Original Article



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

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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

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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). LipofectamineTM 2000 transfection reagent was purchased from Invitrogen. T4 DNA polymerase, T4 DNA ligase, endonucleases ApaI, BgIII, BssHII, HindIII, KpnI, MluI, NheI, PstI, SacII, SalI, Stul, Smal, and Xhol 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 TAGCCCGCAGTGCTGGGCGGGGGGGCGCTGAC-3') and Rezr1 (5'-CCCAAGCTTTCGGTTTCTGGTGAGTATCC TCGATCC-3'), digesting PCR products with NheI/HindIII, and inserting it into NheI/HindIII 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 KpnI/SalI, and inserting it into KpnI/XhoI 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 SmaI to construct a passage vector A, then deleting VIL2 -772/-706 of vector A with PstI/BgIII to construct another passage vector B, and finally digesting vector B with KpnI/SacII 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 HindIII, blunting the fragment with T4 DNA polymerase, and digesting it with XbaI, thus recovering the fragments containing Amp^r gene and VIL2 5'-flanking region; then digesting source vector (ii) with KpnI, blunting the fragment with T4 DNA polymerase, and digesting it with XbaI, 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 1.5×10^5 cells/mL, grown to 50–80% confluency and transfected with the plasmids described above using LipofectamineTM 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)	KpnI/StuI
2	pGL3-hE(-1297/-706)	pGL3-hE(-1541/-706)	KpnI/BssHII
3	pGL3-hE(-1025/-706)	pGL3-hE(-1541/-706)	KpnI/ApaI
4	pGL3-hE(-946/-706)	pGL3-hE(-1541/-706)	KpnI/SmaI
5	pGL3-hE(-768/-706)	pGL3-hE(-1541/-706)	KpnI/PstI
6	pGL3-hE(-1541/-921)	pGL3-hE(-1541/-706)	SacII/BglII
7	pGL3-hE(-1541/-1029)	pGL3-hE(-1541/-706)	ApaI/BglII
8	pGL3-hE(-1541/-1102)	pGL3-hE(-1541/-706)	PstI/BglII
9	pGL3-hE(-1541/-1186)	pGL3-hE(-1541/-706)	SmaI/BgIII
10	pGL3-hE(-1541/-1293)	pGL3-hE(-1541/-706)	BssHII/BglII
11	pGL3-hE(-1541/-1445)	pGL3-hE(-1541/-706)	StuI/BglII
12	pGL3-hE(-1297/-1186)	pGL3-hE(-1541/-1186)	KpnI/BssHII
13	pGL3-hE(-1186/-1102)	pGL3-hE(-1541/-1102)	KpnI/SmaI
14	pGL3-hE(-1025/-921)	pGL3-hE(-1541/-921)	KpnI/ApaI
15	pGL3-P(hE)-hE(-1297/-1186)	(i) pGL3-hE(-1297/-1186)	HindIII/XbaI
		(ii) pGL3-hE(-87/+134)	KpnI/XbaI
16	pGL3-P(hE)-hE(-1186/-1102)	(i) pGL3-hE(-1186/-1102)	HindIII/XbaI
		(ii) pGL3-hE(-87/+134)	KpnI/XbaI
17	pGL3-P(hE)-hE(-1025/-921)	(i) pGL3-hE(-1025/-921)	HindIII/XbaI
		(ii) pGL3-hE(-87/+134)	KpnI/XbaI
18	pGL3-P(hE)-hE(-919/-773)	(i) pGL3-hE(-919/-773)	HindIII/XbaI,
		(ii) pGL3-hE(-87/+134)	KpnI/XbaI
19	pGL3-P(SV40)-hE(-1297/-	(i) pGL3-hE(-1297/-1186)	HindIII/XbaI
	1186)	(ii) pGL3-Promoter	KpnI/XbaI
20	pGL3-P(SV40)-hE(-1186/-	(i) pGL3-hE(-1186/-1102)	HindIII/XbaI
	1102)	(ii) pGL3-Promoter	KpnI/XbaI
21	pGL3-P(SV40)-hE(-1025/-921)	(i) pGL3-hE(-1025/-921)	HindIII/XbaI
		(ii) pGL3-Promoter	KpnI/XbaI
22	pGL3-P(SV40)-hE(-919/-773)	(i) pGL3-hE(-919/-773)	HindIII/XbaI
		(ii) pGL3-Promoter	KpnI/XbaI

