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The Impact of CAG Repeat within the *Androgen Receptor* (*AR*) and Lipid Profile to the Clinicopathological Features of Prostate Cancer in Javanese Population

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Abstract: Androgen is important in the development of prostate glands and defect in its signaling pathway could induce prostate glands carcinogenesis. The CAG repeat polymorphism on exon 1 of *Androgen Receptor* (*AR*) gene and the lipid profile. It has been reported as genetic and environmental risk factors for the development of prostate cancer (PCa). This research aims to investigate the association of CAG repeat of *AR*, lipid profile and combination of both with clinicopathological features of prostate cancer in Javanese population, Indonesia. We conducted a cross-sectional study of prostate cancer from October 2013 to September 2014. Correlation analysis was performed to examine the association between CAG repeat of *AR* and lipid profile with the tumor grade (PSA level) and tumor stage (Gleason score).We observed that there is no association between cholesterol level with either PSA or Gleason Score. Multivariable analysis showed that level of triglyceride was negatively associated with Gleason Score (β = -0.1054, p = 0. 044) but not with the PSA (β = -2,042, p = 0.270). The CAG repeat is not associated either with the level of PSA (β = -12.611, p = 0.524) or with the Gleason score (β = -0.1056s, p = 0.225). It suggests that there is no association between variability of CAG repeat length and the level of cholesterol with the clinicopathological features. The level of triglyceride is negatively associated with the Gleason score. Although not significant, men with shorter CAG repeat tend to develop PCa earlier than those with longer CAG repeat.
Keywords: Prostate Cancer (PCA), CAG repeat, Cholesterol, Triglyceride (TG), PSA, Gleason score.

Introduction

Prostate cancer is the second most common cancer diagnosed and the sixth most common cause of death among men worldwide[1,2]. The incidence of prostate cancer varies between ethnic groups, with the Asian/Pacific Islander have the lowest incidence rate based on data of year 1999 – 2011 from centers for disease control and prevention (www.cdc.gov). Other than ethnicity, advanced age, family predisposition, high

level of Androgen hormone, diet, obesity, smoking history and physical activity levels are known risk factors for development of prostate cancer[3-7]. Androgen plays important role in the development of prostate gland and defect in its receptor has been known to be associated with development of prostate cancer[8,9]. Androgen receptor (*AR)*is a nuclear receptor and member of steroid hormone receptor superfamily which is located on Xq11-12. The AR protein (NP_000035) consists of 920 amino acids and has three functional domains which are: N-terminal trans-activating domain, DNA binding domain and ligand-binding domain[9-11]. The first exon has CAG repeat [(CAG)n CAA] which encodes polyglutamine and located in the trans-activating domain. This repeat ranges from 14 to 35 in healthy group of males, although may vary in different ethnicity[9-12].Interestingly the different of distribution the length of CAG repeat is co-varies with the incidence of prostate cancer. The African-American, for example, have the highest incidence of prostate cancer in the world and they have the short CAG repeat (≤ 22) allele more frequent than other ethnicity[11]. Some studies showed an inverse relationship between the length of the CAG repeat and its transcription activation[13-16]. The shorter the CAG repeat is, the shorter the polyglutamine on the N-terminal part of the AR protein will be. This leads to a stronger transcription activation of AR protein. As a consequence, it increases the expression of target genes such as the *Prostate-Specific Antigen* (*PSA*). However, whether the length of this repeat is associated with the prostate cancer is still a controversy as the results in different studies on different population gave conflicting results[17-24].

In the early 1980s there was a growing consensus that a low concentration of circulating cholesterol, which is very beneficial for cardiovascular health, can increase the risk of non-cardiovascular diseases, especially the risk of cancer incidence[25-27].Recent study showed that the level of total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglyceride are associated with prostate cancer in cohort study of 2842 Dutch men[28]. While some laboratories studies showed that level of total cholesterol and triglyceride associated with prostate cancer and recurrent of the disease, however population-based evidence showed inconsistency results[27-33]. Understanding the association between the lipid profiles with the increase risk of prostate cancer is important as lipid profile is a modifiable factor with diet or Statin (anti-cholesterol) and therefore has important implication on prostate cancer prevention and treatment. Interestingly, recent study showed that the length of CAG repeats was positively correlated with the level of high density lipid (HDL)-cholesterol and fat-free mass (FFM) respectively[34-35]. In this study, we are interested in investigating the correlation between the CAG repeat within the *AR* gene, profile lipid and combination of both with the clinicopathological features prostate cancer (PAS level and Gleason score) in PCa patients of Javanese population, Indonesia.

