Mage-α_x mRNA Level in Lung Cancer of Mice Derived by Coal Tar Pitch

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Abstract: Objective. To investigate the expression of *Mage-a_x* mRNA in lung cancer tissues of mice induced by coal tar pitch (CTP) fume and to discuss the possibility of the lung cancer animal model induced by CTP as a model for lung cancer immunotherapy with *MAGE-A*. **Methods.** Tumor tissue samples of lung cancer and paired non-tumor tissues of the lung were obtained from 8 lung cancer mice. Total RNA was extracted and cDNA was synthesized. Nested PCR amplification using *Mage-a_x* specific primers was then performed to detect the expression of *Mage-a*. The 2 clones of 1 sample of *Mage-a_x* mRNA positive PCR products were DNA sequenced by using DNAs sequencer (PE-377). **Results.** Of 29 mice in the experimental group, 8 were induced to lung cancer. Of 8 lung cancers, 5 (62.5%) expressed *Mage-a_x* mRNA. The expression of *Mage-a_x* gene was not recognized in adjacent lung tissues at all. The DNA sequence confirmed that the target gene fragments in all 2 samples of PCR products were *Mage-a_x* cDNA. **Conclusion.** The *Mage-a* gene was expressed highly in tumor tissues with lung cancer in mice induced by CTP fume. This suggests that this kind of lung cancer mouse model may be an ideal animal model for lung cancer therapeutic experiment by *MAGE-A*. [Life Science Journal. 2006;3(3):29-34] (ISSN: 1097-8135).

Keywords: *Mage-a_x* mRNA; mouse; CTP fume; lung cancer

Abbreviations: CTP: coal tar pitch; CTL: cytotoxic T lymphocyte; TAs: tumor antigens

1 Introduction

Although the enormous manpower and material resources have been spent, there are still no effective methods developed to prevent and treat malignant tumor. The incidence rate of malignant tumor is increasing and the onset age is tending to be younger along with the changes of environment and lifestyle of human being. In China, the increasing magnitude of lung cancer is in the first place in recent 20 years according to the information derived from a more recent national conference on oncology in 2000[1].

Using a gene transfection approach to identify antigens recognized by CTL (cytolytic T lymphocytes) on a human melanoma cell line, Boon *et al* isolated the gene *MAGE-A* family that is located in the Xq28 region and the gene family includes at least 12 related genes[2-4]. *MAGE-B*, including 4 genes, was identified in the Xp21.3 region[5-7]. *MAGE-C1* is on band Xq26[8]. Most of these *MAGE* genes are expressed in a significant proportion of tumors of various histological origins, whereas no expression has been observed in normal tissues except on placenta and male germ cells.

The *MAGE*-encoded antigens are recognized by cytolytic T cells in the form of antigenic peptides presented by HLA class I molecules. Because male germ cells do not express the HLA class I genes, they fail to present MAGE antigens even though they express *MAGE* genes. The *MAGE*-encoded antigens are therefore strictly tumor-specific. Several immunogenic peptide epitopes from tumor-associated antigens (such as MAGE-A3, MAGA-A1 etc.) have served as targets for cellular immune responses in numerous clinical trials for therapeutic vaccinations[9, 10].

In 1999, Boon *et al* found *Mage-a*, a new family of mouse genes homologous to the human *MAGE-A* genes[11]. *Mage-a* genes were mapped on X chromosome. Like human *MAGE-A*, *Mage-a* genes were transcribed in adult testis, but not in other tissues. Expression of some *Mage-a* genes was also detected in tumor cell lines. *Mage-a* genes are higher degree homologous to the human *MAGE-A* genes. Like *MAGE-A* genes, they encode acidic proteins. As the ideal tumor animal model, it is possible to research the immunotherapy by using *MAGE* tumor antigens(TAs). There is, however, little information on their expression in lung carcinoma of mice. This experiment studied the expression of *Mage-a* gene in mice lung cancer and compared its sequence with that in GenBank, then discussed the possibility of the lung cancer ani-

mal model induced by CTP as a model for lung cancer immunotherapy with the use of *MAGE-A*. *Mage-a*$_1$, a_2, a_3, a_5, a_6, a_8 of *Mage-a* are arranged in a cluster located in a region syntenic to Xp22 and they share more than 93% nucleotide identity. The above 6 genes which were amplified in this study were called as *Mage-a*$_x$.

