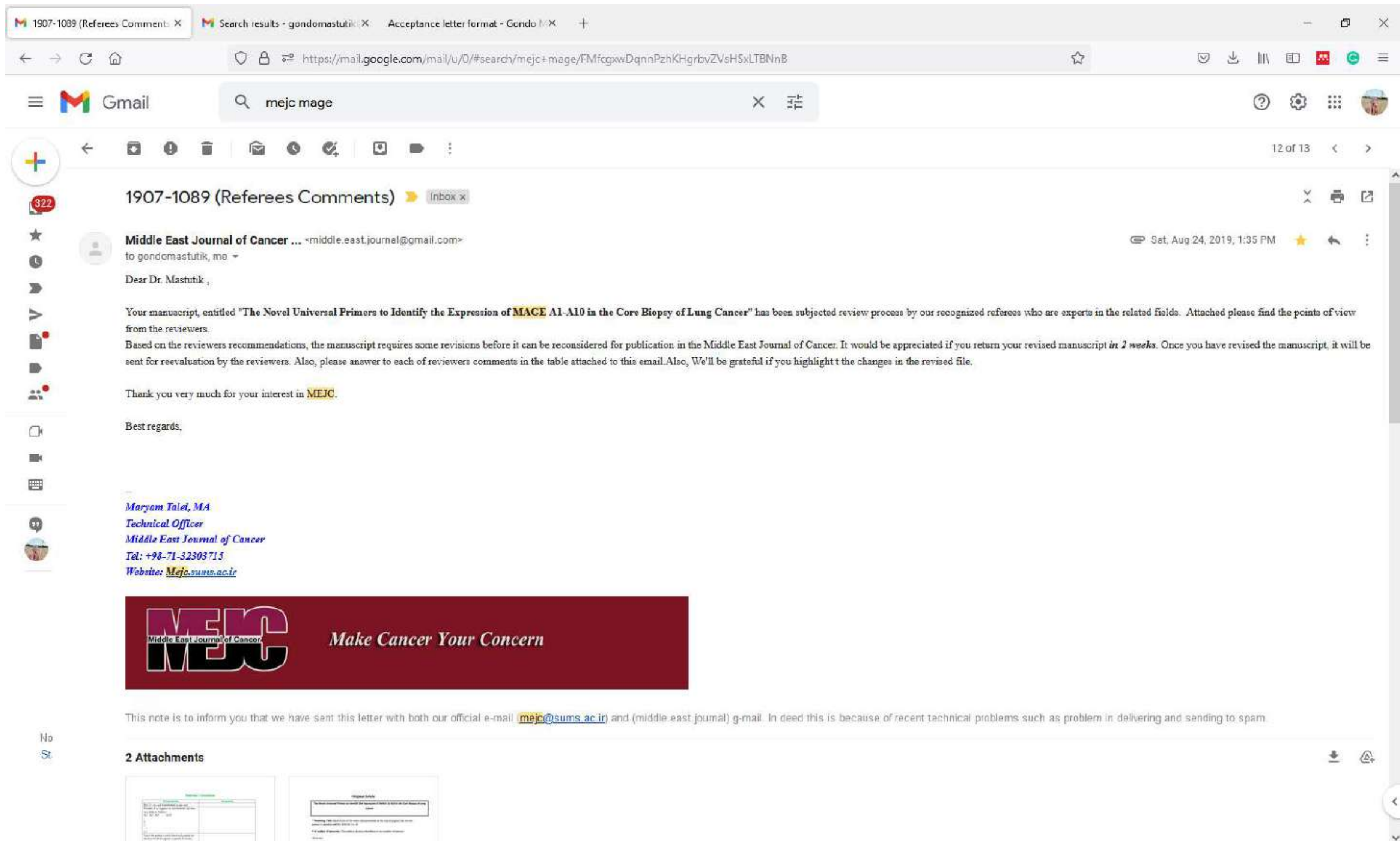
BUKTI CORESPONDING MEJC



1907-1089 (Referees Comments)

Middle East Journal of Cancer ... <middle.east.journal@gmail.com>
to gondomastutik, me

Sat, Aug 24, 2019, 1:35 PM

Dear Dr. Mastutik ,

Your manuscript, entitled "**The Novel Universal Primers to Identify the Expression of MAGE A1-A10 in the Core Biopsy of Lung Cancer**" has been subjected review process by our recognized referees who are experts in the related fields. Attached please find the points of view from the reviewers.
Based on the reviewers recommendations, the manuscript requires some revisions before it can be reconsidered for publication in the Middle East Journal of Cancer. It would be appreciated if you return your revised manuscript ***in 2 weeks***. Once you have revised the manuscript, it will be sent for reevaluation by the reviewers. Also, please answer to each of reviewers comments in the table attached to this email.Also, We'll be grateful if you highlight t the changes in the revised file.

Thank you very much for your interest in MEJC.

Best regards,

Maryam Talei, MA
Technical Officer
Middle East Journal of Cancer
Tel: +98-71-32303715
Website: Mejc.sums.ac.ir

Make Cancer Your Concern

This note is to inform you that we have sent this letter with both our official e-mail (mejc@sums.ac.ir) and (middle east journal) g-mail. In deed this is because of recent technical problems such as problem in delivering and sending to spam.

2 Attachments

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2 Attachments

Referees' Commen... | referees(Highlighte...

gondo mastutik <gondomastutik@gmail.com>
to Middle

Thu, Aug 29, 2019, 3:58 PM

Dear Editors,
Here with, I attach the file that I have repaired according to the referees suggestions.
This manuscript is complete with all of table and figure, including new table (Table 3) and new subtitle figure in Fig 3.
Thank you very much for give us some suggestion for improve our manuscript and give us an opportunity to publish our study in your journal.

Best Regard,
Gondo Mastutik

2 Attachments

Referees' Commen... | Revisi Submit MEJ...

Middle East Journal of Cancer ... <middle.east.journal@gmail.com>
to me

Sat, Aug 31, 2019, 12:16 PM

Dear Dr. Mastutik,

Thanks for your reply, However, we need you to highlight the parts you've changed in the revised version. Please send us the highlighted version as soon as possible.

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12 of 13

Referees' Commen... | Revisi Submit MEJ...

Middle East Journal of Cancer ... <middle.east.journal@gmail.com> Sat, Aug 31, 2019, 12:16 PM
to me

Dear Dr. Mastutik,

Thanks for your reply, However, we need you to highlight the parts you've changed in the revised version. Please send us the highlighted version as soon as possible.

Sincerely,

...

gondo mastutik <gondomastutik@gmail.com> Sun, Sep 1, 2019, 7:10 AM
to Middle

Dear Editors,
Thank you for your replying. here with I attach the file highlighted.
Thank you.

Best Regard,
Gondo Mastutik

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Revisi Submit MEJ...

Reply | Forward

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11 of 13

1907-1089 (Further Revisions) Inbox

Middle East Journal of Cancer ... <middle.east.journal@gmail.com>
to gondomastutik, me, alphania-r, marhanadr, drnilakurniasari, moch.amin, taatputra

Sun, Feb 23, 2020, 6:57 PM

Dear Dr. Mastutik,

Your manuscript entitled " **The Novel Universal Primers to Identify the Expression of MAGE A1-A10 in the Core Biopsy of Lung Cancer**" should be sent to the journal's copy editor. There are several points highlighted in the attached file which should be revised before that. Please perform all the revisions and send it back to us *as soon as possible*. We'll appreciate if you highlight the changes in the revised version.

Sincerely,

--

Maryam Talei, MA
Technical Officer
Middle East Journal of Cancer
Tel: +98-71-32303715
Website: Mejc.sums.ac.ir

This note is to inform you that we have sent this letter with both our official e-mail (mejc@sums.ac.ir) and (middle.east.journal) g-mail. In deed this is because of recent technical problems such as problem in delivering and sending to spam.

checked for P to au...

gondo mastutik <gondomastutik@gmail.com>
to Middle

Mon, Feb 24, 2020, 5:26 PM

1907-1089 (Further Revisions) | Search results - gondomastutik | Acceptance letter format - Gondo

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Tel: +98-71-32303715
Website: Mejc.sums.ac.ir

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gondo mastutik <gondomastutik@gmail.com> Mon, Feb 24, 2020, 5:26 PM
to Middle

Dear Editors,
Here with I attach the revision manuscript file in the green highlight.
Thank You.

Sincerely,
Gondo Mastutik

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9 of 13

1907-1089 (Final Revisions) Inbox ×

Middle East Journal of Cancer ... <middle.east.journal@gmail.com>
to gondomastutik, me, alphania-r, marhenadr, drnilakurniasari, moch.amin, taatputra

Tue, Mar 10, 2020, 4:09 PM

Dear Dr. Mastutik,

Your manuscript entitled "**Novel Universal Primers to Identify the Expression of MAGE A1-A10 in the Core Biopsy of Lung Cancer**" was sent to the journal's copy editor for further revisions. There is one point in the attached file which should be answered. Please revise the script based on the point and send it back to us as soon as possible.
We'll appreciate if you highlight the changed parts after revision and please make sure that you perform changes on the attached file.

Sincerely,

--
Maryam Talei, MA
Technical Officer
Middle East Journal of Cancer
Tel: +98-71-32303715
Website: Mejc.sums.ac.ir

This note is to inform you that we have sent this letter with both our official e-mail (mejc@sums.ac.ir) and (middle.east.journal) g-mail. In deed this is because of recent technical problems such as problem in delivering and sending to spam.

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Website: Mejc.sums.ac.ir

This note is to inform you that we have sent this letter with both our official e-mail (mejc@sums.ac.ir) and (middle east journal) g-mail. In deed this is because of recent technical problems such as problem in delivering and sending to spam.

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gondo mastutik <gondomastutik@gmail.com> Sat, Mar 14, 2020, 2:12 PM

to Middle

Dear Editor,
I attract the file revision.
Thank you

Sincerely yours,
Gondo Mastutik

revised by cp to au...

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Fwd: 1907-1089 (Galley Proof)

Inbox

gondo mastutik <gondomastutik@fk.unair.ac.id>
to me

Tue, Dec 8, 2020, 7:29 PM

---------- Forwarded message ---------
Dari: **Middle East Journal of Cancer ...** <middle.east.journal@gmail.com>
Date: Sab, 5 Des 2020 20:37
Subject: 1907-1089 (Galley Proof)
To: <gondomastutik@fk.unair.ac.id>
Cc: <alphania-r@fk.unair.ac.id>, <marhanadr@yahoo.com>, <drnilakurniasari@gmail.com>, <moch.amin@staf.unair.ac.id>, <taatputra@gmail.com>

Dear Dr. Mastutik ,

Please find attached the Galley proof of your manuscript entitled "**Novel Universal Primers to Identify the Expression of MAGE A1-A10 in the Core Biopsy of Lung Cancer**" for review and confirmation. The Galley proof must be returned to the MEJC office (by responding to this Email) *in 3 days*. Please let us know if there are any errors in the manuscript. As we will be launching the journal very shortly, your prompt response is greatly appreciated.

Thank you very much for your interest in MEJC.

Best regards,

--

Maryam Talei, MA
Technical Officer
Middle East Journal of Cancer
Tel: +98-71-32303715
Website: Mejc.sums.ac.ir

This note is to inform you that we have sent this letter with both our official e-mail (mejc@sums.ac.ir) and (middle.east.journal) g-mail. In deed this is because of recent technical problems such as problem in delivering and sending to spam.

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This note is to inform you that we have sent this letter with both our official e-mail (mejc@sums.ac.ir) and (middle.east.journal) g-mail. In deed this is because of recent technical problems such as problem in delivering and sending to spam.

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Gondo Mastutik <gondomastutik@gmail.com> Wed, Dec 9, 2020, 5:29 AM

to gondo

Dear Editor,

Here with, I have notified "highlight yellow colour" and write some comments as correction for the error thing.

Thank you for your attention.

With Warm Regard,

Dr. Gondo Mastutik, DVM, M.Sc.
Associate Professor Department of Anatomic Pathology
Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.
E-mail: gondomastutik@fk.unair.ac.id, gondomastutik@gmail.com
Mobile +6281231071818

2 Attachments

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7 of 13

Page Proof

Inbox ×

Middle East Journal of Cancer ... <middle.east.journal@gmail.com> Tue, Jan 26, 2021, 11:42 AM

to gondomastutik, me, alphania-r, marhanadr, drnilakurniasari, moch.amin, taatputra

Dear Dr. Mastutik,

It is our great pleasure to inform you that according to the comments of our referees, your manuscript, entitled '**Novel Universal Primers to Identify the Expression of MAGE A1-A10 in the Core Biopsy of Lung Cancer** " is accepted for publication in the Middle East Journal of Cancer (MEJC).

Page proofs of your manuscript is attached for checking and correction. The Page proofs must be returned to the MEJC office *in 3 days*. Furthermore, you are responsible for any error in the published paper due to your oversight.

Thank you very much for your interest in MEJC

--

Maryam Talei, MA
Technical Officer
Middle East Journal of Cancer
Tel: +98-71-32303715
Website: Mejc.sums.ac.ir

This note is to inform you that we have sent this letter with both our official e-mail (mejc@sums.ac.ir) and (middle.east.journal) g-mail. In deed this is because of recent technical problems such as problem in delivering and sending to spam.

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Gondo Mastutik <gondomastutik@gmail.com> Wed, Jan 27, 2021, 9:04 AM

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This note is to inform you that we have sent this letter with both our official e-mail (mejc@sums.ac.ir) and (middle.east.journal) g-mail. In deed this is because of recent technical problems such as problem in delivering and sending to spam.

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Gondo Mastutik <gondomastutik@gmail.com>
to Middle

Wed, Jan 27, 2021, 9:04 AM

Dear Maryam Talei, MA,

Here, I found 2 words for correction.
1. In abstract, "onmost" , it should be "on most".
2. In the title of figure1. it type "fugure", it should be "figure"
I also marker the pdf with yellow light and comment.

Thank you very much for your correction.

Best regard,
Gondo Mastutik

2(1) edit GM.pdf

Reply | Forward

Acknowledgement of Submission (Manuscript #MEJC-1907-1089) Inbox

Middle East Journal of Cancer <mejc@sums.ac.ir> Sun, Jul 14, 2019, 10:38 AM
to gondomastutik, me, taatputra, alphenia-r, moch.amin, drnilakurniasari, marhanadr

Manuscript ID: MEJC-1907-1089

Manuscript Title: **The Novel Universal Primers to Identify the Expression of MAGE A1-A10 in the Core Biopsy of Lung Cancer**

Authors: Gondo Mastutik, Alphania Rahniayu, Isnin Anang Marhana, Nila Kurniasari, Mochamad Amin, Suhartono Taat Putra

Dear **Dr. Gondo Mastutik**

I wish to acknowledge receiving the of the above mentioned manuscript.

It should be noted that the manuscript will be reviewed for possible publication in the Scientific Journals Management System.

Please be sure that the submitted manuscript has not been published previously and will not be submitted elsewhere prior to our decision.

Our editorial decision will be brought to your attention once the paper has been reviewed due the referees consideration.

I wish to take this opportunity to thank you for sharing your work with us.

