

# Neoantigen in esophageal squamous cell carcinoma for dendritic cell-based cancer vaccine development

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**Abstract** Esophageal squamous cell carcinoma (ESCC) is a highly malignant tumor which usually is diagnosed in advanced stages due to its asymptomatic course of tumorigenesis. Current therapeutic modalities are not effective enough and the 5-year survival rate of the disease is still very low which prompts the urgent need for finding novel efficient therapeutic methods. In this study, we evaluated ex vivo immune response of ESCC patients against our newly designed chimeric construct consisting of highly immunogenic cancer-testis antigens. After confirming effective expression of the in vitro transcribed chimeric mRNA in ex vivo electroporated dendritic cells

(DCs) of the ESCC patients, the patients' CTLs were primed by DCs and cytotoxicity assay was performed to evaluate how the primed CTLs can recognize and target the chimeric mRNA-loaded cells. The chimeric protein was strongly expressed relative to the housekeeping gene expression in electroporated cells. The cytotoxicity of the CTLs was significantly higher in DCs loaded with chimeric mRNAs compared to mock DCs ( $p < 0.05$ ) in all of the tested ESCC patients. We are introducing a novel construct that our functional study showed can stimulate and induce an effective immune response in ESCC patients. The designed chimeric mRNA-loaded DCs are capable of priming CTLs effectively and induce cytotoxicity against tumor. Therefore, loading DCs with chimeric epitopes of highly immunogenic antigens, such as cancer-testis antigens, are potentially interesting and effective therapeutic modalities for immunotherapy of ESCC.

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## Introduction

Esophageal cancer is a highly malignant tumor and is globally ranked as the sixth leading cause of cancer-related deaths [1]. The majority of esophageal carcinoma, approximately 95 % worldwide, is squamous cell carcinoma [2]. In majority of cases, the progression of the disease has an asymptomatic course and it is diagnosed in advanced stages with poor prognosis and unrespectable or metastatic disease in many cases. Despite the advancements in the available therapeutic modalities such as surgery, or chemo- and radiotherapy, the 5-year survival rate

of the disease is still very low, and even in localized disease, it is as low as 38 % and does not exceed 3 % in patients with distant metastasis [3]. Therefore, there is an urgent need, and in fact, it is an area of great interest to develop novel therapeutic methods such as hormonal therapy, targeted therapy and immunotherapy [4].

By recognizing tumor cells, immune system can play roles in elimination of malignant cells via innate and adaptive immune responses similar to the immunological processes involved in combating pathogens [5, 6]. Tumor cells can express different types of antigens that are presented as epitopes in grooves of major histocompatibility complex (MHC) class I molecules at the surface of tumor cells and recognizable by the adaptive immune system promoting an effective antitumor immune response [5]. Such tumor antigens (TAs) are recognized as overexpressed antigens, mutated antigens, differentiation antigens, viral antigens, re-expressed embryonic antigens and cancer-testis antigens (CTAs) [4].

Dendritic cell (DC) vaccine-based immuno-gene therapy is one of the effective immunotherapeutic methods, which applies recombinant DNA or mRNA constructs encoding TAs.

Various clinical trials in phases I, II and III are evaluating *ex vivo* DC-loaded vaccines as an anticancer immunotherapeutic intervention against a variety of malignancies (reviewed by Galluzzi et al. [7]) and TAs have been more attractive targets in the trials overall, compared to DCs pulsed with tumor-derived mRNA or tumor cell lysates.

A growing list of TAs is considered in trials including PAP, HER2, MUC1, p53 and CTAs such as MAGEA1, MAGEA3 and NY-ESO1 [7]. Despite existing mysteries about the function of CTAs in different tumor cells, they have some attractive properties which candidate them as an interesting targets for cancer immuno-gene therapy. First of all, their restricted pattern of gene expression in normal tissues decreases their potential to induce autoimmunity when they are applied in adaptive T cell therapy and vaccination. In addition, concomitant overexpression of a variety of CTAs is frequently reported in different malignancies allowing vaccination against multiple targets simultaneously [8–10]. Furthermore, it has been frequently shown that most of CTAs are immunogenic either *in vitro* or *in vivo* eliciting both cellular and humoral responses, while they are self-antigens. Therefore, using such important antigens in tumor immunotherapy, can induce strong T cell response which may confer tumor rejection and clinical improvement [11].

