LAMPIRAN KORESPONDENSI ARTIKEL

The use of high-resolution melting techniques for mutation screening of diseases caused by trinucleotide repeats expansion, with emphasis on the AR gene

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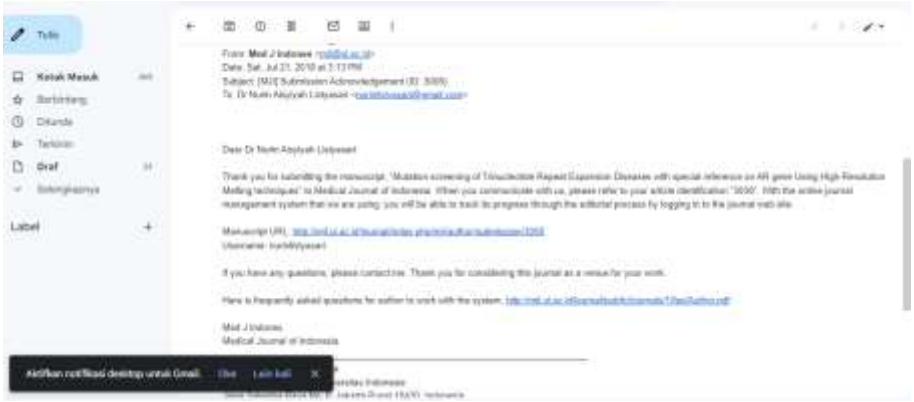

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Dear Dr Nurin Aisyiyah Listyasari:

Thank you for submitting the manuscript, "Mutation screening of Trinucleotide Repeat Expansion Diseases with special reference on AR gene Using High-Resolution Melting techniques" to Medical Journal of Indonesia. When you communicate with us, please refer to your article identification "3008". With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

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Manuscript Review and Revision

From: **Med J Indones** <mji@ui.ac.id>
Date: Thu, Nov 15, 2018 at 12:58 AM
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Dear Dr Nurin Aisyiyah Listyasari:

We have reached a decision regarding your submission to Medical Journal of Indonesia, "Mutation screening of Trinucleotide Repeat Expansion Diseases with special reference on AR gene Using High-Resolution Melting techniques".

After the first round review, your manuscript need to be revised accordingly. Please upload your revision from the system to enable us in tracking your record.

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Mutation screening of Trinucleotide Repeat Expansion Diseases with special reference on AR gene Using High-Resolution Melting techniques

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Disclaimer (if any) :

Conflict of interest :

Running title : Screening of TRE disease using HRM techniques

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Synopsis : The first technique for AR gene screening using HRM, which is useful and less laborious that may applicable to

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~~Synopsis: The first technique for AR gene screening using HRM, which is useful and less laborious that may applicable to other TRE diseases.~~

ABSTRACT

Background: Trinucleotide repeat expansion (TRE) disease~~s~~ is known as a genetic disease caused by an increased number of codons such as CAG, CGG, and CTG. The elongation of CAG repeats in exon 1 AR gene is known to have an association with disorders of sex development (DSD) phenotype variation and SBMA. While the traditional Southern blot for CAG repeats expansion is laborious and time-consuming methods, high-resolution melting (HRM) can be an alternative.

~~**Objective:** To screen CAG repeat expansion of TRE diseases with special reference of ARgene using HRM technique.~~

Methods: Thirty subjects 46, XY DSD, 30 healthy males and additional one SBMA case were included. Estimation of CAG repeat was determined using melt curve analysis. Sanger sequencing was used to confirm the length of CAG repeat.

Results: The melting point (Tm) from both DSD cases and controls were in the normal range from 89°C - 91.05°C, whilst SBMA case had the Tm of HRM at 92.65°C. Kruskal-Wallis H test showed a statistically significant difference Tm between the different length of CAG repeats with the result equal to the rise of Tm (X^2=45.022, p ~~=~~ 0.000). Sanger sequencing confirmed that all DSD cases had normal range from 13 to 27 repeats of CAG repeat and SBMA case had expanded 51 CAG repeats.

Conclusion: Mutation of exon 1 AR gene in DSD and hypospadias cases is rare~~,~~, however PCR-HRM is useful and less time consuming for screening of TRE. This is the first technique for AR gene screening that may applicable to other TRE diseases.

