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Prostate Cancer Aggressiveness Locus on Chromosome 7q32—q33 Identified by Linkage and Allelic Imbalance Studies

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Abstract

The biologic aggressiveness of prostate tumors is an important indicator of prognosis. Chromosome 7q32—q33 was recently reported to show linkage to more aggressive prostate cancer, based on Gleason score, in a large sibling pair study. We report confirmation and narrowing of the linked region using finer-scale genotyping. We also report a high frequency of allelic imbalance (AI) defined within this locus in a series of 48 primary prostate tumors from men unselected for family history or disease status. The highest frequency of AI was observed with adjacent markers D7S2531 (52%) and D7S1804 (36%). These two markers delineated a common region of AI, with 24 tumors exhibiting interstitial AI involving one or both markers. The 1.1-Mb candidate region contains relatively few transcripts. Additionally, we observed positive associations between interstitial AI at D7S1804 and early age at diagnosis (*P*=.03) as well as a high combined Gleason score and tumor stage (*P*=.06). Interstitial AI at D7S2531 was associated with a positive family history of prostate cancer (*P*=.05). These data imply that we have localized a prostate cancer tumor aggressiveness loci to chromosome 7q32–q33 that is involved in familial and nonfamilial forms of prostate cancer.

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Introduction

Prostate cancer is the most commonly diagnosed neoplasm and the second leading cause of cancer mortality in men in the USA, with 30,200 deaths predicted in 2002 [1]. Despite this, little is known regarding the genetic etiology of this disease or why some prostate tumors are biologically more aggressive than others.

Studies to date indicate the presence of multiple prostate cancer susceptibility loci including: *HPC1* located on 1q24–q25 [2], *CAPB* on 1p36 [3], *PCAP* on 1q42.2–q43 [4], 8p22–p23 [5], *HPC2* on 17p [6], 16q23–q24 [7], 20q [8] and *HPCX* on Xq27–q28 [9]. Whereas there is some supporting evidence for many of these candidate loci [10–18], several studies have failed to verify any linkage to these regions [19–25]. These complex and apparently contradictory linkage data strongly suggest a heterogeneous nature of hereditary prostate cancer [26]. Four members of our group (D.V.C., W.J.C., B.K.S. and J.S.W.) have recently reported the identification of chromosomal regions at 5q31–q33, 7q32–q33, and 19q12 that exhibit linkage to more aggressive forms of prostate cancer [27], as determined by Gleason score [28].

We chose to further examine chromosome 7q32–q33 as reports indicate frequent deletions of the long arm of chromosome 7 in prostate cancer [29,30] and many other tumor types including breast [31], pancreas [32], stomach [33], thyroid [34], and ovary [35]. Allelic imbalance (AI) of 7q is also associated with poor outcome in patients with prostate cancer [36]. Specifically, a significant correlation has been reported between 7q AI and higher Gleason score, increased mortality and systemic progression of disease at follow-up [37], all considered markers of more aggressive disease. Loss of heterozygosity (LOH) at 7q31 is the most commonly reported alteration in many tumor types and is often observed in early-stage cancers [29,34,35]. Recent evidence suggests that *ST7* is the tumor suppressor gene associated with LOH at 7q31 [38].

The present study was designed to further characterize the candidate prostate tumor aggressiveness locus on 7q32–q33 by utilizing linkage analysis and AI techniques. In this report, we confirm and narrow the previously reported linkage region on chromosome 7q32–q33 and report a high frequency of AI within this region. The current study provides strong evidence for the presence of a prostate cancer aggressiveness gene mapping to 7q32–q33 that may play a role in both familial and nonfamilial forms of prostate cancer.

