

## Epigenetic silencing of the endothelin-B receptor gene in non-small cell lung cancer

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**Abstract.** Endothelin-1 is overexpressed in several tumor types. Activation of the endothelin-A (ET<sub>A</sub>) receptor may promote cell growth, angiogenesis and invasion, and inhibits the apoptotic process, while activation of the endothelin-B (ET<sub>B</sub>) receptor may induce cell death by apoptosis and inhibit tumor progression. Hypermethylation and subsequent silencing of the ET<sub>B</sub> receptor gene promoter has been reported in some cancer types. As the endothelin pathway is subject to research for pharmacological cancer treatment, we investigated the extent of epigenetic deregulation of the ET<sub>B</sub> receptor gene in non-small cell lung cancer (NSCLC). We scanned 64 NSCLC paired tumor/normal surgical specimens for the ET<sub>B</sub> receptor promoter for methylation by developing four pyrosequencing assays that covered 24 CpGs. The ET<sub>B</sub> receptor promoter was significantly hypermethylated in 31 (48%) of tumor samples, presenting considerably higher methylation in 22/24 CpG sites compared with the normal counterpart tissues. ET<sub>B</sub> receptor mRNA levels were reduced in all lung tumors compared with normal adjacent lung tissue, indicating the potentially important involvement of this gene in lung cancer development. Furthermore, tumor samples with ET<sub>B</sub> receptor gene methylation tended to have lower receptor mRNA levels compared with unmethylated tumor specimens, suggesting a primary epigenetic role in ET<sub>B</sub> receptor silencing. Our results point to a significant

involvement of ET<sub>B</sub> receptor epigenetic deregulation in the pathogenesis of lung cancer making the gene a promising candidate biomarker for response to regimens modulating the endothelin axis.

### Introduction

Endothelin-1 is a 21-amino acid vasoactive peptide that plays an important role in the development and progression of many cancer types (1). Endothelin-1 is overexpressed in several tumor types, including those of the lung, where it is associated with poor prognosis (2,3). Binding of endothelin-1 to the G-protein-coupled endothelin-A (ET<sub>A</sub>) and -B (ET<sub>B</sub>) receptors on the cell surface results in distinct and opposing effects on cell growth and survival. In most cells, activation of the ET<sub>A</sub> receptor promotes cell growth, angiogenesis and invasion, and inhibits the apoptotic process (4), whereas, in contrast, activation of the ET<sub>B</sub> receptor induces cell death by apoptosis and thus inhibits tumor progression (5). Additionally, the ET<sub>B</sub> receptor mediates clearance of endothelin-1 (6), suggesting that ET<sub>B</sub> receptor blockade may increase circulating levels of endothelin-1, resulting in competition for ET<sub>A</sub> receptor binding between receptor antagonists and excess endothelin-1. This, in turn, may lead to increasing activation of the ET<sub>A</sub> receptor.

Any mechanism, therefore, that results in a reduction of ET<sub>B</sub> receptor expression may drive tumor progression by favoring cell survival. DNA methylation at cytosine-guanine (CpG) dinucleotides is a key epigenetic event involved in normal genetic processes including DNA repair, chromosome stability, telomere maintenance and transcriptional control (7). It is now well established that the DNA methylation profile of human tumors is fundamentally distorted by a combination of a global genome hypomethylation, and specific CpG island hypermethylation (8,9). Frequently, this CpG island hypermethylation occurs within the promoter regions of genes whose function is crucial for genome stability and growth control, including genes for DNA damage response, cell cycle arrest and apoptosis (9,10). Numerous studies have

