Aberrant methylation of the vascular endothelial growth factor receptor-1 gene in prostate cancer

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Transcriptional silencing of cancer-related genes by DNA methylation is observed in various cancers. To identify genes controlled by methylation in prostate cancer, we used cDNA microarray analysis to investigate gene expression in prostate cancer cell lines LNCaP and DU145 treated with a methyltransferase inhibitor alone or together with a histone deacetylase inhibitor. We detected significant changes (3.4–5.7%) in gene expression in prostate cancer cell lines with the drug treatments. Among the affected genes, that for the vascular endothelial growth factor receptor 1 (*VEGFR-1*) was re-expressed in LNCaP and DU145 after the drug treatments. Bisulfite sequencing revealed the promoter and exon 1 of the *VEGFR-1* to be hypermethylated in the cell lines. These results support the idea that methylation is associated with loss of VEGFR-1 mRNA expression in prostate cancer cell lines. Combined bisulfite restriction analysis (COBRA) showed the gene to be methylated in 24 (38.1%) of 63 primary local prostate cancer samples, while in all 13 benign prostate samples it was not. These findings indicate that methylation of *VEGFR-1* is related with prostatic carcinogenesis. (Cancer Sci 2003; 94: 536–539)

Prostate cancer is not as commonly diagnosed in Japanese as in Caucasians or African-Americans, but the number of cases is increasing every year. Genetic alterations, such as point mutations, loss of heterozygosity, and homozygous deletions in many tumor suppressor genes, are associated with carcinogenesis.[1)] Many studies have provided evidence of such genetic alterations in prostate cancers.[2)] More recently, it has also been reported that inactivation of tumor suppressor genes by epigenetic promoter methylation plays an important role in carcinogenesis and tumor progression.[3)]

DNA methylation, especially in CpG-rich 5′ regions, inhibits transcription by interfering with the initiation or by reducing the binding affinity of sequence-specific transcription factors.[4)] In prostate cancer, inactivation by aberrant methylation has been reported for many genes, such as *RARβ2*, *GSTP1*, *E-cadherin* and *RASSF1A*.[5–9)] Restriction landmark genomic scanning (RLGS),[9)] methylated CpG island amplification (MCA)/representational difference analysis (RDA),[10)] methylation-sensitive (MS)/RDA,[11)] and differential methylation hybridization (DMH)[12)] have been employed to find genes whose expression is regulated by DNA methylation.

To identify cancer-related genes controlled by epigenetic alteration in prostate cancer, we have performed screening by cDNA microarray analysis in prostate cancer cell lines treated with a methyltransferase or/and a histone deacetylase inhibitor. We detected significant re-expression of the vascular endothelial growth factor receptor 1 (*VEGFR-1*) gene in cells treated with drugs. VEGFR-1, also called Flt-1, is a tyrosine kinase receptor for VEGF which plays an important role in tumor angiogenesis and growth.[13, 14)] Several studies have pointed to altered expression and function of VEGF or its receptor (VEGFR-1, -2) in prostate cancer. In this study, we analyzed the methylation status of the 5′ region of *VEGFR-1* in prostate cancer cell lines and primary prostate samples.

Materials and Methods

Cell lines and primary prostatic samples. Prostate cancer cell lines, LNCaP, DU145 and PC-3, were obtained from the American Type Tissue Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Specimens of 63 local primary prostate cancers and 13 benign prostates with hyperplasia (BPH) were obtained by surgery, snap-frozen and stored at −80°C. Exclusion of prostate cancer from the latter was confirmed histopathologically.

Drug treatments and cDNA microarray analysis. The methyltransferase inhibitor, 5-aza-2′-deoxycytidine (5-Aza-CdR), and the histone deacetylase inhibitor, trichostatin A (TSA), were used (Sigma Chemical Co., St. Louis, MO). LNCaP and DU145 were treated with 1 μM 5-Aza-CdR for 72 h before harvesting, alone or with 100 ng/ml of TSA for the last 24 h. These two cell lines were selected because LNCaP is an androgen-dependent, and DU145 is an androgen-independent cell line. Changes in mRNA expression compared with the non-treatment case were analyzed using the Atlas Human Cancer 1.2 Array (BD Biosciences Clonthech, Tokyo). Expression increases or decreases of at least twofold compared with controls were considered as significant.

