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Original Article

Development of multiplex methylation-specific PCR for detecting methylated/un-methylated PTEN promoter in breast cancer patients using a single closed tube

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Abstract

PTEN is a suppressor gene that methylation in its promoter is potentially used for diagnosis and prognosis for breast cancer and other cancer. We aim to develop a simple, fast, and economical Multiple Methylation-Specific PCR (MMSP) PTEN, recognizing a PTEN promoter gene for methylation and un-methylation developed in one single closed tube. This true-experimental study uses a recombinant plasmid prepared with a TA cloning system. For the development of PTEN with Multiple MSPs, two formulations for MMSP PTEN were designed and compared, in which the best was to study specificity, limit of detection, reproducibility, and repeatability. The methods were validated with a clinical sample that identified its methylation status using Methylation-Specific PCR (MSP). As a result, the best formulation for MMSP PTEN was formulation 2, which comprises of 1:3 methylation and un-methylation primer, with only high specificity for detecting its target and high sensitivity of 1.5625%, while its limit detection was 0.00032 ng/µl. It has high reproducibility and repeatability, which showed consistent data in the experiments with clinical samples in this activity. This simple molecular evidence aids in detecting biomarkers that are potentially used for the diagnosis and prognosis of cancer and the epidemiological study of methylated PTEN promoters.

Keywords: PTEN, Methylation, recombinant plasmid, breast cancer, detection, diagnosis, multiple

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1. Introduction

Breast cancer is the leading cause of death among women in developed and developing countries. According to GLOBOCAN 2020 data, it is the most commonly diagnosed cancer in women. It surpasses lung cancer with new cases estimated at 2.3 million (11.7%), with a mortality rate of 0,7 million (6.9%) (Rustamadji, Wiyartha, Bethania & Kusmardi, 2021; Sung *et al.*, 2021). Therefore, it is essential to identify potential biomarkers for the high-risk screening of patients in combination with classical pathological parameters for the diagnosis and prognosis of breast cancer.

PTEN (phosphatase and tensin homolog deleted on chromosome 10) gene is a suppressor gene found in the region of the chromosome 10q23.3 area, which is also a regulator for P13K/AKT signal with dual protein and lipid phosphatase activity (Fusco *et al.*, 2020). Methylation of the PTEN promoter has shown a high contribution to gene expression and results in the inactivation of PTEN in breast cancer. Furthermore, Khan *et al.* (2004) and Zhang *et al.* (2013) stated that around 60% of breast cancer tissue exhibiting PTEN methylation showed loss of expression. The loss of PTEN was related to aggressive behaviour and the prediction of worse outcomes for breast cancer patients (Xu *et al.*, 2017). PTEN is important in the development of breast cancer since its loss in tissue is significantly higher, thereby supporting the initiation and malignant progression of breast cancer (Li *et al.*, 2017; Ramadan, Hashim, Abouzid & Swellam, 2021). Loss of PTEN in breast cancer corresponds with resistance to trastuzumab. This statement, which was confirmed by Razis *et al.* (2011), reported that trastuzumab therapy in patients with HER-2 positive breast cancer was highly dependent on PI3K/AKT pathway activation. The pathway is induced by PIK3CA mutation or activation of PTEN loss for the therapeutic efficacy of trastuzumab in patients.

Contrary to the previous statement, Stern *et al.* (2015) reported that in HER-2 positive patients loss of PTEN was not associated with resistance to trastuzumab but with worse outcomes. Furthermore, the PTEN promoter's hypermethylation in the early stage of breast cancer carcinogenesis (Luo, Chen, & Mo, 2016) suggests that PTEN is valuable for diagnosing breast cancer (Lu, Cheng, & Teng, 2016). Loss of PTEN expression is commonly reported as a prognostic factor, and has been observed in many human tumors, other than breast cancer such as in gastric tumors, brain tumors, melanomas, hepatocellular carcinomas, thyroid carcinomas, endometrial carcinomas, and lymphoid neoplasia (Kang *et al.*, 2002; Roh *et al.*, 2016).

