World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i14.4001

World J Gastroenterol 2014 April 14; 20(14): 4001-4010
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Epithelial membrane protein 1 negatively regulates cell growth and metastasis in colorectal carcinoma

Guo-Gui Sun, Ya-Di Wang, Da-Wei Cui, Yun-Jie Cheng, Wan-Ning Hu

Guo-Gui Sun, Wan-Ning Hu, Department of Chemoradiotherapy, Tangshan People's Hospital, Tangshan 063000, Hebei Province, China
Ya-Di Wang, Department of Radiotherapy, the Military General Hospital of Beijing PLA, Beijing 100700, China
Da-Wei Cui, Department of Urology, Tangshan Workers Hospital, Tangshan 063000, Hebei Province, China
Yun-Jie Cheng, Department of Radiotherapy, The Fourth Hospital of Hebei Medical University, Shijiazhuang 050017, Hebei Province, China
Author contributions: Sun GG and Wang YD performed the majority of experiments; Cui DW and Cheng YJ provided vital reagents and analytical tools and were also involved in editing the manuscript; Hu WN designed the study and wrote the manuscript.
Correspondence to: Dr. Wan-Ning Hu, Department of Chemoradiotherapy, Tangshan People's Hospital, No. 65, Shengli Road, Lunan District, Tangshan 063000, Hebei Province, China. wanning_hu2008@sina.com
Telephone: +86-315-2880376 Fax: +86-315-2880376
Received: September 13, 2013 Revised: December 24, 2013
Accepted: February 17, 2014
Published online: April 14, 2014

Abstract

AIM: To determine the expression and function of epithelial membrane protein 1 (EMP1) in colorectal carcinoma.

METHODS: Colorectal samples were taken from cancer lesions and adjacent normal tissue in colorectal cancer patients immediately after endoscopic biopsy. A portion of the sample was either fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry or stored in liquid nitrogen for Western blot. In order to determine protein expression of EMP1 in colorectal cancer (n = 63) and normal tissue (n = 31), semi-quantitative immunohistochemistry and Western blot were utilized. For in vitro studies, the human colorectal cancer cell line SW-480 was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. Recombinant lentivirus mediated overexpression of EMP1 in SW-480 cells was quantified by real-time reverse transcription-polymerase chain reaction and Western blot. Control SW-480 cells were transfected with an empty vector. To further study the effect of EMP1 overexpression in SW-480 cells, cell proliferation, apoptosis, migration and invasion assays were conducted.

RESULTS: Expression of EMP1 was significantly lower in colorectal cancer tissue than in normal tissue using both immunohistochemistry (39.7% *vs* 90.3% of tissues, $P < 0.05$) and Western blot (0.126 ± 0.022 *vs* 0.632 ± 0.053, $P < 0.05$). The level of EMP1 protein expression was not correlated with gender, age, or tumor location. Decreased expression of EMP1 was significantly correlated with T stage, lymph node metastasis, clinic stage, and histological grade in patients with colorectal cancer ($P < 0.05$). According to Kaplan-Meier analysis, low EMP1 expression correlated significantly with poor overall five-year survival (34.2% *vs* 64.0% survival, $P < 0.05$). SW-480 cells transfected with EMP1 had a lower survival fraction, higher cell apoptosis (12.1% ± 1.3% *vs* 3.1% ± 0.6%, $P < 0.05$), a significant decrease in migration and invasion (124.0 ± 17.0 and 87.0 ± 12.0, respectively *vs* 213.0 ± 29.0 and 178.0 ± 21.0, respectively, $P < 0.05$), higher caspase-9 (0.635 ± 0.063 *vs* 0.315 ± 0.032, $P < 0.05$), and lower VEGFC protein expression (0.229 ± 0.021 *vs* 0.519 ± 0.055, $P < 0.05$) relative to cells not transfected with EMP1.

CONCLUSION: Low EMP1 expression in colorectal cancer is associated with increased disease severity, suggesting that EMP1 may be a negative regulator of colorectal cancer.

Key words: Epithelial membrane protein 1; Colorectal carcinoma; Caspase-9; Vascular endothelial growth factor C; Prognosis

Core tip: Epithelial membrane protein 1 (EMP1) has a known role in tumor development and progression, and its activity is linked to a number of biological processes including proliferation, apoptosis, invasion, and metastasis of colorectal cancer. We detected expression of EMP1 in colorectal carcinoma and analyzed the biological effect of EMP1 overexpression in a colorectal carcinoma cell line. EMP1 expression was decreased in colorectal cancer and its expression correlated significantly with T stage, lymph node metastasis, clinic stage, histological grade, and poor overall survival. Taken together, our findings suggest that EMP1 may play an important role as a negative regulator of colorectal cancer.

Sun GG, Wang YD, Cui DW, Cheng YJ, Hu WN. Epithelial membrane protein 1 negatively regulates cell growth and metastasis in colorectal carcinoma. *World J Gastroenterol* 2014; 20(14): 4001-4010 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/4001.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.4001

INTRODUCTION

Colorectal cancer is one of the most common malignancies worldwide, and it is a major cause of mortality, with a five-year survival rate of approximately 50%. The main cause of death is metastasis to the liver and lungs, which is present in up to 25% of patients at the time of diagnosis[1,2]. Well-established histopathological factors that influence disease outcome are tumor size, histological type and subtype, the presence of signet ring morphology, the degree of differentiation, the presence of lymphovascular invasion, and lymph node involvement[3,4]. Further understanding of the molecular mechanisms underlying the pathophysiology of metastatic processes will help us not only identify those patients at greatest risk of recurrence but also find novel molecular targets for the development of treatment strategies for colorectal cancer. A preliminary study on the epithelial membrane protein 1 (*EMP1*) gene found that EMP1 is closely linked to tumor development and progression[5,6]. Activation of the *EMP1* gene in particular can prevent tumor proliferation, and it may be a new target for tumor therapy[7,8]. However, to date there is no information available regarding the relationship between EMP1 and colorectal cancer. We studied EMP1 expression in colorectal cancer using immunohistochemistry and Western blot and analyzed the effect of EMP1 overexpression *in vitro* in the colorectal cancer cell line SW-480[9,10].

MATERIALS AND METHODS

Clinical data

All patients enrolled in this study provided informed consent in advance. There were 37 males and 26 females, and they ranged in age from 31 to 78 years, with a median age of 54 years. Of the 63 cases of colorectal cancer, 27 had stages T1 and T2 disease and 36 had stages T3 and T4 disease. Twenty-eight patients did not present with lymph node metastasis (N0), whereas 35 presented with identified lymph node involvement (N+). As for the clinical stage, 25 cases had stage Ⅰ-Ⅱ colorectal cancer and 38 had stage Ⅲ-Ⅳ colorectal cancer. Regarding grade of differentiation, 20 had grade Ⅰ (well differentiated) tumors, and 43 had grade Ⅱ or Ⅲ (moderately to poorly differentiated) tumors. Samples were instantly taken after the endoscopic biopsy, and either fixed in 4% paraformaldehyde solution and embedded in paraffin for immunohistochemistry or stored in liquid nitrogen for Western blot analysis.

