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Improved PCR-RFLP Method for Her-2 Ile655Val Breast Cancer Patients Detection

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***Abstract*— HER2 is a protein that is essential to the process of differentiation, proliferation, tissue development and other physiological processes. HER-2 overexpression was occurred in 20-30% of breast cancer patients. Targeted therapies was performed using trastuzumab, on the basis of the examination by immunohistochemistry. Single nucleotide polimorphisme (SNP) Ile655Val were found on HER-2, which indicates the risk of cancer as well as a predictive factor of treatment with targeted therapies. The method had been used for HER-2 SNP detection is using TaqMan method which is expensive, or PCR RFLP which is cheaper and easier. 148bp PCR product was widely used in PCR RFLP for HER-2 Ile655Val SNP detection confirmed at high concentration of agarose (3%). This study conducted to improve the PCR RFLP methods become more economical and more efficient by increasing the size of the PCR products confirmed with low concentration of agarose which have lower prices. The results showed that PCR RFLP method developed in this study, 480bp PCR product restricted with BsmAI confirmed with 2% agarose, has been successfully performed with cost effective and a high level of reproducibility.**

***Keywords*— Breast cancer; HER-2; Trastuzumab; SNP; PCR-RFLP**

I. INTRODUCTION

Cancer is one of the leading causes of death worldwide. In Indonesia, around 8,2juta deaths caused by cancer in 2012. Nationally, the prevalence of cancer in the population of all ages in Indonesia in 2013 amounted to 1.4 ‰ or estimated to be around 347.792 people. Cervical cancer and breast cancer is disease with the highest prevalence in Indonesia in 2013, which amounted to 0.8 ‰ of cervical cancer and breast cancer by 0.5 ‰. Based on Dharmais national cancer hospital data, during 2010-2013, breast cancer, cervical, lung, ovary, rectum, thyroid, colon, hepatoma, and nasopharyngeal cancer is known as the most common cancer. Breast cancer, cervical cancer and lung cancer are the three most common diseases, which the number of morbidity and mortality continues to increase [1], [2].

It is known that the cell growth cycle is controlled by various oncogenes and suppressor genes. Both of these genes products function as components in signal transduction which allows respond to a simulation of the outside of the cell. Abnormalities in oncogenes or suppressor genes will produce abnormal proteins with abnormal signal transduction functions that result in malignant transformation. It is can be assumed that cancer is not only caused by the uncontrolled cell growth but could also because of wrong signalling processes. The signalling pathway not only regulate the growth but also regulate various cellular processes such as differentiation, angiogenesis, migration, apoptosis, inhibition of apoptosis, and etc. Some of important signaling molecules such as Ras /MAPK, HER-2/PIK3Ca / PTEN/Akt and others. Currently, the signal molecules were reported as a therapeutic target, as a potential prognostic biomarker. The use of molecules in the signaling pathway as a therapeutic target is currently applied in the implementation of the concept of personalized medicine [3], [4], [5].

Her-2 is a proto-oncogene consist of the receptor protein, trans membrane and tyrosine kinase. It is classified to the epidermal growth factor receptor (EGFR) which also known as HER family encoded by the genes her-2 on chromosome 17q21. In normal conditions, these proteins together with other HER proteins form homodimers or heterodimers regulate a variety of cellular functions including cell proliferation. Overexpression of HER-2 occur in 20-30% of breast cancer cases. Breast cancer with HER-2 overexpression is more aggressive and have a poor prognosis. Not only found in the breast cancer, overexpression of HER-2 is also found in the ovarian, lung, and gastric carcinoma.

Monoclonal antibody trastuzumab is a targeted treatment for those overexpression HER-2 breast cancer patients [6] – [9].

As an antitumor agent, treatment with trastuzumab mechanism is not fully understood. Tratsuzumab been reported to induce antibody dependent cellular toxicity (ADCC), inhibits the cleavage domain of extracellular HER-2, inhibit PIK3 / AKT, improve the localization of membrane PTEN, are the target of treatment based on a monoclonal antibody, trastuzumab. Recommended duration of trastuzumab treatment is one year of treatment according to the ESMO (European Society Medical Oncology). NCCN (National comprehensive Cancer Network) and St. Gallen, also recommends treatment with trastuzumab at the same duration for this type of breast cancer. Costs incurred for the purchase of monoclonal antibody trastuzumab per ampoule for each month usage is in the range of thirty million rupiahs. Since the prize quite expensive, it is need mature consideration for trastuzumab treatment. Immunohistochemistry methods are not enough used as a basis for treatment with trastuzumab. It is reported that single nucleotide polymorphism at the trans membrane domain HER-2 Ile655Val can be used as the area to know receptor-drug interaction, drug susceptibility, receptor topology. Thus it can be used as a predictive factor of the patient's response to treatment monoclonal antibody trastuzumab. It was reported that trastuzumab were more effective to patients with overexpression of HER-2 Ile655Val genotype homozygous. The risk of cardiotoxicity will be found in patients with heterozygous Ile655Val [10] – [19].

