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Novel Mutations of Epidermal Growth Factor Receptor in Localized Prostate Cancer

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1. ABSTRACT

We recently demonstrated that EGFR protein overexpression is more common in African American (AA) prostate cancer patients compared to Caucasian patients. We further examine *EGFR* dysregulation by determining *EGFR* mutation status in the tyrosine kinase (TK) domain in prostate cancer patients of different ethnicity. Normal and tumor DNA from 89 radical prostatectomy cases were studied for mutations in the EGFR TK domain using genomic DNA sequencing. We identified 4 novel missense mutations in exons 19, 20 and 21 of EGFR TK domain: 3 in Koreans and 1 in Caucasian but none in AA. We also identified 5 distinct synonymous DNA sequence changes, which did not alter the encoded amino acid, in exons 20 and 21 in 31/89 (35%) patients. Interestingly, these synonymous sequence changes were not observed in normal DNA in 7(23%) patients, indicating the presence of *de novo* somatic mutation to a new synonymous sequence. Our data reveal that *EGFR* missense mutation in the TK domain occurs in localized prostate cancer. Our data also demonstrate the presence of somatic mutation to a new synonymous sequence in a subset of patients. Larger population-based studies are required to define the association between EGFR mutations and the ethnic background of patients.

2. INTRODUCTION

Several groups have demonstrated the role for increased EGFR signaling in prostate carcinogenesis and progression (1-3). This critical role has been further supported by preclinical studies showing that EGFR inhibitors can effectively hinder the growth of both androgen-dependent and androgen-independent prostate cancer xenografts (4-6). Although predictors of clinical response to EGFR inhibitors have not been elucidated fully (7, 8), it has been shown recently that a subset of patients with non–small-cell lung cancer have specific mutations in the *EGFR* gene that correlate with clinical responsiveness to the tyrosine kinase inhibitor, gefitinib (9-11).

EGFR overexpression is identified as a mechanism of *EGFR* dysregulation in both primary and metastatic prostate cancer clinical specimens (12-15). In this context, we recently showed that EGFR is overexpressed more commonly in African American (AA) patients as compared to Caucasian patients, contributing to the worse prognosis of prostate cancer in AA (16). In this study, we further examined prostate cancer patients of differing ethnic backgrounds for *EGFR* mutations in the tyrosine kinase (TK) domain. In addition to the AA and Caucasian

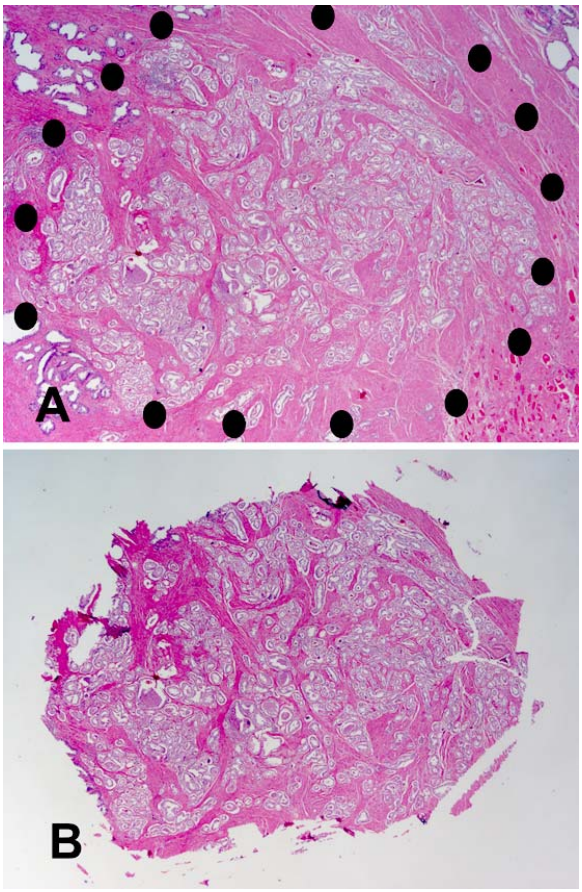


Figure 1. Macrodissection of Prostate Cancer Cells for DNA Sequencing. Macrodissection of prostate cancer cells for DNA sequencing. 1A. The area of interest (prostate cancer) located within the dots was circled by a pathologist. 1B. The undesired area was removed with a scalpel. Shown is the area of interest to be used for DNA extraction. In general, the cancer cells in the area of interest were greater than 90% of total epithelial cells.