Truly yours,

Executive managing Editor of **Middle East Journal of Cancer**

gondo mastutik <gondomastutik@fk.unair.ac.id> Sun, Jul 14, 2019, 10:40 AM
to me

Comment reviewer

Referees' Comments

Comments	Answers
Ref 25 was not mentioned in the text.	
Results: It is suggest to summarize all data in a table as bellow:- A1 A2 A3 …….A10 1 2 // 15	
Since the authors used a forward primer for the first PCR designed to anneal to exon2-exon3 junction, why they used a removal enzyme after mRNA extraction?	
Part of the introduction can be summarized in a table and avoid the complexity (highlighted).	
The naming of the reverse primers in the text (M7,8,9) and table 1 (M8,9,10) does not match.	
Move the description of the images in the text to the subtitle of the images and avoid duplication. Page 6, 7 (highlighted).	
In the discussion section, the material and method are duplicate and should be deleted (highlighted).	

Menjawab comments reviewer

Referees' Comments

Comments	Answers
Ref 25 was not mentioned in the text.	Thank you, I have written in discussion paragraph 5. The expression of MAGE A1-10 in this study showed positive at the lung tissue from patient with diagnozed lung cancer. As reported in previous studies, MAGE-A gene is often expressed in some cancer, such as oral squamous carcinoma,[5,24] gastric cancer,[6,25] renal cancer,[26] pappilary thyroid carcinoma,[12] lung cancer,[14,27] and
Results: It is suggest to summarize all data in a table as bellow:- A1 A2 A3 …….A10 1 2 // 15	Yes, Done To be Table 3
Since the authors used a forward primer for the first PCR designed to anneal to exon2-exon3 junction, why they used a removal enzyme after mRNA extraction?	Aswer; To be carefully and avoid the genomic contamination as other study mentions in 13. Park J, Kyu T, Kim I, Sohn S, Kim Y, et al. A new strategy for the diagnosis of MAGE-expressing cancers. J Immunol Methods 2002;266(1-2):79-86.
Part of the introduction can be summarized in a table and avoid the complexity (highlighted).	I have summarized it
The naming of the reverse primers in the text (M7,8,9) and table 1 (M8,9,10) does not match.	Thank you. I have repaired
Move the description of the images in the text to the subtitle of the images and avoid duplication. Page 6, 7 (highlighted).	Thank you, I have moved it in subtitle of Fig 3. **Fig. 3** Fig. 3. The PCR product from testicular tissue specimen. Marker (lane 1), Negative control (lane 2), GAPDH (+) 320 bp (lane 3), MAGE A1-10 first Round 823 bp (-) (lane 4), MAGE A1-10 second round 461 bp (+) (lane 5), MAGE A1-6

	first round 852 bp (+) (lane 6), MAGE A1-6 seocond round 469 bp (+) (lane 7), MAGE A1 377 bp (+) (lane 8), MAGE A2 523 bp (+) (lane 9), MAGE A3 569 bp (+) (lane 10), MAGE A4 580 bp (+) (lane 11), MAGE A5 478 bp (+) (lane 12), MAGE A8 419 bp (+) (lane 13), MAGE A9 407 bp (+) (lane 14), MAGE A10 464 bp (+) (lane15), Marker (lane 16).
In the discussion section, the material and method are duplicate and should be deleted (highlighted).	Thank you. I have moved it on methods. The MAGE-A gene sequences were retrieved from Genbank data (National Centre for Biotechnology Information). In this study, we design the novel universal primer MAGE A1-10 that carried out based on the mRNA sequences of the target gene by selecting the area between exon 1, 2, and 3 (Fig. 1). The variability areas of MAGE-A gene was at the promoters regions and first exon regions of MAGE A gene,[23] in contrast, the high homology domains were exon 2 and exon 3. The exon 3 was the coding region that had similar sequences among all of MAGE A gene families A. Therefore, the forward primer was designed to bind to exon 2 joining with exon 3, and the reverse primer was designed to attach to exon 3. The expression of MAGE A1-10 all together was identified by nested PCR, using MF10/MR10 primers for the first round and MF10/MR12 primers for the second round (Table 1). The primer MF10 as outer forward primer would bind to cDNA MAGE A1-10 at exon 2 joining with exon 3. For the second round, MF10 and MR12 were used as inner primer to verify that the PCR product would only amplify the cDNA of MAGE A1-10. To compare this method with other study, we identified the expression of MAGE A1-6 by using their primers[13] that are MMRP1/MMRP1 primer for the first round and MMRP3/MMRP4 primer for the second round.

The revision form authors

Red underline and highlight from referees	Page	After repair
The objective of this study is to construct the novel universal primers for detecting mRNA of MAGE A1-10 genes in lung cancer patients.	Abstract, page 1	The objective of this study is to construct the novel universal primers for detecting mRNA of MAGE A1-10 genes in lung cancer patients. **Methods:** The specimen were a testicular tissue and 15 the core biopsy of lung cancer tissues from Dr. Soetomo General Academic Hospital Surabaya Indonesia. The universal primers was designed to bind to mRNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 regions, the assay was done by nested PCR, and continued by direct sequencing.
Methods: The specimen were a testicular tissue and 15 the core biopsy of lung cancer tissues from … General Academic Hospital … Indonesia. The universal primers was designed to bind to cDNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 regions, the assay was done by nested PCR, and continued by direct sequencing.	Abstract, page 1	**Methods:** The specimen were a testicular tissue and 15 the core biopsy of lung cancer tissues from Dr. Soetomo General Academic Hospital Surabaya Indonesia. The universal primers was designed to bind to mRNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 regions, the assay was done by nested PCR, and continued by direct sequencing.
Results: The PCR using the universal primer MAGE A1-10 can detect at least one MAGE A mRNA of 10 subtype of MAGE A from testicular and lung cancer tissues.	Abstract, page 1	**Results:** The PCR using the universal primer MAGE A1-10 can detect the MAGE A mRNA of 10 subtype of MAGE A from testicular and lung cancer tissues.
Introduction Melanoma antigen (MAGE) gene is well known as cancer testis antigen (CTA) expressed in various tumors. Yet, it is silent in normal adult tissues, except the testis.1,2 Based on the tissues-restricted expression and gene structure, the MAGE gene is classified into 2 different types. The first types are MAGE A, MAGE B, MAGE C restricted to male germ-line tissues and located in clusters on the X chromosome that Xq28, Xp21, and Xp26-27,	2-3	Melanoma antigen (MAGE) gene is well known as cancer testis antigen (CTA) expressed in various tumors. Based on the tissues-restricted expression and gene structure, the MAGE gene is classified into 2 different types. The first types are MAGE A, MAGE B, MAGE C restricted to male germ-line tissues and located in clusters on the X chromosome that Xq28,

respectively.3 The second type is MAGE D expressed in body tissues and located in Xp11. MAGE D is expressed in normal tissues, whereas MAGE A, B, C are expressed in tumor cells.4 Type I MAGE expression, including MAGE A, has been detected in a broad variety of malignancies. There are 12 subtypes of MAGE A; MAGE A1, A2, A3, A4, A5, A6, A7 (pseudo gene), A8, A9, A10, A11, A12. The expression of each MAGE-A subtypes, A1-A12, was examined positive in oral squamous cell carcinoma and gastric cancer by immunohistochemically stained.5,6 MAGE-A1 and MAGE-A3 mRNA was expressed in neuroblastoma tumors,7 hepatocellular carcinoma,8 benign and malignant neoplasms of the salivary glands.9 The mRNA of MAGE-A1, -A3, -A4, -A10 were expressed in epithelial ovarian cancer (EOC).10 MAGE A1 and A4 were detected in squamous cell vulvar neoplasms.11 The expressions of MAGE A1-6 was detected in papillary thyroid carcinoma,12 head and neck cancer tissues, breast cancer tissues, and lung cancer tissues.13 MAGE A1 and A3 were expressed at primary non small cell lung cancer (NSCLC).14 Several *MAGE* subgroup genes, such as *MAGE-A5*, *-A7*, *-A8*, *-A9*, *-A11*, *-B3*, *-B4*, *-B10*, *-D2*, *-D3*, *-F1*, *-G1*, *-H1*, and *-L2*, have been first discovered to show differential expression in NSCLC.15		Xp21, and Xp26-27, respectively.[1] The second type is MAGE D expressed in body tissues and located in Xp11. MAGE D is expressed in normal tissues, whereas MAGE A, B, C are expressed in tumor cells.[2] Type I MAGE, including MAGE A, the first known as gene that it is silent in normal adult tissues, except the testis.[3,4] Then the last study showed that the expression of MAGE A has been detected in a broad variety of malignancies. There are 12 subtypes of MAGE A; MAGE A1, A2, A3, A4, A5, A6, A7 (pseudo gene), A8, A9, A10, A11, A12. The expression of each MAGE-A subtypes, A1-A12, was examined positive in oral squamous cell carcinoma,[5] gastric cancer,[6] neuroblastoma tumors,[7] hepatocellular carcinoma,[8] benign and malignant neoplasms of the salivary glands,[9] epithelial ovarian cancer (EOC),[10] squamous cell vulvar neoplasms,[11] papillary thyroid carcinoma,[12] head and neck cancer tissues, breast cancer tissues, and lung cancer tissues.[13] MAGE A1 and A3 were expressed at primary non small cell lung cancer (NSCLC).[14] Several *MAGE* subgroup genes, such as MAGE A, B, D have been first discovered to show differential expression in NSCLC.[15]
The expression of MAGE A1-6 in cancer tissues can be detected by Reverse Transcription Polymerase Chain Reaction (RT PCR) and nested PCR.13,21 Other than MAGE A1-6, there are subtypes MAGE A8, A9, A10, that are also expressed in cancer tissues.15,22 The detection of MAGE A1-10 gene expression in a single reaction	3	The expression of MAGE A1-6 in cancer tissues can be detected by Reverse Transcription Polymerase Chain Reaction (RT PCR) and nested PCR.[13,21] Other than MAGE A1-6, there are subtypes MAGE A8, A9, A10, that are also expressed in cancer tissues.[15,22]

of PCR can be more useful to improve the diagnose, therapy, and prognose of cancer. Therefore, we have decided to develop a novel universal primer in order to identify MAGE-A mRNA subtype A1-10.		Therefore, we have decided to develop a novel universal primer in order to identify MAGE-A mRNA subtype A1-10.
2. Design primer The MAGE-A gene sequences were retrieved from Genbank data (National Centre for Biotechnology Information). Subtypes of the MAGE A gene are MAGE A1, A2, A3, A4, A5, A6, A8, A9, A10, A11, A12, while MAGE A7 is defined as a pseudo gene. MAGE A11 and A12 genes have mRNA sequences that have different homology with the sequence of the MAGE A1-10 gene. The design of the novel universal primer MAGE A1-10 in this study was carried out based on the mRNA sequences of the target gene by selecting the area between exon 1, 2, and 3 (Fig. 1).	4	The MAGE-A gene sequences were retrieved from Genbank data (National Centre for Biotechnology Information). In this study, we design the novel universal primer MAGE A1-10 that carried out based on the mRNA sequences of the target gene by selecting the area between exon 1, 2, and 3 (Fig. 1). The variability areas of MAGE-A gene was at the promoters regions and first exon regions of MAGE A gene,[23] in contrast, the high homology domains were exon 2 and exon 3. The exon 3 was the coding region that had similar sequences among all of MAGE A gene families A. Therefore, the forward primer was designed to bind to exon 2 joining with exon 3, and the reverse primer was designed to attach to exon 3.
The expression of MAGE A1-10 was identified by nested PCR, using MF10/MR10 primers for the first round and MF10/MR12 primers for the second round (Table 1). The expression of MAGE A1-6 was identified by using MMRP1/MMRP1 primer for the first round, and MMRP3/MMRP4 primer for the second round.13 The individual MAGE-A genes were detected by using MMRP3 as forward primer and reverse primer using M1, M2, M3, M4, M5, M6 with product MAGE A1 377 bp, MAGE A2 523 bp, MAGE A3 569 bp, MAGE A4 580 bp, MAGE A5 478 bp, MAGE A8 419 bp.13	4	The expression of MAGE A1-10 all together was identified by nested PCR, using MF10/MR10 primers for the first round and MF10/MR12 primers for the second round (Table 1). The primer MF10 as outer forward primer would bind to cDNA MAGE A1-10 at exon 2 joining with exon 3. For the second round, MF10 and MR12 were used as inner primer to verify that the PCR product would only amplify the cDNA of MAGE A1-10. To compare this method with other study, we identified the expression of MAGE A1-6 by using their primers[13]

		that are MMRP1/MMRP1 primer for the first round and MMRP3/MMRP4 primer for the second round. The individual MAGE-A genes (from MAGE A1 until A10, separately) were detected by using MMRP3 as forward primer and reverse primer using M1, M2, M3, M4, M5, M6 with product MAGE A1 377 bp, MAGE A2 523 bp, MAGE A3 569 bp, MAGE A4 580 bp, MAGE A5 478 bp, MAGE A8 419 bp.[13]
The identification of MAGE A8, A9 and A10 subtypes used MF10 as forward and as reverse used M7, M8, and M9.	4	The identification of MAGE A8, A9 and A10 subtypes used MF10 as forward and as reverse used M8, M9, and M10.
In this research, the DNA amplification reactions for MAGE A1-10, MAGE A1-6, and individual MAGE A1-10, performed by using GoTaq(R) Green Master Mix (Promega, USA).	5	In this research, PCR was performed by using GoTaq(R) Green Master Mix (Promega, USA).
4. Sequences analysis MF10/MR10 and MF10/MR12 primers were used to verify the result of PCR, and so were MMRP1/MMRP1 and MMRP3/MMRP4. The specimen from a testicular tissue was used as a template for PCR of individual MAGE A-10. It was then continued by direct sequencing to confirm the positive result of PCR, using the Genetic Analyzer (Applied Biosystems, USA).	6	To verify the PCR result of MAGE A1-10 and MAGE A1-6 all together, we performed PCR for individual subtype of MAGE A1-10, separately. It was then continued by direct sequencing to confirm the positive result of PCR, using the Genetic Analyzer (Applied Biosystems, USA). The sequences were compared with the sequences from Gene Bank data.
These results showed that PCR using the MF10/MR10 primers for MAGE A1-10 indicated 823-919 base pair (bp), MF10/MR12 primers showed 461-557 bp, and MMRP1/MMRP1 primers for MAGE A1-6 showed 852 bp, and MMRP3/MMRP4 primers showed 469-490 bp (Fig. 2A). The dilution test of the PCR using the MF10/MR10 primers is shown in Fig. 2B, whereas MMRP1/MMRP2 is given in Fig. 2C.	6	Asnwer: MF10/MR10 and MF10/MR12 are pairs common/ universal primer for MAGE A1-10. MMRP 1/MMRP2 and MMRP3/MMRP4 are pairs common/universal primer for MAGE A1-6. Base on the bioinformatic study, the result of those primers is not a band with one size in basepair, but it is