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 μ m, 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 \pm 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

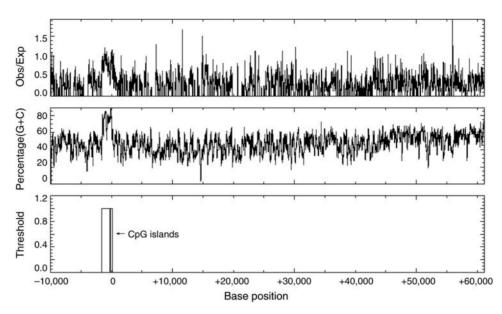


Figure 1 Analysis of CpG islands from -10000 to +61180 of human *VIL2* CpG islands of DNA fragments corresponding to -10000 (relative to transcription start site) to +61180 of human *VIL2* were detected using the program CpGPlot (http://www.ebi.ac.uk/emboss/cpgplot). CpG islands (bottom panel) were defined 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.

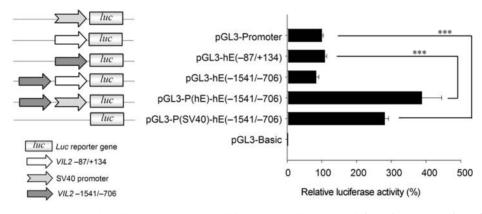


Figure 2 Promoter and enhancer activities of human *VIL2* -1541/-706 in EC109 cells Schematic representation of the plasmids used for transient transfections is shown on the left. pGL3-Promoter, pGL3-hE(-87/+134), pGL3-hE(-1541/-706), pGL3-P(hE)-hE(-1541/-706), pGL3-P(SV40)-hE(-1541/-706), or pGL3-Basic 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 \pm 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, \sim 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 $\sim 2 \text{ kb}$ 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

Cell line	Relative luciferase a	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		

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

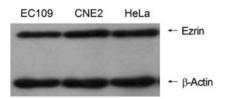


Figure 3 Expression of ezrin in EC109, CNE2, and HeLa cells Whole-cell protein from EC109, CNE2, and HeLa cells was collected and analyzed by western blotting using 20 μ g protein per lane. β -Actin was used as a loading control.

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 luciferas 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 within VIL2 -1541/-706. The existed regions -1293/-1186, -1186/-1102, and -921/-706 positively regulated transcription, and the region -1029/-921negatively regulated transcription of human VIL2 in EC109 cells, which was consistent with the results of the 5'-deletion experiments.

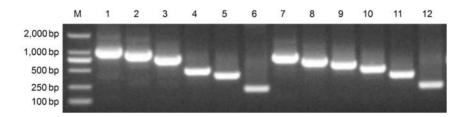


Figure 4 PCR identification of *VIL2* -1541/-706 sequence 5'- and 3'-deletion plasmids DNA fragments were amplified from pGL3-hE(-1541/-706) (lane 1); *VIL2* -1541/-706 5'-deletion plasmids pGL3-hE(-1445/-706) (lane 2), pGL3-hE(-1297/-706) (lane 3), pGL3-hE(-1025/-706) (lane 4), pGL3-hE(-946/-706) (lane 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.

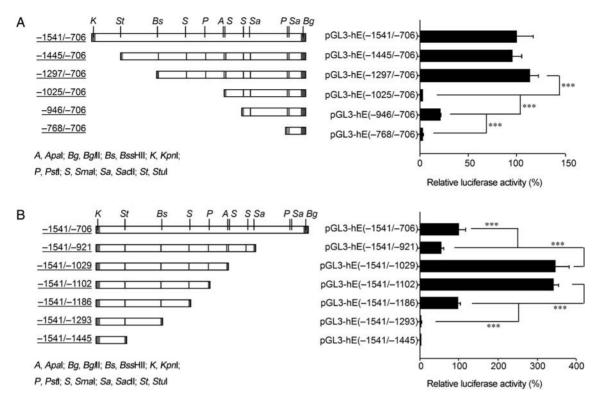


Figure 5 Transcriptional regulatory regions within *VIL2* – 1541/–706 in EC109 cells by 5'-deletion (A) and 3'-deletion (B) analysis Schematic representations of the *VIL2* – 1541/–706 5'- or 3'-deletion constructs used for transient transfections are shown on the left. The 5'- or 3'-deletion constructs 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 \pm 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/-921and -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/-773increased luciferase slightly; activity 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,

