Prevalence of chromosomal rearrangements involving non-ETS genes in prostate cancer

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Abstract. Prostate cancer is characterized by structural rearrangements, most frequently including translocations between androgen-dependent genes and members of the *ETS* family of transcription factor like *TMPRSS2:ERG*. In a recent whole genome sequencing study we identified 140 gene fusions that were unrelated to *ETS* genes in 11 prostate cancers. The aim of the present study was to estimate the prevalence of non-*ETS* gene fusions. We randomly selected 27 of these rearrangements and analyzed them by fluorescence *in situ* hybridization (FISH) in a tissue microarray format containing 500 prostate cancers. Using break-apart FISH probes for one fusion partner each, we found rearrangements of 13 (48%) of the 27 analyzed genes in 300-400 analyzable cancers per gene. Recurrent breakage, often accompanied by partial deletion of the genes, was found for *NCKAP5*, *SH3BGR* and *TTC3* in 3 (0.8%) tumors each, as well as for *ARNTL2* and *ENOX1* in 2 (0.5%) cancers each. One rearranged tumor sample was observed for each of *VCL*, *ZNF578*, *IMMP2L*, *SLC16A12*, *PANK1*, *GPHN*, *LRP1* and *ZHX2*. Balanced rearrangements, indicating possible gene fusion, were found for *ZNF578*, *SH3BGR*, *LPR12* and *ZHX2* in individual cancers only. The results of the present study confirm that rearrangements involving non-*ETS* genes occur in prostate cancer, but demonstrate that they are highly individual and typically non-recurrent.

Introduction

Prostate cancer is the most frequent malignancy in men. Although the majority of patients present with early stage tumors that can be surgically treated in a curative manner, ~20% of the tumors will progress to metastatic and hormone refractory disease, accounting for >250.000 deaths per year worldwide (1). Targeted therapies that would allow for an effective treatment after failure of androgen withdrawal therapy are lacking.

Recent whole genome sequencing studies have shown that the genomic landscape of prostate cancer differs markedly from that of other solid tumor types. Whereas, for example, breast or colon cancer is characterized by high-grade genetic instability and presence of a multitude of mutations, deletions, and amplifications including important therapy target genes such as *HER2* and *EGFR* (2,3), prostate cancers show only comparatively few mutations and almost completely lack amplifications (4-7). In contrast, prostate tumors are typically characterized by translocations, deletions, and gene fusions, the latter of which are recurrently involving androgen-responsive genes and transcription factors of the E-twenty six (ETS) family (8). The most frequent *ETS*-fusion is caused by interstitial deletion or translocation of a 3.7 Mb genomic segment located between the TMPRSS2 serine protease and the *ERG* transcription factor at chromosome 21q22. Approximately 50% of prostate cancers carry the *TMPRSS2:ERG* fusion, which brings *ERG* under the control of the androgen responsive TMPRSS2 promoter and results in permanent expression of *ERG* (9). Accordingly, ETS-fusion proteins have been proposed as putative targets for future gene-specific therapies (10).

In a recent study, which was performed in the context of the International Cancer Genome Consortium (11) (ICGC) project on Early-Onset Prostate Cancer, we have carried out integrated genomic analyses, including whole-genome, transcriptome, and DNA methylome sequencing in 11 early onset prostate cancer (EO-PCA) patients and detected a total of 156 individual gene fusions, 140 of which were non-recurrent and unrelated to *ETS* genes (5). It could be possible that some of these rearrangements result in expressed fusion proteins that could serve as cancer-specific therapy targets, provided that these rearrangements occur at sufficient frequency to justify the efforts of drug development. Accordingly, the aim of the present study was to

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determine the prevalence of rearrangements of 27 genes by fluorescence *in situ* hybridization (FISH) analysis in 500 prostate cancer samples in a tissue microarray format.

Materials and methods

Tissues. A subset of our previously described prostate cancer prognosis tissue microarray (12) was used for the present study, including one TMA block containing one 0.6 mm punch each from formalin-fixed and paraffin-embedded tumor samples of 500 different patients undergoing surgery between 1992 and 2004 at the Department of Urology, University Medical Center Hamburg-Eppendorf. Presence of tumor cells in the tissue spots was confirmed in 478 tissue spots by 34βE12 immunostaining in an adjacent TMA slide (13). The remaining 22 tissue spots were excluded from analysis. The pathological parameters of the TMA spots are described in Table I.