Cell culture and gene transfection

Human colorectal cancer SW-480 cells were maintained in RPMI-1640 medium (Gibco BRL, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (Gibco BRL). Medium was changed every two to three days; when the cultures reached confluence, the cells were subcultured with 0.25% trypsin and 1% ethylenediaminetetraacetic acid (EDTA). Cells were tested every three months for mycoplasma, and mycoplasma removal agent (MRA) (MP Biomedicals Co. Ltd., Shanghai, China) was used to maintain mycoplasma-free cultures. EMP1 cDNA was cloned into the BamHI and AscI sites of the plenti6/V5-DEST vector (Invitrogen, Carlsbad, CA, United States). After amplification and DNA sequence confirmation, this vector was used to overexpress EMP1 in SW-480 cells. Briefly, SW-480 cells were grown and stably transfected with pLenti6-EMP1 or plenti6/V5-DEST for control using Lipofectamine 2000 (Invitrogen) and grown in Blastidicin (5 μg/mL)-containing RPMI-1640 medium.

Immunohistochemistry

Immunohistochemistry was performed as previously described[11]. Briefly, 4 μm sections were prepared from a paraffin-embedded block and dehydrated, incubated in 3% hydrogen peroxide for 10 min, and incubated in trypsin for 20 min. Sections were blocked with 10% goat serum at room temperature for 20 min and treated with a rabbit anti-human EMP1 polyclonal antibody (1:100; Abcam, Cambridge, United Kingdom) overnight at 4 ℃. After rinsing, sections were treated with biotin-conjugated antibodies (4A Biotech Co. Ltd., Beijing, China) for 20 min, and biotin-immune complexes were identified with a diaminobenzidine (DAB) substrate immunochemistry kit (4A Biotech Co. Ltd.) and hematoxylin stain. Sections were mounted and dehydrated with the coverslip sealed. For the negative control, sections were treated identically except that the primary antibody was replaced with PBS. Two pathologists blinded to patient and tissue status assessed the results. Three slides for each specimen were counted, with five fields of view randomly selected for evaluation per section. EMP1 expression level was based

on the percentage of positive cells and staining intensity. The percentage of positive cells was divided into four levels: 0 points: ≤ 5% of positive cells; 1 point: 5%-25%; 2 points: 25%-50%; and 3 points: > 50%. The intensity of staining was classified as: 0 points: no staining; 1 point: weak staining (light yellow); 2 points: moderate staining (yellowish-brown); and 3 points: strong staining (brown). The final score of EMP1 expression was the product of the EMP1 expression level and intensity, graded as 0 for negative, + for 1-3 points, ++ for 4-6 points, and +++ for 7-9 points.

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from SW-480 cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol[12]. Five hundred nanograms of total RNA was reverse transcribed using TaKaRa Reverse Transcriptase Reagents (TaKaRa, Shiga, Japan). Quantitative real-time (q) reverse transcription-polymerase chain reaction (qRT-PCR) was performed with an ABI Prizm 7300 (Applied Biosystems Inc., Carlsbad, CA, United States) according to the standard protocol for SYBR Premix ExTaq (Perfect Real Time; TakaRa). Primers for EMP1 and β-actin for normalization were as follows: EMP1 sense 5'-CCCTCCTGGTCTTCGTGT, antisense 5'- AATAGCCGTGGTGATA; β-actin sense 5'- ATCGTCCACCGCAAATGCTTCTA, antisense 5'-AGCCATGCCAATCTCATCTTGTT. Thermal cycling conditions were 95 ℃ for 1 min, 95 ℃ for 15 s, and 40 cycles at 60 ℃ for one min. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method with SDS 1.3 software (Applied Biosystems Inc.).

Western blot

Western blot was performed as previously described[13]. Samples were lysed in a lysis buffer containing 1% NP-40, 0.1% SDS, 25 mmol/L Hepes, 134 mmol/L NaCl, 1 mmol/L vanadate, 100 mmol/L NaF, and 0.5% Na-deoxycholate. After centrifugation at 12000 r/min for 20 min at 4 ℃, the supernatant was stored at -20 ℃. Protein concentration was detected with the BCA Protein Assay Kit (Tiangen Biotech Co., Ltd., Beijing, China). Fifty milligrams of protein was resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. For EMP1, blots were blocked for 2 h with 5% skim milk and incubated overnight at 4 ℃ with rabbit anti-human EMP1 (1:1000), caspase-9 (1:1000; Abcam) and VEGFC (1:1000; Abcam). For β-actin, blots were blocked in 5% nonfat dry milk for 1 h at room temperature and incubated overnight with a mouse anti-β-actin antibody (Sigma, St. Louis, MO, United States) at 4 ℃. After washing, membranes were either incubated with goat anti-mouse fluorescent secondary antibody (1:20000; IRDye800, LI-COR Bioscience, Inc., Lincoln, NE, United States) or DyLight Fluor conjugated goat anti-rabbit secondary antibody (LI-COR Bioscience, Inc.) in the dark for 1 h at room temperature. The blots were scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR Bioscience Inc.). Western blot data were quantified by normalizing the signal intensity of each sample to that of β-actin[13].

MTT assay

Cell viability was determined using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described[14]. Briefly, cells were plated into 96-well culture plates at an optimal density of 5×10^3 cells/mL in 200 μL of culture medium per well. After 24-96 h of culture, 20 μL of 5 mg/mL MTT was added to each well and incubated at 37 ℃ for 4 h. The medium was then gently aspirated and 150 μL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The optical density of each sample was immediately measured using a microplate reader (BioRad, Hercules, CA, United States) at 570 nm.

Flow cytometry assay

An annexin V-FITC-flow cytometry assay (4A Biotech Co. Ltd.) was used to detect the apoptosis rate in the cells after EMP1 transfection, as previously described[15]. Cells were seeded into 60 mm dishes for 48 h and grown to 70%-75% confluence. After quick detachment from the plate, cells were collected, washed with ice-cold PBS, and resuspended at a cell density of 1×10^6/mL in a binding buffer from the annexin V-FITC apoptosis detection kit (4A Biotech Co. Ltd.). Cells were then stained with 5 μL of annexin V-FITC and 10 μL of propidium iodide (PI, 20 μg/mL). The cells were incubated in the dark at 25 ℃ for 15 min before 10000 cells were analyzed by a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA, United States) and Cellquest software (BD Immunocytometry Systems) for apoptosis rate determination.

Invasion and migration assays

Invasion and migration assays were performed as previously described[16]. For the invasion assay, Costar Transwell 8 μm inserts were coated with 50 μg reduced serum Matrigel (BD Biosciences, Bedford, MA, United States). Invasion Chambers (BD China, Shanghai, China) were coated with Matrigel, and 10×10^5 cells were added per chamber. Medium supplemented with 10% FBS was used in the lower chamber. For migration assays, the same procedure was used excluding the Matrigel. After 12 h, non-invading cells and media were removed, and cells on the lower surface of the membrane were fixed with polyoxymethylene (Sigma) and stained with 0.1% crystal violet (Sigma) for 0.5 h. Stained cells were counted under a microscope in four randomly selected fields, and the average was used to indicate cell migration and invasion.

