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RAPID COMMUNICATION

Expression of Livin and vascular endothelial growth factor in different clinical stages of human esophageal carcinoma

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Abstract

AIM: To investigate the role of Livin and vascular endothelial growth factor (VEGF) in human esophageal carcinoma, and analyze its relationship to clinical stages.

METHODS: Expression of Livin in fresh esophageal cancer tissues was detected by immunohistochemistry (IHC), Western blotting and reverse transcriptase-polymerase chain reaction (RT-PCR), and VEGF by Western blotting and RT-PCR. All statistical analyses were performed by SPSS version 11.0.

RESULTS: Livin positivity was also significantly correlated with tumor stages, increasing with tumor progression. Expression of Livin and VEGF increased with the process of esophageal carcinoma. In the fourth clinical stage, expression of Livin and VEGF was the most significant. Expression of Livin was positively correlated with VEGF.

CONCLUSION: Over-expression of Livin and VEGF contributes to the pathogenesis of esophageal carcinoma.

Key words: Esophageal carcinoma; Livin; Vascular endothelial growth factor

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INTRODUCTION

Livin, also known as inhibitor of apoptosis (IAP) has been identified as a new member of the IAP family proteins[1-3]. Like other IAP family proteins, Livin interacts with downstream caspases, such as caspase-3, caspase-7, and caspase-9, leading to their inactivation and degradation[4,5]. Its overexpression can protect cells from several proapoptotic stimuli. Very importantly, treatment of cancer cells with Livin antisense oligo-DNA causes apoptotic cell death, indicating that Livin expression may be essential for survival of certain cancer cells[6,7]. Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells, and its expression has been correlated with increased tumour angiogenesis[8,9]. VEGF plays a crucial role in tumour expansion by initiating permeabilization of blood vessels, by extravasation of plasma proteins, by invasion of stromal cells, and by causing the sprouting of new blood vessels that supply the tumour with oxygen and nutrients. A number of studies have shown that expression of certain VEGF transcripts are correlated with tumour progression[10]. Although increases of certain VEGF transcripts have been demonstrated to correlate with the progression of various tumours, the actual protein levels of the different VEGF isoforms and their significance during cellular transformation are unknown. Moreover, it has been suggested that elevated protein expression in tumour tissues was mediated by both enhanced transcription and translation. Thus, in order to understand the role of Livin and VEGF in tumour progression, it is important to investigate Livin and VEGF expression of different clinical stages at the protein and mRNA level during tumourigenesis. Esophageal carcinoma is one of the most frequent malignancies in many countries. Despite recent progress in chemotherapeutic, radiotherapeutic, and surgical treatment, the 5-year survival rate of esophageal carcinoma patients is still low, especially in advanced cases. In order to further explore the role of Livin and

VEGF in the development of esophageal carcinoma, we investigated the role of Livin and VEGF in human esophageal carcinoma and analyze its relationship with clinical stages.

MATERIALS AND METHODS

Materials

Specimens of cancer tissues were taken from 67 consecutive patients with esophageal carcinoma from Oct 2004 to Sept 2005 at the Department of Thoracic Surgery, the First Affiliated Hospital of Chongqing Medical University. None of them received irradiation or chemotherapy preoperatively. The patients included 46 men and 21 women with a mean age of 57 ranges from 38-86 years. The clinicopathologic stage was determined according to TNM classification. Six tumors were located in the upper thorax, 6 cases for clinicopathologic stage one, 24 case clinicopathologic stage two, 28 cases clinicopathologic stage three, 9 cases clinicopathologic stage four. All fresh tissues were taken immediately after operation and stored in a nitrogen canister. Informed consent was obtained from all participants, and the study was approved by the ethics committee on human research in Chongqing Medical University, Chongqing, China.