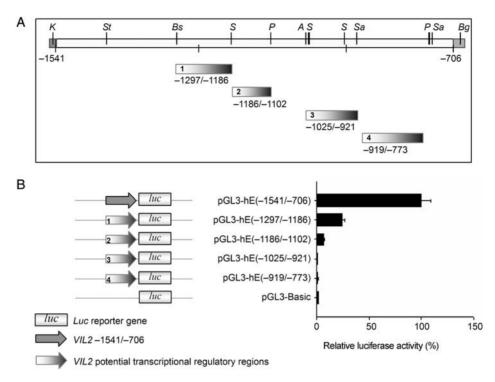


Figure 6 Promoter activity of *VIL2* potential transcriptional regulatory regions in EC109 cells (A) Schematic representation of potential transcriptional regulatory regions within *VIL2* -1541/-706. (B) pGL3-hE(-1541/-706), pGL3-hE(-1297/-1186), pGL3-hE(-1186/-1102), pGL3-hE(-1025/-921), pGL3-hE(-919/-773), 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 \pm 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.

further define the role of Sp1 in То 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/-1186containing 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

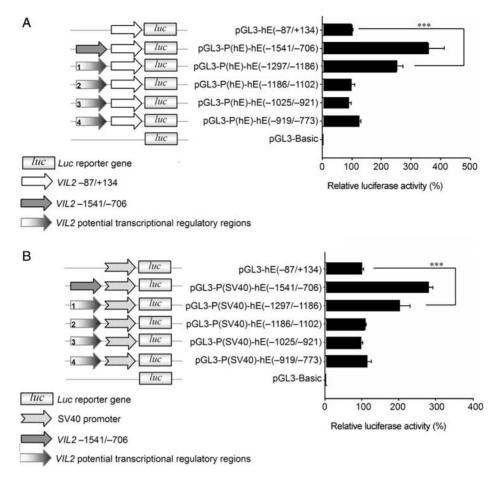


Figure 7 Transcriptional enhancement of *VIL2* potential transcriptional regulatory regions in EC109 cells on *VIL2* (A) and SV40 (B) promoters (A) pGL3-hE(-87/+134), pGL3-P(hE)-hE(-1541/-706), pGL3-P(hE)-hE(-1297/-1186), pGL3-P(hE)-hE(-1186/-1102), pGL3-P(hE)-hE(-1025/-921), pGL3-P(hE)-hE(-919/-773), 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 pRL-TK into EC109 cells. Luciferase activity was normalized to *Renilla* luciferase activity and then shown relative to that of cells 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 pRL-TK into EC109 cells. Luciferase activity was normalized to *Renilla* luciferase activity and then shown relative to that of cells 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 \pm SD. The data were representative of at least two independent experiments. Transfections were carried out in triplicate for each experiment. ***P < 0.001.

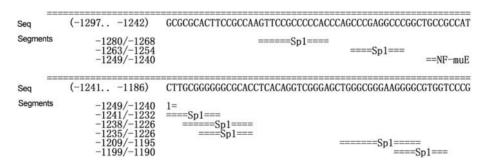


Figure 8 Prediction of potential transcription factor binding sites within VIL2 - 1297/-1186 Potential transcription factor binding sites were predicted using the gene-regulation.com/website (http://www.gene-regulation.com/pub/programs/alibaba2). The parameters were as follows: pairsim to known sites 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.

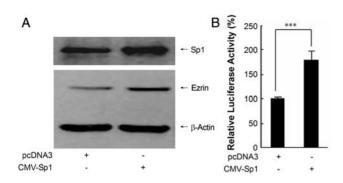


Figure 9 Transient expression of Sp1 upregulates ezrin expression (A) and transcriptional activation of VIL2 -1297/-1186 (B) in **EC109 cells.** (A) EC109 cells were transfected without (-) or with (+)transcription factor expression vectors pcDNA3 or CMV-Sp1. Total protein from EC109 cells was collected and analyzed by western blotting using 20 μ g 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 \pm 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 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.

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