Makara Journal of Health Research

Volume 20
Issue 2 *August*

Article 1

8-1-2016

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Makara J. Health Res., 2016, 20(2): 29-34
doi: 10.7454/msk.v20i2.5522

Development of Sybr Green I-Based Melting Curve Method for *HER2*^{I655V} Polymorphism Detection in Breast Cancer

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Abstract

Background: Currently available molecular method to detect *HER2*^{I655V} polymorphism such as PCR-RFLP is hampered by the costly experimental method, and post-PCR treatment requirement that makes this technique is not meeting for high-throughput analysis purpose. In this study, we developed an accurate, simple, low cost and rapid test to detect polymorphism at *HER2* gene using SBR Green I based-melting curve method. **Methods:** Two forward allele-specific primers and one common reverse primer were used then these primers were tested to discriminate known genotypes of genomic templates (GG type or AA type) and genomic samples retrieved from breast cancer patients. **Results:** Melting curve analysis derived from SYBR Green I-based allele-specific PCR with defined primers concentration and annealing temperature at 54.3 °C showed good discrimination level of Tm peaks in which GG genotype melted at 89 °C slightly higher than AA genotype which melted at 86 °C, while AG genotype harbored both of homozygous Tm characteristics. **Conclusions:** This preliminary result will be as basic for further large-scale typing of *HER2*^{I655V} polymorphism.

Keywords: *breast cancer; HER2, polymerase chain reaction, polymorphism, SYBR Green I*

Introduction

Breast cancer, the second leading cause of cancer-related death for women, has become global health concern with almost 1.5 million women were diagnosed where one-third died due to this illness.[1] It is estimated that the incidence rate of breast cancer will further increase due to 'westernised' lifestyle adaptation in many developing countries.[2] For many years, efforts have been conducted to elucidate the risk factors that contribute to breast tumorigenesis. Nowadays, it was established that environmental and genetic factors are the major keys that implicate to breast cancer risk.[3,4] Indeed, much of studies have clearly shown how the aberrant genetic factors could induce carcinogenesis in the breast, indicating the accumulative effect of such event that collectively increases breast cancer risk.[5]

Elucidating the genetic predisposition of breast cancer risk has risen the importance of genetic variants named single nucleotide polymorphisms to breast cancer development and progression.[6] One of the genes that contains genetic variant whose function in breast cells is very significant named *HER2*.[7] It is believed that the aberrant of this oncogenic gene leads breast cells to uncontrol growth and tumour induction.[8] Genetic variant of this gene is exclusively found at codon 655 (isoleucine or valine) located at the transmembrane domain of the *HER2* protein.[9] This amino acid changing mainly isoleucine to valine was presumed to increase the ability of breast cells to transform through receptor activation by mimicking ligand induction.[10] Indeed, case-control study conducted by Xie, *et al.*[11] in Chinese women population highlighted the role of *HER2* polymorphism as a susceptible biomarker for breast cancer risk. Women whose *HER2* contains Val (GTC)/Val (GTC) or Ile (ATC)/Val (GTC) variant tend to have more breast cancer risk than its wild variant.[12] Independent studies also strengthen how this allelic imbalance significantly contribute to breast cancer development.[13]

Predicting the types of *HER2*^{I655V} polymorphism precisely in a population will significantly impact not only on the development of antibody-based anticancer drugs but also improvement of the care quality for breast cancer patients. Currently, a commonly available method with routine use for detecting *HER2*^{I655V} polymorphism in breast cancer is Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).[11,13-15] Although this method offers superiority in term of sensitivity, and specificity yet post-PCR processing step such as digesting enzyme treatment and agarose electrophoresis to visualise the result is mandatory before the data could be retrieved.[16] These additional steps of method cause the analysing more costly and most importantly that it will be a time-

consuming procedure. Moreover, an involvement of cancer-inducing agent such as ethidium bromide during visualising steps further brings the additional issue such as safety aspect for laboratory workers.

