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Endothelin-2 is upregulated in basal cell carcinoma under control of Hedgehog signaling pathway

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ABSTRACT

Vasoactive peptide endothelins are a group of small peptides with diverse paracrine/autocrine actions and are reported to be involved in the pathogenesis of many human malignancies. Basal cell carcinoma (BCC) is a common malignant skin tumor that frequently has aberrant activation of the Hedgehog (HH) signaling pathway. We show here that endothelin-2 (ET-2) is overexpressed in BCC under the control of HH signaling. By real-time quantitative RT-PCR analysis, significant expression of ET-2 mRNA was observed in 19 of 20 cases (95%) compared to normal skin. In addition, inhibition of the HH signaling pathway in a mouse BCC cell line downregulated endogenous ET-2, and activation of HH signaling in mouse embryonic fibroblast upregulated endogenous ET-2. Moreover, the 3′ promoter region of ET-2 gene contains the GLI-binding site and a 0.8 kb downstream fragment containing GLI-binding sites activates transcription in a reporter assay. These data indicate that ET-2 is a direct target gene of HH signaling in BCC.

Introduction

Basal cell carcinoma (BCC) is a common malignant tumor arising in the skin. Recent studies have shown that BCC frequently has abnormalities of the Hedgehog (HH) signaling pathway. HH signaling plays a key role in vertebrate development as it is involved in multiple biological processes, such as cell differentiation, proliferation, and growth [1]. Also, several genes overexpressed in BCC have been reported and downstream signaling pathways of HH signaling are currently being investigated [2–7]. In one of those investigations, we performed DNA microarray analysis using BCC and identified that G-protein-coupled receptor GPR49 (GPR49) is specifically overexpressed in BCC and plays a significant role in cell proliferation [2]. However, the function of the genes reported to be overexpressed is still not sufficient to explain the characteristic features of BCC, including interstitial invasion and angiogenesis; therefore, further understanding of the molecules expressed in BCC and their relation in HH signaling is required.

To identify other candidate genes that play a pathogenic role in BCC, we reexamined our results of DNA microarray analysis and focused on the vasoactive peptide coding gene endothelin-2. The endothelin (ET) family is a group of 21 amino acid peptides with diverse paracrine/autocrine actions [8]. The ET family is composed of three vasoactive peptides, ET-1, ET-2 and ET-3. ET-1 and ET-2 have similar structures, differing by only two amino acids, whereas ET-3 differs in structure in six amino acids [9]. ETs are processed from inactive precursor pro-polypeptides by a subgroup of membrane-bound zinc metalloproteases, the ET-converting enzymes. The three ET isoforms bind to two cell surface receptors, which are seven-transmembrane domain G-protein-coupled receptors: ET receptor subtype A (ET_AR), and subtype B (ET_BR) [10]. ET-1 has the highest affinity for ET_AR, followed by ET-2 and ET-3, with all ETs exhibiting equal affinity for ET_BR [11]. The major role of ETs is controlling vascular tone by acting on vascular smooth muscle cells [12], but recent reports suggest that they are also involved in the pathohistology of various human malignancies [8,9]. Here we report that ET-2 is markedly upregulated in almost all cases of BCC under the control of the HH signaling pathway and show evidence that ET-2 is a direct target of HH signaling.

Materials and methods

Samples. Tumor samples were collected from patients at Keio University Hospital and other affiliated hospitals. Tumor and normal skin were snap-frozen after surgical removal and stored at −80 °C until use. The experiment was approved by the ethics committee of Keio University School of Medicine and all samples were taken after written informed consent was obtained from the patients.

