Acta Biochim Biophys Sin 2011, 43: 455–464 | © The Author 2011. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr033.

Original Article

Potential transcriptional regulatory regions exist upstream of the human ezrin gene promoter in esophageal carcinoma cells

Shuying Gao[1], Yanpeng Dai[1], Meijun Yin[1], Jing Ye[1], Gang Li[2], and Jie Yu[1,3*]

[1]Central Laboratory, Shenzhen PKU-HKUST Medical Center, Peking University Shenzhen Hospital, Shenzhen 518036, China
[2]Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Peking University, Beijing 10081, China
[3]Laboratory of Male Reproduction, Peking University Shenzhen Hospital, Shenzhen PKU-HKUST Medical Center, Shenzhen 518036, China
*Correspondence address. Tel: +86-755-83923333-3516; Fax: +86-755-83923333-3516; E-mail: yujie007@hotmail.com

We previously demonstrated that the region −87/+134 of the human ezrin gene (*VIL2*) exhibited promoter activity in human esophageal carcinoma EC109 cells, and a further upstream region −1324/−890 positively regulated transcription. In this study, to identify the transcriptional regulatory regions upstream of the *VIL2* promoter, we cloned *VIL2* −1541/−706 segment containing the −1324/−890, and investigated its transcriptional regulatory properties via luciferase assays in transiently transfected cells. In EC109 cells, it was found that *VIL2* −1541/−706 possessed promoter and enhancer activities. We also localized transcriptional regulatory regions by fusing 5′- or 3′-deletion segments of *VIL2* −1541/−706 to a luciferase reporter. We found that there were three positive and one negative transcriptional regulatory regions within *VIL2* −1541/−706 in EC109 cells. When these regions were separately located upstream of the luciferase gene without promoter, or located upstream of the *VIL2* promoter or SV40 promoter directing the luciferase gene, only *VIL2* −1297/−1186 exhibited considerable promoter and enhancer activities, which were lower than those of −1541/−706. In addition, transient expression of Sp1 increased ezrin expression and the transcriptional activation of *VIL2* −1297/−1186. Other three regions, although exhibiting significantly positive or negative transcriptional regulation in deletion experiments, showed a weaker or absent regulation. These data suggested that more than one region upstream of the *VIL2* promoter participated in *VIL2* transcription, and the *VIL2* −1297/−1186, probably as a key transcriptional regulatory region, regulated *VIL2* transcription in company with other potential regulatory regions.

Keywords ezrin; *VIL2*; promoter; enhancer; esophageal carcinoma

Received: January 12, 2011 Accepted: March 4, 2011

Introduction

Ezrin, encoded by *VIL2*, is a membrane–cytoskeleton linker protein involved in a wide variety of cellular processes such as adhesion [1], survival [2], motility [3], and signal transduction [4–6]. Recent data have provided further evidence for the novel roles of ezrin in the control of cyclin A gene transcription and endothelial cell proliferation [7], as a mediator of c-Myc-induced tumorigenesis in prostate cancer cells [8], and in the bacterial uptake by trophoblast giant cells [9].

Ezrin is often aberrantly expressed in human cancers. There is a relationship between high expression of ezrin and metastatic potential of some carcinomas, including medulloblastoma [10], hepatocellular carcinoma [11], lung cancer [12], breast carcinoma [13], pancreatic adenocarcinoma [14], gastric cancer cell [15], and colorectal cancer [16]. We have also demonstrated that ezrin is overexpressed in a malignantly transformed esophageal epithelial cell line compared to an immortalized cell line [17]. Our recent studies on esophageal squamous cell carcinoma (ESCC) samples have shown that ezrin tends to translocate from the plasma membrane to the cytoplasm in the progression from normal epithelium to invasive carcinoma of the esophagus [18], and ezrin overexpression in ESCCs is associated with poor survival [19]. Moreover, both *in vivo* and *in vitro* experiments suggest that ezrin may directly affect tumor formation and tumor invasiveness [20]. These findings of ezrin upregulation associated with tumor metastasis and invasion make ezrin a potentially new prognostic marker and/or therapeutic target for some carcinomas [11,13,21–25].

There are few reports regarding the regulation of ezrin expression. It has been reported that insulin-like growth factor 1 inhibits ezrin expression in human colon cancer cells, while epidermal growth factor and interleukin-11 increases cellular ezrin levels [26]. Moreover, tumor necrosis factor-α treatment of human endothelial cells elevates ezrin expression [27]. In

disseminated osteosarcoma, ezrin is strongly stained by immunohistochemistry and has been proposed as a crucial factor for osteosarcoma metastasis [28]. Ogino *et al*. [29] have demonstrated a high level of ezrin mRNA expression in an osteosarcoma biopsy sample with lung metastasis, which is in agreement with previous reports analyzing ezrin protein levels. These data suggest that ezrin levels are controlled at the transcriptional level. Our recent study on ezrin regulation demonstrated that the cooperativity of Sp1 and AP-1 (c-Jun/c-Fos heterodimer) regulated *VIL2* promoter activity and ezrin expression, and that mitogen-activated protein kinase kinase (MEK1/2) and extracellular signal-regulated kinase (ERK1/2) were upstream kinases that controlled human *VIL2* transcriptional activation in ESCC cells [19]. There are relationships between ezrin expression and transcriptional activation of the *VIL2* promoter in esophageal carcinoma cells. We also found that the region within human *VIL2* −1324/−890 positively regulated transcription. However, the transcriptional regulatory regions upstream of the *VIL2* promoter in ESCC cells remain unclear.

In the present study, we investigated the transcriptional regulatory characteristics of DNA sequence upstream of the *VIL2* promoter, and explored the importance of *VIL2* fragments in regulating luciferase reporter expression in human esophageal carcinoma cells (EC109 cells), which are derived from ESCC. We found that there was more than one region upstream of the *VIL2* promoter participating in *VIL2* transcription. The −1297/−1186 region, exhibited considerable promoter and enhancer activities, probably as a key transcriptional regulatory region, as well as regulated *VIL2* transcription in company with other potential regulatory regions in a cooperative or synergistic manner.

Materials and Methods

Materials

Expression plasmid CMV-Sp1 was kindly provided by Dr Guntram Suske (Philips University, Marburg, Germany). Plasmid pcDNA3 was purchased from Invitrogen (Carlsbad, USA). Plasmids pGL3-Basic, pGL3-Promoter, and pRL-TK, Dual-luciferase® reporter assay systems were purchased from Promega (Madison, USA). Lipofectamine™ 2000 transfection reagent was purchased from Invitrogen. T4 DNA polymerase, T4 DNA ligase, endonucleases *Apa*I, *Bgl*II, *Bss*HII, *Hin*dIII, *Kpn*I, *Mlu*I, *Nhe*I, *Pst*I, *Sac*II, *Sal*I, *Stu*I, *Sma*I, and *Xho*I were purchased from Takara (Dalian, China). Antibodies against Sp1, Ezrin, and β-Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). All other reagents were of analytical grade.

