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Differential expression of galectins in normal, benign and malignant prostate epithelial cells: Silencing of galectin-3 expression in prostate cancer by its promoter methylation

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Abstract

Galectins (gal), a family of soluble β-galactoside-binding proteins present at the cell surface, are involved in cancer progression and metastasis. Here we investigated the expression of several galectins in normal (PrEC), benign (BPH-1), and malignant (LNCaP) prostate epithelial cells and found that all galectins, except gal1 are differentially expressed. The gal3, 7, and 9 are highly expressed in PrEC, but not in LNCaP cells. Out of seven isoforms of gal8, the proto isoform gal8e and our newly discovered proto isoform gal8g were upregulated in LNCaP cells compared to PrEC, whereas the two tandem-repeat isoforms gal8a and gal8b were equally expressed in these cells. To determine if the silencing of gal3 in LNCaP cells was due to promoter methylation, LNCaP cells were treated with azacytidine. Azacytidine treatment induced the expression of gal3 in LNCaP cells, indicating that the gal3 gene was silenced by methylation of its promoter. To examine further, we evaluated cytosine methylation in gal3 promoter in LNCaP, normal prostate and placenta DNA and observed that it is highly methylated in LNCaP but not in normal cells and azacytidine completely abolished this methylation in LNCaP cells. Similar to prostate cancer cells, gal3 promoter was highly methylated in human prostate cancer tissue but not in normal tissue. To our knowledge, this is the first report indicating that gal3 expression is regulated by promoter methylation in LNCaP cells and prostate tumors. The methylation of gal3 promoter may constitute a powerful tool for early diagnosis of prostate cancer.
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Keywords: Galectin-3; Galectin-8 isoforms; Prostate cancer; Promoter methylation; Differential expression; Gene silencing

Cell surface proteins that modulate cell–cell and cell–extracellular matrix interactions are subjects of current intense research in cancer biology including diagnosis, prevention and therapeutics. Galectins (gal), a family of β-galactoside-binding proteins, are involved in cancer progression and metastasis [1,2]. To date, fifteen members of the galectin family have been identified in higher vertebrates displaying either one (proto and chimera types) or two carbohydrate-recognition domains (CRD) (proto and tandem-repeat types) [3]. Gal1, 2, 5, 7, 10, 11, 13, 14, and 15 are examples of the proto type galectins- of which gal5 and 7 are monomers, whereas all others are homodimers. Gal4, 6, 8, 9, and 12 are tandem-repeat type galectins. Gal3 is the only chimera type galectin, with three distinct structural domains: a 12-amino acid NH_2-terminal domain, a collagen-like sequence rich in proline and glycine; and a carboxy-terminal domain containing a CRD [4].

DNA methylation is an enzyme-mediated chemical modification that adds methyl (CH_3) groups at selected sites of DNA. In mammals, DNA methylation is the only known natural modification of DNA, and may affect cytosine (C) only when it is followed by guanosine (G). Thus, in mammals, DNA methylation occurs at CpG sites. Several studies suggest that the transcriptional silencing of tumor suppressor genes with aberrant promoter methylation has an important role in the development of various cancers including prostate cancer [5]. Although most galectins are

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ubiquitously expressed in various human tissues, in most cancers galectins were either silenced or upregulated compared to the normal tissue [6]. For example, gal1 is upregulated in thyroid cancer and uterine sarcoma [7,8], but its expression is decreased in head and neck cancer compared to the normal tissue [9]. Similarly, expression of gal3 is found upregulated in gastric cancer [10], liver cancer [11], and thyroid cancer [7], but its expression is down regulated in head and neck cancer [9] and uterine sarcoma [8] compared to normal tissues. In prostate cancer, the expression of gal3 is found decreased compared to normal prostate tissue [12]. Moreover, Pacis et al. [13] investigated various stages of prostate tumors for gal3 expression and found that the expression was dramatically decreased in early stages of tumors relative to the normal prostate tissue. In contrast, results on expression of gal8, also known as prostate carcinoma tumor antigen-1 (PCTA-1; identified by surface-epitope masking and expression cloning), are conflicting. One study indicates that gal8 is selectively expressed in prostate tumors, but not in normal prostate or benign hyperplasia [14]. On the contrary, histochemical analysis revealed that gal8 is expressed at low levels in normal tissues as well as benign hyperplasia or adenocarcinoma [15]. The latter view is supported by the results from Cancer Genome Anatomy Project (CGAP) library analysis (http://cgap.nci.nih.gov/). The mechanisms of regulation of galectin expression are poorly understood. In general, the regulation of galectin expression is a complex process which depends on cell type, external stimuli and environmental conditions and involves numerous transcription factors and signaling pathways [16]. However, it remains largely unknown until very recently if epigenetic changes of galectin DNA are responsible for differential expression in cancers. Recently Ruebel et al. [17] showed that the expression of the gal3 gene in pituitary tumors was silenced by methylation of its promoter.