There are several methods for the single-nucleotide polymorphisms (SNPs), from the simple way to the complex ones. In several study, HER-2 Ile655Val, was reported tested using TaqMan allelic discrimination which are expensive. The popular methods for HER-2 Ile655Val genotyping which are no special equipment, easy to design, simple and inexpensive is PCR-RFLP. The presence or absence of the restriction enzyme recognition site result in the formation of restriction fragments of different sizes, allele identification which can be done by the electrophoretic fragments separation. In some reports, most of the PCR RFLP of HER-2 Ile655Val genotyping were done with 148bp PCR fragment, continue with digestion of each PCR product with *BsmAI*, which gives 116 and 32 bp fragments for the Val allele and 148bp fragment for the Ile Allele. The PCR product cut result will be too short and should be separate in the high concentration of agarose (3% agarose). This will increase the cost service of the HER-2 SNP detection with PCR-RFLP methods. Here in this research were conducted to improve the HER-2 PCR-RFLP A to G transition (Ile655Val) methods in order for cost effective of HER-2 SNP detection by increasing the PCR product size confirmed in low concentration of agarose [20] - [23].

II. Materials and Methods

A. Materials

The research was conducted using ethical clearance issued by Indonesia Ministry of Health . Breast cancer tissue was provided from several hospital in West Sumatera province in Indonesia. The fresh tissue samples were stored at -80 ° C.

B. HER2 Breast Cancer Tissue Genome Extraction

DNA genom of breast cancer tissue were extracted according to the procedure PureLink® DNA genome extraction kit from Invitrogen. The DNA genome extraction were then confirmed in 1% agarose gel

C. HER-2 PCR Amplification

HER-2 PCR amplification were amplified with below PCR mixed: 22µL Thermo Scientific Nuclease-Free Water, 25µL Thermo Scientific DreamTaq Green PCR Master Mix (2x), 1µL Forward Primer (5'-TGA TCT GCC CAC AGA CTC-3 ') and 1µL Reverse Primer (5'-TCT CAT CGT CCG CTT GTA CC-3') and genome template. PCR was performed using a thermo cycler (Kyratec's SuperCycle, Republic of Korea) under the following PCR conditions: Initian denaturation of 5 min at 95 ° C, 35 cycles of 30 seconds at 95 ° C (denaturation), 30 seconds at 55 ° C (annealing), 1 mins at 72 ° C (extension), followed by a final extension for 5mins at 72 ° C. PCR product were confirmed in 1% agarose stained with EtBR.

D. DNA Sequencing

DNA sequencing were done based on the Sanger's method , with ABI 3100 Genetic Analyzer Applied conducted by PT . Genetic Science. Sequencing result than was analysed with BLAST program for gene identification.

E. Sample Preparation, optimization digestion and analyze data

480bp PCR product were purified with the Wizard SV gel and PCR clean up system kit. The purification methods were following the protocol from the manufactures. Pured DNA PCR product 480bpwere restricted with *Bsm*AI, and incubated at several incubation time 15 min, 30 min, 45 min, 1 hour and 3 hours, incubated at 55 ° C. The *Bsm*AI restricted result than were electrophoresis with 2 different concentrations of agarose, 1.5 % compared with 2 % agarose. Restricted product 480bp DNA fragment were separated with optimum agarose gel. Restricted enzyme *Bsm*AI will not cut 480bp DNA target if there was no SNP. 376bp and 104bp DNA fragment will produced if there was homozygous SNP. While for the heterozygous SNP will produce 480bp, 376bp and 104bp DNA fragment.

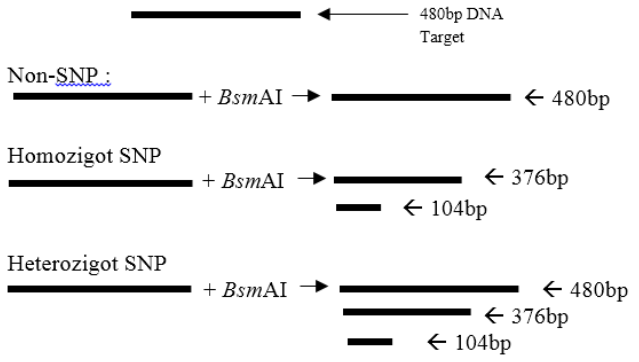

Fig. 1. Illustration Results of *Bsm*AI cuts inside 480bp PCR fragment product.