Our aim in this study was to design a new chimeric construct consisting of highly immunogenic HLA-restricted epitopes of overexpressed CTAs in ESCC patients and to evaluate that how DCs loaded with such chimeric molecule can induce and activate immune response *ex vivo*.

Our results show that pulsing DCs with the designed chimeric construct mRNA can prime CTLs specifically and activate the immune response of T cells.

## Materials and methods

### Construction of recombinant chimeric gene

mRNA expression of MAGE-A4, NY-ESO1 and LAGE1 was previously analyzed in ESCC patients [10]. After showing the tumor-specific pattern of mRNA expression in the selected CTAs, the cytotoxic HLA-restricted epitopes of MAGE-A4, NY-ESO1 and LAGE1 were selected as the target of interest to synthesize a chimeric sequence [12]. The construct was targeted to the endoplasmic reticulum (ER) by ER entry signal peptide where the MHC class I epitopes are processed. The epitopes are arranged using hydrophobic linkers. Furthermore, the Kozak sequence is located adjacent to the ORF to improve protein expression. HA-tag epitope is used as a reporter epitope for analyzing the protein expression. The chimeric construct was analyzed for mRNA and protein structures using bioinformatics tools. The desired properties were verified by GenScript (NJ, USA), and the multimeric gene was synthesized by ShineGene Molecular Biotech, Inc (Shanghai, China).

### Plasmids and cloning

The chimeric construct was synthesized and cloned in pPCR Script Vector by ShineGene Molecular Biotech, Inc (Shanghai, China). The multimeric gene was then sub-cloned and replaced GFP gene in the pGEM4Z/GFP/A64 plasmid containing the poly(A) template (kindly provided by Dr. E. Gilboa, University of Miami, FA, USA) using HindIII and EcoRI restriction sites.

### In vitro transcription

The pGEM4Z/construct/A64 plasmid was linearized using the SpeI restriction site at the end of poly-A region and then purified with phenol/chloroform extraction. Since the chimeric gene was cloned under the control of T7 promoter, *in vitro* transcription was carried out by T7 RNA polymerase using the mMESAGE mMACHINE kit (Ambion, Austin, TX, USA). The purity and the quality of synthesized capped mRNAs were evaluated by spectrometric analysis and gel electrophoresis.

### Cell lines

Two cell lines were selected to analyze chimeric gene expression. An esophageal (KYSE-30) and a leukemia

(K562) cell line were obtained from the National Cell Bank of Iran, Pasteur Institute of Iran (NCBI, IBI, Tehran, Iran). Cell lines were cultured in a complete RPMI-1640 medium supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 mg/mL), amphotericin B (1.25 mg/mL fungizone) and 10 % fetal bovine serum (FBS). Cells were maintained in a logarithmic phase growth at 37 °C in a humidified atmosphere supplemented with 5 % CO<sub>2</sub>. All cell culture reagents were purchased from Gibco BRL (Gibco BRL Life Technology, Gaithersburg, MD, USA).

### Electroporation

The K562 and KYSE-30 cultured cells in a logarithmic phase growth were selected and washed with RPMI1640. After resuspending of the cells in Opti-MEM (Gibco BRL) medium,  $10 \times 10^5$  cells was mixed with 2–4 µg of transcribed mRNAs in a total volume of 100 µl and electroporated in a 2 mm cuvette (BioRad) at 300 Volt for 500 µs, the optimized parameters for the most efficient transfection [13].

### SDS PAGE and Western blotting

Cellular proteins were prepared using NET cell lysis buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA and 0.5 % Triton  $\times 100$ ). Equal amounts of protein (50 µg) were separated by 15 % SDS PAGE and then transferred to Polyvinylidene fluoride (PVDF) membranes (NY, USA) by electroblotting. The membranes were blocked with 2 % BSA in PBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) for 2 h, and then incubated with human HA-tag [horseradish peroxidase (HRP) conjugated] antibody (GenScript, NJ, USA) for 2 h, at 37 °C. After washing for three times, the proteins were visualized using enhanced chemiluminescence reagent (Santa Cruz). The expression level of chimeric protein was analyzed using LabWork 4.0 program (UVP) and normalized to that of  $\beta$ -actin protein.

## Functional study

### Samples

A 26-year-old healthy male donor and three ESCC patients were enrolled in this study. The patients had not experienced any therapeutic intervention such as chemo- or radiotherapy prior to the surgery. The ethics committee of Mashhad University of Medical Sciences (MUMS) approved the study and all patients formally declared their consent to be enrolled. To extract peripheral blood mononuclear cells (PBMCs) of all cases, 30 ml of blood was

collected. Blood sampling for the patients was done closely (approximately 1 h) before the surgery.