Keywords: androgen receptor gene (AR), CAG repeat, disorder of sex development (DSD), high

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resolution melting (HRM), melt curve analysis

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~~INTRODUCTION~~

Trinucleotide repeat expansion diseases (TRE) also known as trinucleotide repeat expansion disorders are codon reiteration exceed the normal and stable size either in coding or non-coding region of the genes that caused genetic disorders.[1] The codon repeat expansion can be polyglutamine (CAG) in some genes (AR gene, ATXN, DRPLA and HTT); and non-polyglutamine such as CGG (FMR1), CCG (FMR2), CTG (DMPK, SCA8 and SCA12 gene).[2]

The androgen receptor gene (*AR*) is localized on the X chromosome at Xq11–12. More than 1000 mutations (http://androgendb.mcgill.ca) in the AR and various functional defects have been found.[3] Eight exons of *AR* gene have four major parts: transcription regulation / N-terminal domain (exon 1), a DNA-binding domain (exons 2 and 3), Hinge region (exon 4) and steroid-binding domain (exon 5,6,7,8). The polymorphism in exon 1 of AR which is the N-terminal domain associated with gene regulation. The common changes is a trinucleotide repeat expansion (TRE) of polyglutamine (CAG).[4]

Androgens action in the target cells are mediated by the androgen receptor (AR). The receptor disruption will cause androgen insensitivity which may lead into varies phenotypes of 46, XY DSD from complete sex reversal (female phenotype), ranges of hypospadias to micropenis.[5] AR gene mutations also known to have an association with variation phenotype from ambiguous genitalia in newborn to azoospermia or oligospermia which contribute to 2-3% male infertility.[6]

Both in-vivo and in-vitro study show the elongation of CAG repeats will decrease the transcription activity of the AR by reducing protein product of the androgen-receptive area. The normal expression of AR is essential for the establishment and development of the genitals. Elongation of CAG repeats is expected to extend the chain of glutamine in receptor and increase the disruption of the structure and function of the N-terminal receptor. In normal people, the number of CAG repeats is 11-31.[7] Among under masculinized man, elongation of CAG repeats showed significantly different results compared to control.[7] Furthermore, pathogenic effects can also be found in expanded CAG repeat

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length as the risk of hypospadias in Caucasians.[8]

Spinal bulbar muscular atrophy (SBMA) or Kennedy's disease is a neurodegenerative disorder that consists of motor neuron degeneration in the brainstem and spinal cord caused by TRE of poly-CAG repeat expansion in the *AR* on the X chromosome.[9] SBMA patient with CAG tract length higher than DSD patient, might also have gynecomastia, oligospermia and erectile dysfunction as a results of mild androgen insensitivity.[10]

Fast and simple method will be useful for screening mutation in 46, XY DSD, especially in population study such as unknown causes of hypospadias and infertility. The traditional Southern blot is laborious, time-consuming, and requires high amounts of DNA template.[11] High-resolution melting (HRM) analysis is a simple method for mutation screening which has been introduced in 2002 performed on the genomic DNA (double stranded DNA) to amplify the DNA region of interest using real time PCR based process. A fluorescence dye applied to the HRM technique to bind specifically on the double stranded DNA as marker. Melting curves are directly created by monitoring the fluorescence level that is fast in sequence differences detection.[12] The HRM machine identified the process and show the graph of melting curve for each sample.[11] Good performance of real time PCR protocol is reported as fast, robust and potential use for diagnosis TRE in Huntington Disease.[13] However, this technique have not been carried out to analyse CAG repeat in AR gene. This study aimed to develop a fast and simple technique of CAG repeat expansion of *AR* gene using HRM technique that will be useful in screening and diagnosis of patients with TRE disease in Indonesia.

METHODS

Samples were 30 patients whose phenotypes characteristics of 46 XY, DSD such as external genitalia abnormality, and severe hypospadias with the diagnosis of under-masculinized ambiguous genitalia. Controls were 30 healthy males whose biological offspring and who had no history of primary

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infertility. One case with clinically diagnosed as SBMA were also included. Blood samples were drawn in heparinized vacutainer for chromosomal analysis and EDTA vacutainer for molecular genetic analysis. All subjects with DSD and SBMA had 46, XY. The chromosomal and molecular analysis had been done in Center for Biomedical Research (CEBIOR) Faculty of Medicine Diponegoro University. Ethical clearance was obtained from Institute Research Board Faculty of Medicine Diponegoro University. Please explain about the informed consent.

