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Protein Phosphatase and TRAIL Receptor Genes as New Candidate Tumor Genes on Chromosome 8p in Prostate Cancer

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Abstract. *Background: Allelic losses on chromosome 8p are common in prostate carcinoma, but it is not known exactly how they contribute to cancer development and progression. Materials and Methods: Expression of 12 genes located across chromosome 8p, including established tumor suppressor candidates (CSMD1, DLC1, NKX3.1), and others from a new microarray-based comparison was studied by quantitative RT-PCR in 45 M0 prostate carcinomas and 13 benign prostate tissues. Results: Significantly reduced expression was observed for two protein phosphatase subunit genes (PPP2CB, PPP3CC) and two TRAIL decoy receptors (TNFRSF10C/DcR1, TNFRSF10D/DcR2), but not for the three established candidates nor for TRAIL death receptor genes. Low expression of PPP3CC and TNFRSF10C located at 8p21.3 was highly significantly associated with tumor recurrence. In addition to allele loss, down-regulation of TNFRSF10C and TNFRSF10D was found to be associated with hypermethylation, although bisulfite sequencing usually revealed it to be partial. Conclusion: Our data strongly support a recent proposal that a segment at 8p21.3 contains crucial prostate cancer tumor suppressors. In addition, they raise the paradoxical issue of why TRAIL decoy receptors rather than death receptors are down-regulated in aggressive prostate cancer.*

It is a basic tenet of tumor biology that recurrent chromosomal alterations in human cancer point to the location of genes functionally important for their development and progression. This conclusion is based on the observation that specific chromosomal losses often inactivate the second copy of tumor suppressors, whereas chromosomal gains or amplifications lead to the overexpression of oncogenes. For instance, loss of 17p often signifies the functional inactivation of TP53, whereas gains or amplifications at 8q24 are often associated with overexpression of oncogenic MYC. Unfortunately, the association between chromosomal changes and the inactivation or oncogenic activation of individual genes is not always straightforward. Thus, in prostate cancer, it has not yet been possible to link the common losses at 8p to the inactivation of any particular gene. Nevertheless, the question which genes are affected by chromosome 8 alterations is important, as a large number of studies has found either gain of 8q, or loss of 8p, or both, to correlate with increased tumor stage, grade, metastasis, recurrence, or death of disease [reviewed in refs. (1-3)].

Alterations at chromosome 8p are heterogeneous among individual prostate cancers. Most commonly, losses are detected in the 8p12-8p21 and in the 8p23.1 region, but independent studies have not reproducibly yielded common consensus regions for deletions within these segments. In addition, some studies have hinted at gains in the pericentromeric region 8p11-12, which are better documented in breast cancer (4, 5). Theoretically, deletion of one copy should reduce gene expression to half its normal level. In the case of classic tumor suppressors, base mutations, deletions, or promoter hypermethylation inactivate the second copy. In prostate cancer, no gene on chromosome 8p is consistently inactivated by any such combination of genetic or epigenetic events. Since deletions in cancer cells usually extend across several loci, it is thus difficult to distinguish which genes down-regulated in prostate cancers are decisive for tumor development and progression and which are 'bystanders' of 8p loss.

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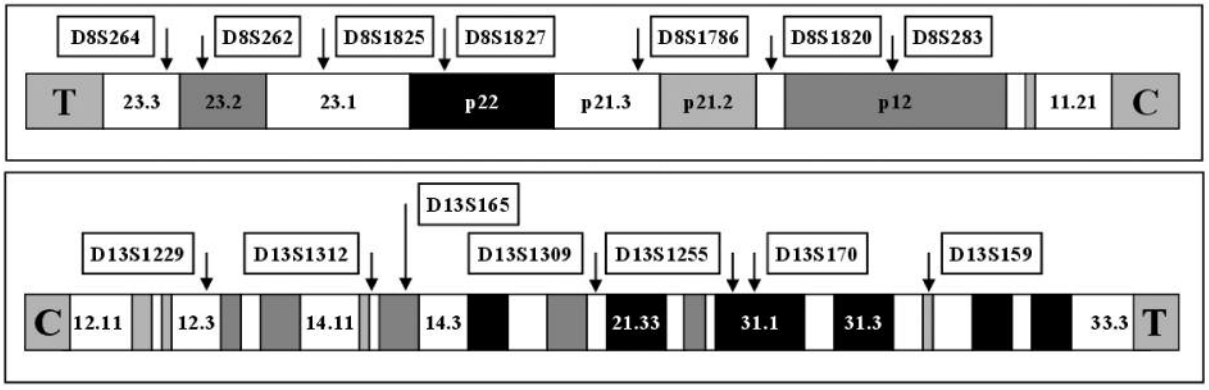

Figure 1. *Localization of microsatellites investigated on chromosomes 8p and 13q.*

The relationship between gene copy number alterations and expression changes in cancer cells is of course not straightforward, since in addition to gene dosage regulatory mechanisms influence expression. Accordingly, comparisons of dosage and expression changes across chromosomal regions in cancer reveal a good, but not perfect correlation (6). Obviously, only those genes that are consistently down-regulated in cancer cases with loss of the respective chromosome region are candidates for tumor suppressors. Conversely, down-regulation of gene expression is not necessarily associated with gene losses and chromosomal alterations, if it is due to epigenetic mechanisms such as promoter hypermethylation. In prostate cancer, the consistent down-regulation of *GSTP1* by biallelic promoter methylation illustrates this case. Thus, a tumor suppressor on 8p important in a majority of prostate cancer cases ought to be down-regulated across a representative series of cancer specimens, but especially in the subset that harbors deletions on 8p.

We therefore investigated the expression of twelve 8p candidate genes drawn from the literature and from our own microarray analysis in prostate cancer tissues compared to benign prostates and their relationship to 8p allelic imbalances.

Materials and Methods

Tissues. Normal and cancerous prostate tissues were obtained as described elsewhere (7). Cancerous tissues were selected and macrodissected to contain a maximum of carcinoma cells in order to obtain reasonable amounts of RNA and DNA for multiple analyses from the same tumor region. Benign prostate tissue samples were selected by their gross and microscopic morphology in cancer-carrying prostates. Fifty-one tumor samples, for which additional DNA from blood leukocytes was available, were investigated for allelic imbalances. Clinical and pathological data for this group were as follows: 21 pT2, 28 pT3, 2 pT4 tumors, 16 with Gleason Scores <7, 23 with 7 and 12 with >7, respectively. Twelve cancer cases were lymph node-positive. Thirteen of these patients suffered a disease relapse evidenced by two successive PSA levels above 0.2 ng/ml. Expression analyses were performed using 45 tumor and 13 normal tissue specimens, for which high quality RNA could be obtained, except for *CSMD1* for which two tumor samples were missing and *DLC1/FGFR1* for which only ten normal tissue specimens were measured. Clinicopathological data for this overlapping group were as follows: 20 pT2, 23 pT3, 2 pT4 tumors, 13 with Gleason Scores <7, 26 with 7 and 6 >7, respectively. Eleven cancer cases were lymph node-positive. Ten patients from this group experienced relapse. The median follow-up period was >5 years. The study was approved by the Ethics Committee of the Heinrich Heine University medical faculty.