To be considered as standard tools for diagnostic purposes, a method must be accurate, cost effective, and fast enough to allow the automated testing of large numbers of samples without omitting its sensitivity, specificity, reproducibility, precision, and high-throughput capability.[17] The invention of real-time PCR the machine has open widely to the new area of method developing for polymorphisms detection.[18] Indeed, such multiplex-based real-time PCR usually involves no/less-carcinogenic dyes such as SYBR Green I as a labelling agent.[19] This automated assay could be performed due to melting curve analysing is based on differentiating double-stranded from single-stranded DNA by changes in fluorescence intensity in the present of DNA-binding dyes at melting temperature condition.[20,21] Although, application of such SYBR Green I-based melting curve analysis has been widely applied in the variety of molecular diagnostics,[22-27] yet studies related to the application for *HER2*^{I655V} polymorphism detection especially in the case of breast cancer experiment, no reports have been published.

In this study, we developed the *HER2*^{I655V} genotyping assay by integrating allele-specific PCR with melting curve analysis using SYBR Green I, avoiding subsequent post-PCR treatments hence far from chemical and physical dangers. This technique also provides multiplex genotyping assay in the single tube format with accurate, rapid and inexpensive cost, allowing high-throughput *HER2*^{I655V} is genotyping.

Methods

Samples collection and genomic DNA extraction. Ten tissue samples of 10 breast cancer patients were collected from M. Djamil Padang hospital, West Sumatra province as frozen section sample. Genomic DNA were extracted using Pure Link Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific Inc., America) followed manual instruction using 10-20 mg of tissue as starting material to be extracted while plasmid extraction was extracted from overnight culture of recombinant *Escherichia coli* DH5α (collection of Molecular Biology and Diagnostics Laboratory, Research Center for Biotechnology-LIPI) using high Speed Plasmid Mini Kit (Geneaid Biotech Ltd. Taiwan) followed manual instruction with 30 µl of ddH_2O applied to elute recombinant plasmid. The integrity of all genomic DNA was checked using 0.8% agarose and the DNA content was also measured using spectrometer (Gene Quant TM Pro RNA/DNA calculator, GE Healthcare Biosciences). Two genomic DNA and pGEM_HER2/AA with known their genotype were also prepared. Their polymorphisms have been checked using Sanger sequencing and allele-specific PCR.[28] For all subject enrolled in this study was approved by local ethics committee issued from Ministry of Health, Republic of Indonesia.

Sensitivity test of Allele-Specific PCR. This test was done using PCR supermix reagent purchased from Invitrogen. The composition for each PCR components followed the instruction except primers; we used 200 pmol each. Two specific allele primers with forwarding direction (HER2_GG: 5'GCGGGCAGGGCGGCGGGG GCGGGGCC-CCAGCCCTCTGACGTCCACCG'3 and HER2_AA: 5'CCAGCCCTCTGACGTCCAGCT'3) and one common reverse primer (HER2_R:5'CGTGTACTT CCGGATCTTCTGCTG'3) were used followed methods Bui, *et al.,*[29] and Germer and Higuci.[30] The Genomic DNA (182 ng/µL stock) with GG genotype and pGEM_HER2 (182 ng/µL stock) with AA genotype were used as a template. Serial dilution was done 10 to 100 times from each stock then mixed to create genomic DNA that contains both of genotype that mimics AG genotype in nature. Amplification condition consisted of a 5 minutes pre-incubation period at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing carried out for 1 minutes at 51.5 °C and extension were at 72 °C for 30 seconds. The PCR product was checked on 2% of gel agarose.

