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Original Article

Chromosome rearrangement associated inactivation of tumour suppressor genes in prostate cancer

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Abstract: Prostate cancer, the most common male cancer in Western countries, is commonly detected with complex chromosomal rearrangements. Following the discovery of the recurrent *TMPRSS2:ETS* fusions in prostate cancer and *EML4:ALK* in non-small-cell lung cancer, it is now accepted that fusion genes not only are the hallmark of haematological malignancies and sarcomas, but also play an important role in epithelial cell carcinogenesis. However, previous studies aiming to identify fusion genes in prostate cancer were mainly focused on expression changes and fusion transcripts. To investigate the genes recurrently affected by the chromosome breakpoints in prostate cancer, we analysed Affymetrix array 6.0 and 500K SNP microarray data from 77 prostate cancer samples. While the two genes most frequently affected by genomic breakpoints were, as expected, *ERG* and *TMPRSS2*, surprisingly more known tumour suppressor genes (TSGs) than known oncogenes were identified at recurrent chromosome breakpoints. Certain well-characterised TSGs, including *p53*, *PTEN*, *BRCA1* and *BRCA2* are recurrently truncated as a result of chromosome rearrangements in prostate cancer. Interestingly, many of the genes residing at recurrent breakpoint sites have not yet been implicated in prostate carcinogenesis such as *HOOK3*, *PPP2R2A* and *TCBA1*. We have confirmed the generally reduced expression of selected genes in clinical samples using quantitative RT-PCR analysis. Subsequently, we further investigated the genes associated with the t(4:6) translocation in LNCaP cells and reveal the genomic fusion of *SNX9* and putative TSG *UNC5C,* which led to the reduced expression of both genes. This study reveals another common mechanism that leads to the inactivation of TSGs in prostate cancer and the identification of multiple TSGs inactivated by chromosome rearrangements will lead to new direction of research for the molecular basis of prostate carcinogenesis.

Keywords: prostate cancer, chromosome rearrangements, chromosome breakpoints, tumour suppressor gene, oncogene, SNP array, FISH, QRT-PCR

Introduction

Prostate cancer is the most common male cancer in Western countries, including Europe, North America and parts of Africa [1]. Despite extensive studies and certain achievements in this disease, many issues still remain regarding the management and treatment of prostate cancer. Prostate cancer has a natural course that is different from many other human tumours. Most early-stage prostate cancers are latent and only approximately 25% of them will become aggressive and life-threatening [2]. However, currently, it is difficult to differentiate between low- and high-risk localised prostate cancers [2-4]. Following the application of the prostate-specific antigen (PSA) test for early detection of prostate cancers, there is a big issue in managing these early stage cancers. It is a dilemma to treat early-stage localised cancers. The current methods commonly used in the US and many other countries may over-treat the majority of early prostate cancer patients who will not develop metastases. However, conservative management, such as watchful waiting and active surveillance, which is used in certain European countries may miss the opportunity to cure the small proportion of aggressive disease at an early stage [3]. Once aggressive cancer has progressed to the metastatic stage,

the chance of survival is low. The treatment strategy for metastatic prostate cancer is androgen deprivation. Although this treatment works efficiently in the majority of patients, most cancers usually relapse after two years [4, 5]. When the disease becomes androgen-resistant, very limited options are left [4]. Chemotherapy for prostate cancer is generally unsuccessful, although recently new developments have been achieved [5]. Therefore, currently, advanced disease is still incurable and it is difficult to predict the progression of early stage cancers [2-5].

Cancer is a genetic disease. Chromosome rearrangements, including translocations, inversions and internal deletions are the hallmarks of human cancer [6]. Fusion genes and the deregulation of oncogenes associated with chromosome rearrangement have been extensively studied in haematological malignancies and soft tissue sarcomas and can frequently be used to define a tumour subtype and are associated with disease prognosis [6]. Gleevec (also known as Imatinib or STI571), which targets the *BCR:ABL* gene fusion product in chronic myeloid leukaemia, was the first successful drug developed for gene-targeted therapy [7]. Carcinomas are the most common human malignancies. However, due to the complexity of the genomic alterations in cells from carcinomas and the difficulty in karyotyping them, only a small number of fusion genes, each occurring at a low frequency, have been reported in tumours of epithelial origin. Until recently, many people believed that gene fusions were not important events in carcinomas [8]. Following the recent discovery of recurrent fusions of the *TMPRSS2* and *ETS* family transcription factor genes in prostate cancer [9-11] and *EML4:ALK* in non-small-cell lung cancer [12], it is now accepted that fusion genes also play an important role in epithelial cell carcinogenesis [6].

Although detected at a much higher frequency in certain types or subtypes of human malignancies, the fusion genes previously identified in haematological malignancies and sarcomas, occur only in relatively rare tumour types. Due to the high incidence of prostate cancer, *TMPRSS2:ERG,* which occurs in about 50% of prostate cancer, is currently the most frequently found fusion gene in human malignancies [6]. The discovery of the high frequency *TMPRSS2* and *ETS* fusion has stimulated huge interest in the search to find more fusion genes and investigation into their roles in carcinomas, particularly in prostate cancer. However, apart from the fusion of *ETS* family genes with *TMPRSS2* and other genes highly active in prostate epithelial cells, including *SLC45A3*, *HERV-K_22q11.23*, *C15orf21* and *HNRPA2B1* [9, 13-15], no other frequent fusion genes have been found in prostate cancer, so far [13-16].