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Quantitative real-time polymerase chain reaction (QRT-PCR). Total RNA was isolated from tissues and cell lines with an RNeasy Mini Kit, including DNAase treatment (Qiagen KK, Tokyo, Japan). cDNA was synthesized with a Prime Script RT reagent kit (Takara, Kyoto, Japan). QRT-PCR analysis was carried out on TaKaRa PCR Thermal Cycler Dice (Takara) using SYBR Premix Ex Taq (Perfect Real Time) (Takara). Primer sequences for QRT-PCR studies are as follows: human GAPDH primers forward 5′-CCAGCCGAGCCACATCGCTC-3′, reversed 5′-ATGAGCCCCAGCCTTCTCCAT-3′; human ET2 primers forward 5′-TTGGACATCATCTGGGTGAA-3′, reverse 5′- GAAATGT CCCTCAGCCTTTG-3′; human GLI1 primers forwards 5′-GAAGACCT CTCCAGCTTGGA-3′, reversed 5′-GGCTGACAGTATAGGCAGAG-3′; human GLI2 primers forwards 5'-TGGCCGCTTCAGATGACA-GATGTTG-3′, reversed 5′-CGTTAGCCGAATGTCAGCCGTGAAG-3′; mouse Gapdhprimers forwards 5′-TGCACCACCAACTGCTTAG-3′, reversed 5′-GGATGCAGGGATGATGTTT-3′; mouse Et2 primers forwards 5′-TTCTGCCATCGAAGACACTG-3′, reversed 5′- TCCTGCAGCT-CATGGTGTTA-3′; and mouse Gli1 primers forwards 5′-CATTCCACA GGACAGCTCAA-3′, reversed 5′-TGGCAGGGCTCTGACTAACT-3′.

Cell culture. The mouse BCC cell line ASZ001 was kindly provided by Dr. Ervin Epstein (Department of Dermatology, University of California, San Francisco, CA) and Dr. Matthew P. Scott (Department of Developmental Biology, Howard Hughes Medical Institute, Stanford University School of Medicine), and was maintained as reported previously [4]. C3H10T1/2 cells, COS-7 cells, were maintained as described elsewhere [13].

Reagents. Cyclopamine (Biomol Int., Philadelphia, PA) was dissolved in 0.19% ethanol and added to ASZ001 culture at a concentration of 2, 5 or 10 μM. As a control, the same amount of 0.19% ethanol was added. Recombinant Mouse Sonic Hedgehog N Terminus (ShhN). (R&D Systems, Oxford, UK) was dissolved in PBS and added to C3H10T1/2 culture at a concentration of 0.5, 1.0, or 2 μM. As a control, the same amount of PBS was added.

Plasmids, transfection and luciferase assay. ET-2 gene reporter plasmids were constructed by PCR using human genomic DNA. Primers used for PCR are listed below. DNA fragments were treated with restriction enzyme Sal I and BamH I, and inserted into PGL-3 promoter vector (Promega, San Luis Obispo, CA).

Table 1
Expression level of ET-2, GLI1 in BCC.*

Case	Pattern	Lesion	ET-2**	GLI1**
1	Nodular	Face	9.95	43.73
2	Nodular	Face	32.36	24.21
3	Nodular	Face	11.88	13.27
4	Nodular	Face	36.53	102.92
5	Nodular	Face	57.08	81.08
6	Superficial	Trunk	3.56	4.00
7	Nodular	Face	71.75	82.72
8	Nodular	Face	31.93	59.42
9	Superficial	Face	50.23	84.68
10	Superficial	Trunk	1.59	1.33
11	Nodular	Face	5.38	7.06
12	Superficial	Trunk	3.99	10.41
13	Superficial	Trunk	30.94	29.80
14	Superficial	Trunk	9.05	19.86
15	Nodular	Face	18.57	66.31
16	Nodular	Face	38.46	63.46
17	Nodular	Face	26.97	70.66
18	Nodular	Face	21.74	32.22
19	Nodular	Face	19.37	33.26
20	Superficial	Trunk	10.10	17.52

* Each gene expression value represents the ratio of mRNA in the tumor to that in normal skin mRNA.

** Pearson correlation coefficient (two-tailed) was calculated pairwise using Statcel2 software for all combinations ($p < 0.01$). A high correlation was seen between the expression levels of *ET-2* and *GLI1* at $r = 0.807$.

Primer sequences constructed for the reporter of ET-2 promoter regions were as follows: ET2 promoter primers forward 5'-GGAT CCTCTGGTTTTTTGTCTTGGCCA-3', reverse #1 5'-GTCGACTCATA CTGCAGTGGTGACTCAT-3', reverse #2 5'-GTCGACTCTTCCTATGAC CACCCAC-3', reverse #3 5'-GTCGACCCTGGCCTCTTTTGAGTCTT-3'.