RT-PCR. From prostate cancer cells treated with 5-Aza-CdR or/and TSA, total RNA was extracted using Isogen (Nippon Gene, Tokyo), according to the manufacturer's instructions. One microgram of total RNA was used for generation of cDNAs using Superscript reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The specific primers applied to detect VEGFR-1 transcripts were as follows: forward, 5′-GCA CCT TGG TTG TGG CTG AC-3′, reverse, 5′-CGT GCT GCT TCC TGG TCC-3′. PCR amplification was performed for 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Human β-actin was used as an internal control. The PCR products were subjected to electrophoresis in 2.0% agarose gels, stained with ethidium bromide and quantitated by the use of NIH Image.

Bisulfite modification. Genomic DNA (2 μg) was treated with sodium bisulfite as described previously.[15)] Briefly, after denaturation in 0.3 *M* NaOH at 37°C for 15 min, sodium bisulfite and hydroquinone were added to give final concentrations of 3.1 *M* and 0.5 m*M*, respectively. The reaction was performed at 55°C for 16 h, and the mixture was desalted using Wizard DNA purification resin (Promega, Madison, WI) according to the manufacturer's instructions. Bisulfite modification was completed by 0.3 *M* NaOH treatment at 37°C for 15 min. Modified DNA was

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precipitated with ethanol, washed in 70% ethanol, dried, and resuspended in 50 μl of distilled water.

Bisulfite sequencing and combined bisulfite restriction analysis (COBRA). The methylation status of the 5′-regulatory region of the *VEGFR-1* gene was analyzed by bisulfite genomic sequencing and COBRA as described.[16, 17] Modified DNAs were amplified with the following primers: forward, GGTGGAGGGAGTTTGTAAGG, reverse, TTCCCCAACCCACTTCCYAC. The PCR reaction was performed for 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. The PCR products were subcloned into the TOPO TA cloning vector (Invitrogen, San Diego, CA). About 10 positive clones in each sample were sequenced using the Big Dye terminator method. With COBRA for VEGFR-1 in prostate samples, we employed the same primers and PCR conditions as for bisulfite sequencing. PCR products were digested with *Taq* I (New England Biolabs, Beverly, MA) at 65°C for 4 h, and then were subjected to electrophoresis in 4.0% agarose gels and visualized by ethidium bromide staining. When digested bands were confirmed, a methylated status was concluded.

Statistical analysis. Associations between methylation status and clinicopathological characteristics (age, stage, Gleason score, androgen dependence) were analyzed by χ^2 test with the criterion of significance set at the level of $P<0.05$. All comparisons were conducted using Survival Tool for StatView (Abacus Concepts, Berkeley, CA).

Results

To identify genes regulated by epigenetic mechanisms such as methylation and histone acetylation, a total of 1176 cancer-related genes in LNCaP and DU145 were analyzed for change of expression induced by 5-Aza-CdR or/and TSA treatments (Table 1). In LNCaP, 24 and 23 genes were up-regulated significantly by 5-Aza-CdR alone and together with TSA, respectively. Table 2 shows the common genes induced by 5-Aza-CdR alone and together with TSA in LNCaP. In DU145, this was the case for 19 and 34 genes. We next sought to confirm the observed gene expression in LNCaP, DU145, and PC-3 cells before and after treatment with drugs by RT-PCR. The *VEGFR-1* was isolated as one example showing re-expression or up-regulated in more than two cell lines (Fig. 1). A CpG island is included in the promoter region/exon 1 of *VEGFR-1*

Table 1. Summary of Atlas Human Cancer 1.2 array results for prostate cancer cell lines after drug treatment

Cell line	Treatment	Changed genes	Direction of shift (ratio)	
			Up (≥2.0)	Down (≤0.5)
LNCaP	5-Aza-CdR	47 (4.0%)	24 (2.0%)	23 (2.0%)
	5-Aza-CdR+TSA	54 (4.6%)	23 (2.0%)	31 (2.6%)
DU145	5-Aza-CdR	40 (3.4%)	19 (1.6%)	21 (1.8%)
	5-Aza-CdR+TSA	67 (5.7%)	34 (2.9%)	33 (2.8%)