In this study, we investigated the expression of several galectins in normal, benign and malignant prostate epithelial cells and showed that most galectins including our newly discovered proto type isoform of gal8 are differentially expressed. Moreover, our results show for the first time that the silencing of the gal3 gene in LNCaP cells is regulated by methylation of its promoter. Similarly, the methylation status of the gal3 promoter isolated from the prostate tumor tissues showed that it was heavily methylated. The methylation pattern of gal3 in prostate tumors may constitute a powerful tool for the development of early diagnostic tests for prostate cancer.

Materials and methods

Cell culture. PrEC cells (Clonetics/Cambrex, North Brunswick, NJ) was cultured in keratinocyte-serum free medium with 2 mM glutamine, 5 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract (Gibco/Invitrogen Corp., Carlsbad, CA) and 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (Sigma, St. Louis, MO). BPH-1 (benign prostatic hyperplasia) cell line (was a gift from Dr. Simon Hayward) was cultured in IMEM (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Quality Biologicals, Gaithersburg, MD), 2 mM glutamine, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. LNCaP (American Type Culture Collection, Manassas, VA) cells were cultured in phenol red free RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. All cells were cultured in the presence of 5% CO_2 at 37 °C.

The LNCaP cells were treated with 10 μM 5-aza-2′-deoxycytidine (Sigma) for 7 days in two-independent experiments following a protocol published elsewhere [18]. Cells were treated with azacytidine on a daily basis and the medium was replaced upon treatment. Control wells received only medium with vehicle. At the end of the treatment, cells were harvested and used for DNA and RNA.

Preparation of RNA, cDNA, and genomic DNA. Total RNA was extracted from each cell line with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Poly(A)$^+$ RNA was isolated from the total RNA on poly(dT)-Dynabeads using an mRNA purification kit (Dynal, Oslo, Norway). First strand cDNAs were generated from the purified mRNA using the cDNA synthesis kit (Life Technologies, St. Petersburg, FL). Genomic DNA from LNCaP was purchased from ATCC. Genomic DNA from human placenta was purchased from Sigma Chemical Co. Genomic DNA from azacytidine-treated LNCaP was extracted using DNAEasy kit (Qiagen, Valencia, CA) following manufacturer's instructions. Genomic DNA from normal and tumor prostate tissue (T2c,NX,MX, Gleason grade $3+4=7$) was custom made by Ambion (Austin, TX).

Expression of galectins in normal, benign, and malignant prostate epithelial cells by reverse transcriptase (RT)-PCR. For reverse transcriptase-PCR analysis, first strand cDNAs were used as templates. All PCR amplifications were carried out with *Taq* DNA polymerase (Promega) in the buffer and Mg^{2+} solution provided by the manufacturer. Annealing temperatures varied from 50 to 68 °C. The non-overlapping galectin-specific primers used were as follows: **gal1:** 5′-CCTGGAGAGTGCCTTCGAGTG-3′ (forward) and 5′-CTGCAACACTTCCAGGCTGG-3′ (reverse), expected product size 220 bp; **gal3:** 5′-CGAGCGGAAAATGGCAGAC-3′ (forward) and 5′-CTGCCCCTTTCAGATTATATCATGG-3′ (reverse), exp. pdt. size 780 bp; **gal4:** 5′-CTGGTCTTCATAGTCCTGGCTGAG-3′ (forward) and 5′-AGGCTGTTCCGGACCACG-3′ (reverse), exp. pdt. size 440 bp; **gal7:** 5′-GTCCCCCACAAGTCCTCGCTG-3′ (forward) and 5′-CTCAGAAGATCCTCACGGAGTCC-3′ (reverse), exp. pdt. size 450 bp; **gal9:** 5′-CGTGTGGACACCATCTCCG-3′ (forward) and 5′-CAGCCCTCCCAGAATGGTG-3′ (reverse), exp. pdt. size 330 bp. For PCR amplification of specific isoforms of gal8, the following reverse primers were used with the same forward primer (5′-CTCTGCTCTATGGCCACAGGATC-3′): **gal8a:** 5′-TGCAGCGAATGGCAGCCTAAGCT-3′ (reverse), exp. pdt. size 200 bp; **gal8b:** 5′-TGGTGTAGACAGTTCTGGGTGCG-3′ (reverse), exp. pdt. size 240 and 360 bp; **gal8e:** 5′-CCCTGCAGCAGCACTTTGACAC-3′ (reverse), exp. pdt. size 300 bp; **gal8g:** 5′-TGAATGAAGCTGCCCTCCACGG-3′ (reverse), exp. pdt. size 270 bp.

