Deletion of a Small Consensus Region at 6q15, Including the MAP3K7 Gene, Is Significantly Associated with High-Grade Prostate Cancers

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Abstract

Purpose: Chromosome 6q14-21 is commonly deleted in prostate cancers, occurring in ~22% of all tumors and ~40% of metastatic tumors. However, candidate prostate tumor suppressor genes in this region have not been identified, in part due to the large and broad nature of the deleted region implicated in previous studies.

Experimental Design: We first used high-resolution Affymetrix single nucleotide polymorphism arrays to examine DNA from malignant and matched nonmalignant cells from 55 prostate cancer patients. We identified a small consensus region on 6q14-21 and evaluated the deletion status within the region among additional 40 tumors and normal pairs using quantitative PCR and fluorescence in situ hybridization. We finally tested the association between the deletion and Gleason score using the Fisher’s exact test.

Results: Tumors with small, interstitial deletions at 6q14-21 defined an 817-kb consensus region that is affected in 20 of 21 tumors. The MAP3K7 gene is one of five genes located in this region. In total, MAP3K7 was deleted in 32% of 95 tumors. Importantly, deletion of MAP3K7 was highly associated with higher-grade disease, occurring in 61% of tumors with Gleason score ≥8 compared with only 22% of tumors with Gleason score ≤7. The difference was highly significant (P = 0.001).

Conclusion: Our study provides strong evidence for the first time that a small deletion at 6q15, including the MAP3K7 gene and four other genes, is associated with high-grade prostate cancers. Although the deletion may be a marker for high-grade prostate cancer, additional studies are needed to understand its molecular mechanisms.

Many if not most prostate cancers do not pose a major health threat to their hosts. The molecular factors responsible for variations in the aggressiveness of prostate cancers are poorly defined. Deletion of DNA sequences from chromosome 6q14-21 is one of the most common deletion events in the genome of prostate tumors (reviewed in ref. 1). In a recent study that estimated the frequency of DNA copy number alterations in the prostate cancer genome based on all published comparative genomic hybridization studies of prostate cancers, about one quarter of the 891 prostate cancers had a deletion at 6q14-21 (2). More importantly, the deletion seemed to be more common in metastatic/advanced tumors (40%) than in localized/primary tumors (19%). Despite this overwhelming evidence for frequent 6q deletions, specific prostate tumor suppressor genes have not been identified in this region. One of the major obstacles is the size of the deleted region implicated in previous studies, due at least in part to limited resolution of the detection methods. In our combined analysis of all published comparative genomic hybridization studies, the deleted region at 6q spans ~30 Mb (2). The large number of genes (>170) located within the broad deletion interval poses a significant challenge to effective searches for tumor suppressor genes in the region. Therefore, efforts are needed to define a smaller candidate region. Higher-resolution detection methods, such as representational oligonucleotide microarray analysis and single nucleotide polymorphism (SNP) arrays, may be helpful by identifying smaller deletions and delineating detailed deletion patterns, such as interstitial deletions (3, 4).

In this study, we used high-resolution Affymetrix SNP arrays, fluorescence in situ hybridization (FISH), and quantitative PCRs (qPCR) to examine deletion patterns at 6q among 95 prostate tumors. Our goal was to identify a small consensus

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region, evaluate candidate genes within the region using various molecular methods, and examine the possible correlation between gene deletion and tumor aggressiveness.

Materials and Methods

Subjects. All tumors analyzed in the study were from independent prostate cancer patients undergoing radical prostatectomy for treatment of clinically localized disease at Johns Hopkins Hospital. We selected a subset of subjects from whom genomic DNA of sufficient quantity (>5 μg) and purity (>70% cancer cells for cancer specimens, no detectable cancer cells for normal samples) could be obtained by dissection of matched nonmalignant (hereafter referred to as “normal”) and cancer containing areas of prostate tissue as determined by histologic evaluation of H&E-stained frozen sections of snap-frozen radical prostatectomy specimens. Genomic DNA was isolated from trimmed frozen tissues as described previously (5).