Plasmids construction

All *VIL2* segments that originated from genomic DNA of EC109 cells were generated by polymerase chain reaction (PCR). Plasmids were constructed using standard methods [30] and are described briefly below and in **Table 1**. Plasmid pGL3-hE(−87/+134) was constructed by amplifying *VIL2* −87/+134 using primers Fezr1 (5′-CTAGC TAGCCCGCAGTGCTGGGCGGGGCGCTGAC-3′) and Rezr1 (5′-CCCAAGCTTTCGGTTTCTGGTGAGTATCC TCGATCC-3′), digesting PCR products with *Nhe*I/*Hin*dIII, and inserting it into *Nhe*I/*Hin*dIII sites of pGL3-Basic. Plasmids pGL3-hE(−1541/−706) and pGL3-P(SV40)-hE(−1541/−706) were constructed by amplifying *VIL2* −1541/−706 using primers Fezr2 (5′-CGGGTACCAAA CGTGCCACTTAACCAGAGCTTCG-3′) and Rezr2 (5′-A CGCGTCGACAAGCCCGTGAGAAGCCGAGCACTC-3′), digesting PCR products with *Kpn*I/*Sal*I, and inserting it into *Kpn*I/*Xho*I sites of pGL3-Basic or pGL3-Promoter. pGL3-hE(−919/−773) was made by first deleting *VIL2* −1187/−947 of pGL3-hE(−1541/−706) with *Sma*I to construct a passage vector A, then deleting *VIL2* −772/−706 of vector A with *Pst*I/*Bgl*II to construct another passage vector B, and finally digesting vector B with *Kpn*I/*Sac*II and self-ligating recovery fragments. In **Table 1**, plasmid Nos 1 to 14 were made by deleting *VIL2* fragments of their source vectors with appointed endonucleases and then self-ligating recovery fragments. Plasmid Nos 15 to 22 were made by first digesting source vector (i) with *Hin*dIII, blunting the fragment with T4 DNA polymerase, and digesting it with *Xba*I, thus recovering the fragments containing Amp^r gene and *VIL2* 5′-flanking region; then digesting source vector (ii) with *Kpn*I, blunting the fragment with T4 DNA polymerase, and digesting it with *Xba*I, thus recovering the fragments containing *VIL2* promoter −87/+134 or SV40 promoter; and finally ligating the two recovery fragments. In the name of plasmids, the letters "P(hE)" and "P(SV40)" indicate that the plasmids carry *VIL2* −87/+134 and SV40 promoter, respectively.

Cell culture and transfection

EC109 and CNE2 cells were maintained in 1640 medium (Invitrogen), and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen), which was supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in a 5% carbon dioxide environment. For transfection, cells were seeded in 96-well plates at 1.5×10^5cells/mL, grown to 50−80% confluency and transfected with the plasmids described above using Lipofectamine™ 2000 Reagent (Invitrogen) according to the manufacturer's protocol. After transfection, cells were incubated for another 48 h before being harvested for the luciferase assay or gene expression assay.

Luciferase assay

Transfected cells were harvested in passive lysis buffer (Promega) and the cell lysates were analyzed for luciferase

Table 1 Construction of plasmids in this study

Plasmid number	Plasmid names	Source vectors	Endonucleases for digesting source vector
1	pGL3-hE(−1445/−706)	pGL3-hE(−1541/−706)	*Kpn*I/*Stu*I
2	pGL3-hE(−1297/−706)	pGL3-hE(−1541/−706)	*Kpn*I/*Bss*HII
3	pGL3-hE(−1025/−706)	pGL3-hE(−1541/−706)	*Kpn*I/*Apa*I
4	pGL3-hE(−946/−706)	pGL3-hE(−1541/−706)	*Kpn*I/*Sma*I
5	pGL3-hE(−768/−706)	pGL3-hE(−1541/−706)	*Kpn*I/*Pst*I
6	pGL3-hE(−1541/−921)	pGL3-hE(−1541/−706)	*Sac*II/*Bgl*II
7	pGL3-hE(−1541/−1029)	pGL3-hE(−1541/−706)	*Apa*I/*Bgl*II
8	pGL3-hE(−1541/−1102)	pGL3-hE(−1541/−706)	*Pst*I/*Bgl*II
9	pGL3-hE(−1541/−1186)	pGL3-hE(−1541/−706)	*Sma*I/*Bgl*II
10	pGL3-hE(−1541/−1293)	pGL3-hE(−1541/−706)	*Bss*HII/*Bgl*II
11	pGL3-hE(−1541/−1445)	pGL3-hE(−1541/−706)	*Stu*I/*Bgl*II
12	pGL3-hE(−1297/−1186)	pGL3-hE(−1541/−1186)	*Kpn*I/*Bss*HII
13	pGL3-hE(−1186/−1102)	pGL3-hE(−1541/−1102)	*Kpn*I/*Sma*I
14	pGL3-hE(−1025/−921)	pGL3-hE(−1541/−921)	*Kpn*I/*Apa*I
15	pGL3-P(hE)-hE(−1297/−1186)	(i) pGL3-hE(−1297/−1186)	*Hin*dIII/*Xba*I
		(ii) pGL3-hE(−87/+134)	*Kpn*I/*Xba*I
16	pGL3-P(hE)-hE(−1186/−1102)	(i) pGL3-hE(−1186/−1102)	*Hin*dIII/*Xba*I
		(ii) pGL3-hE(−87/+134)	*Kpn*I/*Xba*I
17	pGL3-P(hE)-hE(−1025/−921)	(i) pGL3-hE(−1025/−921)	*Hin*dIII/*Xba*I
		(ii) pGL3-hE(−87/+134)	*Kpn*I/*Xba*I
18	pGL3-P(hE)-hE(−919/−773)	(i) pGL3-hE(−919/−773)	*Hin*dIII/*Xba*I,
		(ii) pGL3-hE(−87/+134)	*Kpn*I/*Xba*I
19	pGL3-P(SV40)-hE(−1297/−1186)	(i) pGL3-hE(−1297/−1186)	*Hin*dIII/*Xba*I
		(ii) pGL3-Promoter	*Kpn*I/*Xba*I
20	pGL3-P(SV40)-hE(−1186/−1102)	(i) pGL3-hE(−1186/−1102)	*Hin*dIII/*Xba*I
		(ii) pGL3-Promoter	*Kpn*I/*Xba*I
21	pGL3-P(SV40)-hE(−1025/−921)	(i) pGL3-hE(−1025/−921)	*Hin*dIII/*Xba*I
		(ii) pGL3-Promoter	*Kpn*I/*Xba*I
22	pGL3-P(SV40)-hE(−919/−773)	(i) pGL3-hE(−919/−773)	*Hin*dIII/*Xba*I
		(ii) pGL3-Promoter	*Kpn*I/*Xba*I

activity with the dual-luciferase reporter assay system (Promega) according to the manufacturer's recommendations.

