Genetic Alterations in the PI3K Pathway in Prostate Cancer

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Abstract. *Alterations in the PIK3CA and PTEN genes were assessed in 40 prostate tumors (radical prostatectomy samples). Genetic analyses in glands of the highest Gleason pattern within each tumor revealed PIK3CA amplification in 13% , PIK3CA mutations in 3% , PTEN homozygous deletion in 13% and PTEN hemizygous deletion in 8% of the cases analyzed. Supporting the view that PTEN and PIK3CA act in the same PI3K signaling pathway, genetic alterations in the PIK3CA and PTEN genes were mutually exclusive, except in one tumor. Overall, 13 of the 40 (33%) prostate tumors had alterations in the PI3K pathway. For cases with genetic alterations, other tumor areas with lower Gleason patterns as well as non-tumorous prostate glands were also analyzed. Of nine tumors with Gleason score 7, five cases contained the same genetic alterations in tumor areas of Gleason patterns 3 and 4, whereas in another four cases, genetic alterations were detected only in tumor areas of Gleason 4 but not Gleason 3 patterns. There were no alterations in non-tumorous glands. These results suggest that genetic alterations in the PI3K pathway are common in prostate cancer, and occur mainly through PIK3CA amplification and PTEN hemizygous or homozygous deletion. Glands of Gleason pattern 3 are genetically heterogeneous, some containing the same genetic alterations observed in glands of Gleason pattern 4.*

Prostate cancer is one of the most common malignances among men in developed countries, and the second highest cause of cancer death in males (1, 2). However, the molecular changes underlying its development have not been fully elucidated.

The signaling pathway involving PTEN, PI3K and AKT plays a significant role in the regulation of cell growth and death. Activation of growth factor receptors such as EGFR results in recruitment to the cell membrane of PI3K (phosphatidylinositol 3-kinase), which phosphorylates PIP_2 (phosphatidylinositol-4,5-bisphosphate) to PIP_3 (phosphatidylinositol-3,4,5-triphosphate). PIP3 activates downstream effector molecules such as AKT, leading to cell proliferation and blocking apoptosis (3, 4). PTEN inhibits PIP_3 signaling, inhibiting cell proliferation (5). A variety of human neoplasms show gain of function of the *PIK3CA* gene, that encodes the p110α catalytic subunit of PI3K, and loss of PTEN function (3, 6), and therefore potential strategies for developing therapies targeted to this signalling pathway have emerged (3, 4).

Many studies have shown alterations in the *PTEN* gene in prostate cancer, but frequencies vary significantly across different studies. *PTEN* mutations have been reported in 0-15% of locally confined prostate cancers and 20-30% of their metastases (7-12). LOH 10q, in particular LOH at the *PTEN* locus (10q23), has been observed in 22-60% of prostate cancer (13-19). Homozygous or hemizygous *PTEN* deletion has been reported in 0-26% of locally confined cancers and metastases (8, 9, 15, 20-23). Both homozygous and hemizygous *PTEN* deletions were associated with significantly shorter survival of prostate cancer patients (24). In contrast, there is less information on *PIK3CA* alterations in prostate cancer. One study reported absence of *PIK3CA* mutations in 12 cases of prostate cancer (25), while another, using array CGH, found that 39% of hormone-sensitive tumors and 50% hormone-independent tumors showed *PIK3CA* gene amplification (26). There have been no studies in which both *PTEN* and *PIK3CA* genes were analyzed in the same prostate cancer.

In the present study, prostate tumors were screened for *PIK3CA* alterations (mutations and amplification) and *PTEN* alterations (mutations, hemizygous and homozygous deletion), to assess alterations in the PI3K pathway in prostate cancer.