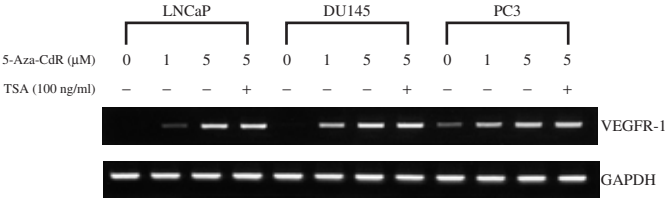

Fig. 1. VEGFR-1 mRNA expression in prostate cancer cell lines. Prostate cancer cell lines were treated with 1 or 5 μM methyltransferase inhibitor (5-Aza-CdR) for 72 h alone or with 100 ng/ml of histone deacetylase inhibitor (TSA) for the last 24 h. In LNCaP and DU145, VEGFR-1 expression was recovered with 5-Aza-CdR. In PC-3, VEGFR-1 expression was up-regulated.

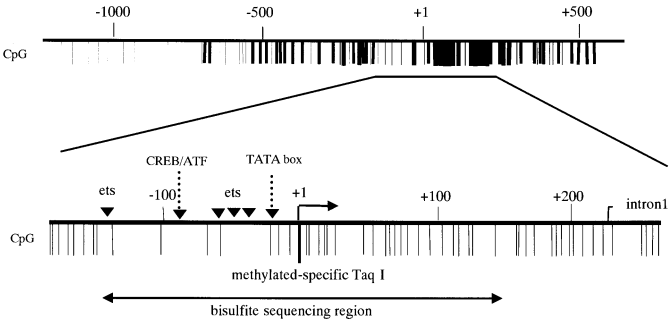

Fig. 2. Map of the *VEGFR-1* gene 5′ CpG island. The CpG density is represented by vertical lines corresponding to each CpG site. The positions of ets binding sites, a CREB/ATF element and a TATA box binding site are indicated.

Table 2. Up-regulated genes in LNCaP treated with drugs

Gene	Accession No.	Ratio	
		5-Aza-CdR	5-Aza-CdR+TSA
Leukocyte antigen-related protein precursor (*LAR*) and intearin-associated protein (*IAP*)	Y00815+X69398	5.9	6.0
WAF1	L25610	4.2	3.4
ATP synthase coupling factor 6 mitochondrial precursor (*F6*)	M37104	4.1	3.8
ADP/ATP carrier protein	J02683	2.9	2.6
ERBB-3 receptor protein-tyrosine kinase precursor epidermal growth factor receptor	M34309	2.9	2.3
Type II cytoskeletal 2 epidermal keratin (*KRT2E*)	M99061	2.8	6.0
Stromelysin 3	X57766	2.8	2.7
Transforming protein rhoA H12 (*ARH12*)	L25080	2.8	2.3
Nucleotide diphosphate kinase B (*NDKB*)+nm23-H2S	L16785+M36981	2.5	3.4
DCHT	AF017635	2.5	2.3
VEGFR1	**X51602**	**2.4**	**2.7**
Insulin-like growth factor binding protein 2 (*IGFB P2*)	M35410	2.3	2.7
rhoC	L25081	2.3	2.3
Transforming protein rhoB (*ARH6*)	X06820	2.2	2.0
Cathepsin D precursor (*CTSD*)	M11233	2.2	2.0