		in a range, depending on MAGE A that express in the specimen.
The results of PCR using the primer of individual MAGE A1-10 for identifying the expression of MAGE A1, A2, A3, A4, A5, A8, A9, A10 from testicular tissue specimen can be seen in Fig. 3. These were used for direct sequencing. The products were GAPDH (+) 320 bp in lane 3, MAGE A1-10 first Round 823 bp (-) in lane 4, MAGE A1-10 second round 461 bp (+) in lane 5, MAGE A1-6 first round 852 bp (+) in lane 6, MAGE A1-6 seocond round 469 bp (+) in lane 7, MAGE A1 377 bp (+) in lane 8, MAGE A2 523 bp (+) in lane 9, MAGE A3 569 bp (+) in lane 10, MAGE A4 580 bp (+) in lane 11, MAGE A5 478 bp (+) in lane 12, MAGE A8 419 bp (+) in lane 13, MAGE A9 407 bp (+) in lane 14, MAGE A10 464 bp (+) in lane15 (Fig. 3). **Fig 3 Before** Fig. 3. The PCR product from testicular tissue specimen. Marker (1), Negative control (2), GAPDH (3), MAGE A1-10 first (4) and second round (5), MAGE A1-6 first (6) and second round (7), MAGE A1 (8), MAGE A2 (9), MAGE A3 (10), MAGE A4 (11) (+), MAGE A5 (12), MAGE A8 (13), MAGE A9 (14), MAGE A10 (15), Marker (16)	6-7	Change in subtitle Fig 3. Fig. 3. The PCR product from testicular tissue specimen. Marker (lane 1), Negative control (lane 2), GAPDH (+) 320 bp (lane 3), MAGE A1-10 first Round 823 bp (-) (lane 4), MAGE A1-10 second round 461 bp (+) (lane 5), MAGE A1-6 first round 852 bp (+) (lane 6), MAGE A1-6 seocond round 469 bp (+) (lane 7), MAGE A1 377 bp (+) (lane 8), MAGE A2 523 bp (+) (lane 9), MAGE A3 569 bp (+) (lane 10), MAGE A4 580 bp (+) (lane 11), MAGE A5 478 bp (+) (lane 12), MAGE A8 419 bp (+) (lane 13), MAGE A9 407 bp (+) (lane 14), MAGE A10 464 bp (+) (lane15), Marker (lane 16).
The result of PCR for MAGE A1-10 showed positive 7/13 (53.8%) specimen for first round PCR and 12/13 (92.3%) specimen for second round PCR, MAGE A1-6 was 1/13 (7.7%) specimen for first round and 3/13 (23.1%) for second round. The GAPDH negative showed negative result for all MAGE A1-10. The result of individual MAGE A indicated that MAGE A1 was positive 2/13 for specimen no 8 and 9, MAGE A3 was positive 1/13 for specimen no 8. MAGE A5 was positive 12/13 for specimen no 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, and 15. MAGE A8 was positive 5/13 for specimen no 3, 4, 5, 6, and 9. MAGE A10 was positive 7/13 for specimen no 2, 3, 5, 6, 7, 9, and 15, while MAGE A2, A4, A6, and A9 were negative	7	The result of PCR for MAGE A1-10 showed positive 7/13 (53.8%) specimen for first round PCR and 12/13 (92.3%) specimen for second round PCR, MAGE A1-6 was 1/13 (7.7%) specimen for first round and 3/13 (23.1%) for second round. The GAPDH negative showed negative result for all MAGE A1-10. The result of individual MAGE A indicated that MAGE A1 was positive 2/13 for specimen no 8 and 9, MAGE A3 was positive 1/13 for specimen no 8. MAGE A5 was positive 12/13 for specimen no 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, and 15. MAGE A8 was

for all specimens.		positive 5/13 for specimen no 3, 4, 5, 6, and 9. MAGE A10 was positive 7/13 for specimen no 2, 3, 5, 6, 7, 9, and 15, while MAGE A2, A4, A6, and A9 were negative for all specimens (Table 3).
The objective of this study was to construct a novel universal primer to identify the expression of MAGE A1-10 which could bind to the cDNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 simultaneously by nested PCR. To avoid any genomic DNA contamination, mRNA was reversed into cDNA prior to nested PCR. In this study, the reverse transcription reaction mix with genomic DNA remover for removing the genomic DNA and reverse transcription. The first step was a genomic DNA degradation, and then it was continued by reverse trancription for synthesis cDNA. This study was performed by RT PCR amplification to evaluate the expression of MAGE A gene. The universal primer of MAGE A1-10 was designed based on the mRNA sequences of target gene by selecting the area between exon 1, 2, and 3 of MAGE-A gene. The variability areas of MAGE-A gene was at the promoters regions and first exon regions of MAGE A gene,23 in contrast, the high homology domains were exon 2 and exon 3. The exon 3 was the coding region that had similar sequences among all of MAGE A gene families A. Therefore, the forward primer was designed to bind to exon 2 joining with exon 3, and the reverse primer was designed	Discussion 8	I have deleted it in this section, and some of it, I have moved in methods The objective of this study was to construct a novel universal primer to identify the expression of MAGE A1-10 which could bind to the cDNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 simultaneously by nested PCR. The pair primers of MF10/MR10 and MF10/MR12 had already been evaluated by RT PCR using the testicular tissue resulted in a single target band. The PCR for the individual MAGE-A subtypes showed positive results for MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A8, MAGE-A9, MAGE-A10, but negative for MAGE-A6. The sequences of MAGE-A6 primers used in this study was the same as published before.[13] The negative result for MAGE-A6 might be due to the athropy testicular tissue which did not expressed MAGE-A6. As stated before, the testicular specimen was collected from the athrophy testis which had received orchidectomy theraphy.

to attach to exon 3. The primer MF10 as outer forward primer would bind to cDNA MAGE A1-10 at exon 2 joining with exon 3. For the second round, MF10 and MR12 were used as inner primer to verify that the PCR product would only amplify the cDNA of MAGE A1-10.		

MF10/MR10 and MF10/MR12 are pairs common/ universal primer for MAGE A1-10. MMRP 1/MMRP2 and MMRP3/MMRP4 are pairs common/universal primer for MAGE A1-6. Base on the bioinformatic study, the result of those primers is not a band with one size in base pair, but it is in a range, depending on MAGE A that express in the specimen.

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Original Article

The Novel Universal Primers to Identify the Expression of MAGE A1-A10 in the Core Biopsy of Lung Cancer

****Running Title** (short form of the main title presented on the top of pages): the novels primer to identify mRNA MAGE A1-10

** **Conflict of interests:** The authors declares that there is no conflict of interest.

Abstract

Background: Recent studies have reported that Melanoma antigen (MAGE) gene is expressed in variety of cancers and testicular tissue. The expression of MAGE-A genes could be used for biomarkers with high tumor specificity but the data is still missing for most solid tumors. The objective of this study is to construct the novel universal primers for detecting mRNA of MAGE A1-10 genes in lung cancer patients.

Methods: The specimen were a testicular tissue and 15 the core biopsy of lung cancer tissues from … General Academic Hospital … Indonesia. The universal primers was designed to bind to cDNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 regions, the assay was done by nested PCR, and continued by direct sequencing.

Results: The PCR using the universal primer MAGE A1-10 can detect at least one MAGE A mRNA of 10 subtype of MAGE A from testicular and lung cancer tissues. The sequences analysis of individual MAGE A1-10 shown the same homology with MAGE A from Genbank data. Among fifteen of lung cancer patients, 13/15 (86.67%) were tested positive for GAPDH and subsequently those were used for MAGE-A gene detection, while the negative for GAPDH were excluded. The PCR results showed 12/13 (92.31%) positive for MAGE A1-10 and 3/13 (23.08%) for MAGE A1-6.

Conclusion: This finding showed that the novel universal primer can be applied as a new tool for detecting the expression of MAGE A1-10 in cancer cells.

Keywords: MAGE A1-10, testicular tissue, universal primer, core biopsy, lung cancer

Introduction

Melanoma antigen (MAGE) gene is well known as cancer testis antigen (CTA) expressed in various tumors. Yet, it is silent in normal adult tissues, except the testis.[1,2] Based on the tissues-restricted expression and gene structure, the MAGE gene is classified into 2 different types. The first types are MAGE A, MAGE B, MAGE C restricted to male germ-line tissues and located in clusters on the X chromosome that Xq28, Xp21, and Xp26-27, respectively.[3] The second type is MAGE D expressed in body tissues and located in Xp11. MAGE D is expressed in normal tissues, whereas MAGE A, B, C are expressed in tumor cells.[4]

Type I MAGE expression, including MAGE A, has been detected in a broad variety of malignancies. There are 12 subtypes of MAGE A; MAGE A1, A2, A3, A4, A5, A6, A7 (pseudo gene), A8, A9, A10, A11, A12. The expression of each MAGE-A subtypes, A1-A12, was examined positive in oral squamous cell carcinoma and gastric cancer by immunohistochemically stained.[5,6] MAGE-A1 and MAGE-A3 mRNA was expressed in neuroblastoma tumors,[7] hepatocellular carcinoma,[8] benign and malignant neoplasms of the salivary glands.[9] The mRNA of MAGE-A1, -A3, -A4, -A10 were expressed in epithelial ovarian cancer (EOC).[10] MAGE A1 and A4 were detected in squamous cell vulvar neoplasms.[11] The expressions of MAGE A1-6 was detected in papillary thyroid carcinoma,[12] head and neck cancer tissues, breast cancer tissues, and lung cancer tissues.[13] MAGE A1 and A3 were expressed at primary non small cell lung

cancer (NSCLC).[14] Several *MAGE* subgroup genes, such as *MAGE-A5*, *-A7*, *-A8*, *-A9*, *-A11*, *-B3*, *-B4*, *-B10*, *-D2*, *-D3*, *-F1*, *-G1*, *-H1*, and *-L2*, have been first discovered to show differential expression in NSCLC.[15]

The high expression of subtype MAGE-A1 until A12 is detected in some malignancies.[8,11,16] The patients with MAGE-A gene expression has a poorer prognose than those with no MAGE-A expression.[5,6,10,17-20] It indicates that the identification of individual subtype of MAGE-A together can increase the value of diagnose and prognose for patient with cancer.

The expression of MAGE A1-6 in cancer tissues can be detected by Reverse Transcription Polymerase Chain Reaction (RT PCR) and nested PCR.[13,21] Other than MAGE A1-6, there are subtypes MAGE A8, A9, A10, that are also expressed in cancer tissues.[15,22] The detection of MAGE A1-10 gene expression in a single reaction of PCR can be more useful to improve the diagnose, therapy, and prognose of cancer. Therefore, we have decided to develop a novel universal primer in order to identify MAGE-A mRNA subtype A1-10.

This study designed a novel universal primer of MAGE A1-10 that could bind to the cDNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10, all together. The PCR results showed that by using the universal primer for MAGE A1-10 for nested PCR can be used to identify MAGE A1-10 gene in the core biopsy samples of lung cancer tissues.

Materials and Methods

1. Specimens

This experiment used 15 specimens from the core biopsy of lung tissues taken from patients with the lung cancer diagnosis at … General Academic Hospital … Indonesia in 2017. As for the positive control, a testicular tissue derived from a patient who received the orchidectomy theraphy was used. The study was approved by the Health Research Ethics Committee of … General Academic Hospital … Indonesia, number … and all subjects signed the informed consent form.

2. Design primer

The MAGE-A gene sequences were retrieved from Genbank data (National Centre for Biotechnology Information). Subtypes of the MAGE A gene are MAGE A1, A2, A3, A4, A5, A6, A8, A9, A10, A11, A12, while MAGE A7 is defined as a pseudo gene. MAGE A11 and A12 genes have mRNA sequences that have different homology with the sequence of the MAGE A1-10 gene. The design of the novel universal primer MAGE A1-10 in this study was carried out based on the mRNA sequences of the target gene by selecting the area between exon 1, 2, and 3 (Fig. 1). The expression of MAGE A1-10 was identified by nested PCR, using MF10/MR10 primers for the first round and MF10/MR12 primers for the second round (Table 1). The expression of MAGE A1-6 was identified by using MMRP1/MMRP1 primer for the first round, and MMRP3/MMRP4 primer for the second round.[13] The individual MAGE-A genes were detected by using MMRP3 as forward primer and reverse primer using M1, M2, M3, M4, M5, M6 with product MAGE A1 377 bp, MAGE A2 523 bp, MAGE A3 569 bp, MAGE A4 580 bp, MAGE A5 478 bp, MAGE A8 419 bp.[13] The identification of MAGE A8, A9 and A10 subtypes used MF10 as forward and as reverse used M7, M8, and M9. The GAPDH PCR was performed using GAPDH-F and GAPDH-R (Table 1).

3. RNA extraction and reverse trancription-PCR

RNA was extracted from testicular tissue and lung tissue from core biopsy using RNAeasy Plus Mini Kit (Qiagen, Germany). The procedures were conducted by following the instruction protocols from the manufacturer. Total RNA was stored at −20°C until further use. Reverse Transcription PCR (RT PCR) was performed using ReverTraAce® qPCR RT Master mix with gDNA remover (Toyobo, Japan). Total volume was 50 µl, content 25 µl of RNA template was kept in ice 65°C for 5 minutes for RNA denaturation, then 12 µl 4 × DN master mix (with genomic DNA remover) was added and 3 µl random primer 37°C for 5 minutes. It was directed for genomic DNA removal step. Finally, 10 µl 5 × RT master mix II was added for cDNA synthesis. The mix reaction was incubated at 37°C for 15 minutes, 50°C for 5 minutes. The reaction stopped at 98°C for 5 minutes. cDNA was stored at 4°C or −20°C until further use.

In this research, the DNA amplification reactions for MAGE A1-10, MAGE A1-6, and individual MAGE A1-10, performed by using GoTaq(R) Green Master Mix (Promega, USA). In the first stage, PCR was performed in total volume 20 µl which content was 10 µl Gotaq green master mix, 1 µl primer forward, 1 µl primer reverse, 5 µl nuclease free water, and 3 µl cDNA template. Primer concentration was 10 pmoles/µl. Amplification PCR condition was pre denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealling at 55°C for 45 seconds, and extention at 72°C for 45 seconds. The final extention was at 72°C for 7 minutes, then visualized on 2% gel electrophoresis. In the second round, PCR, 3 µl PCR product from the first round was used as template. For the second round PCR, the reaction and condition were the same as the first one, except for the primers and

templates. The PCR for housekeeping gene GAPDH was also performed for all samples at the same reaction and condition with PCR for MAGE A1-10 identification.

4. Sequences analysis

MF10/MR10 and MF10/MR12 primers were used to verify the result of PCR, and so were MMRP1/MMRP1 and MMRP3/MMRP4. The specimen from a testicular tissue was used as a template for PCR of individual MAGE A-10. It was then continued by direct sequencing to confirm the positive result of PCR, using the Genetic Analyzer (Applied Biosystems, USA). The sequences were compared with the sequences from Gene Bank data.

Results

1. Analysis of the MAGE A1-10 primers

The analysis indicated several results. These results showed that PCR using the MF10/MR10 primers for MAGE A1-10 indicated 823-919 base pair (bp), MF10/MR12 primers showed 461-557 bp, and MMRP1/MMRP1 primers for MAGE A1-6 showed 852 bp, and MMRP3/MMRP4 primers showed 469-490 bp (Fig. 2A). The dilution test of the PCR using the MF10/MR10 primers is shown in Fig. 2B, whereas MMRP1/MMRP2 is given in Fig. 2C. The total RNA concentration from testicular tissue was measured as 133,4 ng/µl. This was used as dilution test with a ratio of 1;10, 1:100, 1:1000.