Statistical analysis

All statistical analyses were performed using SPSS 16.0 software (IBM, Chicago, IL, United States), as previously described[17]. For the clinicopathologic features, *P* values were calculated using the χ^2 test. Student's *t* test was used

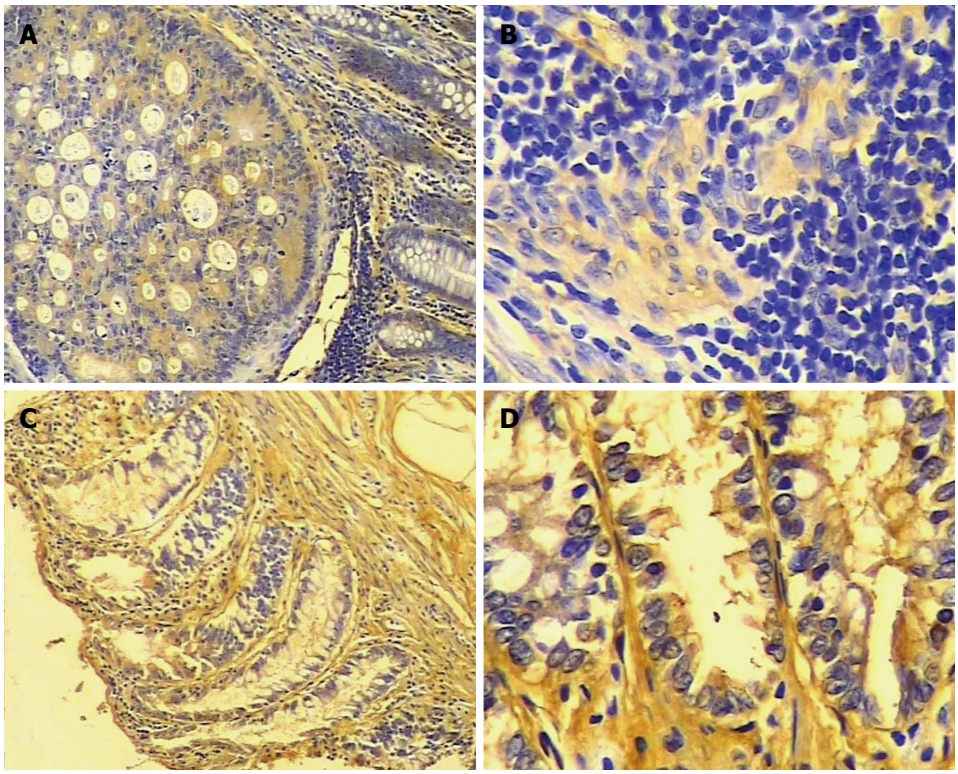

Figure 1 Immunohistochemistry of epithelial membrane protein 1 protein in colorectal carcinoma and adjacent normal tissue. A and B: Representative sample of colorectal carcinoma (A: SP × 100, B: SP × 400). There is little staining for epithelial membrane protein 1 (EMP1); C and D: normal colorectal tissue (C: SP × 100, D: SP × 400). There is intense yellow and yellow-brown staining of EMP1.

Table 1 Expression of epithelial membrane protein 1 in colorectal cancer tissue and normal colorectal tissue

Group	Case	Expression of epithelial membrane protein 1					
		-	+	+ +	+ + +	χ^2	*P* value
Normal tissue	31	3	6	11	12	25.239	0
Cancer tissue	63	38	10	8	7		

to analyze the difference between groups. Survival distributions were estimated by the Kaplan-Meier method and compared using the log-rank test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

EMP1 protein expression in colorectal cancer and normal tissues

EMP1 staining in colorectal cancer tissue was negative or weak relative to normal adjacent colorectal tissues that exhibited light yellow to brown staining. EMP1 expression was significantly lower ($P < 0.05$) in colorectal cancer tissue (expressed in 39.7%, 25/63) than in normal tissue (expressed in 90.3%, 28/31) (Figure 1 and Table 1). Western blot analysis showed that the expression of EMP1 in cancer lesions was significantly less than that in adjacent normal tissue (0.126 ± 0.022 and 0.632 ± 0.053, respectively, $P < 0.05$) (Figure 2). The expression of EMP1 negatively correlated with T stage, lymph node metastasis, clinical stage and pathological differentiation ($P < 0.05$), regardless of age, gender, and tumor location (P

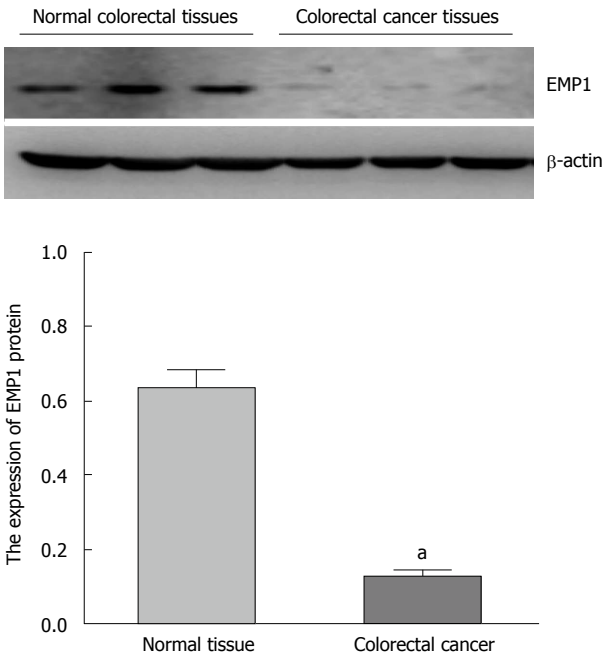

Figure 2 Epithelial membrane protein 1 protein in colorectal carcinoma and normal tissue detected by Western blot. Upper panel, representative blots of normal (left) and cancer colorectal (right) tissue. Lower panel, summary of all samples. Epithelial membrane protein 1 (EMP1) levels are significantly less in colorectal cancer relative to control, $^aP < 0.05$.