Identification of PTEN CpG island is challenging since there is a high similar sequence homology (91%) (Hesson *et al.*, 2012), even 98% with the PTEN pseudogene of PTENP1 (Zysman, Chapman & Bapat, 2002), and this requires careful design consideration for detection. Furthermore, the PTENP1 pseudogene is a single exon gene located at 9p13.3. The methylation of PTEN detection has been developed using various methods, such as combined bisulfite restriction analysis (COBRA) (Xiong & Laird, 1997), allelic bisulfite sequencing, bisulfite pyrosequencing (de Campos, Junior, Winheski, Martin & Lopes, 2020), real-time quantitative methylation-specific PCR, and bisulfite Methylation-specific PCR (MSP). Compared with other methods, MSP is a popular method for detecting PTEN methylation since it is simple economically, does not require expensive equipment, is sensitive, and is faster. In the reported MSP, PTEN methylation and unmethylation gene was reacted and prepared in different tubes, making them inefficient and costly.

This report focuses on developing multiplex methylation-specific PCR (MMSP) for detecting simultaneously methylated/un-methylated PTEN promoters in breast cancer patients using a single closed tube with one-step PCR processing which more economical of operational cost and uses sample quantity. Since PTEN promoter methylation also reported to play a critical role to other carcinomas as described above, this MMSP method could also be applied for other cancers, it has further potentially to be used for the diagnosis and prognosis of cancer and the epidemiological study of methylated PTEN promoters. Further, the MMSP method has been developed for some cancer DNA biomarkers because of its rapid method and high accuracy (Fackler *et al.*, 2004; Nawaz *et al.*, 2014).

2. Materials and Methods

2.1 Study design and ethical declaration

This study is a true-experimental study with ethical clearance number 053/PEP/01/2012 approved by the medical research ethics committee of the Dharmais cancer hospital. The study was undertaken with the understanding and written consent of each subject.

2.2 DNA extraction and bisulfite modification

Genomic DNA was isolated from frozen fresh breast carcinoma samples with a Purelink DNA genome kit (Invitrogen, K1820-01). Furthermore, the extracted DNA was bisulfite modified with Bisulfite Methyledge Conversion System (Promega, TM381), and this was conducted according to the manufacturer's instructions.

2.3 Cloning of methylation and un methylation PTEN small fragment in TA cloning

The PTEN methylation and un-methylation DNA fragments were amplified with Taq Polymerase Thunderbird SYBR qPCR Master MIX (TOYOBO, QPS-201) with a total of 100 µl for each. The mixed composition for each DNA fragment was 33 µl pH 7 TE Buffer, 55 µl Taq Polymerase Thunderbird SYBR qPCR Master MIX (TOYOBO, QPS-201), 5.5 µl for forward, and 5.5 µl reverse primer respectively, and 1 µl DNA genome sample. The DNA genome sample was modified with a bisulfite conversion system, while the non-template control also contained the same composition except that it had no template. We used a DNA genome already identified in the preliminary study with the MSP method. Since PTEN pseudogenes existed, primer is used, concerning Salvesen *et al.* (2001) and Zhang *et al.* (2013), for the forward methylation primer 5' TTCGTT CGTCGTCGTATTT3', reverse primer 5' GCCGCTTAACTC TAAACCGCAACCG 3' and un-methylation primer forward 5' TGTTGGTGGAGGTAGTTGTTT 3', and for reverse primer 5' ACCACTTAACTCTAAACCACAACCA 3'. PCR settings were 95ºC for 3min for PCR predenaturation, 35

cycles for denaturation of 95ºC for 3 min, annealing 55.28ºC for 30 sec, extension 72ºC for 1 min, and post extension 72ºC for 10 min. Therefore, the result for DNA fragment product was confirmed in 1% agarose with electrophoresis devices Mupid-exU system (Takara, AD140), in which the TA cloning process system was followed by the manufacturer's instructions of Promega pGEM-easy vector (Promega, A1360).

