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Phenotype-Specific CpG Island Methylation Events in a Murine Model of Prostate Cancer

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Abstract

Aberrant DNA methylation plays a significant role in nearly all human cancers and may contribute to disease progression to advanced phenotypes. Study of advanced prostate cancer phenotypes in the human disease is hampered by limited availability of tissues. We therefore took advantage of the Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) model to study whether three different phenotypes of TRAMP tumors (PRIM, late-stage primary tumors; AIP, androgen-independent primary tumors; and MET, metastases) displayed specific patterns of CpG island hypermethylation using Restriction Landmark Genomic Scanning. Each tumor phenotype displayed numerous hypermethylation events, with the most homogeneous methylation pattern in AIP and the most heterogeneous pattern in MET. Several loci displayed a phenotype-specific methylation pattern; the most striking pattern being loci methylated at high frequency in PRIM and AIP but rarely in MET. Examination of the mRNA expression of three genes, *BC058385*, *Goosecoid*, and *Neurexin 2*, which exhibited nonpromoter methylation, revealed increased expression associated with downstream methylation. Only methylated samples showed mRNA expression, in which tumor phenotype was a key factor determining the level of expression. The CpG island in the human orthologue of BC058385 was methylated in human AIP but not in primary androgen-stimulated prostate cancer or benign prostate. The clinical data show a proof-of-principle that the TRAMP model can be used to identify targets of aberrant CpG island methylation relevant to human disease. In conclusion, phenotype-specific hypermethylation events were associated with the overexpression of different genes and may provide new markers of prostate tumorigenesis.

Introduction

Prostate cancer (CaP) is the most commonly diagnosed cancer in American men (1). Molecular markers, such as prostate-specific antigen (PSA), have increased chances of diagnosing CaP at its earliest stages (1). Treatment options for primary prostate cancer (PRIM) detected early include radical prostatectomy, radiation therapy, and active surveillance (1). At the time of

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diagnosis, ~30% of men have disease that extends beyond the prostate gland—some have clinically metastatic (MET) disease at the time of diagnosis and others suffer clinical or biochemical recurrence after potentially curative radical prostatectomy or radiation therapy. Advanced CaP is usually treated by testicular androgen deprivation therapy (ADT) using surgical or medical castration (1). However, for most men, CaP will recur in this androgen-depleted environment as a disease commonly called androgen-independent CaP (AIP; refs. 2, 3). There are currently no dependable biomarkers to determine which cancers will be most likely to be highly aggressive and eventually develop into AIP and/or MET disease.

DNA methylation is an epigenetic modification of the DNA that is frequently disrupted in nearly all types of cancer. Hypomethylation of repetitive elements is frequently seen and hypermethylation of specific CpG islands in promoter regions of several tumor suppressor genes are commonly observed to be associated with the transcriptional silencing of the gene (4–8). Although less well-studied, methylation of nonpromoter CpG islands in the 3' ends or bodies of genes has been associated with ectopic, or overexpression of genes such as *PAX6* (9,10), *p16* (11), and others (12).

In recent years, DNA methylation has been touted as an ideal target for the development of cancer biomarkers (8,13,14). The most common CpG island methylated in CaP is the *GSTP1* gene, for which some reports have shown methylation in >90% of primary CaP (15, 16). However, studies focusing specifically on the molecular biology of AIP and/or MET CaP directly in human tissues rather than cell lines are limited. A major reason for this is the difficulty in obtaining these tissues. Given improved screening using digital rectal examination and PSA testing, most men present with indications for the disease before clinically evident MET spread. Those patients that present with MET disease are treated by ADT rather than surgery. Recurrence during ADT is not generally treated surgically as the recurrence typically occurs at multiple MET sites. Even rarer is the collection of MET tumors from patients who have not received ADT. However, because the MET phenotype can be acquired before ADT, the MET potential of a cell and its ability to grow independent of androgen stimulation are separable phenotypes. Therefore, different molecular events may underlie the acquisition of these two phenotypes. The rarity of these tissues available for research and the difficulty in separating the MET and AIP phenotypes has limited investigation into the molecular basis of these phenotypes at the genomic level.

To circumvent some of these difficulties, human CaP cell lines have been used extensively to study the molecular biology of CaP. These cell line models have led to a number of important observations that have been confirmed in human tumor tissues (17,18). However, we have shown that cell lines provide exceptionally poor models for genomic screening to identify CpG island hypermethylation events (19). Recently, several studies have been completed using the Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) model in which disruption of the DNA methylation pathway was observed. Dnmts were found to be overexpressed in late-stage TRAMP primary tumors and metastases to liver, kidney, and lymph node. Furthermore, nonrandom hypermethylation of CpG islands in late-stage TRAMP primary tumors was also reported (11).