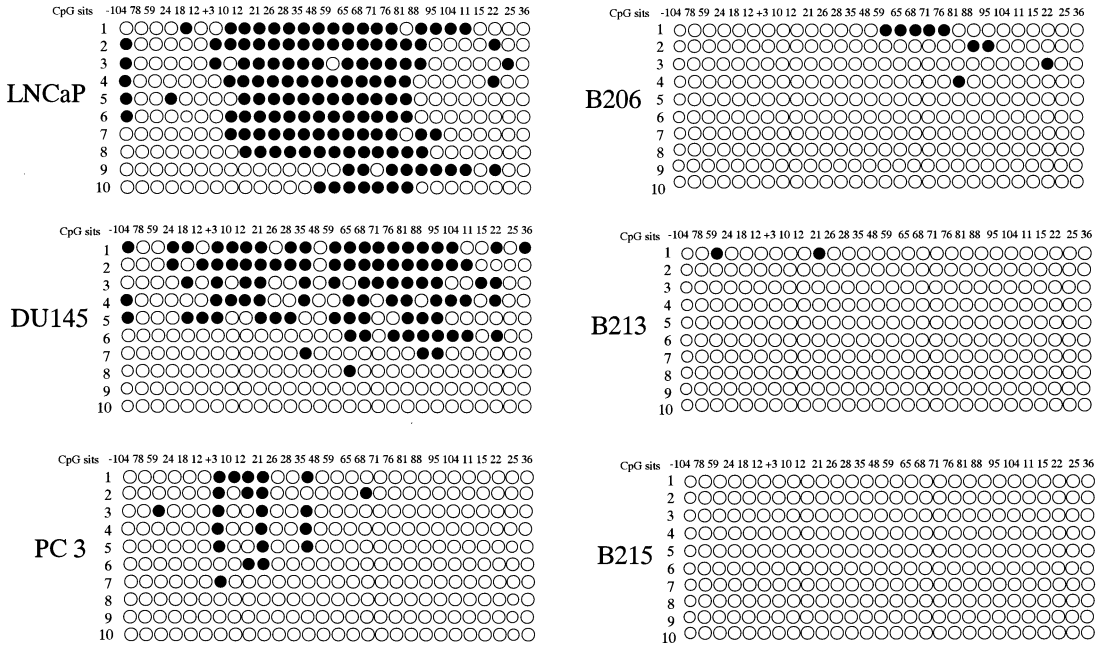

Fig. 3. Methylation status of the 5′ region of the *VEGFR-1* gene. Prostate cancer cell lines (LNCaP, DU145 and PC-3) and benign prostate samples (B206, B213, B215) were analyzed by bisulfite sequencing. For each sample, 10 clones were analyzed for methylation status. Methylated and unmethylated CpG sites are represented by closed and open circles, respectively.

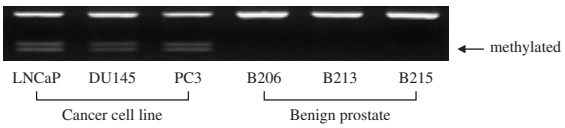

Fig. 4. Methylation analysis by COBRA of the 5′ region of the *VEGFR-1* gene in prostate cancer cell lines and benign prostate samples. PCR products were digested with a restriction enzyme (*Taq I*) that cleaves CpG sites retained after bisulfite treatment because of methylation. The arrow indicates methylated alleles. The prostate cancer cell lines are all methylated, while benign prostates are not.

Table 3. Frequencies of VEGFR-1 methylation during prostatic carcinogenesis

Samples	*n*	Methylation-positive
Prostate cancer cell line	3	3 (100%)
Primary prostate cancer	63	24 (38.1%)
Androgen-dependent	53	19 (35.8%)
Androgen-independent	10	5 (50.0%)
Benign prostate[1)]	13	0 (0%)

1) ALL samples were obtained from benign prostatic hyperplasia, with prostate cancer pathologically excluded.

(Fig. 2). So, we assessed the methylation status of the 5′ CpG island in the promoter region and exon 1 of *VEGFR-1*. Bisulfite sequencing of about 28 CpG dinucleotides revealed aberrant methylation in LNCaP and DU145, which showed re-expression on 5-Aza-CdR treatment, and partial methylation in PC-3, which showed up-regulation (Fig. 3). On the other hand, three benign prostatic tissues tested were all hypomethylated. These results show the importance of methylation in the epigenetic silencing of this gene. COBRA also revealed the three prostate cancer cell lines to be methylated (Fig. 4) and 24 (38.1%) of 63 samples of primary prostate cancers were methylated, but none of 13 benign prostate specimens. The frequency of methylation in androgen-independent prostate cancers, 5 (50%) of 10, was slightly higher than in androgen-dependent cancers, 19 (35.8%) of 53, but this was not significant (P=0.4, Table 3). Other clinicopathological characteristics were not significantly associated with methylation status (data not shown).

