

MAGE-1 cDNA ISOLATION FROM TESTIS WITH RT PCR

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Asia and it is generally diagnosed at advanced stage, during which the prognosis of the patients has been poor and their capability to survive has also been low. Hepatic tissue of HCC patients expresses melanoma antigen-1 (MAGE-1) gene in the form of mRNA that can be identified only by RT PCT examination. mRNA examination with RT PCR is relatively more complicated since RNA is easier to be degraded by RNAase, resulting in difficulties in isolating the gene cDNA. Optimized examination with RT PCR is needed to be able to isolate MAGE-1 cDNA from testicular tissue that definitely expresses MAGE-1 mRNA. However, to date RT PCR optimal RT PCR from testis samples has never been undertaken. This was a laboratory explorative study generally aimed to isolate MAGE-1 cDNA from testicular tissue with RT PCR. The particular objective of this study was to obtain optimum condition for isolating cDNA of MAGE-1 gene from testicular tissue using RT PCR and to obtain MAGE-1 cDNA sequence from testicular tissue. mRNA isolation of MAGE-1 gene was obtained by sample extraction to obtain total RNA, which was subsequently altered into cDNA by means of oligo(T) polymer. Formed cDNA was amplified with polymerase chain reaction (PCR) using the primers GM F421 and GM R421 that synthesized 421bp. PCR product was electrophoresized and visualized with UV transilluminator. PCR product was also sequenced with Genetic Analyzer ABI Prism 310. The result showed that nucleotide sequence of MAGE-1 gene was accessible in Genbank with access number of EU161102. Optimum condition to obtain MAGE-1 cDNA from testicular tissue was predenaturation in 95°C for 5 minutes, denaturation in 95°C for 1 minute, annealing at 58°C or 60°C for 1 minute, extension at 72°C for 1 minute, 35 cycles. The cycle was prolonged at 72°C for 10 minutes. In conclusion, cDNA isolation of MAGE-1 gene can be obtained by using RT PCR from testicular tissue. The result of this study can be used as a basis for further studies in exploring the expression of MAGE-1 gene from Hepatocellular carcinoma (HCC) patient with RT PCR.

Keywords: testis, MAGE-1, RT PCR

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies found in Asia (Chen et al. 2000) and generally diagnosed at advanced stage (Liu 1999), so that the prognosis of the patient is poor and his ability to survive is also low. Recently, HCC diagnosis is established by using ultrasonography (USG) and alpha fetoprotein (AFP) level measurement in patients with high hepatocellular carcinoma risk (Soemohardjo 2004). USG examination is based on the change of tissue structure, which is apparent after it becomes a large mass. Moreover, AFP is also less specific for hepatocellular carcinoma since it can also be produced by other normal cells. Therefore, an examination at molecular level is needed to obtain an early detection. The examination at molecular level can be conducted by

reverse transcription-polymerase chain reaction (RT PCR) to find the gene expression that presents as mRNA. A number of studies have found that hepatic tissue of hepatocellular carcinoma patients expresses the melanoma antigen-1 (MAGE-1) gene in the form of mRNA (Chen et al. 2003; Kobayashi et al. 2000; Peng et al. 2005; Luo et al. 2002; Zhao et al. 2004). MAGE-1 belongs to cancer-testis (CT) antigen, which is expressed by cancer cells only, while it is not expressed in normal tissue except in the testis, so that this gene is specific for cancer. Such specificity can lead to a conclusion that each MAGE-1 expression indicates the presence of cancer cells. MAGE-1 gene expression can be identified from MAGE-1 mRNA that can be detected with RT PCR from the hepatic tissue of hepatocellular carcinoma patients. RNA examination with RT PCR is relatively more difficult since RNA is easily degradable

by RNAase, resulting in difficulties to isolate the cDNA gene. Optimized RT PCR examination is needed to isolate the MAGE-1 cDNA from testicular tissue that definitely expresses MAGE-1. However, until recently optimized examination using RT PCR has never been conducted to testicular samples.