DNA extraction and allelic imbalance analysis. High molecular weight genomic DNA from tissue, cell lines, and whole blood was isolated as described (7). Matched pairs of normal and tumor DNA samples were screened for allelic imbalances at chromosome 8p and 13q at seven microsatellite loci for each chromosome arm (Figure 1) using primer sequences from the Genome DataBase in duplex PCR reactions as described elsewhere (8).

RNA isolation and quantitative RT-PCR. Total mRNA was isolated from cell cultures and tissues and cDNA synthesis was carried out as described elsewhere (9). Real-time PCR assays were either performed using the LightCycler apparatus and the LightCycler-FastStart DNA Master PLUS SYBR Green I (Roche, Mannheim, Germany) for *DLC1, FGFR1*, and *CSMD1* with primers indicated in Table I, or an ABI 7900 instrument (Applied Biosystems, Weiterstadt, Germany) with assays supplied by the same company. For all RNA measurements, *TBP* was employed as a reference gene.

Microarray expression analysis. High-quality total RNA from 6 benign and 22 cancerous prostate samples was analyzed on HG-U133A (Affymetrix) microarrays as described elsewhere (9). Raw expression data were normalized by the VSN normalization procedure (10), using all 28 samples jointly. After normalization, the expression set was restricted to the 736 probesets located at chromosome 8 and the 386 probsets at chromosome 13, respectively. For every probeset separately, a two-sample t-statistic with equal variance was computed between the cancer and the benign prostate specimens. The resulting *p*-values were adjusted for multiple testing according to the false discovery rate (FDR) method (11).

Table I. *Primer sequences and PCR conditions.*

Method	Sequence 5' → 3'	Annealing (°C)	Cycles
	Real-time RT-PCR		
FGFR1 fwd	GGAGGATCGAGCTCACTCGTGG	65.8°C	45
FGFR1 rev	CGGAGAAGTAGGTGGTGTCAC	61.8°C	45
DLC1 fwd	GCTCCATCCTCTACTCCAGTTCAG	64.4°C	45
DLC1 rev	GACTGGCAGTTAATCTGTAGTGA	58.9°C	45
CSMD1 fwd	ACAACCCGCCACTCTAACTG	59.4°C	45
CSMD1 rev	TCTGCCTGGCCTTTAATTTG	55.3°C	45
	Bisulfite sequencing		
DcR2 fwd	TGTTGTTTATAGTTTGGATAGGAT	56.0°C	36
DcR2 rev	CTTTATCCCCAAAATCCCATA	56.0°C	36

fwd: forward, rev: reverse.

DNA methylation analyses. MS-PCR was performed as described elsewhere (7) using primer pairs for DcR1 and DcR2 as given in (12). DNA from normal urothelial cells or blood leucocytes was used as a positive control for U-reactions and DNA from PC3 cells as a control for M-reactions. Bisulfite sequencing was performed essentially as described (7) using the primers DcR2 fwd and DcR2 rev (Table I) at an annealing temperature of 56°C.

Results

Analyses of allelic imbalances at 8p and 13q. Initially, we searched for allelic imbalances of a number of microsatellite markers spaced along chromosome 8p (Figure 1A) in a series of 51 primary prostate cancer samples, all without distant metastases. The assays were conducted as quantitative duplex assays, with one marker from 8p each combined with a different marker from 13q (Figure 1B). This procedure allows the reliable investigation of macrodissected cancer tissues, including the detection of homozygously deleted markers (8). Of these samples, 23 displayed at least one allelic imbalance at 8p. Incidentally, the same number of specimens exhibited allelic imbalances at 13q. On 8p, allelic imbalances were most frequent at D8S1827 (27% of the cases) and D8S1786 (24%). On 13q, allelic imbalances were most frequently detected at D13S1312 and D13S170 (each 18%). No homozygous deletions were detected.

The presence of allelic imbalances at 8p was associated with an increased Gleason score (χ^2 p=0.038), tended to be more frequent when lymph node metastases were present (p=0.086), but was not significantly associated with time to recurrence (log-rank p=0.247). No associations between 13q allelic imbalances and clinical parameters were observed.

Microarray analysis of gene expression changes in prostate cancer. In parallel, a microarray expression analysis of 22 cancer samples compared to 6 benign prostate specimens was conducted. Figure 2 illustrates the observed differences in the expression of genes on chromosomes 8 and 13. Clearly, both chromosomes contained genes that were overexpressed or underexpressed in cancers *vs*. normal tissues.

A number of genes on 8q or 13q highlighted by this comparison have been studied in prostate cancer (13-18). For instance, *TRAM1* at 8q13 encoding the steroid receptor coactivator SRC3 and *RAD21* encoding the securin Scc1 are overexpressed in prostate cancer (13, 14). The most strongly overexpressed gene *YWHAZ* encodes the 14-3-3θ protein, a relative of 14-3-3σ (15). Among the possibly down-regulated genes on 8q, *RUNX1* encoding the AML1 protein may be of particular interest. Of the down-regulated genes at 13q, *FAM48A*, also named C13orf19 (16), *FLT1* (17), *TNFSF11* encoding the cytokine RANKL, and the androgen receptor inhibitor RAP2A (18) are notable.

Analysis of expression changes of 8p candidate genes. Table II details the genes on chromosome 8p arm that were expressed at significantly lower or higher levels in tumor *vs*. benign tissues. From these lists, candidates were chosen according to their potential biological role in prostate cancer and from various chromosomal subregions. Furthermore, several previously reported candidate genes were investigated as well as three genes from the TRAIL receptor cluster at 8p21.3 in addition to *DR5/TNFRSF10B* which had been conspicuous in the microarray analysis (Table III).

