# The Fibroblast Growth Factor Receptor-4 Arg$^{388}$ Allele Is Associated with Prostate Cancer Initiation and Progression

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## ABSTRACT

***Purpose:*** **Increased expression of fibroblast growth factors that can activate the fibroblast growth factor receptor-4 (FGFR-4) occurs in a substantial fraction of human prostate cancers *in vivo*. A germline polymorphism of the FGFR-4 gene resulting in expression of arginine at codon 388 (Arg$^{388}$) is associated with aggressive disease in patients with breast and colon cancer. We therefore sought to determine whether the FGFR-4 Arg$^{388}$ allele was associated with prostate cancer incidence and/or the occurrence of aggressive disease.**

***Experimental Design:*** **The FGFR-4 genotype of men undergoing radical prostatectomy and controls of the same race was determined and the genotype correlated with clinical and pathologic markers of disease aggressiveness. PNT1A cell lines expressing predominantly the FGFR-4 Arg$^{388}$ or Gly$^{388}$ allele were established, and cell migration and invasiveness of these cells were assessed by a wounding assay and by quantitative determination of invasion through Matrigel. Expression of urokinase-type plasminogen activator receptor was determined by quantitative RT-PCR and enzyme-linked immunoabsorption assay.**

***Results:*** **Homozygosity for the FGFR-4 Arg$^{388}$ allele is strongly associated with the occurrence of prostate cancer in white men. The presence of the FGFR-4 Arg$^{388}$ allele is also correlated with the occurrence of pelvic lymph node metastasis and biochemical (prostate-specific antigen) recurrence. Expression of FGFR-4 Arg$^{388}$ in immortalized prostatic epithelial cells results in increased cell motility and invasion through Matrigel and was associated with increased expression of urokinase-type plasminogen activator receptor.**

***Conclusion:*** **The FGFR-4 Arg$^{388}$ allele is associated with both an increased incidence and clinical aggressiveness of prostate cancer and results in changes in cellular motility and invasiveness in immortalized prostate epithelial cells consistent with the promotion of metastasis.**

## INTRODUCTION

Prostate cancer is the second most frequent cause of cancer deaths in North American men. The etiology of human prostate cancer is complex and, although many genetic and epigenetic alterations have been detected in human prostate cancer, the role of many of these changes in prostate cancer initiation and progression remain unclear. Members of the fibroblast growth factors (FGFs) family, a group of >20 structurally related proteins, are known to play an important role as growth factors in the normal human prostate, and alterations of the FGF axis seem to play a key role in prostate cancer progression. FGFs control a multitude of cellular processes in different contexts, including proliferation, differentiation, survival, and motility (1, 2). The FGF/fibroblast growth factor-receptor (FGFR) system also plays a critical role in cancer development attributable to its role in angiogenesis (1). Of particular relevance to prostate cancer is the observation that FGF signaling may be important in the progression of steroid hormone-dependent cancers to a hormone-independent state (3).

A number of FGFs are expressed at increased levels in human prostate cancer. Our laboratory has shown that FGF2 (4) and FGF6 (5) are both overexpressed in human prostate cancer. Other laboratories have found that FGF8 is overexpressed in prostate cancer and such overexpression is correlated with aggressive disease (6–8). Autocrine expression of FGFs and expression of FGF receptors have been reported in all of the commonly used prostate cancer cell lines, *i.e.*, PC-3, DU-145, and LNCaP (9–11). Recent evidence indicates that FGF-binding protein, a secreted factor that enhances FGF mitogenicity, is produced by prostate cancer cell lines and that decreasing FGF-binding protein by stable expression of a FGF-binding protein-specific ribozyme inhibits tumorigenicity of PC-3 cells *in vivo* (12). Thus prostate cancers express a variety of proteins that lead to enhanced FGF signaling *in vitro* and *in vivo*.

FGFs bind to a family of four distinct transmembrane tyrosine kinase receptors (FGFR1–4), and there is strong evidence that these receptors play a role in prostate cancer progression. Yan *et al.* (13) have shown that progression of prostate cancers in the Dunning rat system is associated with expression of FGFs not originally present in the tumors and changes in FGF-receptor expression consistent with autocrine FGF-receptor activation. The same group has shown that expression of FGFR-1 accelerates tumorigenesis in this system (14). In agreement with these findings in animal models, we have shown that there is increased expression of FGFR-1 in poorly differentiated human prostate cancers (4). Our laboratory has also shown that

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expression of dominant-negative FGF receptors, which block FGF-receptor signaling, leads to $G_2$ arrest and cell death in prostate cancer cell lines (2). Thus FGF-receptor signaling plays a key role in the cell cycle and cell survival in human prostate cancer and enhances prostate cancer progression.

The role of FGFR-4 in human prostate cancer has not been systematically examined. We have shown previously that FGFR-4 is expressed in normal human prostate, in prostate cancer cell lines, and in the immortalized human prostate epithelial cell line PNT1A. Of interest is the observation that FGF2, FGF6, and FGF8, which are all overexpressed in human prostate cancer, are potent activators of FGFR-4 (15). A role of FGFR-4 in breast cancer has been more clearly established. There is evidence that increased expression of FGFR4 occurs in breast cancer cell lines (16, 17) and amplification of the FGFR-4 gene occurs in a subset of breast cancers (18). Recently, the occurrence of a germline polymorphism in the FGFR-4 gene, resulting in expression of FGFR-4 containing either glycine ($Gly^{388}$) or arginine ($Arg^{388}$) at codon 388 has been reported (17). Bange *et al.* (17) found that the presence of the FGFR4 $Arg^{388}$ allele has a substantial negative impact on disease-free survival in breast cancer patients with lymph node metastasis, although another group has not observed a similar effect in their patient population (19). In addition, the presence of the FGFR4 $Arg^{388}$ allele was associated with metastasis and poor prognosis in colon cancer (17). By analogy, it seems likely that the presence of this FGFR-4 allele may contribute to disease progression in prostate cancer.

We report here that FGFR-4 is expressed in normal prostate epithelium, prostatic intraepithelial neoplasia, and prostate cancer epithelium *in vivo* and that homozygosity for the FGFR-4 $Arg^{388}$ allele is significantly associated with prostate cancer incidence in white patients. Furthermore, the presence of the FGFR-4 $Arg^{388}$ polymorphism is correlated with the occurrence of pelvic lymph node metastasis and prostate-specific antigen (PSA) recurrence in men undergoing radical prostatectomy. Expression of the FGFR-4 $Arg^{388}$ in immortalized prostate epithelial cells results in increased cell motility and invasion through Matrigel when compared with cells expressing the FGFR-4 $Gly^{388}$ allele. This was associated with up-regulation of the urokinase-type plasminogen activator receptor, which is known to promote invasion and metastasis (20). This may explain, in part, the increased aggressiveness of prostate cancers in men bearing this polymorphism. These findings indicate that FGFR-4 plays a substantial role in prostate cancer initiation and progression.