2.4 PTEN MSP temperature optimization with recombinant plasmid

PTEN methylation and un-methylation DNA fragments were amplified with Taq Polymerase Thunderbird SYBR qPCR Master MIX (TOYOBO, QPS-201) using the recombinant plasmid and primer used above. Furthermore, the PCR reaction was conducted in different PCR tubes with a setting of 95ºC for 3 min for PCR predenaturation, 35 cycles for denaturation of 95ºC for 3 min, annealing 53ºC-60.2ºC for 30 sec, and extension for 1 min, while for post extension was 72ºC for 10 min. The best annealing temperature will show clear and clean one single target PCR.

2.5 MMSP PTEN assay with recombinant plasmid

Multiplex and methylation-specific PCR techniques were combined to develop the MMSP assay to detect the methylation and un-methylation of the PTEN promoter in one single tube. pGEM-T easy PTEN methylation (1ng DNA plasmid concentration) mixed with other prepared recombinant plasmids with a 1:1 proportion. Furthermore, two formulations, in with the first comprise 3.4 µl TE buffer, 5 µl Taq Polymerase Thunderbird SYBR qPCR Master MIX (TOYOBO, QPS-201), 0.1µM Forward and Reverse primer for each PTEN Methylation primer, and 0.2 µM forward and reverse un-methylation PTEN primer for each respectively, with 1 µl DNA template. Meanwhile, the second constitutes 3.2 µl TE buffer, 5 µl Taq Polymerase Thunderbird SYBR qPCR Master MIX (TOYOBO, QPS-201), 0.1 µM Forward and reverse primer for each of PTEN Methylation primer, 0.3 µM forward and reverse primer for PTEN un-methylation PTEN primer for each, with 1 µl DNA template. The best result of PCR temperature optimization from the above activity was used for PCR temperature setting. The best MMSP PTEN will show a clear and clean DNA band of 162 bp for un-methylation and 206bp for methylation.

2.6 MMSP PTEN characterization (Specificity, sensitivity, limit detection, repeatability, and reproducibility)

For MMSP PTEN specificity test activity, a one ng pGEM-T easy PTEN methylation was mixed with other prepared recombinant plasmids, with a proportion of 100%: 50%: 25%: 12.5%: 6.25%: 3.115%: 1.5625%: 0.78%: 0.39%: 0%, the last percentage contains only pGEM-T easy PTEN un-methylation, non-template control also prepared as a negative control. For the limit detection test, the above of both recombinant plasmid was mixed with 1:1 proportion and was diluted to 1, 0.2, 0.04, 0.008, 0.0016, 0.0032, and 0.000064 ng/µl. We prepared double of this limit detection test for its repeatability study. Then on another day, the activity was repeated with another operator to check its reproducibility.

2.7 Validation of MMSP in a clinical sample

The best formulation of MMSP PTEN was then tried on a clinical sample that had already identified its methylation or un-methylation status with popular reported methods, PTEN MSP. In this activity, two different polymerases were compared, Taq polymerase and KOD polymerase (KOD One PCR master Mix Blue, TOYOBO, FX-201). The PCR ingredient and setting the temperature for Taq polymerase and KOD polymerase were the same as described above using the best formulation (formulation two), except for denaturation temperature of KOD polymerase referred to manufacture denaturation determination, 98ºC.

3. Results and Discussion

Methylation in CpG islands is responsible for the gene silencing of PTEN tumor suppression, which significantly affects PTEN expression. Furthermore, according to Shabbir Alam *et al.* (2017), the clinical stage and age of the patients are related to the level of expression of PTEN and influence the existence of breast cancer. Two tube MSP of PTEN uses a set of primers in which one primer binds to a methylated sequence, while another binds to unmethylated sequence. It is currently a popular method for detecting the Methylation of PTEN. In this study, methods for detecting the presence of methylation alleles were designed, un-methylation allele or presence of both alleles in one single closed tube with one step PCR process, that further differentiated the homozygously or heterozygously methylated sample. Therefore, this approach makes the sample quantity and chemical reagent more economical than the popular MSP PTEN detection.