Materials and Methods

Patients

We conducted a Cross-sectional study of 42prostate cancer patients from 5 hospitals in Central Java province from October 2013 to September 2014.The ethnicity of all patients was Javanese and the age is ranging from 42 to 99 years old. The diagnosis of prostate cancer was based on the pathological examination from transurethral resection. Patients who took anti-lipid drugs or anti-androgen therapy were excluded from this study. We collected 3 ml of blood from all of the patients for DNA isolation. The written informed consents were obtained from all participants and this study was approved by the ethic committee of faculty of Medicine, University of Brawijaya, and Central Java, Indonesia.

DNA Isolation

Genomic DNA was extracted from peripheral blood leukocytes from patients using Genomic DNA isolation kit (Roche Life Sciences) in the MagNA Pure LightCycler32 instrument (Roche Life Sciences) according to the manufacturer's protocol.

Genotyping of CAG Repeat Length

The touch-down Polymerase Chain Reaction (PCR) was performed in the final volume of 50 µl containing of 100 ng of DNA, 25 µl of ready-to-use PCR master mix KAPA2G Fast PCR kits(Kapabiosystem, Wilmington, Massachusetts, USA) and 1µl of 20 pmol/µl of each forward primer 5' ACTACCGCAT CATCACAGCC 3' and reverse primer 5' CTTAAGCCGGGGAAAGTGG 3'. Touch-Down PCR of Thirty five cycles were performed as follow: The first part started with the denaturation at 95°C for 5 minutes, followed by 10 cycles of 1 minute at 95°C, 1 minute at 70°C (the annealing temperature was dropped-down 1°C in each cycle) and 1 minute at 72°C. The second part of the PCR program was 25 cycles of 1 minute at 95°C, 1 minute

at 60°C and 1 minute at 72°C. The last elongation step was at 72°C for 10 minutes. Five microliter of PCR product was checked on 2% of Agarose gel. The remaining of PCR products were sent to 1st Base in Malaysia. The PCR products were gel-purified and Sanger sequenced using Big Dye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, USA) on an ABI 3730XL automated sequencer. The length of the CAG repeat was determined by counting the repeat on the electropherogram of Sanger Sequencing using BioEdit software.

Lipid Profile Measurement

Five milliliter of blood was collected and plasma was separated from the sample. The total cholesterol and triglyceride level were performed using enzymatic reaction and measured the results by colorimetric method as previously described[28].

Prostate Specific Antigen (PSA) Level Measurement

PSA level were measured by using ARCHITECT total PSA assay (ARCHITECT reagent kit, Abbot, Irreland) according to the manufacturer's protocol.

Statistical Analysis

Descriptive analysis for examining data distribution was performed by histogram and boxplot. Data normality was tested by Shapiro-Wilk test. To compare the variables with ratio or interval scale between two population groups, we used t-test or Mann-Whitney test, for normal or non-normal distributed data respectively. To estimate association of CAG repeat of *AR* and lipid profile with Gleason score and PSA level, multivariable analysis was conducted by linear regression.

Results

Descriptive Analysis

Clinical characteristics of prostate cancer patients who enrolled in this study are shown in the Table 1. Forty two prostate cancer patients voluntary enrolled in this study, the level of cholesterol or triglyceride of four subjects were outlier hence only data of 38 subjects remained for the analysis. The age was ranging from 42 to 99 years old with the mean was69.34 years old. The mean of total cholesterol and triglyceride were 160 and 99 respectively while the mean of PSA and Gleason score are 80.98 and 7 respectively. The level of cholesterol, triglyceride, PSA and the Gleason score were not significantly different in the group of subjects with the length of CAG repeat < 23 and ≥ 23.

Table 1. Clinical characteristics of subjects.

