ILE655VAL GENOTYPING STUDY OF HER2-POSITIVE BREAST CANCER OF PATIENTS FROM PADANG-INDONESIA WITH HIGH RESOLUTION MELTING TECHNIQUE

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Abstract

Trastuzumab has proven to be a great improvement in the treatment of HER2+ breast cancer patients, but it is associated with relevant adverse cardiac events and significantly elevated cost of treatment. One of the risk factor for cardiotoxicity due to trastuzumab is the I655V HER2 polymorphism (GTC> ATC mutation) in which the allele mutant (Ile/Val or Val/Val) has a higher risk than the wild type (Ile/Ile). The detection of specific alleles is very important for therapeutic decision-making. In this study, our group has developed a high resolution melting (HRM) with EvaGreen dye method to discriminate specific allele of I655V HER2 (wild type, heterozygote mutant or homozygote mutant) in 66 frozen section samples of HER2+ breast cancer patients. Our study revealed that the wild type is the most prevalent allele (77,27%), whereas heterozygous mutation is significantly present in this research (21.21%) and around 1.52% of samples were detected as minor allele. Only one sample was detected as a minor allele (Val/Val) and may have relatively low abundance in the population. This method has been compared to Sanger sequencing and shows 100% of validity.

Keywords: Breast cancer, HER2, I655V, HRM, allele

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Introduction

It was estimated that almost 1.7 million cases of female worldwide diagnosed with breast cancer during 2012 (WHO, 2012). Approximately a quarter (24%) of all those breast cancers were diagnosed within the Asia-Pacific region (approximately 404,000 cases at a rate of 30 per 100,000), with the greatest number of the incidents occurred in China (46%), Japan (14%), and Indonesia (12%) (Youlden *et al.*, 2014). In Indonesia, breast cancer is placed as the first rank of cancer among females which caused around 19.731 died in 2012 (WHO, 2014).

Gene expression profiling studies have identified at least four categories of breast cancer: luminal A, luminal B, HER2 overexpressing, and basal-like or triple negative (TN) (Eliyatkın *et al.,*2015). Around 15% of breast cancer patients were identified as HER2 overexpressing category (Slamon *et al.*, 1987). The extracellular domain of HER2 specifically is recognised by humanized monoclonal antibody called Trastuzumab (Herceptin), which inhibit basal and induce HER2 cleavage. This effect strongly give an impact on the treatment of breast cancers and use as first line therapy for metastatic breast cancer overexpressing HER2 receptor with taxane-based chemotherapy. (De Laurentiis *et al.,* 2005).

HER2 gene polymorphism studies have revealed that the substitution of isoleucine to a valine at codon 655 (I655V, GTC> ATC) is a major risk factor in the development of breast cancer. (Millikan *et al.,* 2005; Lu *et al.,* 2010). Women with the genotype of Ile / Val or Val / Val have a higher risk of breast cancer, although some

other studies suggest that this correlation is controversial (Dahabreh & Murray, 2011). On the other hand, the presence of valine alele was also associated with higher possibility of cardiotoxicity in the use of trastuzumab (Roca et al., 2013; Beauclair et al., 2007).

Therefore, examination on specific alleles of the HER2 gene is crucial in patient care decision making. Low cost method that highly sensitive and specific becomes the main requirements for inspection. Detection of HER2 I655V polymorphism has been widely studied by various methods such as PCR-RFLP (Lee et al., 2008; Desriani et al.,2016). Real Time PCR TaqMan (Siddig *et al.*, 2008; Nelson *et al.*, 2005), Sybr Green I-Based Melting Curve method (Budiarto et al., 2016) and PCR-sequencing (Budiarto & Desriani, 2016). PCR-sequencing is the gold standard of mutation detection but the labor and costs are still limiting factors for routine clinical practice. Meanwhile, HRM is easier, cheaper and quite familiar for mutation detection, but no report on SNP HER2 I655V detection using this method.

The HRM is a post-PCR DNA melt curve analysis used for identifying genetic variation, which relies on the use of fluorescent dyes as DNA helix intercalating dye. Some of the dyes that commonly used for HRM are LCGreen, ResoLight, and SYTO9. Unfortunately, these dyes are expensive and may not be suitable for a large number of samples. EvaGreen is a much cheaper and has been used in quantitative real-time polymerase chain reaction (qPCR) and HRM. (Li *et al.*, 2010). The purpose of our study was to develop HRM method using EvaGreen dye, a cheap intercalating dye, for detection and screening of SNP HER2 I655V polymorphisms among samples of HER2 positive breast cancer isolated in Padang, Indonesia. The results can be used as preliminary data to determine the characteristics of gene mutation pattern of HER2 positive breast cancer patients in Indonesia.