### Generation of dendritic cells

After obtaining of PBMCs from 30 ml of whole blood via Ficoll-Hypaque (Biosera Inc., East Sussex, United Kingdom), the immature DCs were generated from adherent cells and confirmed as previously described [13]. The non-adherent PBMCs were frozen and kept for CTL generation. On the sixth day, the immature DCs were transfected with chimeric mRNAs by electroporation. Induction of cultured DCs maturation was performed with maturation cocktail including IL-1B (10 ng/ml, Invitrogen), IL6 (10 ng/ml, Invitrogen), TNF- $\alpha$  (10 ng/ml, Invitrogen) and PGE2 (10<sup>-7</sup> M, Sigma) 4 h after electroporation. The efficiency of transfection was confirmed as described before [13].

### Fluorescence-activated cell sorting (FACS) analysis of immature DC

To analyze the phenotype of monocyte-derived DCs, immunofluorescence staining of the cells was performed using mouse monoclonal antibodies against differentiation and activation markers including anti-CD1a, CD80, CD83, CD86, FITC-conjugated (fluorescein isothiocyanate), and CD14 PE-conjugated (phycoerytherin) (IQ Company, Netherlands). Appropriate mouse PE/FITC isotype was used as negative control.

### RNA electroporation

On day 6, immature DCs were washed with the Opti-MEM medium (Gibco BRL, Eggenstein, Germany) and transfected with 3 µg of in vitro transcribed chimeric mRNAs per  $10^6$  cells. Transfection was performed as previously explained [13]. Mock DCs were also electroporated without the addition of chimeric mRNAs as negative control.

### Induction of tumor-specific CTL

The T cells were enriched from non-adherent fraction of PBMCs for CTL generation and suspended in RPMI with 10 % fetal calf serum, 25 mm Hepes, L-glutamine, and antibiotics.  $2 \times 10^6$  T cells were combined with  $2 \times 10^5$  transfected matured DCs and cultured in the presence of 10 ng/ml IL-7 at 37 °C in 5 % CO<sub>2</sub> for 8 days. On third day, 20 IU/ml IL-2 was added to the culture. After the first round, re-stimulation of T cells (effectors) was performed with newly transfected DCs for second 8 days period to successfully induce specific CTL against transfected DCs.

## Cytotoxicity test

Evaluation of cytotoxicity was performed using the calcein-AM method, as previously described [13]. Effectors and calcein-labeled targets (DC targets loaded with chimeric mRNAs, the ESCC cell line KYSE-30 and mock DCs as negative control) were co-cultured in 1:1, 3:1 and 9:1 ratios of effector:target (E:T) and measured by fluorescence spectrophotometer (FP-6200, Jasco, Japan; exciting filter:  $485 \pm 9$  nm; band-pass filter:  $530 \pm 9$  nm). Data were expressed as arbitrary fluorescent units (AFU). The cytotoxicity percent of the assay was calculated as described before [13].

## Statistical analysis

The results of cytotoxicity tests were analyzed using the SPSS 19.9 statistical package (SPSS, Chicago, IL) by both unpaired and paired Student's *t* test. *p* values <0.05 were considered statistically significant.

## Results

### Plasmids, subcloning and in vitro transcription

Transcribed chimeric mRNA was visualized as a 433 bp band on electrophoresis gel. Spectrometric and gel electrophoresis analyses confirmed the purity of the in vitro transcribed chimeric mRNAs. The yield of in vitro transcription is approximately 25  $\mu$ g mRNA per reaction for 1  $\mu$ g of digested plasmid.

### Cell lines, electroporation and analysis of chimeric genes expression

Leukemia (K562) and esophageal (KYSE-30) cell lines were selected for analysis of chimeric gene expression. Since dendritic cells of patients are the target cells for ESCC immune-gene therapy, K562 might be the closest known cell line to the DCs in terms of developmentally source. We have applied the previously optimized condition for a highly efficient electroporation of in vitro transcribed mRNA into the K562 cell line [13]. In this study, the introduction efficacy, the percentages of cell viability and successfully transfected viable cells were approximately as same as previous report. The optimized conditions were also reproduced for KYSE-30 cell line, and it was successfully transfected by in vitro transcribed chimeric mRNA. The Western blot analysis was performed on transfected cell lines to identify the fidelity and accuracy of expression of the chimeric protein. The results showed that chimeric protein was strongly expressed relative to the