A set of primers was used to amplify CAG repeat in Exon 1 of AR gene: forward 5'-AGGCACCCAGAGGCCGCGAG-3' and reverse 5'-TAGCCTGTGGGGCCTCTACGAT-3'. qPCR-HRM Rotor Gene Q 5Plex HRM 72-well rotor (Qiagen, California, USA) amplified 203 bp PCR product with respective steps as follows: 95°C initial denaturation for 10 min; 40 cycles of 94°C denaturation for 30 sec, 71°C annealing for 45 sec and 72°C extension for 45 sec; and 80-95°C HRM in rising temperature by 0.1°C each step. Melt curve analysis (MCA) from post HRM step was analysed to estimate the CAG repeat length. Confirmation for CAG repeat length was done using Sanger Sequencing.

Kruskal-Wallis test used for analysis the association between CAG repeat lengths with HRM melting temperature (Tm). The repeat then classified into two categories for ≥ 20 and < 20 repeats for comparing means analysis using Mann-Whitney U test.

RESULTS

The CAG repeats of *AR* varied from all samples of both DSD cases and controls in range from 13(1; 1.67%) to 27 (3; 5.0%). The highest repeat frequency was 22 vs 23 repeat in group of DSD cases and controls, respectively (Figure 1). The mean of CAG repeat in healthy controls was slightly lower than DSD cases group. Mann Whitney-U test showed no significance differences between CAG repeat in healthy controls and DSD cases group (Table 1). The mean Tm is significantly difference ($p<0.05$) in ≥ 20 (n=55) and < 20 (n=5) repeat were 89.82 ± 0.19 and 89.29 ± 0.24 respectively.

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The MCA in DSD cases and controls showed the minimum Tm value was 88.87°C (13 CAG repeat) to the maximum value 89.95°C (27 CAG repeat). The SBMA case showed 51CAGrepeat with Tm at 92.65°C (Figure 2). Sequencing was performed as confirmation the CAG repeat length. The lowest and highest normal range; and the expanded CAG repeat were shown in Figure 3. Kruskal-Wallis H test showed a statistically significant difference Tm between the different length of CAG repeats with the result equal to the rise of Tm (X^2=45.022, p = 0.000). Higher Tm was observed in higher repeat (Figure 4).

DISCUSSIONS

The study of CAG repeats in exon 1 of AR gene is often analysed worldwide in DSD especially androgen insensitivity syndrome, male infertility, prostate cancer and SBMA. Patients with 46, XY DSD born with impairment in sex phenotype have high risk to be infertile male because of androgen receptor disruption.[14] Numerous studies in recent years have attempted to establish the relationship between CAG repeat length variation and on the targets of testosterone action.[7] The screening for CAG repeat polymorphism in AR gene by Adamovic et al (2012) revealed its association with the risk of hypospadias in Caucasians population.[8] Therefore, AR gene screening using simple and fast techniques is needed to find out if this polymorphism in the AR gene could be associated with reproductive males disorders.

CAG repeat lengths in this study were in the normal range with almost similar repeat average size from both the DSD patients and the healthy controls group. This results are in accordance with previous study conducted by Muroya et al (2001) as the CAG repeat length of AR gene did not constitute as a

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major factor in the development of hypospadias.[15] CAG repeat lengths appears to negatively affect the atypical phenotype in DSD cases. However, the finding of normal range of CAG repeat does not exclude the possibility of infertility and histology abnormality of testis in our DSD cases. The observation by Fietz et al (2011) demonstrated no correlation between CAG repeat length with histology of the testis.[16] Further study of higher number population is advised to assess genotype phenotype correlation in CAG repeat of AR gene with 46, XY DSD and male infertility.

The diagnosis for DSD in Indonesia with a multiple steps approach including clinical examination, chromosomal analysis, sonographic evaluation of the internal genitalia and mutation analysis found high prevalence of 46, XY DSD (65.9%).[17] However, establishment of DSD diagnoses in Indonesia currently faces limitations; such as lacking of diagnosis facilities and shortage access of health insurance. In comparation with sanger sequencing as the gold standard for CAG repeat, HRM is cost effective and rapid technique because require only one-step real-time PCR reagents compare to sequencing method.[18,19] This method also time effective and less laborious compare to RFLP-Southern blot analysis in TRE.[11,20] In developing countries with limited molecular laboratory facilities, this technique will be more applicable particularly for research and screening.