Western blot analysis

Whole-cell protein extracts were boiled for 5 min with Laemmli buffer and subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis using standard methodology. Proteins were then transferred electrophoretically onto a polyvinylidene difluoride membrane (Immobilon, pore size 0.45 μm, Millipore, Bedford, USA) using a constant voltage of 60 V for 120 min. The membranes were then blocked in 5% non-fat milk in phosphate-buffered saline containing 0.1% Tween 20 for 1 h at room temperature followed by the addition of the primary antibody for 1 h at room temperature. The membranes were then washed and incubated with a secondary antibody coupled to horseradish peroxidase for 1 h at room temperature. Antigen–antibody complexes were detected by western blot luminol reagent (Santa Cruz Biotechnology).

Statistical analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS 13.0, Inc., Chicago, USA). A two-tailed independent-sample *t*-test was used to determine the significance of differences between groups. Differences were considered statistically significant at $P<0.05$. Data were present as mean ± SD.

Results

DNA sequence upstream of the *VIL2* promoter possesses potential promoter and enhancer activities

To understand DNA structure of *VIL2*, we analyzed DNA sequence of the *VIL2* −10000/+61180 (including 10 kb

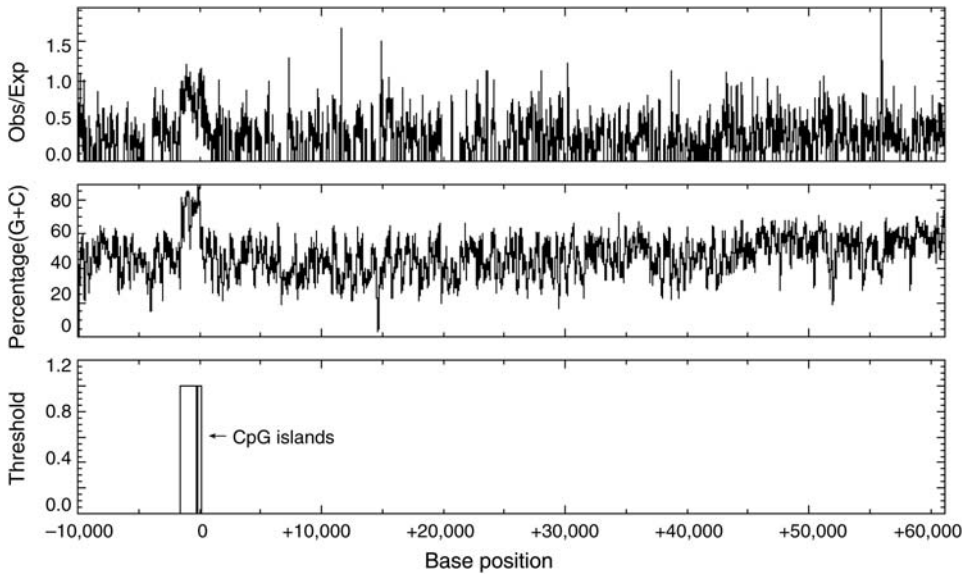

Figure 1 Analysis of CpG islands from −10000 to +61180 of human *VIL2* CpG islands of DNA fragments corresponding to −10000 (relative to transcription start site) to +61180 of human *VIL2* were detected using the program CpGPlot (http://www.ebi.ac.uk/emboss/cpgplot). CpG islands (bottom panel) were defined as regions where the observed/expected ratio (top panel) was >0.60 and the percentage (G+C) (middle panel) was >50% within a 200-bp window.

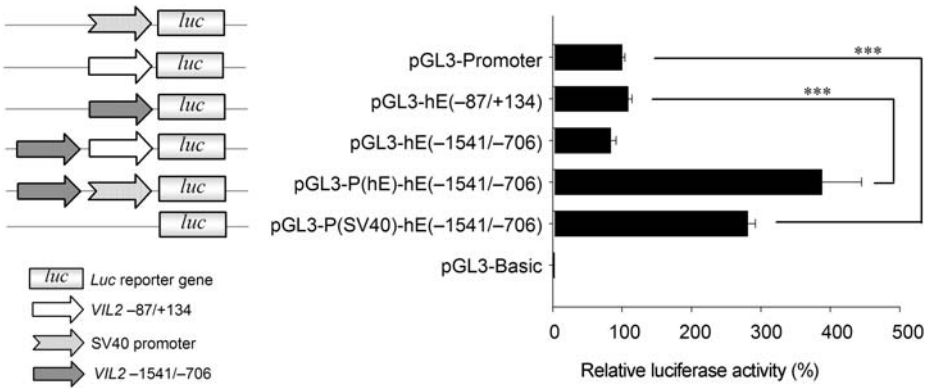

Figure 2 Promoter and enhancer activities of human *VIL2* −1541/−706 in EC109 cells Schematic representation of the plasmids used for transient transfections is shown on the left. pGL3-Promoter, pGL3-hE(−87/+134), pGL3-hE(−1541/−706), pGL3-P(hE)-hE(−1541/−706), pGL3-P(SV40)-hE(−1541/−706), or pGL3-Basic were cotransfected with pRL-TK into EC109 cells. Luciferase activity was normalized to *Renilla* luciferase activity and then shown relative to that of EC109 cells transfected with pGL3-Promoter and pRL-TK, which was set to 100%. Each value was represented as the mean ± SD. The data are representative of at least two independent experiments. Transfections were carried out in triplicate for each experiment. ***$P < 0.001$.

5′-flanking region, ~51 kb transcription region, and 10 kb 3′-flanking region) online (GenBank accession number AL589931). The CpGPlot program (http://www.ebi.ac.uk/emboss/cpgplot) revealed that ~2 kb region upstream of the *VIL2* transcription start site was highly GC-rich and with CpG islands (**Fig. 1**). Our previous study demonstrated that the human *VIL2* promoter located within −87/+134 and the region within −1324/−890 positively regulated transcription [19]. Here, we investigated the transcriptional regulatory properties of DNA sequence upstream of the *VIL2* promoter. The segment −1324/−890 was difficult to be accurately amplified via PCR as its GC content reached 80%. Primer premier 5.0 program revealed that the −1541/−706 was the obtainable shortest segment containing −1324/−890. Therefore, −1541/−706 was chosen and cloned from EC109 cells and analyzed via luciferase assays.