Detection of 6q deletions using Affymetrix SNP arrays. We first used Affymetrix SNP array panels to detect DNA copy number alterations in the genome among 55 prostate cancers, and for this study, we focused entirely on 6q deletions. We used the 100K SNP array for the first 22 subjects (4) and then used the 500K SNP array (Affymetrix, Inc.) for the remaining 41 subjects. Eight of the subjects were analyzed using both 100K and 500K SNP arrays. All of the reagents used for the assay were obtained from the manufacturers recommended by Affymetrix. We digested, ligated, amplified, fragmented, and labeled the samples and hybridized, washed, and stained the SNP mapping arrays according to the manufacturer’s instructions. DNA copy number was calculated based on allele intensity using two different software packages: Copy Number Analyzer for Affymetrix GeneChip® (CNAG2.0; ref. 6) and dChip analyzer (7). Allele-specific analysis was also done to estimate DNA copy number for each chromosome pair using CNAG2.0. The results from all three types of analyses using CNAG2.0 and dChip were consistent, and we show the output from CNAG2.0 in Fig. 1. The physical positions of detected deletions were determined based on the Human hg17 Assembly (National Center for Biotechnology Information Build 35).

Detection of DNA deletion at MAP3K7 using qPCR. qPCR analysis was used to evaluate the deletion status of the MAP3K7 gene in the 55 tumors described above and among 40 additional prostate tumors that were not evaluated by SNP arrays. The qPCR analysis was done using the ABI Prism 7000 Sequence Detection System as described in detail elsewhere (4). A primer set located in the last exon of MAP3K7 was used to amplify the test amplicon (forward primer: 5′-AACGGTCCCAGA-GAATCATGAAGTGC-3′; reverse primer: 5′-GAGGCTCATAGAGTGACG-CAGCAGA-3′), and a primer set located around the junction of intron 2 and exon 3 of MAP3K7 was used to amplify the control amplicon (forward primer: 5′-TCTCATGCGCTCTGCTCTTG-3′; reverse primer: 5′-AAGCCGCCCCATACAGCACAATCT-3′).

Statistical analysis. The difference in the frequencies of DNA deletion at MAP3K7 between lower-grade (Gleason scores ≤7) and higher-grade tumors (Gleason scores ≥8) was tested using Fisher’s exact test.

Confirmation of DNA deletion at MAP3K7 using FISH. A subset of identified MAP3K7 deleted tumors was analyzed using FISH to confirm their deletion status. We obtained the PAC clone RP1-154G14 (~100 kb at 6q15-16.3) for the FISH analysis from the Children’s Hospital Oakland Research Institute.6 The only known gene contained in this clone is MAP3K7. We grew the clone, isolated the DNA, and checked the identity of the insert in the clone as recommended. The hybridization mixture contained 200 ng of PAC RP1-154G14 DNA, which was labeled by nick translation with SpectrumOrange dUTP following the manufacturer’s protocol (Vysis, Inc.). In addition, the hybridization mixture contained two additional probes: the centromeric probe CEPI6 labeled with SpectrumGreen (Vysis) and the LSI MYB probe labeled with SpectrumAqua (Vysis), which bracket the PAC RP1-154G14 clone (Fig. 2B). Paraffin-embedded normal prostate and prostate cancer sections (5 μm) were pretreated, hybridized, and washed following the manufacturer’s protocol (Vysis). FISH analysis was done using fluorescent microscopy with the appropriate filters to visualize the three probes. Slides were blinded, and then for each sample, a total of 200 interphase cells was analyzed independently by two individuals.

MAP3K7 protein expression using immunohistochemistry. For immunohistochemical staining of proteins, sections were deparaffinized by successive incubation in xylene, 100% ethanol, and 90% ethanol following standard procedures. The endogenous peroxidase activity was blocked by incubation for 20 min at room temperature in 0.5% H2O2 in water. Sections were washed thrice in PBS. Retrieval of antigens was done by incubating the sections in antigen retrieval solution (Sigma) for 1 h in a 95°C water bath. After allowing the sections to cool, samples were washed in PBS and blocked with 3% bovine serum albumin in PBS for 30 min at room temperature. After blocking, sections were incubated with anti-transforming growth factor-β activated kinase 1 (Tak1) primary antibody (1:50 dilution; Abcam, Inc.) for 1 h at room temperature and washed. Sections were then incubated with the goat anti-rabbit secondary antibody with a horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, Inc.), washed, and then incubated with diaminobenidine for approximately 2 to 5 min. Following counterstain with hematoxylin (Sigma) and clearing of the sections through ethanol and xylene, coverslips were mounted using Permount medium.