Materials and Methods

Sample. 66 frozen section samples of HER2+ breast cancer were collected from M. Djamil Hospital Padang, Sumatera Province, Indonesia. Genomic DNA from samples was isolated by using *Purelink*® genomic DNA mini kit (Invitrogen) following the manufacturer's protocols. DNA quantity was assessed spectrophotometrically with the NanoDrop ND 1000 (Peqlab, Erlangen, Germany), and the quality of genomic DNA was confirmed by agarose gel electrophoresis. We used pGEM_HER2_AA as wildtype control and pGEM_HER2_AG & pGEM_HER2_GG as mutant controls (homozygous & heterozygous). The plasmids were obtained from recombinant *E. coli* DH5α using High Speed Plasmid Mini Kit (Geneaid) and proceeded following the instruction manual.

Primers and PCR-HRM. Sequence-specific primers were custom designed to flank polymorphisms of the target gene of interest. Primers were designed by using Primer3 software and verified by BLAST on Pubmed database for mismatching. Primer set used for amplification of the I655V (homozygous and heterozygous) in the HER2 genes for PCR-HRM were 5'CCAGCCCTCTGACG-TCCAT3' as forward primer and 5'CACCCCCAAGACCACGACCA3' as reverse primer. PCR - HRM analysis was performed on a *CFX96 real-time PCR* system (*Bio-Rad*) with KAPA HRM FAST PCR Kits (Kapa Biosystems, Cape Town, South Africa) containing EvaGreen as a saturating double-stranded DNA dye optimized for HRM analysis. Optimization run included wild-type (AA), heterozygous mutant (AG), homozygous mutant (GG), and no template control as negative control (NTC) to obtain the reagent composition and optimum reaction conditions for sample testing. All oligonucleotides were tested in triplicate to generate reproducible results.

PCRs were performed in 10 μl reaction volumes, containing 5 μl KAPA HRM FAST Master Mix (2X), 2.5 mM MgCl$_2$, 0.2 μM of forward and reverse primers and ±25 ng template DNA. PCRs have been done under the following cycling conditions: an initial predenaturation at 95°C for 3 min followed by 35 cycles for denaturation at 95°C for 10s, annealing at 60°C for 30s, and then extension at 72°C for 30s. After the PCR amplification steps, HRM ramps were generated by acquiring fluorescence data at a temperature

ramp from 65 to 95°C at 5°C/s intervals. A cooling phase for 1 min at 40°C was finally performed.

Optimization of sample concentration. Prior to testing on clinical samples, optimization of sample concentration was performed using one of the sample with the following 10 concentration variations: 0.019; 0.078; 0.15; 0.3125; 0.039; 0.625; 1.25; 2.5; 5; 10 ng/µl.

PCR-HRM analysis for cluster detection
After the Post-PCR-HRM process was completely performed, a data visualization software was needed. In this study, we used a Precision Melt Analysis software obtained by Bio-Rad. The melting temperature data were visualized as melting curves and classified into different groups (showed in different colors) by the software.

Results

Controls testing. The use of HRM method to see the difference in mutations caused by single-base changes has been widely publicized. The results of these analysis were obtained by using small PCR products. The recommended length of PCR product is about 50-300bp. (Myers RM, Maniatis T and Lerman, LS, 1987). The longer the amplycons, the difficulties in performing separation of sequence variants tends to be greater. Meanwhile, screening unknown sequence differences, optimal results can be achieved using amplicon size around 200 to 500 bp (Sun *et al*., 2016).

HRM testing was initially performed on wild and mutant controls to obtain the reagent composition and optimum reaction conditions for sample testing. Fluorescence data were normalized, and WT controls were user-designated in order to generate different plots to distinguish variant allele from WT.
Three separate groups (WT, HtM and HmM) could be observed in the HRM curve (Fig. 1). Single nucleotide base pair mutations caused a shift in melting temperature of the target amplicon compared to WT control, allowing for visual separation of genotypes with HRM curves. The number of hydrogen bonds between A / T and G / C is strongly related to melting temperature differences. The substitution of adenine to guanine caused an