Cancer-testis (CT) antigen is expressed in several human cancers, but not expressed in normal tissue, except in the testis. The gene was firstly isolated from melanoma, and recognized as melanoma antigen (MAGE) gene (Kumar et al. 2005; van Baren et al. 1999). CT antigen has several particularities, i.e., 1) it is particularly expressed by tumor tissue and not in normal tissue, 2) CT antigen coding gene is located in chromosome X (Zhao et al. 2004). MAGE-1 belongs to the antigens coded by MAGE gene family. MAGE-1 gene is expressed by hepatocellular carcinoma (Chen et al. 2003; Kobayashi et al. 2000; Luo et al. 2002; Peng et al. 2005; Zhao et al. 2004). MAGE-1 expression can be used as a prediction of hepatocellular carcinoma event in high-risk patients, so that it can be applied as a basis for determining the therapy for the patients. MAGE-1 examination can be carried out by using samples from hepatic tissue of patients with hepatocellular carcinoma. However, one of the obstacles found in carrying out MAGE-1 examination from hepatic biopsy tissue is the risk of uncontrolled bleeding. Another alternative is the use of samples from fine needle biopsy (FNAB). Unfortunately, due to limited cell counts aspirable with FNAB, the examination has to be conducted as effective as possible. To overcome these obstacles, examination should be undertaken in optimum condition. Therefore, RT PCR examination should also be optimized. Otherwise, the results would be less convincing.

This study was a laboratory explorative study intended to isolate MAGE-1 cDNA from testicular tissue using RT PCR. Primer was designed to synthesize MAGE-1 cDNA in specific area for MAGE-1, covering exon 1, exon 2, and a part of exon 3. Total products obtained were 421 base pairs. This area is a conserved area for MAGE-1 (Chen et al. 2000). MAGE-1 cDNA amplification products were then sequenced. The results were expected to be useful for obtaining optimum condition for isolating MAGE-1 cDNA so that it can be used as reference for conducting further studies to detect the expression of MAGE-1 gene in hepatocellular carcinoma patients from FNAB samples.

MATERIALS AND METHODS

Samples were obtained from prostate cancer patients aged more than 70 years and received orchidectomy therapy. Testicular tissue was separated from the

covering tunics, and excision of about 30 mg (3x3x3 mm) was immediately carried out. The tissue was kept within RNAlater to stabilize RNA. The tissue was kept in a temperature of 40°C until the time of use. Total RNA extraction was undertaken using RNeasy Protect Mini Kit (Qiagen), while cDNA synthesis was undertaken using First strand cDNA Synthesis kit for RT PCR (AMV) (Roche). Amplification was done using 2X PCR master Mix (Fermentas) with GM F421 primer was 5'-CGGCCGAAGGAACCTGACCCAG-3' and GM R421 was 5'-GCTGGAACCCTCACTGGTTGCC-3', producing 421 base pairs. Primer concentration used for PCR was 50pmol/ml. Protocol was done according to sheat product data. The condition of PCT was referred to that of Chen et al. (2000), in which the predenaturation was at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing was 60°C 1 minute, and extension at 72°C 1 minute, 35 cycles. The cycle was prolonged at 72°C for 10 minutes. The PCR condition was modified by increasing and reducing the annealing temperature of 2°C to become 58°C, 60°C, and 62°C. PCR product was electrophoresized with 2% electrophoresis gel and visualized with UV transilluminator. PCR product was sequenced with Genetic Analyzer ABI Prism 310.

RESULTS

The isolation of MAGE-1 gene mRNA was undertaken by extracting samples to obtain total RNA which was subsequently altered to become cDNA by using oligo(T) primer. Formed cDNA was amplified by means of a pair of MAGE-1-specific primer. The primers used were GM F421 and GM R421 that synthesize 421bp. The sequence of GM F421 primer was 5'-CGGCCGAAGGAACCTGACCCAG-3' and GM R421 primer was 5'-GCTGGAACCCTCACTGGTTG CC-3'. cDNA synthesis was carried out with oligo(T) primer. Poly A presents at the 3' end of mRNA, so that oligo(T) would pair with the poly A. Subsequently, cDNA synthesis was undergone by reverse transcriptase enzyme. The results of cDNA synthesis was visualized by running the 2% electrophoresis gel, and photographed with polaroid camera. cDNA appeared as smear with gel as can be seen in Figure 1.

