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Address : **Airlangga University School of Medicine**
Jl. Prof dr Moestop o 47 Surabaya 60131
Phone: 62 -31- 5013749, 5020251 -3 ext. 135
Fax : 62 -31- 5013749, 62 -31- 5022472
E-mail: folia@fk.unair.ac.id

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CONTENTS

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CLONING OF MELANOMA ANTIGEN-1 (MAGE-1) GENE FROM FINE NEEDLE ASPIRATION BIOPSY OF HEPATIC TISSUE OF HEPATOCELLULER CARCINOMA PATIENTS

Gondo Mastutik[1], Reny I'tishom[5], JH Lunardi[1], Ni Nyoman Tri Puspaningsih[2], Soetjipto[3], Hernomo Ontoseno Kusumobroto[4], Suhartono Taat Putra[1]

[1]Department of Anatomic Pathology, School of Medicine, Airlangga University
[2]Department of Chemistry, Faculty of Science and Techonology, Airlangga University
[3]Department of Biochemistry, School of Medicine, Airlangga University
[4]Department of Internal Medicine , School of Medicine, Airlangga University, Dr Soetomo Hospital, Surabaya
[5]Department of Medical Biology, School of Medicine, Airlangga University

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Asia. HCC expresses mRNA of melanoma antigen-1 (MAGE-1) and not expressed in non-tumor hepatic cells. This expression has been taking place at the early malignant transformation and continuously undergoing its progression. MAGE-1 is the important marker for diagnose HCC. MAGE-1 protein could be detected with antibody to MAGE-1 protein which could be obtained by doing clone of MAGE-1. However, the recombinant plasmid of MAGE-1 clone for developing of the diagnosis of HCC hasn't been obtained. The objective was to clone of coding sequence (CDS) of MAGE-1 from fine needle aspiration biopsy (FNAB) of HCC into vector and to obtain MAGE-1 recombinant plasmid. cDNA of MAGE-1 were extracted then continued by RT PCR and nested PCR. The PCR product was cloned to pET101/D-TOPO and transformed to E. coli Top10. Analyze of recombinant plasmid was undertaken by sequencing and restriction test. The result revealed that CDS of MAGE-1 were isolated and obtained band ±1105 bp at first round and ±931 bp at second round. The recombinant plasmid pETGM/MAGE1-HCC contained 927 nucleotides that encoded 309 amino acids. Partial sequence of MAGE-1 can be accessed in GeneBank EU161102 and ABW06861. Analyze of CDS MAGE-1 from HCC and GeneBank had 100% homology with M77481 and NM_004988 (both from skin melanoma tissue) and 99% with BC017555 (from skin melanoma tissue) and AY148486 (from HCC). This study obtains recombinant plasmid pETGM/MAGE1-HCC from the FNAB of HCC. It reveals that MAGE-1 recombinant plasmid that can be used for developing the diagnosis of HCC

Keywords: *fine needle aspiration biopsy, hepatocellular carcinoma, melanoma antigen-1, cloning*

Correspondence: Gondo Mastutik, Department of Anatomic Pathology, Airlangga University School of Medicine, Jl. Prof Dr Moestopo 47, Surabaya 60131, phone 62-31-5020251 ext. 151, email: mastutikg@yahoo.com

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Asia and it is generally diagnosed at advanced stage, during which the prognosis of the patients has been poor and their capability to survive has also been low (Chen H, et al 2000, Liu BB et al 1999). A number of studies have found that hepatic tissue of hepatocellular carcinoma patients expresses the melanoma antigen-1 (MAGE-1) gene in the form of mRNA between 60-80% (Peng et al 2002, Mou et al 2002, Miyamoto et al 2000, Kariyama et al 1999, Chen et al 2000, Liu et al 1999, Zhao et al 2004). MAGE-1 expressed in tumor with a size of less than 2 cm, normal or abnormal Alfhafeto protein (AFP) level, not expressed in non-tumor hepatic cells, not expressed in infectious of liver cells (Kobayashi et al 2000). The expression of MAGE-1 mRNA in hepatocellular carcinoma is related with the deviation of genome hypomethylation and MAGE-1 promoter domain at element promoter B' and B upstream -63 until -46 (De Smet et al 1996, 1999, 2004, Xiao et al 2005, Zhang et al 2004), This expression has been taking place at the early malignant transformation and continuously undergoing during its progression from hepatocelluler carcinoma.