## MATERIALS AND METHODS

**Immunohistochemistry.** To confirm that FGFR-4 is expressed in the prostate cancer cells *in vivo*, a rabbit polyclonal anti-FGFR4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which has been shown previously to work well in paraffin-embedded material (21), along with 12 prostate cancer tissue samples from radical prostatectomy specimens were used to perform immunohistochemical staining. Immunohistochemistry was done as described previously (22). Primary antibody incubation was carried out at 4°C overnight at a 1:500 dilution, followed by the avidin-biotin peroxidase complex procedure (Vector Laboratories, Inc. Burlingame, CA). Before use, a 5-fold excess of blocking peptide was used to incubate a negative control antibody for 2 hours at room temperature.

Staining was evaluated semiquantitatively in normal epithelium, high-grade prostatic intraepithelial neoplasia and prostate cancer by a pathologist (M. I.). Weak staining was graded as 1+, intermediate staining was considered as 2+, and strong staining was considered 3+.

**Patient Tissue Samples and Nucleic Acid Extraction.** DNA was extracted from benign tissues from a total of 329 prostate cancer patients. Information about patients, such as race, Gleason score, pathologic stage, and PSA recurrence, defined as serum PSA $>$ 0.2 ng/ml, was obtained. Forty-five of these patients were African American and the remainder white. In four cases, some pathologic staging information could not be obtained, and PSA recurrence data were not available on 40 of the white patients. Genomic DNAs were extracted from benign prostatic tissue as described previously (23). According to the protocol supplied by Qiagen (Valencia, CA), DNeasy tissue kit was used to extract seminal vesicle tissue. According to the protocol supplied by Invitrogen (San Diego, CA), Trizol was used to extract RNA from 19 normal peripheral zone and 20 cancer tissues (at least 70% cancer) from radical prostatectomy specimens.

DNAs isolated from immortalized lymphocytes from two control groups of healthy individuals without a history of cancer were obtained from the Baylor Human Polymorphism Resource. The white control group consisted of 97 individuals whereas the African-American group consisted of 94 individuals. The race of each individual for both cancer patients and healthy controls was based on self-assignment.

**FGFR-4 Genotyping.** To determine the distribution of FGFR-4 $Arg^{388}$ allele and $Gly^{388}$ allele in prostate cancer patients and in control groups, the following primers were used: 5′-GACCGCAGCAGCGCCCGAGGCCAG-3′ and 5′-AGAGGGAAGAGGGAGAGCTTCTG-3′ (17). The PCR product of this pair of primer is 168 bp in length and corresponds to the transmembrane domain (exon 9) of FGFR-4. The G to A transition in codon 388 creates a new *Bst*NI restriction site; therefore, genotyping was done by PCR-RFLP analysis with this enzyme (17). Five hundred nanograms of genomic DNA were used in a 50-μl total PCR reaction volume. Annealing temperature was 70°C. After PCR, the product was digested with *Bst*NI (New England BioLabs, Beverly, MA) for 2 hours. Restriction fragments were resolved on a 15% nondenaturing polyacrylamide gel, and DNA was visualized by ethidium bromide staining. Two fragments of 82 and 27 bp characterized the $Arg^{388}$ allele, whereas a single visible band of 109 bp was observed for the $Gly^{388}$ allele with additional fragments of 22 and 37 bp present in both genotypes.

The computer program GENEPOP version 3.3 was used for tests of Hardy-Weinberg equilibrium (24). This program calculates exact *P* values when the number of alleles is $<$5, as for these evaluations. Calculations were made for each set of controls to evaluate for nonrandom sampling of controls, population substructure, non-independence of samples, missed alleles in genotyping, amplification of alternate sites in genotyping, and other defects.

**Tissue Culture.** To establish stably-transfected cell line expressing cDNAs encoding FGFR-4 with either $Arg^{388}$ or $Gly^{388}$, we used the nontumorigenic SV40-immortalized human prostatic epithelial cell line PNT1A (European Collection of Cell Cultures, Cambridge, United Kingdom), which has a Gly/Arg genotype. The cells were cultured in DMEM with high glucose (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum (Life Technologies, Inc.).

**Vector Construction, Transfection, and Isolation of Cell Clones.** We established stably-transfected PNT1A cell lines expressing cDNAs encoding either FGFR-4 $Arg^{388}$ or $Gly^{388}$ in the pCEP4 vector (Invitrogen), in which the cDNA is under control of a cytomegalovirus promoter. The pCEP4 vector also contains the hygromycin resistance gene, which allows selection of the transfected cells. Primers 5′-GTCCCTGAGAGCTGTGAGAAG-3′ and 5′-CCTTGCTCATGTCTGCACCCC-3′ were used to obtain the full-length human FGFR-4 cDNA, which is 2,415 bp in length, by PCR of prostate cancer cell line cDNAs. For cloning into the expression vector pCEP4, RNA from PC-3 cells (which have $Arg^{388}/Arg^{388}$ genotype) or DU145 cells (which have $Gly^{388}/Gly^{388}$ genotype) were initially used to clone the cDNAs into pTOPO vector (Invitrogen) after RT-PCR. In both cases the entire cDNA was sequenced to confirm both the genotype at codon 388 and the absence of mutations attributable to the PCR reaction. *Kpn*I and *Not*I restriction sites were then used to subclone the cDNAs into pCEP4 vector.

PNT1A cells were plated at $3 \times 10^5$ cells/60-mm dish and transfected with 6 μL Fugene6 (Invitrogen) and 2 μg of plasmid for 5 hour in a total volume of 4 ml of DMEM without serum. One milliliter of fetal calf serum was then added to each dish to achieve a final serum concentration of 20%. After an additional 18 hours of incubation, cells were re-fed with complete medium and then split 1:3 after 48 hours. The next day, selection was initiated by the addition of hygromycin at 400 ug/ml. Selection was carried out for 2 weeks, and long-term cultures were routinely maintained in hygromycin (100 ug/ml). Pooled clones of each type were used to carry out experiments.

**Migration (Wound Healing) Assay.** The ability of cells of each genotype to migrate into a defect in a monolayer culture was determined essentially as described by Bange *et al.* (17). Cells of each genotype were seeded at $2 \times 10^6$ in 60-mm-diameter culture dishes and grown to confluence in complete medium. Cells were gently scraped with a plastic tip. The medium was removed, and cells were washed twice with PBS and the medium replaced. The cells were permitted to migrate into the area of clearing for a total of 48 hours, and photomicrographs were taken at 24 hours and 48 hours.

**Matrigel Invasion Assay.** To investigate differences in invasive ability between cells expressing FGFR-4 $Arg^{388}$ or $Gly^{388}$ after transfection, we used BD BioCoat Matrigel Invasion Chamber (BD Bioscience, Bedford, MA). The chamber package was allowed to come to room temperature before use. Medium was added to inserts and wells and removed without disturbing the layer of Matrigel matrix on the membrane after rehydration for 2 hours in humidified tissue culture incubator. Cells ($2.5 \times 10^4$) of each genotype were added to inserts, and 0.75 ml of medium was added to the bottom of each well. After 24 or 48 hours of incubation, the non-invading cells were removed from the upper surface of the membrane. Following the protocol of the manufacturer (IMEB Inc., San Marcos, CA), Dif-Quik stain kit was used to stain the cells on the lower surface of the membrane. After staining, membranes were removed from the insert and mounted on slides, and the invading cells were counted under the microscope. Matrigel assays were performed in triplicate.