The TRAMP model uses the rat probasin promoter to drive expression of SV40 early genes (large and small T antigens) in prostatic epithelium (20). Oncogene expression leads to further genetic and/or epigenetic changes that result in prostate tumor formation and disease progression to metastases at several sites in the mouse (21). Castration of TRAMP mice leads to tumor regression and increased survival (22,23). However, some of the mice develop AIP primary tumors that often are more aggressive and also metastasize (22,23).

In the current study, the TRAMP model system was used to study CpG island hypermethylation in CaP in three tumor phenotypes—PRIM, MET, and AIP—to determine if DNA methylation differences existed among them. Numerous high-frequency methylation events and phenotype-specific events were identified, which included three genes whose expression was increased in methylated samples dependent on tumor phenotype.

Materials and Methods

Animals and tissue samples

Restriction Landmark Genomic Scanning (RLGS) was performed on 4 benign prostate DNAs from nontransgenic mice and a total of 90 TRAMP tumors. All tumors came from TRAMP mouse F₁ generation males from the cross TRAMP × FVB, with the TRAMP mothers on a C57BL/6 background. All androgen-dependent PRIM and MET were collected from different mice without androgen deprivation. For the intraprostatic AIP, animals were castrated at ages 12 wk (after primary tumor formation had begun) and recurring tumors were collected 12 wk later. A total of 30 PRIM, 30 MET (from liver and lung), and 30 AIP tumors were analyzed.

Human tissues

The androgen-stimulated benign prostate (AS-BP) samples were obtained from patients treated for lower urinary tract symptoms by transurethral prostatectomy. The androgen-stimulated PRIM (AS-CaP) samples were obtained from patients treated for CaP by prostatectomy. The recurrent primary tumors (RCaP) were obtained by transurethral resection from patients who presented with urinary retention from recurrent CaP during ADT (24). AS-CaP and AS-BP were enriched to >70% epithelial cells using standard microdissection of 20-μm frozen step sections adjacent to 8-μm sections identified by H&E staining to contain >50% epithelial cells, as previously described (25). RCaP did not require microdissection as it was composed of an average of 92% malignant cells (24).

RLGS and spot cloning

The protocol for extraction of genomic DNA from tissues was described previously (26). The published protocol of Dai et al. (27) was followed for RLGS gels. RLGS spots of interest were cloned as previously described (26,28–30).

Quantitative real-time reverse transcription-PCR

RNA samples were extracted from mouse tissues using Trizol reagent (Invitrogen) and converted to cDNA using random hexamer and oligo-dt primers by First strand cDNA synthesis kit (Fermentas). PCR reactions were conducted using the qPCR MasterMix Plus for SYBR Green I (Eurogentec) Primer sets, shown in Supplementary Table S1, for specific amplification were obtained from IDT. Absolute quantification was used to determine gene expression copy number normalized to 18S rRNA. All reactions were performed in triplicate, and the data are presented as the mean 18S normalized quantity × 10,000.

Sodium bisulfite treatment

Sodium bisulfite treatment to convert unmethylated cytosine to thymidine was completed following the manufacturer's protocol EZ-96 DNA Methylation kit (Zymo Research) using 750 ng in 50 μL of distilled water and M-Dilution Buffer. The treated samples were resuspended in 75 μL of M-Elution Buffer and stored at –20°C.

MassARRAY quantitative methylation analysis

MassArray Quantitative Methylation Analysis (MAQMA) was performed using the MassARRAY Compact system developed by the Sequenome Company, as previously

described (31), using the primers shown in Supplementary Table S2. This system uses mass spectrometry for the detection and quantitative analysis of DNA methylation. This approach has been shown to be a highly accurate and reproducible way to quantitate methylation (32). Regions near the RLGS spots of interest to be analyzed were determined as follows: For spots 2G63 and 3C21, which were within CpG islands, we sequenced a region within the islands. For spot 5F09, which was not within a CpG island, nor near the 5' end of the gene, we sequenced a region encompassing the NotI site.