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Materials and Methods

Radiation Hybrid Mapping

The order of the 11 microsatellite markers across 7q used in the AI study was determined using the Genebridge 4 (GB4) low-resolution radiation hybrid panel (Research Genetics, Huntsville, AL). The GB4 panel [39] contains DNAs from 93 human fibroblast-derived human:hamster hybrids. Individual PCR reactions were performed for each hybrid with each of the 11 markers shown in Table 1. The PCR reactions were performed using a PCR thermal cycler (MWG Biotech, Highpoint, NC). Each 15-μl reaction contained 2 μl of DNA, 1.25 mM of each deoxynucleotide triphosphate, 0.5 μM reverse primer, 0.5 μM forward primer, 0.75 U of *Taq* DNA polymerase (Life Technologies, Rockville, MD), 67 mM Tris–HCl (pH 8.8), 67 mM magnesium chloride, 16.6 mM ammonium sulfate, 10 mM β-mercaptoethanol, and 10% DMSO. The DNA amplification cycle comprised a 5-minute denaturation step at 94°C, 35 cycles of 94°C for 45 seconds, annealing at the appropriate temperature for 1 minute, and extension for 1 minute at 72°C, followed by a final 5-minute extension at 72°C. Ten microliters of each PCR product was resolved on a 6% nondenaturing polyacrylamide gel and visualized following ethidium bromide staining. Each hybrid was scored for the presence or absence of the PCR product with all 11 markers. The results were analyzed using the Map Manager QT program [40] to determine the relative order and distance between markers (Table 1).

Genotyping Methods

A higher density linkage analysis across the 7q32–q34 region was performed on 513 men, from the equivalent of 326 concordant sibships, from the original sibling pair study [27]. Five microsatellite markers were chosen; one of these markers (D7S530) lies distal and the remaining four (D7S2452, D7S640, D7S684, and D7S495) lie proximal to the marker exhibiting the highest degree of linkage (D7S1804) in the previous study [27]. PCR was carried out for each marker using blood lymphocyte DNA from each individual in the study. Each forward primer was designed to include a fluorophore at the 5′ end to enable detection and analysis on an ABI 373 XL DNA sequencer. Before loading onto a 6% denaturing polyacrylamide gel, PCR products were diluted in water and multiplexed according to marker size and fluorophore. One microliter of multiplexed product was then added to 3 μl of formamide loading dye, containing a 350 base pair 6-carboxytetramethylrhodamine size standard (Applied Biosystems, Foster City, CA) and denatured at 95°C for 5 minutes. ABI Collection and ABI Genescan version 3.1 software packages (Applied Biosystems) were used to process each electrophoresis run. Marker allele sizes and intensity for each sample was assessed using Genotyper software (Applied Biosystems).

Table 1. Radiation Hybrid Map Order of 11 Microsatellite Markers on 7q.

Marker	Intermarker Distance* (cR-3000)
D7S3061	
	34.44
D7S1875	
	27.44
D7S530	
	8.06
D7S2519	
	14.45
D7S2531	
	30.62
D7S1804	
	12.68
D7S2452	
	8.92
D7S640	
	63.15
D7S684	
	11.02
D7S495	
	28.44
D7S1824	

*Map distances are based on D7S3061 as 0.

Patient Selection and Evaluation of Tissue

A consecutive series of 51 prostate patients, for whom we had both tumor tissue and comprehensive clinical data available, were identified through the tumor registry at the Cleveland Clinic Foundation. This study was approved by the Institutional Review Board of the Cleveland Clinic Foundation. Each tumor was staged at the time of surgery and graded, using the Gleason system, following assessment of microscopic sections of the surgical specimen. Normal and tumor tissue from each case was microdissected from 5-μm unstained paraffin-embedded tissue sections, as previously described [16], following the review and annotation of a corresponding hematoxylin and eosin stained section by a pathologist (H.L.). DNA was extracted from the microdissected tissue using the Qiagen Tissue Kit (Qiagen, Valencia, CA) and eluted in 100 μl of Tris buffer (pH 9). Final tumor DNA content was estimated to be at least 70%. Three patients were subsequently removed from the study due to insufficient DNA quality for PCR amplification. A total of 48 patients were included in our genetic analysis. Clinical information for all patients is noted in Table 2.

AI Study

Eleven microsatellite markers were used in the AI study (Table 3). Of these, three (D7S3061, D7S1804, and D7S1824) had shown significant linkage to aggressive disease in our prostate cancer sibling linkage study [27]. Information regarding primer sequence was obtained from the Genome Database (http://www.gdb.org). Separate PCR reactions were performed using DNA from microdissected normal and tumor tissue. PCR conditions were as described above, but using a γ-^{32}P end-labeled forward primer. End-labeling with [γ-^{32}P]dATP was carried out using T4 polynucleotide kinase (USB, Cleveland, OH). Two

microliters of PCR product was combined with 4 μl of 95% formamide loading dye containing 20 mM EDTA, 0.5 mg/ml bromophenol blue, and 0.5 mg/ml xylene cyanol. The sample mixtures were denatured at 95°C for 5 minutes and immediately cooled on ice before loading onto a 6% denaturing polyacrylamide gel. Gels were run for approximately 3 hours at 65 W and analyzed following autoradiographic band detection using a STORM phosphorimager (Molecular Dynamics, Sunnyvale, CA). AI was determined by visual examination by two investigators as previously reported [41] and samples that defined AI breakpoints were verified using PCR products from a separate PCR amplification reaction.