prostate cancer patients, we included South Korean patients in view of the published data in non-small cell lung cancer which demonstrated a higher frequency of *EGFR* mutations in the TK domain in Asian patients (17, 18).

This is the first study, we are aware of, that reports on *EGFR* TK domain mutations in prostate cancer patients with various ethnic backgrounds. We demonstrate that *EGFR* missense mutation in the TK domain occurs in localized prostate cancer. We identified 4 novel missense mutations, 3 of which we found in Korean patients. Therefore, larger population-based studies are needed in Asia to determine if there is a true increase in the rate of EGFR TK domain missense mutations in prostate cancer in this population. We also report the novel observation of the presence of *de novo* somatic mutation to a new synonymous sequence in a subset of patients. Nevertheless, functional studies are needed to determine the oncogenic impact of these mutations and its relationship with response to anti-EGFR agents.

3. PATIENTS AND METHODS

3.1. Patients' Characteristics

Patients were identified through review of the Department of Urology database at the Veterans Administration Medical Center (VAMC)/New York University School of Medicine, New York, NY. This prospective database enrolled patients with prostate cancer from 1990 to the present, documenting patient demographics and characteristics, including racial background, stage, and grade of the primary tumor. The study has been conducted under institutional review board approval. We accrued 53 patients from VAMC [26 Caucasians (mean age 65, Gleason <7 n=15, ≥7 n=11, Stage T2 n= 21, ≥T3 n=5), and 27 African Americans (mean age 63, Gleason <7 n=6, ≥ 7 n=21, Stage T2 n=15, ≥T3 n=12)]. The patients self identified themselves as AA or Caucasians during the hospital registration process, and this was confirmed by a review of the clinical notes prior to data analysis. Taking into consideration of the reports of the interethnic variation of *EGFR* mutations in cancers other than prostate, we included 36 patients' specimens from South Korea (mean age 65, Gleason <7 n =1, ≥7 n =35, Stage T2 n=14, ≥T3 n=22). These specimens were obtained from Department of Pathology, Asan Medical Center, Seoul, South Korea. The study was also approved by the Asan Medical Center institutional review board.

3.2. DNA extraction, PCR amplification and Sequencing Methods

Genomic DNA was extracted from macro-dissected paraffin sections of the tumors. The area of interest (prostate cancer) was circled by an attending pathology investigator (Peng Lee) The tissues of interest were isolated by removing the undesired area with a scalpel. In general, the cancer cells in the area of interest were greater than 90% of total epithelial cells (Figure 1A and B). We did not have to perform micro-dissection of our cases as it was technically feasible to separate the tumor from the normal tissues. Another tissue block comprising only normal prostate tissue was chosen for corresponding normal DNA extraction. Qiagen kit was used for DNA isolation according to the manufacture's instructions after deparafinization of the tissue sections. Primers for PCR amplification of *EGFR* exons 19, 20, and 21 and flanking intronic sequences were designed according to the previous study by Paez *et al* (17). PCR products were then sequenced using ABI Big Dye Terminator Chemistry (Version 3.1) and analyzed on an ABI 3730 Capillary Sequencer (Memorial Sloan-Kettering Cancer Center, New York, NY). PCR was performed in a total volume of 10 µl, containing 20 ng of DNA and a reaction mixture of 1.5mM $MgCl_2$, 5 mM dNTP, 0.1 µl Taq DNA polymerase, and 3 µM of each primer with an annealing temperature of 60°C for 30 seconds. The results of DNA sequencing were analyzed using NCBI alignment algorithm (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov, Bethsada, MD).