The melanoma antigen-1 (MAGE-1) gene, belongs to cancer tetis (CT) gene. The gene was firstly isolated from melanoma, and recognized as melanoma antigen (MAGE) gene (Kumar et al 2005, van Baren et al 1999). CT antigen has several particularities, i.e., 1) it is particularly expressed by tumor tissue and not in normal tissue, 2) CT antigen coding gene is located in chromosome X (Zhao et al 2004). MAGE-1 belongs to the antigens coded by MAGE gene family. This gene expressed by cancer cells, while it is not expressed in normal tissue except in the testis (Van Baren et al 1999, Luo et al 2002), so that this gene is specific for cancer. Such specificity can lead to a conclusion that each

MAGE-1 expression indicates the presence of cancer cells. MAGE-1 expression can be used as a prediction of hepatocellular carcinoma so that MAGE-1 is the important marker for diagnoses hepatocelluler carcinoma. MAGE-1 protein could be detected with antibody to MAGE-1 protein which could be obtained by doing clone of MAGE-1. However, the clone of coding sequence of MAGE-1 from the result of FNAB of hepatic tissue of hepatocellular carcinoma patients and recombinant plasmid of MAGE-1 for developing of the diagnosis of hepatocelluler carcinoma has not been obtained.

mRNA of MAGE-1 can be carried out by using samples from hepatic tissue of patients with hepatocellular carcinoma. However, one of the obstacles found in carrying out MAGE-1 examination from hepatic biopsy tissue is the risk of uncontrolled bleeding. Another alternative is the use of samples from fine needle biopsy (FNAB). Unfortunately, due to limited cell counts aspirable with FNAB, the examination has to be conducted as effective as possible. To overcome these obstacles, examination should be undertaken in specific condition.

This study was a laboratory explorative study intended to clone of coding sequence of MAGE-1 from the result of FNAB of hepatic tissue of hepatocellular carcinoma patients into vector and to obtain MAGE-1 recombinant plasmid. Isolated of coding sequence was conducted by nested PCR. The first round was covering exon 1, exon 2, and exon 3 with total product 1105bp. This area is a conserved area for MAGE-1 (Chen et al 2000). The second round was covering in coding sequnce of MAGE-1 into exon 3 with total product 931bp.

MATERIALS AND METHOD

Samples were obtained from hepatocellular carcinoma by with fine needle aspiration biopsy (FNAB). Extraction of mRNA was referred to Takano T, et al (1999) and Giardia E, et al (2002) by lysis into needle. The result of FNAB taken by 22 or 24G needle with CT Scan as direction and directly put into tube containing lysis buffer, then pipetting and aspirate for 5 times for lysis into needle. Total RNA extraction was undertaken using RNeasy Protect Mini Kit (Qiagen) as protocol. cDNA synthesis was undertaken using First strand cDNA Syntesis kit for RT PCR (AMV) (Roche) which total RNA was subsequently altered into cDNA by means of oligo(T) primer.

Formed cDNA was amplified with nested polymerase chain reaction (PCR) and done using 2X PCR master Mix (Fermentas).The first round of PCR was done with GMF421 primer was 5'-CGGCCGAAGGAACCT GACCCAG-3' and GMTOPOR 5'-GCTTTGAGA GAGGAGGAAGAGGGAGTC-3', producing 1105bp. The second round was done with GM TOPO F CACCATGTCTCTTGAGCAGAGGAGTC and GMTOPOR 5'-GCTTTGAGAGAGGAGGAAGA GGGAGTC-3', producing 931bp. Primer concentration used for PCR was 50pmol/ml. Protocol was done according to sheat product data. The condition of PCR was the predenaturation was at 95oC for 5 minutes, denaturation at 95oC for 1 minute, annealing was 58oC 1 minute, and extension at 72oC 1 minute, 31 cycles. The cycle was prolonged at 72oC for 10 minutes. The PCR product was electrophoresized with 2% electrophoresis gel and visualized with UV transluminator. PCR product was sequenced with GeneticSequenzer (ABI Prism 310). The product of second round nested PCR was cloned to the vector pET101/D-TOPO and transformed to *E. coli* Top10 to confirm the accomplishment of insertion. Analyze of recombinant plasmid was undertaken by sequencing to find the nucleotide sequence of target DNA and by restriction test to find cloning accomplishment.