Immunohistochemical (IHC) assay

Tissue samples were collected after surgery and immediately frozen in liquid nitrogen. Prior to IHC assay, frozen sections were prepared with a cryostat (FACS caliber, Becton Dickinson, USA) at -20℃, dried at room temperature, and fixed with acetone. The PBMC were routinely isolated and the slides were prepared with a cytospinner. The ABC immunohistochemical assay was carried out according to the protocols we described before Anti-Livin (Antibody Diagnosis, USA) was prepared in our lab. The second antibody, a goat anti-mouse IgG labeled with biotin, was purchased from Vector Co., USA. Two hundred cells were counted and the intensity of staining for each of those cells was adjusted. Five grades were employed to express the degrees of staining, which represent 5 reaction coefficients respectively. The 5 products of every coefficient and the corresponding cell number were added up, which resulted in the value of a positive score. All slides were measured in duplicate. Those samples with a positive score over 10 or frequency over 5% were considered as positive.

Western blotting

Mouse tissues were dissected and homogenized in T-PER buffer in the presence of protease inhibitors. After homogenization, the lysates were centrifuged at 100 000 × *g*, and the supernatants were saved for Western blot, Ciphergen (BioSource International, Inc., USA) Protein Chip Array. Equal amounts of lysates were subject to SDS-PAGE (Tris-glycine mini gel; 1:2500, BioSource International, Inc., USA) and Western blot analysis using antibodies specific for the following: Livin (1:2500, BioSource International, Inc., USA), VEGF (1:2500, BioSource International, Inc., USA), β-tublin (1:5000; BioSource International, Inc., USA). The optical densities of the specific bands were scanned and measured by image analysis software (HPIAS 2000, Tongji Qianping Company, Wuhan, China).

Table 1 Oligonucleotides used for reverse transcriptase-polymerase chain reaction

Target gene	Primer sequence (5′-3′)	Size (bp)	Annealing temperature (℃)
β-actin	Forward: GTTCGCCATGGA TGACGATATC	266	59
	Reverse: GCCAGATCTTCTC CATGTCGTC		
VEGF	Forward: TGCTCAGCATTC GGACTGACCT	228	61
	Reverse: CAGTATGCATGGA CCATGACGG		
Livin	Forward: CTGGTCAGAGCC AGTGTTCCT	312	61
	Reverse: TCATAGAAGGA GGCCAGACG		

Primers were designed using the Primer Express Program and offered by AuGCT Biotechnology, Beijing, China.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Animals were sacrificed at corresponding time points and total RNA in the treated sections were extracted according to the total RNA extracting kit. 4 μg total RNA was heated at 70℃ for 5 min and then chilled on ice. Samples were incubated at 37℃ for 1 h and the reaction was stopped by heating at 70℃ for 10 min. Specific primers were designed for PCR: *Livin* and *VEGF* (Table 1). PCR was performed using 2 μL cDNA, 2 mmol/L dNTP, a specific pair of primers (20 pmol), 2 U DNA polymerase, 5 × PCR buffer and deionized water were added to the cDNA. The total volume was 25 μL. Amplification was performed for 32 cycles. The PCR products were separated by electrophoresis using a 1.5% (w/v) agarose gel containing 0.5 mg/L of ethidium bromide. Single band corresponding to the predicted size of the amplified product for Livin and VEGF and β-actin were identified under an ultraviolet transilluminator and transferred to a nylon filter membrane, and hybridized with an ECL-labeled probe 10 mL. The probes hybridized only to the bands which corresponded in size to the ethidium bromide stained gels, thereby confirming the amplified PCR products. The band densities were scanned with a densitometer. The relative amount of mRNA in each sample was calculated from the densitometry ratio of Livin and VEGF *A* value/β-actin *A* value.

Statistical analysis

Quantitative data were expressed as mean ± SD. All statistical analyses used the SPSS software for Windows 11.0 (SPSS, Inc., Chicago, IL, USA), using Student's *t*-test for intergroup. For statistical evaluation one-way analysis of variance (ANOVA) were employed. Pearson correlation analysis was also performed to some index. $P < 0.05$ was considered as statistically significant.

