutic strategies.

NEDD9 is expressed in different tissues and play important roles in development, metabolism, and aging [6]. Moreover, some researchers have shown that NEDD9 might also play a role in human carcinogenesis [7-20]. Research suggested that NEDD9 was overexpressed in colorectal cancer [20]. Our data showed that the expression of NEDD9 was markedly increased in colorectal cancer tissues compared with adjacent noncancerous tissues, consistent with the preliminary studies.

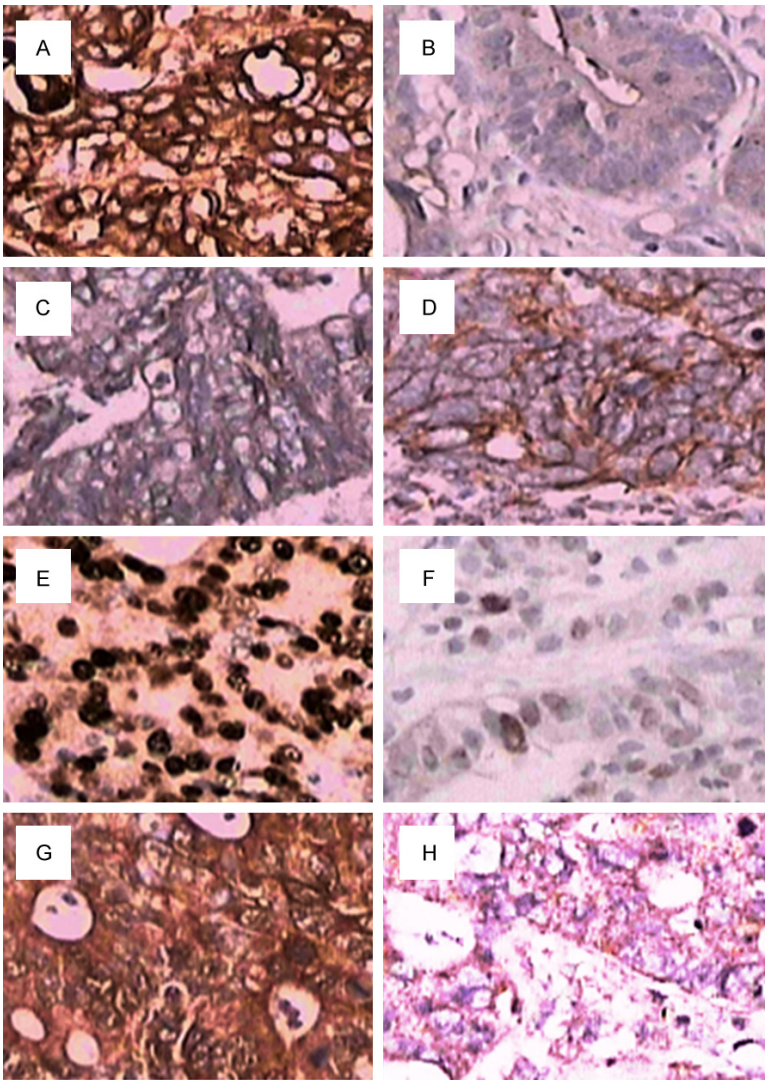

Figure 2. Representative immunohistochemical images of NEDD9 and EMT markers. A. High NEDD9 expression in colorectal cancer tissues; B. Negative NEDD9 expression in adjacent noncancerous tissues; C. Loss of epithelial marker E-cadherin in colorectal cancer tissue samples; D. Positive expression of E-cadherin in colorectal cancer tissue samples; E. β-catenin expression in colorectal cancer tissues; F. Negative β-catenin expression in adjacent noncancerous tissues; G. Acquisition of expression of the mesenchymal marker vimentin in colorectal cancer cells; H. Vimentin expression was absent in colorectal cancer cells.