**Primer Design, cDNA Synthesis, and Quantitative Real-Time PCR.** The Molecular Beacon primer design program (PREMIER Biosoft International, Palo Alto, CA) was used to design primers for real-time PCR. Primers were all carefully designed to cross exon boundaries, avoid the formation of primer-dimers or hairpins, and self-complementarity. Primers were FGFR-4 forward, 5′-GGTGACTCCTTGACCTCCA-3′ and reverse, 5′-GGGGTAACTGTGCCTATTCG-3′; urokinase-type plasminogen activator receptor (uPAR) forward, 5′-CAACGACACCTTCCACTTC-3′ and reverse, 5′-GCACAGCCTCTTACCATATAG-3′; β-actin forward, 5′-AGCACGGCATCGTCACCAACT-3′ and reverse, 5′-TGGCTGGGGTGTTGAAGGTCT-3′ and keratin 18 forward, 5′-AGGGCTCAGATCTTCGCAAAT-3′ and reverse, 5′-GCT-ATCAATGACCTTGCGGAG-5′. RNeasy Mini kit (Qiagen) was used to extract total RNAs from cells. According to the protocol of the manufacturer (Bio-Rad Laboratories, Hercules, CA), iScript cDNA Synthesis kit was used to reverse transcribe 5 μg of each RNA sample into cDNA in total volume of 100 μL. Five microliters of the template cDNA or standard control vector that contained genes of interest was used to carry out real-time PCR in a final reaction volume of 25 μL. The buffer for real-time PCR contained 2 mmol/L $MgCl_2$, 0.4 μmol/L each forward and reverse primers, and 2.5 μL of LC-FastStart DNA Master SYBR GREEN (10×, Roche, Indianapolis, IN). The iCycler iQ instrument (Bio-Rad) was used to perform real-time PCR. The keratin18 plasmid (ATCC no. MGC-9348) and β-actin plasmid (ATCC no. MGC-10559) were purchased from American Type Culture Collection (Manassas, VA). As described by the manufacturer (Invitrogen), a Topo TA Cloning kit was used to clone the FGFR-4 and uPAR plasmids into the PCR 2.1-Topo vector. The Qiagen Spin Mini-prep kit was used to prepare plasmids. Quantification of plasmid was done spectrophotometrically. The plasmid concentration was converted into copy number, and a dilution series of each plasmid from $10^8$ to $10^2$ copies was used as DNA standard for real-time PCR. All real-time PCR efficiencies were controlled in the range of $100\% \pm 5$.

**Enzyme-Linked Immunoabsorption Assay (ELISA).** Human uPAR Immunoassay was purchased from R&D System Inc. (Minneapolis, MN). Culture supernatant was collected by centrifugation, and cells were lysed in radioimmunoprecipitation assay buffer. Bio-Rad Protein Assay was used to obtain protein concentrations. A total volume of 50 μL of each 5-fold-diluted sample and standard was used in ELISA assay following the manufacturer's protocol.

## RESULTS

**Immunohistochemical Analysis of FGFR-4 Expression in Prostate Cancer.** We have shown previously that FGFR-4 mRNA is expressed in normal prostate epithelial cells in culture, immortalized prostate epithelial cells, and prostate cancer cell lines (5). To confirm that FGFR-4 protein is expressed in prostate cancer cells *in vivo*, we carried out immunohistochemistry with an anti-FGFR-4 antibody on 12 paraffin-embedded prostate cancer tissues from radical prostatectomies. FGFR-4 immunoreactivity was found in normal epithelium from the peripheral zone, high-grade prostatic intraepithelial neoplasia, and in cancer epithelial cells. Examples of immunohistochemical staining for FGFR-4 are shown in Fig. 1. Staining of cancer was usually graded as 2+ or 3+, with some variability of staining within any given tumor (Fig. 1*A* and *B*). Staining of cancers was completely abolished by preincubation of antibody with the peptide used for immunization (Fig. 1*C*). High-grade prostatic intraepithelial neoplasia had staining that was similar in intensity to the carcinomas (2–3+). Normal epithelium had weak (1+) to moderate (2+) staining intensity (Fig. 1*E*). Positive immunohistochemical staining was also seen in occasional stromal cells (Fig. 1*F*). To confirm these observations, we analyzed FGFR-4 expression, using quantitative RT-PCR in 20 clinically localized prostate cancers and 19 normal peripheral zone tissues. On the basis of the immunohistochemical analysis, FGFR-4 is expressed primarily in epithelial cells; therefore, keratin 18, an epithelial specific marker expressed in normal and neoplastic prostate epithelial cells, was used to normalize expression. The mean FGFR-4 transcript level was 2.57 FGFR-4 transcripts/$10^2$ keratin 18 transcripts in the cancer samples and 1.51 FGFR-4 transcripts/$10^2$ keratin 18 transcripts in the benign tissue samples. This finding is consistent with our immunohistochemical observation that FGFR-4 expression is higher in the prostate cancer epithelial cells than the normal epithelium.

**FGFR-4 Genotype in Control Groups and in Cancer Patients.** To determine whether the presence of the Arg$^{388}$ allele was associated with increased incidence of prostate cancer, we carried out PCR-RFLP analysis of DNAs derived from benign tissues from control subjects and men with prostate cancer as described previously by Bange *et al.* (17). The frequency of each genotype at codon 388 of FGFR-4 is summarized in Table 1. In the white control group, the overall frequencies of the Gly/Gly, Gly/Arg, and Arg/Arg genotypes were 55, 41, and 4%, respectively. This frequency is not statistically different from that reported by Bange *et al.* (17) for a group of white German and Russian controls ($\chi^2 = 2.33$, $P < 1$). There