2 Materials and Methods

2.1 Sample collection and RNA extraction

Animal : 64 Kunming mice, 32 males and 32 females, were provided by Henan Animal Center (Zhengzhou, Henan, China). The mice were divided into experiment group and control group randomly. The experiment group was exposed to CTP fume 2 h per day for 12 weeks. The mice were killed in the 12th week and the 24th week, respectively. The lung tissues were frozen in liquid nitrogen. All the samples were confirmed by pathology.

Total cellular RNA was isolated using the flash column total RNA preparation kit (QIAGEN, German) according to the manufacture's instructions.

2.2 Nested RT-PCR

For nested RT-PCR analysis of *Mage-a*$_x$ transcripts, 5 μg of total RNA were reverse-transcribed with the first round PCR specific primers: 5′-AATACCAAGTCCTCCCCAG-3′ (forward), 5′-CTTGGGCCCCACAGGAACC-3′ (reverse) in a 30 μl reaction mixture containing reverse transcriptase buffer, 5 mmol dNTP, 25 pmol primer and 10 U AMV reverse transcriptase (Promega, USA). The mixture was incubated at 42 ℃ for 60 min, heated at 95 ℃ for 5 min and then stored under −20 ℃. 3 μl of RT reaction were used in one round of PCR with 1 × Taq buffer, 5 mmol dNTPs, 25 pmol first round PCR specific primers and 2 U of Taq DNA polymerase (Promega, USA). PCR amplification was 35 cycles at 94 ℃ for 50 sec, 55 ℃ for 50 sec and 72 ℃ for 60 sec with an initial one-cycle predenaturation at 94 ℃ for 120 sec and a final elongation cycle at 72 ℃ for 300 sec. Under the same PCR condition, a second round of PCR was performed using nested primer 5′-AGCGGATCC-CTCTCTCCCCAGGCC-3′ (forward), 5′-ACG *AAGCTT*CCAATTTCCGACGACACTCC-3′ (reverse), and 1 μl of first-round PCR products as template. The nested primers were added appropriate *Bam*HI and *Hind* III restricted site respectively.

2.3 Cloning and sequencing of recombinant plasmids

For the construction of recombinant pUC18 plasmids, a PCR fragment of *Mage-a*$_x$ cDNA was obtained with the nested primer. pUC18 plasmids and the target fragments were digested by *Bam*HI and *Hind* III respectively. Bands were isolated from low melting point agarose and purified by UNIQ-5 column DNA gel reextraction kit (Sangon, Shanghai, China) respectively, and then PCR product was ligated directly into pUC18 plasmid with T_4 DNA ligase. The mixture was transfered into *E. coli* cell, strain DH5α according to the method of *Molecular Cloning*[12]. At least 3 positive clones were picked up and amplified. Then, the ligated PCR products were isolated and sequenced by dideoxy method using DNAs sequencer (PE377). The sequencing results were analyzed using DNAsis software.

3 Results

3.1 The results of CTP induced cancer

Of 29 mice in the experiment group, 8 were induced to lung cancer, 7 were carcinoid and 1 was adenocarcinoma within 24 weeks. There were 5 specimens with squamous metaplasia, 2 were hyperplasia and the rest were normal. All the 32 mice in control group were normal. The cancer incidence was significantly different between two groups ($P < 0.01$). Detail results were shown in Table 1.

Table 1. Comparison of tumor induced in control group and experiment group

Group	Cases(n)	Mice with tumor(n)	Mice without tumor(n)	Ratio
Control group	32	0	32	0
Experiment group	29	8	21	27.6

$P<0.01$

3.2 Expression of *Mage-a*$_x$ gene at mRNA level in mice lung cancer tissues

Of 8 mice with cancers, *Mage-a*$_x$ mRNA was expressed in 5 (62.5%). The expression of *Mage-a*$_x$ gene was not recognized in adjacent and normal lung tissues at all. *Mage-a*$_x$ gene was also expressed in one squamous metaplasia. The PCR products were then digested by restriction endonucleas *Sca* I. The two fragments, 300 bp and 200 bp, were shown respectively. Representative gels were shown in Figure 1 and Figure 2. Results were shown in Table 2.

