MAGEA10 gene expression in non-small cell lung cancer and A549 cells, and the affinity of epitopes with the complex of HLA-A*0201 alleles

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ABSTRACT
MAGEA10, a cancer/testis antigens expressed in tumors but not in normal tissues with the exception of testis and placenta, represents an attractive target for cancer immunotherapy. However, suppressive cytoenvironment and requirement of specific HLA-alleles presentation frequently led to immunotherapy failure. In this study MAGEA10 was scarcely expressed in cancer patients, but enhanced by viili polysaccharides, which indicates a possibility of increasing epitopes presentation. Furthermore the correlation of gene expression with methylation, indicated by R2 value for MAGEA10 that was 3 times higher than the value for other MAGE genes tested, provides an explanation of why MAGEA10 was highly inhibited, this is also seen by Kaplan–Meier analysis because MAGEA10 did not change the patients’ lifespan. By using Molecular-Docking method, 3 MAGEA10 peptides were found binding to the groove position of HLA-A*0210 as same as MAGEA4 peptide co-crystallized with HLA-A*0210, which indicates that they could be promising for HLA-A*0201 presentation in immunotherapy.

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1. Introduction

The human MAGE gene family encodes nuclear proteins of which most are expressed in tumor cells but silent in normal tissues except in the germ cells of testis and placenta [1–4]. Because both germ cells are devoid of surface HLA class I molecules, the responses of specific CD8+ cytotoxic T lymphocytes (CTL) to antigens encoded by MAGE genes have been suggested to be strictly tumor specific, but in different cancers, in other words they are cancer antigens [5–8]. MAGE molecules in cancer cells can be degraded by proteasome mediated proteolysis with cellular ubiquitin, and the degraded peptides can be transported to the cell surface by antigen processing protein. These epitopes can be presented by HLA-alleles to cytotoxic T lymphocytes (CD8+ CTL cell), and activated CTL then can release granzymes and perforins to kill the correspondent cancer cells by apoptosis or granulysis if without other barriers. Several peptides of MAGEA3 have been regarded as the most attractive epitopes, and the vaccines containing these peptides are under preparation [9–11]. The antigen presentation and immune eliciting in some situations can be enhanced even though the mechanisms are far from well understood.

It has been reported that some foodborne ingredients, especially lipopolysaccharide-like compounds, can increase innate and adaptive immunity [12]. Viili, a semi-solid yogurt that originated in Finland, has a ropey, gelatinous consistency and a sour taste resulting from the microbial action of lactic acid bacteria (LAB) and a surface-growing fungus Geotrichum candidum, which forms a velvet-like surface. In addition, viili contains yeast: Kluyveromyces marxianus and Pichia fermentans. Among the mesophilic LAB strains, the slime-forming LAB cremoris produces phosphate-containing exopolysaccharides (EPS). The basic
structure of viili EPS (VEPS) is mainly composed of D-glucose, D-galactose, L-rhamnose, and phosphate with an average molecular weight of about 2000 KDa and repeating unit of “→4→β-D-Glcp-(1→4)→β-D-Galp-(1→4)→β-D-Glcp-(1→4)”, as well as groups of α-L-Rhap and α-o-Galp-1-p attached to each side of Galp [13,14]. Because of its similar structure to lipopolysaccharides, viili has been claimed to have various benefits including anti-oxidation, anti-inflammation, anti-cancer, and anti-aging functions and enhancement of natural immunity [15–17], which possibly increases the presentation of MAGE protein for immunogenicity by extracellular factors.

A number of MAGE genes encode shared tumor-specific antigens, which have been detected in different cancers, and some are used in therapeutic vaccination trials for cancer patients [18–20]. MAGE derived HLA ligands have repeatedly been shown to elicit T-cell responses against tumor cells, but with tremendous difficulties in determination for different populations and individuals with their specific HLA-alleles in immunotherapy. Thus it is important to increase the gene expression of CTA or tumor associated antigens (TAAs) and to determine the criteria for peptides in the majority of tumors and the HLA-alleles that limit the epitopes besides the non-specific compounds that promote the presentation. We applied a high resolution structure of HLA-A*0201 co-crystallized with natural MAGEA4 peptide [21] as affinity modeling, and identified 3 top epitopes of MAGEA10 besides the detection of MAGEA10 gene expression in lung cancer patients and in A549 cells. It is anticipated that these analyses will contribute insights into the development of alternative ways to increase the presentation of MAGEA10 peptides for vaccine preparation and immunotherapy.