Statistics

All statistical analyses were performed using the Graph Pad Prism 5 software. To test for normality of distribution of data, D'Agostino and Pearson omnibus normality test was used. To test for differences between two groups with nonnormal distribution, a Student's *t* test with Welch's correction factor was used. To test for differences among more than two groups with nonnormal distribution, the Kruskal-Wallis test was used. To test for correlation between percent methylation and expression levels, the Spearman's Rank Order correlation test was used.

Results

Genomic scanning of CpG island methylation in TRAMP tumors

To identify CpG island hypermethylation in CaP from the TRAMP model, we performed RLGS. RLGS profiles of benign prostatic DNA collected from four different animals from the cross C57BL/6 × FVB _{F1} produced the same pattern of RLGS spots. A region of the profile containing 1,445 spots was found to be resolvable in all tumor gels and was analyzed in all 90 TRAMP tumor RLGS profiles compared with a single benign prostate tissue DNA RLGS profile. Any spot absent or greatly reduced in intensity in the tumor profile was considered lost and, therefore, methylated as previously shown (11,33,34). The numbers of RLGS spots lost for each of the three groups of 30 tumors studied is shown in Supplementary Table S3 and analyzed in Fig. 1. An average of 32, 36, and 31 RLGS spots were lost (hypermethylation events) in PRIM, AIP, and MET tumors, respectively (Supplementary Table S3; Fig. 1A). Although there was no significant difference in the average number of methylated loci in each group, there was significantly more variability among samples in the MET group compared with either the PRIM or AIP groups. The PRIM and AIP groups showed nearly identical distributions of the total number of methylated loci in each of the 30 samples, whereas the MET group exhibited a significantly different distribution with some samples having >80 methylated loci and others with as few as 12 (Supplementary Table S3; Fig. 1A).

Despite the fact that the distribution of total numbers of methylated loci was nearly identical between PRIM and AIP, the frequency at which specific loci were methylated within the groups differed (Fig. 1B). Although the number of loci methylated in any sample was lowest in the AIP group, these loci were methylated at a higher frequency within the AIP group than either the PRIM or MET groups (Fig. 1B). Comparing the three phenotypes, the AIP group displayed the highest percentage of RLGS spots that were methylated in 26 to 30 of the samples (Fig. 1B), whereas the MET group had the largest proportion of loci methylated in only 6 to 10 samples. These data indicate that the AIP tumors have the most homogeneous pattern of RLGS spot methylation, whereas the MET group has the most heterogeneous.

Totals of 222, 212, and 270 RLGS spots were methylated at least once in the PRIM, AIP, and MET groups of samples, respectively. Limiting the analysis to spots methylated minimally thrice of 30 within each phenotype, 89, 93, and 106 such spots, respectively, were found. A total of 48 loci were hypermethylated at least once in all three phenotypes (Fig. 1C). The number of spots lost in two of the three phenotypes decreased to 10, 10, and 15 for the

combinations of PRIM+MET, AIP+MET, and PRIM+AIP, respectively (Fig. 1C). These data indicate that the two primary tumor phenotypes are more similar to each other than they each are to the MET phenotype. In addition, the number of loci hypermethylated in a single phenotype (PRIM, 16; AIP, 20; and MET, 38) suggests more heterogeneity in the MET samples compared with the PRIM samples (Fig. 1C).

Tumor phenotype–specific hypermethylation of RLGS spots

Table 1 shows the RLGS spots of interest based on their pattern of loss among the three phenotypes. A total of 13 RLGS spots were lost in >33% of the samples regardless of phenotype. The most frequently lost spot was 3D22, which was lost in 82 of the 90 samples. This RLGS spot has been identified in the 3' end of the *Cdkn2a* locus. We have previously shown that the promoter region of p19 and p16 were not methylated in TRAMP tumors, and the 3' end methylation detected by RLGS was associated with overexpression (11).

Although no RLGS spot losses were specific to either the PRIM or MET phenotypes, six loci showed significant specificity for the AIP phenotype. More striking was the observation that 13 loci showed a significant association of methylation with the PRIM and AIP phenotypes but little or no methylation in the MET tumors (Table 1). The strongest example of which was spot 3C21 (Table 1, *spot 1*), lost in 20 PRIM and 27 AIP tumors but only 1 MET lesion. Supplementary Fig. S1 shows representative examples of RLGS gel analysis with 4C38 (Table 1, *spot 23*) representing a locus lost at high frequency regardless of phenotype (Supplementary Fig. S1A), spots 3C21 and 2G63 (Table 1, *spot 1*) lost only in PRIM and AIP (Supplementary Fig. S1B and C), and spot 4D69 (Table 1, *spot 4*) lost exclusively in AIP samples (Supplementary Fig. S1D). These observations support the hypothesis that different tumor phenotypes display a specific methylation pattern.