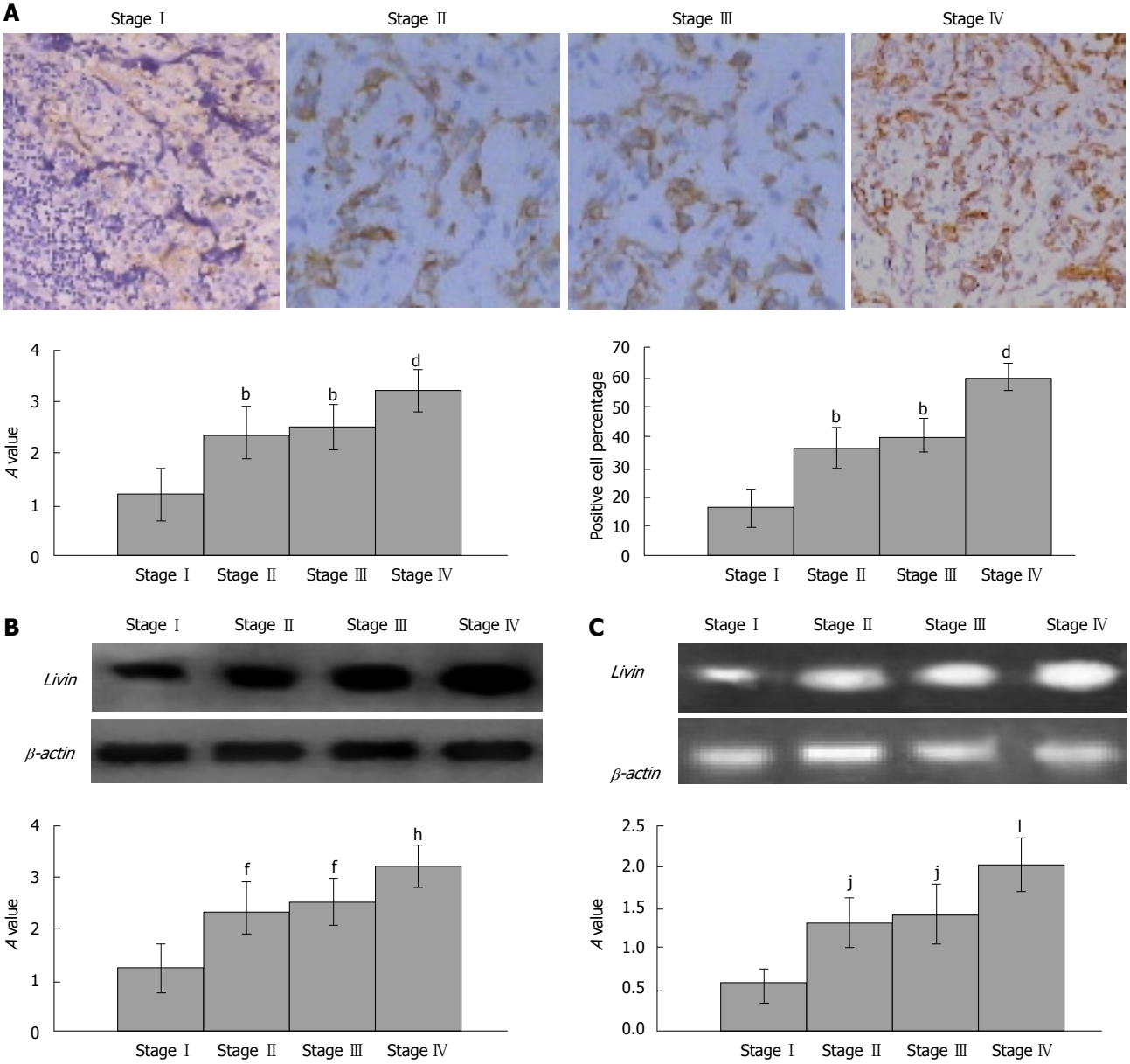

Figure 1 **A**: The expression of Livin was measured by IHC (SP × 400). Optical density value and positive cell percentage in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^{b}P < 0.01$). Furthermore, optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($^{d}P < 0.01$). IHC showed that Livin had significant expression in the cytoplasm and nucleus in the Stage Ⅱ, Ⅲ and Ⅳ (the cytoplasm and nucleus had stained yellow), slight expression in the cytoplasm and nucleus in Stage Ⅰ (the cytoplasm and nucleus had slightly stained yellow); **B**: The expression of Livin by Western blotting showed that expression of Livin in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^{f}P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($^{h}P < 0.01$); **C**: mRNA level of *Livin* was tested by RT-PCR. Up-regulation of *Livin* gene transcription matched with the protein level of Livin that was significantly increased along with the progression of esophageal carcinoma. Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^{j}P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($^{l}P < 0.01$).

RESULTS

Expression of Livin in esophageal carcinoma

The expression of Livin measured by IHC showed that expression of Livin in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Furthermore, optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($P < 0.01$) (Figure 1A). The results by IHC showed that expression of Livin increases along with the progression of esophageal