RESEARCH ARTICLE

Germline Mutations and Polymorphisms of *Androgen Receptor* in Prostate Cancer Patients: Frequency and Results of *in Silico* Analysis

Yunia Sribudiani[1,2]*, Deineke W Marwan[3], Aulanni'am Aulanni'am[4], Muhammad A Widodo[5], Basuki B Purnomo[5], Ramdan Panigoro[1,2], Ahmad B Utomo[6]

Abstract

Background: Germline and somatic polymorphisms and mutations of the *Androgen Receptor (AR)* gene are known to be associated with the incidence of prostate cancer (PCa) in different populations. In this study we assessed germline *AR* polymorphisms and mutations in PCa patients with prediction of pathogenicity of the identified mutations by *in silico* analysis. **Methods:** Diagnosis of PCa was based on histopathology of prostate tissue (Gleason Score criteria) and serum prostate-specific antigen (PSA) levels. Genomic DNA was extracted from peripheral blood of 38 patients. All exons and exon-intron boundaries of *AR* were amplified using polymerase chain reactions (PCR) followed by Sanger sequencing. *In silico* analysis was performed using Polyphen-2 and Mutation Taster®. **Results:** Two polymorphisms, CAG repeat sequence (13-34 repeats in length) and p.Pro214Glu (MAF: 0.0789) located in exon 1 were identified. A missense mutation (c.47C>A/p.Pro146Glu) and in-frame deletion of a CAG sequence leading to loss of Arginine at codon 85 (c.252_254delCAG/p.Arg85-) were identified in a 70 year old patient with a Gleason Score and PSA level of 2 and 2.4ng/dL, respectively. His PSA level decreased to < 0.5 ng/dL after 9 months of androgen deprivation therapy. Identified mutations were predicted to be non-disease causing by Polyphen-2 and Mutation Taster®. **Conclusion:** Our data demonstrated that the frequency of germline mutations of *AR* was low in PCa patients in Indonesia (5.26%: 2/38 alleles), so that they are not likely to be major etiological factors. The *in silico* analysis of identified *AR* mutations in this study corroborated the clinopathology features of the patient.

Keywords: Androgen receptor- germline- mutation- polymorphism- prostate cancer (PCa)

Introduction

The data from the International Agency for Cancer Research (IARC) stated that Prostate cancer count as second most common cancer diagnose in male after lung cancer. It has been reported that the incidence of PCa in Asian countries is lower than that in Western countries (Ferlay et al., 2012; Bray et al., 2013). The incidence of PCa in Indonesia is yet unknown, however the data from several hospitals in Indonesia showed that the number of PCa patients were tripled in the last 10 years. It has been known, as in other types of cancer, the pathophysiology of PCa is multifactorial, including germline mutations in one or more genes or somatic mutations induced by hormonal imbalance, oxidative stress, pollutants from the environment, and chronic inflammation (Sebastiano and Mourtzakis., 2014). It has been reported

that cigarettes, eating pattern, age, family history with PCa and some polymorphism in *Androgen Receptor* (*AR*) gene such as CAG and GGC trinucleotide repeat sequence in the exon 1 of *AR* gene are risk factors for PCa. The shorter CAG repeat (≤ 20) had been reported to be associated with the increase risk of PCa in different populations, while the longer CAG repeat > 20 decrease the risk of PCa (Qin et al., 2017). However this result remains controversial as conflicted results have been reported in different populations (Sircar et al., 2007; Tayeb et al., 2004; Bennett et al., 2002; Beilin et al., 2000; Mittal, Mishra, and Mandhani, 2007).

Androgen Receptor (AR) is a nuclear receptor superfamily, whose function is to regulate the expression of androgens such as testosterone (T) and dihydrotestosterone (DHT). *Androgen Receptor* is a 110 kD protein composed of 919 amino acid and it is encoded by *AR* gene which is

[1]*Department of Biomedical Science, Division of Biochemistry and Molecular Biology,* [2]*Medical Genetics Working Group,* [3]*Graduate School Biomedical Sciences Master Program, Faculty of Medicine, University Padjadjaran, Bandung,* [4]*Faculty of Mathematics and Natural Sciences,* [5]*Faculty of Medicine, Brawijaya University, Malang,* [6]*Departement of Urology, Faculty of Medicine, Muhammadiyah University, Surakarta, Indonesia. *For Correspondence: y.sribudiani@unpad.ac.id*