MAQMA of RLGS loci of interest

RLGS spot loss is based on the methylation of critical CpGs in the target sequence of the *NotI* enzyme. To confirm the specificity of methylation to the PRIM and AIP phenotypes observed by RLGS and, furthermore, to determine if methylation of the *NotI* site indicated methylation in the surrounding region, three of the four RLGS loci that showed the strongest prevalence for methylation in the PRIM or AIP phenotypes were analyzed using MAQMA on all 90 tumor samples. MAQMA gives a quantitative value of percent methylation at each informative CpG dinucleotide. A diagrammatic representation of the data for half the samples in each phenotype for 2G63 and 3C21 is shown in Supplementary Fig. S2. The MAQMA analysis confirmed the phenotype specificity identified by RLGS, demonstrating methylation of these loci specifically in PRIM and AIP tumors but not MET tumors.

To get a single value for comparison purposes between RLGS and MAQMA data, the average percent methylation of all the CpG dinucleotide in each of the 90 samples was plotted in Fig. 2. Methylation of the *NotI* site by RLGS analysis matched quite well with methylation of the surrounding DNA for spots 2G63 and 5F09 (Table 1, *spot 3*). Only a small number of cases exhibited discordance between the two approaches (Supplementary Fig. S2A; Fig. 2A and C). This was particularly clear for 2G63 (Fig. 2A); the only AIP tumor of 30 that did not show RLGS spot loss also showed very low methylation by MAQMA. However, for spot 3C21, we observed that several of the samples expected to be methylated based on RLGS analysis had <50% methylation (Supplementary Fig. S2B; Fig. 2B). Nevertheless, all samples that were unmethylated as shown by RLGS displayed very low levels of methylation by MAQMA.

This and all bisulfite sequencing–based approaches can underestimate methylation due to PCR bias against the methylated alleles. The potential for such PCR bias has been documented by multiple groups and shown to be very amplicon dependent (35,36). To assess the possibility

of bias against amplification of methylated alleles, we *in vitro* methylated (IVM) an aliquot of commercially available peripheral blood lymphocyte (PBL) DNA with SssI methylase, which methylates all CpG dinucleotides. Various ratio mixtures of the normal DNA with the IVM DNA were prepared, creating 100%, 75%, 50%, 25%, and 0% IVM DNA controls. These control DNAs were bisulfite treated and used as template for bisulfite PCR and MAQMA analysis. Examination of the control samples for each of the MAQMA primer sets revealed that, although 2G63 and 5F09 had very little bias, 3C21 displayed a strong bias toward unmethylated product, particularly between 25% and 75% expected methylation (Supplementary Fig. S2A and B; Fig. 2D). This bias resulted in a lower observed percent methylation than expected for 3C21 (Fig. 2D). These data indicate that RLGS analysis is a good predictor of methylation within an entire region surrounding the NotI site.

Expression of phenotype-specific hypermethylated genes in TRAMP

DNA methylation is an important mechanism for regulating gene transcription and has been most commonly associated with gene silencing when found at the 5' ends of genes. Most RLGS loci hypermethylated in cancer come from CpG islands in the 5' ends of genes. However, for the set of eight genes that were specifically methylated in PRIM and AIP, but not MET, >60% were found in the body or 3' end of the gene (Table 1). We chose three such RLGS loci (2G63, 3E30, and 3C21) to study the relationship between body of the gene or 3' end methylation and expression.

Using quantitative reverse transcription-PCR (qRT-PCR), the mRNA expression of the 2G63 transcript, BC058385, where the methylated CpG island surrounds the fourth exon, was measured in Fig. 3. Increased expression correlated with increased methylation (Fig. 3B; Spearman's Rank Order Correlation r , 0.756; $P < 0.0001$) using RNA from 15 samples in each phenotype (samples were chosen to include both methylated and unmethylated examples in each phenotype). No expression and very little methylation were observed in normal prostate. Although methylation of this locus was primarily seen in PRIM and AIP tumors, there were eight MET tumors with methylation. We analyzed expression in six and found that all six showed little or no expression. This is in contrast to the methylated PRIM and AIP samples, approximately half of which showed expression when methylated (Fig. 3B). These results indicate a separate mechanism in conjunction with DNA methylation that may be regulating expression of these genes in a phenotype-specific manner.