HER2^{I655V} **genotyping using SYBR Green I and melting curve analysis.** This analysis was done using CFX96 Real-Time PCR (Biorad Laboratory Inc, USA). Primers concentration applied in this experiment followed formulation as suggested by Darawi, *et al.*[31] Allele-specific PCR composition for each reaction was 10 µL for final volume of each reagent as followed: 1x SsoAdvance Universal SYBR Green Supermix, 0.06 pmol HER2_GG primer, 0.1 pmol HER2_AA, 0.15 pmol HER2_R, DNA template at the range of 0.07-0.158 ng and the rest is dH_2O to gain the final volume of 10 µL as stated in the kit except for primers concentration it was modified slightly.Allele-specific PCR amplification profile was as follows: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 20 seconds, temperature annealing at 54.3 °C, for 20 seconds, and extension at 72 °C for 30 seconds. The fluorescence melting curve was analysed immediately following amplification. After amplification, the fluorescence intensity of the PCR product was measured from 65-95 °C with 0.5 °C/cycle increment. One sample PCR reaction with no contain DNA was also included in the experiment to clarify PCR contamination.

Results

Firstly, the sensitivity of the method was evaluated especially in the case of heterozygote (AG type) using a mix of two genomic DNA template with known genotype by dilution from 10 up to 100 times. The

effect of DNA template dosage followed inversely the ratio of DNA amount added with 100 dilution factor exhibiting the best polymorphism discrimination as marked by the appearance of two allele-specific PCR product sizes which referred to AA type and GG type (Figure 1).

The melting curve analysis for each corresponding *HER2*^{I655V} genotypes showed that GG genotype exhibits Tm value slightly higher than AA genotype while AG genotype produces two different Tm values that refer to each of homozygote type (Figure 2). The extra poly-GC on a primer that was amplified as GG genotype produce higher of GC content that impacts directly on its Tm value and PCR product stability.

To confirm the ability of method in detecting poly-morphism, 10 samples of breast cancer patient with unknown their *HER2*^{I655V} polymorphism were enrolled with three replication to ensure reproducibility of the assay. The integrity and quantity of the genomic DNA vary but overall that genomic DNA is acceptable to be used in method confirmation (Figure 3).

Tm curve analysis on 10 samples tested showed that 50% of samples belong to AA genotype while another half were AG genotype. No GG type was observed from 10 samples tested (Figure 4). Comparing this result with another established method such as Sanger sequencing, allele-specific Tm curve method exhibited closely 100% accordance among genotype tested, except for one sample

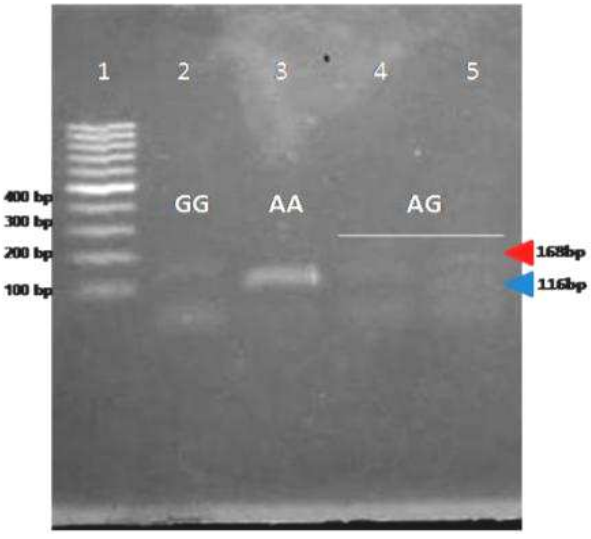

Figure 1. Allele-specific PCR Result of each *HER2*^{I655V} Genotype. Lane 1; 100 bp of DNA Ladder, Lane 2; Genomic DNA with GG Genotype (182 ng/µL stock), Lane 3; Genomic DNA with AA Genotype (182 ng/µL stock), Lane 4; mix of 2 and 3 after 10 Time Dilution, Lane 5; mix of 2 and 3 after 100 Time Dilution. Red Arrow and Blue Arrow Sequentially Refers to GG Type (166 bp) and AA Type (116 bp)