Statistical Analyses

Linkage was statistically evaluated using a multipoint generalized Haseman-Elston (HE) linkage test [42] as described previously [27]. To determine whether AI at any marker was associated with clinical characteristics of the men with prostate cancer (e.g., high Gleason score)

Table 2. Selected Clinical Parameters for the 48 Prostate Cancer Patients in the Study.

Patient ID	Age at Diagnosis	Prostate Cancer Family History	PSA at Diagnosis	Pathology Stage	Surgical Grade
3-104	63	Y	5.4	T2	6
3-130	66	N	6.0	T2	5
3-249	51	Y	5.0	T2	6
3-342	64	N	5.0	T3	6
4-188	54	Y	4.6	T3	7
5-121	55	N	5.7	T3	7
5-187	63	Y	5.7	T3	7
5-189	62	N	3.5	T2	6
5-350	60	Y	6.3	T2	6
5-369	66	N	5.6	T2	7
5-436	63	Y	6.0	T2	6
5-665	65	Y	6.0	T3	5
5-905	63	N	5.4	T2	6
6-201	64	Y	5.4	T2	5
6-322	56	Y	5.6	T2	6
6-350	58	N	5.6	T2	7
6-425	61	N	6.2	T2	6
6-452	64	Y	6.3	T3	7
7-155	65	Y	5.5	T3	7
7-187	63	Y	5.3	T3	7
7-206	71	Y	5.5	T2	7
7-220	56	Y	6.0	T2	6
7-286	57	N	7.2	T3	7
7-293	62	N	5.9	T2	7
7-297	57	N	10.9	T3	6
7-309	68	N	4.4	T2	7
7-310	63	N	7.1	T2	7
7-311	62	N	6.1	T2	6
7-324	58	N	8.0	T2	5
7-341	62	N	12.9	T3	7
7-348	60	Y	17.0	T3	7
7-353	56	N	5.9	T2	7
7-375	57	N	4.8	T2	6
7-392	60	N	13.0	T2	9
7-393	58	N	3.4	T2	7
7-401	59	N	25.0	T3	7
7-404	60	Y	8.0	T2	7
7-410	73	N	5.0	T2	6
7-433	66	N	9.8	T3	7
7-441	69	N	10.0	T3	8
7-451	68	N	5.3	T2	7
7-475	55	Y	6.6	T2	6
7-484	62	N	8.2	T2	7
7-485	63	N	7.6	T2	7
7-491	63	Y	4.3	T2	7
7-684	48	Y	5.9	T2	7
7-923	71	Y	5.0	T3	7
8-501	47	N	18.2	T3	9

Table 3. AI on Chromosome 7q31–q35 Loci in 48 Primary Prostate Tumors.

Marker	Name	AI/Informative (% of AI)	Cytogenetic Location*
D7S3061	CHCL.GGAA6D03	12/43 (28%)	7q31.32
D7S1875	AFMa345wc9	5/30 (17%)	7q31.33
D7S530	AFM249×f9	4/42 (10%)	7q32.1
D7S2519	AFMc024we9	7/35 (20%)	7q32.2
D7S2531	AFM338wd5	16/31 (52%)	7q32.2
D7S1804	CHCL.GATA43C11	12/33 (36%)	7q33
D7S2452	AFMa282wf9	7/39 (18%)	7q33
D7S640	sWSS1204	6/37 (16%)	7q33
D7S684	AFM312wb5	9/42 (21%)	7q34
D7S495	AFM168×c3	6/25 (24%)	7q34
D7S1824	CHLC.GATA32C12	8/41 (20%)	7q35

*According to the NCBI Entrez Genome web site (http://www.ncbi.nlm.nih.gov).

Fischer's exact χ^2 test was used. Analyses were carried out using the statistical software SAS (SAS Institute, Cary, NC).

Results

Radiation Hybrid Mapping of 7q Microsatellite Markers

To confirm the order of the 11 microsatellite markers used in this study, the low-resolution GB4 radiation hybrid panel [39] was used. We found the mapping order of the markers to be in agreement with that given for the Marshfield linkage map with the exception of marker D7S640, which our data places distal to D7S2452, and marker D7S495, which lies distal to D7S684 according to our study. The chromosomal order of these markers, as determined by analysis with Map Manager QT is shown in Table 1.