In EC109 cells, the *VIL2* −1541/−706 region directed luciferase activity similar to those of the SV40 promoter and −87/+134 (**Fig. 2**). In addition, transcription of the luciferase reporter gene increased 2–3 folds when the −1541/−706 segment was located upstream of the *VIL2* promoter or SV40 promoter. These data suggested that *VIL2* −1541/−706 possessed potential promoter and enhancer activities.

Besides in EC109 cells, we also detected the transcriptional regulatory functions of −1541/−706 and expression of ezrin

Table 2 Promoter and enhancer activities of human *VIL2* −1541/−706 in EC109, CNE2, and HeLa cells

Cell line	Relative luciferase activity (%)				
	pGL3-Promoter	pGL3-hE (−87/+134)	pGL3-hE (−1541/−706)	pGL3-P(SV40)-hE (−1541/−706)	pGL3-P(hE)-hE (−1541/−706)
EC109	100.00 ± 4.14	108.39 ± 6.43	83.78 ± 7.99	280.76 ± 11.06	387.87 ± 57.13
CNE2	100.00 ± 10.43	140.77 ± 12.46	66.12 ± 5.98	209.22 ± 13.41	317.75 ± 42.62
HeLa	100.00 ± 3.51	46.62 ± 2.18	7.60 ± 0.57	120.85 ± 9.49	99.66 ± 3.49

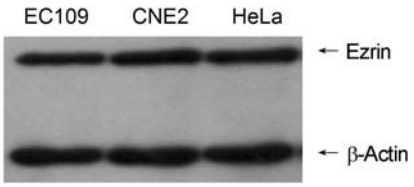

Figure 3 Expression of ezrin in EC109, CNE2, and HeLa cells Whole-cell protein from EC109, CNE2, and HeLa cells was collected and analyzed by western blotting using 20 μg protein per lane. β-Actin was used as a loading control.

in CNE2 and HeLa cells. The transcriptional activations were about 83.8, 66.1, and 7.6% relative to that of the SV40 promoter in EC109, CNE2, and HeLa cells, respectively. When the −1541/−706 was located upstream of the SV40 promoter or *VIL2* promoter, it enhanced the luciferase expression in EC109, CNE2, and HeLa cells in a different degree (**Table 2**). However, the expression levels of ezrin in EC109, CNE2, and HeLa cells were not obviously different (**Fig. 3**). These results suggested that the transcriptional activation and enhancement of *VIL2* −1541/−706 characterized a cell-type specificity, and the transcriptional regulation mechanism in different human tumor cells might not be absolutely identical and remains to be further characterized.

Localization of transcriptional regulatory regions within *VIL2* −1541/−706

Transient transfection of EC109 cells showed that *VIL2* −1541/−706 could drive transcription of a luciferase reporter (**Fig. 2**). To localize regulatory regions within *VIL2* −1541/−706 and avoid the confusion of other promoter activity, a series of 5′-deletion mutants without the *VIL2* promoter or SV40 promoter was constructed from pGL3-hE(−1541/−706) (**Fig. 4**, **Table 1**) and analyzed via luciferase assays. In EC109 cells, when compared with region −1541/−706, further deletions (i.e. −1445/−706 and −1297/−706) did not markedly change the reporter activity [**Fig. 5(A)**]. Sequence 5′-deletions from −1297 to −1025 and from −946 to −768 nearly abolished the activity, which from −1025 to −946 caused a considerable increase in the activity. These data suggested that the regions −1297/−1025 and −946/−768 positively regulated transcription and the region −1025/−946 negatively regulated transcription of human *VIL2* in EC109 cells.

To further confirm the results of the 5′-deletion experiments, a series of 3′-deletion mutants was constructed (**Fig. 4**, **Table 1**) and analyzed. In EC109 cells, sequence 3′-deletions from −706 to −921, −1102 to −1186, and −1186 to −1293 caused a remarkable reduction in luciferase activity, which from −1029 to −1102 did not change the activity, whereas those from −921 to −1029 increased luciferase activity for about 5-fold [**Fig. 5(B)**]. These data showed that several transcriptional regulatory regions existed within *VIL2* −1541/−706. The regions −1293/−1186, −1186/−1102, and −921/−706 positively regulated transcription, and the region −1029/−921 negatively regulated transcription of human *VIL2* in EC109 cells, which was consistent with the results of the 5′-deletion experiments.

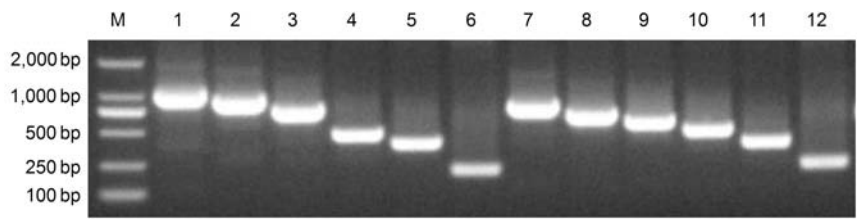

Figure 4 PCR identification of *VIL2* −1541/−706 sequence 5′- and 3′-deletion plasmids DNA fragments were amplified from pGL3-hE(−1541/−706) (lane 1); *VIL2* −1541/−706 5′-deletion plasmids pGL3-hE(−1445/−706) (lane 2), pGL3-hE(−1297/−706) (lane 3), pGL3-hE(−1025/−706) (lane 4), pGL3-hE(−946/−706) (lane 5), and pGL3-hE(−768/−706) (lane 6); and *VIL2* −1541/−706 3′-deletion plasmids pGL3-hE(−1541/−921) (lane 7), pGL3-hE(−1541/−1029) (lane 8), pGL3-hE(−1541/−1102) (lane 9), pGL3-hE(−1541/−1186) (lane 10), pGL3-hE(−1541/−1293) (lane 11), and pGL3-hE(−1541/−1445) (lane 12) by PCR using primers 5′-CTAGCAAAATAGGCTGTCCC-3′ and 5′-CTTTATGTTTTTGGCGTCTTCCA-3′. PCR products were separated on 1.8% agarose gel containing ethidium bromide and detected via ultraviolet illumination.