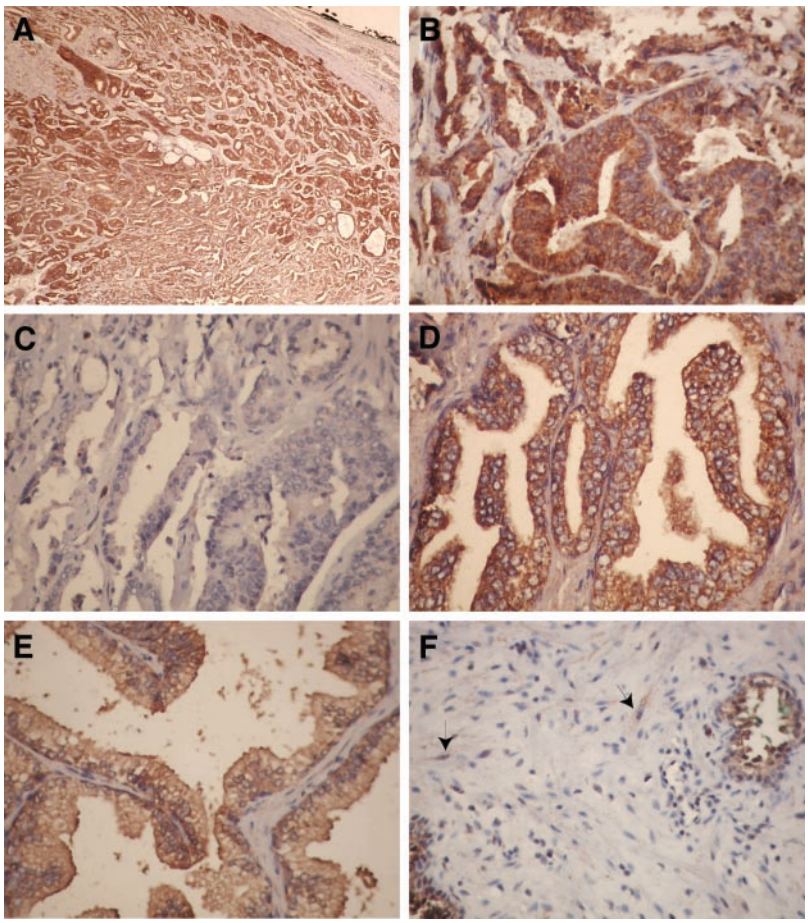


*Fig. 1* Immunohistochemical determination of FGFR-4 expression in prostate cancer, prostatic intraepithelial neoplasia, and normal prostate. Tissue sections from radical prostatectomies were analyzed by immunohistochemistry with an anti-FGFR-4 antibody as described in Materials and Methods. *A*, prostate cancer (original magnification, ×100). Prostate cancer (×400). *C*, Same prostate cancer as in (*B*) but immunohistochemistry done with antibody preincubated with excess peptide antigen. *D*, high-grade prostatic intraepithelial neoplasia (×400). *E*, normal prostatic epithelium (×400). *F*, Normal prostate (×400). Stromal cells expressing FGFR-4 are indicated by *arrows*.

*Table 1* FGFR-4 genotype in prostate cancer patients and control groups

| | FGFR-4 genotype | | | |
|---|---|---|---|---|
| | Gly/Gly (%) | Gly/Arg (%) | Arg/Arg (%) | $P$ ($\chi^2$) |
| White patients ($n$ = 284) | 125 (44) | 117 (41) | 42 (15) | 0.005 |
| White controls ($n$ = 97) | 53 (55) | 40 (41) | 4 (4) | |
| African-American patients ($n$ = 45) | 37 (82) | 6 (13) | 2 (5) | ND |
| African-American controls ($n$ = 94) | 76 (81) | 18 (19) | 0 | |

NOTE. FGFR-4 genotype was determined by PCR-RFLP as described in Materials and Methods. The number of patients or controls is indicated in parentheses, as is the percentage of patients or controls with the given genotype. The statistical significance of the differences in frequency of the Arg/Arg genotype *versus* the combined frequency of the Gly/Gly and Gly/Arg genotypes in patients *versus* controls was determined by Pearson's $\chi^2$ test. ND indicates that statistical tests were not done because of the small number of patients with certain genotypes.

is no evidence for deviation from Hardy-Weinberg equilibrium in the control group ($P > 0.41$). As can be seen in Table 1, the white prostate cancer patients had an almost 4-fold increase in the rate of homozygosity for the FGFR-4 $Arg^{388}$ allele (15%) when compared with white controls (4%). This difference is statistically significant ($P = 0.005$, $\chi 2$).

When African-American controls were analyzed, we found a much lower incidence of the $Arg^{388}$ allele than in white patients. The allelic frequency of the $Arg^{388}$ allele was 24.7% of all FGFR-4 alleles in the white control group *versus* 9.3% in African-American controls. This difference was highly statistically significant ($P < 0.00003$, $\chi^2$). The genotype frequencies, however, within the African-American controls, were as expected based on the allele frequencies; namely, testing for Hardy-Weinberg equilibrium showed complete agreement ($P = 1.0$). It is interesting to note that two of the 45 African-American prostate cancer patients were homozygous for the $Arg^{388}$ allele, whereas none of 94 controls had this genotype. However, given the lower frequency of the $Arg^{388}$ allele in African-American controls and the lower number of such patients in our sample, firm conclusions about the role of the $Arg^{388}$ polymorphism in African-Americans patients would be premature.

**Association of FGFR-4 Genotype with Clinical and Pathologic Characteristics of Clinically Localized Prostate Cancer.** Bange *et al.* (17) have shown a significant correlation between the presence of the $Arg^{388}$ allele and decreased disease-free survival in breast cancer patients with metastasis to axillary lymph nodes and with advanced pathologic stage in colon cancer. To determine the impact of the FGFR-4 $Arg^{388}$on the pathologic stage of prostate cancer at the time of radical prostatectomy for white men with clinically localized prostate cancer, we compared the pathologic characteristics and FGFR-4 genotype in white men. Table 2 shows the correlation between the FGFR-4 genotype and the pathologic stage and Gleason score for this group of 283 patients for which pathologic staging information was available. For the purposes of statistical analysis, men who were heterozygous and homozygous for the $Arg^{388}$ allele were treated as a single group. Combining these two genotypes is logical, based on the prior observations of Bange *et al.* (17) that the $Arg^{388}$ allele appears to act as a dominant factor in the clinical aggressiveness of breast and colon cancer, with the presence of a single $Arg^{388}$ allele significantly worsening prognosis in these two cancers. As can be seen in Table 2, there was a statistically significant association

*Table 2* Association between FGFR-4 genotype pathological parameters in white patients

| | | FGFR-4 genotype | | | | |
|---|---|---|---|---|---|---|
| | | Gly/Gly (%) | Gly/Arg (%) | Arg/Arg (%) | GA/AA (%) | $P(\chi^2)$ |
| Gleason score ($N$ = 283) | | $n$ = 125 | $n$ = 117 | $n$ = 41 | $n$ = 158 | |
| 5, 6 | 120 | 57 (48) | 46 (38) | 17 (14) | 63 (52) | |
| 7–9 | 163 | 68 (42) | 71 (44) | 24 (15) | 95 (59) | 0.33 |
| Pathological stage ($N$ = 280) | | $n$ = 123 | $n$ = 116 | $n$ = 41 | $n$ = 157 | |
| $T_2$ | 155 | 73 (47) | 61 (39) | 21 (14) | 82 (53) | |
| $T_3$ | 125 | 50 (40) | 55 (44) | 20 (16) | 75 (60) | 0.23 |
| LN metastasis ($N$ = 283) | | $n$ = 125 | $n$ = 117 | $n$ = 41 | $n$ = 158 | |
| (−) | 262 | 120 (46) | 104 (40) | 38 (14) | 142 (54) | |
| (+) | 21 | 5 (24) | 13 (62) | 3 (14) | 16 (76) | 0.04 |
| PSA recurrence ($N$ = 244) | | $n$ = 104 | $n$ = 105 | $n$ = 35 | $n$ = 140 | |
| (−) | 171 | 81 (47) | 70 (41) | 20 (12) | 90 (53) | |
| (+) | 73 | 23 (31) | 35 (48) | 15 (21) | 50 (69) | 0.02 |

NOTE. FGFR-4 genotype was determined by PCR-RFLP as described in Materials and Methods. The T stage is the pathological stage based on the American Joint Committee on Cancer staging system for prostatectomy specimens. The presence of lymph node (LN) metastasis was determined by pelvic lymph node dissection at the time of radical prostatectomy. The percentage of patients with the given genotype is indicated in parentheses. Assessment of the difference in frequency of the Gly/Gly genotype *versus* the combined Gly/Arg and Arg/Arg groups was done by Pearson's $\chi^2$ test.

