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Association of Haplotypes in Exon 4 of KLK2 Gene with Raised Serum Prostate-Specific Antigen

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Abstract

The standard diagnostic modalities for Prostate Cancer (PC) include serum Prostate-Specific Antigen (PSA) assay, Digital Rectal Examination (DRE), and histological examination of prostate biopsy. They are limited by low predictive potential and inability to predict which patients are at risk of developing metastatic disease. The aim of this study is to investigate the exon 4 of the KLK2 gene of subjects for changes in its nucleotide sequences (SNPs) and determine the correlation of these changes with serum PSA in an Igbo population of Nigeria. One hundred male subjects aged 40 years and above, who gave their consent, were used for the study. Their PSA determinations were done using ELISA technique while genetic studies were carried out using real-time PCR. tPSA, fPSA, and % fPSA of the subjects ranged between 0.8% - 18.30%, 0.10% - 1.60% and 0.0% - 0.7% respectively. Of the 100 subjects, 28 subjects had tPSA levels above 4.0 ng/ml with a mean of 7.10 (±3.30) ng/ml. Those with tPSA less than 4 ng/ml had a mean of 1.87 (±0.85) ng/m. 15 subjects showed SNPs with a mean tPSA of 6.87 (±4.82) ng/ml while the remaining 85 subjects without SNPs had a mean of 1.86 (±0.80) ng/ml. Results from direct DNA sequencing showed 11 SNPs. Ten subjects are curated in SNP database while one is uncurated. The Chi-square test showed significant association ($p = 0.00$) between tPSA levels and SNPs mutation ($X^2 = 17.35$, $p = 0.00$). A Kruskal-Wallis test demonstrated that the positional arrangement of the SNP mutations had no effect on PSA-total or free-values ($H(10) = 10.92$, $p = 0.28$; $H(10) = 10.07$, $p = 0.38$ respectively). Two SNPs: rs6072 and rs74478031 were associated with elevated PSA levels ($p < 0.05$). Their presence, therefore, has the

potential to serve, in conjunction with raised PSA, as biomarkers of prostate cancer in the study population.

Keywords

Prostate Cancer, Prostate-Specific Antigen, Kallikrein 2 Gene, Genetic Mutations, Haplotypes, Short Nucleotidepolymorphism

1. Introduction

The prostate is a compound tubulo-alveolar exocrine gland of the male reproductive system. It makes a significant contribution to the production and ejaculation of semen during sexual intercourse. It also plays a role in the control of micturition by pressing directly against the part of the urethra it surrounds [1]. Common prostate problems include prostatitis which is caused by infections, Benign Prostate Hyperplasia (BPH), and Prostate Cancers (PCs). Some PCs are slow growing, remaining confined to the gland for many years. During this time, there are little or no symptoms. Other forms of this disease are more aggressive, metastasizing to other organs within a short period of time [2]. The risk of developing PC increases with age more rapidly than any other type of cancer with less than 1% of diagnoses in men below 50 years of age.

PC has become a major health issue in male folk. It is likely to impact the lives of a significant proportion of men that are alive today. The aetiology of PC, like that of most cancers, is not certain, but age, family history, lifestyle, diet, and a number of genetic factors have been implicated. Several reports have shown a strong association between some variants of the hK2 protein gene, KLK2, and the presence of PC at biopsy [3]. PC is curable if diagnosed properly and probably on time. At present, diagnosis is based on the triad of Prostate Specific Antigen (PSA) test, Digital Rectal Examination (DRE), and prostate biopsy for histology. Each of these tests has its strengths and pitfalls. For instance, the PSA, as a biomarker, does not have a strong correlation with prostate malignancy because it is also produced in healthy prostatic cells within the range of 0 - 4 ng/ml and in prostate cells that have undergone benign hyperplasia [4]. As a result of this pitfall, PSA testing, though having been widely employed for four decades for the detection and monitoring of prostate diseases can never be employed as a one-off test for the diagnosis of prostate cancer [5] [6].

The use of total PSA results of above 4 ng/ml as an indicator for prostate biopsy has resulted in a huge increase in the number of prostate biopsies performed in histopathology laboratories. Prostate cancer is detected in only about 30% of these biopsies. This is a major cause of concern because of the psychological and economic burden of unnecessary prostate biopsy on patients and the unnecessary workload that negative biopsy results in an impact on the histopathology laboratories. However, the association of raised serum PSA with a genetic factor that is not affected by other variables might improve the predictive

potential of screening tests and shield the no- or low-risk subjects who may never develop prostate cancer from the psychological and economic trauma of going through prostate biopsy.