Expression of the Goosecoid (*Gsc*) homeobox gene (3E30; Table 1, *spot 2*) was also measured using qRT-PCR. This locus is unique in that the NotI site is not contained within a CpG island but is within the third exon at the 3' end of the gene ~2 kb away from the CpG island encompassing the transcriptional start site (Fig. 3). MAQMA analysis surrounding the transcriptional start site showed no methylation (data not shown) in any of the 90 samples despite methylation of the 3' NotI site in 24 PRIM, 29 AIP, and 5 MET samples. Quantitative expression analysis showed that none of the unmethylated samples showed expression of *Gsc*, but some of the methylated samples did (Fig. 3C). Furthermore, after separating the data by phenotype, it became clear that the few MET samples that had methylation of the 3' NotI site also had the highest levels of expression as a group (Fig. 3D).

DNA methylation and expression from the Neurexin 2 α and β promoters

The most striking example of phenotype specificity of CpG island methylation was RLGS spot 3C21, which was lost in 20 of 30 PRIM, 27 of 30 AIP, and only 1 of 30 MET tumors ($P = 1.0E-12$ for specificity to PRIM and AIP). 3C21 represents a CpG island in exon 2 of the *Nrxn2* locus. There are two promoters for *Nrxn2* (α and β), which is a signature feature of the Neurexin family of genes (37–39), and each promoter is within a CpG island, as shown in Fig. 4 (*CpG islands A and C*). The transcript from the β promoter contains a unique first exon but

otherwise shares the same exons as the transcript from the α promoter from exon 17 onward (39). The NotI site analyzed by RLGS is found within CpG island B, located 9 kb downstream of the α promoter of *Nrxn2*. MAQMA analysis of all three CpG islands showed that the methylation at island B in exon 2 closely matched the methylation observed by RLGS; most of the PRIM and AIP tumors displayed robust hypermethylation (Fig. 2B). However, little or no methylation was observed at either the α or the β promoter (*CpG islands A and C*) in any of the 90 samples (data not shown).

Using qRT-PCR and primers that were specific to each transcript (Fig. 4), mRNA expression originating at both promoters was measured (Fig. 4). We observed a significant increase in expression at the α promoter (Fig. 4A) when CpG island B was hypermethylated 9 kb downstream. Furthermore, consistent with the methylation pattern, the overexpression was only observed in the primary tumors and AIP tumors, not in the METs (Fig. 4B). Expression for the β isoform of *Nrxn2* did not correlate either positively or negatively with methylation at CpG island B. (Fig. 4C and D). No correlation could be detected between usages of the α or β promoters (data not shown).

Aberrant methylation in TRAMP model conserved in human CaP

To determine if novel targets of aberrant CpG island hypermethylation identified in the TRAMP model were relevant to the human disease, the methylation of the human orthologues of two RLGS loci were studied in sets of 13 human AS-BP, 13 AS-CaP, and 12 primary tumor recurrences during ADP (RCaP). MAQMA analysis was performed on the human samples for the orthologous regions in the human genome for the mouse RLGS spots 3C21 and 2G63. For spot 3C21, the human *NRXN2* locus has the same structure as the mouse. However, we found no methylation in any of the human samples for CpG island B (data not shown). For spot 2G63, the orthologous region in the human genome matches a CpG island in the fourth exon of gene BC029292 on chromosome 7q11.23. The MAQMA data in the human samples, as shown in Fig. 5, showed little or no methylation in the AS-BP and AS-CaP (Fig. 5A and B) samples, but >50% methylation was observed in 5 of 12 RCaP samples (Fig. 5B and D). These data show a proof-of-principle that the loci identified as novel targets of methylation in the TRAMP model (Table 1) may be relevant to the human disease.

Discussion

These studies showed that three different tumor phenotypes—PRIM, AIP, and MET—from the TRAMP model exhibited many hypermethylation events throughout the genome as well as phenotype-specific patterns of hypermethylation. Furthermore, the TRAMP model was used to identify CpG island methylation relevant to human CaP. Similar to previous studies (33, 40,41), we found that methylation at the NotI site as measured by RLGS is a good indicator of the methylation in the surrounding region. Our findings led to the identification of gene overexpression coinciding with downstream hypermethylation, which may or may not be contained within a CpG island. This is similar to previous findings of methylation of nonpromoter CpG islands in the 3' ends or bodies of genes associated with ectopic, or overexpression of genes such as *PAX6* (9,10), *p16* (11), *PDX1*, and *OTX1* (12). We showed that the BC058385 (RLGS spot 2G63) and *Nrxn2* (RLGS spot 3C21) transcripts are overexpressed when their downstream CpG islands are hypermethylated. We also found that *Gsc* expression is increased with hypermethylation of a downstream region that does not contain a CpG island, whereas the upstream CpG island showed no methylation in any samples.