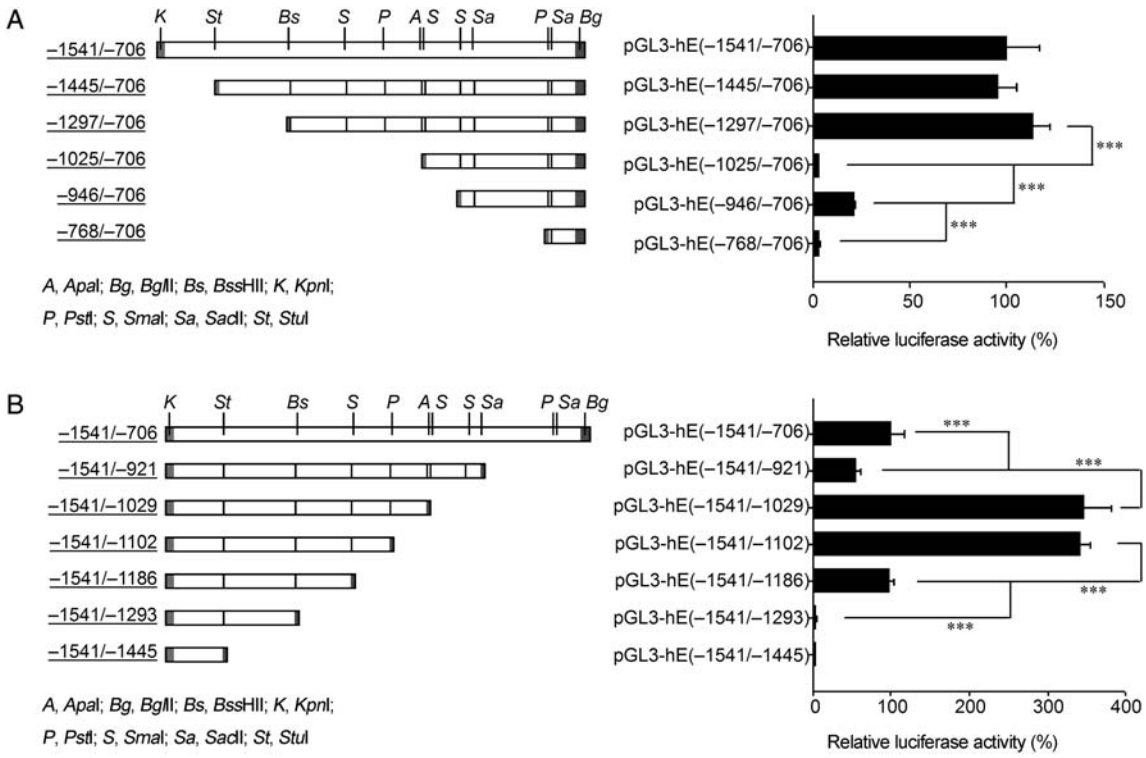

Figure 5 Transcriptional regulatory regions within *VIL2* −1541/−706 in EC109 cells by 5′-deletion (A) and 3′-deletion (B) analysis Schematic representations of the *VIL2* −1541/−706 5′- or 3′-deletion constructs used for transient transfections are shown on the left. The 5′- or 3′-deletion constructs were cotransfected with pRL-TK into EC109 cells. Luciferase activity (right) was normalized to *Renilla* luciferase activity and then shown relative to that of EC109 cells transfected with pGL3-hE(−1541/−706) and pRL-TK, which was set to 100%. Each value was represented as the mean ± SD. The data were representative of at least two independent experiments. Transfections were carried out in triplicate for each experiment. ***$P < 0.001$.

Promoter activity of *VIL2* potential transcriptional regulatory regions

Considering enzyme sites within pGL3-hE(−1541/−706) and the results of deletion experiments, four regions, including three potential positive regulatory regions −1297/−1186, −1186/−1102, and −919/−773, and one potential negative regulatory region −1025/−921, were selected and constructed into plasmids for further study [**Fig. 6(A)**, **Table 1**]. Plasmids carrying different *VIL2* potential transcriptional regulatory regions were transfected into EC109 cells for detecting the promoter activity via luciferase assays. In EC109 cells, *VIL2* −1297/−1186 and −1186/−1102 directed lower luciferase activity. Their promoter activities were respectively 24 and 6% of that of −1541/−706, whereas *VIL2* −1025/−921 and −919/−773 did not exhibit promoter activity [**Fig. 6(B)**]. These *VIL2* segments, although exhibiting significant positive or negative transcriptional regulation in deletion experiments, showed a weak or absent transcriptional activation individually, which suggested that these *VIL2* potential transcriptional regulatory regions participated in transcriptional regulation probably in a cooperative or synergistic manner.

Enhancement of *VIL2* potential transcriptional regulatory regions on the *VIL2* promoter and SV40 promoter

To explore the enhancement of *VIL2* potential transcriptional regulatory regions on the *VIL2* promoter, these four regions were separately located upstream of the *VIL2* promoter in pGL3-hE(−87/+134) to detect the luciferase expression. Comparing with the luciferase activity of EC109 cells transfected with pGLB-hE(−87/+134), −1297/−1186 increased 2-fold luciferase activity, which was lower than that of −1541/−706; −919/−773 increased luciferase activity slightly; whereas −1186/−1102 and −1025/−921 did not markedly change the reporter activity [**Fig. 7(A)**]. These data suggested that −1297/−1186 might act as a key region to enhance transcription of gene controlled by the *VIL2* promoter; other *VIL2* transcriptional regulatory regions might play assistant roles.

To further investigate whether the transcriptional regulatory characteristics of *VIL2* transcriptional regulatory regions on the SV40 promoter are similar to those on the *VIL2* promoter, a series of reporter gene expression vectors carrying the SV40 promoter and *VIL2* −1297/−1186,