In our SBMA patient were found expansion of CAG repeats in exon 1. The amplicon contained higher CAG repeat that caused shifting melt curved to the right was proven in SBMA case. The polyglutamine segment of *AR* contain GC rich base lead to increase Tm in case of CAG repeat expansion.[12] Escalation of Tm in each group was associated with increase of CAG repeat, indicating that higher Tm in HRM analysis accordance with the raise of CAG repeats size (p = 0.00). This finding can be utilized as fast screening of CAG repeat expansion in SBMA and other TRE diseases. Nevertheless, the identification of CAG repeat for androgen insensitivity syndrome (either partial or

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complete type) should not be suggested due to its various mutation in almost all 8 exons.[21]

The expansion of poly-glutamine tract has been reported to have correlation with neurodegenerative genetic diseases such as SBMA, Huntington's disease and spinocerebellar ataxia (SCA). The expansion alters the N-terminal region that cause AR dysfunction leads to partial loss of AR function.[22] The repeat length of CAG in range 38-62 will cause SBMA and develop the signs of androgen insensitivity such as gynecomastia and infertility in addition of neurodegenerative symptoms.[10] In this study we could not find the correlation between atypical phenotype in 46, XY DSD and repeat length of CAG in AR gene, probably because of small samples size, various type of hypospadias or genital anomaly, ethnicity background and many other genes that caused DSD with similar phenotype. Other genes analysis using more advanced molecular testing should be done to deliver a genetic diagnosis of hypospadias or 46, XY DSD.

Similar technique was done in CGG repeats of FMR-1 for Fragile X Syndrome screening with quite high sensitivity and specifity result.[11]. This is the first study of CAG repeat screening using HRM in AR gene. More over screening using triplet repeat prime PCR and HRM for other TRE diseases which is quite common in neurodegenerative diseases can be considered.

What is the limitation of study?

~~CONCLUSION~~

Mutation in exon 1 of AR gene in DSD and hypospadias cases is scarce in Indonesian population. PCR-HRM is useful and effective for screening of CAG repeat expansion. This is the first technique for AR

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gene screening that may be applicable for other TRE diseases.

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ACKNOWLEGDEMENT

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CONFLICT OF INTEREST

The authors whose names are listed certify that they have **no conflict of interest** to declare.

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Table 1 please describe in one table about the patient characteristics that used for this studies.

Table 1. Results of the CAG repeat length analysis

Category	CAG repeats Mean ± SD	Median	*p*
DSD cases	23.23 ± 2.7	23	0.381*
Healthy controls	22.60 ± 2.62	23	

*Mann-Whitney U test not significant ($p > 0.05$)

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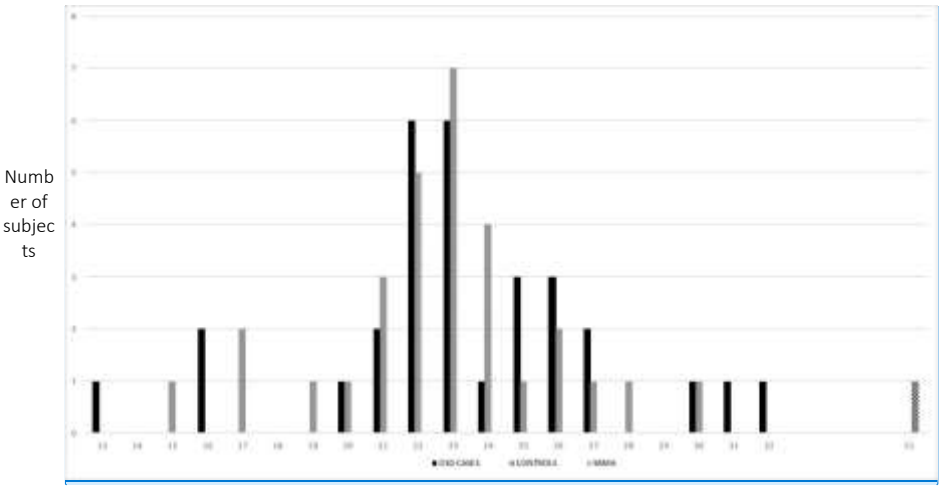


Figure 1 : The distribution of the CAG repeat length at exon 1 of the AR gene in DSD cases, SBMA case and controls.