Patients and Methods

Prostate tumor samples. Forty samples from patients who underwent radical prostatectomy were obtained from the Pathology Department, Innsbruck Medical University, Austria. Whenever possible, cases in

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which tumor areas of different Gleason patterns, in particular Gleason patterns 3, 4 and 5 were clearly separately recognizable were chosen. Tumor areas with glands of different Gleason patterns were marked on formalin-fixed paraffin-embedded sections and were manually microdissected, and DNA was extracted as previously described (27). The mean age of patients with prostate cancer was 62.1 years (range, 49-74 years). Genetic analyses were first carried out on DNA samples extracted from the tumor areas containing glands of the highest Gleason grade within the tumor, and for cases with positive results, further genetic analyses were performed in tumor areas with lower Gleason grades and as well as non-tumorous prostate glands.

PIK3CA mutations. Prescreening for mutations in exons 9 and 20 of the *PIK3CA* gene was carried out by PCR-SSCP followed by direct DNA sequencing, as previously described (28). Primer sequences for PCR amplification were reported previously (28). PCR was performed in a total volume of 10 μL, consisting of 1 μL of DNA solution, 1 U of Taq DNA polymerase (Invitrogen, Cergy Pontoise, France), 1 μCi of α-^{33}P dCTP, 1.5-2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μM of both sense and antisense primers, 1 μL 10× buffer in a T3 thermocycler (Biometra, Archamps, France), with an initial denaturing step at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 1 min, annealing at 60-62˚C for 1 min, polymerization at 72˚C for 1 min and a final extension at 72˚C for 5 min. Samples with mobility shifts were further analyzed by direct DNA sequencing on an automated sequencing system (ABI PRISMTM 3100 Genetic Analyzer, Applied Biosystems, Hitachi, Japan) using an ABI PRISM BigDye Terminator version 1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Branchburg, NJ, USA).

PIK3CA amplification. *PIK3CA* amplification was assessed using quantitative real-time PCR, with an Mx3000P real-time PCR System (Stratagene, La Jolla, CA, USA). The *GAPDH* sequence was used as a reference, as previously reported (28). Sequences of primers and TaqMan probes have been reported previously (28). Primers and TaqMan probes were synthesized by Sigma-Proligo SAS (Paris, France). Each probe was labeled with FAM at the 5' end, and with BHQ1 at the 3' end. The conditions for real-time PCR were as previously reported (28). Briefly, each 20 μL of real-time PCR reaction contained 5 μL of DNA (approx. 3.2 ng/μL), 1× TaqMan Gold PCR Master Mix (Applied Biosystems), 0.3 μM of sense and antisense primers, and 0.1 μM of Taqman probe for *PIK3CA*; 0.1 μM of sense, 0.3 μM of antisense primer and 0.15 μM Taqman probe for *GAPDH*. The PCR reaction was performed in triplicate for each sample in 96-well polypropylene plates (Stratagene). The thermal cycling consisted of preheating at 50˚C for 2 min, followed by an initial denaturing step at 95˚C for 10 min, then 40 cycles consisting of 95˚C for 15 sec and 60˚C for 1 min. The cycle T threshold (Ct) of PCR, the standard curve of *PIK3CA* or *GAPDH* and the calculation of *PIK3CA* copy number were based on previous reports (28). Copy numbers of *PIK3CA* >3.0 were considered to constitute gene amplification with a confidence of 95% , as previously described (28).

PTEN mutations. Prescreening for mutations in exons 1-9 of the *PTEN* gene was carried out by PCR-SSCP. Primer sequences for PCR amplification were as reported previously (29), with the exception of exon 3 (sense primer, 5'-GGT GGC TTT TTG TTT GTT TG-3'; antisense, 5'-ACA ATG CTC TTG GAC TTC TTG AC-3'). Briefly, PCR was performed in a total volume of 10 μL, consisting of 1 μL of DNA solution, 1 U of Taq DNA polymerase (Invitrogen), 1 μCi of α-^{33}P dCTP, $MgCl_2$ (1.5-2 mM) , 0.2 mM of each dNTP, 1 μM of sense and antisense primers, 1 μL 10× buffer in the T3 thermocycler (Biometra), with an initial denaturing step at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 1 min, annealing at 51-62˚C for 30 sec, polymerization at 72˚C for 1 min and a final extension for 5 min at 72˚C. Samples with mobility shifts were further analyzed by direct DNA sequencing on an automated sequencing system.