The formed cDNA was amplified with PCR. PCR conditions were as follows: predenaturation at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 58°C, 60°C, or 62°C for 1 minute, extension at 72°C 1 minute, 35 cycles. The cycle was prolonged at 72°C for 10 minutes. The PCR condition was modified by increasing and reducing the annealing temperature of 2°C to become 58°C, 60°C, and 62°C. The result of PCR product electrophoresis from those three PCR

conditions at 62°C did not reveal any bands, while annealing at 58°C and 60°C revealed bands as can be seen in Figure 2.



Figure 1. Result of cDNA synthesis

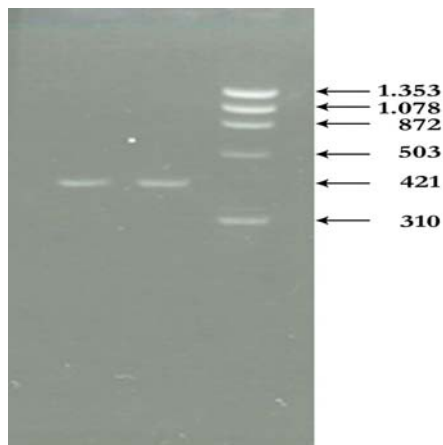


Figure 2. Result of MAGE-1 gene PCR using primers GM F421 and GM R421. Notes: wells from left to right, annealing temperature 58°C, 60°C, and marker.

PCR product was sequenced using Genetic Analyzer ABI Prism 310. Nucleotide sequence of MAGE-1 gene can be accessed in Genbank with access number EU161102. The sequence is as follows: cgccgaagg aacctgacc aggtctgtg agggagcaag gtttcagg gacaggccaa cccagaggac aggtatccct ggaggccaca gaggagcacc aaggagaaga tctgctgtg ggtctcatt gccagctcc tgccacact cctgctgct gcctgacga gagtcacat gtctcttgag cagaggagtc tgcactgcaa gcctgaggaa gcccttgagg cccaacaaga ggccctgggc ctggtgtgtg tgcaggctgc cacctcctcc tctctctc tcgtctctggg caccctggag gaggtgccca ctgctgggtc aacagatcct cccagagtc ctgaggagc ctccgcttt cccactacca tcaattcac tcgacagagg caaccagtg aggttccag c.

DISCUSSION

This was a laboratory explorative study generally aimed to isolate MAGE-1 cDNA from testicular tissue with RT-PCR. The particular objective of this study was to obtain optimum condition of MAGE-1 gene cDNA from testicular tissue with RT-PCR and to obtain MAGE- cDNA sequence from testicular tissue. The benefit for scientific development of this study was to provide information on MAGE-1 cDNA isolation from testicular tissue with RT-PCR that can be used as a basis for studying the detection of MAGE-1 gene expression from HCC patients. Practical benefit from this study was to obtain optimum condition of MAGE-1 gene examination with RT-PCR. The result can be used as a basis for further study on the exploration of MAGE-1 gene expression from HCC patients with RT-PCR.

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

cDNA was synthesized from total RNA with RT PCR (Reverse Transcriptase- Polymerase Chain Reaction). RT PCR is a method designed to amplify cDNA copy from RNA. The use of RT PCR was 1) to obtain and clone mRNA 5' end to 3' end and to derive a large number of cDNA library from a very few total mRNA. 2) to identify mutation and polymorphism in transcript sequence (mRNA). The principle step in RT PCR is the alteration of mRNA into template single strand cDNA with reverse transcriptase enzyme. Primer hybridization (attachment) occurred in mRNA sequence, and sequence elongation by reverse transcriptase enzyme occurred, and then cDNA copy could be amplified with PCR. Primers used for hybridization was RT PCR are 1) Gene Specific Primer (GSP), used for primer antisense (oligonucleotide antisense) from the sequence needed. Maximum result can be obtained if the primer antisense used for amplification in the PCR is located far upstream from the oligonucleotide used for cDNA synthesis. 2) Oligo(T) (dT), which binds endogeneous