located at Xq11-12. *Androgen Receptor* contains four domains: The Amino Terminal Activation Domain (NTD); the DNA Binding Domain (DBD); the hinge region and the Carboxyl Ligand-Binding Domain (LBD). The NTD domain (amino acid 1-556) is known to be the least conserved among the four domains (Lonergan and Tindall, 2011; Eisermann et al., 2013; Koochekpour et al., 2014). Androgens signaling regulates male sexual differentiation, secondary sexual maturation and also essential for many aspects of prostate development and function, both in normal prostate development, and progression to PCa and metastases (Lonergan and Tindall, 2011; Eisermann et al., 2013). Disruption of *AR* protein function and cell signaling could induce uncontrolled cell growth in the prostate gland. The blockade activation of *AR*, deprivation of androgens (androgen deprivation therapy-ADT), orchiectomy, administration of agonists/antagonists of luteinizing hormone releasing hormone (LHRH) or antagonist *AR* are standard therapy for PCa. The therapy initially success in ~80% of patients, however, eventually the PCa relapse and develop into hormonal-independent/Castration resistant Cancer Prostate (CRCP) (Eisermann et al., 2013).

Previous studies showed that both somatic or germline *AR* mutations were identified in prostate cancer patients in different populations (Eisermann et al., 2013). The gain-of-function mutations, rearrangements and polymorphisms of *AR* gene resulting in mutant protein could interfere with the various functions of the *AR*. Frequency of somatic mutations in *AR* gene varies among different studies, 25% in androgen-stimulated tumors and 10 up to 30% on castration resistant tumor or metastasis (Eisermann et al., 2013). Only few studies on analyzing germline polymorphism and/or mutations of *AR* gene in PCa patients had been reported and most of those studies were in familial cases (Koochekpour, 2010; Gruber et al., 2003; Mononen and Schleutker, 2009; Hu et al., 2010). So far, *AR* somatic or germline mutations analysis in PCa patients in Indonesian population has been reported. In this present study, the frequency of germline polymorphism and mutations of *AR* gene will be investigated in sporadic cases of PCa. Furthermore *in silico* analysis of identified mutations will be performed to predict their pathogenicity.

Materials and Methods

Patients

A total of 38 prostate cancer patients from Javanese ethnic group, one of the biggest ethnic group in Indonesia, were enrolled in this study, the age was from 49 to 90 years with the average is 69.7 year-old. The diagnosis of prostate cancer (PCa) was based on the pathological examination from transurethral resection. Three milliliters of peripheral blood was collected from each patient for genomic DNA isolation and measurement of PSA level. The written informed consents from all patients for the use of their samples for diagnostic and scientific purposes were obtained and this study was approved by the ethic committee of faculty of Medicine, University of Brawijaya, and Central Java, Indonesia (No: 90/II/HREC/2015).

PSA level Measurement

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

Genomic DNA Isolation

Genomic DNA was extracted from three milliliter peripheral blood using genomic DNA isolation kit (Roche Life Sciences) and the MagNA Pure LightCycler32 instrument (Roche Life Sciences) according to the manufacturer's protocol. The DNA concentration was measured using NandoDrop™ 1000 (Thermo Scientific).

Polymerase Chain Reaction (PCR) and Sanger Sequencing

Touch-down Polymerase Chain Reaction (PCR) was performed with the annealing temperature was 68°C to 58°C. fifty to 100 ng of genomic DNA was used for PCR template with 25 ml ready-to-use PCR Mastermix KAPA2G fast PCR Kit (Kapabiosystem, Wilmington, Massachusetts, USA), 1µl of each forward and reverse primer (20 pmol/mL) and MilliQ water to the total volume of 50 µl. Forward and reverse primers to amplified exon 1 to 8 of *AR* gene are listed in supplementary Table S1. PCR products were Sanger Sequenced using forward primers and the results were analyzed using Bioedit and BLAST Nucleotide (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

In Silico Analysis

Pathogenicity prediction of identified germline *AR* mutations were analyzed using Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) and Mutation Taster (http://www.mutationtaster. org/). Polyphen-2 can only be used to predict the pathogenicity of missense mutation.

Results

Characteristics of Research Subjects

A total of 33 out of the 38 subjects (86.8%) had a Gleason score ≥ 4 and the PSA levels were ranging
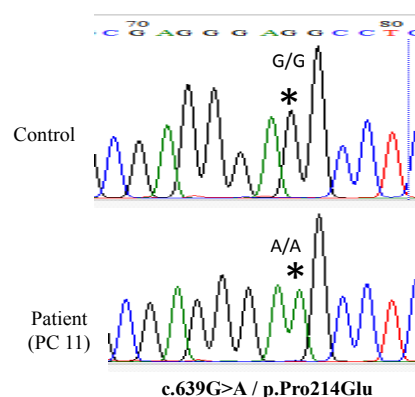

c.639G>A / p.Pro214Glu

Figure 1. Hemizygouse Missense Polymorphism on the Exon 1 of *Androgen Receptor* Gene (c.639G>A) Changes the Amino Acid Proline into Glutamic Acid (p.Pro214Glu) Were Identified in Three Prostate Cancer Patients in Indonesian Population.