Genetic factors that can help stratify individuals into risk groups are continually being investigated [7]. One of the gene families that studies have demonstrated to have a strong association with prostate cancer is the tissue kallikreins which have KLK2 and KLK3 (PSA) genes as members [8] [9]. Single Nucleotide Polymorphisms (SNPs) in the KLK2 gene have been shown to predict the presence of prostate cancer at biopsy thereby aiding the detection of high-risk patients who should be screened [10]. The KLK2 gene is localized in chromosome 19q13.4 and consists of 5 exons. The serine protease hK2 solely secreted by the prostate is known to activate the pro-forms of PSA and urokinase-type plasminogen activator. The proteolytic activities of hK2 are therefore associated with PC growth and metastasis.

Since studies have shown that one out of every four black males will suffer from prostate cancer, being able to pinpoint individuals that have a high risk of developing prostate cancer will enable clinicians to strategize routine prostate cancer screening for such individuals so as to improve early detection and diagnosis of prostate cancer.

Concurrent findings of a genetic risk factor and elevated PSA in an individual without signs and symptoms of cancer might render PC screening more specific, inform prognosis and help in the choice of treatment in the presence of PC.

The aim of this study is to investigate the exon 4 of the KLK2 gene of subjects for changes in its nucleotide sequences (SNPs) and to determine the correlation of these changes with changes in serum PSA.

2. Materials and Methods

The cross-sectional study was given ethical approval by the University of Nigeria Teaching Hospital (UNTH), Enugu Health Research Ethics Committee. The reference number for the Ethical approval is
NHREC/05/01/2008B-FWA00002458-IRB00002323.

The sample size was calculated using Power analysis software (StatMate version 2.0) by www.graphpad.com. The sample size of 100 used has a 99% power to detect an increase of 0.21 in prostate cancer incidence with a significance level (alpha) of 0.05 (two-tailed). Apparently, healthy men aged 50 years and above, residing in Enugu Metropolis, were recruited into the study after completing an Informed Consent Form. Inclusion criteria were: adults aged 50 years and above with no previous history of prostate cancer, not involved in any treatment for prostate disease, and must have signed the Informed Consent Form and willing to donate 5 ml of venous blood. A questionnaire consisting of 21 questions about risk factors associated with prostate cancer and familial predisposition was administered to each enrolled participant.

5 ml blood samples were collected from individuals and half of it dispensed into EDTA bottle for genetic studies while the remaining was dispensed into

clean plain tube and allowed to clot and retract at room temperature. Serum was separated by spinning at 3000 rpm for 5 minutes and stored frozen until required for PSA analysis. The EDTA blood was treated with red cell lysate buffer, (ammonium chloride buffer) and NaCl solution was added to help break up the pellet produced after centrifugation and disperse the cells. Sodium Dodecyl Sulphate (SDS), solution was added to the WBC/NaCl suspension, to lyse the WBCs, releasing the DNA from the cell. The SDS also dissociates protein/DNA complexes. RNase is added to the solution to destroy RNA. Remaining proteins (including the RNase added in the previous step) are precipitated by phenol-chlorofrom-isoamyl alcohol wash. The DNA is precipitated from the clean aqueous phase with isopropyl alcohol, washed with ethanol, and then dissolved in Tris-EDTA solution and extracted using the QiAMP blood mini kit (Qiagen, UK). The quality, quantity and purity of extracted genomic DNA was assessed on eppendorf biophotometer at 260/280 nm. A reading of 1.7 to 2.0 was accepted as representing good quality of DNA.

By direct PCR, the KLK2 Exon 4 was amplified. The primers used for the exon 4 amplification were:

Forward Primer: TGGAGTCTCCCTATCCTCC.

Reverse Primer: CTTCACTCACCTTTCCCCTC. The product size was 424 bp.

Agar gel electrophoresis was performed to check for the presence of DNA in the extracted GITC samples and to test the quality of DNA in the precipitate. The PCR products were sequenced using the ABI 3130 Genetic Analyzer. The analyzer performs fluorescence based capillary electrophoresis for both sequencing and fragment analysis applications on a 16-capillary array. Purification of the sequenced PCR product was done using the Big Dye X Terminator purification kit. All sequenced data from amplified PCR product (424 bp) of the KLK2 exon 4 were aligned using SeqMan Pro alignment of DNA Star software version 11.2. Aligned sequences were compared to a canonical sequence from the National Centre for Biotechnology Information (NCBI), Ref. Seq. (Reference Sequence).