2. Materials and methods

2.1. Patients, tumors and gene expression and methylation microarray datasets

The MAGE genes for analysis of gene expression were from a microarray dataset with an initial 85 lung tumor samples and 21 adjacent cancer-free lung samples of non-small cell lung cancer (NSCLC) patients, and the tumor characteristics files, which were previously reported [22]. The MAGE genes' methylation data was retrieved from a dataset of Illumina methylation 450 k chip analysis, but with the same panel of NSCLC samples used in the gene expression array [23]. Anti-MAGEA10 and anti-β-actin antibodies were purchased from Bioworld Technology (St. Louis Park, MN, USA) and goat anti-rabbit IgG antibody (H+L) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA).

2.2. Cell culture of A549

A549 cells of NSCLC were purchased from American Type Culture Collection (ATCC). The cells were maintained in RPMI1640 medium supplemented with L-glutamine (1 mM), 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in 5% (v/v) CO2 incubator. In general all experiments were carried out when cells reached 80–90% confluence. The cell culture was in less than 20 passages, cell morphology remain normal and health without mycoplasma contamination throughout the experiments.

2.3. Extraction and purification of vEPS

Growth medium used for the production of viili EPS (vEPS) was reconstituted skim milk autoclaved at 121 °C for 15 min. Fermentation was carried out at 28 °C for 18–20 h with 5% inoculums. Viili protein was removed by the Sevage and isoelectric point method, and the ethanol precipitated vEPS was further purified by DEAE - cellulose (OH−) ionic exchange and gel chromatography [24–26]. Elution was performed in a 245 cm × 5 cm × 50 cm column at a flow rate of 25 ml/h with distilled water, 0.05 M, 0.10 M, 0.15 M, and 0.2 M NaCl subsequently. The peak of vEPS-V-1, vEPS-V-2 and vEPS-V-3 polysaccharide was determined by the sulfuric acid-phenol method, followed by dialysis in distilled water for 3 days and then freeze-drying for cell test.

2.4. Methylation analysis

DNA methylation analysis of the same tissue samples and controls was performed with the Illumina Infinium HumanMethylation450 BeadChip according to the manufacturer's standard protocols. This BeadChip contains more than 485,000 methylation sites, covering 99% of RefSeq genes with an average of 17 CpGs per gene distributed across the promoter, 5'-UTR, first exon, gene body, and 3'-UTR regions. In addition, the BeadChip covers 96% of CGI with an average of five CpG sites each, as well as the corresponding shores and shelves. Furthermore, it includes CpGs outside of CGIs, CGIs outside of coding regions, and micro-RNA promoter regions. Methylation value of MAGEA10 was specifically selected for correlation analysis with those positive MAGEA10 gene expression. The Pearson correlation between methylation and gene expression of MAGEA10 was calculated in R.