As a reference reporter, we used phRL-TK (Promega). Mouse *Gli1* expression vector, reporter constructs of HH signaling, 8 × 3′GBS-luc reporter, and 8 × 3′mut GBS-luc reporter were a gift from Dr. Hiroshi Sasaki (Riken, Kobe, Japan) [5]. Transfection was performed against COS-7, C3H10T1/2, and ASZ001 using Fugene6 (Roche Diagnostics) according to the manufacturer's protocol. Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

Statistical analysis. Statistical analysis was performed using Statcel2 software (OMS, Saitama, Japan). Statistically significant differences were determined by Student's *t*-test.

Results

Overexpression of ET-2 in BCC

As previously reported, DNA microarray analysis in our samples also showed *ET-2* expression to be about 8.1-fold higher than

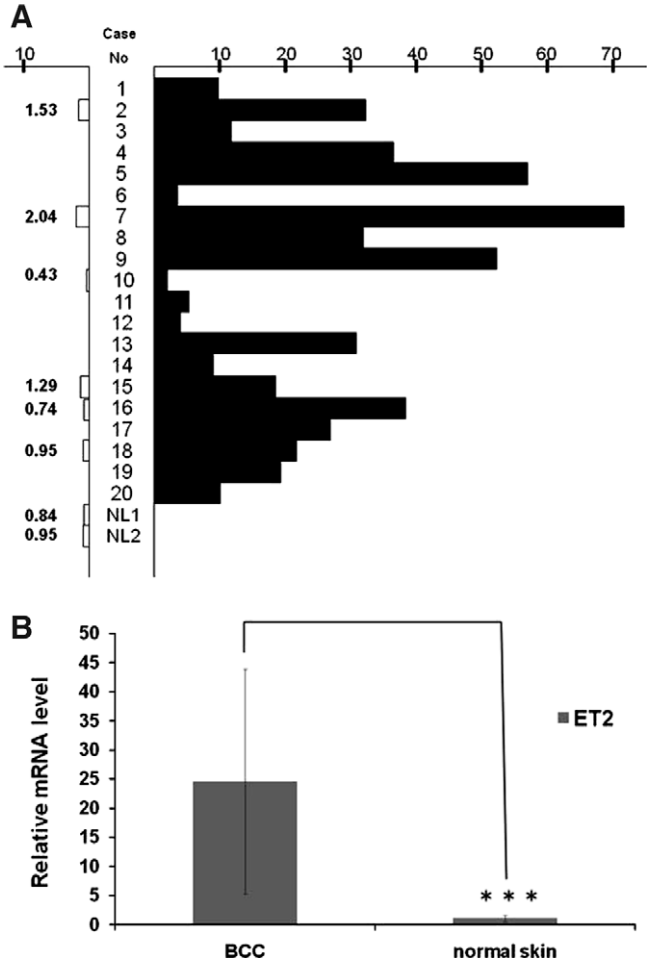

Fig. 1. Overexpression of ET-2 in BCC. (A) QRT-PCR of ET-2. The mRNA levels of ET-2 in 20 BCC and 6 normal skin in the vicinity of BCC, and 2 normal skin samples from non-cancerous patients (NL) are estimated by QRT-PCR (closed column: mRNA level in tumors; open column: mRNA level in normal skin). In normal skin, the expression of ET-2 is negligible compared to BCC. (B) The mean value and SD of each group in QRT-PCR of ET-2. About 24-fold higher levels of ET-2 are shown in BCC in comparison with normal skin.

normal skin [2]. As we could not find or create a specific antibody against ET-2 protein, mainly due to the high similarity with ET-1, we confirmed the expression of *ET-2* by QRT-PCR. Using 20 BCC cases, we analyzed the expression of *ET-2* together with *GLI1*, which were reported to be overexpressed in BCC [3] and to be transcription factors involved in HH signaling (Table 1). Of these 20 cases, 19 showed *ET-2* expression levels more than three times higher than the control (Fig. 1A) (mean increase about 24-fold) (Fig. 1B). On the other hand, the expression by QRT-PCR was low in normal skin samples (Fig. 1A and B).