The results of PCR using the primer of individual MAGE A1-10 for identifying the expression of MAGE A1, A2, A3, A4, A5, A8, A9, A10 from testicular tissue specimen can be seen in Fig. 3. These were used for direct sequencing. The products were GAPDH (+) 320 bp in lane 3, MAGE A1-10 first Round 823 bp (-) in lane 4,

MAGE A1-10 second round 461 bp (+) in lane 5, MAGE A1-6 first round 852 bp (+) in lane 6, MAGE A1-6 seocond round 469 bp (+) in lane 7, MAGE A1 377 bp (+) in lane 8, MAGE A2 523 bp (+) in lane 9, MAGE A3 569 bp (+) in lane 10, MAGE A4 580 bp (+) in lane 11, MAGE A5 478 bp (+) in lane 12, MAGE A8 419 bp (+) in lane 13, MAGE A9 407 bp (+) in lane 14, MAGE A10 464 bp (+) in lane15 (Fig. 3).

The homology analysis showed that all MAGE-A genes from the lung cancer samples had the same homology with MAGE-A from Genbank data with accession number as mention in Table 2. The result of one of the MAGE-A subtypes (MAGE-A3 gene) is depicted in Fig. 4.

2. Identification of MAGE A1-10 from the core biopsy of lung cancer tissues

The fifteen specimens from the core biopsy of lung cancer tissues were as small as a string with the length of approximately a half centimetre. These small specimens were used for PCR with GAPDH primers and the MAGE A1-10 primers. As illustrated in Fig. 5, GAPDH positive was 13/15 (86.7%) specimens which negative result was 2/15 specimen (13.3%). The specimens with negative for GAPDH, then was excluded from this study. The result of PCR for MAGE A1-10 showed positive 7/13 (53.8%) specimen for first round PCR and 12/13 (92.3%) specimen for second round PCR, MAGE A1-6 was 1/13 (7.7%) specimen for first round and 3/13 (23.1%) for second round. The GAPDH negative showed negative result for all MAGE A1-10. The result of individual MAGE A indicated that MAGE A1 was positive 2/13 for specimen no 8 and 9, MAGE A3 was positive 1/13 for specimen no 8. MAGE A5 was positive 12/13 for specimen no 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, and 15. MAGE A8 was positive 5/13 for specimen no 3, 4, 5, 6, and 9. MAGE A10 was positive 7/13 for specimen no 2, 3, 5, 6, 7, 9, and 15, while MAGE A2, A4, A6, and A9 were negative for all specimens.

Discussion

The objective of this study was to construct a novel universal primer to identify the expression of MAGE A1-10 which could bind to the cDNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 simultaneously by nested PCR. To avoid any genomic DNA contamination, mRNA was reversed into cDNA prior to nested PCR. In this study, the reverse transcription reaction mix with genomic DNA remover for removing the genomic DNA and reverse transcription. The first step was a genomic DNA degradation, and then it was continued by reverse trancription for synthesis cDNA.

This study was performed by RT PCR amplification to evaluate the expression of MAGE A gene. The universal primer of MAGE A1-10 was designed based on the mRNA sequences of target gene by selecting the area between exon 1, 2, and 3 of MAGE-A gene. The variability areas of MAGE-A gene was at the promoters regions and first exon regions of MAGE A gene,[23] in contrast, the high homology domains were exon 2 and exon 3. The exon 3 was the coding region that had similar sequences among all of MAGE A gene families A. Therefore, the forward primer was designed to bind to exon 2 joining with exon 3, and the reverse primer was designed to attach to exon 3. The primer MF10 as outer forward primer would bind to cDNA MAGE A1-10 at exon 2 joining with exon 3. For the second round, MF10 and MR12 were used as inner primer to verify that the PCR product would only amplify the cDNA of MAGE A1-10.

The pair primers of MF10/MR10 and MF10/MR12 had already been evaluated by RT PCR using the testicular tissue resulted in a single target band. The PCR for the individual MAGE-A subtypes showed positive results for MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A8, MAGE-A9, MAGE-A10, but

negative for MAGE-A6. The sequences of MAGE-A6 primers used in this study was the same as published before.[13] The negative result for MAGE-A6 might be due to the athropy testicular tissue which did not expressed MAGE-A6. As stated before, the testicular specimen was collected from the athrophy testis which had received orchidectomy theraphy.

The expression of MAGE A1-10 in this study showed positive at the lung tissue from patient with diagnozed lung cancer. As reported in previous studies, MAGE-A gene is often expressed in some cancer, such as oral squamous carcinoma,[5,24] gastric cancer,[6] renal cancer,[26] pappilary thyroid carcinoma,[12] lung cancer,[14,27] and NSCLC.[15] The previous result showed that MAGE-A1 expressed in hepatocelular carcinoma[28] and testicular tissue.[29] The expression of MAGE-A1 was evaluated by Reverse Trancripstion PCR.[30]

The expression of MAGE A1-10 and MAGE A1-6 genes in this study showed the co-expression with minimun for one specimen which was positive for the individual MAGE-A. Other research showed the similar trend that the expression of high MAGE-A co-expression occurred in some cancers. The high frequent of co-expression of multiple members of CT antigens was also reported in hepatocellular carcinoma,[8] in primary and recurrent of vulval tumours,[11] and in medulloblastoma.[16]

The patients with MAGE-A expression had a worse prognosis than those with no MAGE-A expression NSCLC.[18] Expression of MAGE-A1 or -A10 antigens in epithelial ovarian cancer (EOC) resulted in poor progression of free survival.[10] The expression of MAGE A3, -A4, -A5, -A9 and -A11 was significantly associated with lymph node metastasis MAGE A1,-A3, -A4, -A5, -A9 and -A11 was significantly associated with clinically advanced stages of disease and seemed to be of particular

interest of oral squamous cell carcinoma.[5] MAGE-A family was involved in gastric cancer progression that indicated poor prognosis of gastric cancer patients.[6] MAGE A1 expression might be a predictive marker of poor prognosis in gastric cancer.[19] MAGE-A gene expression in peripheral blood served as a poor prognostic marker for patients with lung cancer.[17] MAGE A1-6 expression of bone marrow with lung cancer patient correlated with poor survival rates. MAGE A1-6 positive patients showed poor overall survival and overall diseases free survival rates compared with MAGE A1-6 negative patient. MAGE A1-6 may be considered as a novel prognostic factor for lung cancer which leads to effective follow-up and treatment.[20] This indicated that expression of MAGE A could improve prognose of some cancer.

Conclussion

This study indicates that the novel universal primer could detect the expression of MAGE A1-10 in the core biopsy of lung cancer tissues which expressed at least one of ten subtypes MAGE-A mRNA. This universal primer might be applied as a new tool for detecting the expression of MAGE A1-10 in solid cancer cells.

Conflict of Interest

No conflict of interest is declared.

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Figures and Tables Legend

Table 1.

Table 1. The primer used for identification of the MAGE A1-10 mRNA

Gene Target	**Sequence of Primer (5'→3')**	**F/R**	**Amplicon Lenght (bp)**
GAPDH	GAPDH-F = TCG GAG TCA ACG GAT TTG GTC GTA	F R	320

	GAPDH-R = CAA ATG AGC CCC AGC CTT CTC CA		
MAGE A1-10 (outer)	MF10 = GAA GAY CTG CCW GTG GGT C MR10 = CTC CAG GTA STT YTC CTG CAC	F R	823-919
MAGE A1-10 (inner)	MF10 = GAA GAY CTG CCW GTG GGT C MR12 = CCA GYA TTT CTG CCT TTG TGA	F R	461-557
MAGE A8	M8 = CAC TTT CTC ATC AAG TGC TTC CC	R	419
MAGE A9	M9 = CCT TCA ATT TCA GTG CTT CTT GG	R	407
MAGE A10	M10 =TGG GTA AAG ACT CAC TGT CTG G	R	464

F = forward, R = reverse, bp = base pair

Table 2.

Table 2. Analysis of MAGE A1-10 subtypes with the sequences from GenBank

MAGE A Subtypes	**Homolog with accession number**
MAGE A1	NM_004988.4
MAGE A2	NM_001282501.1, NM_001282502.1, NM_001282504.1, NM_001282505.1, NM_005361.3, NM_175742.2, NM_175743.2, XM_011531159.1

MAGE A2B	NM_001321400.1, NM_001321401.1, NM_001321402.1, NM_001321403.1, NM_001321404.1, NM_153488.4, XM_006724814.2, XM_011531142.1, XM_011531141.1, XM_005277838.3, XM_005277839.2, XM_005277836.1, XM_011531143.1
MAGE A3	XM_005274676.2, XM_006724818.2, XM_011531160.1, XM_011531161.1
MAGE A4	NM_001011548.1, NM_001011549.1, NM_001011550.1, NM_002362.4, XM_005274677.2, XM_005274679.2, XM_005274678.3
MAGE A5	NM_021049.4
MAGE A6	NM_005363.3, NM_175868.2, XM_011531162.1
MAGE A8	NM_001166400.1, NM_001166401.1, NM_005364.4
MAGE A9	NM_005365.4, XM_005262335.2, XM_005262334.2
MAGE A9B	NM_001080790.1, XM_005278193.2, XM_005278192.2
MAGE A10	NM_001011543.2, NM_001251828.1, NM_021048.4

Fig. 1

Fig. 1. The primer position of MF10/MR10 and MF10/MR12 primers

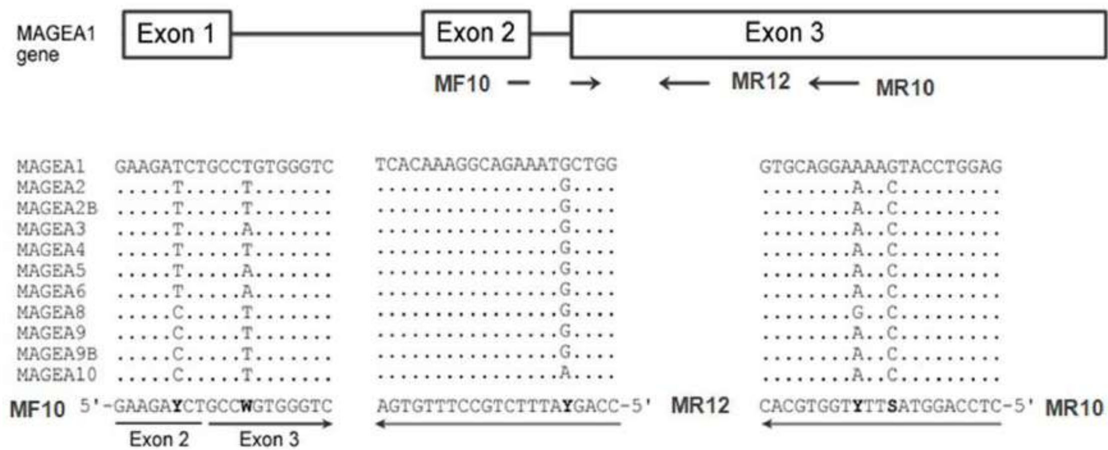

Fig. 2

Fig. 2. PCR product from testicular tissue tested using universal primer for MAGE A1-10 first (1) and second round (2) and MAGE A1-6 first (3) and second round (4)

(2A). Dilution 1:1 (1), 1:10 (2), 1:100 (3) and 1:000 (4) for detecting MAGE A1-10 (2B) compared with MAGE A1-6 (2C). Marker (M), Negative control (NC).

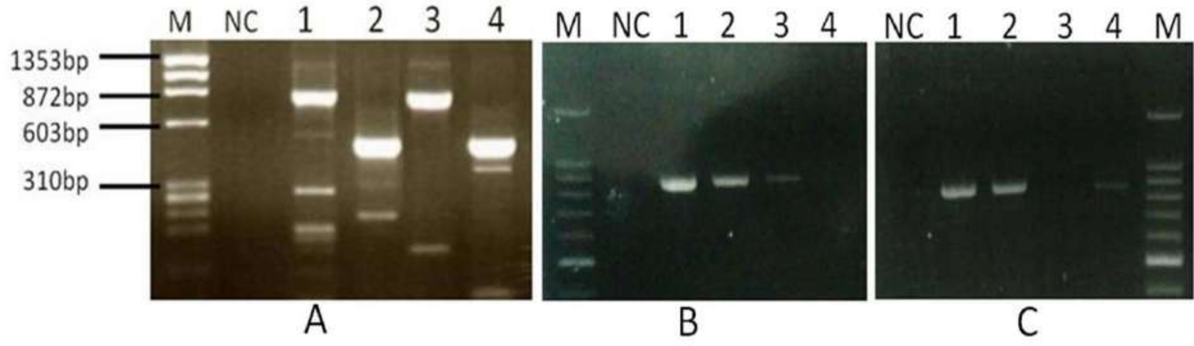

Fig. 3

Fig. 3. The PCR product from testicular tissue specimen. Marker (1), Negative control (2), GAPDH (3), MAGE A1-10 first (4) and second round (5), MAGE A1-6 first (6)

and second round (7), MAGE A1 (8), MAGE A2 (9), MAGE A3 (10), MAGE A4 (11) (+), MAGE A5 (12), MAGE A8 (13), MAGE A9 (14), MAGE A10 (15), Marker (16)