Table 1. Polymorphism and Mutations of Androgen Receptor Identified in Indonesian Population

No.	Patient ID	Exon	Ref	Genotype	AA Change	Nucleotide Change	MAF*	Clinicopathology		
								PSA (ng/dL)	Gleason Score	Domain
Polymorhpisms										
1	PC-11	1	G	A/A	p.Pro214Glu	c.639G>A (rs6152)	0.2387	53.1	6	NTD
	PC-29							151		
	PC-37							156		
Mutations										
1	PC-26	1	C	GCA/-	p.Arg85-	c.252_254delCAG	0.0037	2.24	2	NTD
2			C	C/A	p.Pro146Gln	c.437C>A	0			NTD

AA, Amino acid; Ref, Reference Sequence; MAF*, Minor Alelle Frequency in 1000 Genome; NTD, Amino Terminal Activation Domain (NTD)
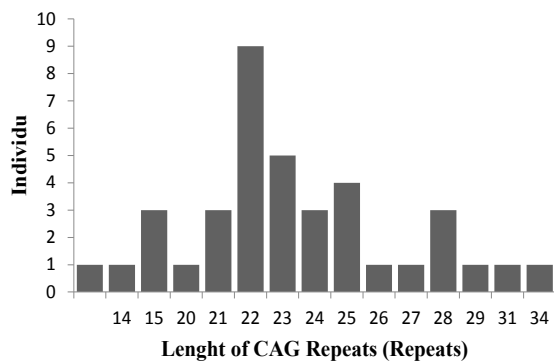

Figure 2. Distributon of CAG Repets (CAGn) Identified in Indonesian Patients with Prostate Cancer

from 0.94 – 1001 ng/dL. The average of age was 69.7 year-old with majority of patients enrolled in this study were 69 to 73 year-old (31.59%). The summary of patients' characteristic enrolled in this study is presented in the Table S2 (supplement).
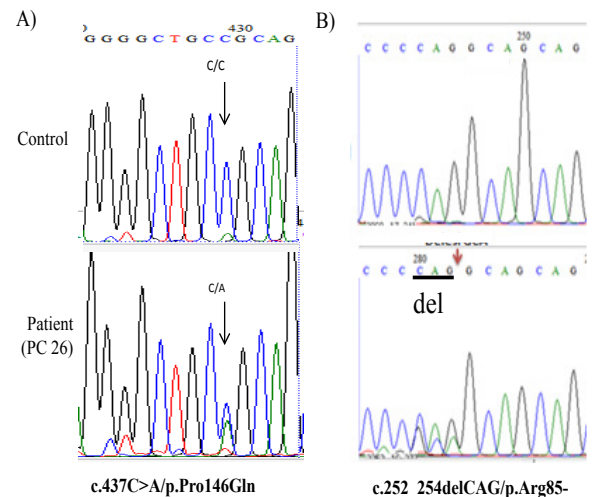

Figure 3. Novel Germline Mutations Were Identified in a Prostate Cancer Patient (ID – PC 26) in Indonesia Population. A) Mosaic missense mutations in the exon 1 (c.437>A/p.Pro146Gln). B) Mosaic in-frame deletion of CAG sequence at the exon 1 lead to deletion of Arginine amino acid (c.252_254delCAG/p.Arg85-) of *Androgen Receptor* gene.

Figure 4. A) *In Silico* Analysis Results on a Novel Missense Mutations p.Pro146Gln Using Polyphen-2 Showed that this Mutation is Non-disease Causing Variant (benign). B) Multiple alignment results of p.Pro146Gln using Mutation Taster® (red bracket/red arrow) showed that this mutation located in non-conserved region across different species.

Androgen Receptor Polymorphisms

Two different polymorphisms were identified in the exon 1 of *AR* gene. Those were CAG repeat sequences (CAGn) encoding Poly-Glutamine (Poly-Q) and hemizygous missense polymorphism which substitute Proline to Glutamic Acid at the codon 214 (p.Pro214Glu) (Figure 1, Table 1). The p.Pro214Glu variant was identified in three patients, hence the MAF of this polymorphism is 0.0789 (3/38 alleles) which is 2 and 3 times lower than MAF in ExAC (0.175) and 1,000 genome (0.239) respectively. All patients carrying this missense polymorphism had PSA > 50 ng/dL (Table 1). The distribution of CAGn repeats sequence length of *AR* gene in this population is from 14 to 34 repeats (Figure 2). The length of CAG repeats of *AR* gene in this population was not correlated with Gleason Scores or with PSA levels (data was not shown).