2.5. RT-PCR

Total RNA was extracted from approximately 2 × 106 cells for each test following the instructions described in the manual (Qiagen, MD, USA). The integrity of the total RNA was determined by 1% agarose gel. Reverse transcription was carried out with 1 μL of Ribolock™ RNase Inhibitor, 1 μL of oligo (dT) 18 primer, 2 μL of 10 mM dNTP Mix, 4 μL of 5 × reaction buffer, 2 μL of template RNA (100 ng/μL), 1 μL of RevertAidTM Reverse Transcriptase, and nuclease-free water added to a final volume of 20 μL. Reagents were mixed, and incubated at 42 °C for 1 h, then at 70 °C for 5 min for termination of the reaction. The cDNA was kept at −20 °C. The PCR product of MAGEA10 (fragment size 179 bp) was detected with primers (Table 1) designed with primer 3 (http://frodo.wi.mit.edu). A unique (virtual) PCR was performed with an e-PCR program at NCBI (http://www.ncbi.nlm.nih.gov/utsilis/e-pcr), using β-actin as the housekeeping gene (285 bp). β-Actin oligomers were synthesized by Invitrogen (Shanghai, China), as shown in Table 1. PCR of MAGEA10 was performed using 5 μL of 10 × Taq reaction buffer, 2 μL of template cDNA, 1.5 μL of primers each, 1 μL of dNTP mix (10 mM), 1 μL of Taq DNA polymerase and nuclease-free water to a final volume of 50 μL. The reaction was carried out at 94 °C for 30 s, then 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 45 s for 35 cycles, and extension at 72 °C for 10 min, then kept at 4 °C. The PCR fragment was confirmed by a commercial sequencing service company (BGI, Beijing, China).

2.6. MAGEA10 protein expression in the presence of vEPS by Western blotting

A549 cells were incubated to log phase and 1 × 106 cells were synchronized for another 10 h, then vEPS were added for final concentration of 0, 10, 25 and 50 mg/L of, and further incubated for 48 h. Cells were collected and washed twice with ice-cold PBS and lysed with lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2 mM PMFS, 1.0% Triton X-100, protease inhibitor cocktail (Sigma)]. Lysates were incubated for 10 min on ice, sonicated and centrifuged for 15 min at 12,000g. After protein concentrations were determined using the Bradford assay, the samples
were boiled for 10 min, equal amounts of protein (20 μg/lane) were separated by SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with a 1:1000 dilution of primary antibody against MAGEA10 and 1:4000 dilution of primary antibody against β-actin at 4 °C overnight. The secondary antibody was goat anti-rabbit IgG antibody (H+L) diluted 1:5000 in blocking solution for 1 h at room temperature. Immunoreactivity was detected using West Pico Mouse IgG Detection kit and visualized by autoradiography. The values of the band density was normalized to β-actin by using Multi-Gauge software (FujiFilm), thus only the nonsaturated signals were quantified, and the background was subtracted.

2.7. Kaplan Meier survival analysis

Survival analysis was performed using the “survival”-package in statistical software R and SPSS (SPSS, Inc.). Overall survival (OS) in the model-predicted high- and low-risk groups were analyzed using the Kaplan–Meier product-limit method and the significance of the difference between the survival curves was measured by the log-rank test.

2.8. Structure of MAGEA10 epitopes in complex with MAGEA4–HLA-A 0201

The high resolution structure of HLA-A’0201 with co-crystallized MAGEA4 epitope (PDB: 1AF) was retrieved from www.PDB.org, which contains heavy α chain: HLA-A’0201 (Chain A), smaller β chain: beta2-microglobulin (Chain B) and a small MAGEA4’s peptide [25]. To find the structure of binding complexes of MAGE10 epitopes in HLA-A’0201, a set of hierarchical methods are employed: I, the sequences of candidate epitopes (P1 to P20) of MAGEA10 (Accession NP_066386) were generated by Bimas at http://www-bimas.cit.nih.gov/molbio/hla_bind/, II, 3D structures of these candidate epitopes were generated by MODELLER [26,27], III, AutoDock4 was employed to find the initial binding pose of candidate epitopes in HLA-A’0201, and re-examined by visual observation, IV, several structures close to natural conformation were selected for further Molecular Dynamics (MD) simulations by using AMBER 10 with Amber99 force field [28]. P4 of MAGEA10 was used for 20 testing docking conformations after reaching equilibrium state of MD simulation. The structures that are in a reasonable binding pose were recognized as correct ones.