Variable	All	CAG <23 N= 17 (45%)	CAG≥23 N=21 (55%)	p-value
Mean (SD)				
Age, in years	69.34(8.97)	66.53(10.11)	71.62(7.43)	0.094*
Median (IQR)				
PSA level, ng/ml	80.98()	199	33	0.082†
Cholesterol Total, mg/dl	160()	144	167	0.082*
Triglyceride, mg/dl	99()	110	89	0.297†
Pathological Gleason score	7()	6	7	0.168†

P-values computed using *t-test or †Mann-Whitney test

The CAG repeat length was not normally distributed as it is shown in the histogram (Figure 1). The CAG length is ranging from 15 to 34 with the median of 23 repeat. Although it was not significantly different, there was a trend that the subjects with the younger age (66.53 years old) have CAG repeat shorter than those with older age (71.62 years old).

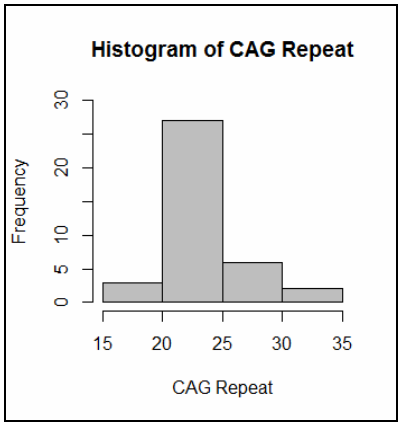

Figure 1. The distribution of the length of CAG repeat in prostate cancer patients in Javanese population, Indonesia.

Univariable Analysis

Most of the subjects (89.5%) had low cholesterol (< 200 mg/dl) and lowtriglyceride (< 160 mg/dl) level. There was no association between high (≥200 mg/dl) and low (< 200 mg/dl) level of cholesterol and high (≥ 160 mg/dl) and low (< 160 mg/dl) level of triglyceride with the PSA and the Gleason scoreit is shown in the Table 2.

Table 2. Association of the level of cholesterol, triglyceride with the PSA and Gleason score.

	Cholesterol (mg/dl)			**Triglyceride (mg/dl)**		
	< 200 N=34 ( )	**≥ 200 N=4 ( )**	**p-value**	**< 160 N=34 ( )**	**≥ 160 N=4 ( )**	**p-value**
PSA, Median(IQR)	80.98( )	228.26( )	0.72	80.98( )	247.22 ( )	0.72
Gleason Score	7.0	6.5	0.73	7.0	6.5	0.65

P-values computed using Mann-Whitney test

Multivariable Analysis

We observed that the level of triglycerideis negatively associated with the Gleason score with p-value 0.044 and β = -0.1054. The CAG repeat is not associated with the Gleason score, however, the magnitude of the effect of CAG repeat (β = -0.1054) to the associationwas stronger than that of the level of triglyceride (β = -0.0156).

Table 3. Multivariable analysis of association of CAG repeat, lipid profiles with PSA level and Gleason Score

	PSA Level		**Gleason Score**	
Variable	**Beta**	**P-value**	**Beta**	**P-value**
Age (years)	-8.302	0.298	0.0009	0.978
CAG Repeat	-12.611	0.524	**-0.1054**	**0.225**
Triglyceride (mg/dL)	-2.042	0.270	**-0.0156**	**0.044**

Discussion

Androgen has been known to be important for the development of prostate glands. The association of the CAG repeat length within exon 1 of *Androgen Receptor* (*AR*)to the risk of prostate cancer (PCa) development had been extensively studied in different population. However, it is still a controversy whether or not this polymorphism is a risk factor for PCa[17-24]. Other than mutations and polymorphism in the *AR* gene, polymorphism in *PSA*, one of the target genes of Androgen, has been known to be associated with the PCa(36,37). Furthermore, the incidences of PCa in developed countries e.g., United States and Scandinavian is six-times fold higher than those in developing countries[2]. Lifestyle and diet could be one of the reasons why the incidence of PCa is higher in more develop countries than those in less develop countries. Overall, this evidence supports the idea that PCA is a multifactorial disease. Combination of mutations or polymorphisms in more

than one gene together with the environmental factors might actually need for the PCa to be developed. Hence, in this study, we are interested in studying the association between CAG repeat of *AR* (as genetic factor), lipid profile (as the environmental factor) with the clinicopathological feature of prostate cancer.