Table 2 Relationship between epithelial membrane protein 1 expression and clinical characteristics of colorectal cancer

Variable	Case	Expression of epithelial membrane protein 1			
		-	+-+++	χ^2	*P* value
Sex					
Male	37	21	16	0.199	0.656
Female	26	15	9		
Age (yr)					
≤ 60	46	27	19	0.187	0.665
> 60	17	11	6		
Tumor location					
Colon cancer	35	19	16	1.197	0.274
Rectal cancer	28	19	9		
Tumor invasion					
T1 + T2	27	11	16	7.566	0.006
T3 + T4	36	27	9		
Lymph node metastasis					
N0	28	11	17	9.314	0.002
N+	35	27	8		
Clinical stage					
Ⅰ-Ⅱ	25	9	16	10.240	0.001
Ⅲ-Ⅳ	38	29	9		
Histological grade					
Ⅰ	20	8	12	5.054	0.025
Ⅱ-Ⅲ	43	30	13		

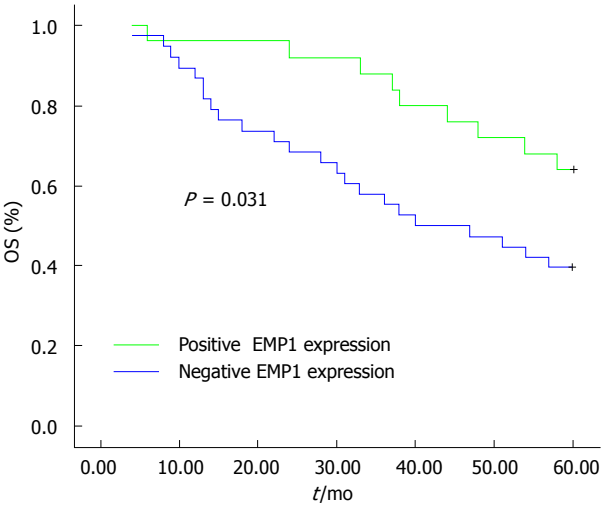

Figure 3 Relationship between epithelial membrane protein 1 expression and five-year survival in colorectal carcinoma by Kaplan-Meier analysis. Overall survival is higher in epithelial membrane protein 1 (EMP1)-positive patients relative to EMP1-negative patients.

> 0.05, Table 2).

EMP1 expression and prognosis

Patients were followed for 60 mo for survival analysis. At the end of the study in 2012, 29 of 63 patients had survived. Patients were divided into two groups according to expression level of EMP1. Of the 25 patients with positive levels of EMP1 expression, 16 were still alive, yielding a survival rate of 64.0%. Of the 38 patients with undetectable levels of EMP1 expression, only 13 were still alive, yielding a survival rate of 34.2%. Patients with high levels of EMP1 had a significantly higher five-year survival rate than those with low levels of EMP1 ($P < 0.05$) (Figure 3).

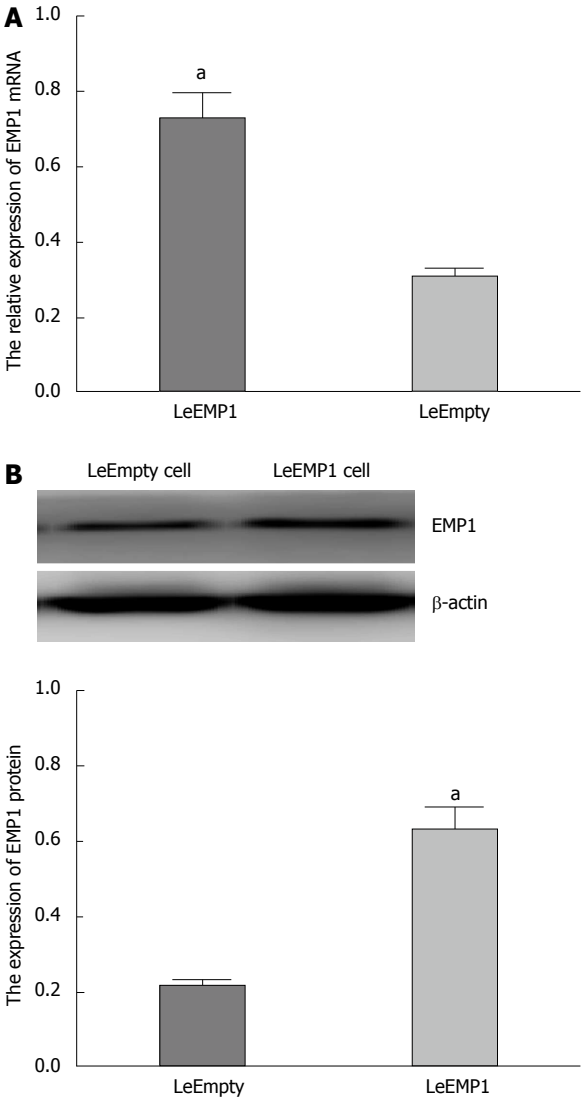

Figure 4 Expression and identification of the epithelial membrane protein 1 gene. A: Reverse transcription-polymerase chain reaction for epithelial membrane protein 1 (EMP1) in LeEmpty cells *vs* LeEMP1 cells; B: Sample Western blots for EMP1 and actin (loading control) in LeEmpty and LeEMP1 cells (top). Summary of Western blot data for EMP1 protein expression (bottom), $^aP < 0.05$ *vs* LeEMP1.

Stable transfection of EMP1 cDNA in colorectal cancer cells

SW-480 cells stably transfected with EMP1 overexpressed EMP1 (named as LeEMP1 cells). Control SW-480 cells were transfected with an empty vector (named as LeEmpty cells). The expression of EMP1 mRNA and protein was significantly elevated in LeEMP1 cells relative to control cells ($P < 0.05$). EMP1 mRNA levels detected by RT-PCR were significantly higher in LeEMP1 cells (0.729 ± 0.066) than in LeEmpty cells (0.305 ± 0.028) ($P < 0.05$; Figure 4A). Western blot analysis found that the level of immunoreactive protein was significantly higher in EMP1 transfected cells (0.631 ± 0.060) relative to controls cells

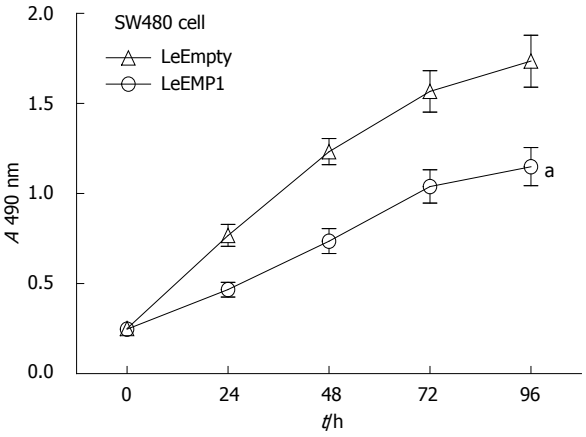

Figure 5 Effects of epithelial membrane protein 1 overexpression on cell proliferation. MTT assay time-course for LeEmpty and LeEMP1 cells. Cells overexpressing EMP1 have a significantly decreased rate of proliferation relative to control cells, $^{a}P < 0.05$. EMP1: Epithelial membrane protein 1.

(0.213 ± 0.018) ($P < 0.05$; Figure 4B).