Prostate cancer is commonly detected with very complex chromosome rearrangements involving many chromosome breakpoints and rejoins [17, 18], the majority of which are unbalanced. It is now clear that unbalanced chromosome translocations can also affect the genes located at or close to chromosome breakpoints [19]. As it is difficult to culture primary prostate cancer cells for karyotyping analysis, new approaches have to be explored to identify genes that are recurrently affected by chromosome rearrangements. The expression outlier analysis was successfully used to identify the common *TMPRSS:ETS* fusions [9-11]. The development of next generation sequencing technology has provided better resolution to detect genetic alterations and has recently been applied for transcriptome sequencing [13-16]. However, it is not necessarily the case that all chromosome rearrangements result in fusion transcripts and/or overexpression of affected genes. Therefore, fusion events that do not result in fusion transcripts or significantly increased level of expression would remain undetected using these approaches focusing on expression level changes. Recently, next-generation sequencing was also applied to whole genomes and many genomic rearrangements have been identified at the DNA base pair level [20]. It is currently still very expensive to sequence the entire genome and analysis of the vast amount of complicated genomic data is challenging. Therefore, only seven prostate cancer samples were analysed in the recent report [20], which is impossible to assess the frequency of genes affected. High-density genomic microarrays provide good coverage of the human genome allowing breakpoints (seen as boundary of DNA copy number changes) to be determined at a sufficiently high resolution (an average of a few kb per SNP). A vast amount of microarray genomic copy number change data already exists and it is simple to identify the genes truncated by chromosome rearrangements- genes located at the genomic gain and loss breakpoints. This offers an opportunity to rapidly identify genes that are frequently trun-

Table 1. Taqman QRT-PCR probes

Gene	Probe ID	Chr.	Chr. Location	Exon Boundary	Amplicon Length (bp)
UNC5C	Hs01031779_m1	4	96083655 - 96470361	9-10	81
SNX9	Hs00212006_m1	6	158244294 - 158366109	2-3	88
PPP2R2A	Hs00160392_m1	8	26149007 - 26230196	1-2	92
HOOK3	Hs00260887_m1	8	42752033 - 42885682	13-14	81
WWOX	Hs03044790_m1	16	78133551 - 79246564	4-5	89
GAPDH	Hs99999905_m1	12	6643657 - 6647536	3-3	122

cated by the chromosome rearrangements.

To investigate the genes recurrently affected by the chromosome breakpoints in prostate cancer, we analysed Affymetrix array 6.0 and 500K SNP microarray data for genes located at genomic copy number change breakpoints. We revealed that many tumour suppressor genes (TSGs) are recurrently truncated as a result of chromosome rearrangements in prostate cancer. Subsequently, we further investigated the inactivation of genes associated with the t(4:6) in LNCaP prostate cancer cell line.

Material and methods

Cell lines

Six prostate cancer cell lines (PC3, DU145, LNCaP, VCaP, 22RV1 and MDAPCa2b) were used in this study. All cell lines were obtained from American Type Culture Collection (ATCC).

Fresh frozen prostate clinical samples

32 UK and 39 Chinese prostate cancer radical prostatectomy samples were collected, snap frozen and stored in liquid nitrogen at local tissue banks. Gleason grading and the percentage of tumour cells in each sample were reviewed using H&E stained frozen sections. Local research ethics committees have approved the use of clinical samples for this study.

Nucleic acid extraction

For DNA extraction, cancer, high-grade prostate intraepithelial neoplasia (HGPIN) and adjacent normal tissue from fresh frozen sections were macro- or micro-dissected to achieve >70% purity of cancer cells. For RNA extraction, only cases suitable for macrodissection were used to increase the purity of cancer cells. DNA and RNA extraction from cell lines were performed following standard protocols. Fresh frozen tissue was cut into 5 μm sections using Cryotome® E electronic cryostat.

Microarray analysis

Microarray data for 3 prostate cancer cell lines and 44 clinical prostate cancer samples have been published previously [17, 21]. In combination with array data from a further 3 prostate cancer cell lines and 27 clinical prostate samples, we analysed all SNP array data (SNP 6.0 and 500K) to identify genes recurrently truncated by chromosome breakpoints. The in-house developed software program, GOLF [21, 22], was used to display the intensity of SNPs along each chromosome and genomic breakpoints were identified where the intensity of SNP signals changed if the signal intensity ratio of the mean of 20 contiguous probes differs 0.4 log2 ratio for two adjacent SNP blocks. Probes were mapped using Ensembl genome build hg18.

Quantitative reverse transcription PCR (QRT-PCR) gene expression analysis

Total RNA was reverse transcribed using Superscript II (Invitrogen) following the manufacturers instructions. QRT-PCR was performed using the ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) keeping the default settings for baselines and thresholds. Predesigned Taqman® gene expression assays were purchased from Applied Biosystems. Specific probes are outlined in **Table 1**. 40 ng cDNA was used for each PCR reaction and each sample was performed in triplicate. Endogenously expressed GAPDH was used as an internal control.

Fluorescence in situ hybridisation (FISH) analysis

FISH on metaphase slides was carried out using standard protocols. Six BAC clones (RP11-

160A16 at proximal 4q22.3, RP11-240J11 at distal 4q22.3, RP11-111J1 at proximal 6q15, RP11-337M11 at distal 6q15, RP3-481C9 at proximal 6q25.3 and RP1-249F5 at distal 6q25.3) were obtained from the Welcome Trust Sanger Institute (Hinxton Hall, Cambridge, UK). BAC DNA was extracted using Qiagen-Tip method following the manufacture suggested protocols. BAC DNA was then labelled directly with fluorescent dyes using a nick-translation method as described previously [23]. RP11-160A16, RP11-240J11 and RP1-249F5 were labelled by Cy5 and RP11-111J1, RP11-337M11 and RP3-481C9 were labelled with Cy3. All labelled probes were cleaned up by G50 columns and precipitated by vacuum drier.