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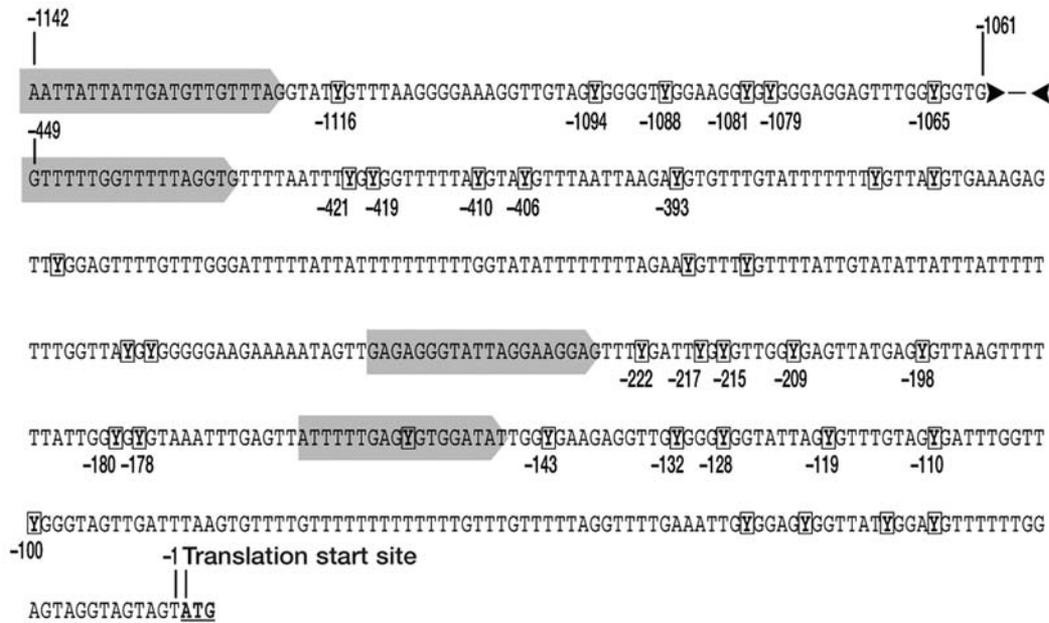


Figure 1. Bisulfite treated ET<sub>B</sub> receptor gene promoter sequence. Potentially methylated cytosine nucleotides (Y) are highlighted. Gray arrows indicate the location of each sequencing primer and the assay direction.

reported epigenetic silencing of such genes in human cancers, with frequent reports of tumor samples carrying abnormal DNA methylation in multiple genes, suggestive of a CpG methylator tumor phenotype (11-14).

Among all human neoplasias, lung cancer has a unique combination of high incidence and mortality that makes it the leading cause of cancer-related deaths worldwide (15). Abnormal epigenetic reprogramming in lung cancer has been investigated extensively, with studies showing a large number of hypermethylated genes (16-21). Of particular importance is the fact that abnormal DNA methylation has been detected in clinical specimens, such as plasma, sputum and bronchial lavage fluid from patients with lung cancer (22-27), suggesting a potential use of DNA methylation biomarkers in early detection and screening strategies (28).

Hypermethylation of the ET<sub>B</sub> receptor gene promoter and resultant silencing of gene expression has been reported in nasopharyngeal (29) and prostate tumors (3) and melanoma cell lines (30). Using Southern blot analysis, Nelson and co-workers found aberrant ET<sub>B</sub> receptor gene hypermethylation in 70% of prostate tumors, while no methylation was detected in morphologically normal tissue (31). Likewise, studies using real-time quantitative methylation-specific polymerase chain reaction (MS-PCR) found only low levels of hypermethylation in prostate tumor specimens and no methylation in normal tissue (32,33). The reasons for this discordance are unclear.

In contrast, other studies using MS-PCR assays have found a similarly high frequency of ET<sub>B</sub> receptor gene hypermethylation in specimens taken from prostate tumor, benign prostatic hypertrophy, and paired morphologically normal tissue (3,34). Methylation data from human lung cancer is limited. There has only been one previous study of ET<sub>B</sub>

receptor gene hypermethylation in lung cancer, in which methylation was detected in 26 out of 79 patients (33%) (6). To clarify the extent of epigenetic deregulation of the ET<sub>B</sub> receptor gene in non-small cell lung cancer (NSCLC), we analyzed methylation levels of the CpG island located in the promoter region of the ET<sub>B</sub> receptor gene in 64 lung tumor samples and compared them with methylation levels in adjacent normal tissue control specimens.