Results and Discussion

The use of high-resolution SNP arrays and allele-specific analyses improved our ability to detect somatic DNA deletions in prostate tumors. Detectable deletions at 6q14-21 were observed in 21 of the 55 prostate cancers (38%) examined using the SNP arrays in this study (Fig. 1). This frequency was considerably higher than the estimate of 25% obtained from a combined analysis of 891 prostate tumors described in published comparative genomic hybridization studies (2). One reason for the higher estimate of deletion in our study is the use of a higher-resolution detection method. For example, we were able to detect a small deletion of ~800 kb at 6q15 in tumor G7-042 using the SNP arrays (Fig. 1). Another potential factor for the higher estimate of deletion was the greater proportion of tumors with high-grade disease (38% with Gleason score ≥8) in our study. We found that the frequency of 6q deletion was significantly higher in tumors with Gleason score ≥8 (12 of 17 tumors, 71%) than that of Gleason scores ≤7 (9 of 38, 24%; P = 0.002). The association between 6q deletion and tumor grade was striking; in fact, it was the strongest among all the association tests between any common recurrent DNA copy number changes (defined as ≥10%) identified in our studies and tumor grade.

The high-resolution SNP arrays and the ability to analyze copy number changes for each chromosome using allele-specific analysis also improved our ability to dissect detailed deletion patterns. We detected three small-size homozygous deletions among these 21 tumors with 6q deletions (Fig. 1). One was ~838 kb (98,588-99,426 kb) in tumor G7-019 and contained the POUSF2 gene, one was ~265 kb (117,394-117,659 kb) in tumor G7-048 and contained no known gene, and the other was ~1.1 Mb (84,868-85,963 kb) in...
tumor G7-030 and contained the Q9Y2L2 gene. These homozygous deletions, however, did not overlap. Although the significance of these homozygous deletions in prostate cancer development is unclear, their nonoverlapping nature may be more consistent with generalized genomic instability than a specific selection for loss of these particular genomic regions.

In addition, we found two additional tumors (tumors G7-026 and G7-028) with interstitial deletions at 6q (Fig. 1). The distal deleted region of tumor G7-026, between 90,451 and 91,310 kb, contained the G8-002 and G8-004 genes. The proximal deleted region of tumor G7-028, between 64 and 119 Mb, contained the G9-003 and G9-006 genes.

Fig. 1. Examples of genomic deletions on chromosome 6q identified in tumor genomes using Affymetrix 100K (a and b) and 500K (c–v) SNP mapping arrays, and CNAG2.0 with genomic smoothing of 10 SNPs. a to s, allele-specific analysis using matched normal DNA as reference. t to v, non–allele-specific analysis using automatically selected references by CNAG2.0 from our database containing 40 normal samples. d, from Nsp array only; i, from Sty array only. Hemizygous deletions outlined by green (allele-specific analysis) or blue (non–allele-specific analysis) horizontal curves below the baseline (solid black line) are marked by green arrows. Homozygous deletions outlined by red and green or blue horizontal curves below the baseline (solid black line) are marked by red arrows. Vertical dotted lines, minimum overlapping deleted region. w, genes in the minimum overlapping deleted region based on the Human hg17 Assembly (University of California at Santa Cruz, National Center for Biotechnology Information Build 35).
97,338 kb, provided information that was critical in defining a minimal overlapping region (see below). When examining the deleted regions shared by each of these 21 tumors, we found one small region, between 90,493 and 91,310 kb, which was shared by all but one tumor. Interestingly, this shared deleted region fell within the 4.3 Mb 6q deleted region observed in LNCaP and seemed to be consistent with the minimum loss of heterozygosity regions reported by Cooney et al. (8), Srikantan et al. (9), Hyytinen et al. (10), and Konishi et al. (11). Only five genes are located within this region of 817 kb, including MDN1, CASP8AP2, CX62, BACH2, and MAP3K7. To further evaluate the significance of these five genes, we examined their differential expression patterns in the ONCOMINE gene expression database. The expression of the MAP3K7 gene was significantly lower in the tumor cells in comparison with the normal tissues of the prostate (P = 0.0001). In contrast, the expressions of the other four genes in prostate tumors were not significantly different from normal. The MAP3K7 gene encodes Tak1, a member of the mitogen-activated protein kinase kinase family and an activator of c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase pathways (12).