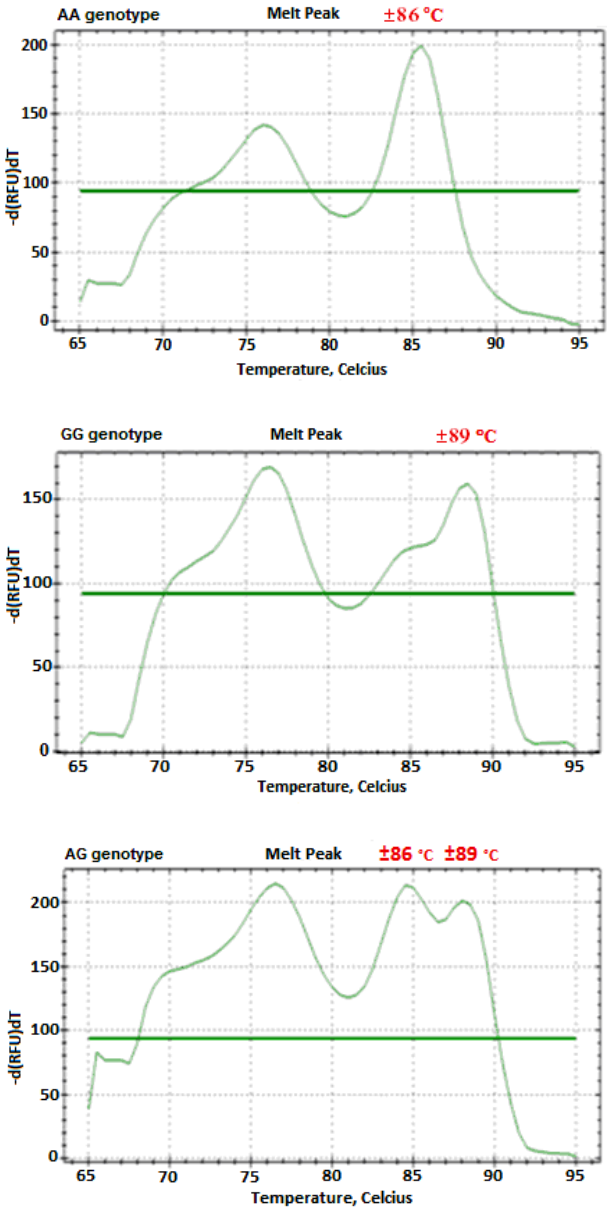

Figure 2. Tm Values for Each Type of *HER2*^{I655V} Poly-morphism

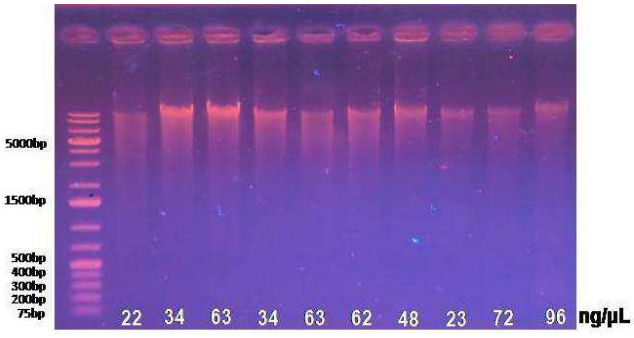

Figure 3. Genomic DNA Extraction Result from 10 Samples of Breast Cancer Patients

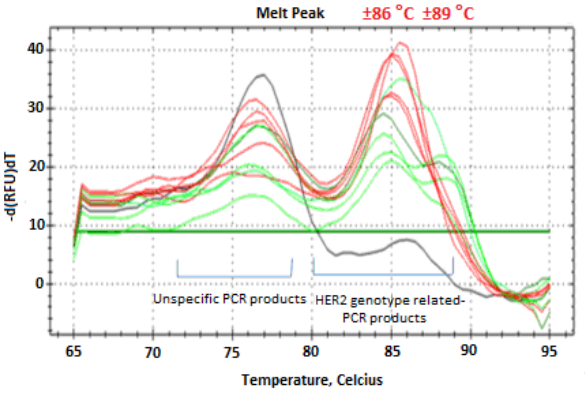

Figure 4. Melting Curve Analysis of *HER2*^{I655V} Polymorphism from 10 Samples of Breast Cancer Patients. Red Line showed Samples with AA Type; Green Line showed Samples with AG Type while NTC (Non-Template Control) was Pointed by Black Line. The Number of Replicate for Each Sample is 3 Times