RESULTS

The isolation of MAGE-1 gene mRNA was undertaken by extracting samles to obtain total RNA which was subsequently altered to become cDNA by using oligo(T) primer. Poly A presents at the 3' end of mRNA, so that oligo(T) would pair with the poly A. Subsequently, cDNA synthesis was undergone by reverse transcriptase enzyme. Formed cDNA was amplified with nested PCR. The first round of PCR was done with GMF421 primer was 5'-CGGCCGAAGGAACCTGACCCAG-3' and GMTOPOR 5'-GCTTTGAGAGAGGAGGAAGA GGGAGTC-3'. The second round was done with GM TOPOF CACCATGTCTCTTGAGCAGAGGAGTC and GMTOPOR 5'-GCTTTGAGAGAGGAG GAAGAGGGAGTC-3'. The condition of PCR was the pre denaturation was at 95oC for 5 minutes, denaturation at 95oC for 1 minute, annealing was 58oC 1 minute, and extension at 72oC 1 minute, 31 cycles. The cycle was prolonged at 72oC for 10 minutes. The results of nested PCR was visualized by runing the 2% electrophoresis gel and documented. The result of this study obtained band + 1105bp at first round and + 931bp at second round, as seen in Figure 1.

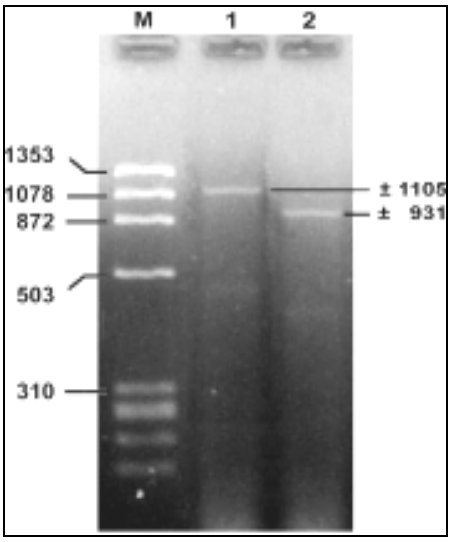

Figure 1. The electroforesis of nested PCR of the coding sequences of MAGE-1 gene from FNAB of hepatic tissue of carcinoma hepatocelluler patients. 1= the first round of PCR product (+ 110¬5 bp), 2= the second round of PCR product (+ 931bp), M= Marker F x 174 RF DNA /Hae III fragments

The product of second round nested PCR was cloned to the vector pET101/D-TOPO and transformed to *E. coli* Top10 to confirm the accomplishment of insertion. The result of transformation into *E. coli* Top 10 is shown at Figure 2. *E. coli* Top 10 growth on the Luria Bertani agar plate medium containing ampicillin for selection. *E. coli* Top 10 sensitive to ampicilin and pET101/D-TOPO was designed with resistance gene for ampicilin so that only coloni which contain recombinant plasmid can grow on the medium.

Figure 2. The plasmid recombinant of pETGM/ MAGE1-HCC in transform cell *E. coli* Top10

Analyze of recombinant plasmid was undertaken by restriction analyzing to find cloning accomplishment and sequencing to find the nucleotide sequence of target DNA. Restriction analyzed was performed using the EcoRV that cut the plasmid at the 545 and 4775 nucleotides. The result of EcoRV restriction had band 4230 and 2340 (1471 + 978). The coding sequence of MAGE-1 gene inserted in the number of 302 nucleotides of pET101/D-TOPO. The full sequence of recombinant plasmid pETGM/MAGE1-HCC had 6680pb (pET101/D-TOPO = 5753 and the coding sequence of mAGE-1= 927; 5773+927=6680). The result is shown at Figure 3.

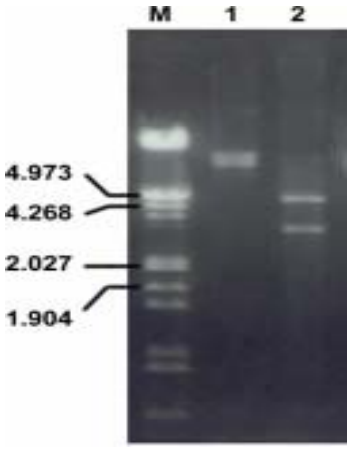

Figure 3. The EcoRV restriction analyze of pETGM/ MAGE1-HCC in transforms cell *E. coli* Top10. M= Marker DNA l cut with HindIII and EcoRI; 1= recombinant plasmid pETGM/MAGE1-HCC and 2= recombinant plasmid pETGM /MAGE1-HCC cut with EcoRV.

