Relationship Between Polymorphisms of DNA Repair Gene XPD 23 With Frequency of Micronuclei in Cervical Cancer Patients

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Abstract. *Cervical cancer is an important health problem, especially in developing countries like Indonesia. Radiotherapy is a major treatment modality for invasive cervical cancer with good treatment outcomes in early-stage patients and plays integral role in the combined-modality management of cervical cancer. Substantial variation in the intrinsic response of individuals to radiation can be seen from the polymorphism of DNA repair genes. DNA repair systems are responsible for maintaining the integrity of the genome and have a critical role in protecting against mutations that can lead to cancer. DNA damage can turn into chromosome alterations that are detected by the micronucleus (MN) assay. Micronuklei (MN) is one indication of structural damage to the chromosomes caused by radiation that can be observed in a cell with two nuclei (binukleat), by blocking the division process at the stage of cytokinesis using sitokalasin B known as cytokinesis Block (CB). The aim of this study is for relationship between polymorphisms of DNA repair gene XPD 23 with micronuclei in cervix cancer patients. From a sample of cervical patients before radiotherapy obtained a strong relationship (r = 0.602) but not significant (p = 0.153)*

Keywords: cervical cancer, xpd23, polymorphism, micronuclei

Introduction

Cervical cancer is the third most common cancer in women, with an estimated 529,828 new cases and 275,128 deaths reported worldwide in 2008. More than 85% of the cases found in developing countries, where it accounts for 13% of all female cancers. In developing countries such as Indonesia, the age standardized mortality rate is 10/10,000, more than three times higher than in developed countries (Irawan, 2008; Colombo, 2012). Cervical cancer deaths are higher in populations around the world where women do not have routine cervical cancer screening. In fact, cervical cancer is the major cause of cancer deaths in women in many developing countries. These women are usually diagnosed with late stage cancers, rather than pre-cancers or early cancers (Colombo, 2012; ACS, 2016).

Radiotherapy is a major treatment modality for invasive cervical cancer with good treatment outcomes in early-stage patients and plays integral role in the combined-modality management of cervical cancer (Kilic, 2015 and Shandiz 2014). The best option for the radiotherapy of cervical squamous cell carcinoma (SCC) is a combination of external radiotherapy and brachytherapy. Previous studies have shown the failure rate of radiotherapy alone around 30% in stage IB-IIA and IIB SCC of the cervix; it increased to 50% in stage III patients (Shandiz, 2014). Radiation is classified in two major forms: ionizing and non-ionizing. Radiotheraphy is one of ionizing radiation. The major types of ionizing radiation are alpha particles, beta particles, X-rays, and gamma rays (Reisz, 2014).

Ionizing radiation has been correlated with detrimental biological effects such as DNA damage, bystander effects, tissue injury, and, in some cases, carcinogenesis (Reisz, 2014, and Desouky, 2015). The biological effect of radiation can be mainly related to damages of the DNA. An ionizing radiation has a potential to directly interact with structures of the target to cause ionization, thus initiating the chain of events to lead to biological changes (Han, 2010). The structure of DNA can be damaged as a result of exposure to ionizing radiation (Maluf, 2004).

Regarding the detection of functional deficiencies, one approach is to use DNA repair defect as a biomarker for prediction of diseases. DNA repair plays a major role in maintaining genetic stability, and so measurement of individual DNA repair capacity should be a valued tool in molecular epidemiology studies (Collins, 2011). Efficient and proficient DNA repair is thus required for the effective maintenance of genome integrity. Ionising radiation is one agent known to cause DNA damage,, including single strand (SSBs) and double strand DNA breaks (DSBs), and it is the mis-repair of DNA damage, particularly DSBs, which results in chromosome aberrations (Wilding, 2005).

Polymorphisms of DNA repair proteins are another group of genetic susceptibility factors that could influence the level of chromosome alterations by affecting the repair of various DNA lesions induced by exogenous and endogenous genotoxic compounds and ionizing and UV radiations. A number of DNA repair protein variants are known in humans, but the significance of many of these polymorphisms is notfully understood (Tuimalaa, 2004).

XPD (xeroderma pigmentosum complementation group D, also known asERCC2) gene encodes an ATP-dependent DNA helicase activity that participates in both nucleotide excision repair and basal transcription as part of the transcription factor, TFIIH. The Lys751Gln (T > G, rs13181) polymorphism at codon 751 of exon 23 causes a non-synonymous substitution of Lysine to Glutamine. A number of case-control studies have shown the association of the 751Gln variant with lung cancer, and melanoma in the male population (Pramani, 2011).