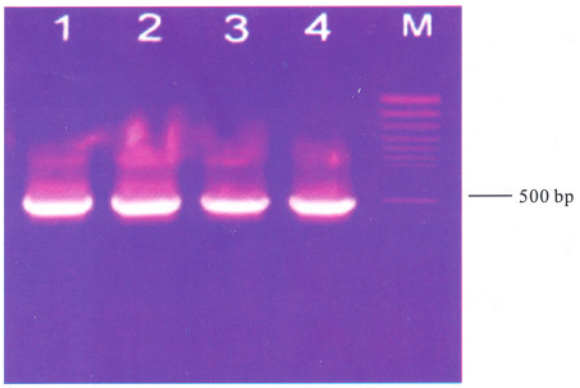

Figure 1. Amplification of target gene
Amplified product of *Mage-α_x* cDNA was 492 bp. Lane 1,2,3,4: lung cancer tissues which expressed *Mage-α_x* mRNA; Lane M: marker

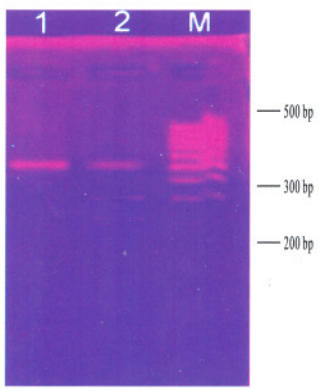

Figure 2. Identification of target gene
Lane 1: amplified target gene; Lane 2: fragments digested with *Sca* I; Lane M: marker

Table 2. Expression of *MAGE-α_x* gene in lung tumors in mice

Pathology	Proportion of positive samples
Carcinoid	4/7 (57.1%)
Adenocarcinoma	1/1 (100%)
Squamous metaplasia	1/5 (20%)
Hyperplasia	0/2
Adjacent lung tissues	0/8
Normal lung tissues	0/32

3.3 Construction, identification and sequencing of mouse *Mage-α_x* gene

The amplified DNA fragment was digested with *Bam* HI and *Hind* Ⅲ. The target fragment was ligated into a predigested (with *Bam* HI and *Hind* Ⅲ) clone vector pUC18. Initial transformation was carried out with *E. coli* DH5α host strain. Positive clone was identified via preparing the plasmid DNA from a number of clones and analyzed by using amplification with universal primer of pUC18 on agarose gel electrophoresis, and was sequenced by using primer M13 (or pUC18 universal primer). The target gene fragments in samples of PCR products were *Mage-α_x* cDNA. The results were shown in Figure 3 and Figure 4.

4 Discussion

The identification of TAs and their recognition by tumor-specific CTL has fuelled the development of immunotherapeutic strategies in cancer[13]. Although numerous TAs and their epitopes have been identified, the majority of these are quite restricted in expression and their clinical utility remains limited. Therefore, it is imperative to evaluate the possibility of tumor immunotherapy by using TAs/epitopes that are widely expressed in tumor. It has many benefit to research the *MAGE* genes' function in mice such as sample got easier, dynamic observation and so on. It may, therefore, be the ideal animal model for the lung cancer's therapeutic experiment by using *MAGE*.

4.1 Reliability, sensitivity and specificity of the experiment

The authors took the following measures to ensure the reliability of the experiment: (1) Remove the necrosis tissues to refrain from RNA degradation caused by necrosis. (2) RNA extracted was all verified by ethidium bromide fluorescence electrophoresis and by ultraviolet radiomete (A260/A280 > 1.9). (3) To ensure RNA was not degraded, a PCR assay with primers specific for β-actin was carried out in each case. (4) To avoid the false positive results, all the samples of extracted RNA were incubated with DNase. (5) Random sampling the positive clones sequenced and confirmed the amplified products being *Mage-α_x* (The sequence is the same as that of GenBank).