Table 1. Comparison Result of Melting Curve with Established Genotyping Method

HER2^{I655V} Genotypes	Samples positivity (% distribution)	
	Melting curve method	Sanger sequencing method
AA	5 (50%)	5 (50%)
GG	-	1 (10%)
AG	5 (50%)	4 (40%)
Total	10 (100%)	10 (100%)

showed AG genotype by this method but presenting GG genotype based on Sanger sequencing, indicating there was a minor difference between methods as result of dissimilarity in their detection sensitivity level (Table 1).

Discussion

The success of genotyping analysis using allele-specific PCR relies on how good enough each of specific PCR products could be differed clearly on gel agarose without producing an ambiguous interpretation. Due to the nature of specific primers designed in this method, the preferential amplification of one of the alleles may occur. As mentioned by von Ahsen, *et al*.[32] The genotyping of heterozygous samples requires special consideration due to the present both of allelic types in a single sample, reducing the amount of template is one of an alternative way to increase the probability of minor allele to be amplified equally during PCR reaction. Although the AG genotype could be well discriminated using this method, yet the thickness of the two PCR products observed on agarose seems imbalanced. Other factors such as the concentration of $MgCl_2$, the unit of *Taq* polymerase and the proportion of primers concentration added into PCR solution are also the essentials substance that determines the success of polymorphism detection using allele-specific PCR.[33]

We have observed that two genotypes exhibited distinct Tm curve profile where GG genotype showed 3 °C higher of Tm value compared with AA genotype although the single peak of specific Tm was only observed in AA genotype. The peaks and its sharpness among genotypes observed on melting curve profile will solely depend on their GC content, sequence length, type of dyes, the concentration of primers annealing temperature of primers and dosage of DNA template used.[6,31,34-36] The important point to be highlighted in this result is that the two peaks between two different genotype still could be seen clearly event though the DNA dosage used in PCR solution is low, Indicating the melting curve method improves the sensitivity of genotype detection. Marin, *et al*.[37] have observed that allele-specific PCR produces a missing result as much as 20-21% while only 1.3% had missing by melting curve analysis. Moreover, they also suggest that to obtain the similar pattern and well discriminated of melting peaks for each genotype, the use of 3-25 ng of genomic DNA as a template in allele-specific PCR recommended. The unspecific peak that arises below 80 °C of Tm value is primer dimer due to too low annealing temperature applied in PCR process. This similar event was observed in another study that stated low annealing temperature used in genotyping human rotavirus generates unspecific PCR product whose Tm is below 77 °C.[38] We predicted that our method improvement in this study is related to the proportion of primers concentration and annealing temperature applied beside DNA dosage usage.

The choice of molecular methods for gene polymorphisms studies must consider several aspects of methods feature, and usually, methods with high sensitivity, specificity, reproducible, robustness, and low-cost are the most researchers used. Beside specific enzymes restriction-based PCR, another method that currently applied for polymorphisms studies is probe-based Real-Time PCR due to high specificity detection produced.[39,40] Nevertheless, such methods require additional primer design that contains costly specific probe that hampers to diagnostics purpose with prerequisite low-cost technology. In our study, we have improved allele-specific PCR for *HER2*^{I655V} polymorphism detection using DNA binding agent such as SYBR Green I dye in PCR-ready used format. This strategy with optimised PCR condition (suitable primers ratio, DNA template dosage, and short DNA target) have been proven to be well performed in discriminating each of *HER2*^{I655V} without any further additional chemicals agent such as a probe. One particular caution in applying SYBR Green