increase in amplicon's Tm. Samples with mutations, which are represented by colored lines, can be easily differentiated from susceptible isolates based on differences in the shapes of the melting curves. Right after PCR amplification and product melting, the dF/dT plot was assessed for one peak per sample, indicating that a single and specific product was amplified and melted.
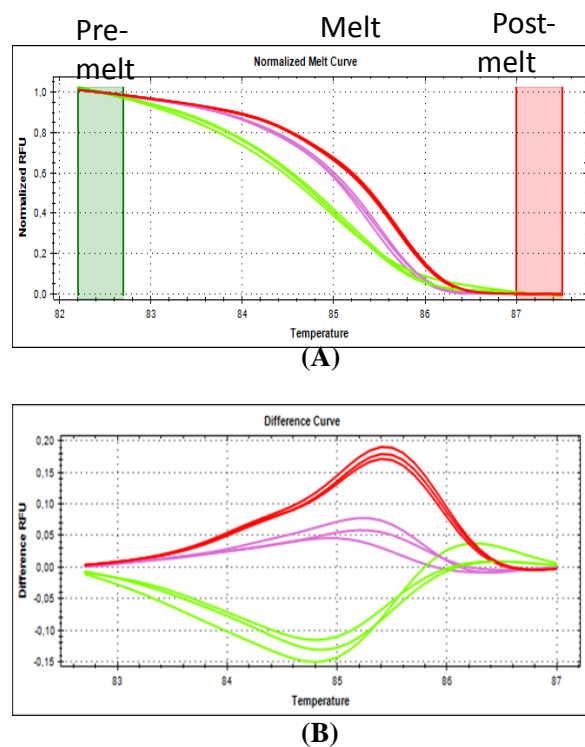

(A)

(B)

Figure 1. HRM analysis for AA, AG and GG used as the reference cluster assignment, panel A shows normalized graph with the green lines depicting the wild type (AA), the purple lines depicting the heterozygous mutant (AG) and the red lines depicting the homozygous mutant (GG) and panel B shows the difference plot of the green lines as wild type (AA), the purple line as heterozygous mutant (AG) and the red line as the homozygous mutant (GG).

Optimization of sample concentration. In order to avoid false wild type or false mutant, optimization of sample concentration were performed prior to clinical samples testing, with the following 10 variations of concentration: 0.019; 0.078; 0.15; 0.3125; 0.039; 0.625; 1.25; 2.5; 5; 10 ng/µl. The results detect the existence of some clusters based on the color of the curve. The red curve shows the correct result (specific) while the others color were not. The limit concentration with specific

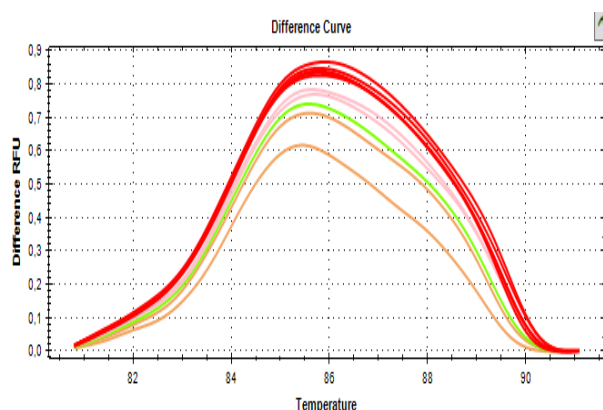result is 0.625 ng/µl which indicates that HRM is a sensitive assay.

Figure 2. Differentiation curve of concentration variation sample for optimization of sample concentration. The red colors indicate the specific result (cluster 1) while the other colors are not specific.

Table 1. Description of Figure 2.

Concentration	Cluster	Color	Percent confidence
10	1		96.5
5	1		97.9
2.5	1		99.4
1.25	1		98.4
0.625	1		98.9
0.3125	2		97.8
0.156	2		98.7
0.078	3		93.2
0.039	4		68.6
0.019	5		100.0

Sample testing

Having validated that wild-type, homozygous and heterozygous mutant types in the HER2 I655V can be identified by HRM, the assays were selected to examine 66 clinical samples that had been analyzed previously by Sanger sequencing for HER2-I655V detection. The Precision Melt Analysis software then compared the melting curve profile of each unknown sample to the control using genotype confidence threshold in order to assign genotypes as either wild type or mutant allele. Compared to Sanger sequencing assay, HRM analysis as frequency of genotypes data shows the same result as seen on table 2. Of 66 samples tested were, we found 52 were Ile/Ile genotype, and 15 were Val carriers. It shows that only one sample was detected as a minor

allele (G/G) and may have relatively low abundance in the population.

Tabel 2. The frequency of genotypes data in HER2-I655V polymorphisms

Genotype frequency, n(%)	HRM n=66	Sequencing N=66
Ile/Ile	51 (77.27)	51 (77.27)
Ile/Val	14 (21.21)	14 (21.21)
Val/Val	1 (1.52)	1 (1.52)

Discussion

The HER2 protein is a tyrosine kinase that phosphorylates downstream serine/ threonine kinases in response to growth factor stimulation. HER2 plays important roles in cell growth, survival, and differentiation in a complex manner. Therefore, HER2 gene amplification and protein overexpression can lead to malignant transformation (Neve *et al*., 2001).