PTEN homozygous deletion. *PTEN* homozygous deletion was assessed by differential PCR using primers for *PTEN* exon 2 (sense, 5'-TTT CAG ATA TTT CTT TCC TTA-3'; antisense, 5'-TGA AAT AGA AAA TCA AAG CAT-3'), together with primers for the *GAPDH* sequence (sense, 5'-AAC GTG TCA GTG GTG GAC CTG-3'; antisense, 5'-AGT GGG TGT CGC TGT TGA AGT-3'). Differential PCR was performed in a total volume of 10 μL, consisting of 6 μL of DNA solution (75 ng/μL), 1 U of Taq DNA polymerase (Invitrogen), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 1 μM of each *PTEN* primer, 0.1 μM of each *GAPDH* primer, 1 μL 10× buffer in the T3 thermocycler (Biometra), with an initial denaturing step at 95˚C for 3 min, followed by 32 cycles of denaturation at 94˚C for 1 min, annealing at 51˚C for 1 min, polymerization at 72˚C for 1 min, and a final extension at 72˚C for 5 min. PCR products were separated on an 8% acrylamide gel and ethidium bromide-stained bands were recorded by Kodak Digital Science ID Image software. Quantitative analysis of the bands for the *PTEN* gene and reference gene (*GAPDH*) was performed using image quantification software. The target gene dosage was calculated relative to normal DNA. A *PTEN:GAPDH* ratio of <0.3, relative to that of the average calculated in normal controls (formalin-fixed, paraffin-embedded sections from normal tissues) were regarded as evidence for homozygous deletion, as previously described (30).

PTEN hemizygous deletion. Quantitative microsatellite analysis was carried out using a microsatellite marker at the *PTEN* locus (10q23; D10S536) to assess loss of heterozygosity (31). PCR reactions were performed in a total volume of 18.75 μL with TaqMan Gold PCR Master Mix, 0.8 mM/L of each primer, 150 nM/L of probe (21 bp oligomer complementary to the microsatellite CA repeat: 5,6-carboxyfluorescein (FAM)-TGT GTG TGT GTG TGT GTG TGT-3,6-carboxytetramethylrhodamine) and approximately 30 ng DNA, with cycling parameters as reported (31). Primers and probes were purchased from Proligo Primers and Probes (Paris, France). TaqMan Gold PCR master mix was purchased from Applied Biosystems. PCR was carried out for each individual DNA sample in triplicate on a 96-well optical plate in MX3000P machine (Stratagene). Amplification of a reference pool of six reference loci served to normalize the differences in the amount of total input DNA, as described previously (31). The value of cycle threshold (Ct) Ct, δCt, δδCt, the relative copy number ($2^{-\delta\delta Ct}$) and the tolerance interval with a confidence of 95% were calculated as previously reported (31). Based on this tolerance interval, copy numbers below 1.33 were considered to represent loss, whereas those above 3.01 were considered to be gain.

Results

Genetic analyses in glands of the highest Gleason pattern within the tumor revealed *PIK3CA* amplification (copy numbers between 3 and 4.95) in 13% of cases, *PIK3CA*

Table I. *Genetic alterations in the PI3K pathway in prostate cancer.*

Case no.	Gleason score	*PIK3CA* amplification	*PIK3CA* mutation	*PTEN* homozygous deletion	*PTEN* hemizygous deletion
62	7	**G4**, G3, N	-	-	-
64	7	**G4**, **G3**, N	-	-	-
140	7	**G4**, **G3**, N	-	-	-
119	9	**G5**, G4, N	-	-	-
183	9	**G5**, **G4**, N	-	-	-
167	7	-	**G4***, G3	-	**G4**, **G3**, N
161	8	-	-	**G4**	-
121	7	-	-	**G4**, **G3**, N	-
153	7	-	-	**G4**, G3, N	-
180	7	-	-	**G4**, G3, N	-
189	7	-	-	**G4**, G3, N	-
85	7	-	-	-	**G4**, G3, N
138	9	-	-	-	**G5**, **G4**, N