High relation between ET-2 and HH signaling

Using 20 cases of BCC, the expression of *ET-2* and *GLI1* was confirmed by QRT-PCR, as shown in Table 1. We therefore analyzed the correlation between the expression level of *ET-2* and *GLI1* to evaluate the relation between HH signaling and ET-2. Statistical analysis using Pearson's coefficient was calculated for all combinations. A high correlation was seen between the expression levels of *ET-2* and *GLI1* at $r = 0.807$. HH signaling is activated in BCC, and we speculate here that the expression of *ET-2* is highly related to HH signaling.

To further evaluate the relation between HH signaling and ET-2, we performed an *in vitro* assay using cell lines. Suppression of HH signaling was confirmed by the reporter constructs of HH signaling, 8 × 3′GBS-luc/8 × mut3′GBS-luc [2]. We treated ASZ001 with cyclopamine, a known inhibitor of HH signaling. Downregulation of mouse ET-2 expression was observed together with downregulation of mouse *Gli1*, a target of HH signaling [3], and this downregulation was dependant on the time after treatment and the concentration of cyclopamine (Fig. 2A and B). Next, to see whether ET-2 expression is upregulated by HH signaling, we treated mouse embryonic fibroblast C3H10T1/2 cells, which are known to respond to HH signaling, with ShhN. The expression of mouse ET-2 was increased together with the expression of mouse Gli1 dependant on the time after treatment and the concentration of ShhN (Fig. 3A and B). Upregulation of mouse ET-2 was also confirmed when we transfected mouse Gli1 expression vector [13] to C3H10T1/2 (Fig. 3C). Activation of HH signaling by mouse Gli1 transfection was confirmed by the reporter constructs of HH signaling, 8 × 3′GBS-luc/8 × mut3′GBS-luc (Fig. 3D). These findings indicate that ET-2 is regulated by HH signaling.

Expression of ET-2 is directly regulated by HH signaling

In order to see the direct transcriptional activation of ET-2 by HH signaling, we analyzed a region approximately 1 kb downstream of the ET-2 for motifs identical to or closely matching the GLI-consensus binding site –TGGGTGGTC- [14]. As expected, we found an identical sequence 797–803 b downstream of the ET-2. Thereafter, we constructed three reporter constructs of the ET-2 3′ promoter region, as shown in Fig. 4A. They were transfected to ASZ001, which has endogenous activation of HH signaling. While the reporter plasmid inserted with the ET-2 downstream fragment from 0 to +543 (reporter plasmid #1) showed less reporter activity, significant reporter activity was observed when the reporter plasmid inserted with the downstream fragment from 0 to +815 (reporter plasmid #2) and 0 to 887 (reporter plasmid #3) was transfected to ASZ001 (Fig. 4B).

Next, they were transfected to COS-7 along with a mouse Gli-1 expression vector or an empty vector. The expression of mouse Gli-1 was confirmed by the reporter constructs of HH signaling, 8 × 3′GBS-luc/8 × mut3′GBS-luc [5] (Fig. 4C). Among them, reporter plasmid #2 and reporter plasmid #3 had significant reporter