It is striking that for *Gsc*, three of the five data points above the median level of expression in the methylated samples (Fig. 3D) come from the MET group, yet this is exactly the group showing the least number of cases with methylation. This is in contrast to the data from *Nrxn2* and the 2G63 transcript (Figs. 3B and 4B), where only methylated samples showed

expression but the MET samples showed no expression even in the few cases where the MET samples were methylated. These data suggest that methylation of the 3' ends of these genes is necessary but not sufficient for their expression because none of the unmethylated samples show any expression, but a number of methylated samples also have no expression. In all three cases, however, tumor phenotype is the key to determining which of the methylated samples show expression. It does not seem likely that the 3' end methylation is driving the overexpression given the large number of cases where methylation is seen without overexpression. However, it may be the case that the 3' end methylation is a stabilizing factor in initiating transcription when other conditions are favorable for transcription of the gene. Nevertheless, what is clear is that only those cases with high level methylation at the 3' end of *Gsc* and the body of *Nrxn2* and the 2G63 transcript showed overexpression in our data set, and tumor phenotype was a strong factor in determining which methylated samples had overexpression.

Gsc is a highly conserved transcription factor that is the most abundantly expressed homeobox gene in the Spemann organizer in *X. laevis* (42). Expression of this gene allows these cells go through epithelial to mesenchymal transition (EMT) during gastrulation and ingress into the interior of the embryo. It was recently shown that ectopic expression of *GSC* is found in human breast tumors, and this expression was shown to be able to induce EMT and increase the ability of cells to form METs (42). We found that 53 of 60 TRAMP tumors at the primary site showed methylation in the 3' end of *Gsc*, yet most showed no expression. The MET tumors, however, showed methylation in only 5 of 30, but 4 of these 5 showed elevated *Gsc* expression, ranging from slightly to highly overexpressed. We propose that the high rate of methylation we see in tumors at the primary site is a requisite early step in overcoming the negative regulation of *Gsc* put in place after embryogenesis but not sufficient for ectopic expression. Those primary tumor cells that do acquire ectopic expression may have better ability to undergo EMT and acquire migration and survival abilities increasing their MET potential. In the MET tumors that develop, continued expression of *Gsc* may not be required, and the negative regulation of this gene may become re-established as reflected by the fact that most MET tumors do not exhibit methylation of the 3' end. In the few MET tumors where we observed 3' end methylation and varied levels of ectopic expression, perhaps the negative regulation of *Gsc* was not yet re-established.

Neurexin 2 has two promoters (α and β); both with CpG islands, plus a third CpG island in exon 2 located 9 kb downstream of the α promoter. We found that transcripts originating from the α promoter were overexpressed when the internal CpG island was hypermethylated. Both the α and β promoters were unmethylated in all 90 samples. Methylation of the internal CpG island does not seem to alter which of the two promoters is used. We could find no correlation between the level of α transcript and β transcript in each sample. Expression of the *Nrxn* family of proteins is highly complex with literally hundreds of different possible proteins arising due to extensive alternative splicing at both the 5' and 3' ends, as well as alternative promoter usage. Much more work will be required to understand how aberrant methylation in CaP affects this complex system.

When we investigated whether some of the methylation events identified in the TRAMP model were conserved in the human disease, we found that the human orthologues to the mouse RLGS spot 2G63 (BC029292) was methylated in 42% of human RCaP with very little methylation in AS-BP and only slightly more in AS-CaP. The human *NRXN2* CpG island, however, was not methylated in any human samples. The *NRXN2* gene product is involved in synaptic adhesion and is expressed in neurons. Interestingly, in the TRAMP mouse, where we observed a high frequency of methylation in PRIM and AIP tumors, a neuroendocrine phenotype is very commonly observed in late-stage primary and AIP tumors but less commonly in MET tumors (43). However, in the human disease, where we did not observe *NRXN2* methylation, a

primarily neuroendocrine phenotype is much less common (43–45). It is likely that the expression of *Nrxn2* in tumors with a high neuroendocrine component is reflective of the fact that a large proportion of the tumor cells have taken on a neuroendocrine phenotype and may in fact prove to be an effective marker of the phenotype. These observations suggest that the TRAMP model may be a very useful tool for the identification of novel targets of aberrant CpG island hypermethylation in human CaP, but that there will also be differences; some of which are likely to be at genes whose function contributes to different characteristics between the mouse model and the human disease.