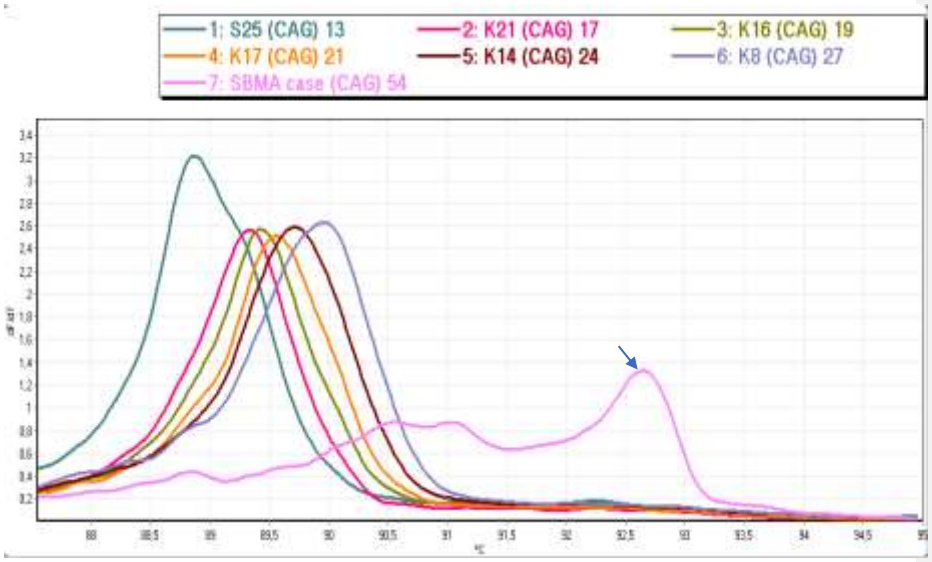
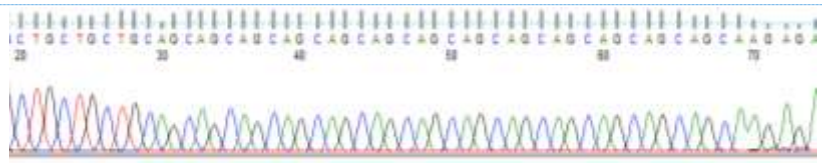


Figure 2. MCA showed Tm differences in each CAG repeat

Highest repeat showed higher Tm in CAG repeat expansion as shown in SBMA case with Tm 92.65^{0}C (arrow). Abbreviation: S = sample with DSD; K = healthy control.

A



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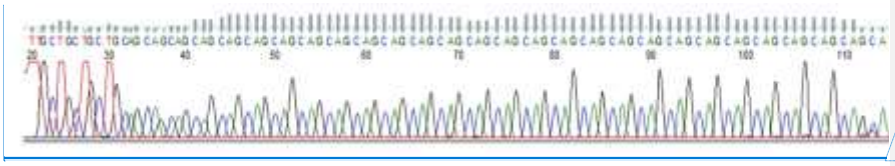

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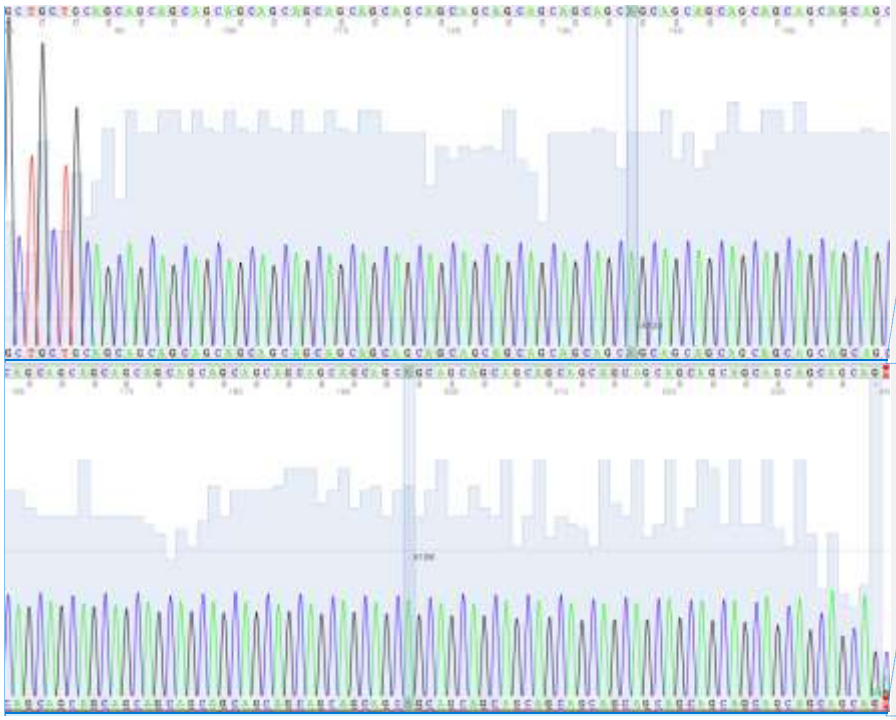