Sequencing of the recombinant plasmid pETGM/MAGE1-HCC was performed using Genetic Sequenzer (ABI Prism 310). The sequence is shown at Figure 4. Partial sequence of MAGE-1 gene coding area can be accessed in GeneBank with accession number EU161102 for nucleotide and ABW06861 for protein. The coding sequence of MAGE-1 gene in the recombinant plasmid pETGM/MAGE1-HCC contained 927 nucleotides that encoded 309 amino acids. The map of recombinant plasmid pETGM/MAGE1-HCC is shown at Figure 5.

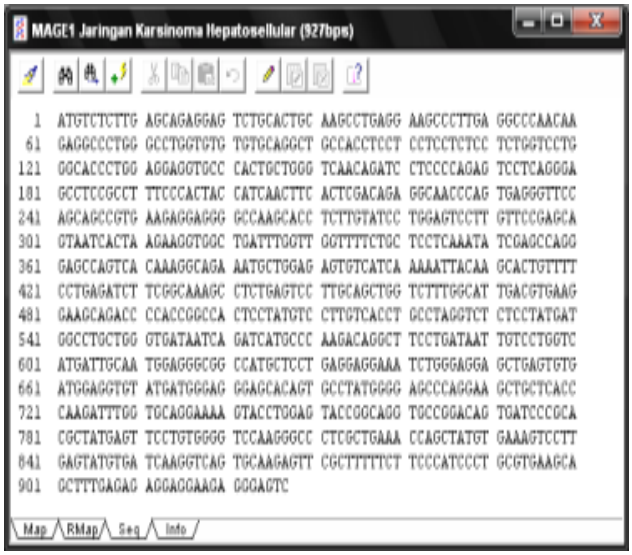

Figure 4. Sequence of the full length of coding sequence of MAGE-1 gene from FNAB of hepatic tissue of carcinoma hepatocelluler patients.

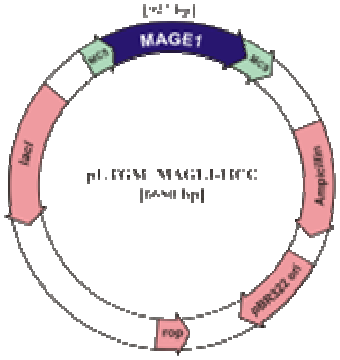

Figure 5. The map of recombinant plasmid pETGM/MAGE1-HCC from the FNAB of the hepatic tissue of carcinoma hepatocelluler patients contained 927 nucleotides that encoded 309 amino acids.

DISCUSSION

This was a laboratory explorative study generally aimed to clone of coding sequence of MAGE-1 from the result of FNAB of hepatic tissue of hepatocellular carcinoma patients into vector and to obtain MAGE-1 recombinant plasmid. The benefit for scientific development of this study was to provide information on sequence of coding sequence of MAGE-from the result of FNAB of hepatic tissue of hepatocellular carcinoma patients. Practical benefit from this study was to obtain recombinant plasmid pETGM/MAGE1-HCC. The result can be used as a basis for further study on the exploration of MAGE-1 gene expression for developing of the diagnosis tools of hepatocelluler carcinoma.

mRNAn isolation in this study used RNesay protect mini kit (Qiagen). Extraction protocol was carried out according to the kit employed in this study. The principle is that the mRNA was removed from cells by tissue lysis, chemically by mixing guanidium isotiocynate and betha mercaptoethanol, contained in buffer lysis, and enzymatically by using proteolytic enzyme, the proteinase K. Ethanol, which was added to prepare RNA binding condition with silica membrane on the column. Total RNA was bound to the column, while contaminants were washed efficiently. Finally, total RNA obtained was dissolved with RNase free water. Resulted RNA concentration using silica membrane column was 100 ug RNA. Total RNA could be kept at -30oC until the usage or could be directly used for cDNA synthesis (Qiagen 2001).