Since Greenburg and Hay first proposed EMT in 1982 [28], accumulating evidence confirmed EMT as a pivotal mechanism contributing to cancer invasion and metastasis. In the progression of malignancy, EMT enabled carcinoma cells to lose their epithelial adherence, undergo cytoskeleton remodeling, facilitate their detaching from the tumor mass, migrate to distant tissue sites, and eventually form metastatic tumor masses. NEDD9 overexpression was associated with a more aggressive phenotype and contributes to migration and invasion through EMT in breast cancer [8] and lung cancer [29]. In the present study, we present several lines of evidence showing that NEDD9 is involved in EMT. Immunohistchemical analysis showed that positive expression of NEDD9 was strongly associated with loss of the epithelial marker E-cadherin and acquisition of the expression of the mesenchymal markers nuclear β-catenin and vimentin, which was in line with other studies showing, that NEDD9 overexpression may be associated with tumor invasiveness [7-20, 29]. Our data also showed that NEDD9 was significantly correlated with tumor differentiation and TNM stage, which were characteristic of tumor invasiveness and metastatic potential as well as independent prognostic factors for survival [29, 30].

In conclusion, NEDD9 was overexpressed in colorectal cancers and associated with tumor differentiation, TNM stage, and EMT marker changes in colorectal cancer, indicating that NEDD9 could be a novel prognostic factor and potential therapeutic target for colorectal cancers.

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Disclosure of conflict of interest

None.

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References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.

[2] Kim J, Huynh R, Abraham I, Kim E, Kumar RR. Number of lymph nodes examined and its impact on colorectal cancer staging. Am Surg 2006; 72: 902-905.

[3] Law SF, Estojak J, Wang B, Mysliwiec T, Kruh G, Golemis EA. Human enhancer of filamentation 1, a novel p130cas-like docking protein, associates with focal adhesion kinase and induces pseudohyphal growth in saccharomyces cerevisiae. Mol Cell Biol 1996; 16: 3327-3337.

[4] Minegishi M, Tachibana K, Sato T, Iwata S, Nojima Y, Morimoto C. Structure and function of Cas-L, a 105-kD Crk-associated substrate-related protein that is involved in beta 1 integrin-mediated signaling in lymphocytes. J Exp Med 1996; 184: 1365-1375.

[5] Kumar S, Tomooka Y, Noda M. Identification of a set of genes with developmentally down-regulated expression in the mouse brain. Biochem Biophys Res Commun 1992; 185: 1155-1161.

[6] Tikhmyanova N, Little JL, Golemis EA. CAS proteins in normal and pathological cell growth control. Cell Mol Life Sci 2010; 67: 1025-1048.

[7] Izumchenko E, Singh MK, Plotnikova OV, Tikhmyanova N, Little JL, Serebriiskii IG, Seo S, Kurokawa M, Egleston BL, Klein-Szanto A, Pugacheva EN, Hardy RR, Wolfson M, Connolly DC, Golemis EA. NEDD9 promotes oncogenic signaling in mammary tumor development. Cancer Res 2009; 69: 7198-7206.

[8] Kong C, Wang C, Wang L, Ma M, Niu C, Sun X, Du J, Dong Z, Zhu S, Lu J, Huang B. NEDD9 is a positive regulator of epithelial-mesenchymal transition and promotes invasion in aggressive breast cancer. PLoS One 2011; 6: e22666.

[9] Tikhmyanova N, Golemis EA. NEDD9 and BCAR1 negatively regulate E-cadherin membrane localization, and promote E-cadherin degradation. PLoS One 2011; 6: e22102.

[10] Kim M, Gans JD, Nogueira C, Wang A, Paik JH, Feng B, Brennan C, Hahn WC, Cordon-Cardo C, Wagner SN, Flotte TJ, Duncan LM, Granter SR, Chin L. Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. Cell 2006; 125: 1269-1281.

[11] Natarajan M, Stewart JE, Golemis EA, Pugacheva EN, Alexandropoulos K, Cox BD, Wang W, Grammer JR, Gladson CL. HEF1 is a necessary and specific downstream effector of FAK that promotes the migration of glioblastoma cells. Oncogene 2006; 25: 1721-1732.

[12] Lucas JT Jr, Salimath BP, Slomiany MG, Rosenzweig SA. Regulation of invasive behavior by vascular endothelial growth factor is HEF1-dependent. Oncogene 2010; 29: 4449-4459.

[13] Chang JX, Gao F, Zhao GQ, Zhang GJ. Expression and clinical significance of NEDD9 in lung tissues. Med Oncol 2012; 29: 2654-2660.