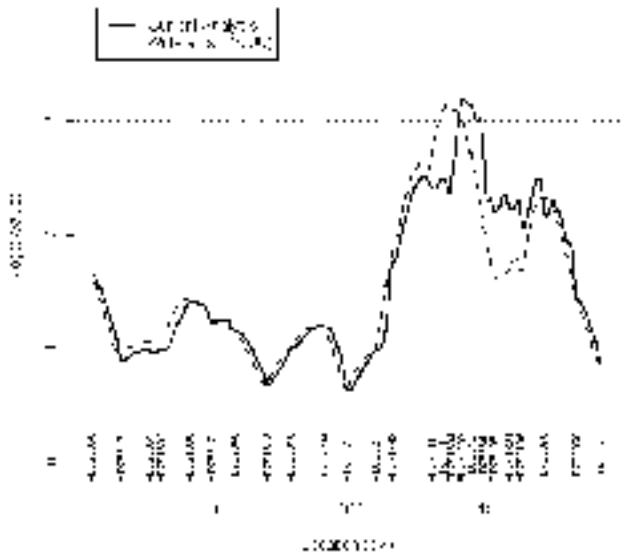

Figure 1. *Results from linkage analysis of prostate cancer aggressiveness on chromosome 7. Broken lines indicate results from the original analysis; solid line denotes results from new analysis. Values on the x-axis show marker positions according to Marshfield Medical Research Foundation, Human Genetics web site and our radiation hybrid mapping data.*

Linkage Analysis

Incorporating the data from the five additional markers into our original linkage analysis strengthened evidence of linkage (Figure 1). We observed a broad region of linkage for which $P<.005$ between markers D7S3061 and D7S495. Within this region, a 6-cM peak ($P<.001$) encompassing markers D7S2452 and D7S640 was identified, supporting the presence of a gene involved in the development of aggressive forms of prostate cancer on 7q32–q33.

AI at 7q32–q33

Overall, 38 of the 48 (79%) samples studied showed AI with at least one marker. The AI frequencies for each marker are shown in Table 3. The highest frequencies of AI were exhibited by adjacent markers D7S2531 (52%) and D7S1804 (36%). Twenty-seven of the 48 samples studied displayed AI involving D7S2531 and/or D7S1804, with 24 tumors showing interstitial deletions across this region (Figure 2). Representative autoradiographs for four of the samples showing interstitial AI across D7S2531/D7S1804 are shown in Figure 3.

Clinical Associations

We examined any potential clinical associations between tumors showing interstitial AI involving D78S2531 and/or D7S1804 compared with 21 samples that showed no AI at either marker. This analysis was designed to remove any bias from surrounding unrelated AI. Parameters such as family history (self-reported and defined as having at least one first-degree affected relative), PSA at diagnosis, Gleason score, and tumor stage were studied (Table 2). A statistically significant association ($P=.03$) was seen between early age at diagnosis (<60 years of age) and interstitial AI at marker D7S1804. A weaker, but still noteworthy, association was observed between interstitial AI involving D7S1804 and a high combined Gleason score (>7) and tumor stage ($\geq$T2c) ($P=.06$). An association between AI with marker D7S2531 and positive

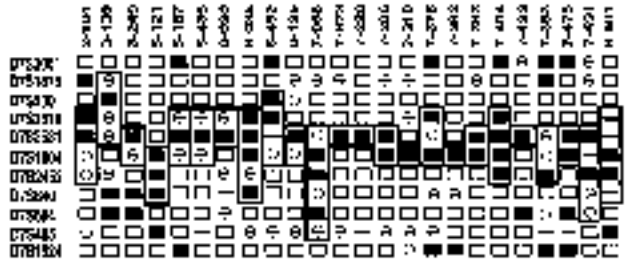

Figure 2. *Summary of 24 primary prostate tumor samples showing interstitial AI on 7q involving D7S2531 and D7S1804. Markers are listed on the left; sample numbers are shown across the top. (□) Informative samples with no AI; (■) informative samples demonstrating AI; (⊖) noninformative (homozygous) samples; (—) did not work. Boxes define maximum extent of AI for each sample.*