Extraction from the FNAB result was done directly into needle by aspirating the lysis buffer and cell from FNAB solution for minimal 5 times. Lysis buffer contains guanidium isotiocynate and betha mercaptoethanol that potent chaotopic agent for lysis cells. Then it continued with enzymatic lysis using proteinase K. The result of extracton is total RNA that ready for reverse to be cDNA by Reverse trancriptase enzyme.

cDNA was synthesized from total RNA with RT PCR (Reverse Trancriptase- Polymerase Chain Reaction). RT PCR is a method designed to amplify cDNA copy from RNA. Primers used for hybridizatoin was RT PCR are 1) Gene Specific Primer (GSP), used for primer antisense (oligonucleotide antisense) from the sequence needed. Maximum result can be obtained if the primer antisense used for amplification in the PCR is located far upstream from the oligonucleotide used for cDNA synthesis. 2) Oligo(T) (dT), which binds endogeneous poly(A) tail that presents at 3' mRNA. This primer can be used as universal primer for synthesizing cDNA. 3) Random hexanucleotide, which can be used to synthesize cDNA from long mRNA template. The primer can attach to all mRNA randomly, so that it can be used as template for cDNA synthesis in all mRNA (Sambrook & Russell 2001). Primer used for RT PCR in this study was oligo(T) as it could obtain total RNA that could be employed as a PCR template for all genes. The result of RT PCR is ready for amplified by nested PCR.

Formed cDNA was amplified with nested PCR. The first round of PCR was done with GMF421 primer was 5'-CGGCCGAAGGAACCTGACCCAG-3' and GMTOPOR 5'-GCTTTGAGAGAGGAGGAA

GAGGGAGTC-3'. The second round was done with GMTOPOF CACCATGTCTCTTGAGCAGAG GAGTC and GMTOPOR 5'-GCTTTGAGAGA GGAGGAAGAGGGAGTC-3'. This second round primer was designed specific for purpose of cloning into pET101/D-TOPO. The result of this study obtained band + 1105bp at first round and + 931bp at second round.

The primer GMTOPOF is forward direction and GMTOPOR is for reverse direction. The forward primer was added CACC sequenceat 5' for direction into clone to pET101/D_TOPO and added ATG for start codon to synthesis protein. The reverse primer was missed stop codon because it uses the vector stop codon. Stop codon of vector was sited down stream of the insert site before histidine taq. The vector was already including the 6x histidine taq for make easy to purify the recombinant protein. The PCR product was purified then inserted into vector, pET101/D-TOPO and transformed into *E. coli* Top 10 for to confirm the accomplishment of insertion. *E. coli* Top 10 grow on the Luria Bertani agar plate medium containing ampicillin for selection. *E. coli* Top 10 sensitive to ampicilin can't growt at medium containg ampicilin. and pET101/D-TOPO was designed with resistance gene for ampicilin so that only coloni which contain recombinant plasmid can grow on the medium.

Analyze of recombinant plasmid was undertaken by restriction analyzing to find cloning accomplishment and sequencing to find the nucleotide sequence of target DNA. EcoRV cut pETGM/MAGE1-HCC at the 545 and 4775 nucleotides. The result of EcoRV restriction had band 4230 and 2340 (1471 + 978). The coding sequence of MAGE-1 gene inserted in the number of 302 nucleotides of pET101/D-TOPO. The full sequence of recombinant plasmid pETGM/MAGE1-HCC had 6680pb (pET101/D-TOPO = 5753 and the coding sequence of mAGE-1= 927; 5773+927=6680).

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MAGE-1 gene contents 3 exons, and 2 introns. The coding sequence of MAGE-1 sited at exon 3, at 188-1117 nucleotide. mRNA MAGE-1 content 1722 nucleotides, coding sequence at nucleotides 188-1117 (927bp nucleotide include start codon without stop codon). This vector pET101/D-TOPO is 5753bp and linier so that it can use as direct cloning from PCR product without restriction before used. The coding sequence of MAGE-1 is 927bp so that the total long of recombinant plasmid pETGM/MAGE1-HCC is 6680bp. The sequence of MAGE-1 gene coding area can be accessed in GeneBank with accession number EU161102 for nucleotide and ABW06861 for protein. Analyze of the coding sequence of MAGE-1 gene from hepatic tissue of hepatocellular carcinoma patient and GeneBank had 100% homology with sequence accession number M77481 (sequence from skin melanoma tissue), NM_004988 (sequence from skin melanoma tissue) and 99% with sequence accession number BC017555 (sequence from skin melanoma tissue) and AY148486 (from hepatocellular carcinoma patient's hepatic tissue).

CONCLUSION

This study obtains recombinant plasmid pETGM/MAGE1-HCC from the FNAB of hepatic tissue of hepatocellular carcinoma. It reveals that MAGE-1 recombinant plasmid that can be used for developing the diagnosis of hepatocellular carcinoma.

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