3.1 TA cloning of methylation and un methylation PTEN DNA small fragment

Because of the limited sample, we prepared and used recombinant plasmid as a target for detecting MMSP PTEN development. Firstly, a bisulfite DNA conversion was performed, and it was further used as a DNA source for isolation of methylation and un methylation DNA fragment, which were differentiated based on its size (Salvesen *et al.*, 2001; Zhang *et al.*, 2013). The recombinant plasmid pGEM-T easy Methylation PTEN and pGEM-T easy un-Methylation PTEN were prepared according to manufacturer instruction from PROMEGA. PCR products of 162 bp for un-methylation and 206bp for methylation were amplified cloned to a pGEM-T easy cloning vector. For plasmid verification, a white-blue colony selection, plasmid extraction, and PCR plasmid (Figure 1) were performed. Plasmid recombinant provides an advantage for molecular detection development, especially in limited sample conditions.

3.2 MMSP PTEN assay construction

According to Salvesen *et al.* (2001), who have already tested and reported the used primer, it detects the

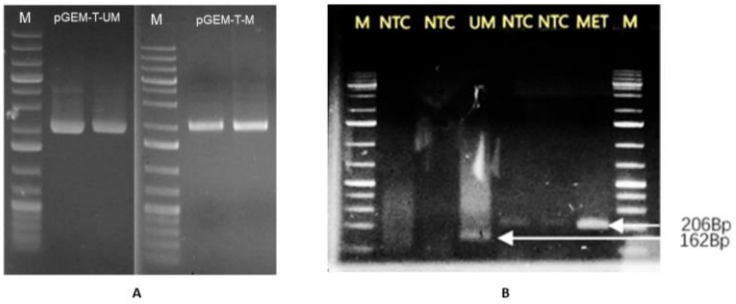

Figure 1. Recombinant plasmid extraction and its PCR verification. (A) pGEM-T easy un-Methylation and pGEM-T easy Methylation extraction. (B) Recombinant plasmid verification with PCR. (UM: un-Methylation; M: Methylation; NTC: Non-Template Control)

sequence of the PTEN promoter sequence, not the PTEN pseudogene. This primer was also used by Zhang *et al.*, (2013) for a PTEN methylation study in breast cancer. In order to determine the MMSP temperature setting, in the preliminary study, we optimized the annealing temperature start from 53ºC – 60, 2ºC for each methylation and unmethylation PTEN. A similar annealing temperature for each primer in multiplex PCR will support all optimum primer performance to recognize its DNA target (Sint *et al.*, 2012). We first determined with the clinical sample, then replaced it and continued with recombinant plasmid as the MSP template. Based on this optimation, it was observed that similar annealing temperatures for two different templates have 55.3ºC as the best annealing temperature, which showed a specific PCR product target of 162 bp for unmethylation and 206bp for methylation PCR product (data not shown). This preliminary study also used the DNA genome, which does not modify bisulfite conversion as an MSP template, to confirm and study the MMSP contamination pattern possibility with a DNA genome. We found no PCR product around 162 bp for unmethylation or 206bp for methylation; the PCR product is reported to be around 450bp for methylation PCR reaction and 510bp for the unmethylation PCR reaction at an annealing temperature of 45ºC to 51.6ºC annealing temperature, while higher annealing temperature showed no PCR product (data not shown). Furthermore, an annealing temperature optimization developed by the MSP method was used to create and compare two formulations of MMSP PTEN promoter in one single closed tube with one step PCR process. Based on the above formulation, we found formulation 2 showed clear and clean results, as this second formulation was used further (Figure 2).

MMSP PTEN in one single tube contains a cocktail of specific primer, and PCR chemical reagents were developed with one-step PCR, not with two-step PCR, to avoid a risk of contamination. The two determined formulations can discriminate the target (Figure 2). These two formulated were determined based on our laboratory's empiric experience, considering GC content, primer length, and primer specificity. This study discovered that formulation 2 shows clearer and clean results with no spurious amplification, common problems encountered in multiplex PCR because of primer interaction (Markoulatos, Siafakas, & Moncany, 2002). MMSP PTEN was developed in such a way that it improves MSP PCR PTEN detection efficiency and reduces cost. At 55.3ºC, the annealing primer reaction showed that the primers bind to both methylated or unmethylated or both templates, according to the existing sample in the PCR tube. This finding generally showed that each primer efficiency is equal, working specifically to its target in the optimum multiplex PCR temperature setting. Salvesen *et al.*, (2001) stated that loading the methylated and unmethylated PCR product in the same well helps its analysis. This finding is still inefficient since the reaction was separated in a different tube for each methylated and un-methylated reaction.