Bisulfite modification of genomic DNA and CD-PCR. Cytosine deamination (CD) by bisulfite treatment of single-stranded DNA (non-methyaled C changes to U) and subsequent PCR amplification (CD-PCR) were performed as previously described [19]. Briefly, genomic DNA (8 μg) was denatured in 0.3 M NaOH for 15 min at 37 °C in a volume of 100 μl, and after adding 60 μl of 10 mM hydroquinone and 1.04 ml of 3.6 M sodium bisulfite (pH 5), the reaction mixture was incubated at 50 °C for 16 h in the dark. The DNA was purified on a desalting column (Magic DNA Clean-Up System; Promega), denatured with 0.3 M NaOH for 15 min at 37 °C, neutralized with 3 M ammonium acetate (pH 7), and ethanol-precipitated. Occasionally, bisulfite modification of genomic DNA was performed using the EZ Gold Methylation kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. All PCR amplifications were carried using Multiplex PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions at annealing temperature 60 °C. The primers for gal3 promoter amplification after taking into account the bisulfite conversion reaction were: (a) forward primer 5′-GGAGAGGGTGGGGGATAG-3′ derived from the wild-type sequence 5′-GGAGAGGGCGGGGGACAG-3′ between −277 and −260 nt [20]; and (b) reverse primer 5′-ACACCCTCTCCCCTACCC-3′ derived from the wild-type sequence 5′-GCGCCCTCTCCCCTGCCC-3′ between +90 and +107 [20].

Cloning and sequencing. The galectin gene and methylated DNA products obtained from RT-PCR and CD-PCR experiments, respectively, were cloned into the pGEM-T vector (Promega, Madison, WI). Plasmids for DNA sequencing were prepared using the QIAprep Miniprep Kit (Qiagen) or GenElute Five-Minute Plasmid Miniprep kit (Sigma). DNA sequences were determined by the dye termination cycle sequencing method using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3130 XL instrument equipped with 16 capillary columns (50 cm length) (Applied Biosystems). For analysis of the gal3 promoter methylation, at least 20 independent clones for each CD-PCR fragment were sequenced by using the T7 and sp6 primers (Novagen). All other manipulations of nucleic acids such as ligation, transformation, gel electrophoresis, gel elution, and preparation of buffers were carried out following standard protocols [21].

Results and discussion

Identification of a novel proto type isoform of gal8 (gal8g)

The gal8 gene (also known as prostate carcinoma tumor antigen-1, PCTA-1) encodes numerous mRNAs by alternate splicing, mostly on intron VIII [22,23; Fig. 1]. These mRNAs encode six different isoforms of gal8 representing various intron VIII lengths; three belong to the tandem-repeat type (containing two CRDs) and three to the proto type group (one CRD). To investigate the expression of these gal8 isoforms in normal and tumor prostate cell lines by RT-PCR, we used the primers (forward primer 5′-CTC TGCTCTATGGCCACAGGATC-3′ in the exon 6 and the reverse primer 5′-GTTCGTCCAGGGCCCATGG-3′ in the exon 9, see Fig. 1) to amplify the linker region that represent all known isoforms of gal8. The PCR is expected to amplify products, if present all isoforms, ranging from 234 to 859 bp. Interestingly, the experiment resulted a PCR product of higher molecular size (approximately 1 kb) than expected (Fig. 2A). Cloning and sequencing of this product resulted in a novel gal8 transcript that contains the entire intron VIII (see Fig. 1). Analysis of this transcript revealed a stop codon at the start of the spliced intron VIII, yielding a proto type isoform of gal8 (GenBank Accession No. EF467046, named gal8 g in accordance with Cooper's review, Ref. [3]), which is different from all previously known proto isoforms [22,23]. To confirm the authenticity of the gal8g, gene-specific primers were designed from its non-overlapping sequence and a specific product was amplified from PrEC, BPH-1 and LNCaP cells by RT-PCR (Fig. 2B). Moreover, gal8g was also amplified (confirmed by cloning and sequencing) during PCR amplification of isoforms gal8b, gal8d, and gal8f with the primers tested (see Fig. 1 for position of primers) (data not shown).