Effects of EMP1 overexpression on colorectal cancer cells

Next, we assessed the effect of EMP1 expression on the regulation of colorectal cancer cell viability. MTT assay showed that relative proliferative capacity of LeEMP1 cells grew significantly slower at 24, 48, 72, and 96 h relative to LeEmpty cells ($P < 0.05$; Figure 5). Meanwhile, there was a significant increase in the early apoptosis rate in LeEMP1 cells (12.1% ± 1.3%) relative to control cells (3.1% ± 0.6%) ($P < 0.05$; Figure 6). SW-480 cells transfected with EMP1 or empty vector were transferred to transwell chambers or Matrigel-coated transwell chambers to evaluate the effect of EMP1 on cell invasion potential. Overexpression of EMP1 significantly decreased cell migration and invasion of SW-480 cells (124.0 ± 17.0 and 87.0 ± 12.0, respectively) relative to control cells (213.0 ± 29.0 and 178.0 ± 21.0, respectively) ($P < 0.05$; Figure 7).

To further study the mechanisms by which EMP1 inhibited colorectal cancer cell proliferation, apoptosis, migration, and invasion, we analyzed the expression of two proteins with critical roles in these processes, caspase-9 and VEGFC. Western blot analysis revealed that overexpression of EMP1 in SW-480 cells significantly upregulated caspase-9 protein expression (0.635 ± 0.063) relative to control cells (0.315 ± 0.032) ($P < 0.05$; Figure 8). In contrast, the level of VEGFC protein expression was significantly lower in SW-480 cells overexpressing EMP1 (0.229 ± 0.021) than in control cells (0.519 ± 0.055) ($P < 0.05$; Figure 8).

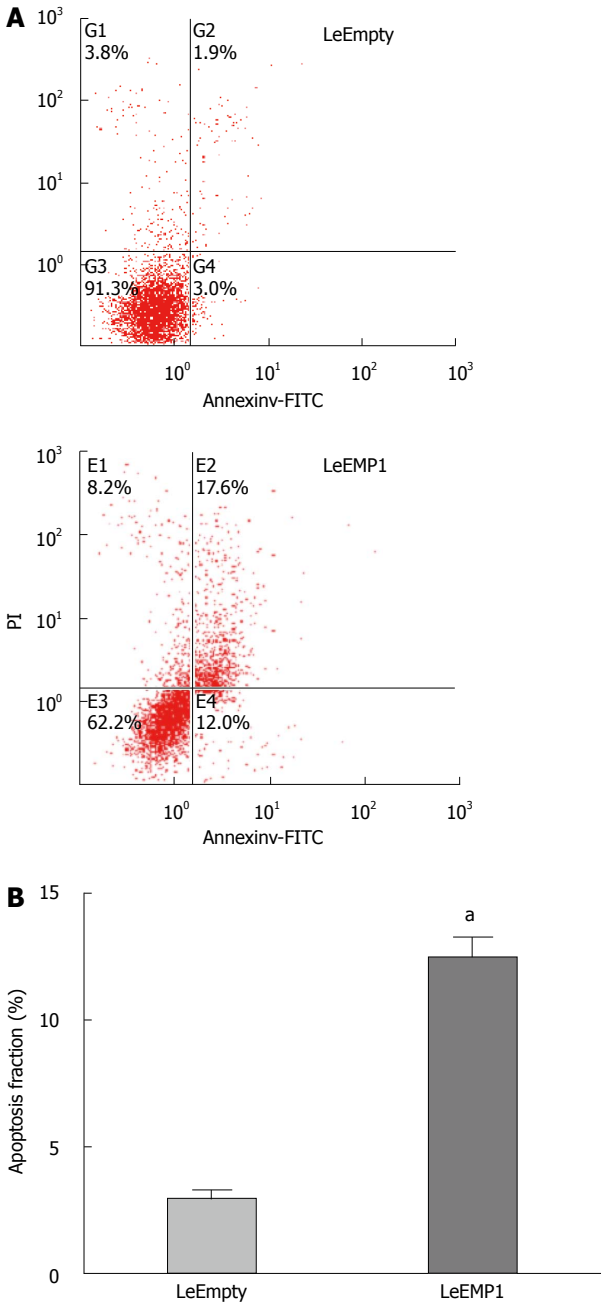

Figure 6 Effects of epithelial membrane protein 1 overexpression on cell apoptosis. A: Cells were stained with 5 μL annexin V-FITC and 10 μL PI (20 μg/mL). Samples were acquired on a FACScan flow cytometer and 10000 cells analyzed with Cellquest software; B: Colorectal cancer cells overexpressing epithelial membrane protein 1 (LeEMP1) exhibit significantly more apoptosis than empty vector transfected cells (LeEmpty), $^{a}P < 0.05$

DISCUSSION

Several studies have shown that the *EMP1* gene is expressed in a number of normal tissues[7,18-23]. In this study, we localized and quantified for the first time EMP1 protein expression in colorectal cancer tissue and normal colorectal tissue using immunohistochemistry and immunoblotting. EMP1 protein levels were significantly lower in colorectal carcinoma than in normal tissue, and EMP1 protein levels correlated with T stage, lymph node metastasis, and clinical stage of colorectal cancer. Since dedifferentiation is a hallmark of tumor cells, our findings suggest that a decline in EMP1 level is a factor in the development and progression of colorectal cancer. In a study evaluating several types of human breast cancer cells with different metastatic characteristics, *EMP1* gene expression was correlated with cell invasion and other properties of metastasis[24]. *EMP1* gene expression was