poly(A) tail that presents at 3' mRNA. This primer can be used as universal primer for synthesizing cDNA. 3) Random hexanucleotide, which can be used to synthesize cDNA from long mRNA template. The primer can attach to all mRNA randomly, so that it can be used as template for cDNA synthesis in all mRNA (Sambrook & Russell 2001). Primer used for RT PCR in this study was oligo(T) as it could obtain total RNA that could be employed as a PCR template for all genes.

There are three types of RNA-dependent DNA polymerase (reverse transcriptase) enzymes, i.e. 1) mesophilic enzyme coded by AMV (Avian myeloblastosis virus) and strain Moloney Murine Leukemia virus (MO-MLV). 2) Variant of Mo-MLV reverse transcriptase that has no RNase H activity. 3) Thermostable Tth DNA polymerase, coded by thermophilic bacteria, *Thermus thermophilus*, and indicates reverse transcriptase enzyme by the presence of Mn²⁺ (Sambrook & Russell 2001). cDNA synthesis was undertaken using First strand cDNA Synthesis kit for RT-PCR (AMV) (Roche). Enzyme used was mesophilic enzyme coded by AMV (Avian Myeloblastosis Virus). The result of RT PCR was cDNA ready for use as template for amplifying cDNA copy with PCR.

Polymerase Chain Reaction (PCR) is a method to amplify specific DNA sequence. This method was firstly introduced by Kary Mullis in 1984. PCR is undertaken by adding several components to solution that contains target DNA sequence. The component is a primer that is able to hybridize with target DNA sequence, all deoxyribonucleotide triphosphates (dNTP), and DNA polymerase that is stable to heating (Berg et al., 2002). The vital components in PCR, according to Sambrook & Russell (2001), are thermostable DNA polymerase, synthetic oligonucleotide, deoxynucleoside triphosphates (dNTP), divalent cation, buffer, monovalent cation, and template DNA. Thermostable DNA polymerase is used to catalyze DNA synthesis that is dependent on template. The selection of enzyme to be used has variety in fidelity, efficiency, and capability to synthesize large-size DNA products. Taq polymerase used for routine PCR is approximately 0.5-2.5 unit per 25-50 ul reaction. This heat-stable polymerase is obtained from thermophilic bacteria (*Thermus aquaticus*) living in hot springs. Synthetic oligonucleotide is used as DNA synthesis primer. It is vital since the primer determines the success of PCR. Sometimes it is also necessary to notice the primer brand used for PCR reaction. Standard PCR contains dATP, dGTP, dTTP, and dCTP at the same amount (equimolar). The concentration of each deoxynucleoside triphosphates (dNTP) of 200-250 uM is recommended for Taq polymerase in reaction

containing 1.5 mM MgCl₂. The dNTP concentration is able to synthesize DNA 6-6.5 ug in 50 ul PCR reaction. Divalent cation is usually Mg²⁺. All thermostable DNA polymerase requires free divalent cation. The Mg²⁺ concentration commonly used is 1.5 mM. Tris Cl is added to maintain pH of 8.3 - 8.8 in room temperature. The standard for PCR is at the concentration of 10 mM. In regard with monovalent cation, PCR standard buffer contains 50 mM KCl to amplify DNA segment of more than 500 bp length, and a concentration of 70-100 mM to amplify shorter DNA segment. Template DNA containing sequence target can be added to PCR in single stranded or double stranded form. Circular DNA is more difficult to be amplified than linear DNA.