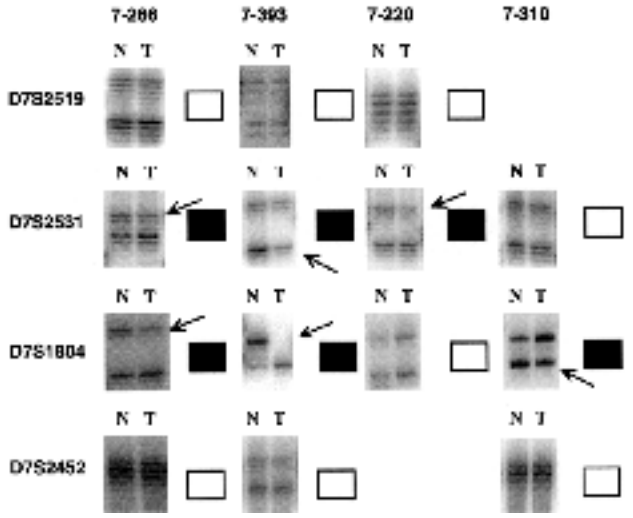

Figure 3. *Examples of autoradiographs from AI studies on matched normal (N) and prostate tumor (T) pairs. Samples 7–286 and 7–393 both exhibit AI at markers D7S2531 and D7S1804 but retain D7S2519 and D7S2452. Tumor 7–220 shows AI at D7S2531 but no AI at any neighboring marker. Sample 7–310 demonstrates AI at D7S1804 but not at any flanking marker. (■) AI; (□) no AI; arrows indicate AI.*

family history for prostate cancer (P=.05) was also observed.

Discussion

The first aim of this project was to provide further evidence for a prostate cancer tumor aggressiveness locus on chromosome 7q32–q33. In our original genome-wide multipoint linkage study [27] we found three microsatellite markers that defined linkage to Gleason score (P<.01) across 7q31–q35 covering a 28 cM region. The linkage region with P<.0001 spanned approximately 8 cM and included the marker D7S1804. In the present study, we undertook finer-scale genotyping in this region using five additional markers (D7S530, D7S2452, D7S640, D7S684, and D7S495) with the same sibling population used in the original study. We found that linkage was maintained on chromosome 7q32–q33, when data were analyzed with respect to Gleason score (Figure 1) and strengthened (P<.005) across the 28 cM region. Furthermore, the highest linkage peak was reduced in size to approximately 6 cM (P<.001) between markers D7S2452 and D7S684. These data imply that this region harbors a gene involved in increased risk for developing more aggressive forms of prostate cancer.

Hereditary chordoma was recently mapped to this region on chromosome 7q32–q33 [43]. Chordomas are rare, locally invasive primary tumors of the bone believed to develop from notochordal remnants. Although these tumors are slow growing, they frequently recur following surgery or radiotherapy [44], implying some aggressive biologic behavior. Three families affected by chordoma showed linkage between markers D7S512 and D7S684, which span the region observed in the present study [43]. Furthermore, another form of familial chordoma has been mapped to 1p36 [45], also reported as a prostate cancer susceptibility locus *CAPB* [3]. Although there is a moderate male predominance (1.7:1) for this disease [43], there are no reports of an association with prostate cancer. Reported analysis of four chordoma tumor samples from affected family members did not reveal any LOH suggesting that this gene and the prostate cancer aggressiveness gene may not be the same [43] or do not undergo the same forms of gene inactivation. However, additional studies would be needed to determine this.

A second goal of the study was to provide evidence of a tumor suppressor gene in this region by applying AI analyses to 48 prostate tumors from patients unselected for family history or clinical status of disease. These studies demonstrated a high frequency of AI within this region. Overall, 79% (38 of 48) of the tumors showed AI of at least one marker. The highest frequency of AI was found at markers D7S2531 (52%) and D7S1804 (36%). Furthermore, 24 tumors (50%) showed interstitial AI involving D7S2531 and/or D7S1804 (Figure 2) and defined a common region of deletion between these two markers of approximately ~1.1 Mb in size, based on build 28 of the public genome sequencing database at NCBI. This pattern of interstitial AI also suggests that the region we have defined in this study is distinct from that containing the *ST7* candidate tumor suppressor gene on 7q31.3 (Figure 4).

The common fragile site FRA7H maps between markers D7S2519 and D7S2531 [46,47]. Fragile sites are regions of the genome particularly prone to breakage and often are associated with regions of AI. Several candidate tumor suppressor genes have been mapped to fragile sites including *FHIT* on 3p14.2 (FRA3B) [48], the multiple candidate tumor suppressor *ST7* on 7q31 (FRA7G) [38,49] and the putative prostate cancer susceptibility locus at 16q23 (FRA16D) [16,50,51].