PSA (total and free) was determined using ELISA method and % free calculated using the formula:

$$\%\ \text{Free PSA} = \text{Free PSA}/\text{Total PSA} \times 100$$

Data were analyzed using Google sheets and were presented as tables. Categorical variables were expressed as numbers (frequencies, f). The difference between continuous variables was determined using independent student's "t" test. Association between variables was determined using Chi-square test (X^2). Differences between the positional arrangement of SNPs and associated PSA values were determined by the Kruskal-Wallis test (H). All reported p-values are two tailed and statistical significance was set at 0.05 α level.

3. Results

The subjects had a mean age of 59 (±9) and 97% of them were of Igbo ethnic

origin. 58% were professionals and 30% were artisans. A third of them had regular daily physical exercise while 43% had physical exercise up to 4x a week. Less than 10% and 1% take alcohol and coffee respectively daily. Red wine was not popular among the respondents and only about 19% ate fruit daily but none ate uncooked tomatoes daily.

The ranges of tPSA, fPSA and %fPSA obtained were 0.8 - 18.3 ng/ml, 0.01 - 1.6 ng/ml, and 0.0% - 0.7% respectively with means of 3.2 ng/ml, 0.4 ng/ml, and 0.2% and SD of 2.9, 0.3, and 0.1 respectively. The corresponding median figures were 2.0 ng/ml, 0.3 ng/ml, and 0.1% respectively (Table 1). 28% of the subjects have elevated total PSA; above 4.0 ng/ml. The range of values for subjects with tPSA >4.0 ng/ml was 4.1 - 18.3 ng/ml; mean tPSA 7.1 ng/ml (±3.3); fPSA 0.8 ng/ml (±0.4); %fPSA 0.1 (Table 2). Chi-square test revealed significant association between tPSA and SNPs mutation (X^2 = 17.35) (p = 0.00). Subjects without SNPs had mean tPSA 1.86 ± 0.80 ng/ml while those with SNPs had mean tPSA of 6.87 ± 4.81 ng/ml., (p = 0.00). After the analysis of the sequenced exon 4 of the KLK2 gene of each subject, the SNPs were identified (Table 2). The SNPs nomenclature, frequency, genotype and their position in the gene segment are as presented in Table 3.

The tPSA values did not correlate with either the number of SNPs or the age of the subjects, (Table 4). Kruskal-Wallis test demonstrated that positional arrangement of SNP mutation had no effect on either tPSA or fPSA values (p = 0.28 and 0.38 respectively).

4. Discussions

Prostate cancer is the second most common cancer in men, with a lifetime prevalence of about 17 percent worldwide [11] [12]. Prostate cancer symptoms generally

Table 1. Showing total, free and % PSA (ng/ml) levels of all subjects.

Parameter	tPSA	fPSA	% fPSA
Minimum	0.8	0.10	0.0
Maximum	18.3	1.60	0.7
Median	2.0	0.30	0.1
Mean	3.2	0.40	0.2
Standard Deviation	2.9	0.3	0.1

Table 2. Showing the descriptive characteristics of subjects with tPSA > 4.0 (ng/ml).

Parameter	tPSA	fPSA	% fPSA
Minimum	4.10	0.20	10.0
Maximum	18.30	1.60	0.3
Median	6.00	0.7	0.1
Mean	7.10	0.8	0.1

Table 3. Segment positions of SNP occurrence, SNP nomenclature, frequency and genotype.

Segment Position	SNP Nomenclature	Frequency	Genotype
11	rs139948121	4	C/T
12	rs6072	12	A/C/G
25	rs74478031	7	A/G
30	rs61750342	1	C/T
52	rs148558632	4	A/G
70	Unknown/Uncurated	1	C/T
72	rs112137063	3	-/C
80	rs10422897	2	C/T
10	rs142114516	7	A/G
14	rs367617400	1	A/G
17	rs371665279	1	A/G

Table 4. Showing the tPSA (ng/ml) associated SNPs and their number and age of subjects.