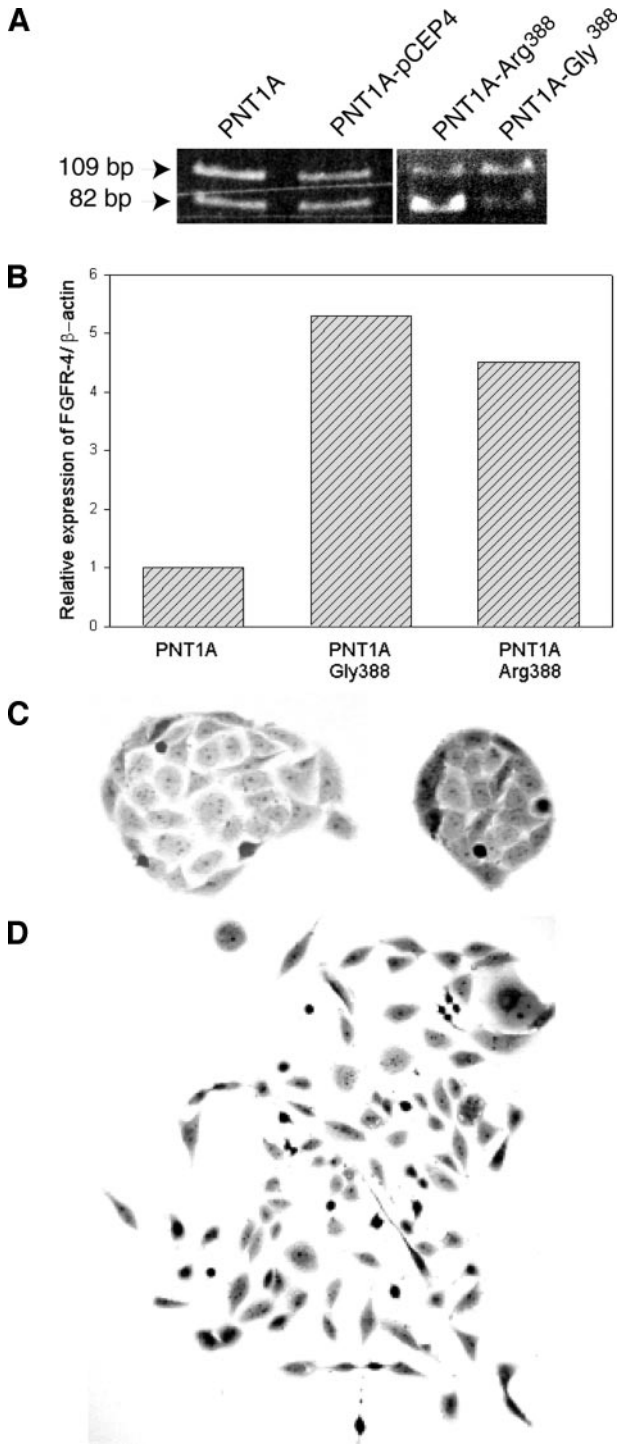


*Fig. 2* Characterization of PNT1A cells stably transfected with FGFR-4 Arg$^{388}$ or Gly$^{388}$ cDNA. Immortalized but nontumorigenic prostatic epithelial cells were stably transfected with cDNAs encoding either FGFR-4 Arg$^{388}$ or Gly$^{388}$ in the pCEP4 expression vector. After selection in hygromycin, pooled clones were characterized. *A*, PCR-RFLP of cDNAs of mRNAs extracted from parental PNT1A and PNT1A transfected with vector only (pCEP4) or with pCEP4 containing either FGFR-4 Arg$^{388}$ or Gly$^{388}$ were done as described in Material and Methods. Parental PNT1A have a Gly/Arg genotype, and both allelesare equally expressed, as indicated by the presence of two bands of equal intensity, one of 109 corresponding to the Gly$^{388}$ allele and one of 82 bp corresponding to the Arg$^{388}$ allele. A similar relative intensity is seen in PNT1A cells transfected with vector only. In PNT1A cells transfected with FGFR-4 Arg$^{388}$ cDNA, the relative intensity of the 82 bp band is markedly increased, whereas in cells transfected with FGFR-4 Gly$^{388}$ cDNA, the 82 bp band was barely visible, indicating that the majority of the FGFR-4 mRNA in the transfected cells arose from the transfected cDNA. *B*, relative expression of FGFR-4 in the transfected cell lines and parental PNT1A cells. FGFR-4 and β-actin RNA levels were determined by quantitative RT-PCR as described in Materials and Methods. The FGFR-4/β-actin ratio in the parental cells was then compared with the same ratio in the transfectants, and the fold increase is shown. *C*, typical rounded, well demarcated colonies observed in PNT1A cells transfected with FGFR-4 Gly$^{388}$ cDNA (×400) *D*, discohesive, irregular colony typical of PNT1A cells transfected with FGFR-4 Arg$^{388}$ cDNA.

($P = 0.02$, $\chi^2$) between the presence of at least one FGFR-4 Arg$^{388}$ allele and the presence of lymph node metastasis at the time of radical prostatectomy. In addition, we examined the association between the presence of the FGFR-4 Arg$^{388}$ allele and PSA recurrence in those 244 patients for whom these data were available. There was a statistically significant increase in the rate of PSA recurrence in men bearing the Arg$^{388}$ allele ($P = 0.02$, $\chi^2$). The presence of pelvic lymph node metastasis and PSA recurrence are both associated with distant metastasis, and in many cases, mortality from prostate cancer (25); therefore, the FGFR-4 Arg$^{388}$ allele is strongly associated with the occurrence of aggressive disease.