The expression of these 12 genes was then compared by quantitative real-time PCR in an extended set of 45 prostate cancer and 13 benign tissue samples (Figure 3). Of the investigated 8p genes, six showed significant differential expressions between normal and tumor tissue samples. Expression of *PPP2CB, PPP3CC, DcR1/TNFRSF10C* and *DcR2/TNFRSF10D* was significantly down-regulated (*t*-test: p=0.023, p=0.002, p=0.0016, and p=0.048, respectively). Of note, the microarray results had predicted

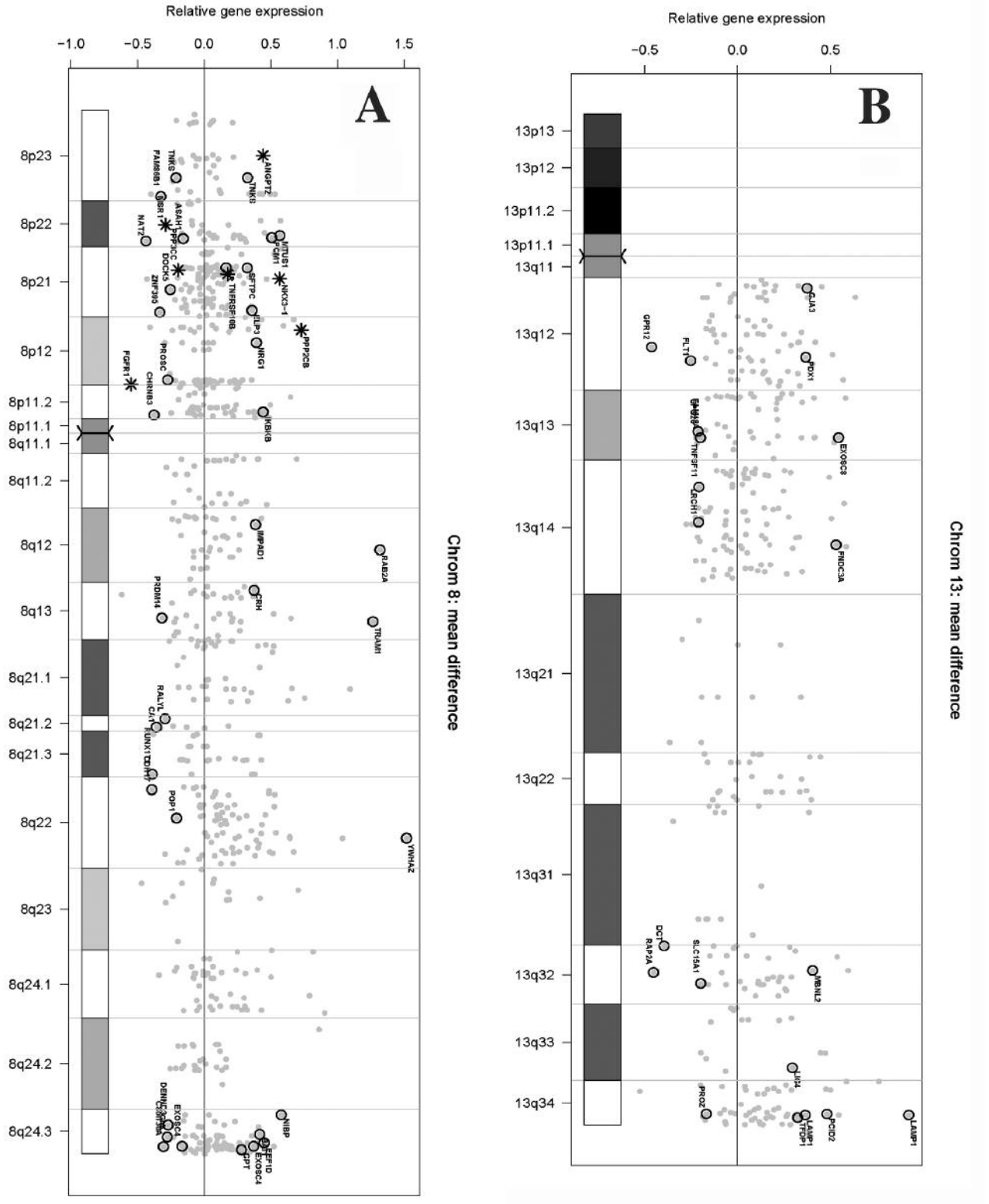

Figure 2. *Differential expression of genes on chromosomes 8 and 13 between cancerous and benign prostate tissues. Median expression of genes along chromosome 8 (A) and chromosome 13 (B). The top 10 significantly up-regulated and down-regulated genes per chromosome arm each are indicated. On chromosome 8p, the genes chosen for further analysis are signified by asterisks. Note that the expression scale is logarithmic, the zero line corresponds to the median of the normal samples.*

PPP2CB as up-regulated (Table II). In contrast, *DLC1* and *NKX3.1* were slightly, but significantly overexpressed (p=0.004 and p=0.006).

For each gene, we additionally compared the expression levels in cancer samples containing any allelic imbalance on 8p and in those cancer samples in which allelic imbalance had been observed at the microsatellite marker located most closely to the gene in question (Figure 4). For PPP2CB, DcR1 and DcR2, the significance levels for the difference increased if tumor samples with allelic imbalances on 8p were considered. For PPP2CB, restriction to samples showing allelic imbalances for the adjacent microsatellite markers resulted in an even more significant p-value. Similarly, the significance of NKX3.1 expression differences increased if only tumor samples with allelic imbalances on chromosome 8p were considered.

Correlations of the investigated genes with Gleason score (<6 *vs.* ≥7), tumor stage (<pT3 *vs.* ≥pT3) and lymph node status (N0 *vs.* N1) yielded three significant associations. *CSMD1* expression was diminished in tumor samples with Gleason scores 7 or higher (p=0.025). *MSR1* expression was significantly elevated in tumor samples with positive lymph node status (p=0.032). *DcR1* was particularly strongly down-regulated in specimens with locally advanced stage (p=0.049) (data not shown).

For the four genes *PPP3CC, DR5/TNFRSF10B, DcR1/TNFRSF10C, NKX3.1*, the expression levels differed significantly between patients with or without disease recurrence. These differences also reached significance in Kaplan-Meyer survival analysis (Figure 5), except for *DR5* (log-rank p=0.077).

In addition to four TRAIL receptor genes (*TNFRSF10A-D*) on chromosome 8p, a further potential TRAIL-binding protein is encoded by the OPG gene located at 8q24.12 in a region gained or amplified in many prostate cancer cases. The expression of *OPG* was therefore measured in the same series. Overall, expression tended to be slightly lower in tumor samples compared to normal tissues (t-test: p=0.061). No significant association of *OPG* expression with clinical parameters was observed; the smallest p-value (0.066) was observed for the difference in expression between recurrent and non-recurrent tumors. Indeed, Kaplan-Meier analysis revealed a significant association of reduced OPG expression with biochemical relapse (Figure 5) (p=0.0358).