carcinoma. To further determine that Livin contributes to the pathogenesis of esophageal carcinoma, the expression of Livin was tested by Western blotting. In coincidence with IHC results, the expression of Livin by Western blotting showed that expression of Livin in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value and

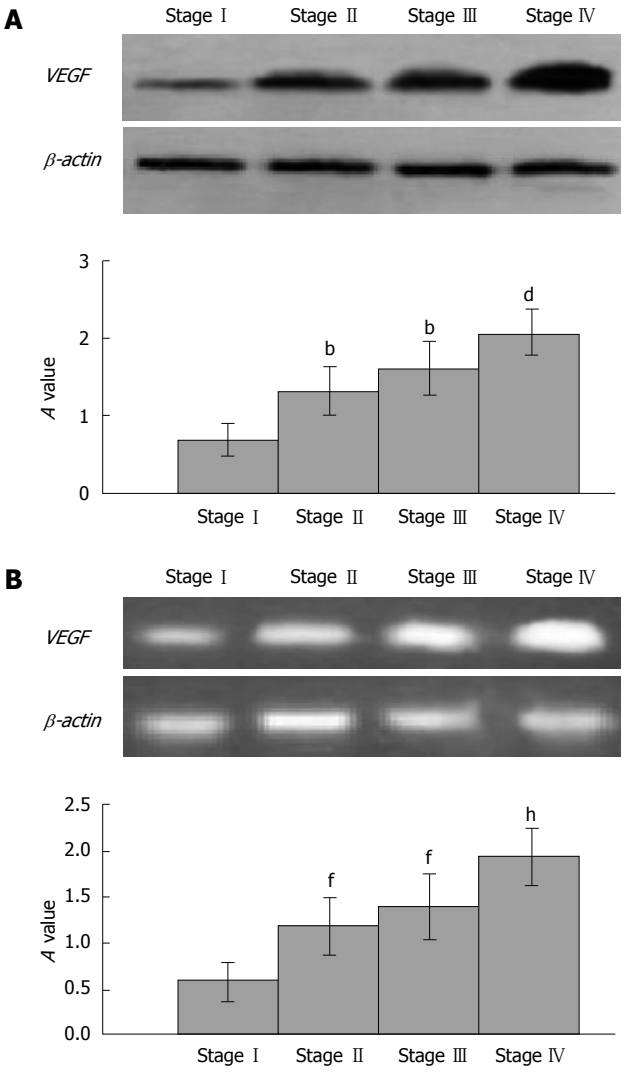

Figure 2 Assay of level of VEGF. **A**: The expression of VEGF by Western blotting showed that expression of VEGF in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^bP < 0.01$). Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^bP < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($^dP < 0.01$); **B**: Up-regulation of VEGF gene transcription matched with the protein level of VEGF that was significantly increased along with the progression of esophageal carcinoma. Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($^fP < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($^hP < 0.01$).

positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($P < 0.01$) (Figure 1B). Previously, the protein level of Livin increases the progression of esophageal carcinoma. To further evaluate that Livin contributes to the pathogenesis of esophageal carcinoma, the mRNA level of *Livin* was tested by RT-PCR. Up-regulation of *Livin* gene transcription matched with the protein level of Livin that was significantly increased along with the progression of esophageal carcinoma. Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($P < 0.01$) (Figure 1C).