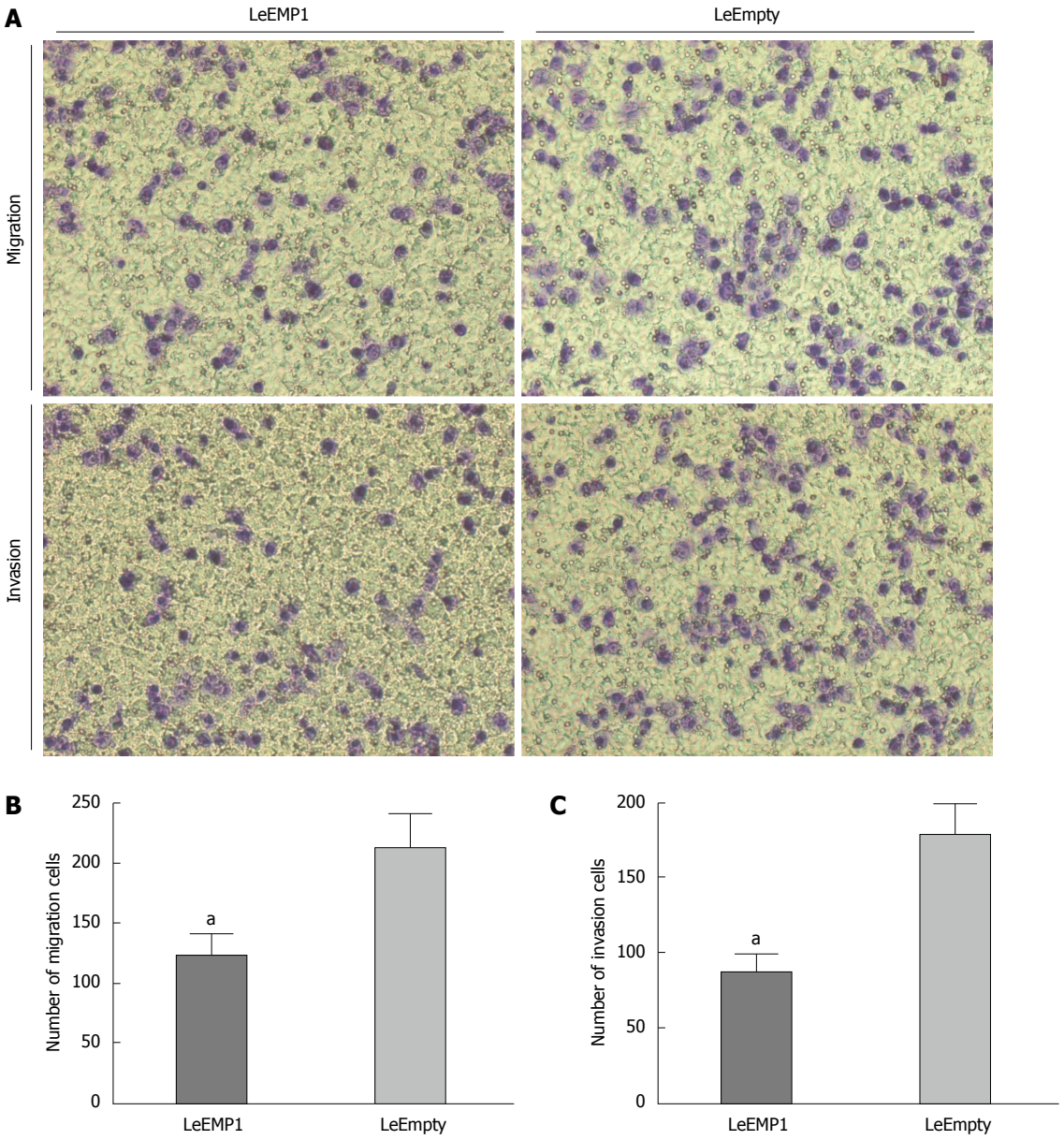

Figure 7 Effects of epithelial membrane protein 1 overexpression on cell migration and invasion. A: Histological sections of cell migration and invasion in LeEmpty and LeEMP1 cells; B: The number of migrating cells is significantly greater in LeEmpty cells than in LeEMP1 cells; C: Number of invading cells is greater in LeEmpty than in LeEMP1 transfected cells, $^{a}P < 0.05$. EMP1: Epithelial membrane protein 1.

down-regulated in oral squamous cell carcinoma and this down-regulation was correlated with lymph node metastasis[25]. Therefore, the EMP1 gene may be an important factor for the regulation of cell signaling, cell communication, and adhesion[26].

Currently an effective treatment paradigm for colorectal cancer is extended surgical resection of the lesion, accompanied by chemotherapy and/or radiotherapy before and after surgery. However, the five-year survival rate with this strategy is only 40%[27,28]. Therefore, efforts should be directed toward early detection of colorectal cancer and the refinement of individual based treatment strategies. Conventional treatment and prognosis of colorectal cancer rely mainly on TNM classification[29]. This system is subjective and not informative for early colorectal cancer, and offers limited information about disease severity, prognosis, and response to treatment. Early detection of colorectal cancer is the most effective way to improve survival[30]. Using survival analysis, we found that EMP1 expression-positive patients had a significantly higher five-year overall survival rate than patients with undetectable EMP1 expression. Thus, combining information from the TNM classification system and EMP1 expression scores may provide valuable information for clinicians regarding prognosis, prediction of disease severity, and selection of treatment regimens.

Furthermore, in vitro experiments demonstrated for the first time that colorectal cancer cells with high EMP1 expression had significantly weakened proliferation, significantly increased apoptosis, and markedly reduced

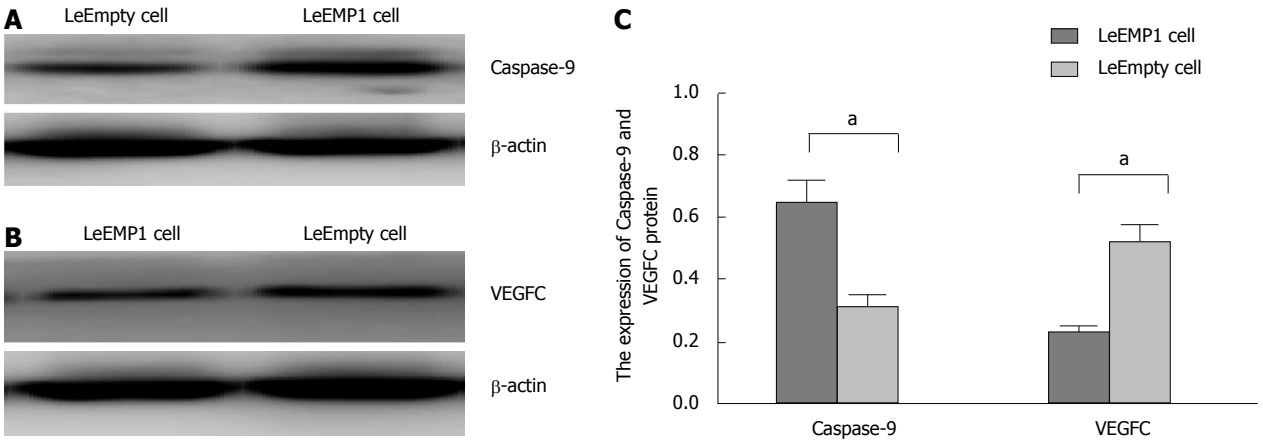

Figure 8 Effects of epithelial membrane protein 1 overexpression on caspase-9 and vascular endothelial growth factor-factor C expression. A: Sample blot for caspase-9 and actin (loading control) of colorectal cancer cells transfected with LeEMP1 and LeEmpty; B: Sample blot for vascular endothelial growth factor-factor C (VEGFC) and actin; C: Quantification of caspase-9 and VEGFC expression in LeEMP1 and LeEmpty cells. $^{a}P < 0.05$ beteween the two groups.

caspase-9 and VEGFC protein levels. Previously, overexpression of EMP1 in an esophageal cancer cell line slowed esophageal cancer cell growth and yielded fewer S-phase cells and more G_1-phase cells[26]. Together with our findings, these data suggest that low levels of EMP1 affect cellular processes that are abnormally regulated in cancer. Mitochondria are not only the site of cellular respiration and oxidative phosphorylation, but also the regulation center of apoptosis. Cytochrome C released from mitochondria to the cytoplasm associates with apoptotic protease activating factor (Apaf-1) to form a multiservice complex in the presence of deoxyribonucleotide triphosphate (dNTP)[31]. This complex interacts with pro-caspase-9 to form an apoptosome and, following dimerization, results in autoactivation of caspase-9. This activated caspase-9 stimulates other caspases, such as caspase-3 and caspase-7, culminating in apoptosis *via* signaling cascades[32-34]. We found in this study that high expression of EMP1 is associated with significantly higher expression of caspase-9 protein, implicating a mitochondrial apoptosis pathway in EMP1-induced apoptosis.