**Table 1** Clinicopathological characteristics of the enrolled ESCC patients

Patient	Patient 1	Patient 2	Patient 3
Sex	Male	Female	Male
Age	57	51	49
Tumor characteristics			
Size	2 cm	3.5 cm	5.5 cm
Location	Middle	Middle	Middle
Stage	IIa	III	III
Grade	MD*	WD	MD
TNM	T3N0M0	T3N1M0	T3N1M0
<i>N</i> <sub>involved</sub> **	0 of 5	3 of 8	2 of 7
mRNA expression***			
LAGE1	1.47	17.35	4.62
MAGE-A4	14.61	21.79	15.34
NY-ESO1	8.52	0.93	9.37

\* MD represents moderately differentiated and WD represents well differentiated

\*\* Number of involved lymph node metastasis

\*\*\* Tumor/normal fold change of mRNA expression of interested genes. More than twofold is considered as overexpression

housekeeping gene expression. The analysis showed a single sharp immunoreactive band at approximately 14 kDa for recombinant chimeric protein.

## Functional study

### Generation of dendritic cells and FACS analysis of immature DCs

On the fifth day of monocytes culturing, the immunophenotype of the immature DCs was confirmed by detection of specific CD markers including CD1a, CD14, CD80, CD83 and CD86. Based on the FACS results, the majority of monocytes were transformed to DCs as evidenced by a decrease in CD14 to less than 3 % and an increase in CD1a to the average of approximately 96 %. Maturation of DCs was confirmed by an increase in CD83 and CD86 to 69.5 and 73.5 %, respectively, on day 8 (Table 1).

### Generation of in vitro transcribed mRNAs and electroporation

The chimeric mRNA pool was in vitro transcribed and electroporated into the monocyte-derived DCs successfully. We confirmed transfection efficacy and efficiency and high percentage of cell viability after electroporation by transfection of in vitro transcribed GFP mRNA into DCs followed by fluorescent microscopy and FACS analyses, as previously described [13].

Cytotoxicity test

After priming of lymphocytes with chimeric mRNAs-loaded DCs for healthy donor and three ESCC patients, the cytotoxicity of CTLs was tested against DC targets loaded with chimeric mRNAs, the ESCC cell line KYSE-30 and mock DCs as negative control. In the healthy donor, the cytotoxicity percentage against DCs loaded with chimeric mRNAs was escalated as we increased the effector:target ratios, ranging from 22.0 (1:1) to 56.3 (1:9). The results of CTL cytotoxicity against KYSE-30 cell line revealed a lower percentage of cytotoxicity in the same ratios ranging from 11.3 to 34.7. Significant differences were observed between cytotoxicity against targets (DC targets loaded with chimeric mRNAs and the ESCC cell line KYSE-30) and mock DCs ( $p < 0.05$ ).

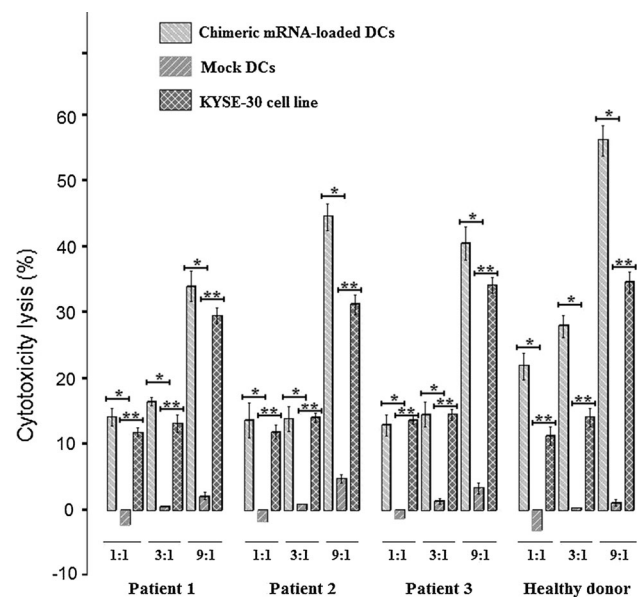
The cytotoxicity of the CTLs was significantly higher in either DCs loaded with chimeric mRNAs or KYSE-30 cell line, compared to mock DCs ( $p < 0.05$ ) in all of the tested ESCC patients. Although the cytotoxicity percentage against patients' DCs loaded with chimeric mRNAs ranged from 13.0 to 44.7 %, there were no significant differences between the cytotoxicity percentages in 1:1 and 3:1 ratios of effector:target in all samples and the cytotoxicity was shown to be significantly higher when the ratio of effector:target was 9:1. Cytotoxicity percentage against KYSE-30 cell line was significantly lower than the DCs loaded with chimeric mRNAs for each patient separately and when the average of the two groups were compared with paired *t* test (Mean  $\pm$  SD;  $19.8 \pm 6.4$  vs.  $34.7 \pm 5.4$ ,  $p < 0.05$ ). The cytotoxicity results of the healthy donor and the ESCC patients are shown in Table 2 and depicted in Fig. 1.