Analysis of TNFRSF10C and TNFRSF10D methylation changes. Down-regulation of the *TNFRSF10C* and *TNFRSF10D* genes has been described as being associated with promoter hypermethylation in various types of human cancer, including prostate carcinoma. We therefore investigated the methylation status of these genes by the methylation-specific PCR technique described in (12). For *TNFRSF10C*, we observed partial methylation in both benign and cancerous prostate tissues, as revealed by positive signals with primers specific for the unmethylated sequence as well as for the methylated sequence, whereas control DNAs yielded only expected bands with primers specific for unmethylated or methylated DNA (data not shown). For *TNFRSF10D/DcR2*, the methylation differences between benign and cancerous tissues were more clear-cut. Of the cancer specimens with normal level *DcR2* expression, five out of eight showed an unmethylated promoter-region along with three out of five specimens of benign tissue. Of the specimens with diminished *DcR2* expression, seven of eight were methylated (data not shown).

In our experience (7, 19), weak signals in MS-PCR with primers specific for the methylated sequence are often caused by 'patchy' methylation. Bisulfite sequencing of the region surrounding the gene transcription start site was therefore performed for selected samples (Figure 6). In accord with the MS-PCR results, DNA from normal leukocytes was completely unmethylated and DNA from the PC3 prostate carcinoma cell line was completely methylated in a dense pattern. Most cancer tissues were partially methylated, mostly at five adjacent CpG sites around –150 bp. To a lesser extent, patchy methylation was also found in the benign samples and the LNCaP cell line. Only one tumor sample with low DcR2 expression yielded densely methylated alleles.

Discussion

Allelic imbalances at 8p, typically representing sequence loss, are common in prostate cancer. In the present study, 45% of the specimens in a series of M0 primary carcinomas displayed changes at at least one locus. The presence of 8p allelic imbalances was associated with higher Gleason scores and a tendency toward lymph node metastasis. These findings are in line with a wealth of other studies. As part of the duplex PCR experimental setup, we determined the frequency of 13q allelic imbalances which likewise was in accord with that in other studies [reviewed in refs. (1-3)].

Given the consistency with which 8p losses are observed in different studies, one might expect that changes in the expression of tumor suppressors 'targeted' by deletions are also consistent. However, this is not the case. For instance, an excellent candidate for a tumor suppressor on 8p, *NKX3.1*, was not down-regulated in most cancer specimens of the present series, not even if allelic imbalances were observed at markers close to the gene. In fact, this finding is no exception. A search of the ONCOMINE database (20) reveals that only a single microarray study comparing benign prostate to carcinoma tissues yielded unequivocal and highly significant down-regulation of the gene (21), whereas others found slight and marginally significant increases as in our investigation [*e.g.* (22-24)] or observed no significant

Table II. *Differentially regulated genes at chromosome 8p according to microarray analysis. Genes down-regulated and up-regulated between cancerous and benign tissues. The last columns present the FDR-adjusted p-values computed based on all arrays.*

	Genes down-regulated in cancer tissues	
Gene symbol	Gene name	Adjusted *p*-value
PROSC	Proline synthetase co-transcribed homolog (bacterial)	8.4e-06
FAM86B1	Family with sequence similarity 86, member B1	9.3e-06
NAT2	N-acetyltransferase 2 (arylamine N-acetyltransferase)	1.1e-05
CHRNB3	Cholinergic receptor, nicotinic, beta 3	3.7e-05
DOCK5	Dedicator of cytokinesis 5	9.7e-05
MSR1	Macrophage scavenger receptor 1	9.7e-05
ZNF395	Zinc finger protein 395	9.7e-05
FGFR1	Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	0.00017
ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	0.00044
TEX15	Testis-expressed 15	0.00051
DLGAP2	Discs, large (Drosophila) homolog-associated protein 2	0.00054
VDAC3	Voltage-dependent anion channel 3	0.00068
PPP3CC	Protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform	0.00084
MTMR7	Myotubularin-related protein 7	0.00240
ZMAT4	Zinc finger, matrin type 4	0.00249
FKSG2	Apoptosis inhibitor	0.00461
REEP4	Receptor accessory protein 4	0.00461
TACC1	Transforming, acidic coiled-coil containing protein 1	0.00566
SPAG11B	Sperm-associated antigen 11B	0.00566
WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1	0.01037
DEFA5	Defensin, alpha 5, Paneth cell-specific	0.01501
MSRA	Methionine sulfoxide reductase A	0.01735
BLK	B lymphoid tyrosine kinase	0.01753
EPB49	Erythrocyte membrane protein band 4.9 (dematin)	0.02212
	Genes up-regulated in cancers	
Gene symbol	Gene name	Adjusted *p*-value
PCM1	Pericentriolar material 1	3.9e-08
NRG1	Neuregulin 1	2.1e-07
PPP2CB	Protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	3.7e-07
ANGPT2	Angiopoietin 2	1.3e-06
SFTPC	Surfactant, pulmonary-associated protein C	1.2e-05
TNKS	Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase	1.5e-05
HR	Hairless homolog (mouse)	2.6e-05
MTUS1	Mitochondrial tumor suppressor 1	2.8e-05
ELP3	Elongation protein 3 homolog (S. cerevisiae)	4.1e-05
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	0.00017
DCTN6	Dynactin 6	0.00067
FNTA	Farnesyltransferase, CAAX box, alpha	0.00105
ATP6V1B2	ATPase, H+ transporting, lysosomal 56/58 kDa, V1 subunit B2	0.00105
PDLIM2	PDZ and LIM domain 2 (mystique)	0.00106
CHMP7	CHMP family, member 7	0.00106
XPO7	Exportin 7	0.00205
KIAA1967	KIAA1967	0.00212
PTK2B	PTK2B protein tyrosine kinase 2 beta	0.00242
NKX3.1	NK3 homeobox 1	0.00259
MYST3	MYST histone acetyltransferase (monocytic leukemia) 3	0.00348
ASH2L	Ash2 (absent, small, or homeotic)-like (Drosophila)	0.00366
SH2D4A	SH2 domain containing 4A	0.00492
PSD3	Pleckstrin and Sec7 domain containing 3	0.00592
BMP1	Bone morphogenetic protein 1	0.00609
LEPROTL1	Leptin receptor overlapping transcript-like 1	0.00723
GSR	Glutathione reductase	0.00810
DUSP4	Dual specificity phosphatase 4	0.00941