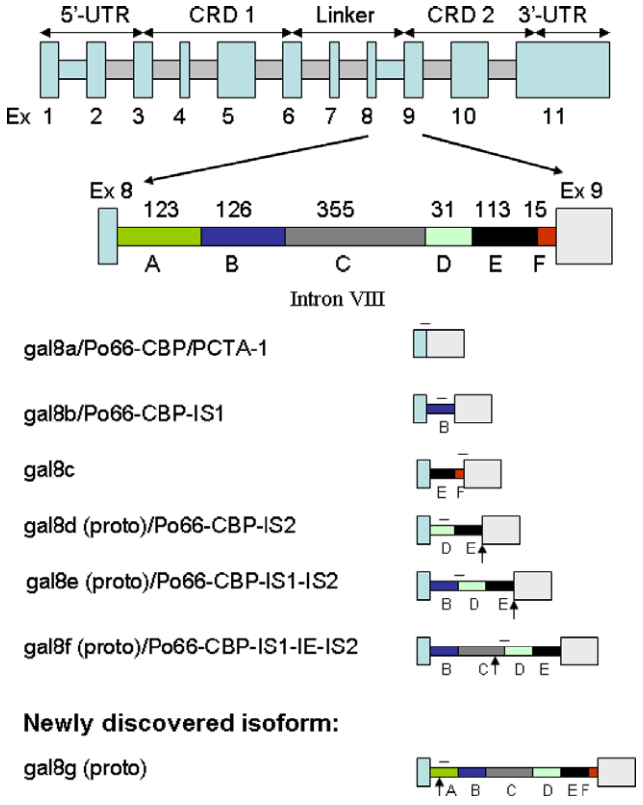

Fig. 1. Gene organization of human gal8. Schematic representation of gal8 gene and cDNAs for several isoforms of gal8. Isoforms (three tandem-repeat type gal8a to gal8c and four proto type gal8d to gal8g) are produced by alternate splicing of intron VIII. The arrow in gal8d to gal8g indicates the stop codon. The horizontal line on each isoform represents approximate location of reverse primers tested on RT-PCR with the same forward primer located in exon 6 for amplification of each isoform. Isoforms of gal8 are named here gal8a–g in accordance with Cooper's review, Ref. [3]. Alternative names for gal8 isoforms are based on Refs. [14,22,23].

Galectins are differentially expressed in normal and tumor prostate cells

As gal1, 3, 4, 7, 8, and 9 are known to express in human prostate, we examined expression of these galectins in normal, benign and malignant prostate cells by RT-PCR. The gal3, 7, and 9 are highly expressed in PrEC, but poorly or not in BPH-1 and LNCaP cells (Fig. 2B).

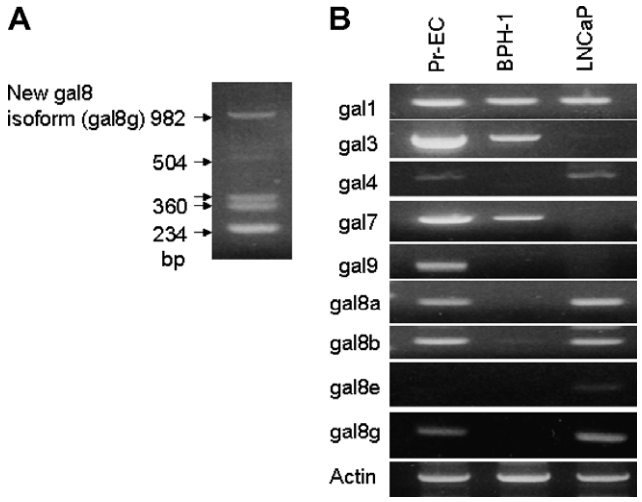

Fig. 2. (A) RT-PCR for expression of gal8 isoforms in LNCaP using a forward primer in exon 6 and a reverse primer in exon 9. (B) RT-PCR analysis of galectin expression in PrEC, BPH-1, and LNCaP cell lines.