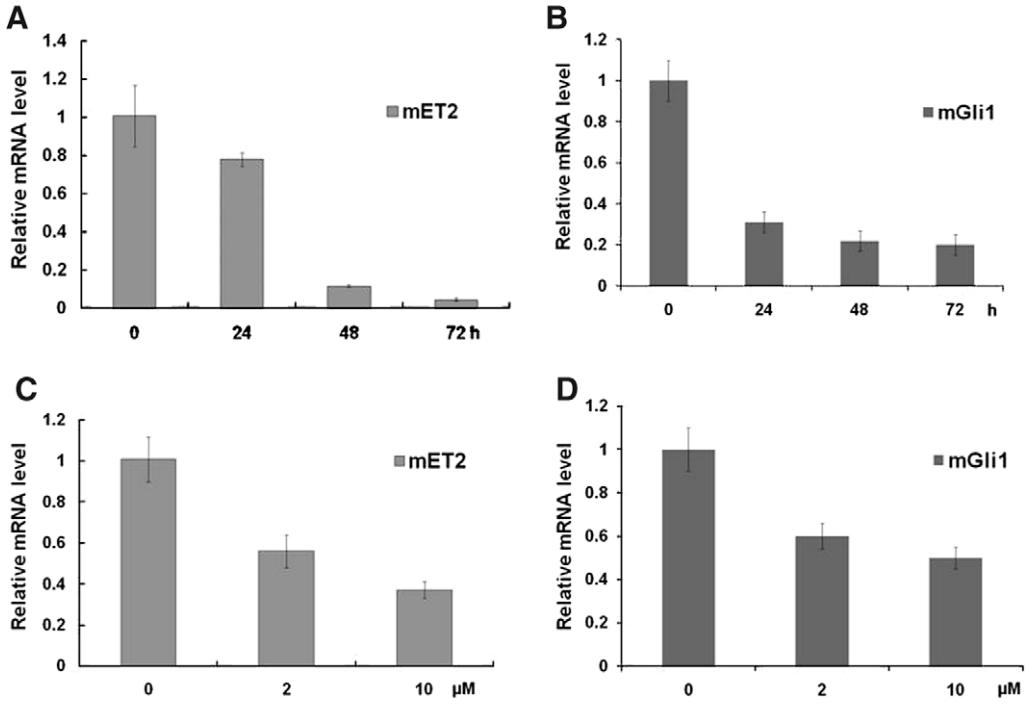

Fig. 2. Suppression of HH signaling decreases ET-2 expression in the mouse BCC cell line ASZ001. (A) Time course of mRNA expression of endogenous mouse ET-2 (m ET-2) and mGli1 in ASZ001 cells treated with cyclopamine. Cells were treated with 10 μM cyclopamine, and expression of m ET-2 (left) and mGli1 (right) mRNA was measured by QRT-PCR at the times shown in the figure. The fold decrease of mRNA levels in cyclopamine-treated to non-treated cells at each sampling time was normalized by setting the baseline value at 1. The figure shows one of the three repeated experiments. Suppression of m ET-2 and mGli1 expression was dependent on the time course. (B) Dose dependency of mRNA expression of m ET-2 and mGli1 on the concentration of cyclopamine. ASZ001 cells were treated with 0, 2, and 10 μM cyclopamine, and expression of m ET-2 (left) and mGli1(right) was measured by QRT-PCR 48 h after treatment. Values are shown as a ratio relative to cyclopamine 0 μM. Suppression of mRNA levels of mET-2 and mGli1 was dependent on the concentration of cyclopamine.