2.9. Molecular dynamic simulation of MAGEA10 epitopes in HLA-A 0201 complex

The selected binding complexes are solvated in water boxes where the water layers are kept no less than 12 angstroms in thickness. Counter-ions (Na+/Cl–) are added to keep the system neutral in charge. The protein complexes are constrained in the first few simulation steps to make sure that the water molecules and counter-ions are heated up to 300 K and fully relaxed [29]. Then the constrains on proteins are removed and a MD simulation of the whole system is carried out until the system reaches equilibrium state, i.e. the changes on temperature, density, total energy and root mean squared deviation (RMSD) are small enough and fluctuating around certain value. Generally, the system will equilibrate state in about 1–2 nano-seconds, and the real simulation takes about 18–36 h on a computer with 2Xeon E5620 CPUs (multi-thread to 24 cores) [30]. Then the structures are examined to make sure that the epitopes are still in complex with HLA-A’0201 and in a reasonable binding pose after reaching equilibrium. Selected complexes are put forward for a productive 2 ns MD simulation to generate a trajectory for MM-PBSA analysis [31]. Finally, the MM-PBSA module of AMBER is employed to analyze the MD trajectory and give out calculated binding free energy (ΔG) [32,33].

2.10. Statistics

Experimental results were expressed as the mean ± standard deviation (SD). Data was analyzed using the T-test, and statistical calculations were performed using Statistical Software SAS 9.1 (SAS Institute Inc., Cary, USA). The level of statistical significance employed in all cases was p < 0.05.

3. Results

3.1. Unequal gene expression of MAGEA10 in NSCLC patients in microarray assay

Microarray analysis revealed that MAGE genes were not expressed in control samples, with an exception of MAGEB10, however, since the control samples were from cancer patients, this may represent a pathology error, or possible early stage carcinogenesis (red hollow arrow, Fig. 1A). Expression of MAGEA10 in all controls was 0%, indicating clean cancer-free samples. Out of the 85 NSCLC patient samples tested, the MAGEA10 gene was present in only 26% of the samples, whereas MAGED1 was highest at 62%, and MAGEC1 was lowest at 22% (Fig. 1B). This may suggest the regulation of MAGE gene family is independent.

3.2. The inverse correlation of MAGEA10 gene expression with methylation

MAGEA10 gene expression was correlated with the methylation data using Microsoft Excel 2007. $R^2$ value of MAGEA10 was 0.1436 (y = -3.5945x + 3.176), the highest among the 26 MAGE genes tested, which means most highly inverse, or negatively correlated to methylation (Fig. 2). The scatter plot clearly shows the tendency that methylation inhibits MAGEA10 genes expression. The correlation was much lower for the other MAGE genes (average $R^2$ value = 0.0472), which is about 3 times lower than MAGEA10 (data not shown). The scarcity of MAGEA10 gene expression in lung cancer cells may at least in part be attributed to high degree of methylation including in the coding region.

3.3. Tangible gene expression of MAGEA10 by vEPS

Since viili polysaccharides are traditionally regarded as immune boosters, the similarity of vEPS with lipopolysaccharides were reported (Kitazawa et al. 2007; Kekkonen et al. 2008). With e-PCR at http://www.ncbi.nlm.nih.gov/sutils/e-pcr/ a specific unique single fragment was confirmed (Fig. 3A), then the RT-PCR was demonstrated by Thermal Cyclers LeCycler-2000 with the

<table>
<thead>
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<th>Genes</th>
<th>Primer (5’-3’)</th>
<th>Primer (5’-3’)</th>
<th>Size</th>
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<tr>
<td>MAGEA10</td>
<td>TTGCCCAAAATTTCAGGAG</td>
<td>TAAATCAGGTCCTCCACTCC</td>
<td>178bp</td>
<td>NM_021048.4</td>
</tr>
<tr>
<td>β-Actin</td>
<td>AGCGGACATCCCCCAAGTT</td>
<td>GGCACAGGAGCCATCATT</td>
<td>285bp</td>
<td>NM_001101.3</td>
</tr>
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Table 1: Primer sequences used for PCR.
subtraction of β-actin, a housekeeping gene (Fig. 3B). By measuring the optical density, relative quantitation shows that MAGEA10 gene expression was significantly increased with increase of vEPS (Fig. 3C).