Because of the decreased expression of MAP3K7 in prostate cancer and its potential tumor suppressor role, we targeted the MAP3K7 gene in the remaining analyses.

To confirm that the MAP3K7 gene was implicated in these 6q14-21 deleted prostate tumors, we did a qPCR analysis using a probe located in the last exon of the MAP3K7 gene. As expected, qPCR analysis detected a hemizygous deletion in the 20 tumors that shared the 817-kb minimal overlapped deleted region (Fig. 2A) and also confirmed that the remaining tumor (tumor G7-028) does not have a deletion at this region. To further confirm the SNP array and qPCR results, we did FISH analysis in a subset of samples (Fig. 2B-D). The RP1-154G14 (~100 kb and includes the MAP3K7 gene), CEP6, and LSI MYB probes were hybridized to tumors G8-005, G9-003, and G7-019, and the probe signal was analyzed in 200 interphase cells for each sample (Fig. 2C and D). This analysis revealed a pattern consistent with a hemizygous interstitial deletion of the MAP3K7 region of chromosome 6q15-q16.3 (2G1O2A signal pattern, as shown in Fig. 2D) in tumor G8-005 (54%), tumor G9-003 (82%), and tumor G7-019 (55%), whereas the normal prostate control revealed a normal signal pattern (2G2O2A; Fig. 2C) in 94% of the cells. This analysis confirmed the SNP array and qPCR results, which also identified a hemizygous interstitial deletion of the MAP3K7 region of chromosome 6q15-q16.3.

To obtain a better estimate of MAP3K7 deletion frequency in prostate tumors and assess their association with Gleason scores, we used the same qPCR assay to examine the deletion status in 40 additional tumors that have not been analyzed by SNP arrays. The vast majority of these tumors had a Gleason score of 6 or 7. We observed the MAP3K7 gene deletion in nine of these tumors. In total, the MAP3K7 gene deletion was detected in 30 of the 95 (32%) tumors in our study using either SNP array or qPCR method. Importantly, the deletion was considerably more common in higher-grade tumors (14 of 23 tumors with Gleason score ≥8, 61%) than in lower-grade tumors (16 of 72 tumors with Gleason score ≤7, 22%; Table 1). The difference was highly significant (P = 0.001). Most strikingly, the frequency of the MAP3K7 deletion was highest in Gleason 9 tumors, occurring in 9 of the 12 tumors (75%).

As a test of our genetic data, we immunostained representative prostate tissue samples with anti-Tak (alias MAP3K7) antibody. Figure 3 shows immunostaining of benign versus high-grade tumor (Gleason 9) in a sample (tumor G9-001) with confirmed hemizygous deletion of MAP3K7. Benign tissue shows staining in the cytoplasm and at the plasma membrane.

**Fig. 2.** Confirmation of MAP3K7 deletions in various tumors using qPCR and FISH. A, examples of validating MAP3K7 deletions by real-time qPCR. Solid lines, controls; dotted lines, tumors; blue, subject G8-005; red, subject G9-003. B, ideogram of chromosome 6 showing the physical map location of the FISH probes. Green, CEP6 probe; orange, PAC RP1-154G14 clone; aqua, LSI MYB. C, normal prostate control with a normal signal pattern (2G2O2A). D, tumor G8-005 with a signal pattern indicating a hemizygous deletion of the PAC RP1-154G14 clone (2G1O2A).