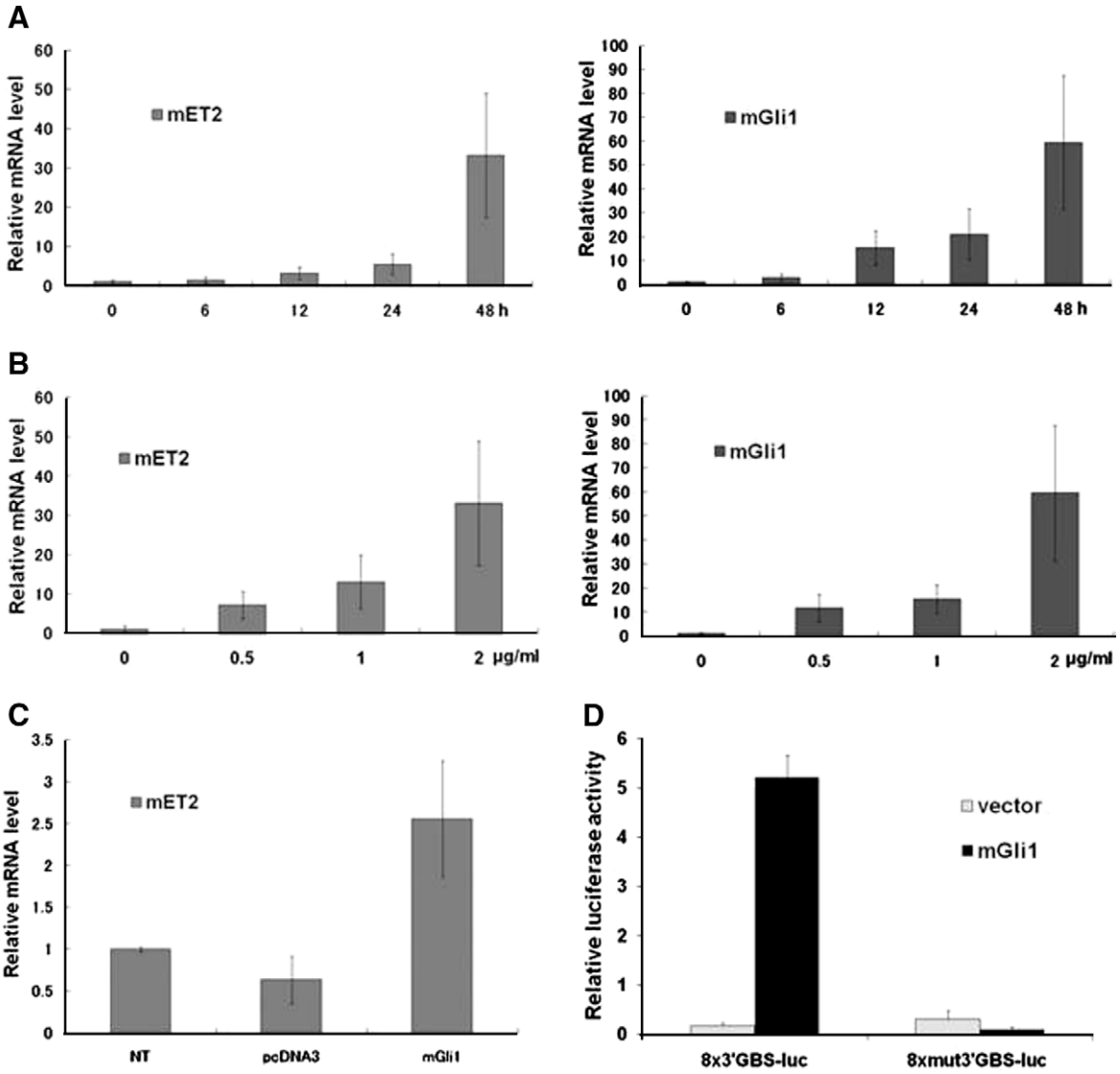

Fig. 3. Activation of HH signaling induces ET-2 expression. (A) Quantitative RT-PCR analysis of endogenous m ET-2 and mGli1 was performed in C3H10T1/2 cells after treatment with ShhN. Cells were treated with 2 μg/ml ShhN for the time indicated in the figure. Upregulation of m ET-2 expression (left) was dependent on the time course, as with mGli1 (right). (B) Dose dependency of mRNA expression of m ET-2 and mGli1 on the concentration of ShhN. C3H10T1/2 cells were treated with 0, 0.5, 1.0 and 2 μg/ml of recombinant N-terminal SHH peptide, and the expression of mET-2 (left) and mGli1 (right) was measured by QRT-PCR 48 h after treatment. Values are shown as a ratio relative to ShhN 0 μg/ml. Expression of mRNA levels of mET-2 and mGli1 was dependent on the concentration of cyclopamine. (C) QRT-PCR analysis of mET-2. Mouse Gli1 expression vector was transfected to C3H10T1/2 cells. mRNA levels of mET-2 were assayed at the time indicated in the figure. The ratios of mRNA level of mGli1-transfected to vector-transfected cells were estimated. When Gli1 is expressed in C3H10T1/2 cells, the gene expression level of mET-2 was elevated. (D) Activation of HH signaling by mGli1-expression vector in C3H10T1/2 cells. Cells were transfected with an mGli1-expression vector, 8 × 3′GBS-luc or 8 × 3′mutGBS-luc, and with phRL-TK as a reference. Luciferase activity was assayed 48 h after transfection. Activity was normalized with those of phRL-TK as a reference. Expression of Gli1 significantly activated the Gli-consensus reporter gene, while a mutant promoter was not affected.

activity when cotransfected with mouse Gli-1 expression vector. On the other hand, reporter activity of the plasmid inserted with fragments from 0 to +543 (reporter plasmid #1) was negligible (Fig. 4D).

Discussion

Our studies showed that ET-2 is overexpressed in BCC. DNA microarray analysis in our samples showed ET-2 expression to be about 8.1-fold higher than normal skin. In QRT-PCR study, ET-2 was markedly overexpressed in 19 of 20 BCC samples of nodular and superficial types in comparison with normal tissue samples. These results suggest that the expression of ET-2 is a characteristic feature in BCC. Expression of ET-2 has not been fully analyzed in the past literature and this is the first report that shows the specific expression of ET-2 in BCC.