3.4. MAGEA10 protein expression was increased by vEPS

MAGEA10 protein expression following treatment with 0, 10, 25 and 50 mg/L vEPS, respectively, was dose dependently increased compared with the controls (Fig. 4). Taken together with RT-PCR our data shown that vEPS is able to increase MAGEA10 gene and protein expression, thus possible to increase MAGEA10 epitope presentation.

3.5. MAGEE2 extends lifespan by Kaplan Meier analysis

Kaplan–Meier analysis of the model-predicted risk groups for 85 patients was performed. The difference between the survival rates in the high and low risk groups was apparently involved in
MAGEA2 gene, and was significant \((p = 0.0007, \text{ using the log-rank test})\), but not with MAGEA10, MAGEC3 and MAGEB18 (Fig. 5).

### 3.6. Fitting of MAGEA10 epitope with MAGEA4–HLA-A*0201 complex

For the purposes of screening novel MAGEA10 epitopes the fitting of a known epitope of MAGEA10 (P4) was applied. The peptide was docked to c-chain position of HLA-A*0201 in different conformation likely because of highly similarity between c-chain and the peptides (Fig. 6C). In our simulation the horse shoe like \((C_2H_4O)_6\) from PEG400 could be removed, but the conformation of whole structure was not affected. The structure is sufficient to test the other unknown peptides from MAGEA10.

### 3.7. The simulated affinity of MAGEA10 epitopes with HLA-A*0201

Twenty MAGEA10 peptides generated by Bimas were autodocked with the conformation originated to HLA-A*0201–MAGEA4 peptide GVYDGREHTV [13] that is similar to GLYDGMEHL (P4) of MAGEA10 (Fig. 7). With the hierarchical methods stated in methods, three epitopes, epitope 1 (SLLKFLAKV), and epitope 3 (KVTDLVQFL, and epitope 4 (GLYDGMEHL) are identified with the high affinity, which could form complexes with HLA-A*0201, and the calculated delta-G is \(-5.85 \text{kcal/mol}, -7.92 \text{kcal/mol}, \text{and } -4.29\), respectively (fully free energy data not shown).

### 4. Discussion

In a previous study MAGEA10 was found to be one of 3 top genes, which was decisive for 1000 days' survival, and may indicate a better prognosis [22]. With the continuation of the array analysis we found that the MAGEA10 gene was expressed in 26% of Estonia NSCLC patients only, and MAGEA10-derived peptide-specific CTL were reported to be induced from the peripheral blood mononuclear cells in several cohorts [34,35]. The strong MAGEA10 antigenicity points to the development of a possible immunotherapy, however, to date most immunotherapy has been limited or unsuccessful, one of the reasons being scarcity of the gene expression. The low gene expression both in NSCLC patients and A549 cells may indicate MAGEA10 is a decisive gene in general, especially in highly lethal diseases. It is common that the frequency of MAGE gene expression in cancer is also low [36–38].

Fig. 3. Gene expression of MAGEA10 in A549 cells and induced with VEPS. (A) MAGEA10 primers validation with e-PCR, (B) RT-PCR of MAGEA10 with housekeeping gene β-actin, (C) relative optical density (ROD) for semi-quantitation, lane 1, 5 µg/mL, lane 2, 25 µg/mL, lane 3, 50 µg/mL of vEPS. * \(p < 0.05\), ** \(p < 0.01\) compared with control were revealed. Results are shown as mean ± SEM of three independent experiments.
Thus it may be important to seek some factors that would increase the gene expression of the MAGE family for immunotherapy. In vitro analysis, MAGEA10 was shown to be upregulated in A549 cells under the influence of viili exopolysaccharides, as demonstrated by RT-PCR and Western blotting. Methylation both in regulatory and coding regions is known to inhibit gene expression [39]. The correlation of MAGEA10 with methylation was almost 3 times higher than the other 25 MAGE genes on average, and shown an inverse correlation in the NSCLC cohort. This is consistent with the low gene expression in normal or tumor samples, which may also tribute to the minimal influence of lifespan demonstrated by gene signatural method [40]. However, it remains unclear that the up regulation of MAGEA10 by viili EPS was associated to hypomethylation. MAGEA10 as specific CTA, has been successfully reported to increase survival. The viili EPS slightly modulates MAGEA10 expression, which indicates viili polysaccharides, or other similar ones could increase MAGEA10 expression and presentation, which may increase NSCLC patients’ survivals. Several MAGE peptides have been shown to be presented by HLA-A’0201 with an extraordinary frequencies of CD8+ T-cell responses [41].