Androgen Receptor Mutations and in Silico Analysis

Two germline mosaic mutations located in the exon 1 of *AR* gene were identified in only one PCa patient in this population, hence the frequency of germline *AR* mutations in this population was only 5.26% (2/38 alleles). One of the mutations was an in-frame deletion of CAG sequence (c.252_254delCAG) resulting in deletion of Arginine at the codon 85 (p.Arg85-). Second mutation was a missense mutation (c.437C>A) lead to substitution of Proline by Glutamine at codon 146 (p.Pro146Gln) (Table 1, Figure 3). Both mutations have never been reported before (novel mutations) and were not found either in 1,000 Genome and EXAC databases. *In silico* analysis using Mutation Taster® showed that those two identified mutations were predicted as a non-disease causing variant (polymorphism). Analysis using Polyphen-2 and Mutation Taster® performed on the c.437C>A/p. Pro146Gln missense mutation showed that this mutation located at the non-conserved region of DNA across different species and predicted to be benign (Figure 4).

Discussion

Androgen receptor (*AR*) has been known to play an important role in the development of prostate tissue. Damage to the *androgen receptor* induced by mutations or polymorphisms in *AR* gene has been known to be associated with an increased risk of PCa. Mutations and polymorphism analysis (both somatic and germline) of all exons *AR* gene in PCa patients in Indonesian population have never been reported before. In this study, we reported for the first time germline polymorphisms and mutations identified in PCa patients from Indonesia.

Two polymorphisms have been identified in this population. One of those polymorphisms is CAG repeat sequence located in the beginning of exon 1. A shorter length of CAG repeats (≤ 18-20 repeats) sequence has been known to be associated with the increasing risk of getting PCa compared to that with the higher length of CAG repeats (≥ 18-26 repeats) (Mononen et al., 2002; Nam et al., 2000). Some studies also showed that the length of CAGn repeats was correlated with PSA levels

and Gleason Score (Mononen et al., 2002; Wang et al., 2003). However, in this study the CAG repeats sequences in PCa patients was not correlated with either Gleason score or PSA level (data was not shown).

Two germline mutations in exon 1 of *AR* gene were identified only in one patient. Those mutations are an in-frame deletion of CAG sequence (c.252_254delCAG/p.Arg85-) and a missense mutation from Proline to Glysin at codon 146 (p.Pro146Gln). Both mutations have never been reported before (novel) and located in the Amino Activating Terminal Domain (NTD). The NTD domain plays important role in the initiation of gene target transcription by binding to the co-factor. Amino acid changes caused by missense mutation, deletion or insertion might affect the protein folding and hence disturb the binding affinity of *AR* to Co-factor. This event could reduce transcription efficiency of target genes. However, *In silico* analysis using two different tools (Polyphen-2 and Mutation Taster) showed that none of identified mutations was predicted to be a disease-causing mutation. Furthermore, The results of *in silico* analysis of identified mutations were corroborating with the clinicopathology features of the patient whom diagnosed with PCa at the age of 70 year old (late onset PCa) with the Gleason score 2 and low level of serum PSA (2.4 ng/dL). The patient also gave a good response to the Androgen hormone deprivation therapy as the level of PSA constantly decreasing and reached < 0.5 ng/dL after 9 months of the therapy. This showing that the prostate carcinogenesis in this patient was not progressive. If the changes does not affect the protein folding and function drastically usually this changes will be kept in the genome through the evolution. This leads to the existence of DNA sequence variation and non-conserved region in the DNA or protein level. As the mutations identified in this study are located in a non-conserved region, this might partly explain why these mutations did not lead to progressive PCa in this patient.

However, germline mutation analysis by using DNA isolated from leucocytes has disadvantage, especially if the mutations identified are mosaics as we could not predict which allele present in the prostate tissue. There is still a possibility that only wild type allele present in the prostate tissue, therefore the PCa in this patient was not progressive. On the other hand, the advantage of preforming germline mutations analysis, if we identified known pathogenic mutations, this could be a powerful tool to early diagnose certain disease including prostate cancer.

In conclusion, the frequency of germline *AR* mutations was low in sporadic PCa in Indonesian population (5.26%) and most likely it is not the major cause of PCa in this population. This study also shows that *in silico* analysis using at least two different prediction tools (polyphen-2 and Mutations Taster®) could predict the pathogenicity of identified mutations accurately.

Conflict Of Interest

The Authors declare that there is no conflict of interest.

Acknowledgments

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