Cytogenetic alterations in cultured peripheral blood lymphocytes, such as chromosomal aberrations(CAs),

sister chromatid exchanges (SCEs), and micronuclei (MN), have for many years been applied as biomarkers of genotoxic exposure and early effects of genotoxic carcinogens (Noorpa, 2004).

Micronuclei assay has been extensively used to evaluate the presence and the extent of chromosomal damage in human population exposed to genotoxic agents in various occupational and environmental setup. In addition, the frequency of micronuclei increases in various chronic diseased conditions. It is known that cytogenetic damage accumulates in humans with age, either due to the prolonged exposure to oxidative damage, chemicals as well as occupational, therapeutic or accidental radiation (Joseph, 2004).

DNA damage can turn into chromosome alterations that are detected by the micronucleus (MN) assay (Maluf, 2004). Another well known bio-indicator of radiation damage is micronuclei in peripheral blood lymphocytes. Micronucleus are or chromosome fragments that lag behind during anaphase and are not included in the main nucleus during telophase. They appear as a small nuclei and can identified during binucleated cell in the division of mitogen-activated human lymphocytes by blocking cytokinesis (Purnami, 2015).

MN frequency was shown to be a reliable biomarker in many biomonitoring studies among human populations therapeutically, occupationally and accidentally or environmentally exposed to ionizing radiation. Most of these studies reported significantly higher MN rates inexposed populations than in controls (Sari-Minodiera, 2007). Because DNA repair and frequncy of micronulei important for detect DNA damage, so we study the relationship between polymorphisms of DNA repair gene XPD 23 with micronuclei in cervix cancer patients.

Materials and Methods
Study Population

The study population consisted of 7 cervical cancer patient with IB due to IIB stadium. Average radiation exposures is 2 Gy. From each donor, a single blood sample (5ml) was taken by venipuncture into heparinized tubes (Vacutainer) and EDTA tube. All donors gave informed written consent for participation in the study.

Processing of blood samples\

Blood samples of cervical cancer patients were isolated with the procedures of DNA isolation kit (Roche) to obtain pure DNA. 200 mL samples inserted into nuclease-free microtube and added 200 mL of binding buffer and 40 mL proteinase-K. Furthermore, homogenized and incubated at 70ºC for 10 minutes. After that, added and mixed 100 mL of isopropanol. Furthermore, the sample is pipetted into the upper

reservoir of high pure filter-receiver tube is then centrifuged 8000 g for 1 minute. Receiver tube was removed and the high pure filter is placed into a new collection tube. Then added 500 mL wash buffer into the high pure filter and centrifuged at 8000 g for 1 minute (1x repeated again). After that, wash buffer in the receiver tube was removed and the high pure filter is placed back into the collector tube is then centrifuged 13,000 g for 10 seconds. After centrifugation, the tube collectors of high pure filter removed and placed into a sterile micro tube. After that, added to the elution buffer that has been warmed to 70ºC in the upper reservoir and centrifuged at 8000 g for 1 minute. After centrifugation, the tube containing pure DNA.

DNA repair gene polymorphism analysis with RFLP

PCR reactions were performed in a volume of 20 mL reaction consisting of 0.5 mL of forward primer and reverse primer, 5 mL of DNA and DNA Essential Faststart Green Master 10 mL, 4 mL Water PCR Grade, and forward primer (5'-GGT-CCT-TCT-CCG-ACT-CCC-3'), reverse primer (5'-CTG-CCT-TCT-CCT-GCG-ATT-3') with annealing temperature of 61ºC. PCR cycle starting from pre-denaturation at a temperature of 95ºC for 10 minutes. PCR cycles were 30 cycles consisting of a denaturation step at 95ºC for 20 seconds, annealing at 55ºC for 20 seconds and elongation at 72ºC for 20 seconds. Afterwards, consisting of initial melting stage at 60°C for 20 seconds and the final stage at 95ºC for 20 seconds. The results of PCR and RFLP do electrophoresis method. Sample results of PCR amplification decampur with 0.5 ul enzyme PstI and incubated for 2 h at 37°C. Then separated on 2% agarose gel. Electrophoresis tool set at a voltage of 50 V for 2.5 hours. The results read using GelDoc. And the genotype is determined by the size of fragment DNA.