We have shown that aberrant DNA methylation plays a significant role in the TRAMP mouse model. The finding of phenotype-specific methylation events, particularly those events unique to tumors found at the primary site, but not at the MET sites, suggests that epigenetics in general, and DNA methylation in particular, may play an important role in advanced CaP. To a lesser degree, we also showed differences in methylation patterns between primary tumors and AIP recurrences. These loci identified by genomic scanning in this mouse model can be used in a candidate gene approach to study the advanced phenotypes of the human disease in the very limited samples that are available and generally not amenable to genome wide screens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Global analysis of RLGS data. *A*, plot showing the number of RLGS spots lost in each of the 30 tumor sample RLGS profiles in each of the three phenotypes: primary, AIP primary, and MET tumors. *Dotted lines*, median number of spots lost in each group; *bars*, interquartile range. *B*, frequency distribution for RLGS spot loss showing the percentage of spots lost in the number of tumors indicated on the X axis. The percentages are taken from the total number of spots lost at least once in each of the three phenotypes (222, 212, and 270 spots each for primary, AIP primary, and MET tumors, respectively). *C*, Venn diagram of RLGS spot loss comparing the specific loci methylated in the three tumor phenotypes and limited to those spots lost three or more times within a phenotype.

Figure 2.

MAQMA of three RLGS loci on the entire sample set. *A* to *C*, the average level of methylation detected by MAQMA across the sequenced fragment of the CpG islands for each samples is shown on the *Y* axis. This value comes from taking the average of MAQMA values for each CpG dinucleotide sequenced for each sample. The samples are divided up categorically by phenotype (PRIM, MET, or AIP) and the RLGS status [methylated (*meth*) or unmethylated (*unmeth*)] for each spot along the *X* axis. Each symbol represents a single sample. *D*, to test for bisulfite PCR bias, we used PBL DNA as a 0% methylation control and an IVM aliquot of the same DNA as a 100% methylated control. These DNAs were mixed at appropriate ratios to generate 75%, 50%, and 25% methylated controls, and all the controls were bisulfite treated and used as template for bisulfite PCR and MAQMA analysis. The data are presented in a XY scatter plot with the observed methylation values (average MAQMA values as in *A–C*) on the *Y* axis plotted against the expected methylation values based on the ratios of PBL DNA to IVM PBL DNA used as template. *Straight line with closed circles*, results of a theoretically perfect experiment. All data points below this line represent PCR bias against the methylated alleles.

Figure 3.

Genomic structure, DNA methylation, and expression of genes. *A*, cartoon of the genomic structure of the BC058385 mRNA 57-kb locus and of the of the *Gsc* 2-kb locus. The CpG island and the NotI site represented by the RLGS spot 2G63 are shown and fall in exon 4. The CpG island and the NotI site represented by the RLGS spot 3C21 are shown. The MAQMA and qRT-PCR primers are shown. *B*, SYBR green qRT-PCR results plotted against MAQMA results in an XY scatter plot. SYBR green qRT-PCR was performed on 15 samples from each of the 3 phenotypes and normal prostate cDNA, and normalized to 18s rRNA levels. *C*, SYBR green qRT-PCR results for *Gsc* plotted against RLGS status. SYBR green qRT-PCR was

performed on 10 samples from each of the three phenotypes and normalized to 18s rRNA levels. *D*, same as in *C* except plotted by phenotype and RLGS status.

Figure 4. Genomic structure, DNA methylation, and expression of the *Nrnx2* locus. Cartoon of the genomic structure of the *Nrnx2* 105 kb locus. **A**, SYBR green qRT-PCR results for *Nrnx2*α plotted against RLGS status. **B**, same as in **A** except plotted against phenotype. **C**, SYBR green qRT-PCR results for *Nrnx2*β plotted against RLGS status. **D**, same as in **C** except plotted against phenotype.