PCR process according to Sambrook & Russell (2001) comprises three steps, template denaturation (separation) by heating, annealing of oligonucleotide primer to single stranded target sequence, and the extension of primer that has been annealed to thermostable DNA polymerase. During denaturation, double-stranded DNA template was denaturated at high temperature. If GC content is high, higher temperature and longer time is needed since GC has strong bond (three hydrogen bonds), while in lower temperature and shorter time, the only area that will be denaturated is that containing AT only. PCR standard denaturation temperature is 94-95°C, 45 seconds, with GC content of less or equal to 55%. During primer annealing to DNA template, the annealing temperature is critical. If the temperature is high, less primer will anneal, so that the amplified templates will be few. If annealing temperature is too low, primers may anneal to non-specific targets, resulting in amplification of unexpected segments. During extension, the extension of primer annealed to the template is catalyzed with Taq DNA polymerase enzyme, whose optimum action is at the temperature of 72-78°C. The first two cycles are to extend the primer and form the template. The subsequent cycle is to amplify the sequence target. Total cycles depend on the number of the number of DNA template copies at the beginning of the reaction and the efficiency of primer extension and amplification. Generally, 30 PCR cycles produce 105 DNA copies (Sambrook & Russell 2001).

MAGE-1 cDNA amplification was carried out using 2X PCR master mix (Fermentas). Mix reaction was 2X PCR master mix 25 ml, forward primer 1 ml (50 pmol/ul), reverse primer 1 ml (50 pmol/ul), cDNA template 10 ml, distilled water up to total volume of 50 ml. The composition of 2x PCR master mix was 0.05 unit/ml Taq DNA polymerase in reaction buffer, 4mM MgCl₂, and dNTP (dCTP, dGTP, dATP, dTTP), each 0.4 mM. PCR condition used in this study was predenaturated at 95°C for 5 minutes, denaturated at

95°C for 1 minute, annealing at 58°C, 60°C, or 62°C for 1 minute, and extension at 72°C for 1 minute, 35 cycles. The cycle was prolonged at 72°C for 10 minutes. The PCR condition was modified by increasing and decreasing 2°C of the annealing temperature to become 58°C, 60°C, and 62°C. The result of electrophoresis of PCR products from the three PCR conditions at 62°C did not reveal any band, while annealing at the temperatures of 58°C and 60°C produced bands as seen in Figure 2. The temperature of 62°C was too high for annealing so that primer did not anneal to the template, resulting in no cDNA amplification so that the band in PCR product electrophoresis running was not apparent. The temperatures of 58°C and 60°C were ideal for annealing, so that primers could anneal to the template and cDNA amplification occurred as can be seen in bands resulted from PCR product electrophoresis. Thus, the optimum PCR condition to obtain MAGE-1 cDNA from testicular tissue was predenaturation at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 58°C or 60°C for 1 minute, extension at 72°C 1 minute, 35 cycles. The cycle was prolonged at 72°C for 10 minutes. PCR results were sequenced using Genetic

Analyzer ABI Prism 310 and analyzed with Bioedit and Clone Manager softwares. Sequencing was done using GM F421 primer to obtain MAGE-1 sequence from 5' direction and GM R421 to obtain sequence from 3' direction. Sequence result using GM F421 primer could be directly used for analysis since its direction had been from 5', while that using GM R421 primer should first be turned from 5' direction. The sequence resulted from sequencing did not describe all sequences since the sequence at 5' was unreadable starting from the first nucleotide in the primer. Therefore, to find the first nucleotide of each sequence needed, the sequencing should be conducted from 5' direction and 3' direction. The result of sequencing with GM F421 primer was cut using GM R421 primer, while the result of sequencing with GM R421 primer was cut using GM F421 primer. This was carried out to obtain the last nucleotide from the sequence needed. The resulting sequence was then aligned with the actual MAGE-1. The source of MAGE-1 product sequence was accessed from Genbank with access number EU161102. The sequence can be seen in Figure 3.