Figure 3. Sequencing analysis of samples (forward direction). CAG repeat was identified from exon 1 of AR gene. (A) The lowest length (13 repeats) was found in sample S8 and (B) the highest length (27 repeats) was found in K8 (healthy control). (C) The expanded 51 CAG repeat was found in SBMA sample.

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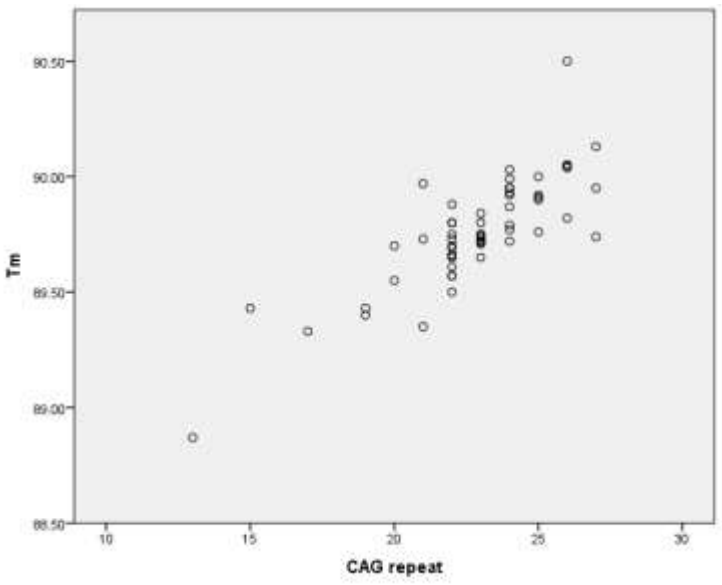
Figure 4. Characteristics of Tm in each repeat



*Kruskal-Wallis test significant (p<0.05).

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~~Tables~~

~~Table 1. Results of the CAG repeat length analysis~~

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~~Figure Legends~~

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Figure 2. MCA showed Tm differences in each CAG repeat

Higher repeat showed higher Tm in CAG repeat expansion.