G3, Gleason pattern 3; G4, Gleason pattern 4; G5, Gleason pattern 5. N, non-tumorous prostate glands. Numbers in bold letters indicate that alterations were present in tumor areas with the respective Gleason pattern. *Missense mutation at codon 545 of the *PIK3CA* gene (GAG->GCG; Glu->Ala).

mutations in one (3%) case (GAG->GCG at codon 545; Glu->Ala), *PTEN* homozygous deletion in 13% of cases, and *PTEN* hemizygous deletion in 8% of cases analyzed (Figures 1 and 2). These alterations were largely mutually exclusive except for one case. No tumor contained a *PTEN* mutation. Overall, 13 out of 40 (33%) prostate tumors showed at least one alteration in the PI3K pathway.

For cases with genetic alterations, other tumor areas with lower Gleason patterns as well as non-tumorous prostate glands were further analyzed. Of nine Gleason score 7 cases with genetic alterations, five cases contained the same genetic alterations in both tumor areas with glands of Gleason 3 and 4 patterns, while four cases contained genetic alterations in only tumor areas with glands of Gleason 4 pattern (Table I). Of three Gleason score 9 cases with genetic alterations, two cases contained the same genetic alterations in both tumor areas with glands of Gleason 4 and 5 patterns, while one case contained genetic alterations in only tumor areas with glands of Gleason 5 pattern (Table I). None of the non-tumorous prostate glands contained genetic alterations in the PI3K pathway.

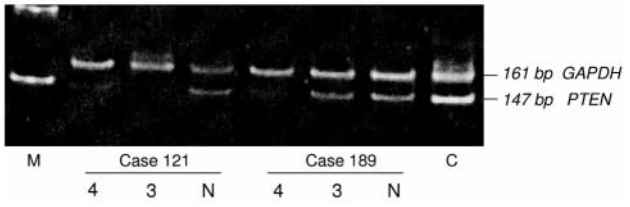

Figure 1. *PTEN homozygous deletion, detected by differential PCR. In one case (case 121), both tumor areas with Gleason patterns 3 and 4 show PTEN homozygous deletion, whereas in another case (case 189), PTEN homozygous deletion was observed only in a tumor area with Gleason pattern 4. M, Molecular size marker; 4, Gleason 4 pattern; 3, Gleason 3 pattern; N, non-tumorous prostate glands; C, control DNA.*

Discussion

The present study shows that alterations in the PI3K signalling pathway are common in prostate cancer, and occur mainly through *PIK3CA* amplification and *PTEN* deletion. Supporting the view that *PTEN* and *PIK3CA* genes act in the same signaling pathway, genetic alterations of these genes in prostate cancer were largely mutually exclusive. A similar reciprocal association between *PTEN* hemizygous deletion and *PIK3CA* amplification in gastric cancer has been reported (32).

It was noted in the present study that in all the prostate tumor samples with *PIK3CA* amplification, the level of amplification was relatively low (gene copy numbers of 3-4.95). Low-level amplification of *PIK3CA* (copy numbers 3-4) has also been reported to be common in other neoplasms. The majority (15/16) of primary cervical tumors had *PIK3CA* amplification with copy numbers >2.5 (33). Another study showed that 18 of 28 cervical cancers with *PIK3CA* amplification had copy numbers <4 (34). Even a low level of *PIK3CA* amplification may have significant functional consequences: cervical cancer cells with *PIK3CA* copy numbers 2.5-3.7 had increased p110α expression and kinase activity of PI3K, subsequently affecting aberrant cell proliferation and apoptosis (33); gastric cancer cells showing *PIK3CA* amplification (<5-fold) were associated with elevated levels of phospho-AKT (32).