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      10      20      30      40      50
PRODUK SEKUENSING 421  C G G C C G A A G G A A C C T G A C C A G G C T C T G T G A G G A G G C A A G G T T T T C A G G G

      60      70      80      90     100
PRODUK SEKUENSING 421  G A C A G G C C A A C C C A G A G G A C A G G A T T C C T G G A G G C C A C A G A G G A G C A C C

      110     120     130     140     150
PRODUK SEKUENSING 421  A A G G A G A A G A T C T G C C T G T G G G T C T T C A T T G C C C A G C T C C T G C C C A C A C T

      160     170     180     190     200
PRODUK SEKUENSING 421  C C T G C C T G C T G C C C T G A C G A G A G T C A T C A T G T C T C T T G A G C A G A G G A G T C

      210     220     230     240     250
PRODUK SEKUENSING 421  T G C A C T G C A A G C C T G A G G A A G C C C T T G A G G C C C A A C A A G A G G C C C T G G G C

      260     270     280     290     300
PRODUK SEKUENSING 421  C T G G T G T G T G T G C A G G C T G C C A C C T C C T C C T C C T C T G G T C C T G G G

      310     320     330     340     350
PRODUK SEKUENSING 421  C A C C C T G G A G G A G G T G C C C A C T G C T G G G T C A A C A G A T C C T C C C C A G A G T C

      360     370     380     390     400
PRODUK SEKUENSING 421  C T C A G G G A G C C T C C G C C T T T C C C A C T A C C A T C A A C T T C A C T C G A C A G A G G

      410     420
PRODUK SEKUENSING 421  C A A C C C A G T G A G G G T T C C A G C

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Figure 3. MAGE-1 product sequence

CONCLUSIONS

cDNA isolation of MAGE-1 gene can be obtained by using RT PCR technique of testicular tissue. Nucleotide sequence of MAGE-1 gene can be accessed from Genbank with access number EU161102. The sequence is as follows:

1	cggccgaagg	aacctgacc	aggtctgtg	aggaggcaag	gtttcaggg	gacagccaa
61	cccagaggac	aggattccct	ggaggccaca	gaggagcacc	aaggagaaga	tctgcctgtg
121	ggctctcatt	gccacgtcc	tgcccacact	cctgcctgct	gccctcacga	gagtcactat
181	gtctcttgag	cagaggagtc	tgcactgcaa	gcctgaggaa	gcccttgagg	cccaacaaga
241	ggccctgggc	ctgggtgtg	tgcaagtgcc	cactctctcc	tcctctcttc	tggtctctgg
301	caccttgagg	gaggtggcca	ctgctgggtc	aacagatcct	cccagagtc	ctcaggagc
361	ctcgccttt	cccactacca	tcaattcac	tcgacagagg	caaccagtg	agggttcag
421	c					

The optimum PCR conditions to obtain MAGE-1 cDNA from testicular tissue are predenaturation at 95°C for 5 minutes, denaturation at 95°C for 1 minute, annealing at 58°C, or 60°C for 1 minute, extension at 72°C 1 minute, 35 cycles, and final extension at 72°C for 10 minutes.