3.3. Immunohistochemical Analysis of EGFR Expression

We have previously reported on the EGFR expression status of the African American and Caucasian

Table 1. Baseline Characteristics of 89 Prostate Cancer Patients Studied for *EGFR* TK Domain Mutation

	Caucasian (n=26)	African American (n=27)	Korean (n=36)
Age Mean (Range)	65.5 (48-81)	63.4 (49-72)	65 (54-76)
Pathologic Stage			
T2	21	15	14
≥T3	5	12	22
Gleason Score			
<7	15	6	1
7	8	18	11
>7	3	3	24

Table 2. Clinicopathological Profile and EGFR Expression Status of Prostate Cancer Patients with Missense Mutations in *EGFR* TK Domain

Patient	Ethnicity	Age	Gleason	pT	EGFR Expression	Exon	Nucleotide	Amino Acid Location	Amino Acid Change
1	Korean	67	9	2	Overexpression	20	2386 G>A	G796S	Glycine to Serine
2	Korean	62	9	3	Normal	20	2411 A>G	E804G	Glutamic Acid to Glycine
3	Korean	53	7	2	Normal	19	2203 G>A	G735S	Glycine to Serine
4	Caucasian	71	7	3	Normal	21	2522 G>A	R841K	Arginine to Lysine

patients (16). We performed immunohistochemistry for the Korean patients using the same methodology. EGFR expression was dichotomized as 0 and 1 (for negative for overexpression) or 2 and 3 (for positive for overexpression) (16).

3.4. Statistical Analysis

Missense and synonymous sequence changes were scored in three groups (AA, Caucasian, and Korean). Age was dichotomized as <65 and ≥ 65. Gleason grade was dichotomized into <7 and ≥ 7. Stage was dichotomized as 2 and ≥3. We did not examine the possible association between missense mutation and baseline characteristics because of the infrequent nature of the missense mutation. The association between the presence of synonymous mutations and ethnic background was evaluated using a logistic regression model by treating mutation as the dependent variable given a binary value with 1 indicating the presence of a mutation and 0 indicating wild type, and the ethnic group as the independent variable. The association between the presence of synonymous mutations and age, Gleason grade, stage, and EGFR expression were determined using a Fisher's exact test. Five patients with non-interpretable EGFR expression status were excluded from the analysis of EGFR expression.

4. RESULTS

To identify mutations in TK domain of *EGFR* and determine their frequency in prostate tumors from patients of varying ethnicity, we sequenced DNA from a total of 89 cases of primary prostate cancer encompassing three different patient cohorts (AA, Caucasians and Koreans). Table 1 summarizes the patients' baseline characteristics. We identified 4 missense mutations and 5 different synonymous sequence changes.