Fluorescence in situ hybridization (FISH). FISH was used to detect rearrangements of the 27 selected target genes. For all genes, dual color FISH break-apart probes were manufactured from Spectrum Orange/Spectrum Green labeled bacterial artificial chromosomes (BACs) corresponding to the 5' and 3' flanking regions of the individual genes. A list of the target genes, BAC clones, and labeling schemes is provided in Table II. For FISH analysis, freshly cut 4 μm TMA sections were de-waxed and pre-treated using a commercial kit (paraffin pretreatment reagent kit; Abbott Molecular, Wiesbaden, Germany), followed by dehydration in 70, 80 and 96% ethanol, air-drying and denaturation for 10 min at 72˚C in 70% formamide-2X SSC solution. Hybridization was done overnight at 37˚C in a humidified chamber; slides were then washed, counterstained with 0.2 μmol/l 4'-6-diamidino-2-phenylindole in mounted in antifade solution.

Scoring of FISH. The stained slides were visually inspected under an epifluorescence microscope. A rearrangement was assumed if at least one split signal consisting of a separate orange and green signal was observed in ≥60% of the tumor cell nuclei (indicating balanced translocations) or if individual orange and green signals from the overlapping orange/green signal were lost (indicating deletions with breakpoint inside the gene or imbalanced translocations). Presence of only one overlapping orange/green signal in >60% of tumor cells were considered heterozygous deletion. Tumors with complete lack of overlapping orange/green signals were regarded as homozygous deletions provided that FISH signals were present in adjacent normal cells.

Table I. Composition of the prognosis TMA containing 500 prostate cancer specimens.

	No. of patients	
	Study cohort on TMA (n=500)	Biochemical relapse among categories (n=130)
Follow-up		
Mean	37 months	-
Median	33 months	-
Age (years)		
<50	16	6
50-60	179	44
>60-70	279	73
>70	26	7
Pretreatment PSA (ng/ml)		
<4	73	9
4-10	282	64
10-20	112	42
>20	33	15
pT category (AJCC 2002)		
pT2	310	38
pT3a	126	46
pT3b	63	45
pT4	1	1
Gleason grade		
≤3+3	195	15
3+4	241	68
4+3	59	42
≥4+4	5	5
pN category		
pN0	202	70
pN+	15	14
Surgical margin		
Negative	356	85
Positive	144	45

Numbers do not always add up to 500 in the different categories because of cases with missing data. AJCC, American Joint Committee on Cancer.

Results

Rearrangements were detected for 13 (48%) of the 27 tested genes. Recurrent breakage was found for *NCKAP5, SH3BGR* and *TTC3* in 3 tumors each, as well as for *ARNTL2* and *ENOX1* in 2 cancers each. One rearranged tumor sample was observed for each of *VCL, ZNF578, IMMP2L, SLC16A12, PANK1, GPHN, LRP1* and *ZHX2*. All but four rearrangement were unbalanced, i.e. either the 5' or the 3' part of the FISH probe was lost. For *ZNF578, SH3BGR, LPR12* and *ZHX2* a split signal was found suggesting balanced translocation. Deletions were markedly more frequent than translocations. The most frequently deleted genes were *NCKAP5* (7.5%), *VCL* (6.8%), *PANK1* (5.9%), *ARNTL2* (5.8%), *SLC16A12* (5.6%), *SH3BGR* (3.0%) and *PCNXL2* (1.6%). All detected deletions were heterozygous. No alternations were found for *C11orf41, MLLT4, ALDH7A1, EPN1, NR3C1, PACRG, LYRM4, DPF3, FAM154A* and *WDR67*. The number of successfully analyzed samples per target gene, and the frequency and type of rearrangements and deletions for all analyzed genes is summarized in Table III. Representative FISH images are shown in Fig. 1.