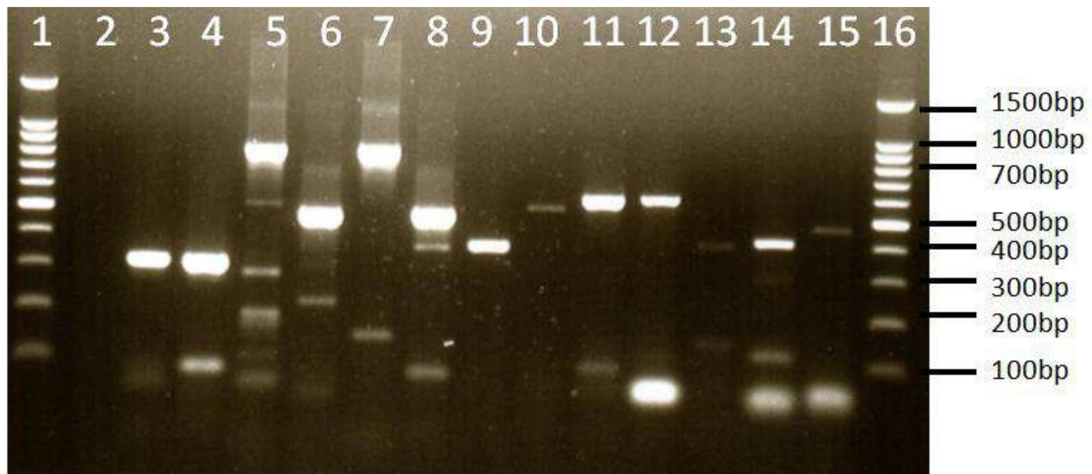

Fig. 4

Fig. 4. The alignment MAGE-A3 from testicular tissue with sequence from the Genbank data

```
XM_005274676-X1    1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC
XM_011531160-X2    1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC
XM_006724818-X3    1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC
MAGEA3-seq         1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC

XM_005274676-X1   61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT
XM_011531160-X2   61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT
XM_006724818-X3   61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT
MAGEA3-seq        61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT

XM_005274676-X1  121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA
XM_011531160-X2  121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA
XM_006724818-X3  121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA
MAGEA3-seq       121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA

XM_005274676-X1  181 CCAGATCCTCCCCAGAGTCCTCAGGGAGCCTCCAGCCTCCCCACTACCATGAACTACCCT
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XM_006724818-X3  181 CCAGATCCTCCCCAGAGTCCTCAGGGAGCCTCCAGCCTCCCCACTACCATGAACTACCCT
MAGEA3-seq       181 CCAGATCCTCCCCAGAGTCCTCAGGGAGCCTCCAGCCTCCCCACTACCATGAACTACCCT

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XM_006724818-X3  241 CTCTGGAGCCAATCCTATGAGGACTCCAGCAACCAAGAAGAGGAGGGGCCAAGCACCTTC
MAGEA3-seq       241 CTCTGGAGCCAATCCTATGAGGACTCCAGCAACCAAGAAGAGGAGGGGCCAAGCACCTTC

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XM_006724818-X3  301 CCTGACCTGGAGTCCGAGTTCCAAGCAGCACTCAGTAGGAAGGTGGCCGAGTTGGTTCAT
MAGEA3-seq       301 CCTGACCTGGAGTCCGAGTTCCAAGCAGCACTCAGTAGGAAGGTGGCCGAGTTGGTTCAT

XM_005274676-X1  361 TTTCTGCTCCTCAAGTATCGAGCCAGGGAGCCGGTCACAAAGGCAGAAATGCTGGGGAGT
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MAGEA3-seq       361 TTTCTGCTCCTCAAGTATCGAGCCAGGGAGCCGGTCACAAAGGCAGAAATGCTGGGGAGT

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XM_006724818-X3  481 CAGCTGGTCTT
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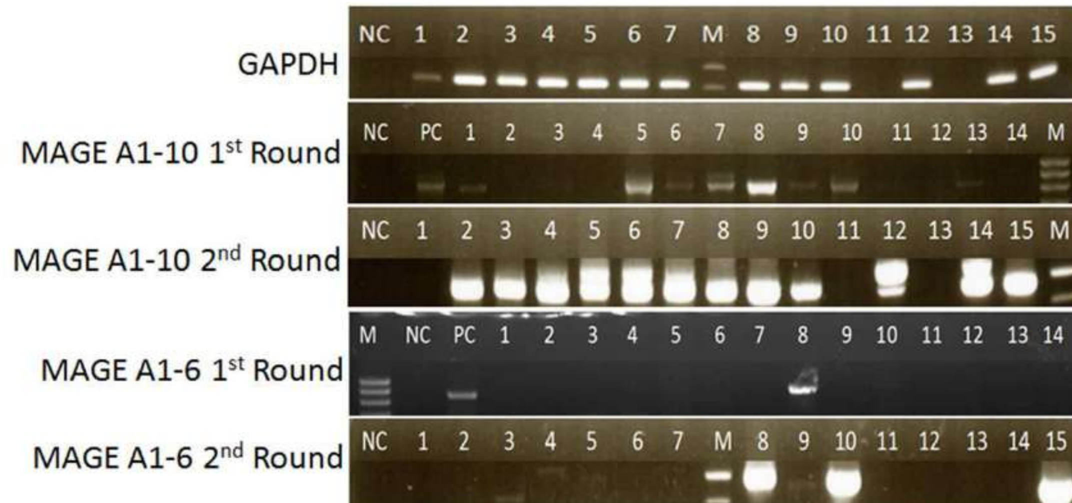
Fig. 5

Fig. 5. Analysis expression of GAPDH, MAGE A1-10, and MAGE A1-6 from the core biopsy of lung cancer patients. PC = positive controle for PCR with template total RNA from testicular tissue.



Menjawab komentar reviewer

Original Article

The Novel Universal Primers to Identify the Expression of MAGE A1-A10 in the Core Biopsy of Lung Cancer

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Abstract

Background: Recent studies have reported that Melanoma antigen (MAGE) gene is expressed in variety of cancers and testicular tissue. The expression of MAGE-A genes could be used for biomarkers with high tumor specificity but the data is still missing for most solid tumors. The objective of this study is to construct the novel universal primers for detecting mRNA of MAGE A1-10 genes in lung cancer patients.

Methods: The specimen were a testicular tissue and 15 the core biopsy of lung cancer tissues from Dr. Soetomo General Academic Hospital Surabaya Indonesia. The universal primers was designed to bind to mRNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 regions, the assay was done by nested PCR, and continued by direct sequencing.

Results: The PCR using the universal primer MAGE A1-10 can detect the MAGE A mRNA of 10 subtype of MAGE A from testicular and lung cancer tissues. The sequences analysis of individual MAGE A1-10 shown the same homology with MAGE A from Genbank data. Among fifteen of lung cancer patients, 13/15 (86.67%) were tested positive for GAPDH and subsequently those were used for MAGE-A gene detection, while the negative for GAPDH were excluded. The PCR results showed 12/13 (92.31%) positive for MAGE A1-10 and 3/13 (23.08%) for MAGE A1-6.

Conclusion: This finding showed that the novel universal primer can be applied as a new tool for detecting the expression of MAGE A1-10 in cancer cells.

Keywords: MAGE A1-10, testicular tissue, universal primer, core biopsy, lung cancer

Running Title: the novels primer to identify mRNA MAGE A1-10

Introduction

Melanoma antigen (MAGE) gene is well known as cancer testis antigen (CTA) expressed in various tumors. Based on the tissues-restricted expression and gene structure, the MAGE gene is classified into 2 different types. The first types are MAGE A, MAGE B, MAGE C restricted to male germ-line tissues and located in clusters on the X chromosome that Xq28, Xp21, and Xp26-27, respectively.[1] The second type is MAGE D expressed in body tissues and located in Xp11. MAGE D is expressed in normal tissues, whereas MAGE A, B, C are expressed in tumor cells.[2]

Type I MAGE, including MAGE A, the first known as gene that it is silent in normal adult tissues, except the testis.[3,4] Then the last study showed that the expression of MAGE A has been detected in a broad variety of malignancies. There are 12 subtypes of MAGE A; MAGE A1, A2, A3, A4, A5, A6, A7 (pseudo gene), A8, A9, A10, A11, A12. The expression of each MAGE-A subtypes, A1-A12, was examined positive in oral squamous cell carcinoma,[5] gastric cancer,[6] neuroblastoma tumors,[7] hepatocellular carcinoma,[8] benign and malignant neoplasms of the salivary glands,[9] epithelial ovarian cancer (EOC),[10] squamous cell vulvar neoplasms,[11] papillary thyroid carcinoma,[12] head and neck cancer tissues, breast cancer tissues, and lung cancer tissues.[13] MAGE A1 and A3 were expressed at primary non small cell lung cancer (NSCLC).[14] Several *MAGE* subgroup genes, such as MAGE A, B, D have been first discovered to show differential expression in NSCLC.[15]

The high expression of subtype MAGE-A1 until A12 is detected in some malignancies.[8,11,16] The patients with MAGE-A gene expression has a poorer

prognose than those with no MAGE-A expression.[5,6,10,17-20] It indicates that the identification of individual subtype of MAGE-A together can increase the value of diagnose and prognose for patient with cancer.

The expression of MAGE A1-6 in cancer tissues can be detected by Reverse Transcription Polymerase Chain Reaction (RT PCR) and nested PCR.[13,21] Other than MAGE A1-6, there are subtypes MAGE A8, A9, A10, that are also expressed in cancer tissues.[15,22] Therefore, we have decided to develop a novel universal primer in order to identify MAGE-A mRNA subtype A1-10. This study designed a novel universal primer of MAGE A1-10 that could bind to the mRNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10, all together. The PCR results showed that by using the universal primer for MAGE A1-10 for nested PCR can be used to identify MAGE A1-10 gene in the core biopsy samples of lung cancer tissues.

Materials and Methods

1. Specimens

This experiment used 15 specimens from the core biopsy of lung tissues taken from patients with the lung cancer diagnosis at Dr. Soetomo General Academic Hospital Surabaya Indonesia in 2017. As for the positive control, a testicular tissue derived from a patient who received the orchidectomy theraphy was used. The study was approved by the Health Research Ethics Committee of Dr. Soetomo General Academic Hospital Surabaya Indonesia, number 497/Panke.KKE/VII/2017 and all subjects signed the informed consent form.

2. Design primer

The MAGE-A gene sequences were retrieved from Genbank data (National Centre for Biotechnology Information). In this study, we design the novel universal primer MAGE A1-10 that carried out based on the mRNA sequences of the target gene by selecting the area between exon 1, 2, and 3 (Fig. 1). The variability areas of MAGE-A gene was at the promoters regions and first exon regions of MAGE A gene,[23] in contrast, the high homology domains were exon 2 and exon 3. The exon 3 was the coding region that had similar sequences among all of MAGE A gene families A. Therefore, the forward primer was designed to bind to exon 2 joining with exon 3, and the reverse primer was designed to attach to exon 3.

The expression of MAGE A1-10 all together was identified by nested PCR, using MF10/MR10 primers for the first round and MF10/MR12 primers for the second round (Table 1). The primer MF10 as outer forward primer would bind to cDNA MAGE A1-10 at exon 2 joining with exon 3. For the second round, MF10 and MR12 were used as inner primer to verify that the PCR product would only amplify the cDNA of MAGE A1-10. To compare this method with other study, we identified the expression of MAGE A1-6 by using their primers[13] that are MMRP1/MMRP1 primer for the first round and MMRP3/MMRP4 primer for the second round. The individual MAGE-A genes (from MAGE A1 until A10, separately) were detected by using MMRP3 as forward primer and reverse primer using M1, M2, M3, M4, M5, M6 with product MAGE A1 377 bp, MAGE A2 523 bp, MAGE A3 569 bp, MAGE A4 580 bp, MAGE A5 478 bp, MAGE A8 419 bp.[13] The identification of MAGE A8, A9 and A10 subtypes used MF10 as forward and as reverse used M8, M9, and M10. The GAPDH PCR was performed using GAPDH-F and GAPDH-R (Table 1).

3. RNA extraction and reverse trancription-PCR

RNA was extracted from testicular tissue and lung tissue from core biopsy using RNAeasy Plus Mini Kit (Qiagen, Germany). The procedures were conducted by following the instruction protocols from the manufacturer. Total RNA was stored at –20°C until further use. Reverse Transcription PCR (RT PCR) was performed using ReverTraAce® qPCR RT Master mix with gDNA remover (Toyobo, Japan).

Total volume was 50 μl, content 25 μl of RNA template was kept in ice 65°C for 5 minutes for RNA denaturation, then 12 μl 4 × DN master mix (with genomic DNA remover) was added and 3 μl random primer 37°C for 5 minutes. It was directed for genomic DNA removal step. Finally, 10 μl 5 × RT master mix II was added for cDNA synthesis. The mix reaction was incubated at 37°C for 15 minutes, 50°C for 5 minutes. The reaction stopped at 98°C for 5 minutes. cDNA was stored at 4°C or –20°C until further use.

In this research, PCR was performed by using GoTaq(R) Green Master Mix (Promega, USA). In the first stage, PCR was performed in total volume 20 μl which content was 10 μl Gotaq green master mix, 1 μl primer forward, 1 μl primer reverse, 5 μl nuclease free water, and 3 μl cDNA template. Primer concentration was 10 pmoles/μl. Amplification PCR condition was pre denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealling at 55°C for 45 seconds, and extention at 72°C for 45 seconds. The final extention was at 72°C for 7 minutes, then visualized on 2% gel electrophoresis. In the second round, PCR, 3 μl PCR product from the first round was used as template. For the second round PCR, the reaction and condition were the same as the first one, except for the primers and templates. The PCR for housekeeping gene GAPDH was also performed for all samples at the same reaction and condition with PCR for MAGE A1-10 identification.

4. Sequences analysis

To verify the PCR result of MAGE A1-10 and MAGE A1-6 all together, we performed PCR for individual subtype of MAGE A1-10, separately. It was then continued by direct sequencing to confirm the positive result of PCR, using the Genetic Analyzer (Applied Biosystems, USA). The sequences were compared with the sequences from Gene Bank data.

Results

1. Analysis of the MAGE A1-10 primers

The analysis indicated several results. These results showed that PCR using the MF10/MR10 primers for MAGE A1-10 indicated 823-919 base pair (bp), MF10/MR12 primers showed 461-557 bp, and MMRP1/MMRP1 primers for MAGE A1-6 showed 852 bp, and MMRP3/MMRP4 primers showed 469-490 bp (Fig. 2A). The dilution test of the PCR using the MF10/MR10 primers is shown in Fig. 2B, whereas MMRP1/MMRP2 is given in Fig. 2C. The total RNA concentration from testicular tissue was measured as 133,4 ng/µl. This was used as dilution test with a ratio of 1;10, 1:100, 1:1000.

The results of PCR using the primer of individual MAGE A1-10 for identifying the expression of MAGE A1, A2, A3, A4, A5, A8, A9, A10 from testicular tissue specimen can be seen in Fig. 3. These were used for direct sequencing.

The homology analysis showed that all MAGE-A genes from the lung cancer samples had the same homology with MAGE-A from Genbank data with accession number as mention in Table 2. The result of one of the MAGE-A subtypes (MAGE-A3 gene) is depicted in Fig. 4.

2. Identification of MAGE A1-10 from the core biopsy of lung cancer tissues

The fifteen specimens from the core biopsy of lung cancer tissues were as small as a string with the length of approximately a half centimetre. These small specimens were used for PCR with GAPDH primers and the MAGE A1-10 primers. As illustrated in Fig. 5, GAPDH positive was 13/15 (86.7%) specimens which negative result was 2/15 specimen (13.3%). The specimens with negative for GAPDH, then was excluded from this study. The result of PCR for MAGE A1-10 showed positive 7/13 (53.8%) specimen for first round PCR and 12/13 (92.3%) specimen for second round PCR, MAGE A1-6 was 1/13 (7.7%) specimen for first round and 3/13 (23.1%) for second round. The GAPDH negative showed negative result for all MAGE A1-10. The result of individual MAGE A indicated that MAGE A1 was positive 2/13 for specimen no 8 and 9, MAGE A3 was positive 1/13 for specimen no 8. MAGE A5 was positive 12/13 for specimen no 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, and 15. MAGE A8 was positive 5/13 for specimen no 3, 4, 5, 6, and 9. MAGE A10 was positive 7/13 for specimen no 2, 3, 5, 6, 7, 9, and 15, while MAGE A2, A4, A6, and A9 were negative for all specimens (Table 3).

Discussion

The objective of this study was to construct a novel universal primer to identify the expression of MAGE A1-10 which could bind to the cDNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 simultaneously by nested PCR. The pair primers of MF10/MR10 and MF10/MR12 had already been evaluated by RT PCR using the testicular tissue resulted in a single target band. The PCR for the individual MAGE-A subtypes showed positive results for MAGE-A1, MAGE-A2,

MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A8, MAGE-A9, MAGE-A10, but negative for MAGE-A6. The sequences of MAGE-A6 primers used in this study was the same as published before.[13] The negative result for MAGE-A6 might be due to the athropy testicular tissue which did not expressed MAGE-A6. As stated before, the testicular specimen was collected from the athrophy testis which had received orchidectomy theraphy.

The expression of MAGE A1-10 in this study showed positive at the lung tissue from patient with diagnozed lung cancer. As reported in previous studies, MAGE-A gene is often expressed in some cancer, such as oral squamous carcinoma,[5,24] gastric cancer,[6,25] renal cancer,[26] pappilary thyroid carcinoma,[12] lung cancer,[14,27] and NSCLC.[15] The previous result showed that MAGE-A1 expressed in hepatocelular carcinoma[28] and testicular tissue.[29] The expression of MAGE-A1 was evaluated by Reverse Trancripstion PCR.[30]

The expression of MAGE A1-10 and MAGE A1-6 genes in this study showed the co-expression with minimun for one specimen which was positive for the individual MAGE-A. Other research showed the similar trend that the expression of high MAGE-A co-expression occurred in some cancers. The high frequent of co-expression of multiple members of CT antigens was also reported in hepatocellular carcinoma,[8] in primary and recurrent of vulval tumours,[11] and in medulloblastoma.[16]

The patients with MAGE-A expression had a worse prognosis than those with no MAGE-A expression NSCLC.[18] Expression of MAGE-A1 or -A10 antigens in epithelial ovarian cancer (EOC) resulted in poor progression of free survival.[10] The expression of MAGE A3, -A4, -A5, -A9 and -A11 was significantly associated with lymph node metastasis MAGE A1,-A3, -A4, -A5, -A9 and -A11 was significantly

associated with clinically advanced stages of disease and seemed to be of particular interest of oral squamous cell carcinoma.[5] MAGE-A family was involved in gastric cancer progression that indicated poor prognosis of gastric cancer patients.[6] MAGE A1 expression might be a predictive marker of poor prognosis in gastric cancer.[19] MAGE-A gene expression in peripheral blood served as a poor prognostic marker for patients with lung cancer.[17] MAGE A1-6 expression of bone marrow with lung cancer patient correlated with poor survival rates. MAGE A1-6 positive patients showed poor overall survival and overall diseases free survival rates compared with MAGE A1-6 negative patient. MAGE A1-6 may be considered as a novel prognostic factor for lung cancer which leads to effective follow-up and treatment.[20] This indicated that expression of MAGE A could improve prognose of some cancer.

Conclussion

This study indicates that the novel universal primer could detect the expression of MAGE A1-10 in the core biopsy of lung cancer tissues which expressed at least one of ten subtypes MAGE-A mRNA. This universal primer might be applied as a new tool for detecting the expression of MAGE A1-10 in solid cancer cells.

Acknowledgment

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Conflict of Interest

No conflict of interest is declared.

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Figures and Tables Legend

Table 1.

Table 1. The primer used for identification of the MAGE A1-10 mRNA

Gene Target	**Sequence of Primer (5'→3')**	**F/R**	**Amplicon Lenght (bp)**
GAPDH	GAPDH-F = TCG GAG TCA ACG GAT TTG GTC GTA GAPDH-R = CAA ATG AGC CCC AGC CTT CTC CA	F R	320
MAGE A1-10 (outer)	MF10 = GAA GAY CTG CCW GTG GGT C MR10 = CTC CAG GTA STT YTC CTG CAC	F R	823-919
MAGE A1-10 (inner)	MF10 = GAA GAY CTG CCW GTG GGT C MR12 = CCA GYA TTT CTG CCT TTG TGA	F R	461-557
MAGE A8	M8 = CAC TTT CTC ATC AAG TGC TTC CC	R	419
MAGE A9	M9 = CCT TCA ATT TCA GTG CTT CTT GG	R	407
MAGE A10	M10 =TGG GTA AAG ACT CAC TGT CTG G	R	464

F = forward, R = reverse, bp = base pair

Table 2.

Table 2. Analysis of MAGE A1-10 subtypes with the sequences from GenBank

MAGE A Subtypes	Homolog with accession number
MAGE A1	NM_004988.4
MAGE A2	NM_001282501.1, NM_001282502.1, NM_001282504.1, NM_001282505.1, NM_005361.3, NM_175742.2, NM_175743.2, XM_011531159.1
MAGE A2B	NM_001321400.1, NM_001321401.1, NM_001321402.1, NM_001321403.1, NM_001321404.1, NM_153488.4, XM_006724814.2, XM_011531142.1, XM_011531141.1, XM_005277838.3, XM_005277839.2, XM_005277836.1, XM_011531143.1
MAGE A3	XM_005274676.2, XM_006724818.2, XM_011531160.1, XM_011531161.1
MAGE A4	NM_001011548.1, NM_001011549.1, NM_001011550.1, NM_002362.4, XM_005274677.2, XM_005274679.2, XM_005274678.3
MAGE A5	NM_021049.4
MAGE A6	NM_005363.3, NM_175868.2, XM_011531162.1
MAGE A8	NM_001166400.1, NM_001166401.1, NM_005364.4
MAGE A9	NM_005365.4, XM_005262335.2, XM_005262334.2
MAGE A9B	NM_001080790.1, XM_005278193.2, XM_005278192.2
MAGE A10	NM_001011543.2, NM_001251828.1, NM_021048.4

Table 3.

Table 3. Identification of MAGE A1-10 from the core biopsy of lung cancer tissues

No Sample	GAPDH	MAGE A1-10	MAGE A1-6	MAGE A1	MAGE A2	MAGE A3	MAGE A4	MAGE A5	MAGE A6	MAGE A8	MAGE A9	MAGE A10
1	+	–	–	–	–	–	–	–	–	–	–	–
2	+	–	–	–	–	–	–	+	–	–	–	+
3	+	–	–	–	–	–	–	+	–	+	–	+
4	+	–	–	–	–	–	–	+	–	+	–	–
5	+	–	–	–	–	–	–	+	–	+	–	+
6	+	–	–	–	–	–	–	+	–	+	–	+
7	+	–	–	–	–	–	–	+	–	–	–	+
8	+	–	+	+	–	+	–	+	–	–	–	–
9	+	–	–	–	–	–	–	+	–	+	–	+
10	+	–	+	+	–	–	–	+	–	–	–	–
11	–	×	×	×	×	×	×	×	×	×	×	×
12	+	+	–	–	–	–	–	+	–	–	–	–
13	–	×	×	×	×	×	×	×	×	×	×	×
14	+	+	–	–	–	–	–	+	–	–	–	–
15	+	+	+	–	–	–	–	+	–	–	–	+
Positive result	13/15 (86.7%)	12/13 (92.3%)	3/13 (23.1%)	2/13 (15.4%)	–	1/13 (33.3%)	–	12/13 (92.3%)	–	5/13 (38.5%)	–	7/13 (53.8%)

Note: + symbol for positive result, – symbol for negative result, × symbol for excluded sample

Fig. 1

Fig. 1. The primer position of MF10/MR10 and MF10/MR12 primers

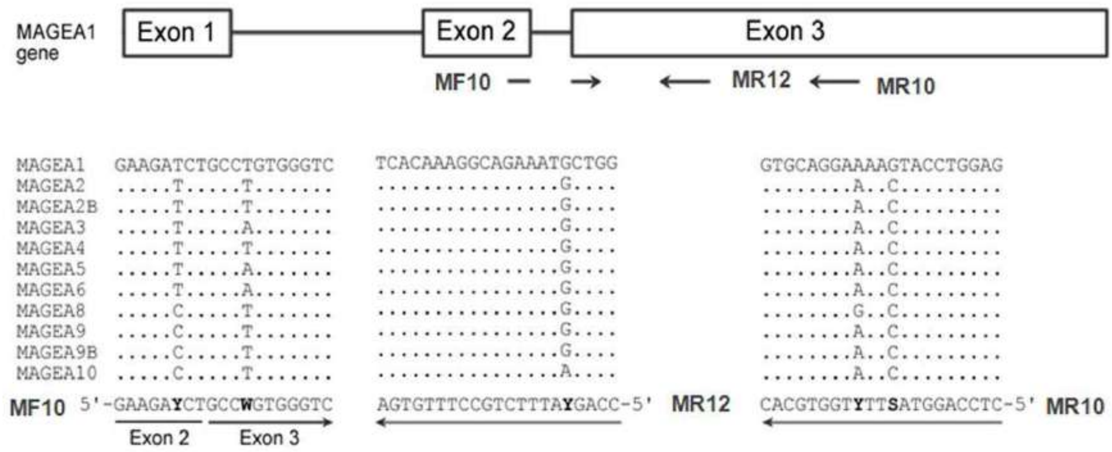

Fig. 2

Fig. 2. PCR product from testicular tissue tested using universal primer for MAGE A1-10 first (1) and second round (2) and MAGE A1-6 first (3) and second round (4) (2A). Dilution 1:1 (1), 1:10 (2), 1:100 (3) and 1:000 (4) for detecting MAGE A1-10 (2B) compared with MAGE A1-6 (2C). Marker (M), Negative control (NC).

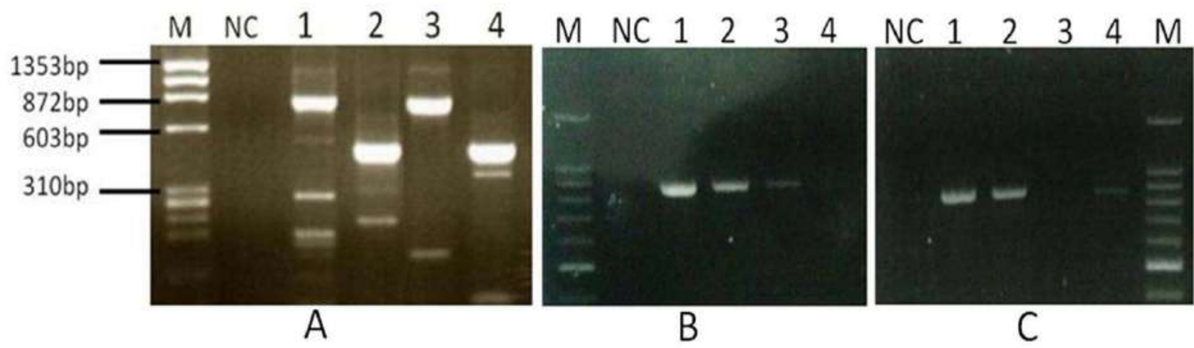

Fig. 3

Fig. 3. The PCR product from testicular tissue specimen. Marker (lane 1), Negative control (lane 2), GAPDH (+) 320 bp (lane 3), MAGE A1-10 first Round 823 bp (-) (lane 4), MAGE A1-10 second round 461 bp (+) (lane 5), MAGE A1-6 first round 852 bp (+) (lane 6), MAGE A1-6 seocond round 469 bp (+) (lane 7), MAGE A1 377 bp (+) (lane 8), MAGE A2 523 bp (+) (lane 9), MAGE A3 569 bp (+) (lane 10), MAGE A4 580 bp (+) (lane 11), MAGE A5 478 bp (+) (lane 12), MAGE A8 419 bp (+) (lane 13), MAGE A9 407 bp (+) (lane 14), MAGE A10 464 bp (+) (lane15), Marker (lane 16).

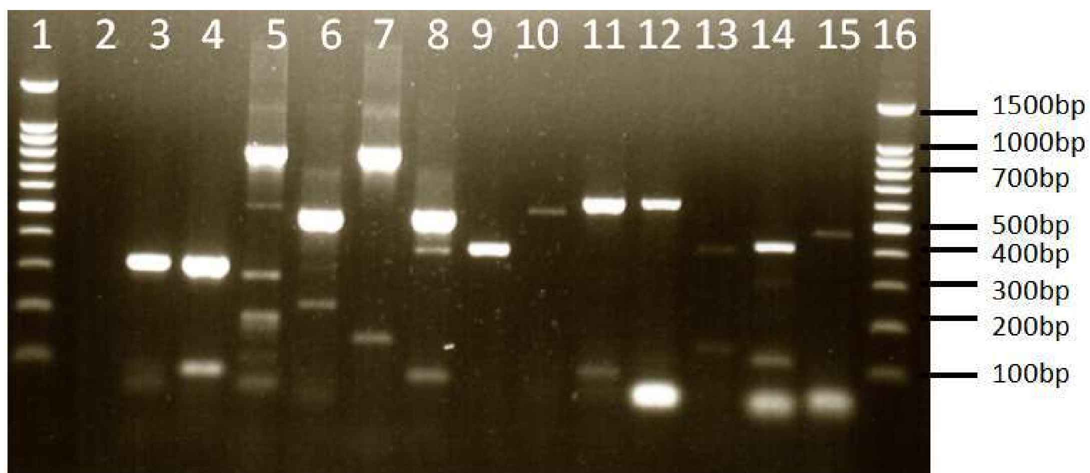

Fig. 4

Fig. 4. The alignment MAGE-A3 from testicular tissue with sequence from the Genbank data