tPSA (ng/ml)	SNPs	Number of SNPs	Age (Years)
12.54	rs139948121, rs6072, rs74478031, rs148558632, rs371665279	5	64
18.32	rs6072, rs74478031, rs142114516, uncurated	4	65
11.14	rs6072, rs112137063, rs142114516	3	59
1.99	rs6072, rs74478031, rs142114516	3	66
4.86	rs6072, rs74478031, rs148558632	3	68
5.13	rs6072, rs148558632	2	53
5.99	rs6072, rs142114516	2	-
3.89	rs6072, rs367617400	2	-
5.17	rs6072, rs74478031	2	-
1.88	rs142114516	1	48
4.63	rs142114516	1	54

occur in advanced stages, making early detection desirable. Digital rectal examination and prostate-specific antigen testing are the most commonly used screening tools. The goal of screening is to detect clinically significant prostate cancers at a stage when intervention reduces morbidity and mortality; however, the merits and methods of screening continue to be debated because of their limitations which give rise to under-diagnosis (DRE and PSA), over-diagnosis and over-treatment (PSA) [13]. There is need for new biomarkers that may increase diagnostic and prognostic information, so that better predictions can be made and treatments may be tailored [14]. The results of this study showed that out of the

100 subjects studied, 28% had total PSA levels of above 4.0 ng/ml. This agrees with the statement that more than 1 out of every 4 men of African descent are at risk of developing prostate disease [15].

28% of the subjects had total PSA levels of above 4.0 ng/ml. This is the conventional cutoff of tPSA used in clinical practice in Nigeria that would require further investigations to rule out prostate cancer or prostatic nodular hyperplasia. Analysis of the administered questionnaire revealed 14% of respondents had relations who suffered prostate cancer. This supports the work of Freeland [16], which states that heredity may account for nearly 15% of prostate cancer cases.

A number of predisposing factors like diet, exercise and lifestyle have been implicated by existing literature in the development of PC but from the questionnaire, no significant effect on PSA values was deduced as a result of alcohol and coffee intake by of the respondents, this agrees with the work of Bostwick [17].

PSA values were observed to be higher in older men. This supports the advocacy that a single value should not be used as the diagnostic cut-off value for all men that are above 40 years. Instead, cut-off values should be considered according to the age of the men in intervals of 10 years for better diagnostic accuracy [18]. In addition to that, factors like life expectancy and the presence of co-morbidities should also be considered when evaluating treatment options for prostate diseases [19].

SNPs give rise to genetic variations which determine a person's susceptibility to diseases, response to drugs, pathogens and other environmental factors [20]. SNPs in the KLK2 gene are especially implicated in the development of prostate diseases [21]. Results from direct DNA sequencing of the exon 4 of the KLK2 gene showed defined 11 SNPs; 10 were curated while 1 was not curated in SNP database. The Kruskal Wallis Results test of variance showed that there was a statistically significant difference ($p < 0.0001$) between the PSA levels of subjects with SNPs and those without SNPs. Two SNPs-rs6072 and rs74478031 were significantly associated with elevated PSA levels ($p < 0.05$). This supports the results of a previous study by Nam *et al.* [22] which had shown that some SNPs are associated with prostate diseases. The presence of the identified SNPs may be used as a genetic tool for stratifying patients into genetically high risk, medium and low risks subjects. This will influence the frequency and suitability of both mass PSA screening and individual prostate cancer screening [19]. We observed a significant correlation between the presence of elevated total PSA values in subjects who expressed SNPs rs6072 and rs74478031 and this indicates that the use of PSA results together with the results of genetic sequencing may prove a better diagnostic tool than the utilization of PSA results only. This has the potential to stratify subjects with elevated PSA results into BPH patients and prostate cancer patients and thus tailor their management strategies effectively. Some subjects with total PSA values above the cut-off point had no SNPs that may predispose them to prostate cancer. This shows that not all men with high PSA

levels are at risk of developing prostate cancer and these may be spared the urinary, sexual, and bowel side effects of biopsy if a test that will stratify them from those at risk of prostate cancer is exploited [23]. On the other hand, some subjects with total PSA values less than 4.0 ng/ml expressed SNPs which if adopted as a potential predictor of prostate cancer will be an indication of their tendency to develop prostate cancer in spite of their normal PSA values. This supports the work of Thompson *et al*. [24], which showed that prostate cancer is not rare in men whose PSA values are below 4.0 ng/ml. However, is possible that SNPS on other exons than exon 4 could influence the results of this study.

5. Conclusion

The total PSA levels of subjects with SNPs rs6072 and rs74478031 were found to be significantly higher than those of the subjects without SNPs. Their presence, therefore, has the potential to serve, in conjunction with raised PSA, as biomarkers of prostate cancer in the population.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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