Table II. List of the genes that were analyzed for rearrangements using FISH break-apart probes.

		FISH break apart probe composition		Whole genome sequencing results[a]	
Gene	Chromosomal locus	5' BAC(s)	3' BAC(s)	Rearrangement type	Fusion partner genes
ALDH7A1	5q23.2	SO RP11-772E11	SG RP11-517I3	Translocation	*ANKRD27:ALDH7A1*
				Translocation	*ZNF480:ALDH7A1*
				Translocation	*ELAVL1:ALDH7A1*
NR3C1	5q31.3	SG RP11-614D16	SO RP11-738H11	Translocation	*NR3C1:HOXA9*
SLC16A12	10q23.31	SG RP11-788M08	SO RP11-168O10	Translocation	*SLC16A12:TESC*
FAM154A	9p22.1	SG RP11-151J10	SO RP11-220B22	Translocation	*FAM154A:IRAK3*
				Translocation	*FAM154A:LRP1*
PANK1	10q23.31	SG RP11-626K2	SO RP11-705K1	Translocation	*CCNT1:PANK1*
ARNTL2	12p11.23	SG RP11-546C06	SO RP11-529A16	Translocation	*ARNTL2*
ZNRF3	22q12.1	SO RP11-436H02, SO RP11-493M06	SG RP11-664C16, SG RP11-213L15	Translocation	*ZNRF3:FBXO16*
IMMP2L	7q31.1	SG RP11-365F8, RP11-148C1	SO RP11-75O20, RP11-154C19	Translocation	*IMMP2L:LYST*
ENOX1	13q14.3	SG RP11-75G24,	SO RP11-364B16,	Translocation	*ENOX1:ANO2*
		RP11-671N06	RPRP11-64J21	Translocation	*WWOX:ENOX1*
LYRM4	5p25.1	SO RP3-520B18	SG RP11-284B11	Translocation	*-:LYRM4*
CNOT10	3p22.3	SO RP11-1005I1	SG RP11-301L7	Translocation	*-:CNOT10*
HLCS	21q22.13	SG RP11-383L18	SO RP11-169M12	Translocation	*C1orf151:HLCS*
				Inversion	*HLCS:TTC3*
				Inversion	*HLCS:ERG*
				Translocation	*TTC3:CCDC21*
TTC3	21q22.13	SO RP11-674C12	SG RP11-70N15	Inversion	*TTC3:ERG*
				Inversion	*HLCS:TTC3*
PCNXL2	1q42.2	SO RP11-740C10	SG RP11-125H16	Translocation	*ENSG00000253819:PCNXL2*
				Deletion	*DISC1:PCNXL2*
				Deletion	*C11orf41:RAG1*
C11orf41	11p13	SG RP11-528E21	SO RP11-60G13	Deletion	*C11orf41:OR51E2*
MLLT4	6q27	SO RP11-351J23	SG RP11-359F23	Deletion	*MLLT4:KIF25*
GPHN	14q23.3	SG RP11-107B06, SG RP11-100A18	SO RP11-205I6, SO RP11-769O05	Deletion	*GPHN:RGS6*
				Deletion	*GPHN:DPF3*
VCL	10q22.2	SG RP11-417O11	SO RP11-178G16	Deletion	*VCL:ZNF503*
DPF3	14q24.2	SO RP5-1140N14,	SG RP11-437J15,	Deletion	*GPHN:DPF3*
		SO RP11-326F24	SG RP3-514A23	Inversion	*RGS6:DPF3*
				Inversion	*ZNF578:EPN1*
ZNF578	19q13.41	SO RP11-108N06	SG RP11-207K02	Inversion	*ANKRD27:ZNF578*
				Inversion	*KDM4B:ZNF578*
SH3BGR	21q22.2	SG RP11-749C05	SO RP11-165H11	Inversion	*SH3BGR:RIPK4*
LRP12	8q22.3	SO RP11-77K11	SG RP11-437B02	Inversion	*LRP12:ENSG00000253350*
ZHX2	8q24.13	SO RP11-94L20	SG RP11-263A19	Inversion	*-:ZHX2*
WDR67	8q24.13	SG RP11-263A19	SO RP11-54J08	Inversion	*ENSG00000254303:WDR67*
EPN1	19q13.42	SO CTD-2537I9	SG CTD-2611O12, RP11-107J22	Inversion	*ZNF578:EPN1*
NCKAP5	2q21.2	SO RP11-736B01, SO RP11-789J19	SG RP11-351L15, SG RP11-393D01	Inversion	*NCKAP5:MGAT5*
PACRG	6q26	SG RP11-57O22, SG RP11-621H02	SO RP11-308E20, SO RP3-495O10	Inversion	*PACRG:LOC285796*
				Duplication	*IPCEF1:PACRG*