```
XM_005274676-X1    1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC
XM_011531160-X2    1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC
XM_006724818-X3    1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC
MAGEA3-seq         1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC

XM_005274676-X1   61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT
XM_011531160-X2   61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT
XM_006724818-X3   61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT
MAGEA3-seq        61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT

XM_005274676-X1  121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA
XM_011531160-X2  121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA
XM_006724818-X3  121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA
MAGEA3-seq       121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA

XM_005274676-X1  181 CCAGATCCTCCCCAGAGTCCTCAGGGAGCCTCCAGCCTCCCCACTACCATGAACTACCCT
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XM_006724818-X3  241 CTCTGGAGCCAATCCTATGAGGACTCCAGCAACCAAGAAGAGGAGGGGCCAAGCACCTTC
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XM_005274676-X1  301 CCTGACCTGGAGTCCGAGTTCCAAGCAGCACTCAGTAGGAAGGTGGCCGAGTTGGTTCAT
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XM_006724818-X3  301 CCTGACCTGGAGTCCGAGTTCCAAGCAGCACTCAGTAGGAAGGTGGCCGAGTTGGTTCAT
MAGEA3-seq       301 CCTGACCTGGAGTCCGAGTTCCAAGCAGCACTCAGTAGGAAGGTGGCCGAGTTGGTTCAT

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XM_006724818-X3  361 TTTCTGCTCCTCAAGTATCGAGCCAGGGAGCCGGTCACAAAGGCAGAAATGCTGGGGAGT
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XM_005274676-X1  421 GTCGTCGGAAATTGGCAGTATTTCTTTCCTGTGATCTTCAGCAAAGCTTCCAGTTCCTTG
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MAGEA3-seq       421 GTCGTCGGAAATTGGCAGTATTTCTTTCCTGTGATCTTCAGCAAAGCTTCCAGTTCCTTG

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XM_011531160-X2  481 CAGCTGGTCTT
XM_006724818-X3  481 CAGCTGGTCTT
MAGEA3-seq       481 CAGCTGGTCTT
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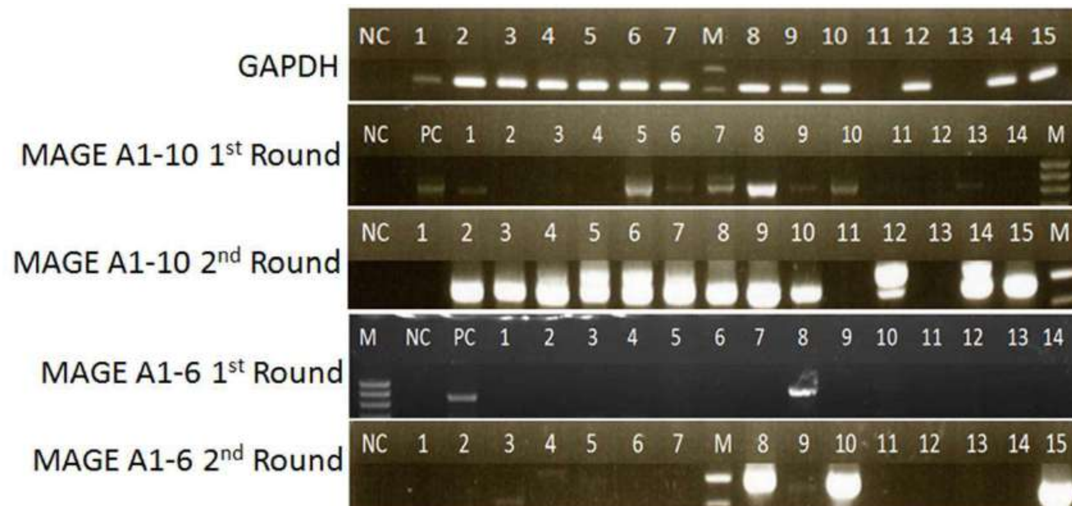
Fig. 5

Fig. 5. Analysis expression of GAPDH, MAGE A1-10, and MAGE A1-6 from the core biopsy of lung cancer patients. PC = positive controle for PCR with template total RNA from testicular tissue.