REFERENCES

- Chen, H, Cai, S, Wang, Y, Zhao, H, Peng, J, Pang, X, Zhu, J, Cong, X, Rui, J, Leng, X, Chen, W 2000, 'Expression of the MAGE-1 gene in human hepatocellular carcinomas', *Chinese Medical Journal*, vol. 113, no. 12, pp. 1112-1118.
- Chen, HS, Qin, LL, Cong, X, Wang, Y, Fei, R, Jiang, D, Cao, J, Su, JJ, Wei, L, Chen, WF, Chen, WF, Wang, Y 2003, *Zhonghua Gan Zang Bing Za Zhi*, vol. 11, no. 3, pp. 145-148.
- Chen, XH, Liu, BY, Zhang, DQ, Zhang, Y, Li, JF, Zhu, ZG 2004, 'Expression of MAGE-1 and MAGE-3 genes in gastric cancer and gastric tissue and its clinical significance', *Xi Bao Yu Fen Zi Mian Yi Za Zhi*, vol. 20, no. 3, pp. 310-313.
- Chen, YT, Stocker, E, Chen, Y, Garin-Cesa, P, Rettio, WJ, Van Der Bruggen, P, Boon, T, Old, LJ 1994, 'Identification of the MAGE-1 gene product by monoclonal and polyclonal antibodies', *Proc Natl Acad Sci*, vol. 91, pp. 1004-1008.
- Chi, SN, Cheung, NK, Cheung, IY 2002, 'Expression of SSx-2 and SSX-4 genes in neuroblastoma', *Int J Biol Markers*, vol. 17, no. 4, pp. 219-223.
- Gillespie, AM, Rodgers, S, Wilson, AP, Tidy, J, Rees, RC, Coleman, RE, Murray, AK 1998, 'AGE, BAGE and GAGE : tumor antigen expression in benign and malignant ovarian tissue', *Br J Cancer*, vol. 78, no. 6, pp. 816-821.
- Kim, YM, Lee, YH, Shin, SY, Kim, EH, Choi, YW, Lee, KM, Park, JH, Lee, YU, Seel, DJ, Kim, MC 2001, 'Expression of MAGE-1, -2 and -3 genes in gastric carcinomas and cancer cell lines derived from Korean patients', *J Korean Med Sci*, vol. 16, pp. 62-68.
- Kobayashi, Y, Higashi, T, Noso, K, Nakastukasa, H, Kaneyoshi, T, Toshikuni, N, Kariyama, K, Nakayama, E, Tsuji, T 2000, 'Expression of MAGE, GAGE and BAGE genes in human liver diseases : utility as molecular markers of hepatocellular carcinoma', *J Hepatol*, vol. 32, no.4, pp. 612-617 [Abst]
- Kumar, V, Abbas, AK, Fausto, N 2005, *Robbins and Cotran's Pathologic Basic of Disease*, Elsevier Saunders, Philadelphia, pp. 328-330.
- Li, J, Yang, J, Fujie, T, Baba, H, Ueo, H, Mori, M, Akiyashi, M 1996, 'Expression of BAGE, GAGE, and MAGE genes in human gastric carcinoma', *Clin Cancer Res*, vol. 2, no. 9, pp. 1619-1625.
- Li, M, Yuan, YH, Han, Y, Liu, YX, Yan, L, Wang, Y, Gu, J 2005, 'Expression of cancer testis genes in 121 human colorectal tissue and adjacent normal tissue', *Clin Cancer Res*, vol. 11, no. 5, pp. 1809-1814.
- Luo, G, Huang, S, Xie, X, Stockert, E, Chen, Y, Kubuschok, B, Pfreundschuh, M 2002, 'Expression of cancer testis genes in human hepatocellular carcinomas', *Cancer Immunity*, vol. 2, p. 11.
- Peng, JR, Chen, HS, Mou, DC, Cao, J, Cong, X, Qin, LL, Wei, L, Leng, XS, Chen, WF 2005, 'Expression of cancer/testis (CT) antigens in Chinese hepatocellular carcinoma and its correlation with clinical parameters', *Cancer Lett*, vol. 219, no. 2, pp. 223-232 [Abst]
- Qiagen, 2001, *A Handbook RNeasy Mini Kit*. 4th edn.
- Ries, J, Schultze-Mosgau, S, Neukam, F, Diebil, E, Wiltfang, J, 2005, 'Investigation of the expression of melanoma antigen-encoding genes (MAGE-A1, to A6) in oral squamous cell carcinomas to determine potential targets for gene-based cancer immunotherapy', *Int J Oncol*, vol. 26, pp. 817-824.
- Sakata, M 1996, 'Expression of MAHGE gene family in lung cancers', *Kurume Med J*, vol. 43, no. 1, pp. 55-61.
- Sambrook, J & Russell, DW 2001, *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 3rd edn.
- Sumohardjo, S 2004, *Hepatocellular Carcinoma, The Strategy of Therapy, in Viral Infection and Cancer*, Faculty of Medicine, Gadjah Mada University.
- Yamanaka, K, Miyake, H, Hara, I, Gohji, K, Arakawa, S, Kamidono, S, 1998 'Expression of MAGE genes in renal cell carcinoma', *Int J Mol Med*, vol. 2, no. 1, pp. 57-60.
- Zhao, L, Mou, DC, Leng, XS, Peng, JR, Wang, WX, Huang, L, Li, S, Zhu, JY 2004, 'Expression of cancer testis genes in hepatocellular carcinoma', *World J Gastroenterol*, vol. 10, no. 14, pp. 2034-2038.