Metaphase slides were prepared from cell lines using standard cytogenetic methods and stored at -20°C. Before hybridisation, slides were pre-treated with 70% acetic acid for 10 min and neutralised by PBS washes. Metaphase slides and labelled BAC probes re-suspended in hybridisation buffer (2xSSC, 10% dextran sulphate, 50% formamide, 1% Tween 20, pH 7) were denatured separately. 10 μl of hybridisation solution containing 200 ng of each labelled BAC probe was applied onto the denatured slide and covered with a 22X22 mm coverslip. Hybridisation was performed at 37°C over-night and then slides were washed using standard formamide wash protocol. Finally, 20 μl Vectashield antifade solution (containing DAPI) was added to each slide after dehydration and mounted with coverslips. FISH signals were reviewed and captured using an Olympus fluorescent microscope equipped with a CCD camera and red/green/blue three-colour filter wheel, controlled by a computer using MacProbe 4.3 software (Applied Imaging, CA).

Results

Identification of recurrent breakpoint in prostate cancer and the associated genes by SNP array analysis

In this study, SNP array data from 71 clinical prostate cancer cases and 6 prostate cancer cell lines were manually analysed with our in-house software, GOLF, and 41 recurrent breakpoints (n≥2) were detected within putative TSGs, oncogenes and/or genes previously identified as a partner gene in gene fusion events (**Table 2**). As expected, the two most frequent breakpoints identified resided on chromosome 21, where the *ERG* and *TMPRSS2* gene are located (18/77 and 15/77 cases, respectively). The *HOOK3* gene was also found at the breakpoints at a considerably high frequency (7/77). Surprisingly, this breakpoint analysis of microarray data revealed preferential involvement of TSGs (n=27) as compared to oncogenes (n=6). Four of the identified TSGs, *PPP2R2A*, *ETV6*, *WWOX* and *BRCA1*, occurred at the breakpoints in at least 4 samples. Representative array images are shown in **Figure 1**. The well-characterised TSGs *p53, PTEN* and *BRCA2* were also found at recurrent breakpoints of copy number changes. Twenty of the genes located on the recurrent breakpoints have previously been reported as fusion partner genes.

From the list of genes recurrently located on the breakpoints, we investigated the expression level of three genes, *HOOK3*, *PPP2R2A* and *WWOX* using QRT-PCR. *HOOK3* expression varies slightly in non-malignant prostate epithelial cells and is downregulated in 7 prostate cancer or HGPIN samples as compared to their matched normal controls (**Figure 2A**). In one paired tumour and normal and one paired HGPIN and normal comparison, *HOOK3* expression was lower in the normal cells. In 8 of 9 paired samples analysed, *PPP2R2A* expression was reduced in tumour samples as compared to their case-matched normal controls (**Figure 2B**). Unfortunately, none of the paired samples available for QRT-PCR analysis were from cases where genomic breakpoints in the *PPP2R2A* had been identified by SNP array analysis. *WWOX* gene expression was also reduced in the tumour/HGPIN lesions from 8 of 9 paired samples. However, the only sample (WX94) analysed by QRT-PCR where a genomic breakpoint was detected in the *WWOX* gene by SNP array analysis, showed higher *WWOX* expression in the tumour sample than the normal (**Figure 2C**).

Characterisation of the t(4;6) in LNCaP and the downregulation of UNC5C and SNX9 expression

We further investigated the previously identified t(4;6) translocation [24] and its impact on genes located at the breakpoints. From our array data, while only one deletion occurred on chromosome 4 (4q22.3: 158,266-160,264 kb) (**Figure 3A**), two deletions occurred on chromosome 6q (6q15: 87,631-91,744 kb and 6q25.3: 158,266-160,264 kb). This correlated

Table 2. Recurrent TSGs, oncogenes and fusion partner genes found in prostate cancer SNP array breakpoint analysis

Gene	Chr	Start	End	Samples (n=77)	TSG	Oncogene	Known fusion partner
ERG	21	38673819	38955488	18		√	√
TMPRSS2	21	41758351	41801948	15			√
HOOK3	8	42871190	42994084	7		√	√
TCBA1	6	124166985	125188502	5			√
PPP2R2A	8	25098204	26284562	5	√		√
SMAD2	18	43613464	43711510	5	√		
ETV6	12	11694055	11939588	4	√		√
WWOX	16	76691052	77803532	4	√		
BRCA1	17	38449840	38530994	4	√		
ARSF	X	2969512	3040767	4		√	
LRP1B	2	140705468	142604768	3	√		
ROBO2	3	76069335	77779351	3	√		
SMAP1	6	71434200	71628435	3			√
NRG1	8	31617043	32741608	3	√	√	√
PTEN	10	89613175	89718511	3	√		
BRCA2	13	31787617	31871809	3	√		
GPC6	13	92677711	93853948	3			√
CDH11	16	63535157	63713440	3	√		√
P53	17	7512464	7531642	3	√		
DISC1	1	229829184	230243637	2			√
RARB	3	25190893	25614424	2	√		
GOLGA4	3	37259742	37383240	2			√
LSAMP	3	117011853	117646568	2	√		
SMARCAD1	4	95348217	95431462	2	√		
MSH3	5	79986050	80208387	2	√		
BAI3	6	69401980	70156124	2	√		
CNTNAP2	7	145444902	147749019	2	√		√
DLC1	8	12985243	13416766	2	√		
IKBKB	8	42247986	42309122	2		√	√
PRKDC	8	48848222	49035296	2	√		
PAG1	8	82042605	82186858	2	√		
ZFPM2	8	106400323	106885939	2			√
PTPRD	9	8304246	9008735	2	√		
OPCML	11	131790085	132907429	2	√		
PIK3C2G	12	18305741	18692617	2			√
RAD51L1	14	67356262	68187315	2	√		√
MKL2	16	14072697	14268130	2			√
ADAMTS18	16	75873527	76026512	2	√		
ACCN1	17	28364221	29507664	2	√		√
SETBP1	18	40514861	40898771	2			√
PTPN1	20	48560298	48634495	2	√	√	
PARVB	22	42726424	42896434	2	√		

to the two translocation breakpoints on chromosome 6 and one translocation breakpoint on chromosome 4 in the t(4;6) chromosome translocation revealed by multiplex fluorescent *in situ* hybridisation (M-FISH) karyotyping (**Figure 3C**). FISH co-localisation analysis of chromosome 4 and 6 probes flanking the translocation breakpoints was performed to confirm the t(4;6) chromosome rearrangement. Using a distal 4q22.3 and proximal 6q15 probe we observed co-localisation on der(6)t(4;6) (**Figure 4A**). However, proximal 4q22.3 and distal 6q15 did not co-localise on der(4)t(4;6). The latter probe hybridisation signal was located sub-telomerically, far away from the 4q22.3 probe (**Figure 4B**). This revealed an inverted configuration of the