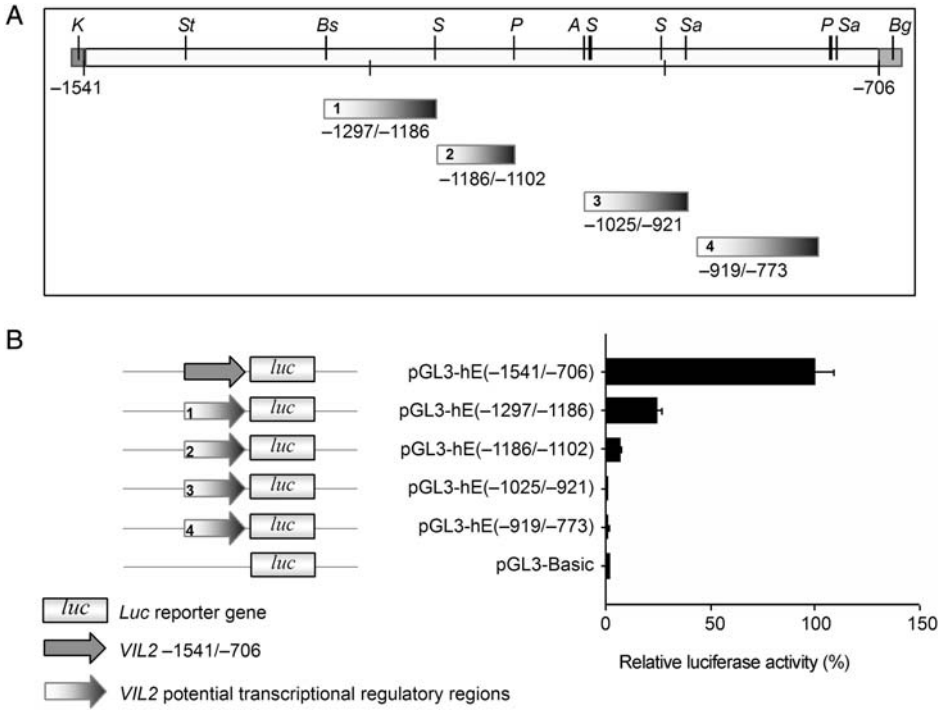

Figure 6 Promoter activity of *VIL2* potential transcriptional regulatory regions in EC109 cells (A) Schematic representation of potential transcriptional regulatory regions within *VIL2* −1541/−706. (B) pGL3-hE(−1541/−706), pGL3-hE(−1297/−1186), pGL3-hE(−1186/−1102), pGL3-hE(−1025/−921), pGL3-hE(−919/−773), or pGL3-Basic were cotransfected with pRL-TK into EC109 cells. Luciferase activity was normalized to *Renilla* luciferase activity and then shown relative to that of EC109 cells transfected with pGL3-hE(−1541/−706) and pRL-TK, which was set to 100%. Each value was represented as the mean ± SD. The data were representative of at least two independent experiments. Transfections were carried out in triplicate for each experiment.

−1186/−1102, −1025/−921, or −919/−773 were constructed. Similarly, the *VIL2* potential transcriptional regulatory regions were separately located upstream of the SV40 promoter in pGL3-Promoter. Transient transfection of EC109 cells showed that −1297/−1186 increased the transcription activity of the luciferase gene controlled by the SV40 promoter, which was lower than that of −1541/−706; whereas −1186/−1102, −1025/−921, and −919/−773 did not markedly change the reporter activity [**Fig. 7(B)**]. These data testified again that *VIL2* −1297/−1186 acted as a key region in transcriptional regulation.

Transient expression of Sp1 upregulates ezrin expression and the transcriptional activation of *VIL2* −1297/−1186

Potential transcription factor binding sites within human *VIL2* −1297/−1186 were predicted using the gene-regulation.com website (http://www.gene-regulation.com/pub/programs/alibaba2). The analysis revealed many potential Sp1 binding sites in this fragment (**Fig. 8**). To explore the effect of transcription factor Sp1 on ezrin expression, EC109 cells were transfected with expression vectors CMV-Sp1. Backbone vector pcDNA3 was used as a negative control. Total protein was extracted for analysis of Sp1 and ezrin expression by western blotting. Transfection with CMV-Sp1 increased Sp1 and ezrin expression [**Fig. 9(A)**]. These data indicated that Sp1 upregulated ezrin expression in EC109 cells.

To further define the role of Sp1 in *VIL2* −1297/−1186 transactivation, EC109 cells were transfected with the expression vectors CMV-Sp1or control vector pcDNA3 in combination with pGL3-hE(−1297/−1186) and pRL-TK. Transfection with the expression vector for Sp1 significantly increased the luciferase expression directed by the *VIL2* −1297/−1186 containing many Sp1 sites [**Fig. 9(B)**]. These results suggest that Sp1 may bind to certain Sp1 sites within *VIL2* −1297/−1186 to regulate human *VIL2* transcription and ezrin expression.

Discussion

In our previous study, we found that ezrin overexpression in ESCCs was associated with poor survival, and the human *VIL2* sequence contained a promoter within −87/+134 and a positive regulatory region within −1324/−890 [19]. We have studied the regulatory region for *VIL2* promoter activity and shown that a consensus Sp1 binding site at −75/−69 and a consensus AP-1 binding