## Materials and methods

**ET<sub>B</sub> receptor gene methylation.** This study was carried out in accordance with ethics review board approval and all patients gave written informed consent. Frozen NSCLC tumor and matched adjacent normal control samples were obtained from 64 patients in two panels. Panel 1 consisted of 48 patients [22 female, 26 male; mean age 65 years (range, 45-82)] with NSCLC recruited at the Broadgreen Cardiothoracic Centre (University of Liverpool Cancer Research Centre, Liverpool, UK). All tumor specimens were of advanced stage (T2, n=44; T3, n=4) and were paired to normal adjacent tissue. Twenty-four patients (50%) had a histological diagnosis of adenocarcinoma; the other 24 patients (50%) had a diagnosis of squamous cell carcinoma.

Panel 2 consisted of 16 patients [1 female, 15 male; mean age 63 years (range 51-73)] with NSCLC recruited at Asterand PLC (Royston, Hertfordshire, UK) and GE Healthcare (Little Chalfont, Buckinghamshire, UK). Samples were of mixed stage (T1-T3). Nine patients had a histological diagnosis of adenocarcinoma, 3 of squamous cell carcinoma, and 4 were not classified.

Tissue samples were frozen immediately after biopsy and stored at -80°C. Tumor specimens were mounted and 6 μm sections were obtained and stained with hematoxylin and

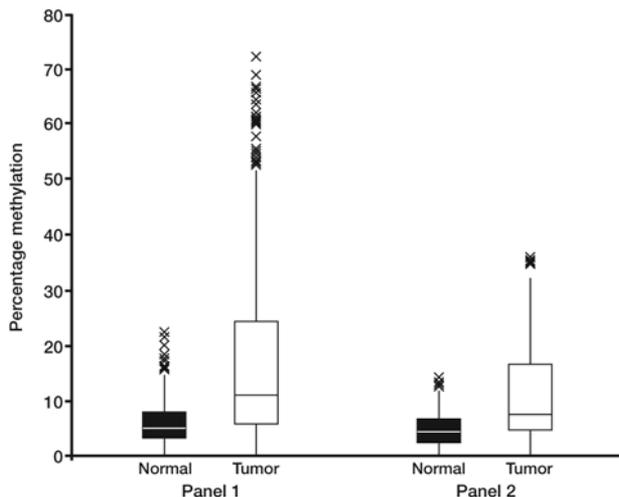


Figure 2. Summary statistics for percentage methylation over 24 CpG sites of the  $ET_B$  receptor gene, according to panel and tissue type. Each box represents the range from the 25th to 75th percentile. The vertical lines extend to 1.5 interquartile range, and values outside this are shown as crosses. Horizontal lines represent the median. The results from one normal sample were excluded from Panel 1, due to assay failure.

eosin. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Crawley, UK) and underwent sodium bisulfite treatment using the EZ DNA Methylation-Gold Kit™ (Zymo Research, CA, USA).

For analysis of  $ET_B$  receptor gene methylation, pyrosequencing assays spanning 24 CpG sites were developed using the primers shown in Fig. 1. As far as possible, the primers were designed to flank CpG sites of interest so that the PCR products accurately represented the methylation status of the source DNA. The pyrosequencing reactions were undertaken using the SQA-Gold kit (Biotage, Uppsala, Sweden) and quantitative, reproducible methylation data across the  $ET_B$  receptor gene CpG island were obtained using pyrosequencing with the Pyro Q-CpG™ software package (Biotage).

**$ET_B$  receptor expression.** Tissue of suitable quality for  $ET_B$  receptor expression analysis was available from 11 lung tumors (5 with methylated receptor genes and 6 with unmethylated receptor genes) from Panel 2. The specimens were thawed at  $-20^{\circ}\text{C}$  in RNAlater® ice (Ambion, TX, USA) and homogenized with the TissueLyser Mixer Mill MM 300 (Qiagen) in RLT buffer (Qiagen) containing  $\beta$ -mercaptoethanol for 6 min at 20 Hz. RNA was extracted from the 11 tumor samples and 4 normal lung tissue samples, and digested using the RNeasy Mini Kit and Rnase-free DNase set (Qiagen) and was quantified using a NanoDrop ND-1000 Spectrophotometer (LabTech International, Ringmer, UK). Samples were adjusted to  $100\text{ ng}/\mu\text{l}$ .