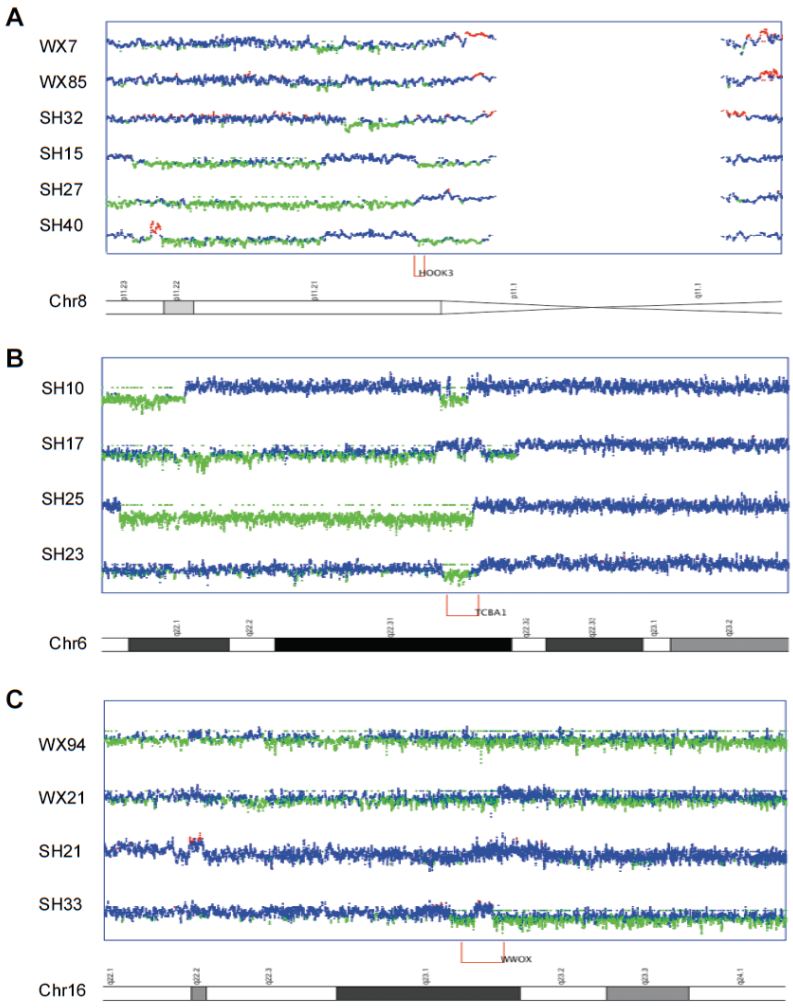


Figure 1. Representative SNP array profiles to show the recurrent genomic breakpoints at gene loci. **A.** Breakpoints at *HOOK3* gene. **B**. Breakpoints in *TCBA1* gene. **C**. Breakpoints at *WWOX* gene.

6q15-25.3 fragments in the der(4)t(4;6;10) where the proximal breakpoint of 6q25.3 was fused to 4q22.3 on der(4)t(4;6) and the 6q15 breakpoint was fused to chromosome 10. This was further confirmed by FISH analysis using proximal 4q22.3 and proximal 6q35.3 probes (data not shown). The small telomeric 6q region had translocated to 10q, which was confirmed by FISH analysis using BAC RP1-249F5 located at distal 6q25.3 breakpoint (data not shown). Fusion of 4q22.3 and 6q25.3 results in the fusion of *SNX9* and *UNC5C* in the same orientation. However, using various pairs of primers to PCR amplify the potential *UNC5C:SNX9* fusion gene, no PCR product was detected. Using QRT-PCR, we further investigated the expression level of *UNC5C* and *SNX9*. Both *SNX9* and *UNC5C* were expressed at a relatively low level in LNCaP cells compared to the other cell lines (**Figure 5**). Analysis of *UNC5C* and *SNX9* expression in clinical prostate cancer samples revealed that in most cases both genes were down-regulated in tumour samples as compared to their adjacent morphologically normal epithelial cells (**Figure 5**). All cell lines analysed expressed low level of the two genes, compared to the clinical samples.

Discussion

Recent studies of genomic rearrangements in prostate cancer have been successful in identifying fusion genes, particularly the *TMPRSS2:ERG* fusion, which is detected in half of prostate cancer samples and is the most commonly found fusion in human malignancies [6]. However, the approaches used, including expression outlier and next generation transcriptome sequencing, have mainly focused on the identification of fusion transcripts. It is now evident that genomic rearrangements have consequences other than gene fusion or deregulation of oncogenes, such as inactivation of TSGs [6]. The complex genomic rearrangements observed in prostate cancer may affect the function of many TSGs. As inactivation of TSGs by genomic rearrangements will not result in fusion transcripts or over-expression of the affected genes, genomic analyses may be necessary to reveal the TSGs affected by these rearrangements.