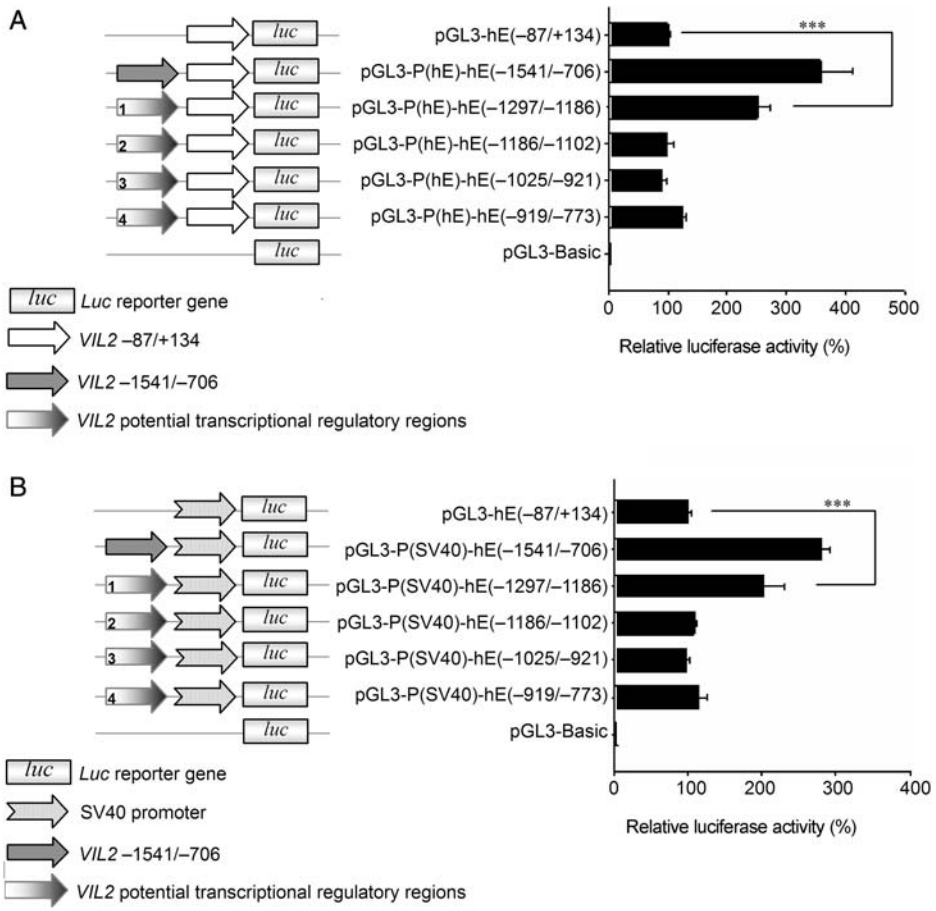

Figure 7 Transcriptional enhancement of *VIL2* potential transcriptional regulatory regions in EC109 cells on *VIL2* (A) and SV40 (B) promoters (A) pGL3-hE(−87/+134), pGL3-P(hE)-hE(−1541/−706), pGL3-P(hE)-hE(−1297/−1186), pGL3-P(hE)-hE(−1186/−1102), pGL3-P(hE)-hE(−1025/−921), pGL3-P(hE)-hE(−919/−773), or pGL3-Basic were cotransfected with pRL-TK into EC109 cells. Luciferase activity was normalized to *Renilla* luciferase activity and then shown relative to that of cells cotransfected with pGL3-hE(−87/+134) and pRL-TK, which was set at 100%. (B) pGL3-Promoter, pGL3-P(SV40)-hE(−1541/−706), pGL3-P(SV40)-hE(−1297/−1186), pGL3-P(SV40)-hE(−1186/−1102), pGL3-P(SV40)-hE(−1025/−921), pGL3-P(SV40)-hE(−919/−773), or pGL3-Basic were cotransfected with pRL-TK into EC109 cells. Luciferase activity was normalized to *Renilla* luciferase activity and then shown relative to that of cells cotransfected with pGL3-Promoter and pRL-TK, which was set at 100%. Each value was represented as the mean ± SD. The data were representative of at least two independent experiments. Transfections were carried out in triplicate for each experiment. ***$P < 0.001$.

```
Seq        (-1297.. -1242)   GCGCGCACTTCCGCCAAGTTCCGCCCCCACCCAGCCCGAGGCCCGGCTGCCGCCAT
Segments     -1280/-1268                ======Sp1====
             -1263/-1254                          ====Sp1===
             -1249/-1240                                   ==NF-muE

Seq        (-1241.. -1186)   CTTGCGGGGGGCGCACCTCACAGGTCGGGAGCTGGGCGGGAAGGGGCGTGGTCCCG
Segments     -1249/-1240  1=
             -1241/-1232  ====Sp1===
             -1238/-1226      ======Sp1====
             -1235/-1226         ====Sp1===
             -1209/-1195                          =======Sp1=====
             -1199/-1190                                 ====Sp1===
```

Figure 8 Prediction of potential transcription factor binding sites within *VIL2* −1297/−1186 Potential transcription factor binding sites were predicted using the gene-regulation.com website (http://www.gene-regulation.com/pub/programs/alibaba2). The parameters were as follows: pairsim to known sites 64, matrix width 10 bp, minimum number of sites 5, minimum matrix conservation 80%, similarity to sequence matrix 1%, and factor class level 5. The putative binding sites and their locations are listed below the sequence.

site at −64/−58 contributed to *VIL2* promoter activity in EC109 cells. Here, we cloned the *VIL2* −1541/−706 segment, which contained the positive regulatory region −1324/−890, and identified the potential transcriptional regulatory regions upstream of the*VIL2* promoter in EC109 cells.

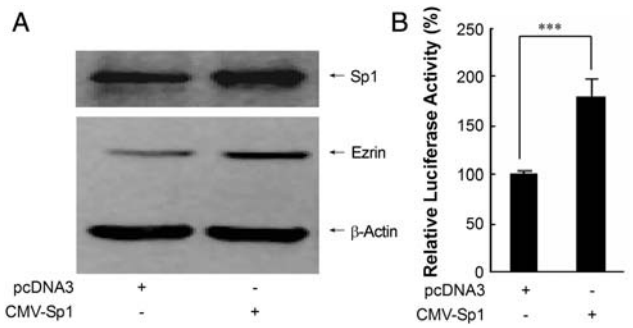

Figure 9 Transient expression of Sp1 upregulates ezrin expression (A) and transcriptional activation of *VIL2* −1297/−1186 (B) in EC109 cells. (A) EC109 cells were transfected without (−) or with (+) transcription factor expression vectors pcDNA3 or CMV-Sp1. Total protein from EC109 cells was collected and analyzed by western blotting using 20 μg protein per lane. β-Actin is shown as a loading control. (B) Constructs pGL3-hE(−1297/−1186) and pRL-TK were cotransfected into EC109 cells without (−) or with (+) pcDNA3 or CMV-Sp1. Luciferase activity was normalized to *Renilla* luciferase activity and then shown relative to that of cells cotransfected with pGL3-hE(−1297/−1186), pRL-TK, and pcDNA3, which was set at 100%. Each value was represented as the mean ± SD. The data were representative of at least two independent experiments. Transfections were carried out in triplicate for each experiment. ***$P < 0.001$.

Transient transfection of EC109 cells showed that *VIL2* −1541/−706 could not only direct transcription of a luciferase reporter but also enhance the expression of luciferase controlled by the *VIL2* promoter or SV40 promoter, suggesting that it owned both promoter and enhancer activities (**Fig. 2**). From deletion experiments, some potential transcriptional regulatory regions within *VIL2* −1541/−706 were screened out. Further investigation revealed that, among these potential regulatory regions, only *VIL2* −1297/−1186 exhibited considerable promoter and enhancer activities. Other regions, −1186/−1102, −1025/−921, and −919/−773, although exhibiting significantly positive or negative transcriptional regulation in deletion experiments, showed a weak or absent transcriptional regulation. We also detected the effect of transfection of Sp1 expression vector on ezrin expression and the transcription activation of *VIL2* −1297/−1186. It was found that Sp1 overexpression upregulated ezrin expression and the transactivation of *VIL2* −1297/−1186. Sp1 might regulate ezrin expression through Sp1 binding sites located within not only the *VIL2* promoter but also the *VIL2* −1297/−1186. Further evidential documents are investigating. It has been reported that the SV40 enhancer is composed of at least two DNA domains that exhibit very little enhancing activity on their own, whereas their association results in a 400-fold enhancement of transcription [31]. Another report about the SV40 enhancer has indicated that the enhancer is composed of multiple functional elements that could compensate for one another [32]. Similar to the SV40 enhancer, the transcriptional activity of each potential regulatory region was weaker than that of −1541/−706, suggesting that the transcriptional regulation of DNA sequence upstream of the *VIL2* promoter needs several domains or elements to work together.

In conclusion, our data indicated that more than one potential transcriptional regulatory region upstream of the *VIL2* promoter participated in *VIL2* transcription. The segment −1297/−1186, probably as a key region, regulated *VIL2* transcription in company with other potential regulatory regions in a cooperative or synergistic manner.

Funding

This work was supported by grants from the China Postdoctoral Science Foundation (Nos. 20090450100 and 201003361), the Natural Science Foundation of Guangdong Province (Nos. 9152800001000017 and 9151030002000008) and the Basic Research Projects of Shenzhen Bureau of Science, Technology, and Information (Nos. JC200903180676A, 200901012, and 200801001).