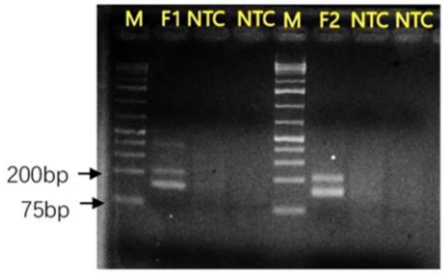

Figure 2. MMSP PTEN formulation verified in 2% Agarose (M*:* DNA Ladder F1: formulation 1 which contains 0.1 µM methylation primer for both forward and reverse + 0.2 µM un-methylation primer for both forward and reverse primer; F2: Formulation 2, which contain 0.1 µM methylation primer for both forward and reverse + 0.3 µM un-methylation primer for both forward and reverse rimer; NTC: Non-Template Control)

3.3 MMSP PTEN assay characterization (specificity, sensitivity, repeatability, and reproducibility)

The developed MMSP PTEN assay was further reviewed for its sensitivity and specificity. For sensitivity or limit detection study, the recombinant plasmid with 1:1 proportion was mixed and diluted to 1, 0.2, 0.04, 0.008, 0.0016, 0.0032, 0.000064ng/µl (Double for each mixed tube). Based on this activity, a template of 0.00032 ng/µl DNA1 has still clearly visible, and this was repeated on the same day by the same operator or a different day by other operators and showed a similar pattern. Since both activities show a consistent and 100% similar result, which means this developed MMSP PTEN assay has high reproducibility and repeatability (Figure 3).

We mixed 1ng of pGEM-T easy PTEN methylation and unmethylation, with proportions 100%: 50%: 25%: 12.5%: 6.25%: 3.115%: 1.5625%: 0.78%: 0.39%: 0% for PTEN methylation recombinant plasmid (Duplo for each mixed tube). This activity discovered that the developed method has a high specificity of 1.5625% for the detection of PTEN methylation, a single 206bp band for 100% pGEM-T

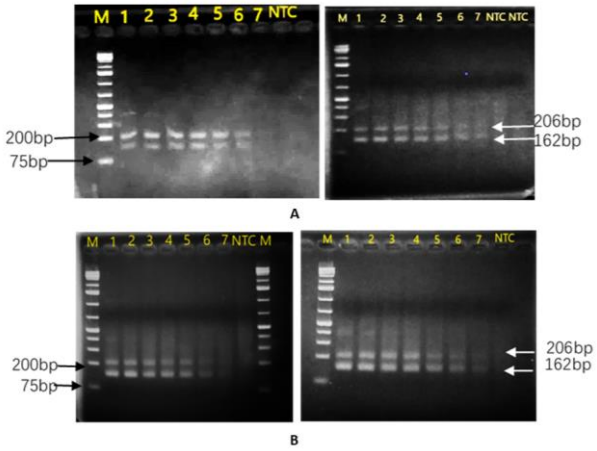

Figure 3. Limit detection and reproducibility assay of Multiplex MSP PTEN with. (A) Limit detection was done by mixed 1:1 of pGEM-T easy UM and pGEM-T easy as DNA template target done with a triple. (B). Reproducibility test of MMSP PTEN with mixed same as above. (M: DNA Marker; 1: 1 ng/µl; 2: 0.2 ng/µl; 3: 0.04 ng/µl; 4: 0.008 ng/µl; 5: 0.0016 ng/µl; 6: 0.00032 ng/µl ; 7: 0.000064ng/µl

easy PTEN methylation template, and 162bp for 0% pGEM-T easy PTEN un-methylation, which also mean no cross-reactivity between the primer (Figure 4).