Consistently with previous reports (8, 9, 15, 19-23), the present study showed that hemizygous or homozygous *PTEN*

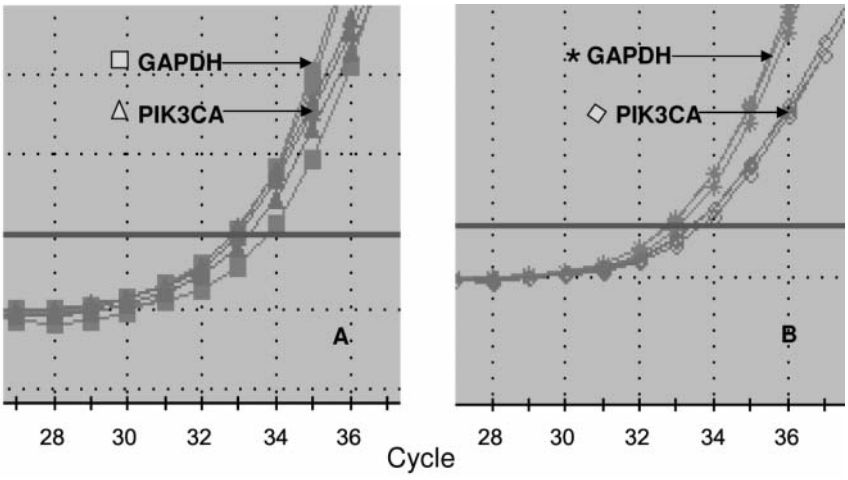

Figure 2. *PIK3CA amplification, detected by quantitative real-time PCR. The fluorescence from PIK3CA and GAPDH is plotted against cycle numbers. A, Prostate cancer (case 183, glands of Gleason 5 pattern) with PIK3CA amplification. B, Non-tumorous prostate glands without PIK3CA amplification. Each experiment was performed in triplicate and gave overlapping amplification curves.*

deletions are common in prostate cancer. Both hemizygous and homozygous *PTEN* deletions appear to have significant biological consequences. PTEN haploinsufficiency (hemizygous deletion) significantly promoted progression of prostate cancer in TRAP mice (35), and accelerated the formation of high-grade astrocytomas in mice lacking *Nf1* and *p53* (36). Yoshimoto *et al*. (24) reported that both homozygous and hemizygous *PTEN* deletions were significant prognostic markers of poor clinical outcome in prostate cancer patients.

The Gleason scoring system based on glandular differentiation is widely used for prostate cancer diagnosis, with Gleason patterns 3 and 4 being most common. Gleason pattern 3 is characterized by glands which are infiltrative between adjacent non-neoplastic glands, but each gland has an open lumen and is circumscribed by stroma. In contrast, the glands of Gleason pattern 4 appear to be fused or cribriform, and are composed of a group of glands that are no longer completely separated by stroma. Within the same tumor, separate areas with glands of Gleason 3 and 4 patterns may be observed, or glands of Gleason 3 and 4 patterns may be co-present in the same tumor area. Glands of Gleason 4 pattern may have evolved from neoplastic cells of Gleason pattern 3, or glands of Gleason 3 and 4 patterns may develop from independent cancer clones.

It is currently not clear whether differentiation status represented by different Gleason patterns reflects different genetic alterations. Using array CGH, Postma *et al*. (37) assessed tumor areas of Gleason patterns 3 and 4, and showed that there were no significant differences in genome-wide chromosomal imbalance between Gleason patterns 3 and 4, or between Gleason grades within one cancer. In the present study, carefully selected prostate cancer samples in which separated tumor areas of different Gleason patterns were recognized were used to show that glands of Gleason 3 pattern are genetically heterogeneous, with some but not all showing the same genetic alterations observed in those of Gleason 4 pattern within the same tumor.

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