Table II. *continued*

Table II. *continued*

	Genes up-regulated in cancers	
Gene symbol	Gene name	Adjusted *p*-value
CNOT7	CCR4-NOT transcription complex, subunit 7	0.00995
WRN	Werner syndrome	0.00998
LZTS1	Leucine zipper, putative tumor suppressor 1	0.01090
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	0.01115
SFRP1	Secreted frizzled-related protein 1	0.01115
EXTL3	Exostoses (multiple)-like 3	0.01193
KIF13B	Kinesin family member 13B	0.01299
ENTPD4	Ectonucleoside triphosphate diphosphohydrolase 4	0.01436
ADAM28	ADAM metallopeptidase domain 28	0.01816
TUSC3	Tumor suppressor candidate 3	0.02109
GATA4	GATA binding protein 4	0.03151

Table III. *Candidate genes at 8p investigated by quantitative RT-PCR.*

Gene name	Region	Source	Expression in microarray	Function
FGFR1	8p12	Candidate oncogene	Down	Growth factor receptor
PPP2CB	8p12	Microarray	Up	Protein phosphatase (PP2A)
NKX3.1	8p21.2	Candidate tumor suppressor	Up	Prostate differentiation
TNFRSF10A				
DR4	8p21.3	location in cluster	Not represented	Apoptosis induction by TRAIL
TNFRSF10B				
DR5	8p21.3	Microarray	Up	Apoptosis induction by TRAIL
TNFRSF10C				
DcR1	8p21.3	location in cluster	Unchanged	Inhibition of apoptosis induction by TRAIL
TNFRSF10D				
DcR2	8p21.3	location in cluster	Unchanged	Inhibition of apoptosis induction by TRAIL
PPP3CC	8p21.3	Microarray	down	Protein phosphatase (calcineurin)
DLC1	8p22	Candidate tumor suppressor	Unchanged	Rho GTPase
MSR1	8p22	Microarray hereditary PCa candidate	Down	Immune regulation
CSMD1	8p23.2	Candidate tumor suppressor	Not represented	Adhesion molecule
ANGPT2	8p31.1	Microarray	Up	Angiogenesis regulation

difference (25, 26). Of note, all studies agree on the down-regulation of NKX3.1 in metastatic cases. Perhaps the lack of association between chromosome loss and gene expression should not be regarded as too surprising, as NKX3.1 expression not only depends on gene dosage, but also on androgens (27) and retinoids (28). Loss of *NKX3.1* also occurs during progression in the mouse TRAMP model in which prostate cancer is elicited by SV40 large-T antigen (29). Together, these findings suggest that loss of *NKX3.1* expression may be primarily related to loss of differentiation in late stages of prostate cancer. At any rate, the association between chromosome 8p loss and expression of this gene does not seem always straightforward. Rather, in some cases, chromosomal losses may be responsible for its down-regulation, whereas regulatory mechanisms adding to or antagonizing dosage changes may be responsible for its up-regulation in some prostate cancer cases and down-regulation in others.

Similar considerations may apply to other candidate genes investigated in this study. Some microarray studies found *DLC1* down-regulation in carcinoma compared to benign prostate tissues (22, 30), whereas others observed increased expression (31, 32), as in the present study. Guan *et al*. (33) reported down-regulation of the gene in prostate cancer compared to normal prostates from young men (<30 years), associated with promoter hypermethylation. However, the gene was also dramatically down-regulated in benign hyperplastic prostates of elderly men. Methylation was found in both BPH and prostate cancer samples and was often patchy. Moreover, the gene actually has three transcripts

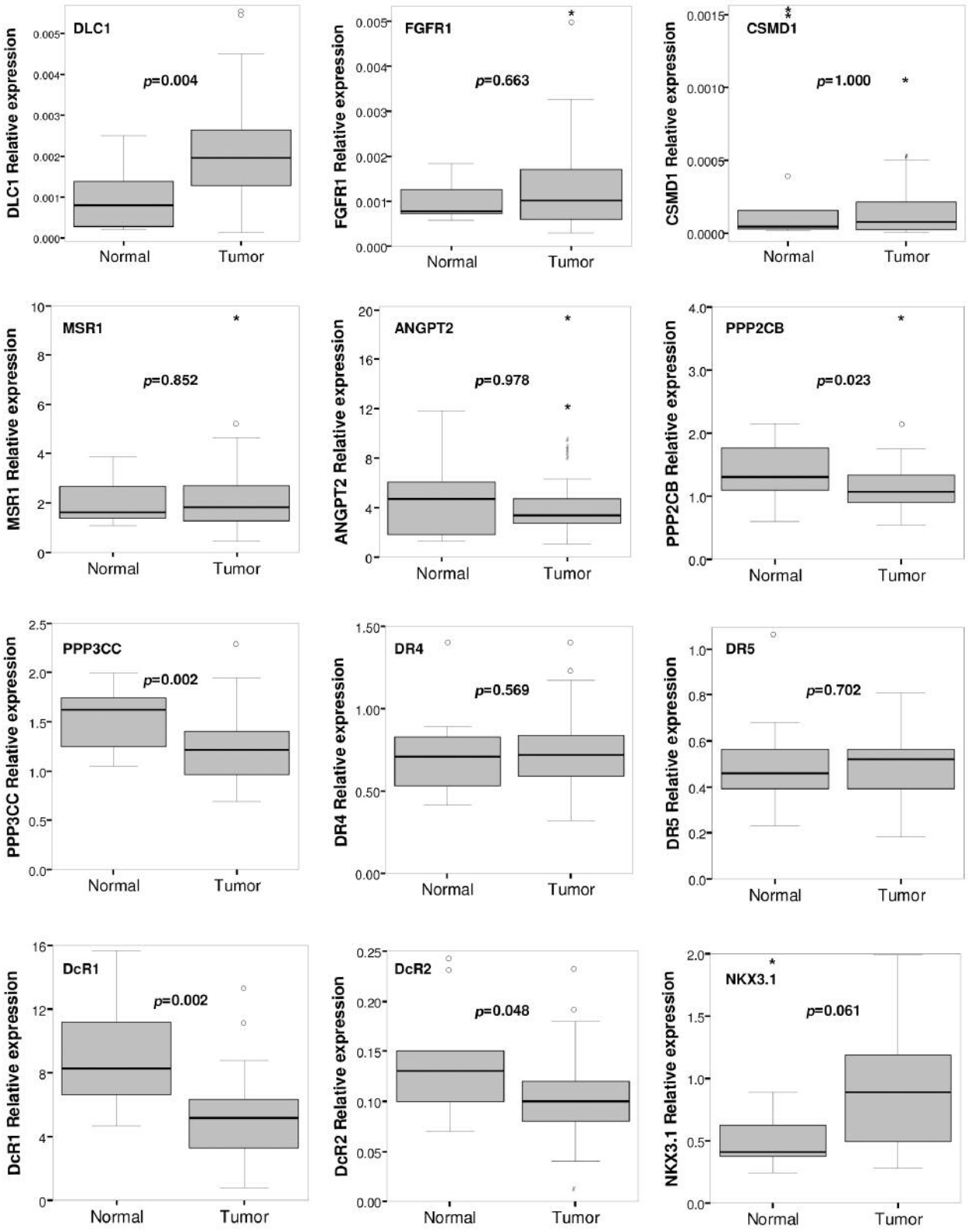

Figure 3. *Quantitative RT-PCR analysis of 8p gene expression. Box plot representation of gene expression measured by real-time RT-PCR in 45 prostate cancer (tumor) and 13 benign (normal) tissues comparing their expression in normal and tumor tissue samples. P-values are from t-tests.*