Highlight check sebelum published

Original Article

Running Title: the novels primer to identify mRNA MAGE A1-10

The Novel Universal Primers to Identify the Expression of MAGE A1-A10 in the Core Biopsy of Lung Cancer

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Abstract

Background: Recent studies have reported that Melanoma antigen (MAGE) gene is expressed in variety of cancers and testicular tissue. The expression of MAGE-A genes could be used for biomarkers with high tumor specificity but the data is still missing for most solid tumors. The objective of this study is to construct the novel universal primers for detecting mRNA of MAGE A1-10 genes in lung cancer patients.

Methods: This was a cross sectional study conducted at Dr. Soetomo General Academic Hospital Surabaya Indonesia in 2017. The specimen were a testicular tissue and 15 the core biopsy of lung cancer tissues. The universal primers was designed to bind to mRNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 regions, the assay was done by nested PCR, and continued by direct sequencing.

Results: The PCR using the universal primer MAGE A1-10 can detect the MAGE A mRNA of 10 subtype of MAGE A from testicular and lung cancer tissues. The sequences analysis of individual MAGE A1-10 shown the same homology with MAGE A from Genbank data. Among fifteen of lung cancer patients, 13/15 (86.67%) were tested positive for GAPDH and subsequently those were used for MAGE-A gene detection, while the negative for GAPDH were excluded. The PCR results

showed 12/13 (92.31%) positive for MAGE A1-10 and 3/13 (23.08%) for MAGE A1-6.

Conclusion: This finding showed that the novel universal primer can be applied as a new tool for detecting the expression of MAGE A1-10 in cancer cells.

Keywords: MAGE A1-10, testicular tissue, universal primer, core biopsy, lung cancer

Introduction

Melanoma antigen (MAGE) gene is well known as cancer testis antigen (CTA) expressed in various tumors. Based on the tissues-restricted expression and gene structure, the MAGE gene is classified into 2 different types. The first types are MAGE A, MAGE B, MAGE C restricted to male germ-line tissues and located in clusters on the X chromosome that Xq28, Xp21, and Xp26-27, respectively.[1] The second type is MAGE D expressed in body tissues and located in Xp11. MAGE D is expressed in normal tissues, whereas MAGE A, B, C are expressed in tumor cells.[2]

Type I MAGE, including MAGE A, the first known as gene that it is silent in normal adult tissues, except the testis.[3,4] Then the last study showed that the expression of MAGE A has been detected in a broad variety of malignancies. There are 12 subtypes of MAGE A; MAGE A1, A2, A3, A4, A5, A6, A7 (pseudo gene), A8, A9, A10, A11, A12. The expression of each MAGE-A subtypes, A1-A12, was examined positive in oral squamous cell carcinoma,[5] gastric cancer,[6] neuroblastoma tumors,[7] hepatocellular carcinoma,[8] benign and malignant neoplasms of the salivary glands,[9] epithelial ovarian cancer (EOC),[10] squamous cell vulvar neoplasms,[11] papillary thyroid carcinoma,[12] head and neck cancer tissues, breast cancer tissues, and lung cancer tissues.[13] MAGE A1 and A3 were expressed at primary non small cell

lung cancer (NSCLC).[14] Several *MAGE* subgroup genes, such as MAGE A, B, D have been first discovered to show differential expression in NSCLC.[15]

The high expression of subtype MAGE-A1 until A12 is detected in some malignancies.[8,11,16] The patients with MAGE-A gene expression has a poorer prognose than those with no MAGE-A expression.[5,6,10,17-20] It indicates that the identification of individual subtype of MAGE-A together can increase the value of diagnose and prognose for patient with cancer.

The expression of MAGE A1-6 in cancer tissues can be detected by Reverse Transcription Polymerase Chain Reaction (RT PCR) and nested PCR.[13,21] Other than MAGE A1-6, there are subtypes MAGE A8, A9, A10, that are also expressed in cancer tissues.[15,22] Therefore, we have decided to develop a novel universal primer in order to identify MAGE-A mRNA subtype A1-10. This study designed a novel universal primer of MAGE A1-10 that could bind to the mRNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10, all together. The PCR results showed that by using the universal primer for MAGE A1-10 for nested PCR can be used to identify MAGE A1-10 gene in the core biopsy samples of lung cancer tissues.

Materials and Methods

Specimens

This was a cross sectional study used 15 specimens from the core biopsy of lung tissues taken from patients with the lung cancer diagnosis at Dr. Soetomo General Academic Hospital Surabaya Indonesia in 2017. As for the positive control, a testicular tissue derived from a patient who received the orchidectomy theraphy was used. The study was approved by the Health Research Ethics Committee of Dr.

Soetomo General Academic Hospital Surabaya Indonesia, number 497/Panke.KKE/VII/2017 and all subjects signed the informed consent form.

Design primer

The MAGE-A gene sequences were retrieved from Genbank data (National Centre for Biotechnology Information). In this study, we design the novel universal primer MAGE A1-10 that carried out based on the mRNA sequences of the target gene by selecting the area between exon 1, 2, and 3 (Figure 1). The variability areas of MAGE-A gene was at the promoters regions and first exon regions of MAGE A gene,[23] in contrast, the high homology domains were exon 2 and exon 3. The exon 3 was the coding region that had similar sequences among all of MAGE A gene families A. Therefore, the forward primer was designed to bind to exon 2 joining with exon 3, and the reverse primer was designed to attach to exon 3.

The expression of MAGE A1-10 all together was identified by nested PCR, using MF10/MR10 primers for the first round and MF10/MR12 primers for the second round (Table 1). The primer MF10 as outer forward primer would bind to cDNA MAGE A1-10 at exon 2 joining with exon 3. For the second round, MF10 and MR12 were used as inner primer to verify that the PCR product would only amplify the cDNA of MAGE A1-10. To compare this method with other study, we identified the expression of MAGE A1-6 by using their primers[13] that are MMRP1/MMRP1 primer for the first round and MMRP3/MMRP4 primer for the second round. The individual MAGE-A genes (from MAGE A1 until A10, separately) were detected by using MMRP3 as forward primer and reverse primer using M1, M2, M3, M4, M5, M6 with product MAGE A1 377 bp, MAGE A2 523 bp, MAGE A3 569 bp, MAGE A4 580 bp, MAGE A5 478 bp, MAGE A8 419 bp.[13] The identification of MAGE A8,

A9 and A10 subtypes used MF10 as forward and as reverse used M8, M9, and M10. The GAPDH PCR was performed using GAPDH-F and GAPDH-R (Table 1).

RNA extraction and reverse trancription-PCR

RNA was extracted from testicular tissue and lung tissue from core biopsy using RNAeasy Plus Mini Kit (Qiagen, Germany). The procedures were conducted by following the instruction protocols from the manufacturer. Total RNA was stored at –20°C until further use. Reverse Transcription PCR (RT PCR) was performed using ReverTraAce® qPCR RT Master mix with gDNA remover (Toyobo, Japan).

Total volume was 50 μl, content 25 μl of RNA template was kept in ice 65°C for 5 minutes for RNA denaturation, then 12 μl 4 × DN master mix (with genomic DNA remover) was added and 3 μl random primer 37°C for 5 minutes. It was directed for genomic DNA removal step. Finally, 10 μl 5 × RT master mix II was added for cDNA synthesis. The mix reaction was incubated at 37°C for 15 minutes, 50°C for 5 minutes. The reaction stopped at 98°C for 5 minutes. cDNA was stored at 4°C or –20°C until further use.

In this research, PCR was performed by using GoTaq(R) Green Master Mix (Promega, USA). In the first stage, PCR was performed in total volume 20 μl which content was 10 μl Gotaq green master mix, 1 μl primer forward, 1 μl primer reverse, 5 μl nuclease free water, and 3 μl cDNA template. Primer concentration was 10 pmoles/μl. Amplification PCR condition was pre denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealling at 55°C for 45 seconds, and extention at 72°C for 45 seconds. The final extention was at 72°C for 7 minutes, then visualized on 2% gel electrophoresis. In the second round, PCR, 3

μl PCR product from the first round was used as template. For the second round PCR, the reaction and condition were the same as the first one, except for the primers and templates. The PCR for housekeeping gene GAPDH was also performed for all samples at the same reaction and condition with PCR for MAGE A1-10 identification.

Sequences analysis

To verify the PCR result of MAGE A1-10 and MAGE A1-6 all together, we performed PCR for individual subtype of MAGE A1-10, separately. It was then continued by direct sequencing to confirm the positive result of PCR, using the Genetic Analyzer (Applied Biosystems, USA). The sequences were compared with the sequences from Gene Bank data.

Results

Analysis of the MAGE A1-10 primers

The analysis indicated several results. These results showed that PCR using the MF10/MR10 primers for MAGE A1-10 indicated 823-919 base pair (bp), MF10/MR12 primers showed 461-557 bp, and MMRP1/MMRP1 primers for MAGE A1-6 showed 852 bp, and MMRP3/MMRP4 primers showed 469-490 bp (Figure 2A). The dilution test of the PCR using the MF10/MR10 primers is shown in figure 2B, whereas MMRP1/MMRP2 is given in figure 2C. The total RNA concentration from testicular tissue was measured as 133,4 ng/μl. This was used as dilution test with a ratio of 1;10, 1:100, 1:1000.

The results of PCR using the primer of individual MAGE A1-10 for identifying the expression of MAGE A1, A2, A3, A4, A5, A8, A9, A10 from testicular tissue specimen can be seen in figure 3. These were used for direct sequencing.

The homology analysis showed that all MAGE-A genes from the lung cancer samples had the same homology with MAGE-A from Genbank data with accession

number as mention in Table 2. The result of one of the MAGE-A subtypes (MAGE-A3 gene) is depicted in figure 4.

Identification of MAGE A1-10 from the core biopsy of lung cancer tissues

The fifteen specimens from the core biopsy of lung cancer tissues were as small as a string with the length of approximately a half centimetre. These small specimens were used for PCR with GAPDH primers and the MAGE A1-10 primers. As illustrated in figure 5, GAPDH positive was 13/15 (86.7%) specimens which negative result was 2/15 specimen (13.3%). The specimens with negative for GAPDH, then was excluded from this study. The result of PCR for MAGE A1-10 showed positive 7/13 (53.8%) specimen for first round PCR and 12/13 (92.3%) specimen for second round PCR, MAGE A1-6 was 1/13 (7.7%) specimen for first round and 3/13 (23.1%) for second round. The GAPDH negative showed negative result for all MAGE A1-10. The result of individual MAGE A indicated that MAGE A1 was positive 2/13 for specimen no 8 and 9, MAGE A3 was positive 1/13 for specimen no 8. MAGE A5 was positive 12/13 for specimen no 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, and 15. MAGE A8 was positive 5/13 for specimen no 3, 4, 5, 6, and 9. MAGE A10 was positive 7/13 for specimen no 2, 3, 5, 6, 7, 9, and 15, while MAGE A2, A4, A6, and A9 were negative for all specimens (Table 3).

Discussion

The objective of this study was to construct a novel universal primer to identify the expression of MAGE A1-10 which could bind to the cDNA of MAGE A1, A2, A2B, A3, A4, A5, A6, A8, A9, A9B, and A10 simultaneously by nested PCR. The pair primers of MF10/MR10 and MF10/MR12 had already been evaluated by RT PCR using the testicular tissue resulted in a single target band. The PCR for the

individual MAGE-A subtypes showed positive results for MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A8, MAGE-A9, MAGE-A10, but negative for MAGE-A6. The sequences of MAGE-A6 primers used in this study was the same as published before.[13] The negative result for MAGE-A6 might be due to the athropy testicular tissue which did not expressed MAGE-A6. As stated before, the testicular specimen was collected from the athrophy testis which had received orchidectomy theraphy.

The expression of MAGE A1-10 in this study showed positive at the lung tissue from patient with diagnozed lung cancer. As reported in previous studies, MAGE-A gene is often expressed in some cancer, such as oral squamous carcinoma,[5,24] gastric cancer,[6,25] renal cancer,[26] pappilary thyroid carcinoma,[12] lung cancer,[14,27] and NSCLC.[15] The previous result showed that MAGE-A1 expressed in hepatocelular carcinoma[28] and testicular tissue.[29] The expression of MAGE-A1 was evaluated by Reverse Trancripstion PCR.[30]

The expression of MAGE A1-10 and MAGE A1-6 genes in this study showed the co-expression with minimun for one specimen which was positive for the individual MAGE-A. Other research showed the similar trend that the expression of high MAGE-A co-expression occurred in some cancers. The high frequent of co-expression of multiple members of CT antigens was also reported in hepatocellular carcinoma,[8] in primary and recurrent of vulval tumours,[11] and in medulloblastoma.[16]

The patients with MAGE-A expression had a worse prognosis than those with no MAGE-A expression NSCLC.[18] Expression of MAGE-A1 or -A10 antigens in epithelial ovarian cancer (EOC) resulted in poor progression of free survival.[10] The expression of MAGE A3, -A4, -A5, -A9 and -A11 was significantly associated with

lymph node metastasis MAGE A1,-A3, -A4, -A5, -A9 and -A11 was significantly associated with clinically advanced stages of disease and seemed to be of particular interest of oral squamous cell carcinoma.[5] MAGE-A family was involved in gastric cancer progression that indicated poor prognosis of gastric cancer patients.[6] MAGE A1 expression might be a predictive marker of poor prognosis in gastric cancer.[19] MAGE-A gene expression in peripheral blood served as a poor prognostic marker for patients with lung cancer.[17] MAGE A1-6 expression of bone marrow with lung cancer patient correlated with poor survival rates. MAGE A1-6 positive patients showed poor overall survival and overall diseases free survival rates compared with MAGE A1-6 negative patient. MAGE A1-6 may be considered as a novel prognostic factor for lung cancer which leads to effective follow-up and treatment.[20] This indicated that expression of MAGE A could improve prognose of some cancer.