Method of nested PCR can improve the sensitivity and specificity of the experiment. The first round PCR will be carried out using the exo-primers firstly, and then the second round PCR will amplify the smaller regions of the first round PCR products by using the nested-primers. So the continuous twice enlargement may increase both the sensitivity and the specificity of PCR greatly. For the extreme-trace target fragments, it's very difficult to get the better results for once amplification, but satisfactory results can be obtained using nested PCR.

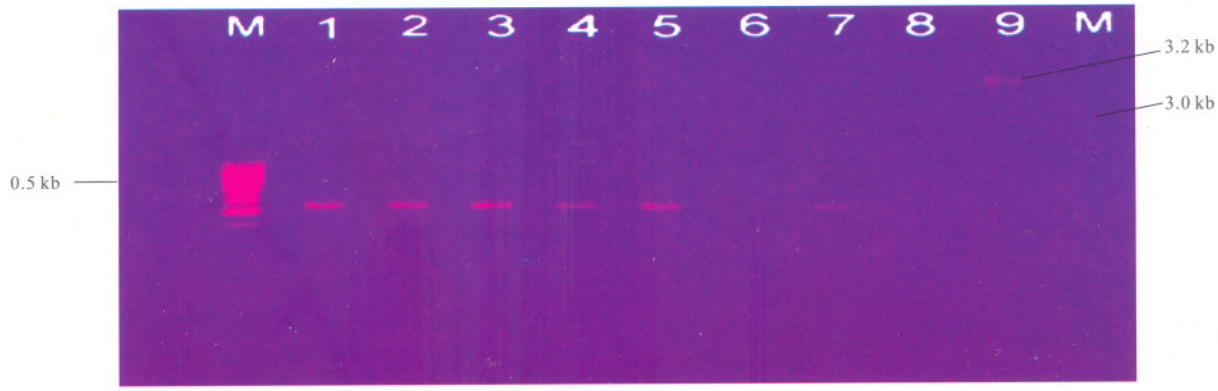

Figure 3. Identification of recombinant-plasmid amplified by pUC18 universal primer
Lane 1,2,3,4,5,7: the inserted target fragments amplified by universal primer; Lane 6: plasmid without inserted the target gene; Lane 8: the negative control; Lane 9: plasmid inserted the target gene