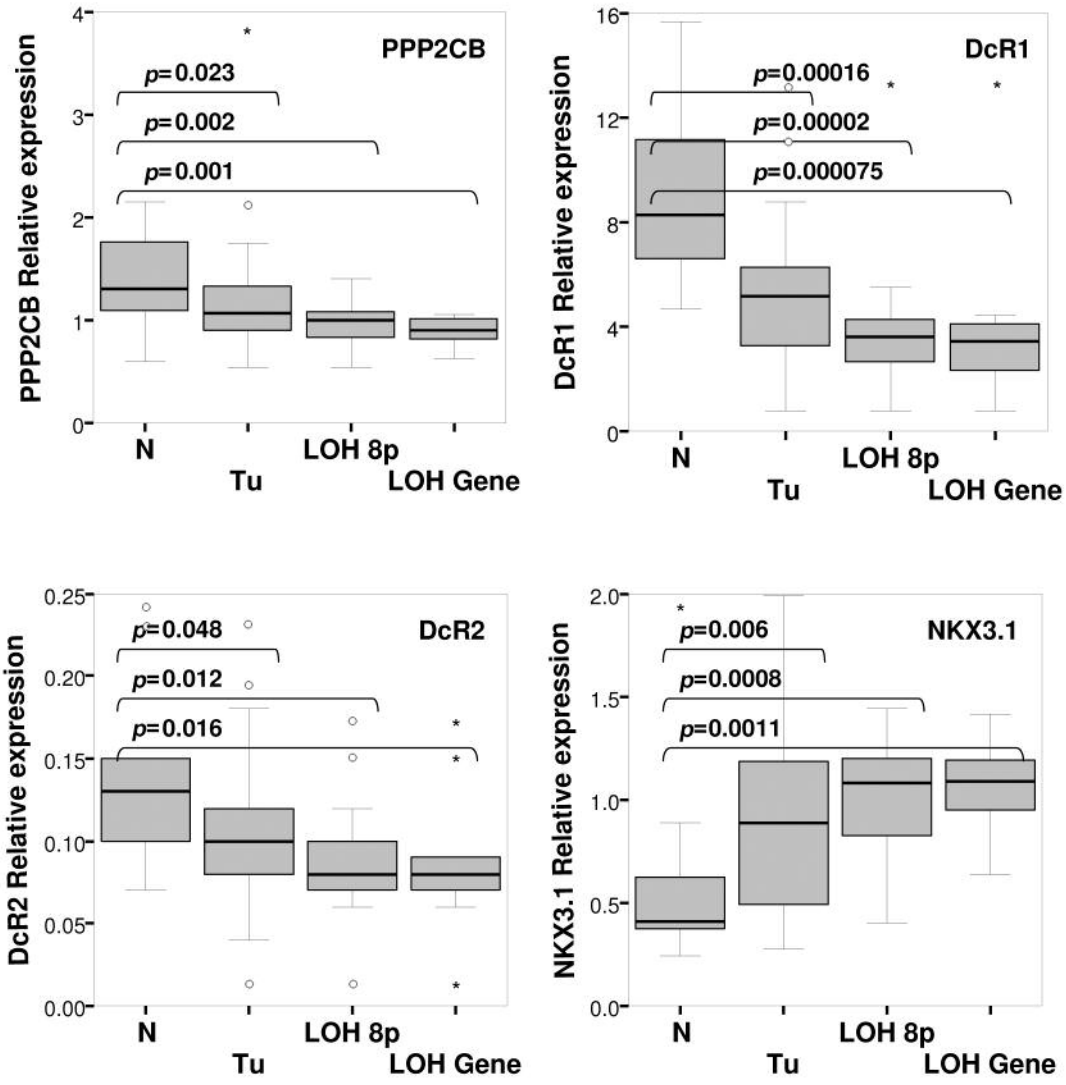

Figure 4. *Relation of expression of 8p genes to allelic loss. Box plot representation of relative expression of PPP2CB, DcR1, DcR2, and NKX3.1 in benign tissues (N), cancer overall (Tu), or cancer samples exhibiting allelic imbalances anywhere on 8p (LOH 8p) or close to the gene location (LOH Gen), respectively. P-values are from t-tests.*

originating from three different promoters, of which only one is located in a CpG-island. *CSMD1* at 8p23.2 was suggested as a candidate tumor suppressor from the analysis of common deletions in several cancer types, including prostate cancer (34). The region containing the gene was independently linked to a high risk of metastasis and recurrence in prostate cancer (35). However, our study did not show differences in the expression of this gene, even in cases with allelic imbalances in the distal part of chromosome 8p.

In breast cancer, 8p deletions in general are likewise common, but a region close to the centromere tends to be rather amplified (4, 5). For this reason, we included *FGFR1* and *PPP2CB* located at 8p12 in our study. Neither gene turned out to be significantly overexpressed. According to RT-PCR, *PPP2CB*, which encodes a subunit of protein phosphatase 2A, was rather down-regulated. According to our microarray data, another interesting candidate might be *NRG1* which appeared to be strongly overexpressed in cancer compared to normal tissues. This gene encodes a variety of peptides through at least 12 different splice variants, among them activators of ERBB receptors. In spite of its biological relevance, it is under-researched in prostate cancer, perhaps because of its complexity (36).