Total RNA quality was assessed using Agilent's 2100 Bioanalyser Instrument with the RNA 6000 Nano Assay (Agilent, CA, USA). A TaqMan® Low Density Array Endogenous Control Panel (Applied Biosystems, CA, USA) was run on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and the two most appropriate

endogenous control assays UBC (HS00824723\_m1) and B2M (HS99999907\_m1) were selected using the geNorm macro software (Ghent University, Ghent, Belgium) for Microsoft Excel (Microsoft Corporation, USA). Real-time quantitative PCR was carried out using a TaqMan® Gene Expression Assay (Applied Biosystems) for the  $ET_B$  receptor (Hs0024 0747\_m1). The relative quantification of  $ET_B$  receptor expression was calculated using the comparative Ct method, where  $\Delta\text{Ct}$  is  $\text{Ct}(\text{ET}_B \text{ receptor}) - \text{Ct}(\text{control})$ . The  $\Delta\text{Ct}$  values were used to calculate  $2^{-\Delta\Delta\text{Ct}}$ .

**Statistical analysis.** The Wilcoxon signed rank test was used to assess the differences in receptor gene methylation between tumor and normal tissue samples at each CpG site. Although the study was designed with individually matched tumor and normal samples, the distribution of the paired differences in percentage methylation between the tumor and normal samples was highly skewed. As a result, the Wilcoxon non-parametric test was preferred to a paired Student's t-test. Minimal loss of power was expected from selecting a non-paired analysis method because the variation between the normal samples was relatively low.

Heatmaps and line graphs were used to visualize the receptor gene methylation patterns across all CpG sites. In order to allow comparison of all tumor and normal tissue samples from the two panels, values in the two datasets were normalized by subtracting the mean methylation value from the raw methylation value and dividing the result by the standard deviation for that panel.

Spearman rank correlation was used to determine the relationship between  $ET_B$  receptor gene methylation and relative  $ET_B$  receptor expression in the 11 lung tumor specimens available for RNA extraction.

The frequency of hypermethylated tumor samples in each panel was calculated by counting the number of samples that had a mean methylation value (mean across all 24 CpG sites) greater than the mean + 3 standard deviations of all the matched normal mean methylation values, and dividing by the total number of tumor samples. All calculations were conducted on a log scale. The difference in hypermethylation frequency between tumor and normal samples was tested using Fisher's exact test.

## Results

**$ET_B$  receptor gene methylation.** In both patient panels, the proportion of samples showing  $ET_B$  receptor gene hypermethylation was significantly higher in tumors compared with the corresponding normal tissues [25/48 versus 1/48, respectively, for Panel 1 ( $P<0.001$ ) and 6/16 versus 0/16, respectively, for Panel 2 ( $P=0.03$ ); Fig. 2]. It was of note that the relative methylation of the 24 individual CpG sites varied within samples creating patterns. Normalized individual methylation profiles across the two panels are shown in Fig. 3. When comparing methylation of individual CpGs, 22 of the 24 CpG sites demonstrated significantly higher levels in tumor specimens (Table I). A heatmap visualization of the  $ET_B$  receptor gene methylation data (Fig. 4) showed that the tumor tissues exhibited a variety of promoter region methylation patterns. Broadly, there

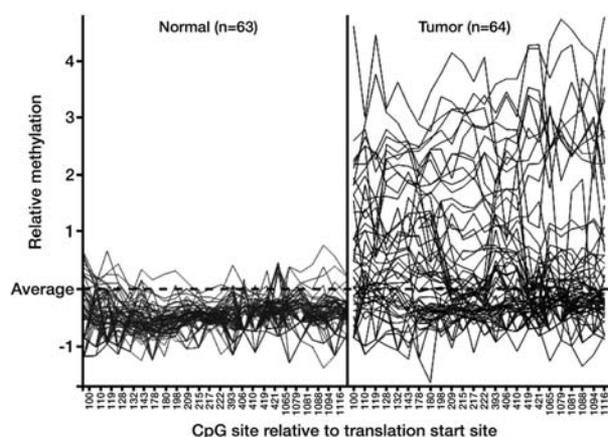


Figure 3. Individual methylation profiles normalized across the two panels.

were four categories: a) consistently high methylation across all 24 CpG sites; b) consistently moderate methylation; c) variable methylation across the 24 CpG sites, resulting in a moderate overall level of methylation; and d) consistently low methylation.