Above specificity and sensitivity data showed that the developed method has a high specificity that could recognize the sample until 1.5625%. Based on the limit detection study, up to 0.00032 ng/µl DNA concentration was observed. The high sensitivity and specificity means, thisdevelopment method can aid the detection of a minimal concentration of DNA target, especially for methylated sequence among tumor heterogeneity. Pyrosequencing has been discovered to be more sensitive than genomic sequencing for methylation detection but is unsuitable for daily use because of its high operational cost with 10% specificity (Colella *et al.*, 2003).

3.4 MMSP PTEN Validation with a clinical sample

The MMSP PTEN assay was validated on a clinical sample treated with sodium bisulfite and confirmed with MSP PTEN, which used two different PCR tubes for each methylation and un methylation. With a confirmed sample (methylated and un-methylated alleles based on the MSP method), we further validated the developed MMSP PTEN, which used only one tube. As a result, two targets are similar to the MSP PTEN assay, 162bp representative for the unmethylated product and 206bp for the methylated DNA product of PTEN (Figure 5). To get a more clean and clear result, we replaced the above MMSP polymerase with KOD polymerase and showed better results showing strict PCR products for each target. (Figure 5)

An excellent concordance was discovered between MMSP in one single closed tube and MSP with two tubes. Based on two different types of polymerase, Hot start KOD polymerase showed better results to differentiate between methylation and un-methylation PTEN gene. According to Markoulatos *et al.*, (2002), Hot start PCR eliminates nonspecific reactions. Although the developed methods needs post-PCR processing with agarose DNA electrophoresis for data interpretation and analysis, this finding could indicate that the developed methods are potentially suitable for prognostic and diagnostic applications. Further, a vast sample is needed to evaluate this MMSP PTEN in the future for its validation.

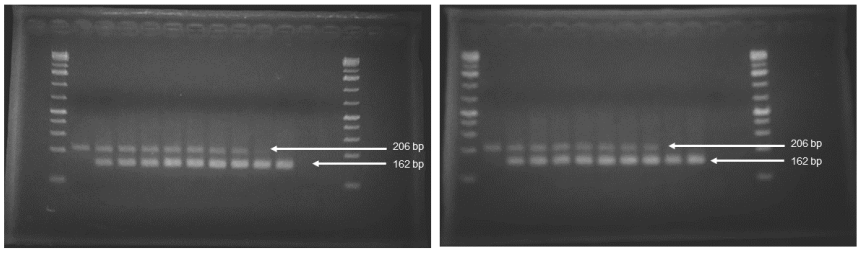

Figure 4. Specificity assay with proportion 100%: 50%: 25%: 12.5%: 6.25%: 3.115%: 1.5625%: 0.78%: 0.39%: 0% respectively from left to right orientation (We prepared double).

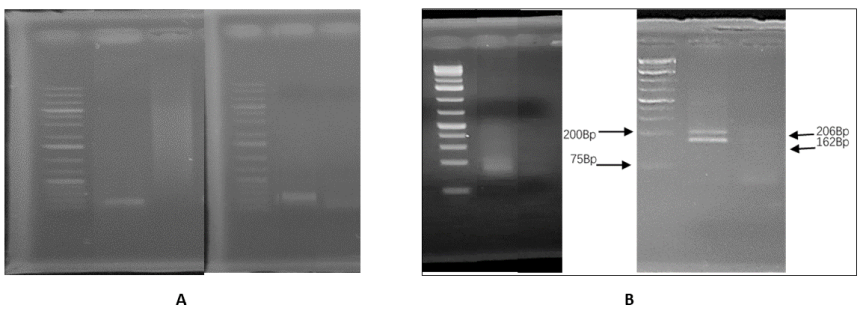

Figure 5. MMSP PTEN formulation 2 test clinical sample, validated with PTEN MSP PCR. (A) Clinical sample test with MSP PCR which amplified with Taq Polymerase. Un-Methylation (left) and Methylation DNA band (right), (B) The same clinical sample test with MMSP PTEN amplified with Taq Polymerase (left) and KOD Polymerase (right).

4. Conclusions

This study provided simple and more economic methods for detecting the prognostic and diagnostic biomarker PTEN and the epidemiological study of methylated PTEN promoters.

Acknowledgements

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