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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 R^2 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.

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 granulysin 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

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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-Glcp-(1 → 4)-β-D-Galp (1 → 4)-β-D-Glcp-(1→", as well as groups of α-L-Rhap and α-D-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-crystalized 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) CO_2 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 2×10^6 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 RibolockTM 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 20μL. 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/sutils/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 for45 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 1×10^6 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 PMSF, 1.0% Triton X-100, protease inhibitor cocktail (Sigma)]. Lysates were incubated for 10 min on ice, sonicated and centrifuged for 15 min at 12,000*g*. After protein concentrations were determined using the Bradford assay, the samples