Expression of VEGF in esophageal carcinoma

The expression of VEGF measured by Western blotting and RT-PCR showed expression of VEGF increases along with the progression of esophageal carcinoma. The expression of VEGF by Western blotting showed that expression of VEGF in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($P < 0.01$) (Figure 2A). Previously, the protein level of VEGF increases the progression of esophageal carcinoma. To further evaluate that VEGF contributes to the pathogenesis of esophageal carcinoma, the mRNA level of *VEGF* was tested by RT-PCR. Up-regulation of *VEGF* gene transcription matched with the protein level of VEGF that was significantly increased along with the progression of esophageal carcinoma. Optical density value in clinicopathologic stage two, three and four was significantly higher than that of stage one ($P < 0.01$). Optical density value and positive cell percentage in clinicopathologic stage four was significantly higher than that of stage two and three ($P < 0.01$) (Figure 2B). Pearson correlation analysis showed that the level of VEGF by Western blotting has a positive correlation with Livin ($r = 0.384$, $P < 0.05$), and VEGF by RT-PCR a positive correlation with Livin ($r = 0.452$, $P < 0.05$) as well. The hypothesis has been made that Livin and VEGF play such an inter-enhancement role in the progress of esophageal carcinoma.

DISCUSSION

Livin may be essential for survival of certain cancer cells[11-13]. Of the IAP family members, CARD-RING domain of cIAP1, CARD-RING domain of cIAP2, X-linked IAP, and NAIP are expressed in normal adult tissues[14,15], whereas Survivin expression is limited to tumor tissues[16-18]. It has been reported that Livin was expressed in some tumor cells and several fetal tissues but not in normal adult tissues. Hence, its expression profiles seem to be very similar to those of Survivin, a cancer-specific IAP family protein. In the present study, we investigated expression of Livin in human esophageal carcinoma and analyze its relationship with clinical stages. The results showed that Livin positivity was also significantly correlated with tumor stages, increasing with tumor progression. Expression of Livin increased with the process of esophageal carcinoma. In the fourth clinical stage, expression of Livin was the most significant. Therefore, over-expression of Livin contributes to the pathogenesis of esophageal carcinoma. Livin is known to play an important role in antiapoptotic cell survival by suppression

of caspase family proteins. The other antiapoptotic proteins, including IAP family and Bcl-2 family[19,20], were also reported to be overexpressed in esophageal carcinoma cells. Namely, Survivin, a member of the IAP family, was overexpressed in esophageal carcinoma specimens, and patients with Survivin expression had significant unfavorable prognosis. Survivin was one of the tumor-associated antigens recognized by both humoral and cellular immunity of cancer patients, and could become a target of CTL. It has been reported that Livin might be involved in the progression of superficial bladder cancer and used as a marker of early recurrence[19,21,22]. Because loss of Livin expression could lead to apoptotic cell death in cervical cancer cells, suppression of Livin should have much advantage in cancer treatment. From this perspective, Livin might be a good candidate as a molecular target for treatment as well as having a prognostic value for esophageal carcinoma.