The gal4 is expressed in PrEC and LNCaP, but not in BPH-1 cells. However, gal1 is equally expressed in PrEC, BPH-1, and LNCaP cells. The expression of gal8 is interesting. Of seven isoforms of gal8, the proto isoforms Po66-CBP-IS1-IS2 (named gal8e) and our newly discovered isoform gal8g are upregulated in LNCaP compared to PrEC. However, the two tandem-repeat isoforms of gal8, PCTA-1 (or Po66-CBP, named gal8a) and Po66-CBP-IS1 (named gal8b) are equally expressed in PrEC and LNCaP cells. Interestingly, little or no expression of these gal8 isoforms was observed in BPH-1 cells. The isoforms gal8c, d, and f were not detected in our RT-PCR studies from these three cells (data not shown). The earlier conflicting report on gal8 expression in prostate tumors [14,15] are probably due to the domain organization complexity of the multiple isoforms (see Fig. 1), and the type of probes used in those studies. The mechanisms of upregulation of gal8e and gal8g in LNCaP are not known from this study, but these proto isoforms (single CRD) may be responsible for modulating gal8-mediated cell adhesion and cell growth during prostate cancer progression [24]. The mechanisms of down regulation of most galectins especially gal4, 8, and 9 in BPH-1 cells are also unknown, but these galectins may be used as biomarkers for BPH in human.

Expression of gal3 gene is transcriptionally regulated by DNA methylation

In order to determine if DNA methylation is responsible for silencing or reduced expression of gal3, 7, and 9 in LNCaP, we treated the cells with azacytidine. We hypothesized that since azacytidine blocks cytidine methyl transferase activity, treatment of cells with azacytidine should reactivate the genes. As shown in Fig. 3A, the treatment of azacytidine induced the expression of gal3 in LNCaP cells indicating that the expression of gal3 is transcriptionally regulated by DNA methylation. Interestingly, no change in expression of gal7 and 9 was observed in azacytidine treated LNCaP compared to the untreated cells (see Fig. 3A) indicating that alternative mechanisms other than DNA methylation are involved in regulating the expression of these galectins in LNCaP. To demonstrate that gal3 silencing is not cell type or prostate cancer specific, we investigated if the gal3 promoter is methylated in gal3 null breast cancer cell line BT549. Treatment of azacytidine induced expression of gal3 in BT549 cells (Fig. 3B) indicating that promoter methylation may be a common mechanism for gal3 gene silencing in cancer.

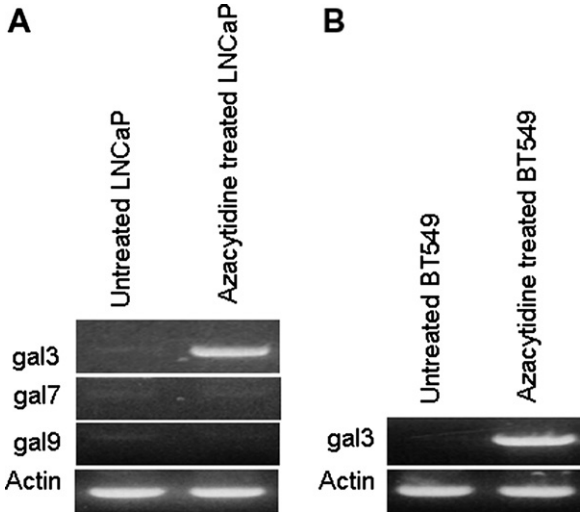

Fig. 3. (A) Expression of galectins in azacytidine-treated LNCaP and (B) gal3 in azacytidine-treated BT549 cells.

To confirm that methylation occurred at the cytosine residues of the gal3 promoter of LNCaP, we treated genomic DNA with sodium bisulfite and then sequenced the PCR amplification product (CD PCR). This method allows precise analysis of methylation in a selected region by converting all non-methylated cytosines (C) into uracil (U), while methylated cytosines remain unchanged. Fig. 4A shows a partial chromatogram of the gal3 promoter sequence from bisulfite treated LNCaP DNA where cytosines remain unchanged. In 400 bp long gal3 promoter PCR amplified product from bisulfite treated LNCaP DNA, about 50 cytosines were found methylated (Fig. 4B). As expected, no methylation of the gal3 promoter was observed in azacytidine-treated LNCaP or in human placenta DNA.