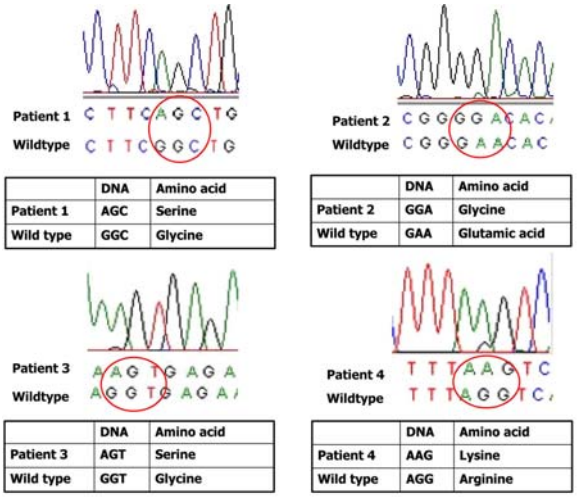
The four distinct missense mutations are all novel (Figure 2). Two of the missense mutations converted the non-phosphorylatable amino acid at that position to a phosphorylatable Serine: GGC was changed to AGC at codon 796 of exon 20 resulting in an amino acid substitution from Glycine to a phosphorylatable Serine (G796S) and GGT was changed to AGT resulting amino acid substitution from another Glycine to a phosphorylatable Serine at codon 735 of exon 19 (G735S). In the third missense mutation, GAA was changed to GGA from Glutamic acid to Glycine at codon 804 of exon 20 (E804G). Finally, AGG was changed to AAG resulting in a change of Arginine to Lysine at codon 841 in exon 21 (R841K) (Table 2, Figure 2). The 3-D rendering of the TK domain (Figure 3) suggests possible mechanisms for *EGFR* dysregulation. The first is structural. Three of the mutations, G735S, G796S, and E804G, are located on external portions of the TK domain structure. G735S is located on the beta-strand of the N-terminal lobe. G796S and E804G are located within alpha helices of the C-terminal lobe. These oncogenic substitutions may induce a conformational change of the kinase domain, leading to its activation. The last mutation, R841K, is located within the catalytic loop. The side chain of the wild type arginine 841 binds with the side chain of the aspartate at codon 837 with a distance of 3.06 Å. Substituting a lysine for an arginine disrupts this binding and lead to a change in tyrosine kinase activity. Of note, 3 of the 4 missense EGFR mutations were detected in Korean patients while only 1 was found in Caucasian patients. None of the AA patients had mutations. Of note, the 4 missense mutations were observed in patients with poorly differentiated tumors and only one patient showed protein overexpression.

Five synonymous sequence changes were found in 31 of the 89 patients, 7 of whom did not have the same sequence change in their normal DNA indicating that they were *de novo* tumor events. Table 3 shows the baseline characteristics of these 7 patients (3 Caucasians, 2 AA and 2 Koreans). Of note, these 7 patients are different than the patients with missesnse mutations.

The first synonymous sequence change (787 Glutamine G>A) was found in 26 (14 Caucasian, 8 AA, 4 Korean) patients and corresponds to NCBI SNP database ID#: rs10251977. In 16 patients the A allele was also observed in the germline DNA. In 5 (2 AA, 2 Caucasian and 1 Korean) patients, this sequence change was a *de novo* event in the tumor and was not observed in the patient's normal tissue DNA. The status of the normal DNA for the remaining 5 patients is unknown because we did not have enough normal tissue available for sequencing. The observed frequency of the A allele of rs10251977 in the

Table 3. Clinicopathological Profile and EGFR Expression Status of Prostate Cancer Patients with *de novo* somatic mutation to a new synonymous sequence in *EGFR* TK Domain

Ethnicity	Age	Gleason	pT	EGFR EXP	Exon - Codon
Korean	54	8	3	3	21 - 858
Korean	73	7	2	2	20 - 783
African American	65	7	2	0	20 - 783
African American	52	6	2	1	20 - 787
Caucasian	69	6	2	0	21 - 875
Caucasian	69	7	3	2	20 - 787
Caucasian	67	5	2	0	20 - 787



	DNA	Amino acid
Patient 1	AGC	Serine
Wild type	GGC	Glycine

	DNA	Amino acid
Patient 2	GGA	Glycine
Wild type	GAA	Glutamic acid

	DNA	Amino acid
Patient 3	AGT	Serine
Wild type	GGT	Glycine

	DNA	Amino acid
Patient 4	AAG	Lysine
Wild type	AGG	Arginine

Figure 2. Nucleotide Sequence of the EGFR TK Domain Resulting in Amino Acid Substitutions with the Corresponding Normal DNA Showing Wild-type Sequence. Nucleotide sequence of the EGFR TK domain in tumor specimens from the four patients with missense mutations resulting in amino acid substitutions. The corresponding normal DNA wild-type sequence and amino acid is shown.

germline DNA of the prostate cancer patients was similar to the racial distribution of the allele frequencies as reported in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).

The second synonymous sequence change, identified in 2 patients (1 Korean, 1 AA), was an ACC to ACT conversion at codon 783 (threonine) in exon 20 13bp upstream of the rs10251977 polymorphism. It was not identified in the corresponding normal DNA of these two patients indicating that it is a somatic mutation in the tumor.