Not surprisingly, from our microarray analysis the two genes most frequently affected by genomic breakpoints are the *ERG* and *TMPRSS2* genes. Fusion events, caused by translocation or deletion of the intervening DNA between the Chromosome 21 *TMPRSS2* and *ERG* genes, are

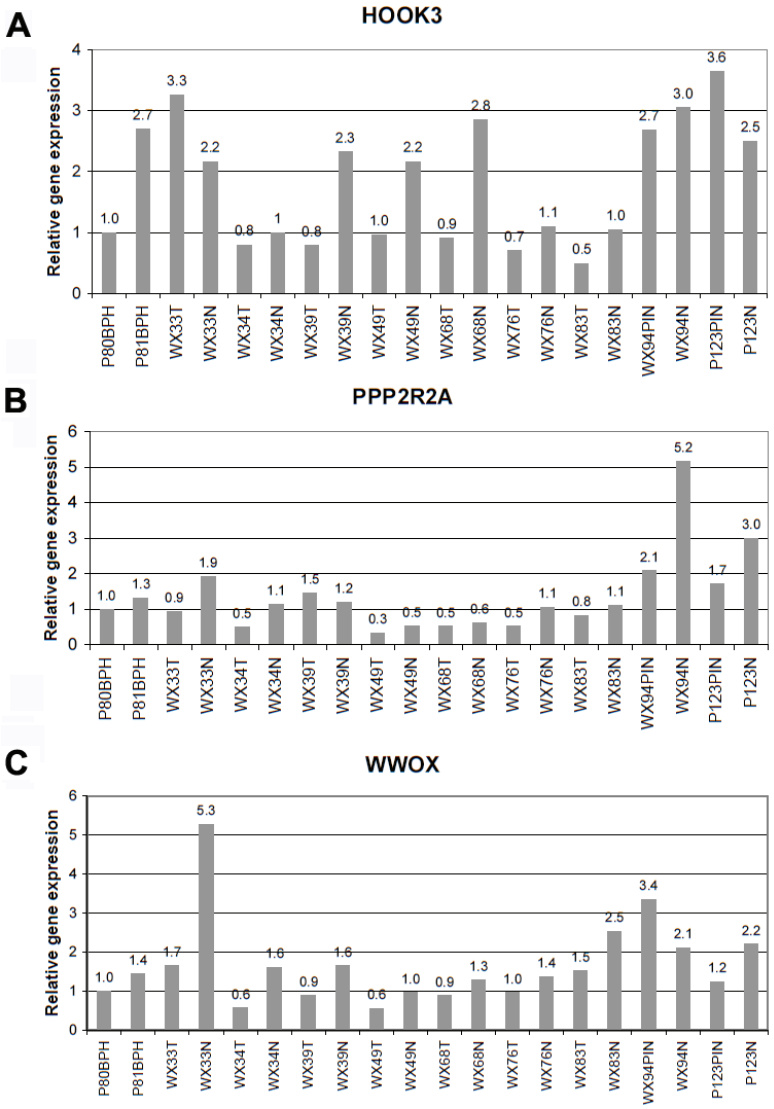


Figure 2. Decreased expression of *HOOK3*, *PPP2R2A* and *WWOX* genes in prostate cancer and HGPIN samples detected by QRT-PCR analysis. **A.** *HOOK3* gene; **B.** *PPP2R2A* gene and **C.** *WWOX* gene. P80BPH and P81BPH are macrodissected samples from benign prostate hyperplasia cases. N: macrodissected morphologically normal gland regions; T: macrodissected cancer lesions.

found in approximately 50% of prostate cancer cases [21, 25]. The frequency of *TMPRSS2* and *ERG* breakpoints observed in our sample set is lower than the recorded frequency. This is because our sample set consists of clinical samples taken from both Chinese and UK prostate cancer patients. We have recently reported that the deletions of chromosome 21 (causing *TMPRSS2:ERG*) and 10q (inactivating *PTEN*), which have been reported as frequent events in prostate cancer, are detected far less frequently in the Chinese population [21].

Interestingly, many of the other genes residing at sites of recurrent breakpoints have not yet been implicated in prostate carcinogenesis, such as *HOOK3, PPP2R2A* and *TCBA1*. These genes, which have previously been characterised as gene fusion partners, should be further investigated and some of them may be novel gene-fusion partners in prostate cancer. Genomic breakpoints within the *HOOK3* gene were found at a considerably high frequency (7 cases). QRT-PCR analysis using primers spanning exon 13-14 showed that *HOOK3* is down-regulated in clinical prostate cancer cases as compared to their matched normal controls. In a case of papillary thyroid carcinoma, a fusion product was identified between exon 11 of *HOOK3* and exon 12 of the *RET* gene [26]. *HOOK3* provides an active promoter to drive the expression of the tyrosine kinase domain of *RET*, thereby rendering the *HOOK3:RET* fusion product with oncogenic properties. We speculate that truncation of the *HOOK3* in prostate cancer may have the same consequence. However, it is also possible that decreased gene expression may result in reduced activity of *HOOK3*. Further investigation is required to identify its fusion partner and active role in prostate cancer development or progression.

During this microarray analysis we identified many known or putative TSGs located at recurrent genomic rearrangement breakpoints. To our surprise, we found more known/putative TSGs residing at breakpoints than known/putative oncogenes. Although many recurrent breakpoints were identified that do not harbour genes with known TSG/oncogene roles, the