**Biological Effects of FGFR-4 Arg$^{388}$ Expression.** To determine the biological effect of FGFR-4 Arg$^{388}$ expression we established stable cell lines expressing either the FGFR-4 Gly$^{388}$ or Arg$^{388}$ alleles in the pCEP4 expression vector in the immortalized but nontumorigenic prostatic epithelial cell line PNT1A. The PNT1A cell line has Gly/Arg genotype, but the expression of FGFR4 is modest in this cell line. PNT1A cells were transfected with cDNAs encoding FGFR-4 with an Arg$^{388}$ or Gly$^{388}$ and after selection in hygromycin, individual cell clones were pooled. The relative expression of the transfected FGFR-4 construct, which is under the control of the strong cytomegalovirus promoter, and the endogenous FGFR-4 gene were assessed by PCR-RFLP of reverse-transcribed cDNAs from each set of pooled clones. As can be seen in Fig. 2*A*, the majority of FGFR-4 transcript in the pools of clones transfected with the FGFR-4 Arg$^{388}$ or Gly$^{388}$ construct is derived from the transfected gene, as indicated by the relative intensity of the PCR band corresponding to the genotype of the transfected cDNA in the RFLP analysis of the cDNAs from these pools. We also carried out quantitative RT-PCR to evaluate the relative expression of FGFR-4 in the parental cell line and two transfected cell lines. The two transfected cell lines both expressed approximately 5-fold higher levels of FGFR-4 than the parental cells (Fig. 2*B*). One difference between these two pools of cells that was immediately apparent was the colony morphology. We observed that cells expressing FGFR-4 Gly$^{388}$ often grew tightly connected to each other and formed circular or ovoid colonies (Fig. 2*C*). In fact, these cells appeared more tightly cohesive than the parental PNT1A cells. In contrast, cells expressing FGFR-4 Arg$^{388}$ usually grew in a scattered fashion, were loosely adherent, and had a more irregular morphology (Fig. 2*D*).

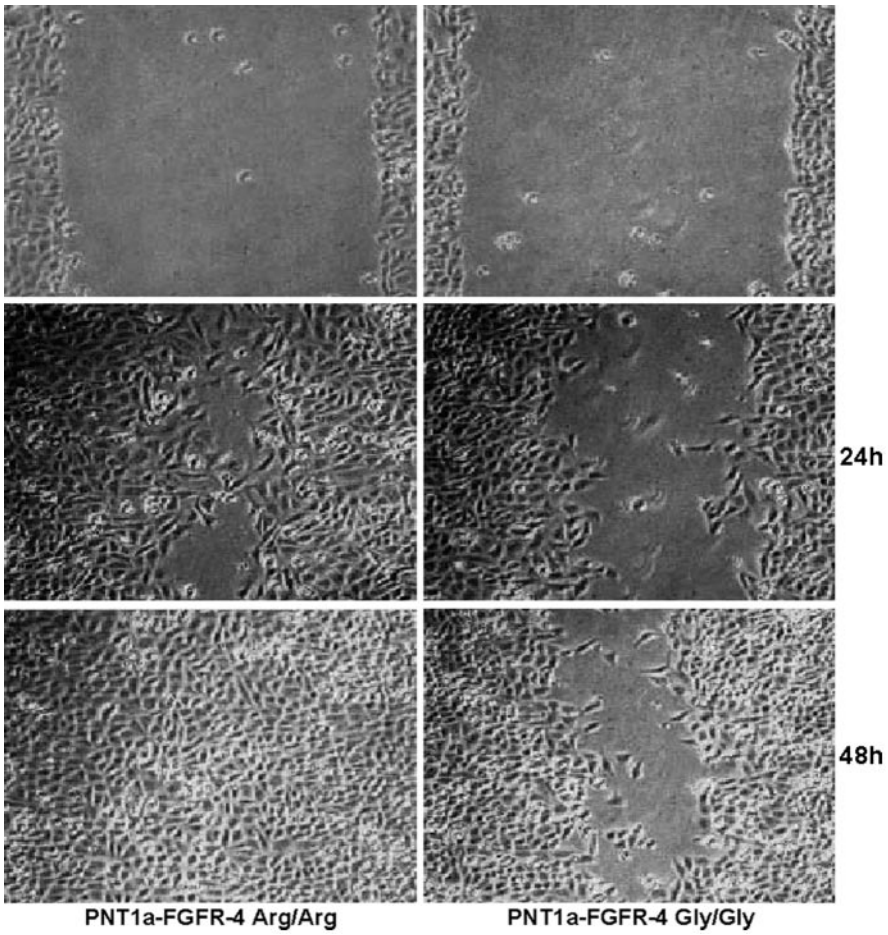


*Fig. 3* Migration (wound healing) assay with PNT1A cells expressing predominantly FGFR-4 $Arg^{388}$ or $Gly^{388}$. PNT1A cells stably transfected with either FGFR-4 $Arg^{388}$ or $Gly^{388}$ cDNAs were pooled, seeded at $2 \times 10^6$ in 60-mm-diameter culture dishes and grown to confluence. Cells were gently scraped with a plastic tip. The medium was removed, and cells were washed twice with PBS, and the medium was replaced. The cells were permitted to migrate into the area of clearing for a total of 48 hours, and photomicrographs were taken at 24 hours and 48 hours. Results are typical of three separate experiments.

Increased cell motility is one of the characteristics associated with malignancy and is involved in the genesis of metastatic disease. Therefore, we evaluated cell motility on plastic substrate by determining the rate of wound closure after scraping cells from an area of monolayer cultures, using PNT1A cells expressing predominantly FGFR-4 $Arg^{388}$ or $Gly^{388}$. Confluent monolayer cells were scraped, and cells were allowed to migrate for 48 hours. Cells expressing $Gly^{388}$ showed an obviously slower closure rate at both 24 and 48 hours when compared with cells expressing FGFR-4 $Arg^{388}$ (Fig. 3).

As an additional measure of cell motility and invasiveness, we assessed the ability of cells expressing FGFR-4 $Arg^{388}$ or $Gly^{388}$ to invade through Matrigel using a BioCoat Matrigel Invasion Chamber (Becton Dickinson, Franklin Lakes, NJ). These chambers contain inserts with an 8-μm pore-size membrane with a thin layer of Matrigel basement membrane matrix. The Matrigel matrix serves as a reconstituted basement membrane *in vitro*. The layer occludes the pores of the membrane, blocking noninvasive cells from migrating through the membrane. In contrast, invasive cells are able to detach themselves from and invade through the Matrigel matrix and the 8-μm membrane pores. Cells were plated on one side of the membrane, and after 24 or 48 hours cells on the opposite side of the membrane were stained and counted. Results of such experiment are shown in Fig. 4. Cells expressing the FGFR-4 $Arg^{388}$ had consistently higher invasiveness through Matrigel when compared with the cells expressing FGFR $Gly^{388}$.