III. Result and Discussion

A. PCR HER2 transmembrane domain with the Target Product 480bp

For optimization of PCR conditions were used gradient PCR from 50-60°C Thermo Scientific DreamTaq Green Polymerase.

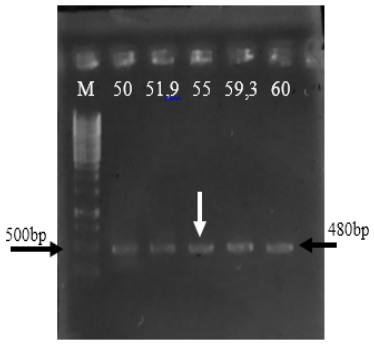

Fig. 2. Optimization of PCR by using using Thermo Scientific DreamTaq Green (right). DNA samples were amplified shows 480bp size.

From the above results optimization of PCR using annealing temperature gradient between 50 ° C to 60 ° C, 55°C shown as the best annealing temperature at 480bp corresponding to the target. The PCR product then confirmed with sequencing methods, and shown as targeted ones. (data was not shown).

B. PCR RFLP optimization

The optimization process conducted on the optimization of incubation time were: 15 min, 30 min, 45 min, 60 min and 180 min, incubated at 55 ° C. Process optimization was performed using the known constituent sequence DNA sample nucleotides, non SNP HER-2 and heterozygous SNP HER-2. The *Bsm*AI restricted result than were electrophoresis compared between 2 different concentrations of agarose, 1.5 % and 2 % agarose.

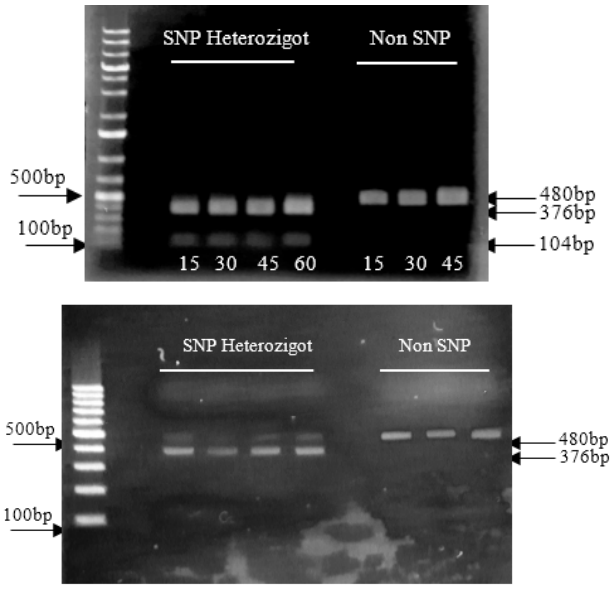

Fig. 3. RFLP Using 4 Incubation Time In contrast to Sample No. 1 and 2 with Marker 1kb DNA Ladder Plus, Agarose 1.5 % (above) and Agarose 2 % (below)

From figure 3 and 4 can be seen that the *Bsm*AI restricted the DNA template and separated well at 2 % agarose accordance to the known DNA sequence template. The incubation time did not show a significant difference in each incubation time: 15, 30, 45 and 60min. Furthermore 180 min incubation time the restricted DNA shown fade, this is possible because the some of the DNA template partially degraded during the incubation. It is suggested incubation time for *Bsm*AI restricted DNA were in 15 min incubation, confirmed at 2% agarose.

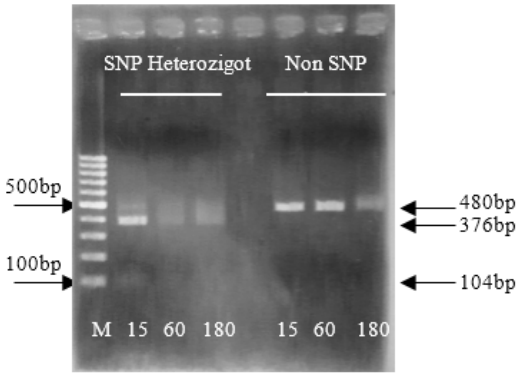

Fig 4. RFLP using 3 Incubation Time In contrast to Sample No. 1 and 2 with Marker 100bp DNA Ladder, Agarose 2 % .