The remaining 3 synonymous sequence changes were identified in 3 patients: one Korean patient had a GTG to GTA conversion at codon 819 (Valine) of exon 20 and another Korean patient had a CTG to TTG conversion at codon 858 (Leucine) in exon 21 previously described (19). The third was an Caucasian patient who had a novel AAA to AAG conversion at codon 875 (Lysine), the last codon of exon 21 in addition to conversion to the A allele of rs1025197 (Table 4). These 3 synonymous sequence changes were not identified in the corresponding normal DNA indicating that it is a somatic mutation in the tumor.

Immunohistochemical results of EGFR protein expression revealed that EGFR overexpression was significantly less common in Caucasian patients, 4 of 22 (18%) Caucasian compared to 12 of 26 (46%) AA and 26 of 36 (72%) Korean patients ($p < .001$). Based on a Fisher's exact test, there was a significant association between the presence of synonymous sequence changes and positive EGFR expression ($p = 0.03$), but no significant association between the presence of a synonymous sequence changes and Gleason grade ($p = 0.20$), or stage ($p = 0.36$). In a multivariate analyses, the association between presence of synonymous sequence changes and EGFR expression was not significant after controlling for race ($p=0.45$).

5. DISCUSSSION

We report here on our study of *EGFR* TK domain in prostate cancer patients of differing ethnic backgrounds. Our study demonstrates several important points. First, *EGFR* missense mutation in the TK domain does occur in prostate cancer; however, it is an infrequent event in patients with localized disease. We identified 4 missense mutations that have not been described before, three of them in Korean patients. Second, synonymous sequence changes in the *EGFR* TK domain is a common event in general and in a subset of patients, it represents a *de-novo* somatic mutation which suggest that it might be related to transformation in the tumor. Third, the patients who had *de-novo* somatic mutations were different than those who had the missense mutations which suggest that the two alterations are independent. Lastly, there is no evidence that *EGFR* mutation, in our studied cohort, has an association with EGFR expression. Taken together, our data suggest that several independent alterations of *EGFR* occur in prostate cancer.

The first alteration is missense mutation in the *EGFR* TK domain that despite its infrequency in general, was observed in predominately Korean patients (3 of 4 patients). This is consistent with what has been reported in lung cancer patients (17, 20, 21). In fact, our analyses suggest that prostate cancer patients of Korean background have an *EGFR* mutation frequency (8% , 3 of 36) similar to what has been recently described in colorectal cancer patients from Japan (12%, 4 of 33 patients) (19) and head and neck cancer from South Korea (7.3%, 3 of 41 patients) (22). While these studies are limited in sample size, precluding us from making any strong conclusions, the reproducibility of the observation of greater tendency of

Table 4. Description of Synonymous Sequence Changes of EGFR in 31 Prostate Patients

Exon Affected	Codon, Sequence Change	Amino Acid Affected	Number of Patients (Ethnicity)	Number of Patients with Somatic Mutation (Ethnicity)
20	783, ACC to ACT	Threonine	2 (1 Korean, 1 AA)	2 (1 Korean, 1 AA)
	787, CAG to CAA	Glutamine	26 (14 Caucasian, 8 AA, 4 Korean)	3 (2 Caucasian, 1 AA)
	819, GTG to GTA	Valine	1 (Korean)	1 (Korean)
21				
	858, CTG to TTG	Leucine	1 (Korean)	1(Korean)
Combination[1]				
20 21	787, CAG to CAA 875, AAA to AAG	Leucine Lysine	1 (Caucasian)	1(Caucasian)

Abbreviations: Nucleotide -G, guanine; A, adenine C, cytosine; T, thymine; Amino acid – G, glycine; S, serine, E, glutamic acid; R, arginine; K, lysine. 1. One patient has a synonymous sequence change at both exon 20 (787) and exon 21 (875).