I-based melting curve method for poly-morphisms studies should keep in mind that the DNA target must be 50-200 bp in length to prevent unspecific binding of the dye due to influence of dye concentration and DNA dosage used.[6,35,41]

Another important issue regarding single nucleotide polymorphisms studies using DNA binding dye-based Tm shift genotyping method is possibility occurrence of variety in Tm values for the same genotype due to the different in PCR instruments usage. This event have been observed in genotyping study of sickle cells anemia-related genes using High Resolution DNA Melting Analysis (HRMA) where the resolution of the melting curve varied among PCR instruments tested, with a 15-fold difference in *T*m SD (0.018 to 0.274 °C) and 33 fold (SYBR Green I) difference in the signal-to-noise ratio.[42] Based on this finding, the Tm values of each *HER2*^{I655V} genotype obtained in our study may produce slight different Tm value when another type of PCR instruments applied, yet the predicted Tm values will probably be around at 85.98-86.27 °C for AA genotype and at 88.92-89.27 °C. for diagnostics purpose, we strongly recommended for the user to do a comparison study of different PCR instruments before the real test done.

The allele frequency of genes in genotyping studies is a very important aspect to obtain the solid conclusion in term of SNP association to some diseases. We have shown that allele-specific PCR in our study is as good as Sanger sequencing in *HER2*^{I655V} polymorphism detection. Both of methods produce the same allelic frequency distribution which is 0.5 for Ile (AA genotype) and 0.5 for Val (AG combined with GG genotype). However, Xie, *et al.*[11] obtained values which are 0.84 for Ile and 0.16 for Val from 339 patients with breast cancer from a Chinese population. This discrepancy probably due to the small size of sample enrolled in our study. Keshava, *et al.*[7] found that the frequency allelic of *HER2*^{I655V} varied significantly by race. This is the limitation of our study which used only 10 DNA samples of breast cancer. Further study needs to be conducted using adequate samples, adding more breast cancer samples to comply statistics prerequisite to clarify and validate our result. Melting curve method has been applied successfully to detect a BDNFVal66Met polymorphism,[41] chicken Mx gene G2032A SNP, and GSTT1, and GSTM1 null polymorphism.[37] Overall, this result showed that melting curve method in our study well performed to discriminate each type of *HER2*^{I655V} polymorphism in breast cancer.

Conclusions

SYBR Green I-based melting curve method has been developed with success, and it well performed to detect *HER2*^{I655V} polymorphism in breast cancer. This preliminary result will be a basic for further large-scale *HER2*^{I655V} genotyping in breast cancer.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Acknowledgements

This research was financially supported by Riset Unggulan LIPI 2015 grand.