We have screened, as an additional control, DNA from the peripheral blood of 16 healthy individuals. The level of methylation in all these samples was similar to that of normal adjacent lung.

*ET<sub>B</sub> receptor expression.* With the exception of one tumor tissue sample (sample number 11),  $ET_B$  receptor mRNA levels were reduced in all lung tumors compared with normal lung tissue, indicating a clear relationship between tissue type and receptor expression (Fig. 5).

Table I. Median percentage methylation at each  $ET_B$  receptor gene CpG site in normal tissue controls and NSCLC tumor specimens.

CpG site	Panel 1				Panel 2			
	Normal (n=47)		Tumor (n=48)		Normal (n=16)		Tumor (n=16)	
	Median	Range	Median	Range	Median	Range	Median	Range
100	5	0-17	16 <sup>a</sup>	0-67	5	0-11	8 <sup>b</sup>	4-22
110	5	0-12	16 <sup>a</sup>	0-43	5	0-9	8 <sup>c</sup>	0-25
119	5	0-18	15 <sup>a</sup>	0-64	3	0-6	7 <sup>b</sup>	0-21
128	6	0-18	19 <sup>a</sup>	5-55	6	0-11	11 <sup>b</sup>	3-35
132	8	0-15	23 <sup>a</sup>	0-66	7	4-12	14 <sup>b</sup>	6-36
143	9	0-21	26 <sup>a</sup>	9-62	8	5-14	14 <sup>c</sup>	4-35
178	9	0-20	22 <sup>a</sup>	0-62	7	6-13	15 <sup>a</sup>	6-35
180	13	6-22	22 <sup>a</sup>	11-61	9	7-12	14 <sup>a</sup>	9-32
198	9	4-20	18 <sup>a</sup>	6-66	8	6-13	14 <sup>b</sup>	6-35
209	5	0-12	10 <sup>a</sup>	0-60	4	0-6	6 <sup>b</sup>	0-25
215	4	0-8	8 <sup>a</sup>	0-62	3	0-6	5 <sup>a</sup>	3-24
217	8	5-15	14 <sup>a</sup>	6-73	4	2-6	7 <sup>a</sup>	0-28
222	5	0-12	9 <sup>a</sup>	0-67	3	0-6	7 <sup>a</sup>	3-25
393	8	0-14	17 <sup>a</sup>	5-61	0	0-11	10 <sup>b</sup>	0-27
406	0	0-9	11 <sup>a</sup>	0-58	0	0-7	6 <sup>b</sup>	0-23
410	4	0-9	11 <sup>a</sup>	0-60	5	0-8	9 <sup>b</sup>	0-25
419	4	0-8	6 <sup>a</sup>	2-54	2	0-5	3	0-21
421	5	0-14	7 <sup>a</sup>	0-56	0	0-5	4	0-17
1065	4	0-11	7 <sup>a</sup>	0-52	4	0-7	5 <sup>b</sup>	3-19
1079	0	0-7	4 <sup>a</sup>	0-43	2	0-4	4 <sup>b</sup>	0-18
1081	4	0-11	7 <sup>a</sup>	0-46	3	2-6	5 <sup>b</sup>	0-25
1088	5	0-16	8 <sup>a</sup>	0-50	5	3-9	8 <sup>b</sup>	4-25
1094	3	0-11	6 <sup>a</sup>	0-41	3	0-7	5 <sup>b</sup>	2-18
1116	3	0-8	5 <sup>a</sup>	0-41	3	0-5	4 <sup>c</sup>	0-27

<sup>a</sup>P<0.001 versus normal; <sup>b</sup>P<0.01 versus normal; <sup>c</sup>P<0.05 versus normal.