Abbreviation: S = sample with DSD; K = healthy control

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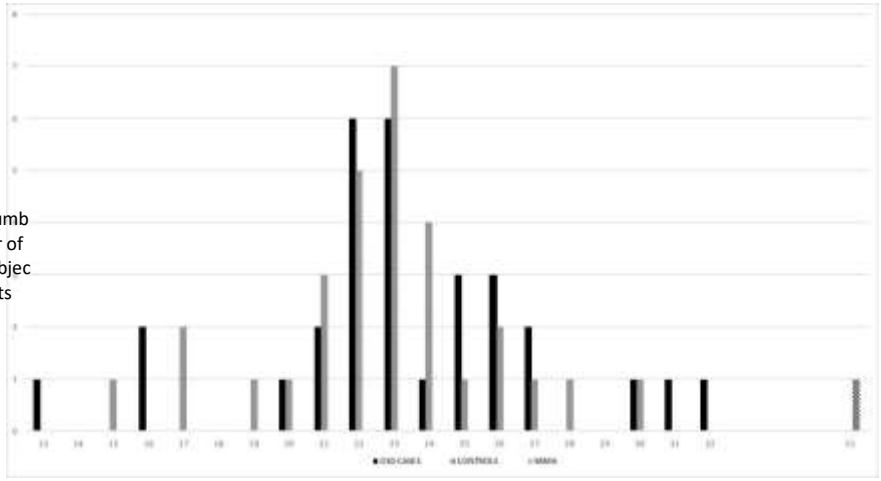
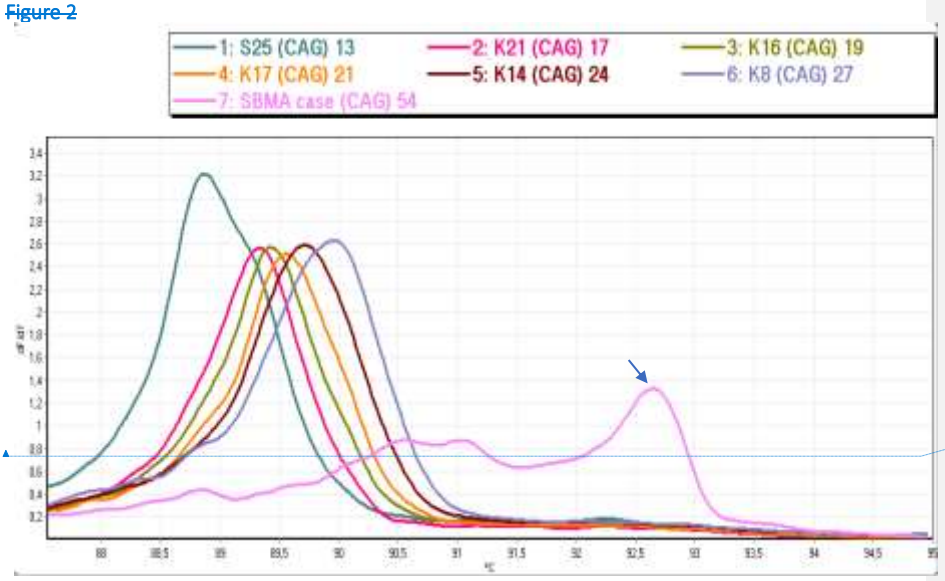


CAG repeat length

Figure 1 : The distribution of the CAG repeat length at exon 1 of the AR gene in DSD cases, SBMA case and controls

~~Figure 2~~



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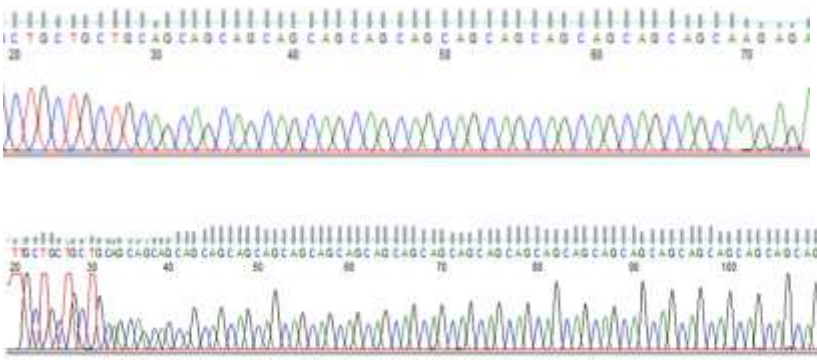
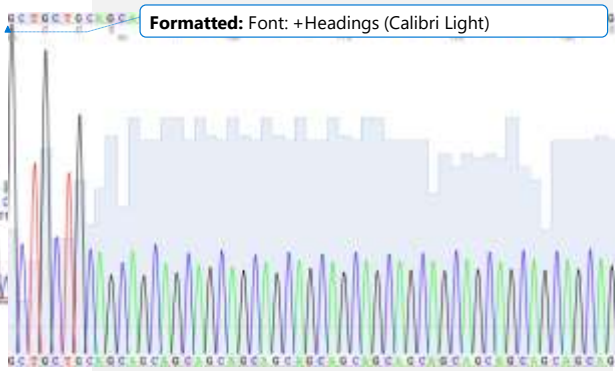
~~Figure 3~~