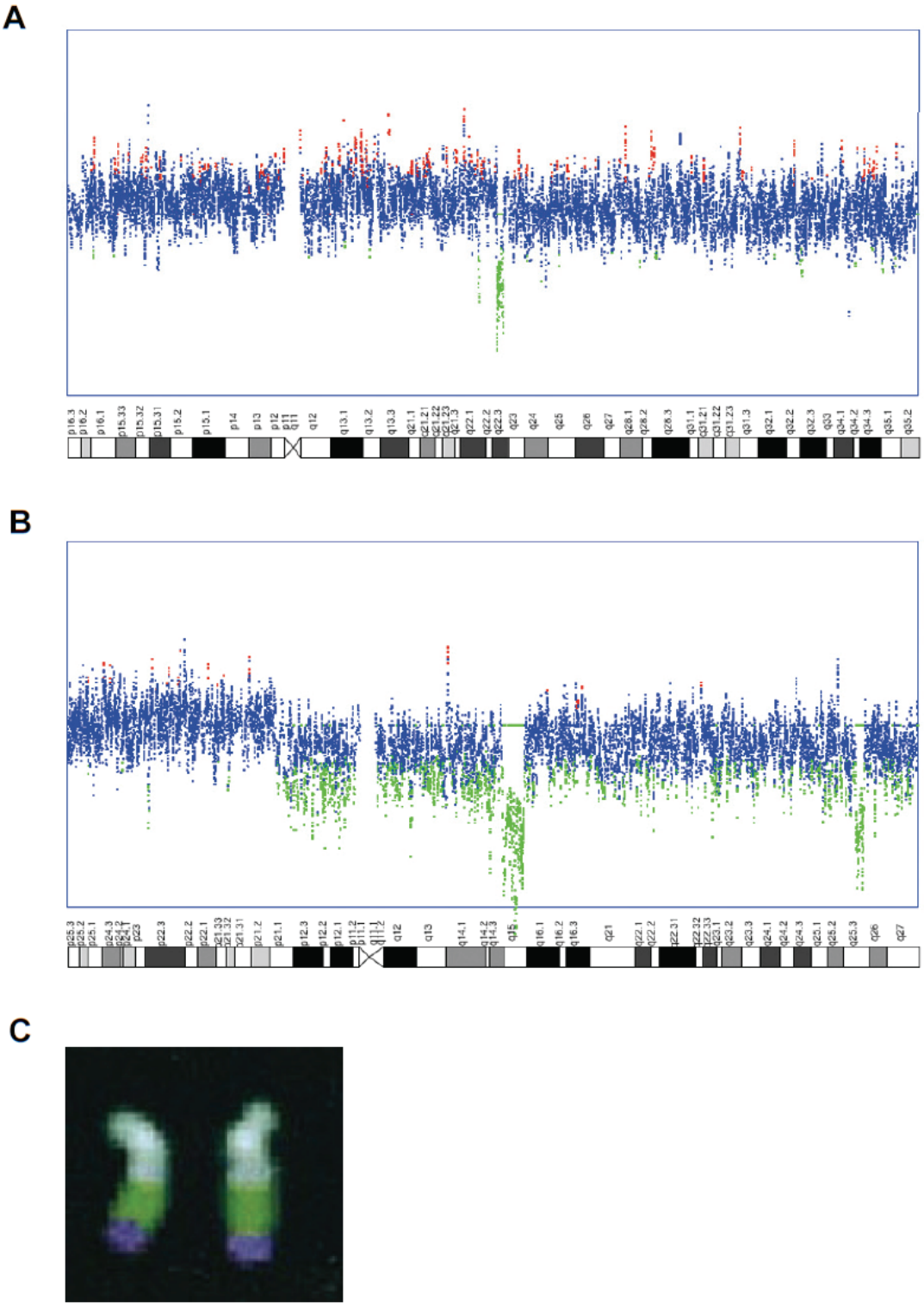


Figure 3. Association of one breakpoint on 4q and two breakpoints on 6q with small chromosome deletions revealed by SNP array analysis. **A.** SNP array data show one small deletion on 4q. **B.** SNP array data show two small deletions on 6q. **C.** The der(4)t(4;6;10) chromosomes from a M-FISH metaphase show one breakpoint on 4q and two breakpoints on 6q.

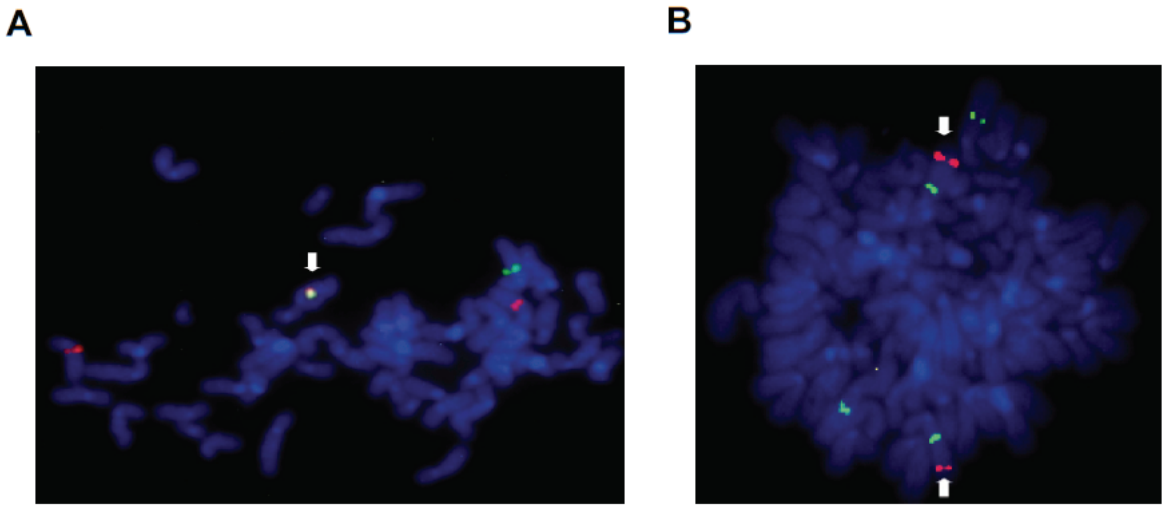
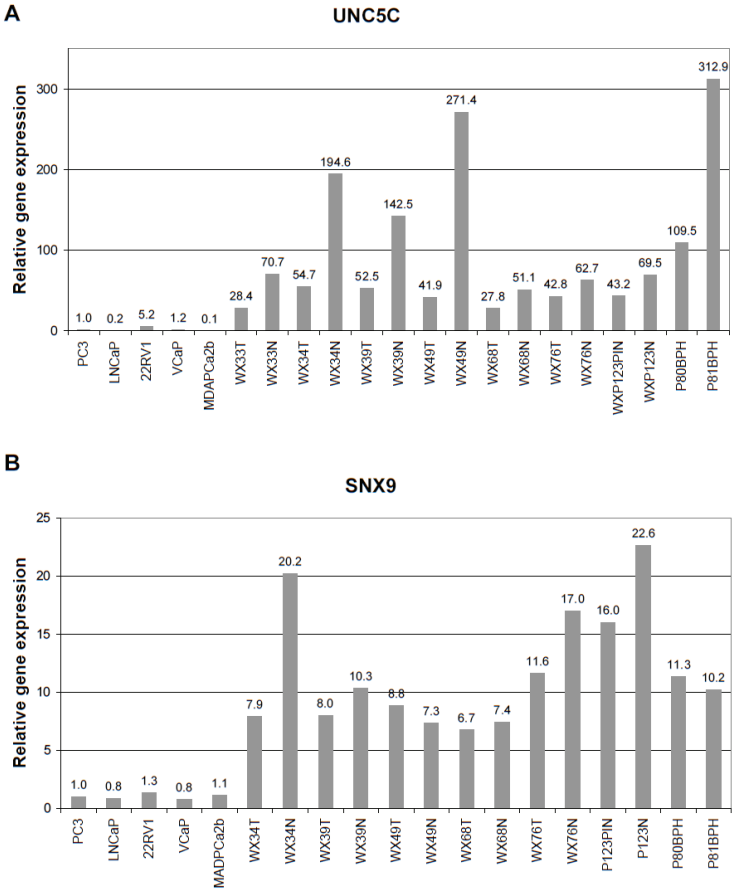