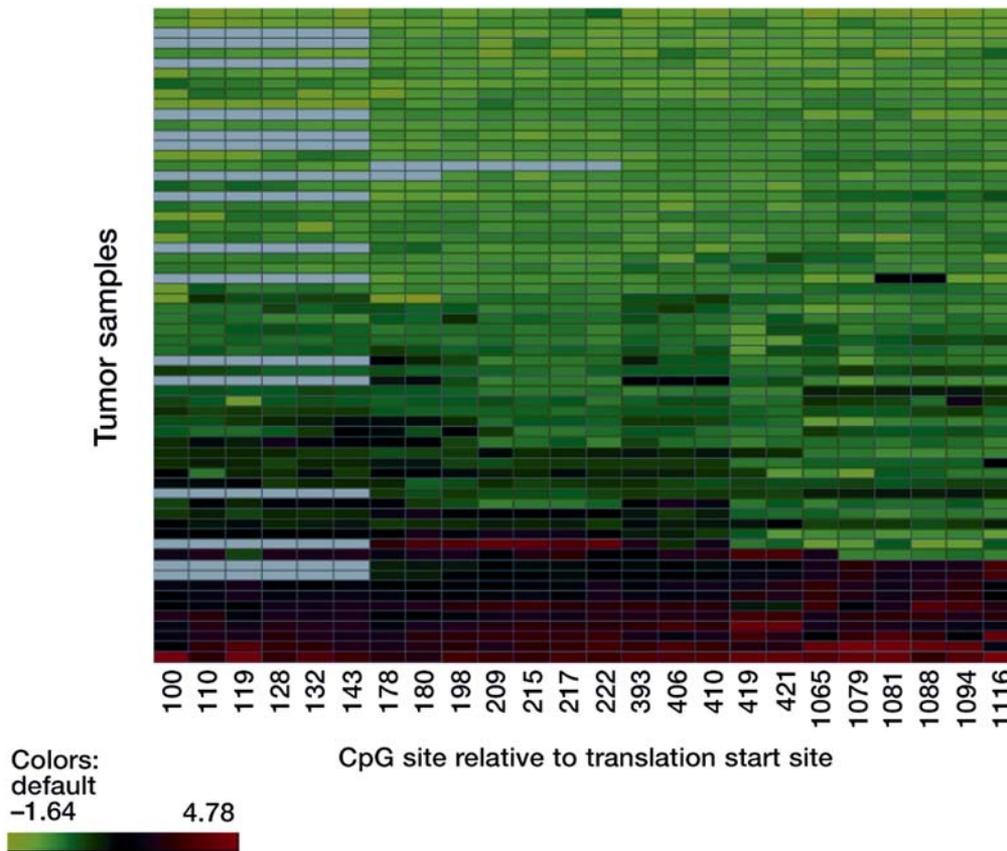


Figure 4. Heatmap visualization of normalized  $ET_B$  receptor gene promoter methylation in tumor samples. Each column represents one CpG site and each row represents one tumor sample. Red indicates hypermethylation; green indicates absence of methylation; gray indicates no data. Samples are ranked by increasing average methylation across all CpG sites.

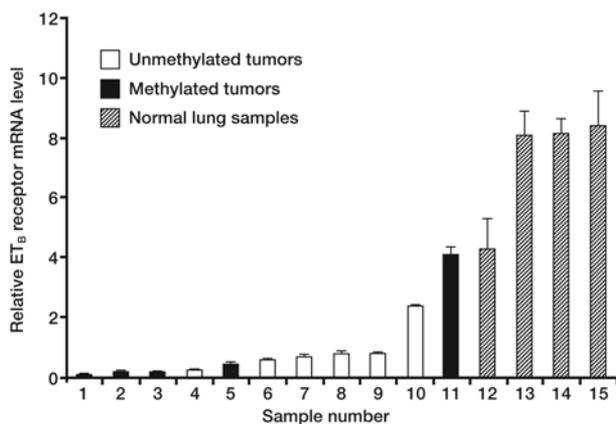


Figure 5. Relative  $ET_B$  receptor mRNA levels in unmethylated tumors, methylated tumors and normal lung samples. Samples are ranked by increasing relative receptor expression.

Within the tumor specimens, samples with  $ET_B$  receptor gene methylation demonstrated lower receptor mRNA levels compared with unmethylated tumor specimens (Spearman rank correlation coefficient,  $-0.63$ ;  $P=0.039$ ).

No association was observed between hypermethylation and histology, nodal metastasis, or differentiation in Panel 1, while no such data was available for Panel 2.