Based on those optimization condition, another 30 DNA sample of breast cancer then were PCR, purified, and restricted. The result was shown in below figure. Those 30 DNA sample were had been checked for the sequence for each sample.

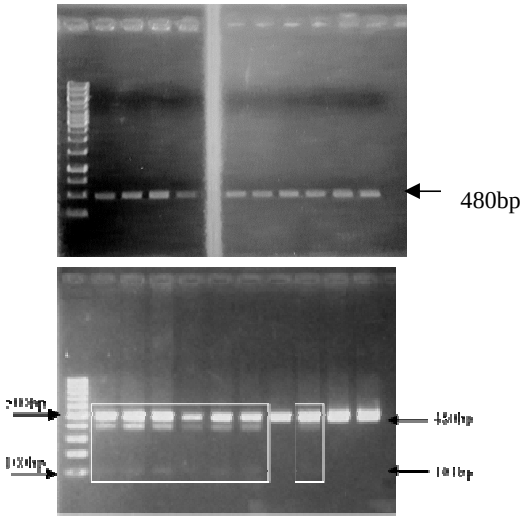

Fig. 5. Some of purified PCR product (above) and PCR RFLP result electrophoresis with 2 % Agarose (below).

Comparing with the sequence result, shown 100% same result as those PCR RFLP result which developed in this research.

Human epidermal growth factor receptor (HER-2) is located on chromosome 17q21 and encodes a transmembrane glycoprotein exhibiting tyrosine kinase activity. Overexpression of HER-2 has been observed in 20-30% of breast cancer, and associated with poor prognosis. Immunohistochemistry (IHC) is the most widely used

methods for the HER-2 overexpression detection. Based on IHC result than as the basis of recommendation for the targeted therapy for those patients. Humanized monoclonal antibody trastuzumab was HER-2 targeted therapy approved for clinically use. Trastuzumab show clinical benefit, but in many patients HER-2 over expressing do not shown respond to these agents {[9], [18], [19]}. Additional diagnostic method was needed for identifying patient for targeted therapy.

PCR-RFLP is a popular method for SNP or mutation genotyping. PCR RFLP methods need no special equipment, easy to design, simple and inexpensive. In some report shown that PCR RFLP has been applied for the detection of mutations associated with tumorigenesis. This method has been successfully applied for N-ethyl-N-nitosourea-induced mutation in codon 12 of cH-rasl (*Msp*I site 1695-1698) and codon 248 of the p53 tumor suppressor gene (*Msp*I site from 14,067 to 14,070) in human skin fibroblasts [25]. For PCR-RFLP HER-2, also have been reported by several researcher which then confirmed in 3% agarose for PCR-RFLP {[26], [27], [28]}. The high concentration of agarose will increase the cost of HER-2 genotyping. In development countries such as Indonesia, lower cost for detection were very consider and needed. Standard protocol for PCR-RFLP HER-2 than were improved by reducing the agarose concentration by increasing the size of the PCR products amplified HER-2 into 480bp. Restricted enzyme *Bsm*AI will not cut 480bp DNA target if there was no SNP. 376bp and 104bp DNA fragment will produced if there was homozygous SNP. While for the heterozygous SNP will produce 480bp, 376bp and 104bp DNA fragment.

In order to get faster result of HER-2 PCR-RFLP, optimization also performed by optimization of the restricted enzyme incubation times. As the result of optimization, 15 minutes as the best incubation time and 2% agarose as best agarose concentration to distinguish homozygous or heterozygous genotypes. In below Table was shown the timeframe in each step of HER-2 PCR RFLP

TABLE I
TIME FRAME IN EACH STEP OF HER-2 PCR-RFLP ANALYSIS

Procedure	Time required (min)
DNA extraction	30
PCR amplification	
• Set up	15
• incubation	90
Restriction digestion	
• Set up	15
• Incubation	15
Gel electrophoresis	
• Gel set up	60
• Run	30
• Gel staining	15

The time needed for genotyping HER2 to determine HER SNP is approximately 4 hours, with shown high reproducibility. Other 30 breast cancer patient which treated with HER-2 PCR RFLP optimize conditions obtained in this study notify 100% the same result as sequencing. Results of the study then could be recommended to be applied to the HER-2 genotyping, assist the oncologist in determining a right patient getting the right targeted therapy to support personalized medicine. This improved PCR RFLP method could be useful for clinical and epidemiological purposes.

IV. CONCLUSIONS

HER-2 PCR-RFLP methods have been succeed improved to be cost effective by increasing the PCR product in order to decrease the agarose concentration. Improved HER-2 PCR RFLP shown high reproducibility, 100% similarity as sequence results.

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