The urokinase plasminogen activator system has been implicated in invasion and metastasis in many types of cancer (20), including prostate carcinoma (26). A key component of this system is the uPAR. This glycosylphosphatidyl inositol-anchored cell surface protein is a cellular receptor for urokinase-type plasminogen activator, which is expressed by many cell types, including prostate cancer cells. Through a variety of mechanisms including promotion of proteolysis and modulation of cell-extracellular matrix interactions (for review see ref. 20) the interaction of urokinase-type plasminogen activator and uPAR increases tumor migration and invasion. Of note is the observation than autocrine expression of FGF1 or FGF2 by rat NBTII cells was associated with both increased uPAR expression and increased invasiveness (27), suggesting that FGF-receptor activation may induce expression of uPAR. We therefore compared expression of uPAR mRNA and protein in PNT1A cells expressing FGFR-4 $Arg^{388}$ or $Gly^{388}$. As shown in Fig. 5, the expression of uPAR mRNA, as determined by quantitative real-time PCR, was approximately 2-fold higher in the FGFR-4 $Arg^{388}$ expressing cells. Measurement of uPAR protein in cell lysates and culture

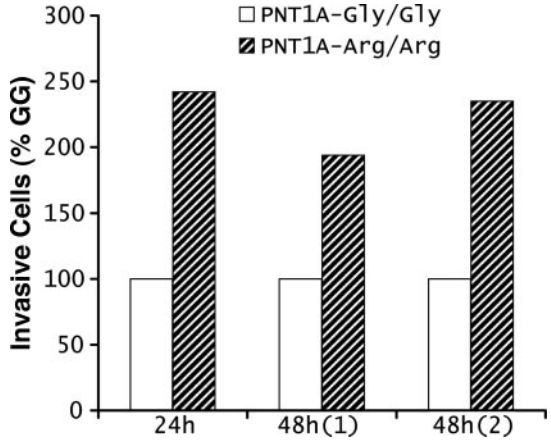


*Fig. 4* Matrigel invasion assay with PNT1A cells expressing predominantly FGFR-4 Arg$^{388}$ or Gly$^{388}$. PNT1A cells stably transfected with FGFR-4 Arg$^{388}$ or Gly$^{388}$ cDNAs were added to inserts of BioCoat Matrigel Invasion Chambers, and medium was added to the bottom of each well. After 24 or 48 hours of incubation, the non-invading cells were removed from the upper surface of the membrane. Diff-Quik stain kit was used to stain the cells on the lower surface of the membrane, the membranes removed from the insert and mounted on slides, and the number of invading cells was determined. The number of cells on the membrane of the PNT1A FGFR-4 Gly$^{388}$-transfected cells was considered as 100% in each experiment, and the number of cells on the membrane of the FGFR-4 Arg$^{388}$-transected cells is expressed relative to this value. Three separate experiments are shown, one with 24-hour incubation and two with 48-hour incubations.

medium showed a similar 1.5- to 1.8-fold increase (Fig. 5*B*). Thus, expression of the FGFR-4 Arg$^{388}$ is associated with increased expression of uPAR, which may account, at least in part, for the increased motility and invasiveness seen in these cells relative to control FGFR-4 Gly$^{388}$ expressing PNT1A cells.

## DISCUSSION

Germline DNA polymorphisms leading to alterations in the coding sequence of the encoded protein have been associated with cancer initiation and/or progression in a variety of human malignancies including prostate cancer. For example, the D104N polymorphism of endostatin (28) and the gly$^{50}$ allele of kallikrein 10 (29) are associated with an increased incidence of prostate cancer. Other DNA polymorphisms are associated with aggressive prostate cancer in men bearing the polymorphism. For example, the C154T polymorphism of the NKX3.1 gene is associated with increased Gleason score and advanced stage in prostate cancer (30). To date these polymorphisms have not been validated as adjuncts to existing markers used in screening for prostate cancer or incorporated into the planning of treatment for prostate cancer patients, but they potentially might add useful information to optimize diagnosis and treatment of men with prostate cancer.

One important property of DNA polymorphisms is that their incidence can vary substantially between different racial or ethnic groups. We found a highly significant difference in the prevalence of the FGFR-4 Arg$^{388}$ polymorphism in African Americans when compared with white American patients. Because of the low incidence of the Arg$^{388}$ polymorphism in the African-American population, larger numbers of African-American patients and controls will need to be assessed to determine whether the presence of the Arg$^{388}$ allele has a similar impact on disease incidence and/or progression in this group of men. If it does, this creates a paradox, in that African-American men have both a higher incidence of prostate cancer and a higher mortality rate from this disease. Given the lower prevalence of the FGFR-4 Arg$^{388}$ allele in African-Americans, it is likely that other factors (genetic and/or environmental) play a role in the higher prostate cancer incidence and mortality in African-American men and that these factors overwhelm the positive effect of the lower incidence of the FGFR4 Arg$^{388}$ allele in the African-American population, if indeed the Arg$^{388}$ allele has the same effect on prostate cancer in this group.

Homozygosity for the FGFR-4 Arg$^{388}$ allele was significantly associated with prostate cancer incidence in white patients. The odds ratio for the development of prostate cancer in men homozygous for the FGFR-4 Arg$^{388}$ allele is 4.04 (95% confidence interval, 1.41–11.56). To place this result in perspective, it should be noted that the relative risk of prostate cancer for men carrying deleterious BRCA-2 mutations is 4.6-fold (31).

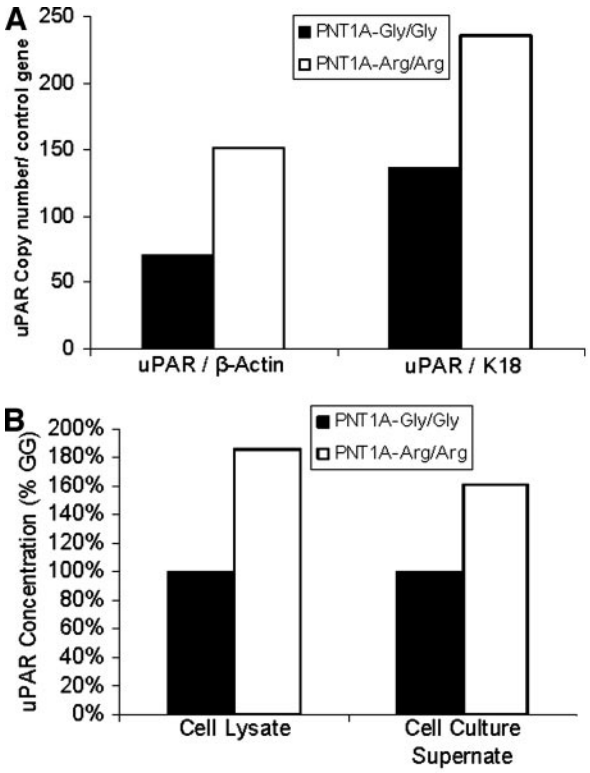


*Fig. 5* Expression of uPAR in PNT1A cells expressing predominantly FGFR-4 Arg$^{388}$ or Gly$^{388}$. *A*, mean uPAR mRNA copy number was determined by quantitative real-time PCR in triplicate and normalized to β-actin (per $5 \times 10^5$ copies) or keratin 18 (per $10^5$ copies) mRNA level. *B*, mean uPAR protein concentration per mg protein was determined in duplicate by ELISA. The uPAR concentration of PNT1A FGFR-4 Gly$^{388}$-transfected cells was considered as 100% in each experiment, and the uPAR concentration of the PNT1A FGFR-4 Arg$^{388}$ is expressed relatively to this value.