SO, Spectrum Orange-labeled; SG, Spectrum Green-labeled. [a]Data taken from Weischenfeldt *et al* (5).

Table III. Prevalence and type of detected structural rearrangements.

		Rearrangement			Deletion	
Gene	Chromosomal locus	Analyzable	Unbalanced	Balanced	Analyzable	Deletion
PCNXL2	1q42.2	436	0	0	436	7 (1.6)
NCKAP5	2q21.2	377	3 (0.8)	0	374	28 (7.5)
CNOT10	3p22.3	382	0	0	382	4 (1.0)
IMMP2L	7q31.1	320	1 (0.3)	0	320	1 (0.3)
LRP12	8q22.3	321	0	1 (0.3)	321	0
ZHX2	8q24.13	389	0	1 (0.3)	389	0
VCL	10q22.2	338	1 (0.3)	0	176	12 (6.8)
SLC16A12	10q23.31	363	1 (0.3)	0	250	14 (5.6)
PANK1	10q23.31	355	1 (0.3)	0	188	11 (5.9)
ARNTL2	12p11.23	316	2 (0.6)	0	171	10 (5.8)
ENOX1	13q14.3	435	2 (0.5)	0	435	0
GPHN	14q23.3	406	1 (0.2)	0	406	0
ZNF578	19q13.41	393	0	1 (0.3)	393	0
HLCS	21q22.13	360	0	0	360	2 (0.6)
TTC3	21q22.13	385	3 (0.8)	0	385	0
SH3BGR	21q22.2	368	2 (0.5)	1 (0.3)	368	11 (3.0)
ZNRF3	22q12.1	273	0	0	273	4 (1.5)

Discussion

The results of the present study demonstrate that most chromosomal rearrangement, including balanced translocations and partial deletions characterized by intragenic breaks, represent very rare events in prostate cancer. The prevalence of breakage events affecting the 27 analyzed genes in this study was usually below 1%.

Based on our data, obtained in a cohort of over 500 tumors, it is not surprising that whole genome sequencing studies on prostate cancer found only few recurrent rearrangements (except *TMPRSS2:ERG*) in a total of 18 cancers (4,5). Although >250 individual non-ETS gene fusion events (resulting from translocations, inversions and duplications) were identified in these two studies in total, only 16 non-ETS genes in the study by Berger *et al* (4) and 1 gene in the study by Weischenfeld *et al* (5) were recurrently hit by structural rearrangements, however, in each case there was a different fusion partner. Only *ETS*-fusions were highly recurrent in these studies, with 4/7 tumors (4) and 8/11 tumors (5) carrying the *TMPRSS2:ERG* fusion.

Little is known about the prevalence of individual gene rearrangements (except *TMPRRS2:ERG*) in prostate cancer. Two studies performed by Reid *et al* (14) and us analyzed breakage of the *PTEN* tumor suppressor, and reported 7% (13/187) (14) and 3% (162/5,404) (5) of *PTEN* breakage, which was typically (3 out of 4 affected cases) associated with deletions of the second *PTEN* allele. In addition, we have previously studied breakage of the 3p13 tumor suppressor *FOXP1* (15) and found 1.2% of rearrangements. These data suggest that rearrangements are infrequent even for genes with a key role including *PTEN*. The 0.2-1% of rearrangements found for half of the genes analyzed in the present study fit well to these numbers.