## Discussion

In view of its potential implication on endothelin axis-based cancer treatments, we investigated the extent of epigenetic deregulation of the  $ET_B$  receptor gene. More specifically, we scanned the CpG island located in the promoter region, in order to map its methylation pattern in NSCLC. We demonstrated a significant aberrant methylation across the  $ET_B$  receptor gene promoter region, suggesting methylation plays an important role in lung tumorigenesis. Hypermethylation of the  $ET_B$  receptor has been also described in prostate (31,35,36), nasopharyngeal (29), bladder (37) and renal (38) tumors, while there is a single previous report for lung cancer (6). This indicates that epigenetic deregulation of the  $ET_B$  receptor is most probably a common feature of many epithelial tumor types. There was no difference in hypermethylation observed between histological types, namely adenocarcinomas and squamous cell carcinomas, which contradicts the previous report (6). A larger confirmatory study is required to confirm the absence or presence of such a difference.

It is of note that methylation levels across the promoter were frequently subject to significant differences creating specific methylation patterns that can be visualized in the heatmap analysis (Fig. 4). The significance of the different patterns of methylation observed in tumors is not yet understood. While some tumors showed consistently high levels

of methylation, which may be expected to lead to down-regulation of ET<sub>B</sub> receptor expression, others showed variable, moderate, or even low levels of methylation across all CpG sites. The variable methylation patterns observed in some tumor samples may therefore lead to incorrect assignment in analyses based on a small number of representative CpG sites. These data highlight the importance of quantitative, comprehensive analysis of CpG sites across the promoter region, prior to design of a targeted assay.

In this study, tissue of sufficient quality for RNA extraction was only available from a small subset of samples. Nevertheless, ET<sub>B</sub> receptor mRNA levels were significantly lower in tumors with methylated gene promoters compared with tumors with unmethylated gene promoters. These results demonstrate for the first time in lung tumors the ET<sub>B</sub> receptor gene epigenetic repression of transcription, confirming previous findings in lung tumor cell lines (6). This is important not only because ET<sub>B</sub> receptor gene methylation is implicated in the pathogenesis of lung cancer, but also because patients with methylation and resultant loss of receptor expression may respond differently to treatment with ET<sub>A</sub> receptor antagonists.

Absence of the active form of the ET<sub>B</sub> receptor reduces the opportunity for apoptotic signaling elicited by activation of the ET<sub>B</sub> receptor. Moreover, downregulation of the ET<sub>B</sub> receptor may prevent internalization and removal of endothelin-1. In the tumor cell environment, this lack of compensatory apoptotic influence coupled with an inability to remove endothelin-1 from the circulation may permit unabated activation of the ET<sub>A</sub> receptor, leading to increased cell survival, proliferation and invasion. Thus, tumors with hypermethylated ET<sub>B</sub> receptor gene promoter regions may be more aggressive and progressive, and lead to metastatic disease more quickly than those tumors where such hypermethylation is absent.

The highest levels of endothelin-1 expression have been recorded around tumor vasculature and in tumor tissue specimens from patients with NSCLC (2,39). While endothelin-1 expression has been demonstrated in all lung cancer types, the highest levels have been observed in adenocarcinoma and squamous cell carcinoma (40,41), the type analyzed in our study. Hence, in tumors where ET<sub>B</sub> receptor gene hypermethylation prevails, this may lead to an increased local concentration of endothelin-1, which will drive the survival of that tumor via unabated activation of the ET<sub>A</sub> receptors.

The role of ET<sub>B</sub> receptor gene hypermethylation in response to treatment with endothelin receptor antagonists is currently unclear. However, one may hypothesize that patients with an unmethylated ET<sub>B</sub> receptor gene could gain a clinical benefit as blocking the ET<sub>A</sub> receptor would redirect signaling via a still-active pro-apoptotic pathway mediated by the ET<sub>B</sub> receptor. On the other hand, patients with a methylated ET<sub>B</sub> receptor gene would receive the majority of their endothelin-1 signaling in tumor tissue via the ET<sub>A</sub> receptor pathway. Inhibition of that pathway may therefore have a more dramatic impact on disease progression in patients with a methylated ET<sub>B</sub> receptor. The role of ET<sub>B</sub> receptor gene hypermethylation is currently being explored

in cell lines and in clinical trials in patients receiving treatment with a specific ET<sub>A</sub> receptor antagonist.

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