It is of interest to note that Bange *et al.* detected an increase in the incidence of homozygosity for the FGFR-4 $Arg^{388}$ allele in 145 white women with breast cancer (11%) *versus* 123 white controls (6%). Although this difference is not statistically significant, it does suggest that there may be an association of homozygosity for the FGFR-4 $Arg^{388}$ allele with breast cancer incidence that is weaker than that observed for prostate cancer. In fact, if our controls are combined with those of Bange *et al.*, the calculated odds ratio for the occurrence of breast cancer in women homozygous for the $Arg^{388}$ allele is 2.15 (95% confidence interval, 0.98–4.69), but the validity of combining the controls in these two studies is not clear. Obviously, a larger number of breast cancer patients and controls will need to be examined by a single group to determine whether indeed women homozygous for the FGFR-4 $Arg^{388}$ allele are at increased risk for breast cancer.

The presence of the FGFR-4 $Arg^{388}$ allele in men with prostate cancer was also associated with pathologic and clinical characteristics indicative of aggressive disease. Examination of white patients with prostate cancer revealed a strong association of the FGFR-4 $Arg^{388}$ allele with the occurrence of pelvic lymph node metastasis. The occurrence of pelvic lymph node metastasis is associated with the occurrence of systemic metastasis and death from prostate cancer (25). In addition, we noted a strong association of the presence of the $Arg^{388}$ allele with PSA recurrence after radical prostatectomy, which is also associated with increased mortality from prostate cancer (32). Larger studies will be needed to determine whether the presence of the $Arg^{388}$ allele is an independent prognostic parameter of pathologic stage, PSA recurrence, and/or survival in prostate cancer patients on multivariate analysis. Of note is the fact that the presence of even a single $Arg^{388}$ allele appears to be associated with aggressive disease, similar to the observations of Bange *et al.* in colon and breast cancer. This is in contrast to the observation above that homozygosity of the $Arg^{388}$ allele is associated with increased risk of cancer incidence in white men. The reason for this difference is unclear, but may reflect different biological mechanisms by which the FGFR-4 $Arg^{388}$ allele promotes tumor initiation as opposed to tumor progression, or differences in the cellular context between normal and neoplastic epithelial cells.

The biochemical mechanism by which the $Arg^{388}$ polymorphism leads to increased incidence of, and more aggressive clinical behavior in, prostate cancer is unclear. An analogous mutation of FGFR-3 at codon 380, in which a charged amino acid is introduced into the transmembrane domain, results in increased FGFR-3 signaling and is associated with a developmental disorders of the skeleton (33). A similar mutation in the Her-2/*neu* receptor, in which a hydrophobic amino acid is replaced by a charged amino acid in the transmembrane domain, results in increased tyrosine kinase activity and cellular transformation (34). However, Bange *et al.* (17) report that they were unable to show increased tyrosine kinase activity in breast cancer cells attributable to the $Arg^{388}$ polymorphism. We have also tried repeatedly to detect either increased basal FGFR-4 tyrosine phosphorylation or increased FGFR-4 tyrosine phosphorylation in response to exogenous FGF2 in FGFR-4 $Arg^{388}$ expressing cells (relative to FGFR-4 $Gly^{388}$ expressing cells) and have been unable to detect any differences (data not shown). It may be that currently available techniques are too subtle to reliably detect the differences. Alternatively, it is possible that the effect of the $Arg^{388}$ polymorphism may be attributable to other changes, such as alterations of ligand affinity or changes in the interaction with components of intracellular signal transduction pathways or other cell surface proteins.

The possibility must be considered that the FGFR-4 $Arg^{388}$ allele may be in linkage disequilibrium with some other genetic alteration that contributes to the increased risk of prostate cancer observed *in vivo*. However, it is clear that in comparison to cells expressing the FGFR-4 $Gly^{388}$ form of the receptor, cells bearing the $Arg^{388}$ allele have both increased motility and invasiveness, similar to the observations of Bange *et al.* (17) in breast cancer cells. In addition, we have shown increased expression of uPAR in prostate epithelial cells expressing the FGFR-4 $Arg^{388}$ allele in comparison to cells expressing FGFR-4 $Gly^{388}$allele. It is believed that uPAR can increase invasiveness by concentrating urokinase-type plasminogen activator at the cell surface, in particular at the leading edge of migrating cells. Furthermore, uPAR can regulate cell adhesion by direct high-affinity interaction with vitronectin, which in turn can promote cell motility. Finally, uPAR can modulate the function of integrins (20). These alterations would all be advantageous for both local invasion and distant metastasis. Thus, although we cannot exclude linkage disequilibrium of FGFR-4 $Arg^{388}$ with another genetic alteration that promotes prostate cancer progression, the occurrence of relevant biological and biochemical changes in response to FGFR-4 $Arg^{388}$ expression make this explanation less likely.

On the basis of our immunohistochemical studies, FGFR-4 is expressed in low to moderate levels in normal prostatic epithelial cells and at variable, but generally increased, levels in prostate cancer. Our quantitative RT-PCR analysis of FGFR-4 mRNA expression in normal and cancer tissues support these observations. Ligands for FGFR-4 such as FGF2, FGF6, and FGF8 are expressed at increased levels in prostate cancer (4–8), and although this does not prove that increased activation of FGFR-4 occurs in prostate cancer, it is certainly consistent with this idea. Johnston *et al.* (35) have shown that FGFR-4 is the only FGFR that can promote membrane ruffling when tranfected into COS-7 cells. Such membrane ruffling is associated with changes in the actin cytoskeleton related to increased motility. Thus, FGFR-4 activation may be more important in altering motility when compared with similar stimulation of other FGFRs. It is possible that synergistic effects among increased FGFR-4, increased FGFR-4 ligand expression, and the presence of the FGFR4 $Arg^{388}$ allele might result in prostate cancers with highly aggressive behavior. Systematic analysis of all of these factors and correlation of these findings with clinical and biological characteristics of disease aggressiveness should reveal whether such synergism in fact occurs.

Prostate cancer cells express all four types of FGF receptor. To date, most studies have focused on the role of FGFR-1 and its ability to promote prostate cancer progression. Our findings indicate that FGFR-4 may also play a role in prostate cancer progression. Additional studies to elucidate the biological roles of each of these receptors in prostate cancer progression and to define the extent to which their activities overlap are needed. In addition, studies to define the potential clinical utility of iden-

tification of alterations of FGFR-4, its ligands, and the presence of the Arg$^{388}$ polymorphism in predicting patient outcome and for optimizing treatment for patients with this common malignancy are also of critical importance. Finally, these studies support the idea that therapies targeting FGF-receptor signal transduction may have a role in the prevention and treatment of prostate cancer.

## ACKNOWLEDGMENTS

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