Discussion

The unique capacity of DC to capture, process and present antigens to naive T lymphocytes and induce immune response has made them interesting tools in the field of immunotherapy. A rising number of published clinical studies have been showing that ex vivo generation of antigen-loaded DCs is applicable in a clinical setting and DC vaccines are relatively safe and well tolerated [14–17]. It has been shown that RNA transfection of DCs provides more advantages over transferring other molecules [18–21]. Ex vivo or in vivo loading of DCs, the professional antigen-presenting cells (APCs), with such molecules enhances antigen processing and presentation to CD8 T cells [22] activates both cellular and humoral arms of the adaptive immune system, launching the long-lasting immune response [15]. In addition, DCs can be loaded ex vivo with tumor cell lysates or apoptotic bodies, purified

**Table 2** Results of cytotoxicity assay in different ratios of effector:target cells of the patients

Target cells	Effector:target ratio	Cytotoxicity lysis percentage			
		Patient 1	Patient 2	Patient 3	Healthy donor
Chimeric mRNAs-loaded DCs	1:1	14.2	13.7	13.0	22.0
	3:1	16.5	13.9	14.5	28.1
	9:1	34.0	44.7	40.6	56.3
Mock DCs	1:1	-2.3	-1.8	-1.3	-3.1
	3:1	0.5	0.9	1.4	0.3
	9:1	2.1	4.8	3.4	1.1
ESCC cell line KYSE-30	1:1	11.8	11.9	13.7	11.3
	3:1	13.2	14.1	14.6	14.2
	9:1	29.6	31.3	34.2	34.7



**Fig. 1** Cytotoxicity assay results representing cytotoxic activity of primed CTLs by chimeric mRNA-loaded DCs as effectors against different target cells including chimeric mRNA-electroporated DCs, mock DCs and KYSE-30, an ESCC cell line, at different ratios of effector:target (1:1), (3:1) and (9:1)

TAs, and tumor-derived mRNA, or can be fused ex vivo with tumor cells to elicit immune responses in vivo [7]. Compared to in vivo, ex vivo loading of DCs shows proper stimulation and controlled maturation leading to high degree of specificity in assays [23]. Loading DC with antigen-encoding mRNA is safe and feasible in clinic due to short half-life of mRNA molecules in cytoplasm and lack of their integration into the host genome [24].

The FDA-approved cell-based vaccine Provenge; Dendreon is prepared of autologous PBMCs including professional antigen-presenting cells (DCs) that are activated with a specific antigenic prostate cancer marker



(prostatic acid phosphatase, PSA) fused to the immunostimulant factor GM-CSF [25]. A valuable significant advantage of this therapeutic method is the long-lasting of the induced clinical responses in patients which is shown to have a major impact on patient's survival [26].

Experiences with Provenge indicate that specificity of therapeutic target is critical in immunotherapy so that activation of DCs with specific immunogenic tumor antigen which is expressed in a majority of tumor cells can prime effective CTLs against the cancer cells continuously. Indeed, the first and very important step in immunotherapy is the molecular identification of specific antigens of human cancers and using such molecules in antigen-specific-based immunotherapy can improve clinical progress and development of the method [15].

It has been shown that DC transfection with total tumoral mRNA encoding tumor antigens could establish tumor-specific cytotoxic T Lymphocytes [13]. Loading total tumoral mRNA decreases the probability of immune escape via polyclonal activation of T cells against varied range of tumor-specific antigens and minimizes the consequences of antigen loss in mutant tumor cell clones. However, there is always a concern for presentation of self normal antigens which could break the self-tolerance and stimulate the immune system against normal antigen. Alternatively, a tumor-specific antigen may be able to similarly induce immune response while there would not be a risk of stimulating the immune response toward self.