Conclussion

This study indicates that the novel universal primer could detect the expression of MAGE A1-10 in the core biopsy of lung cancer tissues which expressed at least one of ten subtypes MAGE-A mRNA. This universal primer might be applied as a new tool for detecting the expression of MAGE A1-10 in solid cancer cells.

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Conflict of Interest

None declared.

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Table 1. The primer used for identification of the MAGE A1-10 mRNA

Gene Target	**Sequence of Primer (5'→3')**	**F/R**	**Amplicon Lenght (bp)**
GAPDH	GAPDH-F = TCG GAG TCA ACG GAT TTG GTC GTA GAPDH-R = CAA ATG AGC CCC AGC CTT CTC CA	F R	320
MAGE A1-10 (outer)	MF10 = GAA GAY CTG CCW GTG GGT C MR10 = CTC CAG GTA STT YTC CTG CAC	F R	823-919
MAGE A1-10 (inner)	MF10 = GAA GAY CTG CCW GTG GGT C MR12 = CCA GYA TTT CTG CCT TTG TGA	F R	461-557
MAGE A8	M8 = CAC TTT CTC ATC AAG TGC TTC CC	R	419
MAGE A9	M9 = CCT TCA ATT TCA GTG CTT CTT GG	R	407
MAGE A10	M10 =TGG GTA AAG ACT CAC TGT CTG G	R	464

F = forward, R = reverse, bp = base pair

Table 2. Analysis of MAGE A1-10 subtypes with the sequences from GenBank

MAGE A Subtypes	Homolog with accession number
MAGE A1	NM_004988.4
MAGE A2	NM_001282501.1, NM_001282502.1, NM_001282504.1, NM_001282505.1, NM_005361.3, NM_175742.2, NM_175743.2, XM_011531159.1
MAGE A2B	NM_001321400.1, NM_001321401.1, NM_001321402.1, NM_001321403.1, NM_001321404.1, NM_153488.4, XM_006724814.2, XM_011531142.1, XM_011531141.1, XM_005277838.3, XM_005277839.2, XM_005277836.1, XM_011531143.1
MAGE A3	XM_005274676.2, XM_006724818.2, XM_011531160.1, XM_011531161.1
MAGE A4	NM_001011548.1, NM_001011549.1, NM_001011550.1, NM_002362.4, XM_005274677.2, XM_005274679.2, XM_005274678.3
MAGE A5	NM_021049.4
MAGE A6	NM_005363.3, NM_175868.2, XM_011531162.1
MAGE A8	NM_001166400.1, NM_001166401.1, NM_005364.4
MAGE A9	NM_005365.4, XM_005262335.2, XM_005262334.2
MAGE A9B	NM_001080790.1, XM_005278193.2, XM_005278192.2
MAGE A10	NM_001011543.2, NM_001251828.1, NM_021048.4

Table 3. Identification of MAGE A1-10 from the core biopsy of lung cancer tissues

No Sample	GAPDH	MAGE A1-10	MAGE A1-6	MAGE A1	MAGE A2	MAGE A3	MAGE A4	MAGE A5	MAGE A6	MAGE A8	MAGE A9	MAGE A10
1	+	–	–	–	–	–	–	–	–	–	–	–
2	+	–	–	–	–	–	–	+	–	–	–	+
3	+	–	–	–	–	–	–	+	–	+	–	+
4	+	–	–	–	–	–	–	+	–	+	–	–
5	+	–	–	–	–	–	–	+	–	+	–	+
6	+	–	–	–	–	–	–	+	–	+	–	+
7	+	–	–	–	–	–	–	+	–	–	–	+
8	+	–	+	+	–	+	–	+	–	–	–	–
9	+	–	–	–	–	–	–	+	–	+	–	+
10	+	–	+	+	–	–	–	+	–	–	–	–
11	–	×	×	×	×	×	×	×	×	×	×	×
12	+	+	–	–	–	–	–	+	–	–	–	–
13	–	×	×	×	×	×	×	×	×	×	×	×
14	+	+	–	–	–	–	–	+	–	–	–	–
15	+	+	+	–	–	–	–	+	–	–	–	+
Positive result	13/15 (86.7%)	12/13 (92.3%)	3/13 (23.1%)	2/13 (15.4%)	–	1/13 (33.3%)	–	12/13 (92.3%)	–	5/13 (38.5%)	–	7/13 (53.8%)

Note: + symbol for positive result, – symbol for negative result, × symbol for excluded sample

Figure 1. The position of MF10/MR10 and MF10/MR12 primers base on the sequences of MAGE A1-10 gene

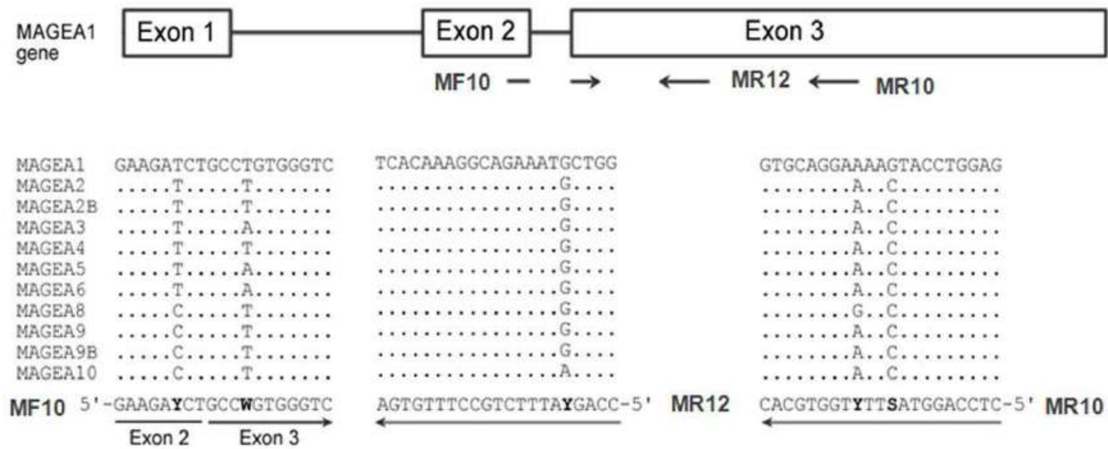

Figure 2. PCR product from testicular tissue tested using universal primer for MAGE A1-10 first (1) and second round (2) and MAGE A1-6 first (3) and second round (4) (2A). Dilution 1:1 (1), 1:10 (2), 1:100 (3) and 1:000 (4) for detecting MAGE A1-10 (2B) compared with MAGE A1-6 (2C). Marker (M), Negative control (NC).

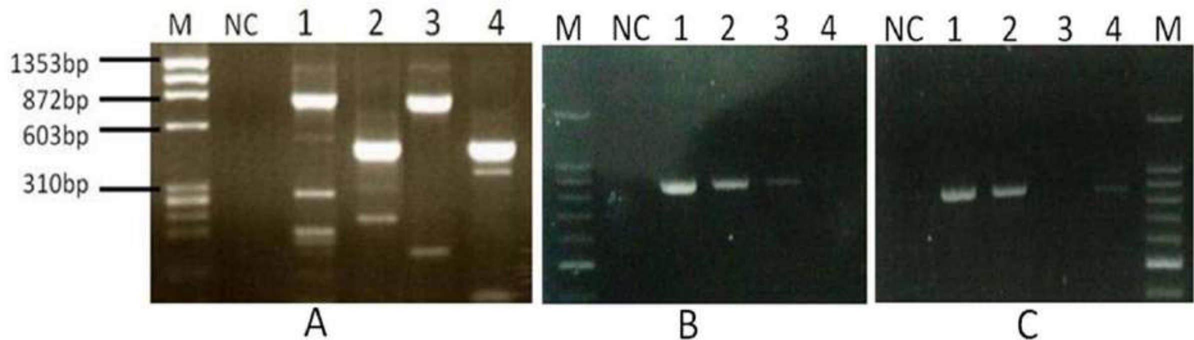

Figure 3. The PCR product from testicular tissue specimen. Marker (lane 1), Negative control (lane 2), GAPDH (+) 320 bp (lane 3), MAGE A1-10 first Round 823 bp (-) (lane 4), MAGE A1-10 second round 461 bp (+) (lane 5), MAGE A1-6 first round 852 bp (+) (lane 6), MAGE A1-6 seocond round 469 bp (+) (lane 7), MAGE A1 377 bp (+) (lane 8), MAGE A2 523 bp (+) (lane 9), MAGE A3 569 bp (+) (lane 10), MAGE A4 580 bp (+) (lane 11), MAGE A5 478 bp (+) (lane 12), MAGE A8 419 bp (+) (lane 13), MAGE A9 407 bp (+) (lane 14), MAGE A10 464 bp (+) (lane15), Marker (lane 16).

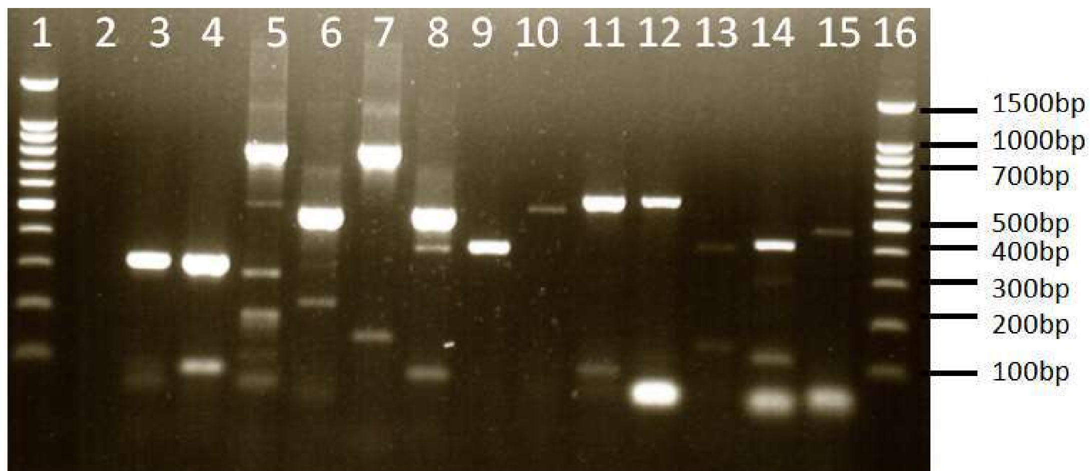

Figure 4. The alignment analysis of MAGE-A3 gene from testicular tissue with the MAGE A3 sequences from the Genbank data

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XM_005274676-X1     1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC
XM_011531160-X2     1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC
XM_006724818-X3     1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC
MAGEA3-seq          1 GTCATCATGCCTCTTGAGCAGAGGAGTCAGCACTGCAAGCCTGAAGAAGGCCTTGAGGCC

XM_005274676-X1    61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT
XM_011531160-X2    61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT
XM_006724818-X3    61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT
MAGEA3-seq         61 CGAGGAGAGGCCCTGGGCCTGGTGGGTGCGCAGGCTCCTGCTACTGAGGAGCAGGAGGCT

XM_005274676-X1   121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA
XM_011531160-X2   121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA
XM_006724818-X3   121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA
MAGEA3-seq        121 GCCTCCTCCTCTTCTACTCTAGTTGAAGTCACCCTGGGGGAGGTGCCTGCTGCCGAGTCA

XM_005274676-X1   181 CCAGATCCTCCCCAGAGTCCTCAGGGAGCCTCCAGCCTCCCCACTACCATGAACTACCCT
XM_011531160-X2   181 CCAGATCCTCCCCAGAGTCCTCAGGGAGCCTCCAGCCTCCCCACTACCATGAACTACCCT
XM_006724818-X3   181 CCAGATCCTCCCCAGAGTCCTCAGGGAGCCTCCAGCCTCCCCACTACCATGAACTACCCT
MAGEA3-seq        181 CCAGATCCTCCCCAGAGTCCTCAGGGAGCCTCCAGCCTCCCCACTACCATGAACTACCCT

XM_005274676-X1   241 CTCTGGAGCCAATCCTATGAGGACTCCAGCAACCAAGAAGAGGAGGGGCCAAGCACCTTC
XM_011531160-X2   241 CTCTGGAGCCAATCCTATGAGGACTCCAGCAACCAAGAAGAGGAGGGGCCAAGCACCTTC
XM_006724818-X3   241 CTCTGGAGCCAATCCTATGAGGACTCCAGCAACCAAGAAGAGGAGGGGCCAAGCACCTTC
MAGEA3-seq        241 CTCTGGAGCCAATCCTATGAGGACTCCAGCAACCAAGAAGAGGAGGGGCCAAGCACCTTC

XM_005274676-X1   301 CCTGACCTGGAGTCCGAGTTCCAAGCAGCACTCAGTAGGAAGGTGGCCGAGTTGGTTCAT
XM_011531160-X2   301 CCTGACCTGGAGTCCGAGTTCCAAGCAGCACTCAGTAGGAAGGTGGCCGAGTTGGTTCAT
XM_006724818-X3   301 CCTGACCTGGAGTCCGAGTTCCAAGCAGCACTCAGTAGGAAGGTGGCCGAGTTGGTTCAT
MAGEA3-seq        301 CCTGACCTGGAGTCCGAGTTCCAAGCAGCACTCAGTAGGAAGGTGGCCGAGTTGGTTCAT

XM_005274676-X1   361 TTTCTGCTCCTCAAGTATCGAGCCAGGGAGCCGGTCACAAAGGCAGAAATGCTGGGGAGT
XM_011531160-X2   361 TTTCTGCTCCTCAAGTATCGAGCCAGGGAGCCGGTCACAAAGGCAGAAATGCTGGGGAGT
XM_006724818-X3   361 TTTCTGCTCCTCAAGTATCGAGCCAGGGAGCCGGTCACAAAGGCAGAAATGCTGGGGAGT
MAGEA3-seq        361 TTTCTGCTCCTCAAGTATCGAGCCAGGGAGCCGGTCACAAAGGCAGAAATGCTGGGGAGT

XM_005274676-X1   421 GTCGTCGGAAATTGGCAGTATTTCTTTCCTGTGATCTTCAGCAAAGCTTCCAGTTCCTTG
XM_011531160-X2   421 GTCGTCGGAAATTGGCAGTATTTCTTTCCTGTGATCTTCAGCAAAGCTTCCAGTTCCTTG
XM_006724818-X3   421 GTCGTCGGAAATTGGCAGTATTTCTTTCCTGTGATCTTCAGCAAAGCTTCCAGTTCCTTG
MAGEA3-seq        421 GTCGTCGGAAATTGGCAGTATTTCTTTCCTGTGATCTTCAGCAAAGCTTCCAGTTCCTTG

XM_005274676-X1   481 CAGCTGGTCTT
XM_011531160-X2   481 CAGCTGGTCTT
XM_006724818-X3   481 CAGCTGGTCTT
MAGEA3-seq        481 CAGCTGGTCTT
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Figure 5. Analysis expression of GAPDH, MAGE A1-10, and MAGE A1-6 from the core biopsy of lung cancer patients. PC = positive controle for PCR with template total RNA from testicular tissue.