~~A.~~

~~B.~~



~~C.~~



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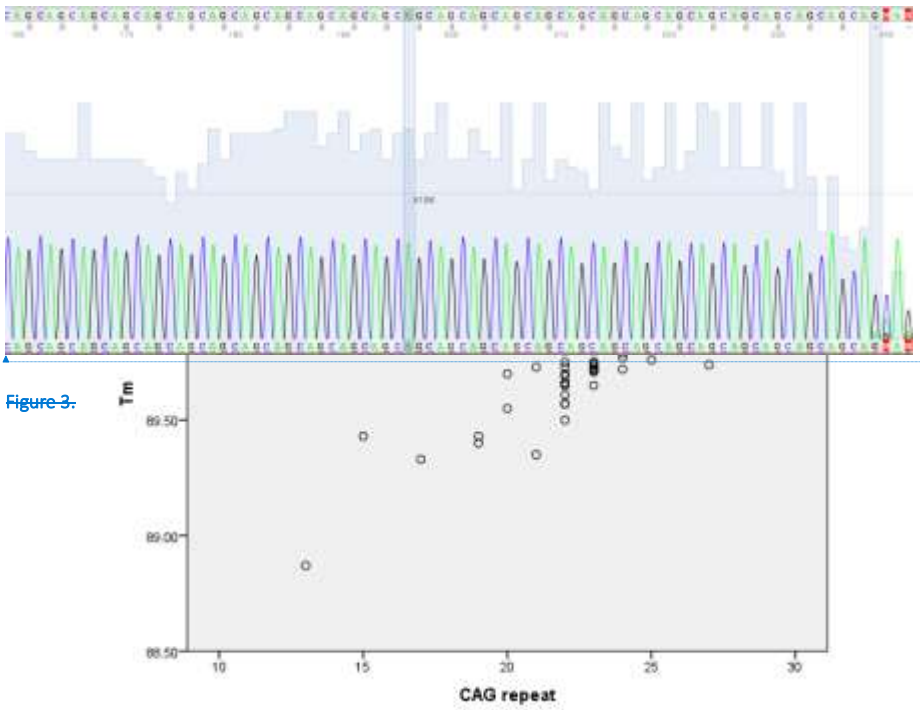

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From: **Med J Indones** <mji@ui.ac.id>
Date: Tue, Dec 18, 2018 at 10:08 AM
Subject: [MJI] Editor Decision: Revision Required (ID:3008)
To: Dr Nurin Aisyiyah Listyasari <nurinlistyasari@gmail.com>
Cc: Mahayu Dewi Ariani <mahayudewi@gmail.com>, Stefani Harumsari <dr.stefanisari@gmail.com>, Agustini Utari <agustiniutari@gmail.com>, Achmad Zulfa Juniarto <zulfa_juniarto@fk.undip.ac.id>, Sultana MH Faradz <sultanafaradz@gmail.com>

Dear Dr. Nurin Aisyiyah Listyasari:

We have reached a decision regarding your submission to Medical Journal of Indonesia, "Mutation screening of Trinucleotide Repeat Expansion Diseases with special reference on AR gene Using High-Resolution Melting techniques".

After the first round review, your manuscript needs to be revised accordingly. Please upload your revision from the system to enable us in tracking your record.

Here is frequently ask questions for the author to work with the system, http://mji.ui.ac.id/journal/public/journals/1/faq/Author.pdf

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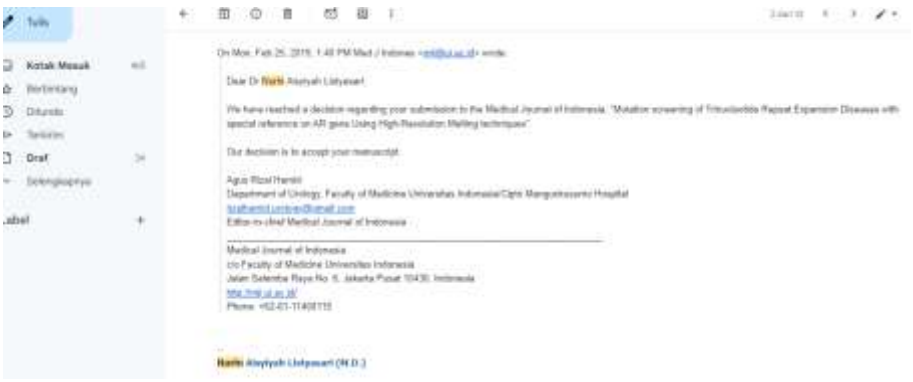
Nurin Aisyiyah Listyasari (M.D.)

Accepted



On Mon, Feb 25, 2019, 1:40 PM Med J Indones <mji@ui.ac.id> wrote:

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We have reached a decision regarding your submission to the Medical Journal of Indonesia, "Mutation screening of Trinucleotide Repeat Expansion Diseases with special reference on AR gene Using High-Resolution Melting techniques".

Our decision is to accept your manuscript.

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