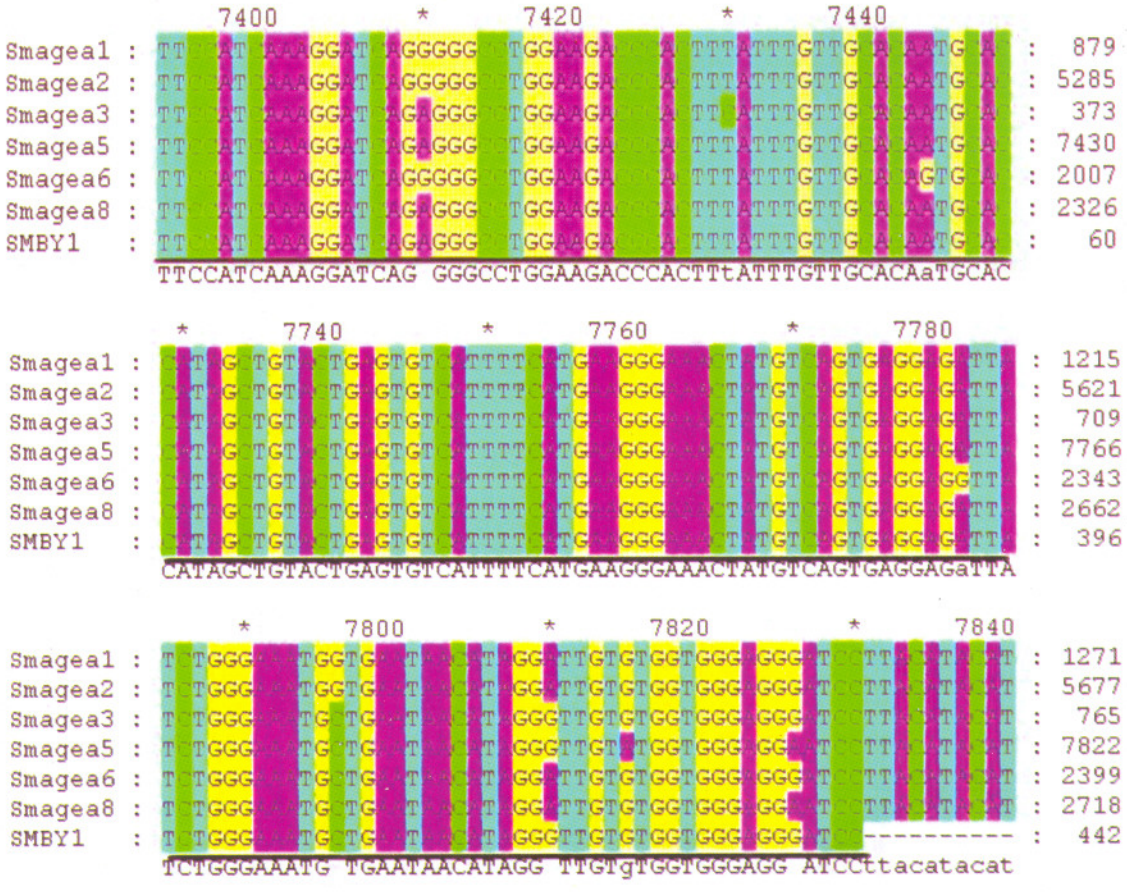

Figure 4. Comparing the sample sequence with *Mage-α_x* sequence in GenBank (The underlined parts were the sample sequences)

4.2 Expression of *Mage-α_x* mRNA of mouse lung cancer tissues

As the results of CTP fume inducing lung cancer among 29 mice in the experiment group within 24 weeks, 8 were induced to lung cancer, 7 were carcinoid and 1 was adenocarcinoma, respectively. PCR results showed that the majority of lung tumors in mice (5 of 8 tested) were positive for the *Mage-α_x* gene by gene-specific primers and the nested-PCR method. These results suggested that more than 60% of lung cancers were expressed *Mage-α_x* gene, which was also expressed in one

squamous metaplasia, but not recognized in adjacent and normal lung tissues at all. Although there are some mouse models used to evaluate human immune responses to *MAGE*-based tumor vaccine, there is no other information about the expression of *Mage-a* in lung carcinoma in mice. Gravekamp *et al*. found high expression levels of *Mage-b* (another *Mage* family of mouse genes homologous to human *MAGE-B* genes) in almost all metastases, regardless of age. The expression levels were 2- to 3-fold higher in the metastases than in the primary 4TO7cg breast tumors[14]. It suggests that *Mage*-encoded tumor antigen will be used as model to study various anti-tumor immunization modalities *in vivo*.

MAGE-A Ags were detected in primary and metastatic tumors of various histological types including melanoma, lung, bladder, ovarian, and breast carcinomas. Individual *MAGE-A* expression varies from one tumor type to another, but overall the majority of tumors express at least one of the *MAGE-A* family. Targeting epitopes shared by all *MAGE-A* Ags would be of interest against a broad spectrum of cancers. At present, *MAGE* peptide-based vaccines have been used in clinical trials with tumorous patients, but with limited success[15,16]. A suitable animal tumor model that would permit the optimization of *MAGE*-encoding cancer vaccines in mice is very necessary to immunotherapy of tumors using *MAGE* gene products. Researchers have used different tumor animal models to evaluate the possibility of tumor immunotherapy by using *MAGE* Ags. Eggert *et al* demonstrated the immunogenicity of two Kb-restricted peptide epitopes derived from mouse MAGE proteins which may serve as valuable tool for preclinical evaluation of vaccination strategies[17]. Ni found that rSFV vaccine could elicit human MAGE-3-specific antibody and CTL response in the Trimera mice[18]. The results of Gravekamp indicated that the metastatic and nonmetastatic breast tumor models could be useful model systems to analyze how breast cancer vaccines for humans[14].

However, a suitable mouse tumor model of lung cancer that would permit the optimization of *MAGE*-encoding cancer vaccines in mice is currently not available. In this study, it shows that *Mage-α_x* gene was expressed highly in tumor tissues with lung cancer induced by CTP fume. This suggests that this lung tumor mice expressing *Mage-α_x* may be an ideal animal model for lung cancer therapeutic experiment by using *MAGE-A*.

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