We also investigated any clinical associations with occurrence of AI at markers D7S2531 and D7S1804 by comparing tumors with interstitial AI at D7S2531 and/or D7S1804 and those without AI at either marker. A statistically significant association (P=.05) was observed between AI at D7S2531 and positive family history. AI at D7S1804 was associated with early age at diagnosis (P=.03) and a high combined Gleason score and tumor stage (P=.06). A similar trend was seen when Gleason score (P=.14) or tumor stage (P=.16) were analyzed separately. These data strongly suggest that this region contains a gene associated with prostate tumor aggressiveness that is implicated in the

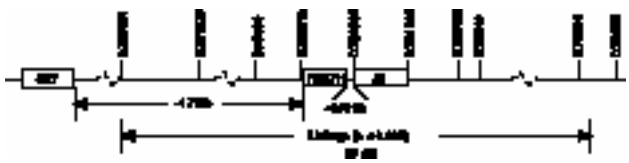

Figure 4. *Schematic representation of 7q32–q33 region showing positions of linked region, AI, fragile site and ST7 tumor suppressor gene. Not shown to scale. ST7=supression of tumorigenicity 7 gene; FRA7H=fragile site 7H; AI=common region of allelic imbalance.*

etiology of both hereditary and nonfamilial forms of prostate cancer.

Sequence coverage of the chromosome 7q32–q33 region is almost complete with three small gaps in the NCBI database. The region contains relatively few transcripts, but contains some intriguing candidates for a prostate cancer tumor aggressiveness gene, including two known genes, muskelin-1 (*MKLN1*) [52] and podocalyxin-like protein (*PODXL*), and four hypothetical genes, *KIAA1550*, *LOC91583*, *LOC91584* (similar to mouse plexin 3), and *LOC96016* (similar to Eukaryotic Translation Elongation Factor 1 Beta 2).

Muskelin-1 is a novel intracellular protein that acts as a positive mediator of cell-spreading, adhesion, and cytoskeletal responses to the extracellular matrix component thrombospondin-1 (TSP-1) [53]. TSP-1 is a potent antiangiogenic molecule [54] and has been shown to inhibit tumor growth and metastasis [55]. TSP-1 is upregulated by p53 and depleted in primary prostate tumors that express mutant *p53* [56]. Evidence suggests that muskelin (MKLN1) is required for cellular responses to TSP-1 [53], implying a role for this protein in the regulation of the biologic aggressiveness of tumors.

Podocalyxin-like protein is a transmembrane sialomucin involved in adhesion in renal glomerular podocytes and vascular endothelium [57]. PODXL was recently shown to be identical to the testicular germ cell tumor marker, gp200, which is associated with highly malignant tumors [58].

Several regions of homology to both mouse and human plexins are identified within the hypothetical genes including PSI (domain found in *p*lexins, *s*emaphorins, and *i*ntegrins) and IPT (*i*mmunoglobulin-like fold shared by *p*lexins and *t*ranscription factors) domains common to the plexin family of proteins [59]. Plexins have been reported to act as cell surface receptors for semaphorins [60], and are implicated in axon repulsion, angiogenesis regulation, and tumor growth and metastasis [61]. The PSI domain found within the extracellular regions of both plexins and semaphorins shows homology to part of the oncoprotein MET. MET and other scatter factor receptors have been shown to play a role in cell motility and invasion [62].

To provide evidence of a role in prostate tumor aggressiveness, we are currently performing germline mutation analyses of candidate genes in men with prostate cancer that show linkage to this region. In addition, we hope to identify more linked families for which there is tumor tissue available and perform AI studies to further confirm and narrow this region.

In summary, we have further refined a region of linkage on chromosome 7q32–q33 associated with prostate cancer tumor aggressiveness. We have also demonstrated a high frequency of AI within this region and have mapped the smallest region of AI to approximately 1.1 Mb between markers D7S2531 and D7S1804. Furthermore, we found that AI at marker D7S1804 was associated with early age of onset of prostate cancer and high combined Gleason score/tumor stage and that marker D7S2531 was associated with family history of prostate cancer in an unselected series of prostate cancer patients. These data support the mapping of a gene to 7q32–q33 associated with aggressive forms of both familial and nonfamilial prostate cancer.

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