Figure 4. Representative FISH images showing the fusion of distal 4q22.3 and proximal 6q15 but not proximal 4q22.3 and distal 6q15 in the t(4;6) of LNCaP cells. **A.** Hybridisation of clones RP11-240J11 (distal 4q22, green) and RP11-111J1 (proximal 6q15, red) to LNCaP cells. The co-localised RP11-240J11 and RP11-111J1 signals on the der (6) are indicated by an arrow. **B.** Hybridisation of clones RP11-160A16 (proximal 4q22, green) and RP11-337M11 (distal 6q15, red) to LNCaP cells. The RP11-160A16 and RP11-337M11 signals located far away on the der(4) are indicated by arrows.



large bias towards TSGs, as compared to oncogenes (27:6), indicates that further clarification of the genes at recurrent breakpoints would not reverse this general trend. Chromosome loss, mutation and promoter methylation are mechanisms that frequently lead to TSG inactivation. Now, chromosome rearrangements and translocations have been found as another potential common cause of TSG inactivation in prostate cancer.

In five cases, breakpoints were identified in the *PPP2R2A* gene, which is located at the frequently deleted chromosome region,

Figure 5. Decreased expression of *UNC5C* and *SNX9* genes in prostate cancer cell lines, clinical cancer and HGPIN samples detected by QRT-PCR analysis. **A.** *UNC5C* gene and **B.** *SNX9* gene. P80BPH and P81BPH are macrodissected samples from benign prostate hyperplasia cases. N: macrodissected morphologically normal gland regions; T: macrodissected cancer lesions.

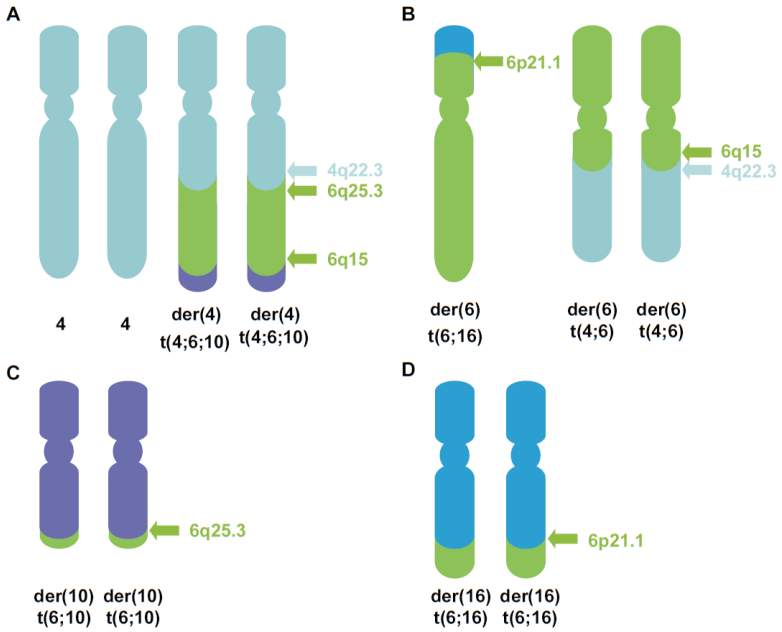


Figure 6. Schematic representation of chromosome 4 and 6 material in LNCaP cells. **A.** Two normal chromosome 4 and two der(4)t(4;6;10)(q22.3::q25.3q15::q23.33). The chromosome 4 and 6 breakpoints are indicated by the arrows. **B.** One der(6)t(6;16)(p21.1::q22.2) and two der(6)t(4;6)(q22.3::q15). The chromosome 4 and 6 breakpoints are indicated by arrows. **C.** Two der(10)t(6;10)(q25.3::q23.33). The chromosome 6 breakpoint is indicated by the arrows. **D.** Two der(16)t(6;16)(p21.1::q22.2). The chromosome 6 breakpoint is indicated by the arrow.

8p21.2. *PPP2R2A* is a putative TSG that induces apoptosis [27, 28]. Like many of the other genes identified at frequent breakpoints, *PPP2R2A* has been identified as a fusion partner gene [29]. Fusion of the *PPP2R2A* and *CHEK2* genes, resulting in the balanced chromosome translocation t(8;22)(p21;q12), was found in an intrathoracic mature teratoma [29]. The *PPP2R2A:CHEK2* fusion transcript does not result in an in-frame chimeric open reading frame, but the open reading frame of the TSG *CHEK2* is maintained, suggesting that promoter swapping leads to deregulated *CHEK2* expression. In this case, the function of both genes with tumour suppressor roles was disrupted. Using QRT-PCR, we show that in 8 of 9 paired samples analysed, *PPP2R2A* expression was reduced in tumour samples as compared to their case-matched normal controls. These suggest that *PPP2R2A* may play a tumour suppressor role in prostate cancer and it is frequently inactivated by various mechanisms including gene truncation.