Figure 5. MAQMA data for the human orthologue to mouse 2G63 CpG island on human prostate tissues. Each line represents a single sample, with each circle representing a CpG dinucleotide. The percent methylation at each CpG is indicated by the gray scale shading of each circle according to the key at the top of the figure. *Gray dashed circle*, CpG dinucleotides for which no data could be obtained. The number to the left of each line indicates the sample number. *A*, data for 13 AS-BP; *B*, data for 13 AS-CaP; *C*, data for 12 primary tumor recurrences after ADP (RCaP); *D*, the average level of methylation detected by MAQMA across the sequenced fragment of the CpG islands for each samples is shown on the Y axis. This value comes from taking the average of MAQMA values for each CpG dinucleotide sequenced for each sample. The samples are divided up categorically by phenotype (AS-BP, AS-CaP, or RCaP) along the X axis. Each symbol represents a single sample.

Table 1

RLGS spots of interest

No	Spot	PRIM	MET	AIP	Total	Class	P*	CGI [†]	Context	Gene homology [‡]
		n = 30	n = 30	n = 30	n = 90					
1	3C21	20	1	27	48	Prim or AIP [§]	1.0E-12	Y	Body	Nrxn2
2	3E30	24	5	29	58	Prim or AIP	2.3E-11	N	3'end	Gsc
3	5F09	19	2	21	42	Prim or AIP	2.5E-08	N	Body	Cacna1a
4	2G63	22	8	29	59	Prim or AIP	7.1E-08	Y	Body	BC058385
5	2B37	14	1	14	29	Prim Or AIP	1.0E-05	—	—	—
6	5B30	20	6	22	48	Prim or AIP	1.2E-05	Y	Body	AK139829
7	4D27	16	6	19	41	Prim or AIP	7.0E-04	Y	5'end [¶]	Hoxa2
8	3E16	8	0	8	16	Prim or AIP	9.1E-04	—	—	—
9	4C01	18	9	20	47	Prim or AIP	3.0E-03	Y	5'end	AK044818
10	3D01	7	0	7	14	Prim or AIP	3.0E-03	—	—	—
11	2G10	8	0	6	14	Prim or AIP	3.8E-03	—	—	—
12	2C17	7	0	5	12	Prim or AIP	7.0E-03	—	—	—
13	4C17	13	8	20	41	Prim or AIP	1.4E-02	Y	5'end	Lhfp14
14	4D69	0	0	9	9	AIP specificity ^{**}	2.0E-05	Y	5'end	Nfyb
15	4E04	2	4	15	21	AIP specificity	5.0E-05	Y	5'end	Tpm2
16	4D54	0	5	14	19	AIP specificity	6.0E-05	Y	5'end	Pawr
17	4E16	0	1	9	10	AIP specificity	1.0E-04	Y	5'end	Tpm2
18	3C39	0	0	7	7	AIP specificity	2.0E-04	Y	5'end	Mid1ip1
19	3G88	5	2	13	20	AIP specificity	1.0E-03	Y	5'end	Il6st
20	3D22	29	23	30	82	Frequency ^{††}	—	N	3'end	Cdkn2a
21	4C11	21	25	28	74	Frequency	—	N	Body	4932416N17Rik
22	4E25	23	25	26	74	Frequency	—	—	—	—
23	4C38	19	15	28	62	Frequency	—	Y	5'end	Lhfp14
24	4C31	23	11	25	59	Frequency	—	N	Body	Adey5
25	4C13	23	9	26	58	Frequency	—	Y	5'end	AK039621
26	3D67	18	17	21	56	Frequency	—	Y	3'end	Oprd1
27	2C28	16	14	22	52	Frequency	—	Y	5'end	AK004006

No	Spot	PRIM	MET	AIP	Total	Class	P*	CGI†	Context	Gene homology‡
		n = 30	n = 30	n = 30	n = 90					
28	2E04	19	9	13	41	Frequency	—	—	—	—
29	2D21	13	8	15	36	Frequency	—	Y	3' end	BC025575
30	3E56	9	11	15	35	Frequency	—	Y	5' end	Zfp787
31	3E07	7	12	16	35	Frequency	—	Y	5' end	U2af1-rs1
32	6D10	6	12	15	33	Frequency	—	N	Intergenic	Intergenic

* Fischer's Exact test (two tailed) for class membership.

† Is the RLGS spot NotI site within 200 bp of a CpG island.

‡ Annotated gene, mRNA, or spliced EST within 5 kb of the CpG island or NotI site.

§ Spots lost significantly in primary tumors plus AIP primary tumors (n = 60) but not MET tumors (n = 30).

¶ RLGS spot is unidentified.

¶¶ Within 5 kb of transcriptional start site and/or including exon 1.

** Spots lost significantly in AIP primary tumors (n = 30) but not in primary plus MET tumors (n = 60).

†† Spots lost in >30 samples from all tumor types (n = 90).