VEGF is a member of the platelet-derived growth factor (PDGF) family and is the most important vascular endothelial growth-stimulating factor during tumor angiogenesis. VEGFC is a recently identified member of the VEGF family, which promotes the proliferation of endothelial cells, increases vascular permeability, and functions as a key factor in tumor angiogenesis, invasion, and metastasis[35,36]. We found in this study that overexpression of EMP1 is associated with a significant decrease in VEGFC expression. This finding suggests that EMP1 may inhibit tumor angiogenesis by suppressing VEGFC expression and hence tumor metastasis.

In summary, we demonstrated that EMP1 protein levels were significantly reduced in colorectal carcinoma and were associated with T stage, lymph node metastasis, clinical stage, and cell differentiation. EMP1 is involved in a number of biological processes including proliferation, apoptosis, invasion, and metastasis of colorectal cancer. Given the complexity of carcinogenesis, further research is needed to understand the molecular mechanism underlying EMP1 regulation of this process. Our findings identify a novel potential therapeutic target for colorectal cancer and suggest that EMP1 may be a reliable biomarker for prognosis of colorectal cancer.

COMMENTS

Background

Colorectal carcinoma remains one of the leading causes of global cancer mortality. Further understanding of the molecular mechanisms underlying the metastatic process will help us to identify those at highest risk of recurrence and to identify novel tumor targets to prevent disease progression. Although epithelial membrane protein 1 (EMP1) has been implicated in tumor development and progression, its role in colorectal carcinoma remains unknown.

Research frontiers

EMP1 protein is believed to be in the same protein family as peripheral myelin 22 (PMP22) and shares high sequence homology with PMP22 (approximately 40%). EMP1 is also found in the liver, heart, lung, bone, muscle, kidney, spleen, prostate, testis, ovary, placenta and thymus. EMP1 is highly expressed in undifferentiated embryonic stem cells and lowly expressed in differentiated adult cells, prolonging the transition of Schwann cells from G-phase to S + G_2/M-phase. It has been suggested that this membrane glycoprotein family is closely related to cell proliferation and differentiation.

Innovations and breakthroughs

The authors report for the first time that EMP1 protein levels were significantly reduced in colorectal carcinoma. EMP1 expression was associated with T stage, lymph node metastasis, clinical stage, and cell differentiation. EMP1 is involved in a number of biological processes including proliferation, apoptosis, invasion, and metastasis of colorectal cancer, suggesting that EMP1 may play important roles as a negative regulator of these processes in colorectal cancer cells.

Applications

The results of this study contribute to a better understanding of the association between the loss of EMP1 in colorectal cancer and tumorigenesis and progression of this cancer. The findings identify a novel therapeutic target for colorectal cancer and suggest that EMP1 may be a reliable biomarker for the prognosis of colorectal cancer.

Terminology

Lentiviral vectors derived from the human immunodeficiency virus (HIV-1) have become major tools for gene delivery in mammalian cells. The primary advantage of using lentiviral vectors is the ability to mediate potent transduction and stable expression into dividing and non-dividing cells both *in vitro* and *in vivo*. Lentiviral vectors are typically produced in HEK 293T cells. Essential lentiviral (*HIV-1*) genes must be expressed in these cells to allow the generation of lenti-

viral particles.

Peer review

Sun *et al* analyzed the expression, clinical significance of EMP1 in colorectal carcinoma and the biological effect in a colorectal carcinoma cell line by EMP1 overexpression using cell and molecular biological and biochemical techniques. The study was well designed and contributed to the understanding that EMP1 protein levels were significantly lower in colorectal carcinoma than in normal tissue.

REFERENCES

1 **Bolocan A**, Ion D, Ciocan DN, Paduraru DN. Prognostic and predictive factors in colorectal cancer. *Chirurgia* (Bucur) 2012; **107**: 555-563 [PMID: 23116828]

2 **Ferlay J**, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893-2917 [PMID: 21351269 DOI: 10.1002/ijc.25516]

3 **Dimofte G**, Târcoveanu E, Taraşi M, Panait C, Lozneanu G, Nicolescu S, Porumb V, Grigoraş O. Mean number of lymph nodes in colonic cancer specimen: possible quality control index for surgical performance. *Chirurgia* (Bucur) 2011; **106**: 759-764 [PMID: 22308913]

4 **Fleming M**, Ravula S, Tatishchev SF, Wang HL. Colorectal carcinoma: Pathologic aspects. *J Gastrointest Oncol* 2012; **3**: 153-173 [PMID: 22943008 DOI: 10.3978j/issn.2078-6891.2012.030]

5 **Lai S**, Wang G, Cao X, Li Z, Hu J, Wang J. EMP-1 promotes tumorigenesis of NSCLC through PI3K/AKT pathway. *J Huazhong Univ Sci Technolog Med Sci* 2012; **32**: 834-838 [PMID: 23271282 DOI: 10.1007/s11596-012-1043-1]

6 **Wang HT**, Liu ZH, Wang XQ, Wu M. [Effect of EMP-1 gene on human esophageal cancer cell line]. *Ai Zheng* 2002; **21**: 229-232 [PMID: 12451984]

7 **Lee HS**, Sherley JL, Chen JJ, Chiu CC, Chiou LL, Liang JD, Yang PC, Huang GT, Sheu JC. EMP-1 is a junctional protein in a liver stem cell line and in the liver. *Biochem Biophys Res Commun* 2005; **334**: 996-1003 [PMID: 16036215 DOI: 10.1016/j.bbrc.2005.06.194]

8 **Kasher R**, Bajayo A, Gabet Y, Nevo N, Fridkin M, Katchalski-Katzir E, Kohen F, Bab I. Restrain of bone growth by estrogen-mimetic peptide-1 (EMP-1): a micro-computed tomographic study. *Peptides* 2009; **30**: 1181-1186 [PMID: 19463753 DOI: 10.1016/j.peptides.2009.02.019]

9 **Bozec A**, Peyrade F, Milano G. Molecular targeted therapies in the management of head and neck squamous cell carcinoma: recent developments and perspectives. *Anticancer Agents Med Chem* 2013; **13**: 389-402 [PMID: 23092267 DOI: 10.2174/1871520611313030003]

10 **Suzuki K**, Nakamura K, Kato K, Hamada H, Tsukamoto T. Exploration of target molecules for prostate cancer gene therapy. *Prostate* 2007; **67**: 1163-1173 [PMID: 17516570 DOI: 10.1002/pros.20613]

11 **Turashvili G**, Bouchal J, Ehrmann J, Fridman E, Skarda J, Kolar Z. Novel immunohistochemical markers for the differentiation of lobular and ductal invasive breast carcinomas. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 2007; **151**: 59-64 [PMID: 17690741 DOI: 10.5507/bp.2007.010]

12 **Muller PY**, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* 2002; **32**: 1372-1374, 1376, 1378-1379 [PMID: 12074169]

13 **Ranganathan V**, De PK. Western blot of proteins from Coomassie-stained polyacrylamide gels. *Anal Biochem* 1996; **234**: 102-104 [PMID: 8742090 DOI: 10.1006/abio.1996.0057]