Furthermore, our results demonstrated that in BCC, the expression of ET-2 is regulated by the activity of the HH signaling pathway. Our results showed that inhibition of the HH signaling pathway in a mouse BCC cell line downregulated endogenous ET-2, and activation of HH signaling in mouse C3H10T1/2 cells upregulated endogenous ET-2. The presence of a single copy of the GLI-binding site in the putative 3′ promoter region of ET-2, together with the activation of reporter gene expression by this region in the presence of mGli1, points to direct regulation of ET-2 by mGli1. These findings strongly suggest that the expression of ET-2 is regulated by HH signaling directly.

ETs are expressed in a broad range of tumors, and are suggested to play a crucial role in tumor growth, progression, and angiogenesis [15]. Among them, most reports focus on the role of ET-1, which is also reported to be expressed in BCC, and blockade of the ET-1/ETR_A pathway can reduce cell survival *in vitro* [16]. ET-1

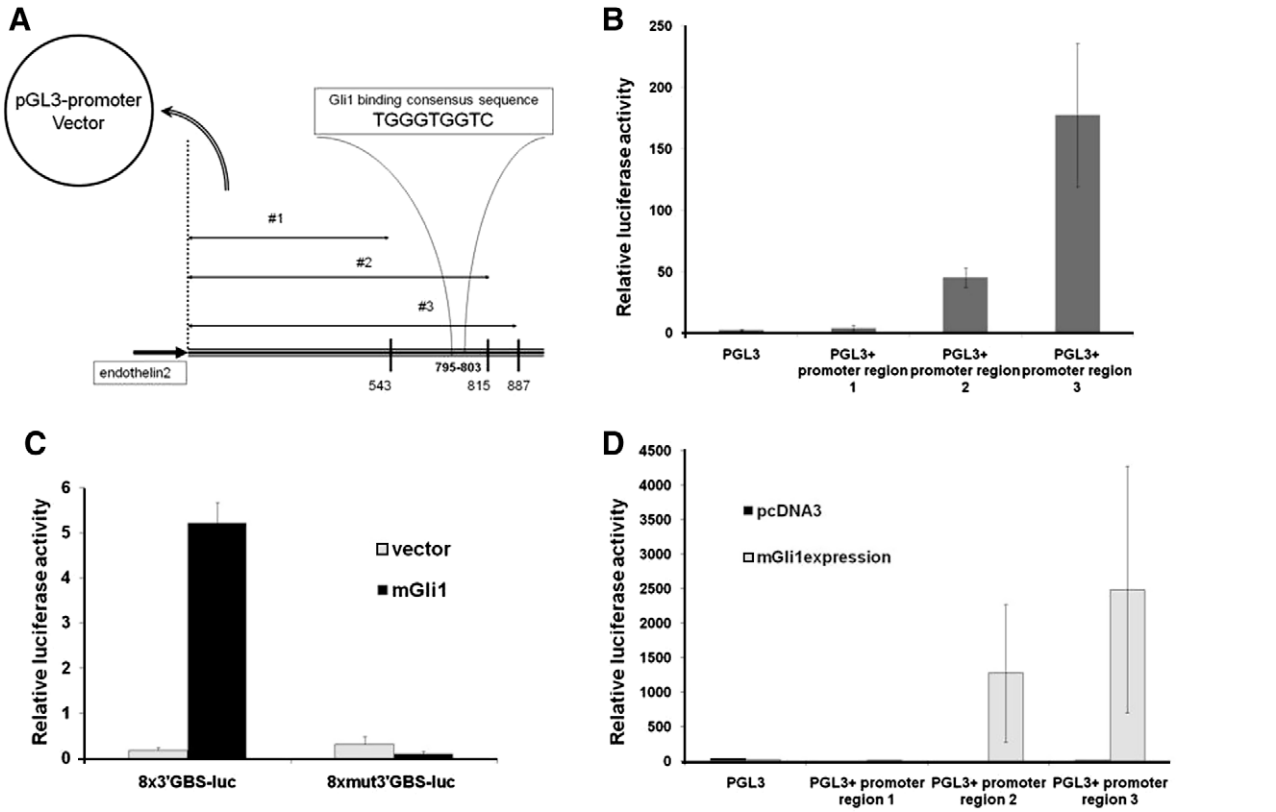

Fig. 4. Analysis of 3′ promoter region of ET-2. (A) The promoter of the human ET-2 gene contains a consensus Gli-binding site. Construction of reporter construct of ET-2 3′ promoter regions. Region between 797 and 803 includes a consensus binding sequence of Gli-1, -TGGGTGGTC-. (B) Reporter activity of ET-2 3′ promoter region in ASZ001. The reporter construct of ET-2 3′ promoter regions was transfected into ASZ001 cells. The relative luciferase activity of the reporter, including the ET-2 downstream fragment from 0 to +815 (reporter plasmid #2) and 0 to 887 (reporter plasmid #3) showed significant luciferase activity. Reporter activity of plasmid inserted with the fragment from 0 to +543 (reporter plasmid #1) was negligible. (C) Reporter activity of HH reporter construct in COS-7. 8 × 3′GBS-luc reporter showed significant reporter activity under the co-transfection of mGli-1 expression vector whereas reporter activity of 8 × 3′mutGBS-luc reporter was negligible. (D) Reporter activity of ET-2 3′ promoter region and HH reporter construct in COS-7. The reporter construct of ET-2 3′ promoter regions and a mouse Gli expression plasmid or empty vector (pcDNA-3) were cotransfected into COS-7 cells. The relative luciferase activity of the reporter including ET-2 downstream fragment from 0 to +815 (reporter plasmid #2) and 0 to 887 (reporter plasmid #3) showed significant luciferase activity when cotransfected with mouse Gli-1 expression vector. Reporter activity of plasmid inserted with fragment from 0 to +543 (reporter plasmid #1) was negligible.