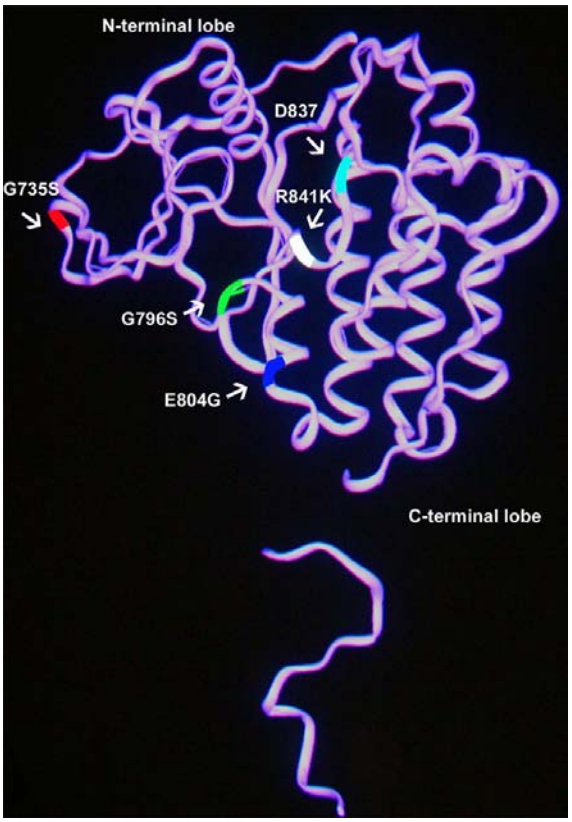


Figure 3. Computational 3D structure of TK domain showing the physical location of four identified novel missense mutations. Computational 3D structural analysis suggests possible mechanisms for the four missense mutations of the TK domain in EGFR dysregulation. Structurally, the amino acids of the three mutations, G735, G796, and E804, are located on the external surface of the tyrosine kinase domain . G735S (red) is located on the beta-strand of the N-terminal lobe. G796S (green) and E804G (blue) are located within alpha helices of the C-terminal lobe. The oncogenic substitutions, G735S, G796S, and E804K, may induce a conformational change of the kinase domain, leading to its activation. The last mutation, R841K (White), is located within the catalytic loop. The guanidinium ion of the side chain of the wild type Arg 841 forms an ion pair with the carboxylate side chain of Asp 837 (cyan) (distance of 3Å). Substituting a lysine for an arginine may disrupt this binding and lead to a change in tyrosine kinase activity.

Asians towards *EGFR* TK domain mutations in tumors by three independent groups on 3 different types of tumor suggests a difference in germline genetic background which interacts with the *EGFR* gene as has been recently been proposed (20). Nevertheless, a recent report from a Korean group, who screened 536 patients, did not reveal *EGFR* mutations in breast, gastric or hepatocellular carcinomas (23). This adds to the complexity of our limited understanding of the correlation of racial background and tumor type, if any, with *EGFR* TK tumor mutations.

The four missense mutations in the TK domain of *EGFR* gene identified in our study have not been described before. Two of the 4 mutations converted a nonphosphorylatable amino acid into a phosphorylatable Serine. As protein phosphorylation is important in regulating its activity, (24) the change from a non phosphorylatable amino acid to a Serine capable of being modified by phosphorylation might be important in *EGFR* signaling. In addition, the 3-D rendering of the TK domain suggests two possible mechanisms for *EGFR* dysregulation as a result of these 4 missense mutations. The first is structural as 3 of the mutations are located on external portions of the TK domain structure. Substituting these amino acids may induce a conformational change. The second mechanism is via the disruption of autoregulation as the remaining mutation is located within the catalytic loop (Figure 3). However, it is unclear how this disruption could promote constitutive oligomerization or increase the sensitivity of the receptor to its ligand. While the mechanism of how missense mutation of *EGFR* gene might lead to cell transformation is unclear at this point, the mechanism where by missense mutation in a gene can result in cell transformation is well studied for a number of proteins involved in cell cycle regulation like ras-p21. Missense mutations resulting in amino acid substitutions at critical positions in the polypeptide chain, such as at Gly 12 and Gln 61, cause hyperactivation of ras- p21 protein resulting in cell transformation (25). On the other hand, overexpression of normal ras-p21 can also result in malignant transformation due to mutations in its gene promoter, resulting in overexpression of the wild-type protein that is also oncogenic (25). Both of these phenomena can occur independently, resulting in a hyper-oncogenic protein, i.e., an over-expressed and mutated protein (26). One other example is missense mutation in p53 gene causes protein accumulation in the cell, though the protein is inactivated (27). In this study, we observed 4 missense mutations where one case showed EGFR overexpression. Functional studies are required in the