Gal3 promoter is heavily methylated in prostate tumor

It is known that expression of gal3 is dramatically decreased in early stage of prostate tumor relative to the normal prostate tissue [13]. To determine if the decreased expression of gal3 in early stage of tumor is related to the cytosine methylation, we examined the methylation patterns of the gal3 promoter (−269 to +89 nt, 20) in normal and tumor tissues after bisulfite treatment of DNA followed by CD-PCR using the same primers used for the cell line DNA. Results showed that the gal3 promoter in tumor was heavily methylated throughout its entire length (Fig. 4B). To our knowledge, this is the first report that gal3 expression is regulated by promoter methylation in LNCaP cells and human prostate tumor tissues. The role of decreased expression of gal3 in early stage of prostate tumor is not known. However, in normal cells gal3 is believed to interact members of Nkx homeodomain family [25], especially to a prostate tumor suppressor, Nkx3.1 [26] and decrease the expression of the cancer phenotype [25]. Therefore, the silencing of gal3 gene during the development of prostate tumor could be necessary to suppress the influence of Nkx3.1 gene and thereby to help tumor cells to proliferate.

In summary, our study shows for the first time that the gal3 expression is regulated by promoter methylation in prostate cancer cells (LNCaP) and tissues. Therefore, methylation specific PCR assay could be used for gal3

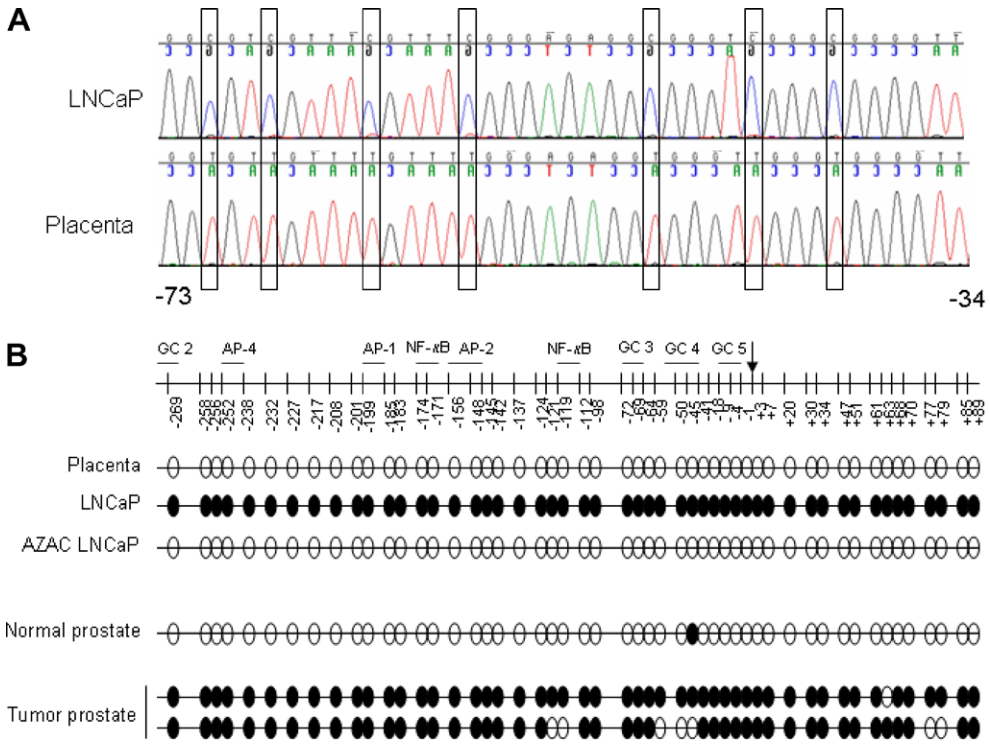

Fig. 4. (A) Sequencing chromatogram of bisulfite treated DNA. Partial chromatogram (−73 to −34 nt) showing cytosine methylation (indicated by box) of the gal3 promoter in LNCaP (upper panel). The lower panel shows the corresponding partial chromatogram of gal3 promoter sequence from human placenta. Note that all boxed nucleotides in the lower panel corresponding to the methylated C (upper panel) have changed to T, which represents U in the promoter. (B) Methylation profile of gal3 promoter region from untreated and azacytidine-treated LNCaP DNA, and normal and tumor prostate DNA. Each oval in each row represents a single CpG site (open oval, non-methylated; closed oval, methylated). Representative sequence from at least 20 clones of each sample is shown. The numbering in the schematic diagram at the top represents the position relative to the published transcription site [20] (+1, indicated by arrow). Two representative sequences from 20 clones of tumor prostate DNA are shown.

promoter using prostate tumor biopsies for early diagnosis of prostate cancer.

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