References

1 Hiscox S and Jiang WG. Ezrin regulates cell-cell and cell-matrix adhesion, a possible role with E-cadherin/beta-catenin. J Cell Sci 1999, 112: 3081–3090.
2 Gautreau A, Poullet P, Louvard D and Arpin M. Ezrin, a plasma membrane–microfilament linker, signals cell survival through the phosphatidylinositol 3-kinase/Akt pathway. Proc Natl Acad Sci USA 1999, 96: 7300–7305.
3 Crepaldi T, Gautreau A, Comoglio PM, Louvard D and Arpin M. Ezrin is an effector of hepatocyte growth factor-mediated migration and morphogenesis in epithelial cells. J Cell Biol 1997, 138: 423–434.
4 Louvet-Vallee S. ERM proteins: from cellular architecture to cell signaling. Biol Cell 2000, 92: 305–316.
5 Pujuguet P, Del Maestro L, Gautreau A, Louvard D and Arpin M. Ezrin regulates E-cadherin-dependent adherens junction assembly through rac1 activation. Mol Biol Cell 2003, 14: 2181–2191.
6 Hatzoglou A, Ader I, Splingard A, Flanders J, Saade E, Leroy I and Traver S, *et al*. Gem associates with ezrin and acts via the Rho-GAP Protein Gmip to down-regulate the Rho pathway. Mol Biol Cell 2007, 18: 1242–1252.
7 Kishore R, Qin G, Luedemann C, Bord E, Hanley A, Silver M and Gavin M, *et al*. The cytoskeletal protein ezrin regulates EC proliferation and angiogenesis via TNF-alpha-induced transcriptional repression of cyclin A. J Clin Invest 2005, 115: 1785–1796.
8 Chuan YC, Iglesias-Gato D, Fernandez-Perez L, Cedazo-Minguez A, Pang ST, Norstedt G and Pousette A, *et al*. Ezrin mediates c-Myc actions in prostate cancer cell invasion. Oncogene 2010, 29: 1531–1542.
9 Watanabe K, Tachibana M, Kim S and Watarai M. Participation of ezrin in bacterial uptake by trophoblast giant cells. Reprod Biol Endocrinol 2009, 7: 95.
10 Osawa H, Smith CA, Ra YS, Kongkham PI and Rutka JT. The role of the membrane cytoskeleton cross-linker ezrin in medulloblastoma cells. Neuro Oncology 2009, 11: 381–393.
11 Kang YK, Hong SW, Lee H and Kim WH. Prognostic implications of ezrin expression in human hepatocellular carcinoma. Mol Carcinog 2010, 49: 798–804.

12 Deng X, Tannehill-Gregg SH, Nadella MV, He G, Levine A, Cao Y and Rosol TJ. Parathyroid hormone-related protein and ezrin are up-regulated in human lung cancer bone metastases. Clin Exp Metastasis 2007, 24: 107–119.
13 Elliott BE, Meens JA, SenGupta SK, Louvard D and Arpin M. The membrane cytoskeletal crosslinker ezrin is required for metastasis of breast carcinoma cells. Breast Cancer Res 2005, 7: R365–R373.
14 Akisawa N, Nishimori I, Iwamura T, Onishi S and Hollingsworth MA. High levels of ezrin expressed by human pancreatic adenocarcinoma cell lines with high metastatic potential. Biochem Biophys Res Commun 1999, 258: 395–400.
15 Wang HJ, Zhu JS, Zhang Q, Guo H, Dai YH and Xiong XP. RNAi-mediated silencing of ezrin gene reverses malignant behavior of human gastric cancer cell line SGC-7901. J Dig Dis 2009, 10: 258–264.
16 Wang HJ, Zhu JS, Zhang Q, Sun Q and Guo H. High level of ezrin expression in colorectal cancer tissues is closely related to tumor malignancy. World J Gastroenterol 2009, 15: 2016–2019.
17 Shen ZY, Xu LY, Chen MH, Li EM, Li JT, Wu XY and Zeng Y. Upregulated expression of ezrin and invasive phenotype in malignantly transformed esophageal epithelial cells. World J Gastroenterol 2003, 9: 1182–1186.
18 Zeng H, Xu L, Xiao D, Zhang H, Wu X, Zheng R and Li Q, *et al*. Altered expression of ezrin in esophageal squamous cell carcinoma. J Histochem Cytochem 2006, 54: 889–896.
19 Gao SY, Li EM, Cui L, Lu XF, Meng LY, Yuan HM and Xie JJ, *et al*. Sp1 and AP-1 regulate expression of the human gene *VIL2* in esophageal carcinoma cells. J Biol Chem 2009, 284: 7995–8004.
20 Xie JJ, Xu LY, Xie YM, Zhang HH, Cai WJ, Zhou F and Shen ZY, *et al*. Roles of ezrin in the growth and invasiveness of esophageal squamous carcinoma cells. Int J Cancer 2009, 124: 2549–2558.
21 Yeh TS, Tseng JH, Liu NJ, Chen TC, Jan YY and Chen MF. Significance of cellular distribution of ezrin in pancreatic cystic neoplasms and ductal adenocarcinoma. Arch Surg 2005, 140: 1184–1190.
22 Kobel M, Langhammer T, Huttelmaier S, Schmitt WD, Kriese K, Dittmer J and Strauss HG, *et al*. Ezrin expression is related to poor prognosis in FIGO stage I endometrioid carcinomas. Mod Pathol 2006, 19: 581–587.
23 Park HR, Cabrini RL, Araujo ES, Paparella ML, Brandizzi D and Park YK. Expression of ezrin and metastatic tumor antigen in osteosarcomas of the jaw. Tumori 2009, 95: 81–86.
24 Brambilla D and Fais S. The Janus-faced role of ezrin in 'linking' cells to either normal or metastatic phenotype. Int J Cancer 2009, 125: 2239–2245.
25 Kim C, Shin E, Hong S, Chon HJ, Kim HR, Ahn JR and Hong MH, *et al*. Clinical value of ezrin expression in primary osteosarcoma. Cancer Res Treat 2009, 41: 138–144.
26 Jiang WG and Hiscox S. Cytokine regulation of ezrin expression in the human colon cancer cell line HT29. Anticancer Res 1996, 16: 861–865.
27 Kishore R, Qin G, Luedemann C, Bord E, Hanley A, Silver M and Gavin M, *et al*. The cytoskeletal protein ezrin regulates EC proliferation and angiogenesis via TNF-α-induced transcriptional repression of cyclin A. J Clin Invest 2005, 115: 1785–1796.
28 Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A and Yeung C, *et al*. The membrane–cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. Nat Med 2004, 10: 182–186.
29 Ogino W, Takeshima Y, Mori T, Yanai T, Hayakawa A, Akisue T and Kurosaka M, *et al*. High level of ezrin mRNA expression in an osteosarcoma biopsy sample with lung metastasis. J Pediatr Hematol Oncol 2007, 29: 435–439.
30 Sambrook J and Russell D. Molecular Cloning: A Laboratory Manual. 3rd edn. New York: Cold Spring Harbor Laboratory Press, 2000.
31 Zenke M, Grundstrom T and Matthes H. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J 1986, 5: 387–397.
32 Herr W and Clarke J. The SV40 enhancer is composed of multiple functional elements that can compensate for one another. Cell 1986, 45: 461–470.