By modeling of HLA-A’0201 with original MAGE4 peptide and MAGEA10 peptides and the horseshoe like molecules in structure [42] apparently show enough space for some parts of the molecular chain of polysaccharides to be able to access to the groove as well, of course it remains unknown whether likely increase the affinity of HLA-alleles and epitopes even though T-cell proliferation could be promoted [43]. However, the cancer antigens can also

![Fig. 4. Enhancement of MAGEA10 protein expression of A549 cells under influence vEPS by Western blot analysis. Lane 1, control; lane 2, 10 µg/mL, lane 3, 25 lane 4 50 µg/mL) of vEPS. *p < 0.05, **p < 0.01 compared with control were revealed.](image)

![Fig. 5. Kaplan–Meier plot of the survival probability in the high and low risk groups predicted by the Bayesian model. The high risk group (short survival) consisting of 24 patients and the low risk group (long survival) consisting of 22 patients. Vertical drops indicate deaths and ticks on the solid lines are censored survival times. The survival rates of the two groups for MAGEE2 are significantly different (*p = 0.0007), however, MAGEA10 apparently show no difference, while MAGEC3 and MAGEB18 have difference, but with *p > 0.05.*](image)
arise from many different proteins that many peptides bound to HLA-A*0201 also exhibit degenerate binding (binding to multiple other alleles), which led to application of the bigger population for at least A2 supertype multi-epitope vaccine that could be designed to provide broad population coverage [44].

By Molecular Docking simulation the peptide P1 (SLLKFLAKV), P3 (KVTDLVQFL) and P4 (GLYDGMEHL) of MAGEA10 with HLA-A*0201 present a low free energy and correct groove binding position suggests that they could be tumor CTL clones and choices of antibody for immunotherapy. Among 3 peptides P4 epitope of MAGEA10 was already determined experimentally with high avidity of antigenicity and antitumor activity [45]. Thus it is possible P1 and P3 predicted here could be also a potential ligands for HLA-A*0201 presentation and cancer antigens for immunotherapy.

Characterization of tumor-associated Ag (TAA)-derived peptides efficiently presented by tumor cells and recognized by tumor-reactive CTL is critical for the development of TAA-specific T cell therapies. This study has shown that MAGEA10 is expressed in some NSCLC patients of a small Estonia corhort, but not necessarily influence the lifespan by single gene alone because they are possible to be under inhibition through methylation in most patients. Foodborne polysaccharides test in vitro apparently show that gene expression of MAGEA10 could be modified, suggesting potential corresponding antibody production. From all above, cell
suppression frequently causes failure of CTL activation. However, if the MAGEA10 epitopes, such as the ones computed by our simulated binding of HLA-A*0201 and 3 peptides, or other TAs are selected, corresponding CTLs are elicited, such autologous immunotherapy could happen if they are able to be present to cell membrane surface in the mean time.

Finally, it is perhaps worthy to point out that MAGEE2 would be further to be analyzed since its expression was correlated with survival. In addition, MAGEA10 has been recently found in about 1/3 of cancers, which also showed strong correlation between MAGE-A10 staining score with tumor grade and stage [46,47], and the massive amplification of MAGEA10 (254–262) epitopes by engineered bacteriophages to induce strong antitumor CTL responses in vivo and in vitro [48], which all show that MAGEA10 remain to be an important target from research to clinic, especially for cancer immunotherapy.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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