It has been shown recently that CTAs MAGE-A4, NY-ESO1 and LAGE1 are specifically and frequently overexpressed in ESCC [10, 27, 28]. MAGE-A4 is identified as a new tumor-specific molecular biomarker that is not only significantly overexpressed in the majority of ESCC patients, but also is associated with the development of tumors through advanced stages, playing a probable oncogenic role in ESCC tumorigenesis. Furthermore, the expression of MAGE-A4 is correlated with the levels of LAGE1 and NY-ESO1 expression and their significant clinical consequences suggested a possible functional interaction between these CTAs [10]. Due to immunogenicity of CTAs in cancer patients and their restricted pattern of expression, CTAs are the favorable candidates for cancer vaccine [29] and a combination of the mentioned CTAs could be a potent target for designing a polyvalent cancer vaccine for ESCC immunotherapy. We designed and synthesized a chimeric gene by arranging the selected HLA-restricted epitopes of MAGE-A4, NY-ESO1 and LAGE1 for MHC class I [12]. The construct was targeted to ER where the MHC class I epitopes are processed. This polytope molecule was designed and configured based on *in silico* techniques. The epitopes were linked together by hydrophobic linkers and the structure of the chimeric gene, related mRNA, translated protein and also their stabilities

were analyzed. Furthermore, the solvent accessibility and posttranslational modifications of the protein, the cleavage sites, T cell epitopes and MHC-binding affinity of peptides in the chimeric protein were predicted. This *in silico* approach defined solubility, immunogenicity and accessibility of the new combination of immunogenic epitopes of CTAs and presented a new vehicle to develop a chimeric gene as an effective structural model for cancer immune-gene therapy. To evaluate the potential applicability of the designed chimeric construct as a target for immunotherapy, here we showed how DCs loaded with such chimeric mRNAs could prime-specific CTLs response in ESCC patients.

In this study, we showed that the chimeric mRNA-loaded DCs are capable of priming CTLs effectively and induce cytotoxicity against tumor. The cytotoxicity was even more significant than previous design of loading DC with total mRNA [13]. The new concept of loading the DCs with a chimeric mRNA of tumor-specific antigens has more advantages over the other targets like total mRNA. Loading DCs with a homogeneous pool of a single chimeric molecule enriched with immunogenic epitopes of highly overexpressed mRNAs can, more effectively, present the immunogenic epitopes. This homogeneous pool provides a huge amount of chimeric molecules that can be uniformly expressed and allows the DCs to encounter a high level of chimeric protein and eventually present them effectively. In tumoral mRNA-loaded DCs, the amount of mRNAs depends on the number of the sourced tumor cells and due to heterogeneity of gene expression in tumor cells, it will be very heterogeneous with various amounts of different antigenic mRNAs.

Furthermore, *in vitro* synthesized chimeric immunogenic mRNAs warrants a higher quality of transfected mRNAs. This happens when the pool of mRNAs is uniform in quality without any artifact or unwanted mRNA molecules. Such quality cannot be achieved by loading DCs with total tumoral mRNAs. These differences in quality and quantity of expression and presentation of tumor-specific antigens in chimeric mRNAs-loaded DCs could have a critical impact on CTLs priming and activity (as the results show).

Our results showed that CTLs which are primed with chimeric construct-loaded DCs can lyse the electroporated DC target cells better than the ESCC cell line, KYSE-30. DCs are professional APCs that will present antigens to the immune system more effectively than KYSE-30 cells. Furthermore, the expression of MHC molecules in tumoral cell lines are decreased significantly to escape of cells from immune system [30] and this may explain lower cytotoxicity of CTLs facing cell lines compared to DCs.

In conclusion, we demonstrated a new approach to use DC-based vaccine for immuno-gene therapy of ESCC.

CTA profiling in ESCC patients allows revealing highly specific overexpressed antigens and subsequently state of the art in silico techniques provide us with a powerful tool to design an effective chimeric construct comprising highly immunogenic epitopes of such CTAs which are confirmed to be capable of inducing immune response. We are introducing a novel construct that our functional study showed can stimulate and induce an effective immune response in ESCC patients. Loading dendritic cells with chimeric epitopes of highly immunogenic antigens, such as cancer-testis antigens, are potentially interesting and effective therapeutic modalities for immunotherapy of ESCC and will be considered in future studies to design a cancer vaccine.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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