The Her2/neu gene has a low expression in normal tissues, while it is amplified and overexpressed in ~30% of invasive breast carcinomas which associated with increased metastatic potential and poor prognosis (Klapper *et al*., 2000). The use of trastuzumab with adjuvant chemotherapy become the standard care for human epidermal growth factor receptor 2 (HER2)-positive in early breast cancer patients as it proven to reduce the risk of recurrence (Viani *et al.*, 2007).

The HER2 gene may have both somatic mutations and germinal polymorphisms such as one located in the transmembrane coding region at codon 655. The existing I655V mutation suggests an increased dimerization, autophosphorylation of HER2 and tyrosine kinase activity, which may cause the transformation of cells (Takano *et al.,* 1995). Some study revealed that allelic variation of I655V has been reported in women which suggests that the genotype of Ile/Val or Val/Val has a high risk in developing breast cancer. In addition, the presence of Val allele may become a risk factor for cardiac toxicity

in patients treated with trastuzumab because no cardiac toxicity was observed in the group contains Ile/Ile homozygous subjects (Beauclair *et al.,* 2007; Lemieux *et al.,* 2013; Xue *et al.,* 2014). Thus, the detection of specific alleles is very important for therapeutic decision-making to avoid useless treatments that could be harmful or expensive. Currently there are various SNP detection methods for detecting the specific allele types with the lowest to highest price comparison are as follows: qPCR labeled with probes (such as Taqman or FRET)> PCR seq> HRM> SYBR Green-Real Time PCR. In this study, we developed a HRM test with EvaGreen dye which allows rapid and reliable detection of specific allele of HER2 I655V. The SYBR green, the cheaper dye, is not a specific dye. SYBR green has quite low of optimum dye concentration (e.g. < 0.5 μM) because of the dye's high will inhibit PCR and promote mispriming. It makes the dyes unsuitable for HRM analysis because DNA melt curve data is unreliable. Compared to the SYBR Green, EvaGreen dye can be used at a higher dye concentration resulting in more robust PCR signal (Karsai *et al*., 2002 and Giglio *et al.*, 2003).

In 1997, Wittwer and colleagues introduced melting curve analysis in conjunction with real-time PCR for genotyping and six years later (2003) he used HRM to identify both heterozygous and homozygous sequence variants. Recently, HRM has been widely used to screen various mutations relevant to cancer (Ririe *et al*, 1997; Lay *et al*, 1997;Witter *et al*, 2003)

HRM analysis is highly suitable for the detection of a single base variantion, small insertion or deletion and detecting allelic variations(Gonzalez-Bosquet *et al.*, 2011; Capper *et al*., 2015).

Homozygous allelic/sequence variants are typically characterized by the temperature shift observed in the HRM melting curve, whereas heterozygotes are commonly characterized by a change in melting curve shape generated from base-pairing mismatch. The HRM data are often plotted using a difference curve to magnify the melting profile differences between different clusters of the same genotype (Garritano *et al*., 2009).

Good sample preparation is needed to get result in solid and quantifiable data. The quality of good DNA samples has a ratio of A260 / 280 and A260 / 230 in the range of 1.8-2.2 and 1.6-2.4, respectively. The concentration of sample used for testing also affect the accuracy of results. In this study, accurate results were obtained from 0.625 ng/μl -10 ng/μl of sample concentration. We did not get data about the maximum amount of DNA concentrations that could be used for HRM analysis, but according to Chambliss, HRM analysis displayed accuracy over input DNA concentrations ranging from 10 to 200 ng/μL (Chambliss *et al.*, 2017).

Our study reveals that the wild type is the most prevalent allele (77.27%) of breast cancer patient with HER2+ category in this research, whereas heterozygous mutation (A/G) is significantly present in this research (21.21%). Only 1.52% of sample (or one sample) was detected as minor allele (G/G). Minor allele may have relatively low abundance in the population, but the detection of this allele has significant clinical utility. Some papers revealed that homozygous for the allele G/G may be a negative prognostic marker of adjuvant trastuzumab response in women who suffer from HER2-positive BC than those who were homozygous for the WT allele (A) or were heterozygous for the allele (A/G). Therefore, the described screening approach serves to rule out patients at risk for adverse drug reactions (Roca *et al*., 2013; Toomey *et al.,* 2016).

In conclusion, we succeeded in discriminating specific alleles of HER2+ I655V in breast cancer by HRM method. However, this study has some limitations. Firstly, the size of our sample is small therefore it needs further validation in a larger sample set. Secondly, this study provides information on the distribution pattern of HER 2-I655V genotype in Padang area only, whilst there are no publication data for other regions in Indonesia. By comparing the existing data in Padang alone with other countries, then based on mentioned that the frequency of alleles in Padang is similar from Asian and European while with African looks very different (Budiarto & Desriani, 2016). In order to provide a better insight into such polymorphisms in Indonesia, data from different regions are required because of the possibility of different genetic variations between ethnicities.

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