Discussion

The present cDNA microarray analysis demonstrated about 3–6% of genes to be up-or-down regulated in prostate LNCaP and DU145 cancer cell lines on treatment with 5-Aza-CdR alone or in combination with TSA. Up-regulated genes included DNA repair genes such as *hMLH1*, and cell cycle regulatory genes such as *p21*, expression of which is known to be suppressed by promoter methylation in several cancers.[17, 18)] Thus, our data are in agreement with these reports. The screening method using microarrays for prostate cancer cells with drug treatment has advantages for identification of candidate genes, although it is limited by the genes targeted. Array chips allowing analysis of many more genes, such as plastic or glass arrays, are being developed.

Liang *et al*. reported the use of a gene expression chip for 6600 human genes after treatment of human fibroblasts or bladder cancer cells with 5-Aza-CdR, and showed the IFN pathway to be highly inducible.[19)] Changes in expression with drug treatment are thought to be caused by at least three processes.[20)] The first is direct change of DNA demethylation or histone acetylation by drugs. The second is change downstream of demethylated or acetylated genes. The third is cytotoxic effects. Therefore, we have to distinguish epigenetically regulated genes.

In the present study, we showed that the *VEGFR-1* is re-expressed in LNCaP and DU145, and up-regulated in PC-3 treated with 5-Aza-CdR. The 5′-flanking region of the *VEGFR-1* gene contains a CpG island, and in this region, there are putative transcription factor binding sites such as a TATA box, a cAMP response element binding protein/activating transcription factor (CREB/ATF) element, and ets binding sites (Fig. 2).[21)] Our bisulfite sequencing showed aberrant methylation of the 5′ region of the *VEGFR-1* in LNCaP and DU145, but only

partial methylation in PC-3. This suggests that promoter methylation of *VEGFR-1* plays a key role in silencing of the gene in prostate cancer cells. To our knowledge, this is the first report providing evidence for such epigenetic regulation of VEGFR-1. VEGF also binds to VEGFR-2, known as Flk-1/KDR.[22] Application of monoclonal antibodies against VEGF resulted in decreased growth and metastasis of DU145 prostate cancer xenografts in severe combined immuno-deficiency mice, indicating that VEGF is a critical factor for progression.[23]

It is still unclear what role the *VEGFR-1* plays in the development and progression of prostate cancer. Nevertheless, VEGFR-1 mutant ES cell cultures and embryos exhibit vascular overgrowth, suggesting that the receptor may act as a negative regulator of blood vessel formation.[24] Several studies on the expression of *VEGFR-1* in prostate tissues have been reported.[25–28] One immunohistochemical investigation showed that the *VEGFR-1* was expressed in normal prostatic epithelium, while 56% of cancer samples were negative, including all those with a Gleason score of >8.[26] Huss *et al*. conducted *in situ* analyses of prostate tissues from the transgenic adenocarcinoma of mouse prostate (TRAMP) model, and identified decreased expression of the *VEGFR-1*, but up-regulation of *VEGFR-2* during progression, calling this a "progression switch."[27] More recently, Jackson *et al*. found that the expression of the *VEGFR-1* is increased in prostatic intraepithelial neoplasia and malignant cells in well- and moderately differentiated neoplasms in comparison with normal glands, but is decreased in poorly differentiated lesions.[28] Since the prostate cancer cell lines, LNCaP, DU145 and PC-3, were derived from lymph node, brain and bone metastases, respectively, they would be expected to be advanced. Methylation of *VEGFR-1* was detected in 38.1% of primary prostate cancer samples, but in none of the benign prostate samples. Interestingly, the frequency of *VEGFR-1* methylation in androgen-independent prostate cancers was higher than in non-refractory prostate cancers (50% vs. 38.1%), although the relationship was not significant. These results suggest that methylation of the *VEGFR-1* may be related with carcinogenesis of the prostate.

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