VEGF plays a crucial role in tumour expansion by initiating permeabilization of blood vessels, by extravasation of plasma proteins, by invasion of stromal cells, and by causing the sprouting of new blood vessels that supply the tumour with oxygen and nutrients. A number of studies have shown that expression of certain VEGF transcripts are correlated with tumour progression[23,24]. Although increases of certain VEGF transcripts have been demonstrated to correlate with the progression of various tumours[25-27], the actual protein levels of the different VEGF isoforms and their significance during cellular transformation are unknown. Moreover, it has been suggested that elevated protein expression in tumour tissues was mediated by both enhanced transcription and translation. Thus, in order to understand the role of VEGF in tumour progression, it is important to investigate VEGF expression of different clinical stages at the protein and mRNA level during tumourigenesis. In the present study, we investigated expression of VEGF in human esophageal carcinoma and analyze its relationship with clinical stages. The results showed that VEGF positivity was also significantly correlated with tumor stages, increasing with tumor progression. Expression of VEGF increased with the process of esophageal carcinoma. In the fourth clinical stage, expression of VEGF was the most significant. Because previous evidence indicated that VEGF plays a crucial role in tumour expansion by initiating permeabilization of blood vessels, our results suggest that over-expression of VEGF contributes to the pathogenesis of esophageal carcinoma. Allowing that VEGF introduces the sprouting of new blood vessels that supply the tumour with oxygen and nutrients, it is a novel strategy for treating esophageal carcinoma, and the results of the ongoing clinical trials in patients with esophageal carcinoma are eagerly awaited. Although encouraging data have emerged to support the use of antiangiogenic therapy in some cancers such as myeloma and glioma, poor tumor response has been reported in others[28-31]. One major problem confronting clinical trials of antiangiogenic therapy is the lack of an established surrogate marker to measure antiangiogenic activity *in vivo* in cancer patients. Tumor response in terms of shrinkage alone might not be an appropriate index of treatment efficacy because of the cytostatic nature of the treatment. Instead, the ability of an antiangiogenic drug to induce prolonged stabilization of the disease and increase survival might be more meaningful end points for clinical trials on antiangiogenic therapy.

Taken together, over-expression of Livin and VEGF contributes to the pathogenesis of esophageal carcinoma. The level of VEGF has a positive correlation with Livin. The hypothesis has been made that Livin and VEGF played such an inter-enhancement role in the progress of esophageal carcinoma. Inhibitors of Livin and VEGF may be potential targets for the prevention or treatment of human esophageal carcinoma.

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COMMENTS

Background

Livin expression may be essential for survival of certain cancer cells. Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells, and its expression has been correlated with increased tumour angiogenesis. It is important to investigate Livin and VEGF expression of different clinical stages at the protein and mRNA level during tumourigenesis.

Research frontiers

In order to further explore the role of Livin and VEGF in the development of esophageal carcinoma, it is critical to investigate the role of Livin and VEGF in human esophageal carcinoma and analyze its relationship with clinical stages.

Innovations and breakthroughs

Over-expression of Livin and VEGF contributes to the pathogenesis of esophageal carcinoma. The level of VEGF has a positive correlation with Livin. The hypothesis has been made that Livin and VEGF have an inter-enhancement role in the progress of esophageal carcinoma.

Applications

Inhibitors of Livin and VEGF may be potential targets for the prevention or treatment of human esophageal carcinoma.

Terminology

Livin may be essential for survival of certain cancer cells. Of the IAP family members, Livin interacts with downstream caspases, such as caspase-3, caspase-7, and caspase-9, leading to their inactivation and degradation. Its overexpression can protect cells from several proapoptotic stimuli. Very importantly, treatment of cancer cells with Livin antisense oligo DNA causes apoptotic cell death, indicating that Livin expression may be essential for survival of certain cancer cells. VEGF is a potent mitogen for endothelial cells, and its expression has been correlated with increased tumour angiogenesis.

Peer review

This is an interesting report showing increased expression of Livin and VEGF in late stages (Ⅱ Ⅲ Ⅳ) of esophageal carcinoma compared to early stage (Ⅰ) of esophageal carcinoma. Since Livin expression may affect apoptosis, correlation of Livin with assays of apoptosis will be of interest. In addition, correlation of Livin expression with patient survival in various stages of esophageal carcinoma will also be interesting.

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