[14] Budhu A, Forgues M, Ye QH, Jia HL, He P, Zanetti KA, Kammula US, Chen Y, Qin LX, Tang ZY, Wang XW. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. Cancer Cell 2006; 10: 99-111.

[15] Sima N, Cheng X, Ye F, Ma D, Xie X, Lü W. The overexpression of scaffolding protein NEDD9 promotes migration and invasion in cervical cancer via tyrosine phosphorylated FAK and SRC. PLoS One 2013; 8: e74594.

[16] Zhang Q, Wang H, Ma Y, Zhang J, He X, Ma J, Zhao ZS. Overexpression of Nedd9 is a prognostic marker of human gastric cancer. Med Oncol 2014; 31: 33.

[17] Liu Y, Wang D, Zhao KL, Zhu JW, Yin HB, Wei YZ, Wu ZJ, Cheng GJ, Wang F, Ni F, Wang XL, Cao GX, Huang J, Cai J. NEDD9 overexpression correlates with poor prognosis in gastric cancer. Tumour Biol 2014; 35: 6351-6356.

[18] Shi R, Wang L, Wang T, Xu J, Wang F, Xu M. NEDD9 overexpression correlates with the progression and prognosis in gastric carcinoma. Med Oncol 2014; 31: 852.

[19] Xue YZ, Sheng YY, Liu ZL, Wei ZQ, Cao HY, Wu YM, Lu YF, Yu LH, Li JP, Li ZS. Expression of NEDD9 in pancreatic ductal adenocarcinoma and its clinical significance. Tumour Biol 2013; 34: 895-899.

[20] Li P, Zhou H, Zhu X, Ma G, Liu C, Lin B, Mao W. High expression of NEDD9 predicts adverse outcomes of colorectal cancer patients. Int J Clin Exp Pathol 2014; 7: 2565-2570.

[21] Singh M, Cowell L, Seo S, O'Neill G, Golemis E. Molecular basis for HEF1/NEDD9/Cas-L action as a multifunctional co-ordinator of invasion, apoptosis and cell cycle. Cell Biochem Biophys 2007; 48: 54-72.

[22] Law SF, O'Neill GM, Fashena SJ, Einarson MB, Golemis EA. The docking protein HEF1 is an apoptotic mediator at focal adhesion sites. Mol Cell Biol 2000; 20: 5184-5195.

[23] Fashena SJ, Einarson MB, O'Neill GM, Patriotis C, Golemis EA. Dissection of HEF1-dependent

functions in motility and transcriptional regulation. J Cell Sci 2002; 115: 99-111.
[24] Bui LC, Tomkiewicz C, Chevallier A, Pierre S, Bats AS, Mota S, Raingeaud J, Pierre J, Diry M, Transy C, Garlatti M, Barouki R, Coumoul X. Nedd9/Hef1/Cas-L mediates the effects of environmental pollutants on cell migration and plasticity. Oncogene 2009; 28: 3642-3651.
[25] Kim MA, Lee HS, Lee HE, Kim JH, Yang HK, Kim WH. Prognostic importance of epithelial-mesenchymal transition-related protein expression in gastric carcinoma. Histopathology 2009; 54: 442-451.
[26] Parkin DM. Global cancer statistics in the year 2000. Lancet Oncol 2001; 2: 533-543.
[27] Weitz J, Koch M, Debus J, Höhler T, Galle PR, Büchler MW. Colorectal cancer. Lancet 2005; 365: 153-165.
[28] Greenburg G, Hay ED. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J Cell Biol 1982; 95: 333-339.
[29] Miao Y, Li AL, Wang L, Fan CF, Zhang XP, Xu HT, Yang LH, Liu Y, Wang EH. Overexpression of NEDD9 is associated with altered expression of E-Cadherin, β-Catenin and N-Cadherin and predictive of poor prognosis in non-small cell lung cancer. Pathol Oncol Res 2013; 19: 281-286.
[30] He R, Yang Q, Dong X, Wang Y, Zhang W, Shen L, Zhang Z. Clinicopathologic and prognostic characteristics of alpha-fetoprotein-producing gastric cancer. Oncotarget 2017; 8: 23817-23830.