The *PPP3CC* gene located next to the TRAIL receptor gene cluster at 8p21.3 has not yet been specifically

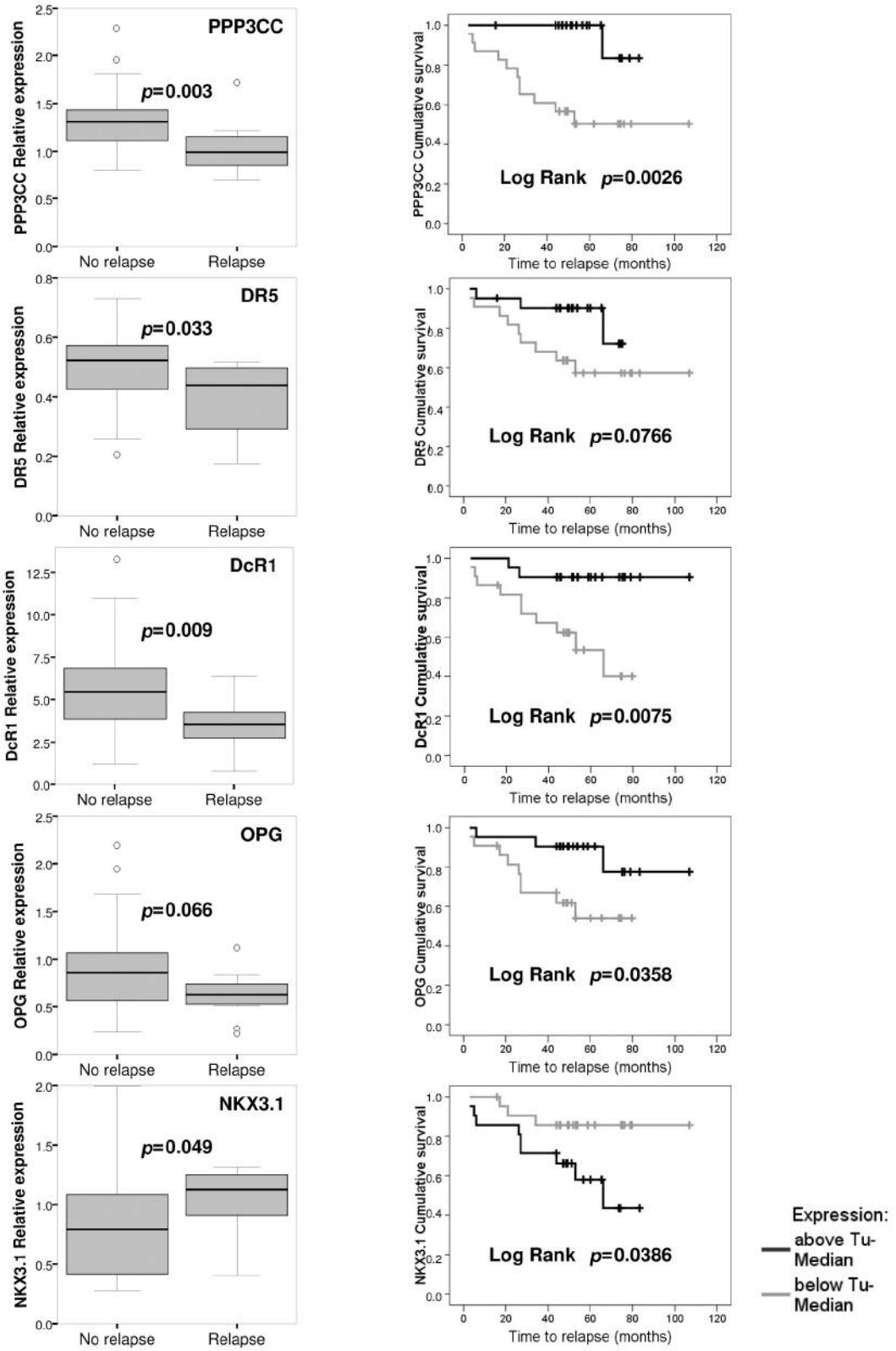

Figure 5. *Association of the expression of selected genes to disease recurrence. Left: t-test comparison of expression values in recurring and non-recurring tumors. Right: Kaplan-Meier survival analysis; cases were grouped by expression above (black) or below (grey) median values. P-values from log-rank tests.*

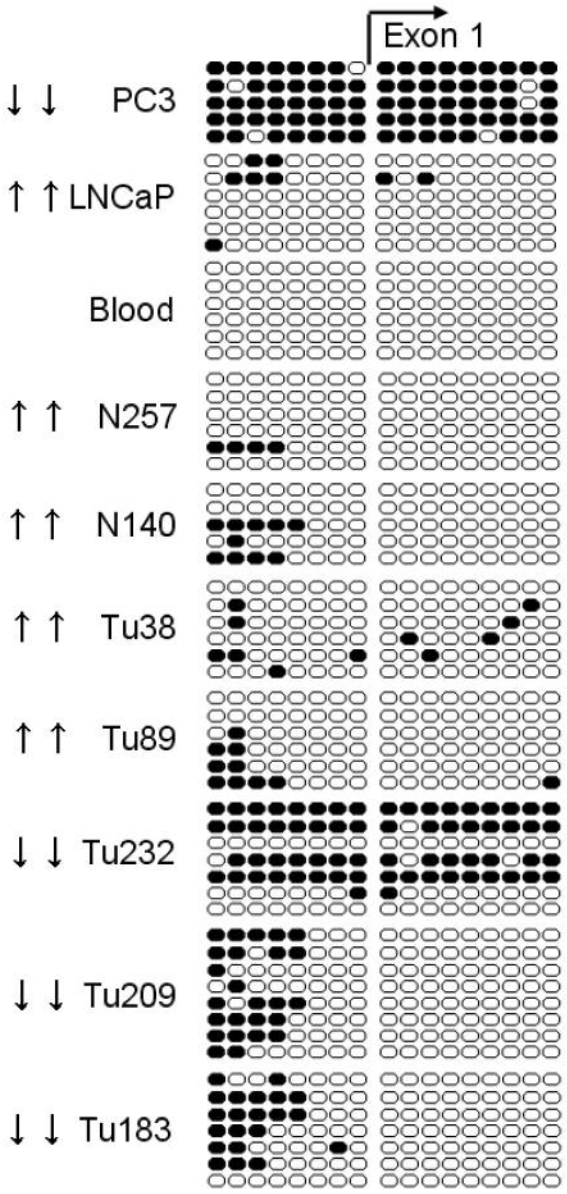

Figure 6. *Bisulfite sequencing analysis of DcR2 promoter methylation. Bisulfite sequencing analysis of 17 CpG sites around the DcR2 transcriptional start site (indicated by the angular arrow) in prostate cancer cell lines (PC3, LNCaP), blood leukocytes, five prostate cancer tissues (T) and two benign prostate tissues (N). Each line corresponds to one cloned PCR product; white circles represent unmethylated, black circles methylated CpGs. Arrows indicate DcR2 mRNA expression levels in the corresponding samples: upward: high, downward: low. Expression in leukocytes was not measured.*