In five cases, breakpoints were found within the *TCBA1* gene at 6q22.31. Deletions of 6q are frequently found in human cancers, including prostate cancer, acute lymphoblastic leukaemia and non-Hodgkin's B-cell lymphomas [30-32]. Tagawa *et al* found that *TCBA1* was involved in 6q aberrations in both T-cell lymphoma and leukaemia cell lines [33]. In a T-cell lymphoblastic lymphoma cell line, HT-1, *TCBA1* was found fused to *SUSP1* (SUMO-1-specific protease), creating a *SUSP1:TCBA1* chimeric gene, the function of which is not yet known [33]. Translocations t(1;6)(q32.3;q22.3) and t(2;6)(q24.3;q22.31) resulting in constitutional inactivation of the *TCBA1* gene have also been detected and are associated with developmental delay [34] and neurological disorders [35.]. These data support that *TCBA1,* which was found frequently inactivated by deletion or chromosome translocation, may be a candidate TSG in prostate cancer. The consequence of the truncation of *TCBA1* in prostate cancer should be further investigated.

Chromosomal common fragile sites are specific mammalian genomic regions that show an increased frequency of gaps and breaks when cells are exposed to replication stress *in vitro [36, 37].* Fragile sites are often involved in deletions and translocations. The two most active fragile sites in the human genome are FRA3B and FRA16D, respectively, where the TSGs *FHIT* and *WWOX* are located on chromosomes 3 and 16, respectively. The *FHIT* gene, which maps to the chromosomal region of *FRA3B* is frequently deleted, or involved in translocation breakpoints in a large number of tumour types [38-41]. Like *FHIT, WWOX* is also downregulated in many human cancers, including prostate cancer [42]. From our array data we can see that in four

cases, breakpoints can be found within the *WWOX* gene. However, no chromosome breakpoints were found within *FHIT*. The reduced expression of the *WWOX* gene in the majority (8/9) of paired samples indicated that *WWOX* may be commonly inactivated in prostate cancer. In one case, where a genomic breakpoint in *WWOX* was detected by SNP array analysis, *WWOX* expression level was higher in the tumour sample than its matched normal control. In rare cases, new oncogenic proteins may be formed by fusion with a TSG. For example the *PAX5* and *ETV6* TSGs have oncogenic properties when found as part of a fusion gene [19]. The mechanism leading to over-expression of *WWOX* in this cancer sample should be further analysed.

From our analysis of the recurrent breakpoints in prostate cancer, and the genes associated with them, it is clear that there are more known/putative TSGs residing at breakpoints than known/putative oncogenes. A number of well-characterised TSGs, including *p53*, *PTEN*, *BRCA1* and *BRCA2*, were found at recurrent copy number change breakpoints. However, other well-known TSGs, such as *RB*, *WT1*, *NF1*, *NF2*, *APC*, *CDH1* and *VHL* were not identified with recurrent breakpoints, indicating that the TSGs identified may be specifically targeted in prostate cancer.

Using M-FISH, we have previously identified several chromosomal alterations in the LNCaP cell line, including a complex t(4;6) translocation, which we have mapped in detail [24, 43]. FISH analysis of patient samples on tissue microarrays confirmed that t(4;6)(q22;q15) is a recurrent chromosomal translocation in prostate cancer [44]. A single gene, *UNC5C*, is interrupted by the t(4;6) breakpoints, leading to loss of the *UNC5C* promoter and exon 1 [24]. From our array data, and further FISH analysis, we can now reveal that, in LNCaP, 4q22 is fused to 6q25.3 but not 6q15 in the derivative chromosome 4. Together with our previous findings, we can now fully interpret the fusion events involving chromosome 4 and 6 in LNCaP cell line (**Figure 6**).

Among the five breakpoints on chromosome 6 and two breakpoints on chromosome 4, only 3 of them have been identified with known genes. The fusion gene *tpc/hpr*, caused by t(6;16), has been reported many years ago [45], but has not been identified as a recurrent fusion gene. The remaining two genes are *SNX9* and *UNC5C*. Although our data showed that *SNX9* and *UNC5C* are genomically fused together in the same gene orientation as a consequence of the 4q22.3:6q25.3 chromosome recombination, no fusion transcript of *SNX9:UNC5C* can be detected. *UNC5C* has been suggested to be a TSG [46, 47] and our QRT-PCR gene expression data supports this TSG role in prostate cancer. The reduced expression of *SNX9* in cancer samples compared with their matched normal controls suggests that *SNX9* may also has a potential TSG role. Therefore, the genomic fusion of *SNX9:UNC5C* in LNCaP cells may lead to the disruption of the activity of two TSGs.

This study reveals that many TSGs are recurrently affected by genomic rearrangements. Their potential to be developed as cancer prognostic markers or therapeutic targets should be further investigated. This finding will significantly enhance our understanding of the genetic alterations of prostate cancer, which will consequently improve the strategies for prostate cancer treatment/management. We speculate that by using this microarray analysis approach to investigate other cancers we would also identify many TSGs at recurrent chromosomal breakpoints. Using an array approach to identify genes affected by genomic breakpoints is not without its limitations; 1) it is not possible to identify whether truncated genes are fused to other genes 2) only genes at breakpoints associated with copy number changes can be identified. Balanced translocations and other rearrangements that do not result in genomic copy number changes cannot be detected. However, a limited number of truly balanced rearrangements exist in solid tumours [17]. Next generation sequencing of the cancer genome will fully reveal the features and consequence of genomic rearrangements.

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