The selection of the 27 genes analyzed in this study was based on the findings of our International Cancer Genome (ICGC) project, where we employed the paired end deep sequencing strategy (16) to specifically identify gene breakages, translocations and gene fusions. In the present study we found a total of 140 non-*ETS* gene rearrangements. For the present study, we randomly selected genes that were potentially involved in non-*ETS* fusions between protein-coding genes or gene inactivation by translocation or gene breakage (5). Such genes are candidates for a dual tumor relevant function, including a putative tumor suppressor function based on inactivation by gene breakage, as well as a putative oncogenic in case of expressed fusion genes.

In this study, deletion of the analyzed region was more frequent than rearrangement. This fits well with the known relevance of many of the analyzed genes, which were located at chromosomal regions that are frequently deleted on prostate cancer, including for example *PANK1*, *VCL* and *SLC16A12* (10q22-q23, deleted in 20-30%) (17-19), *NCKAP5* (2q21, deleted in 10-30%) (17-19), or *ARNTL2* (12p11-p12, deleted in 15-60%) (17,19), explaining the markedly higher frequency of deletions as compared to rearrangements. The deletion frequencies observed in the present study were markedly lower than in these studies, which can be explained by the fact that we did not use a deletion-specific FISH assay including a combination of a locus-specific and a centromere reference probe. With the break-apart probe used in this study, we only called absolute deletions showing unequivocal loss of one red-green signal pair but missed relative deletions, which frequently occur in aneuploid cancers.

Several of the genes analyzed in this study, including *NCKAP5:MGAT5*, *C11orf41:RAG1*, *SH3BGR:RIPK4*, *FAM154A:IRAK3* and *CCNT1:PANK1*, were involved in