considered in the context of prostate cancer. However, down-regulation of the gene in prostate cancer compared to benign tissues in microarray studies is comparatively consistent [*e.g.* (24)]. Our results are in accordance with Glinsky *et al*. (37) who observed a significant decrease of *PPP3CC* expression in recurrent prostate cancer. The gene encodes the γ isoform of the catalytic subunit of calcineurin. Calcineurin is best known for its role in the activation of T-cells. It dephosphorylates and activates the NF-AT transcription factor required for the activation and maturation of T-cells. This function is antagonized by prolyl-isomerase PIN1, encoded by a gene at 19p13.2. The calcineurin pathway is however not restricted to T-cells. In particular, calcineurin activation occurs as a consequence of an alternative pathway of WNT signaling, termed the WNT/calcium pathway, which in general antagonizes the canonical WNT/β-catenin pathway, favoring cell differentiation and polarization (38). Increased activity of the canonical pathway occurs during the progression of prostate cancer, but with the twist that β-catenin stimulates androgen receptor function rather than TCF transcription factors (39, 40). Interestingly, PIN1 is overexpressed in many prostate cancer cases and strong overexpression is a very good indicator of disease recurrence, as observed for *PPP3CC* down-regulation in our series. The questions whether these changes are complementary to each other or synergistic and how they relate to WNT signaling seem worthwhile addressing.

More than 10 microarray studies in the ONCOMINE database (20) reveal a significant down-regulation of *PPP2CB* in prostate carcinomas compared to benign tissues, which is usually aggravated in metastatic cases. The gene encodes a catalytic subunit of protein phosphatase 2A (PP2A). 'PP2A' is really a family of multifunctional protein phosphatases consisting of a regulatory and catalytic subunits (α, β, γ) each encoded by several different genes. PP2A is generally regarded as possessing an antioncogenic function, since it was identified as the target of the SV40 small-T oncoprotein (41). A major physiological substrate of PP2A is activated PKB/Akt (42), which is thought to play a crucial role in the development and progression of prostate cancer. Interestingly, a recent study reported down-regulation of *PPP2CA* located at 5q31.1 in prostate cancer associated with increasing tumor stage and Gleason grade (43).

The effects of the TNF-related factor TRAIL on prostate cancer cells have been extensively studied (44), whereas, surprisingly, the four TRAIL receptor genes (*TNFRSF10A-D*) at 8p21.3 have received relatively little attention. One exception is a study reporting moderately strong and often heterogeneous up-regulation of all four proteins during the progression of prostate cancer (45). In comparison, osteoprotegerin encoded by the *OPG* gene has been studied rather well, because it may exert an important function during bone metastasis by binding RANKL [reviewed in (46)]. To what extent its TRAIL binding ability is relevant *in vivo* and in particular in prostate cancer is, however, unclear.

Relatively recently, surveys of cancer cell lines (12, 47) and tissues (48) have revealed hypermethylation of two TNFRSF10 genes in prostate cancer. Because of the interest

of our group in methylation changes in prostate cancer, this study prompted us to include the genes of the *TNFRSF10* cluster in our analysis, although only one of them had been differentially expressed in the microarray analysis. Meanwhile, the gene cluster was observed to be located within a consensus region for deletions in prostate cancer identified by analysis of allelic imbalances in sporadic cases and linkage in hereditary cases that also contains *PPP3CC* (49). We observed little change in the expression of the *TNFRSF10A* and *TNFRSF10B* genes, but significant down-regulation of the *TNFRSF10C* and *TNFRSF10D* genes, especially in tumors with 8p losses. Moreover, *TNFRSF10C* down-regulation was strongly associated with tumor recurrence. In addition, whereas *OPG* expression was not significantly changed overall in the cancerous tissues, tumors with lower expression were more likely to recur. We also confirmed the hypermethylation of *TNFRSF10C* and *TNFRSF10D* in prostate cancer tissues and its association with gene down-regulation. However, methylation of these genes, especially *TNFRSF10C*, was not restricted to cancerous tissues, but also found in benign tissues. Hypermethylation of genes in the aging prostate is a common finding, prominent examples being *RASSF1A* (8) and *DLC1* (32). Often, such genes are patchily methylated in benign hyperplasia, but become more intensely methylated in the carcinoma cells, suggesting that hypermethylation contributes to a preneoplastic 'field' change. On another note of caution hypermethylation of the *TNFRSF10* genes in prostate cancer tissues as opposed to cell lines (12) is rarely dense, as we demonstrated by bisulfite sequencing of the TNFRSF10D gene promoter. Partial methylation could nevertheless contribute to the down-regulation of the genes by blocking crucial transcription factor binding sites. Indeed, the promoter of the related *TNFRSF6* gene, encoding FAS, is exquisitely sensitive to partial methylation at potential binding sites for NFκB (50).

Taken together, the findings on altered expression, copy number losses, hypermethylation, and association with tumor recurrence point to the functional importance of changes in TRAIL receptor expression in prostate cancer. However, while the findings on the changes are concordant, their function is not straightforwardly evident. The *TNFRSF10A* and *TNFRSF10B* genes with unchanged expression in prostate cancer encode the DR4 and DR5 receptors that mediate the apoptotic action of TRAIL. In contrast, the *TNFRSF10C* and *TNFRSF10D* genes code for the DcR1 and DcR2 receptors, of which DcR1 is certainly and DcR2 likely a decoy receptor. The overall effect of the observed changes should therefore be an enhancement of TRAIL activity, perhaps further strengthened by loss of osteoprotegerin. If so, one would expect prostate carcinomas to be particular sensitive to TRAIL therapy. In fact, there are dramatic differences in the ability of TRAIL to induce apoptosis in prostate cancer cell lines and xenografts (44). In cell lines, these differences do not appear to correspond to the expression pattern of the TRAIL receptors, but rather to the degree to which apoptotic signaling pathways are blocked by mutations or overexpression of antiapoptotic factors. The association of DcR1 down-regulation with tumor recurrence suggests however that *in vivo* the expression levels of the receptors may matter. Accordingly, on the basis of an immuno-histochemical study of TRAIL receptors Sanlioglu *et al.* (45) have proposed that individual prostate cancer cases might react very differently to TRAIL therapy. Moreover, it should be considered that DR4 and DR5, like several other members of the TNFRSF class, in addition to eliciting apoptosis induce activation of NFκB. In some cell types and other types of cancer, it has been observed that sufficiently strong blockade of TRAIL-induced apoptotic signaling uncovers a mitogenic potential of the cytokine (51, 52). Our findings could mean that this occurs in a more aggressive subclass of prostate cancer too.

In conclusion, we suggest that several genes located closely to each other at 8p21 deserve detailed exploration for their role in prostate cancer. This may be a demanding task, because their mode of action is complex, as in the case of TRAIL receptor genes, or because they encode one of many subunits of multifunctional protein phosphatases, such as the *PPP2CA* and *PPP3CC* genes.

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