References

1. GLOBOCAN. Estimated Cancer Incidence, Mortality, and Prevalence Worldwide 2012. 2012. (internet) [cited 2015 September 16]. Available from: http://globocan.iarc.fr/Default.aspx.
2. Youlden DR, Cramb SM, Dunn NA, Muller JM, Pyke CM, Baade PD. The descriptive epidemiology of female breast cancer: An international comparison of screening, incidence, survival and mortality. *Cancer Epidemiol*. 2012;36:237-48.
3. Coyle YM. The effect of environment on breast cancer risk. *Breast Cancer Res. Treat*. 2004;84:273-88.
4. Njiaju UO, Olopade OI. Genetic determinants of breast cancer risk: A review of current literature and issues pertaining to clinical application. *Breast J*. 2012;18:436-42.
5. Turnbull C, Rahman N. Genetic predisposition to breast cancer: Past, present, and future. *Ann Rev Genom Hum Genet*. 2008;9:321-45.
6. Tao W, Wang C, Han R, Jiang H. *HER2* Codon 655 polymorphism and breast cancer risk: A meta-analysis. *Breast Cancer Res. Treat*. 2009;114:371-6.
7. Kurebayashi J. Biological and clinical significance of *HER2* overexpression in breast cancer. *Breast Cancer*. 2001;8:45-51.
8. Moasser MM. The oncogene *HER2*: Its signaling and transforming functions and its role in human cancer pathogenesis. *Oncogen.* 2997;26:6469-87.
9. Papewalis J, Nikitin AY, Rajewsky MF. G to A polymorphism at amino acid codon 655 of the human *erbB-2/HER2* gene. *Nucleic Acids Res.* 1991;19:5452.
10. Weiner DB, Liu J, Greene MI. A point mutation in the *neu* oncogene mimics ligand induction of receptor aggregation. *Nature.* 1989;339:230-1.
11. Xie D, Shu XO, Deng Z, Wen WQ, Creek KE, Dai Q, *et al*. Population-based, case-control study of *HER2* genetic polymorphism and breast cancer risk. *J Natl Cancer Inst*. 2000;92:412-7.
12. Siddig A, Mohamed AO, Kamal H, Awad S, Hassan AH, Zilahi E, *et al*. *HER2*/neu Ile655Val polymorphism and the risk of breast cancer. *Ann NY Acad Sci*. 2008;1138:84-94.
13. Lee SC, Hou MF, Hsieh PC, Wu SH, Hou LA, Ma H, *et al*. A Case–control study of The *HER2* Ile655Val polymorphism and risk of breast cancer in Taiwan. *Clin Biochem*. 2008;41:121-5.