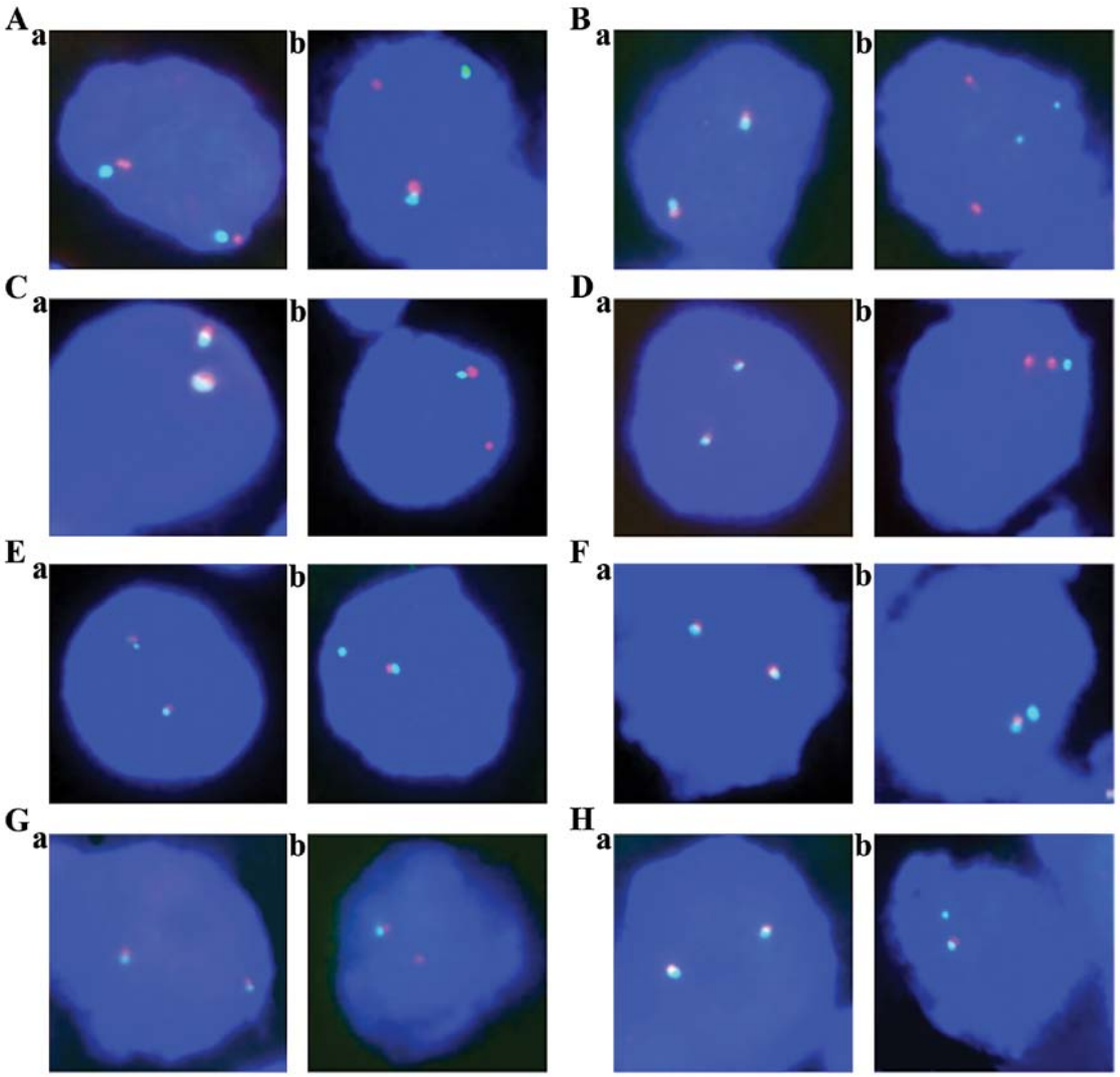

Figure 1. Examples of FISH findings using the break-apart probes. (Aa-Ha) Intact gene locus of *LRP12*, *ZHX2*, *ARNTL2*, *ENOX1*, *IMMP2L*, *GPHN*, *ZNF578* and *NCKAP5* with two adjacent green and orange FISG signals corresponding to the 3' and 5' flanking regions of these genes. (Ab) Breakage of one *LRP12* allele as indicated by a split signal (separate red and green signals) while the second allele is still intact. (Bb) Breakage of two *ZHX2* alleles as indicated by two separate red and green signals. (Cb-Hb) Breakage of one gene allele as indicated by a loss of one red signal of *IMMP2L* (Eb), *GPHN* (Fb) and *NCKAP5* (Hb) or by a loss of one green signal of *ARNTL2* (Cb), *ENOX1* (Db) and *ZNF578* (Gb).

gene fusions leading to overexpression of the fusion partner according to our previous study (5). Such fusion genes may represent suitable targets for new gene specific therapies, since they are specific for the cancer cells. However, the vast majority of gene breakages detected in this study were unbalanced, with loss of either the 3' or the 5' fraction of the gene, suggesting a partial deletion of these genes. Only 4 genes, *ZNF587*, *SH3BGR*, *LRP12* and *ZHX2*, showed balanced rearrangements that might have led to gene fusions. These findings suggest that intragenic breaks may in most cases indicate a deletion break point located inside a coding gene, while formation of a specific rearrangement with a possible functional fusion gene seems to be a comparatively rare event.

We manufactured break-apart probe assays to detect rearrangements of the 27 candidate genes in a tissue microarray format. The use of our tissue microarray format in combination with FISH enables a fast and cheap analysis of gene rearrangements to detect common recurrent gene changes. Break-apart assays are capable of detecting all types of rearrangements of a probed gene, including translocation, (partial) deletion and inversion, and are thus optimally suited to estimate the prevalence of rearrangements for a given gene. We selected a cut-off level of ≥60% affected tumor cell nuclei for the detection of rearrangements in order to avoid false-positive findings due to truncated cell nuclei in 4 μm tissue sections. This cut-off was based on our previous studies analyzing breakage of *ERG* (20) and *PTEN* (5,17). Using this threshold we found a high (>95%) correlation between *ERG* breakage by FISH and ERG expression be immunohistochemistry (20), supporting the validity of our approach to screen for recurrent gene rearrangements.

In summary, the present study shows that a multitude of genes can be affected by chromosomal rearrangements in prostate cancer, but the frequency of specific rearrangements is typically in the range of 1% or less. In most cases, these rearrangements will result in gross deletions inactivating the affected gene. True translocations, potentially resulting in fusion genes, are comparatively rare.

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