By multivariable analysis, in which we considered the age, the length of CAG repeat and the level of triglyceride as co-variates, we observed that the level of triglyceride was negatively associated with the Gleason score, however the length of CAG repeat was not associated either with the Gleason score or with PSA level (Table 3).One of the plausible explanations why we did not observed an association of the length of CAG repeat with clinicopathological feature of PCa because we use small sample size in this analysis, hence we did not have enough power to measure the effect of this polymorphism. Increasing the number of samples to a total 4,272 cases and 5,272 controls in the meta-analysis of 19 published studies only showed a modest association of CAG repeat to the risk of PCa[19]. In 2013, Sun JH, et al., performed a bigger meta-analysis comprising of 13,346 cases and 15,172 controls from 47 published-studies in different population. They observed that particularly in Asians with CAG repeat < 22 have a higher risk of getting PCa (OR 2.06, 95% CI 1.00-4.24) compare to those with CAG repeat ≥ 22[38]. These two studies support the idea that the CAG repeat within the *AR* gene has a low-penetrance and clearly showed that this polymorphism alone will not be enough to cause the disease. We notice from the clinical characteristic shown in the table 1, although not significant, the patients with younger age (66.53 years) tend to have the short CAG repeat (≤23) allele compare to those who are older (71.62 years). The result of this observation is in line with the fact that the length of CAG repeat is correlated with the age of onset[39,40]. This result implies that indeed CAG repeat within *AR* is a genetic component for prostate cancer, although the effect seems to be not as big as we and others thought it would be.

Our finding that the level of triglyceride negatively associated with the tumor stages (Gleason score)[41]. We observed that men whose level of triglyceride < 160 mg/ml were more likely to develop high-grade prostate cancer (Gleason score > 7).However, most studies on cholesterol (other lipoprotein profile) showed conflicting results in which men whose level of cholesterol is in desirable (< 200 mg/dl) or border line level (200 - < 240 mg/dl) were less likely to develop high-grade prostate cancer[27,31].Lipid/cholesterol profile is a modifiable parameter, the level could be really fluctuated depend on the amount of lipid/cholesterol produce endogenously and those we adsorb from daily diet. Lipid profile on prostate cancer patients themselves could be really fluctuated during different time points such as: before the cancer develop, during the carcinogenesis, on early-stage or on late-stage of cancer. Studies performed previously and our study could have totally different time point for blood sampling touse for measuring the level of the lipid profile. Differences on time point of blood sampling, lifestyle and diet in different population could be some of the reasons why the conflicting results are produced on different studies. Furthermore, we could not forget that genetic plays a role in the cholesterol/lipid metabolism. This makes even more difficult to measure precisely the effect of lipid profile/cholesterol to the risk or staging of prostate cancer. One could not be100 percent sure what is the effect of lipid profile to the prostate cancer actually is, unless they could performed an experiment in such a way that the level of lipid profile is measured on different time points and check for the association with the risk of getting prostate cancer risk and/or with prostate cancer staging in the uniform genetic background such as in animal model.

Prostate cancer is a multifactorial and complex disease in which many variants with low- to moderate effect or rare variants with strong effect together with environmental factors synergistically cause the disease. Most of studies performed so far were more to a gene-targeted approach rather than unbiased genome-wide approach. Two strategies could be applied to reveal the genetic factor of PCa, either we focus on big samples size of sporadic PCa cases and performed genome-wide screening such as GWAS, or we could also focus on few familial cases and go deep with the analysis by using exome sequencing or whole-genome sequencing.

In summary, our study suggest that although it is not significant, men with CAG repeat shorter than 23 repeats tend to develop PCa earlier in life compare to those with CAG repeat longer than 23 repeats. We also observed that men with low level of triglyceride were more likely to develop high-grade of PCa. Further investigation onbigger sample size with genome-wide approach strategy needs to be performed to reveal the genetic background of PCa in Javanese population.

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