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of prostatic epithelial cells. The intensity of staining ranges from moderate (data not shown) to intense (Fig. 3A). There are also moderate to strongly staining stromal cells present. In the sample depicted in Fig. 3, the immunoreactivity present in high-grade tumor is greatly diminished (Fig. 3B), consistent with the hemizygous genotype of this sample. Also note the strong immunoreactivity of stromal cells in the panel of the high-grade specimen. Two additional samples with verified hemizygous genotype showed similar Tak1 immunoreactivity (data not shown). These results are consistent with the findings that MAP3K7 RNA expression level is greatly reduced in prostate cancers in comparison with the level in normal prostate tissues.7

Our finding that a small consensus deleted region in prostate cancers, including the MAP3K7 gene, is strongly associated with Gleason score is significant. The MAP3K7 gene encodes Tak1. Tak1 is a member of the mitogen-activated protein kinase kinase family that was originally identified as a key regulator of mitogen-activated protein kinase activation in transforming growth factor-β-induced signaling pathways (12). Mutations and alterations in several layers of the transforming growth factor-β signaling cascade have been identified, including ligand, receptors, and intracellular signaling events (13). Before our genetic study, Tak1 has not been implicated in this process. Our demonstration that the MAP3K7 locus is deleted in prostate tumor specimens adds another step in transforming growth factor-β signaling that is abrogated in prostate tumorigenesis and further strengthens the role of this pathway in prostate cancer development.

Although Gleason score is perhaps the most reliable predictor for prostate cancer behavior, it is far from ideal. Many low- to intermediate-grade cancers may be metastatic, whereas a few of the high-grade tumors still have an indolent course. Thus, molecular markers that could further define the disease prognosis are greatly needed. Markers that can be used, either alone or in conjunction with Gleason score, to more precisely identify prostate cancers capable of progression to disseminated disease would be extremely useful in determining which patients to treat and how aggressively. It is interesting to note that the association between MAP3K7 deletion and Gleason grade is stronger than that of between p53 and Gleason grade in our study. Deletion of the p53 region at 17p13 occurs in 52.94% of tumors with Gleason score ≥8 and only in 22% of tumors with Gleason score ≤7 (P = 0.001). Further work in larger patient populations with follow-up information will be needed to assess the potential of MAP3K7 deletion as a prognostic marker.

Although we have shown the significance of the MAP3K7 deletion in prostate cancer, these results are subject to several caveats. First, our data only provided a strong statistical evidence for association between a consensus deleted region, including the MAP3K7 gene, and tumor grade; we did not assess the causal relationship between MAP3K7 gene and aggressive prostate cancer. Other in vitro, in vivo, and animal studies are needed to understand its molecular mechanisms. Second, the current study targeted only the MAP3K7 gene, and therefore, the roles of four other genes at this minimum overlap region in prostate cancer development remain to be

Table 1. MAP3K7 deletion in prostate tumors

<table>
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<tr>
<th>Group</th>
<th>Gleason sum</th>
<th>No. tumors examined</th>
<th>MAP3K7 deletion</th>
<th>No. tumors</th>
<th>% tumors</th>
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<td>4 + 3</td>
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Fig. 3. Tak1 immunoreactivity is decreased in high-grade prostate tumors. Sections (5 μm) were stained with rabbit anti-Tak1 antibody followed by labeling protocols using goat anti-rabbit horseradish peroxidase secondary as described in Materials and Methods. Controls that lacked primary antibody were clear (data not shown). The images shown were from different areas of the same section of a specimen verified by 100K SNP and q-PCR to be hemizygous for the MAP3K7 locus.

A. benign region. B. Gleason 5 tumor cells. Note benign gland in lower-right hand corner of (B) showing positive Tak1 immunoreactivity.
further investigated. Finally, our results differ from a previous study that found no correlation between loss of heterozygosity at 6q and MAP3K7 transcript expression (11). The differences in the deletion detection methods [loss of heterozygosity using microsatellite markers in the study of Konishi et al. (11) versus SNP arrays and qPCR in our study] and sample size [21 subjects in the study of Konishi et al. (11) versus 95 patients in our study] may contribute to the contradictory findings.

In summary, our study provides evidence that deletion of an 817-kb region of 6q15, including the MAP3K7 gene, is one of the most consistent genetic alterations occurring in the genome of high-grade prostate cancers. Although MAP3K7 represents an important candidate prostate tumor suppressor gene affected by this genomic alteration, further studies will be necessary to establish a causal relationship between this gene and/or the other genes in this interval and prostate cancer initiation and/or progression.

References
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