Micronuclei Assay

Cultures were set up in of Roswell Park Memorial Institute (RPMI 1640) culture medium supplemented with HEPES and L-Glutamine, 20% Fetal Bovine Serum (FBS), Penicillin Streptomycin, and Phytohaemagglutinin (PHA). Culture was maintained in a 5% humidified CO_2 incubator at 37°C for 72 hours. After incubation for 44 h, 0.015 ml of cytochalasin B (Sigma Aldrich) solution inDMSO (Sigma Aldrich) was added to cultures, and cultivation was continued for another 24 h. The cultures were then treated with cold hypotonic solution (0.075 M KCl) to lyse red blood cells. The supernatant was removed and replaced with fixative consisting of methanol:acetic acid (10:1) with Ringer;s solution. The cells were washed with three changes of fixative then resuspended gently. Tthe suspension dropped onto clean glass slides and allowed to dry. Slides then stained with 4% Giemsa solution in a potassium phosphate buffer (pH 7.3) and allowed to dry overnight. The slides were mounted with cover slip and allowed to dry completely before scoring. The slides

then were analyzed and identification of micronuclei was done according to the scoring criteria in IAEA publication. At least 1000 binucleated cells with well-preserved cytoplasm were scored for the MN frequency.

Results and Discussion

Real-time PCR can detect the amount of PCR product (amplicon) during each cycle of PCR by using fluorescence. In the final stages of real-time PCR can be programmed to generate a melting temperature curve and calculate the melting temperature (Tm) PCR products (Sambrook, 2001). Analysis on melting analysis has become part of a real-time techniques. Each DNA has a melting point (melting temperature/Tm). The results of the patient's DNA sample amplification is shown in Figure 1.
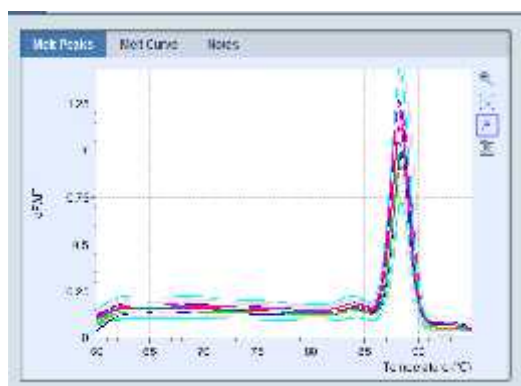

Figure 1. Cervical Cancer Results of Real Time PCR

The results of DNA amplification in cervical cancer samples using annealing 61 $^{\circ}$C indicates there is only one curve for each sample. It is marked that the

sample successfully amplified well and does not come contamination. PCR products can be analyzed in accordance with the information to be obtained as to determine whether or not the length of target DNA or DNA products. From the results of subsequent ampli-fication electrophoresis using RFLP electrophoresis method. Results visualization of PCR by electrophoresis shown in Figure 2.

From visualization of results was obtained DNA with size 207 bp and 244 bp is normal homozygote (genotype AA) and 181 bp, 207 bp and 244 bp are heterozygous (genotype AC). Results of electrophoresis samples of cervical cancer and mikronuklei shown in Table 1.
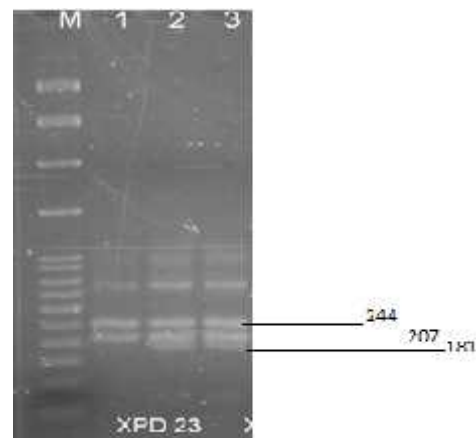

Figure 2. Electrophoresis results in patients with cervical cancer

Table 1. Elektroforesis Results and Micronuklei Frequency

No sample	Size DNA (bp)	Genotip	Frekuensi Mikronuklei
1	207 244	AA	33
2	181 207 244	AC	37
3	181 207 244	AC	54
4	181 207 244	AC	41
5	181 207 244	AC	59
6	181 207 244	AC	46
7	181 207 244	AC	53

Scoring of micronuclei in cells with a nucleus duan (binukleat) is relatively easy, accurate and quick procedure and is therefore as a sensitive biomarker for assessing cellular response to irradiation. It has been reported the radiation dose may increase the frequency of micronuclei in human lymphocytes in vivo and in vitro.

Based on the analysis using SPSS Pearson correlation obtained r = 0.602 with a significance of variables p = 0.153. This shows a strong correlation (r> 0.5-0.75) but not significant (p> 0.05). According Lebana (2004), mikronuklei frequency increases in a variety of chronic pain conditions. It is also known that radiation alone may not always be the only factor affecting cytogenetic damage and may interact with other factors or agents.

Conclusion

Analysis results show that r correlation is 0.551 with a significance of variables p = 0.2. This shows a strong correlation (r> 0.5-0.75) but not significant (p> 0.05).

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