has also been implicated to participate in the pigmentation process of BCC [17]. We also performed QRT-PCR analysis of ET-1 in our BCC samples and confirmed its overexpression (data not shown); however, we could not find a significant relation between ET-1 expression and HH signaling activity. Mouse endogenous ET-1 expression did not change by either inhibiting the HH signaling pathway in ASZ001 cells or activating HH signaling in C3H10T1/2 cells (data not shown). Moreover, we could not find a consensus GLI-binding site in the promoter lesion of human and mouse ET-1; therefore, we speculate that ET-2 rather than ET-1 will play a significant role as a downstream mediator of HH signaling in BCC.

The role of ET-2 in carcinogenesis is still not fully understood. In breast carcinoma, ET-2 is reported to be a hypoxia-induced autocrine survival factor and in invasive ductal carcinoma of the breast, the anti-apoptotic function of ET-2 is reported [18]. With the aim of analyzing the function of ET-2 in BCC, we treated a mouse BCC cell line with BQ-123 (Sigma–Aldrich, St. Louis, MO); a selective ET-RA antagonist, BQ-788 (Sigma–Aldrich); and a selective ET-RB antagonist and ET-2 recombinant protein (Sigma–Aldrich) [19]. However, we could not identify the direct oncogenic role of ET-2 in ASZ001 cells. There was no difference in cell proliferation, cell invasion and cell morbidity between non-treated cells and BQ-123-, BQ-788-, and ET-2-treated cells (data not shown). Therefore, ET-2 may function as a factor affecting the tissues surrounding BCC cells. As one of its putative functions, ET-2 will promote the angiogenesis of BCC. It is known that ETs are angiogenic factors that stimulate the growth of endothelial cells, vascular smooth muscle cells, fibroblast and pericytes in various cancers [20]. BCC also frequently shows a characteristic aberrant angiogenic feature called ‘‘arborizing vessels” in dermoscopic findings, which is histopathologically characterized by the proliferation and dilation of dermal capillaries [21]. We speculate that aberrant expression of ET-2 may play some role in this characteristic vascular formation.

Conclusion

We have characterized ET-2 as a novel target gene of the HH signaling pathway in BCC. Functional analysis of ET-2, especially in its role of tumor and interstitial interaction will shed new light on the pathogenesis of BCC.

Conflict of interest

None declared.

Acknowledgments

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