future to elucidate the association between missense mutations and EGFR overexpression and function in prostate cancer.

Another important observation in this study is the identification of synonymous sequence changes in the *EGFR* TK domain that represent a mixture of *de novo* somatic mutation to a new synonymous sequence and common germline polymorphism. This observation led us to postulate that these synonymous sequence changes might confer some transformation advantage to the cells that carry them or alternatively they may be secondary to the disruption of genomic proofreading mechanisms during tumorigenesis. One possibility is that they might lead to changes in *EGFR* mRNA splicing. To assess this possibility we used the Automated Splice Site Analysis tool developed at Mercy Hospital (https://splice.cmh.edu, Information analysis of human splice site mutations, Human Mutation). This analysis revealed that the five *de novo* synonymous changes are likely to decrease the efficiency with which the normal splicing recognition sites for these exons will be used during mRNA processing (data not shown). This could lead to the production of abnormal mRNA splice variants with missing or added sequence. An example of a deleterious common polymorphism which alters splicing and deletes two codons has been reported in the *XRCC4* gene, which participates in DNA double strand break repair, and is supported by multiple Genbank entries of both transcripts (28). Although functional studies will be required to determine whether this event will alter the distribution of splice variants produced in prostate tumor cells and what the coding sequences of these putative splice variants may be, these data suggest that this issue merits further study.

Our study demonstrates differences in EGFR expression among the 3 cohorts, representing different ethnic backgrounds, with Caucasian patients expressing less EGFR protein compared to both AA and Korean patients. We have previously reported that EGFR overexpression was observed in 75 of 202 (37%) prostate cancer patients. In addition, we reported a strong association between EGFR overexpression and African American race (45% of AA patients had EGFR overexpression compared with 18% in Caucasian patients, p = .0006) (16). In our current study, the frequency of EGFR overexpression in AA and Caucasian cohorts are similar to the frequency observed in our previous study (46% in AA compared to 18% in Caucasian patients. Korean patients have relatively high rate of EGFR overexpression (72%). This is most probably due to the relatively high rate of advanced local disease ($\geq$T3= 61%) and poor differentiation in Korean patients (Gleason >7 =66%) compared to both Caucasian and AA patients (Table 1). This is in concordance with recently published reports by several groups including ours that showed a strong correlation between EGFR overexpression and worse pathological features of the prostate cancer (13, 16). In this regard, recent studies have reported worse clinicopathological features of prostate cancer in South Korea (29, 30). Our findings taken together suggest that there are three different EGFR alterations that may contribute to its dysregulation in prostate cancer: missense mutation, synonymous sequence change, and protein overepression.

There are several potential limitations in our study. First, the relatively small sample size limits the generalization of our results. We planned our pilot study to determine if there was any difference in the mutation rate of *EGFR* TK domain of prostate cancer in patients of differing ethnic background in order to justify embarking on large population-based studies in prostate cancer. Our data suggest that there might be some differences in the mechanism of *EGFR* dysregulation, which depend at least in part on the patients' genetic background. Therefore, larger studies are required to prove our observations. Second, determining the magnitude of the impact of the identified novel TK domain mutations on response to EGFR inhibition will require functional studies. Third, we studied prostate tissues from patients with localized prostate cancer only. A more comprehensive study would include patients with more advanced metastatic castrate-resistant prostate cancer disease. This is of particular importance to determine with confidence the possible role of *EGFR* TK domain mutation in response to EGFR inhibition in prostate cancer patients.

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