14 **van Meerloo J**, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. *Methods Mol Biol* 2011; **731**: 237-245 [PMID: 21516412 DOI: 10.1007/978]

15 **Rasola A**, Geuna M. A flow cytometry assay simultaneously detects independent apoptotic parameters. *Cytometry* 2001; **45**: 151-157 [PMID: 11590627 DOI: 10.1002/1097-0320(20011001)45]

16 **Kramer N**, Walzl A, Unger C, Rosner M, Krupitza G, Hengstschläger M, Dolznig H. In vitro cell migration and invasion assays. *Mutat Res* 2013; **752**: 10-24 [PMID: 22940039 DOI: 10.1016/j.mrrev.2012.08.001]

17 **Richards RJ**. Responsibility for statistical analyses. *Endocr Pract* 2003; **9**: 329 [PMID: 14569976]

18 **Taylor V**, Welcher AA, Program AE, Suter U. Epithelial membrane protein-1, peripheral myelin protein 22, and lens membrane protein 20 define a novel gene family. *J Biol Chem* 1995; **270**: 28824-28833 [PMID: 7499407 DOI: 10.1074/jbc.270.48.28824]

19 **Lobsiger CS**, Magyar JP, Taylor V, Wulf P, Welcher AA, Program AE, Suter U. Identification and characterization of a cDNA and the structural gene encoding the mouse epithelial membrane protein-1. *Genomics* 1996; **36**: 379-387 [PMID: 8884260 DOI: 10.1006/geno.1996.0482]

20 **Wulf P**, Suter U. Embryonic expression of epithelial membrane protein 1 in early neurons. *Brain Res Dev Brain Res* 1999; **116**: 169-180 [PMID: 10521561 DOI: 10.1016/S0165-3806(99)00092-9]

21 **Zoidl G**, Blass-Kampmann S, D'Urso D, Schmalenbach C, Müller HW. Retroviral-mediated gene transfer of the peripheral myelin protein PMP22 in Schwann cells: modulation of cell growth. *EMBO J* 1995; **14**: 1122-1128 [PMID: 7720703]

22 **Jetten AM**, Suter U. The peripheral myelin protein 22 and epithelial membrane protein family. *Prog Nucleic Acid Res Mol Biol* 2000; **64**: 97-129 [PMID: 10697408 DOI: 10.1016/S0079-6603(00)64003-5]

23 **Yu XM**, Li CW, Li YY, Liu J, Lin ZB, Li TY, Zhao L, Pan XL, Shi L, Wang de Y. Down-regulation of EMP1 is associated with epithelial hyperplasia and metaplasia in nasal polyps. *Histopathology* 2013; **63**: 686-695 [PMID: 24033346 DOI: 10.1111/his.12211]

24 **Gnirke AU**, Weidle UH. Investigation of prevalence and regulation of expression of progression associated protein (PAP). *Anticancer Res* 1998; **18**: 4363-4369 [PMID: 9891493]

25 **Zhang J**, Cao W, Xu Q, Chen WT. The expression of EMP1 is downregulated in oral squamous cell carcinoma and possibly associated with tumour metastasis. *J Clin Pathol* 2011; **64**: 25-29 [PMID: 20980531 DOI: 10.1136/jcp.2010.082404]

26 **Wang HT**, Kong JP, Ding F, Wang XQ, Wang MR, Liu LX, Wu M, Liu ZH. Analysis of gene expression profile induced by EMP-1 in esophageal cancer cells using cDNA Microarray. *World J Gastroenterol* 2003; **9**: 392-398 [PMID: 12632483]

27 **Walker J**, Quirke P. Prognosis and response to therapy in colorectal cancer. *Eur J Cancer* 2002; **38**: 880-886 [PMID: 11978512 DOI: 10.1016/S0959-8049(02)00044-8]

28 **Guastadisegni C**, Colafranceschi M, Ottini L, Dogliotti E. Microsatellite instability as a marker of prognosis and response to therapy: a meta-analysis of colorectal cancer survival data. *Eur J Cancer* 2010; **46**: 2788-2798 [PMID: 20627535 DOI: 10.1016/j.ejca.2010.05.009]

29 **Quirke P**, Williams GT, Ectors N, Ensari A, Piard F, Nagtegaal I. The future of the TNM staging system in colorectal cancer: time for a debate? *Lancet Oncol* 2007; **8**: 651-657 [PMID: 17613427 DOI: 10.1016/S1470-2045(07)70205-X]

30 **Jiexian J**, Xiaoqin X, Lili D, Baoguo T, Ting S, Xianwen Z, Cunzhi H. Clinical assessment and prognostic evaluation of tumor markers in patients with gastric cancer. *Int J Biol Markers* 2013; **28**: 192-200 [PMID: 23787496]

31 **Sen S**, Kawahara B, Chaudhuri G. Mitochondrial-associated nitric oxide synthase activity inhibits cytochrome c oxidase: implications for breast cancer. *Free Radic Biol Med* 2013; **57**: 210-220 [PMID: 23089229]

32 **Zhang JM**, Wang HC, Wang HX, Ruan LH, Zhang YM, Li JT, Tian S, Zhang YC. Oxidative stress and activities of caspase-8, -9, and -3 are involved in cryopreservation-induced apoptosis in granulosa cells. *Eur J Obstet Gynecol Reprod Biol*

2013; **166**: 52-55 [PMID: 23006767]
33 **Johnson CR**, Jarvis WD. Caspase-9 regulation: an update. *Apoptosis* 2004; **9**: 423-427 [PMID: 15192324 DOI: 10.1023/B:APPT.0000031457.90890.13]
34 **Fearnhead HO**, Rodriguez J, Govek EE, Guo W, Kobayashi R, Hannon G, Lazebnik YA. Oncogene-dependent apoptosis is mediated by caspase-9. *Proc Natl Acad Sci USA* 1998; **95**: 13664-13669 [PMID: 9811857]
35 **Xie LX**, Zhai TT, Yang LP, Yang E, Zhang XH, Chen JY, Zhang H. Lymphangiogenesis and prognostic significance of vascular endothelial growth factor C in gastro-oesophageal junction adenocarcinoma. *Int J Exp Pathol* 2013; **94**: 39-46 [PMID: 23317352]
36 **Zhang Y**, Meng X, Zeng H, Guan Y, Zhang Q, Guo S, Liu X, Guo Q. Serum vascular endothelial growth factor-C levels: A possible diagnostic marker for lymph node metastasis in patients with primary non-small cell lung cancer. *Oncol Lett* 2013; **6**: 545-549 [PMID: 24137365]

P- Reviewers: Lu JC, Ravi M **S- Editor**: Song XX
L- Editor: Wang TQ **E- Editor**: Ma S

百世登
Baishideng®

Published by **Baishideng Publishing Group Co., Limited**
Flat C, 23/F., Lucky Plaza,
315-321 Lockhart Road, Wan Chai, Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

ISSN 1007-9327