14. Pinto D, Vasconcelos A, Costa S, Pereira D, Rodrigues H, Lopes C, *et al*. *HER2* polymorphism and breast cancer risk in Portugal. *Eur J Cancer Prev*. 2004;13:177-81.
15. Papadopoulou E, Simopoulos K, Tripsianis G, Tentes I, Anagnostopoulos K, Sivridis E, *et al*. Allelic imbalance of *HER2* Codon 655 polymorphism among different religious/ethnic populations of northern greece and its association with the development and the malignant phenotype of breast cancer. *Neoplasma*. 2006;54:365-73.
16. Ota M, Fukushima H, Kulski JK, Inoko H. Single nucleotide polymorphism detection by polymerase chain reaction-restriction fragment length polymorphism. *Nat. Protoc*. 2007;2:2857-64.
17. Kim S, Misra A. SNP genotyping: Technologies and biomedical applications. *Ann Rev Biomed Eng*. 2007;9:289-320.
18. Baris I, Etlik O, Koksal V, Ocak Z, Baris ST. SYBR green dye-based probe-free SNP genotyping: Introduction of T-Plex Real-Time PCR assay. *Anal Biochem*. 2013;441:225-31.
19. Singer VL, Lawlor TE, Yue S. Comparison of SYBR® green I nucleic acid gel stain mutagenicity and ethidium bromide mutagenicity in The Salmonella/Mammalian microsome reverse mutation assay (Ames Test). *Mutat Res*. 1999;439:37-47.
20. Lipsky RH, Mazzanti CM, Rudolph JG, Xu K, Vyas G, Bozak D, *et al*. DNA melting analysis for detection of single nucleotide polymorphisms. *Clin. Chem*. 2001;47:635-44.
21. Hladnik U, Braida L, Boniotto M, Pirulli D, Gerin F, Amoroso A, *et al*. 2002. Single-tube genotyping of *MBL-2* polymorphisms using melting temperature analysis. *Clin Exp Med*. 2002;2:105-8.
22. Balboni A, Dondi F, Prosperi S, Battilani M. Development of a SYBR green real-time PCR assay with melting curve analysis for simultaneous detection and differentiation of *Canine adenovirus* Type 1 and Type 2. *J Virol Methods*. 2015;222:34-40.
23. Yadav BK, Oh SY, Shin BS. Asymmetric real-time PCR improves the SNP detection of VEGF+ 936C>T by melting curve analysis in light cycler. *Biomed. Res*. 2013;24:509-14.
24. Khan SA, Sung K, Nawaz MS. Detection of *aacA-aphD*, *qacEδ1*, *marA*, *floR*, and *tetA* genes from multidrug-resistant bacteria: Comparative analysis of real-time mutiplex PCR assays using evagreen® and SYBR® Green I Dyes. *Mol Cell Probes*. 2011;25:78-86.
25. Lorente A, Mueller W, Urdangarín E, Lázcoz P, Von Deimling A, Castresana JS. Detection of methylation in promoter sequences by melting curve analysis-based semiquantitative real time PCR. *BMC Cancer*. 2008;8:61.
26. Tiwawech D, Chindavijak S, Sornprom A, Sumetcho-timaytha W, Prasitthipayong A, Jarratwisarutporn Y, *et al*. Detection of *GSTT1* polymorphisms in cancer patients by real-time PCR. *Thai Cancer J*. 2010;28:172-83.
27. Mori S, Sugahara K, Uemura A, Akamatsu N, Tutsumi R, Kuroki T, *et al*. Rapid, simple, and accurate detection of K-Ras mutations from body fluids using real-time CR and DNA melting curve analysis. *Lab Med*. 2006;37:286-9.
28. Budiarto BR, Desriani. Detection of HER2 gene polymorphism in breast cancer: PCR optimization study. *Sci Pharm*. 2016;84:103-11.
29. Bui M, Liu Z. 2009. Simple Allele-discriminating PCR for cost-effective and rapid genotyping and mapping. *Plant Methods*. 2009;5:1-8.
30. Germer S, Higuchi R. Single-tube genotyping without oligonucleotide probes. *Genome Res*. 1999;9:72-8.
31. Darawi MN, Ai-Vyrn C, Ramasamy K, Hua PPJ, Pin TM, Kamaruzzaman SB, *et al*. Allele-specific polymerase chain reaction for the detection of Alzheimer's disease-related single nucleotide polymorphisms. *BMC Med Gen*. 2013;14:1-8.
32. von Ahsen N, Oellerich M, Schütz E. Limitations of genotyping based on amplicon melting temperature. *Clin Chem*. 2001;47:1331-2.
33. Zainuddin Z, The LK, Suhaimi AWM, Salleh MZ, Ismail R. A simple method for the detection of *CYP2C9* polymorphisms: Nested allele-specific multiplex polymerase chain reaction. *Clin Chim Acta*. 2013;336:97-102.
34. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem*. 1997;245:154-160.
35. Monis PT, Giglio S, Saint CP. Comparison of SYTO9 and SYBR green I for real-time polymerase chain and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. *Anal Biochem*. 2005;30:24-34.
36. Gudnason H, Dufva M, Bang DD, Wolff A. Comparison of multiple DNA dyes for real-time PCR: Effects of dye concentration and sequence composition on DNA amplification and melting temperature. *Nucleic Acids Res*. 2007;35:1-8.
37. Marín F, García N, Munoz X, Capella G, González CA, Agudo A, *et al*. Simultaneous genotyping of GSTT1 and GSTM1 null polymorphisms by melting curve analysis in presence of SYBR Green I. *J Mol Diagn*. 2010;12:300-4.
38. Tong Y, Lee BE, Pang XL. Rapid genotyping of human rotavirus using SYBR green real-time reverse transcription-polymerase chain reaction with melting curve analysis. *World J Virol*. 2015;4:365.
39. Shen GQ, Abdullah KG, Wang QK. The TaqMan method for SNP genotyping. *Methods Mol Biol*. 2009;578:293-306.
40. Schleinitz D, DiStefano JK, Kovacs P. Targeted SNP genotyping using the TaqMan® assay. *Methods Mol Biol*. 2011;700:77-87.
41. Sánchez-Romero MA, Dorado P, Guarino E, Llerena A. Development of a new genotyping assay for detection of the BDNF Val66Met polymorphism using melting-curve analysis. *Pharmacogenom*. 2009;10:989-95.
42. Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. *Clin Chem*. 2006;52:494-503.