

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 polymorphisms (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.

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.

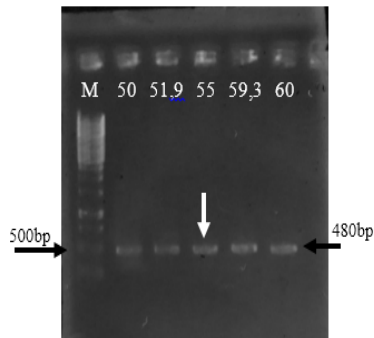


Fig. 2. Optimization of PCR by using using Thermo Scientific DreamTaq Green (right). DNA samples were amplified shows 480bp size.

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.

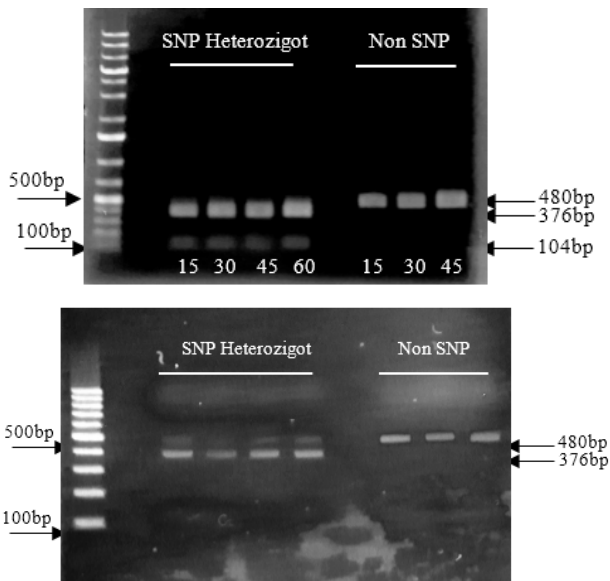


Fig. 3. RFLP Using 4 Incubation Time In contrast to Sample No. 1 and 2 with Marker 1kb DNA Ladder Plus, Agarose 1.5 % (above) and Agarose 2 % (below)

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.

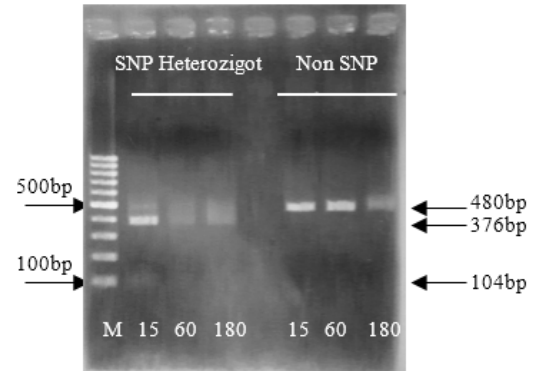


Fig 4. RFLP using 3 Incubation Time In contrast to Sample No. 1 and 2 with Marker 100bp DNA Ladder, Agarose 2 % .

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.

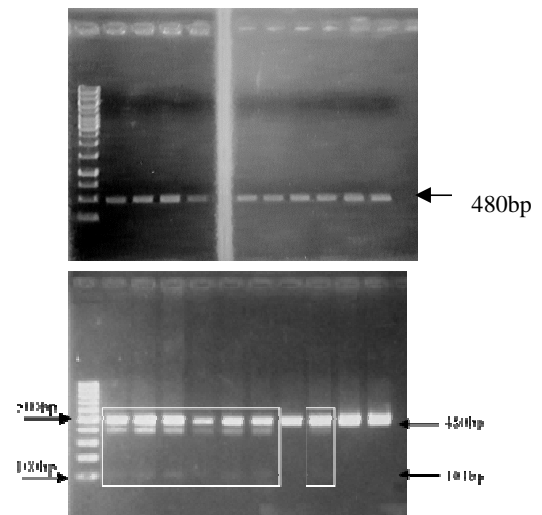


Fig. 5. Some of purified PCR product (above) and PCR RFLP result electrophoresis with 2 % Agarose (below).

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-nitrosourea-induced mutation in codon 12 of cH-ras (*MspI* site 1695-1698) and codon 248 of the p53 tumor suppressor gene (*MspI* 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 *BsmAI* 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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