Articles

Glypican3 in genetically modified human monocyte-derived dendritic cells induced specific cytotoxity against glypican3 overexpressing human hepatocellular carcinoma cells in vitro

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ABSTRACT

الأهداف: من أجل التحول الجيني لمستضد الجين المحدد الجديد (GPC3) للخلايا البشرية (HCC) إلى (DCs)، ومن أجل مراقبة أثر السمية العضوي المحرض بواسطة الجين المعدل (DCs) مقابل الخلايا البشرية (HCC) في (HepG2).

الطريقة: أجريت هذه الدراسة في جامعة الصين الطبية – الصين، خلال الفترة ما بين سبتمبر 2007م وحتى فبراير 2008م. كان تصميم الدراسة من أجل تعديل الجين (DCs) مع الجين (GPC3)، على أن يتم استعماله لتفعيل الخلايا البشرية التائية (T cells) ولاستنباط استجابة تحصين الخلايا المتوسطة مقابل الجين (HepG2) في الخلايا العضوية. تم تحديد ظهور الجين (GPC3) بواسطة اللطخة الغربية وكيمياء الخلايا المناعية. تم اختبار استجابة تشعب الخلايا والتسميم مقابل الجين (HepG2) بواسطة طريقة (WST–1) و (LDH) على التوالي. تم اكتشاف فرز (IFN–γ) بواسطة طريقة إليسا (ELISA).

النتائج: أكدت طريقتي اللطخة الغربية وتحليل كيمياء الخلايا المناعية صحة تحول الجين (GPC3). كان تعديل الجين (GPC3) إلى (DCs) أساسي في تحريض استجابة تشعب الخلايا وإنتاج (IFN–γ). بلغت نسبة التسميم لدى المجموعة التي تحول الجين لديها (GPC3) إلى (38.90±0.95%) (DCs) عند معدل (30.83±1.24%) (E/T: 100:1) عند معدل (E/T: 50:1) و (23.84±0.65%) عند معدل (E/T: 10:0) على التوالي، وتعتبر نسبة ملحوظة بالمقارنة مع المجموعات الأخرى (*p*<0.001). أظهر تعديل الجين (GPC3) إلى (DCs) القدرة على تحريض تسميم محدد عالي مقابل الجين (HepG2) في الخلايا العضوية.

خاتمة: حفزت الخلايا المتأثرة مع الجين (DCs) الذي حولناه مع بلازميد (pEF–hGPC3) الإمكانية في التأثير بفعالية على ظهور (GPC3) وخلايا (HepG2)، الذي يرجح وجود كمون أساسي لدى التولد الجيني للجين (DCs)، ويخدم كتحصين مناعي للخلايا البشرية (HCC).

Objective: To transduce the new hepatocellular carcinoma (HCC) specific antigen gene glypican3 (GPC3) into dendritic cells (DCs) and to observe the in vitro cytotoxic effect induced by the genetically modified DCs against the hepatocellular carcinoma cell line (HepG2).

Method: This study was performed in China Medical University Shenyang, China from September 2007-February 2008. The design of the study was to modify DCs with GPC3 and to be used to activate human T cells and elicit a cell-mediated immune response against HepG2 in vitro. The GPC3 gene expression was identified by western blot and immunocytochemistry. The proliferation of responder cells and cytotoxicity against HepG2 were examined by water-soluble tetrazolium salt -1 and lactate dehydrogenase assay respectively. The interferon γ (IFN-γ) secreted was detected by ELISA assay.

Results: Both Western blot and immunocytochemical analysis assured the validity of GPC3 transfection. Glypican3 modified DCs were potent in inducing responder cells proliferation and IFN-γ production. The cytotoxicity in the group of GPC3 transfected DCs were (38.90±0.95%) at the ratio of effector cells/target cells E/T:100:1, 30.83±1.24% at the ratio of E/T:50:1, and 23.84±0.65% at the ratio of E/T:10:1, respectively (which is significant compared with other groups, $p<0.001$). And the GPC3 modified DCs showed ability to induce high specific cytotoxicity against HepG2 in vitro.

Conclusion: The effector cells stimulated with DCs that were transfected with pEF-hGPC3 plasmid could effectively lyse GPC3 expressing HepG2 cells, which suggested that those genetically engineered DCs have the potential to serve as novel vaccine for HCC.

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Hepatocellular carcinoma (HCC) is one of the major malignancies worldwide. Most of the HCC patients are inoperable at the time of diagnosis. Despite several palliative therapeutic options, there is a high rate of recurrence or of intra-hepatic metastases. Therefore, novel treatment strategies such as immuno-gene therapy are necessary to decrease the frequency of tumor recurrence. Dendritic cell (DC) represents the class of antigen presenting cells that initiates most immune responses. And the unique efficiency at capturing, transporting, and presenting antigen, and at attracting and activating specific T cells, make the mature DCs the most potent APCs known. It is vital to know the fact that DCs can present tumor-associated antigens in the context of both major histocompatibility complex class I and II, in this way, they can stimulate and activate not only cytotoxic T lymphocytes (CTLs) but also the helper T cells.[1-3] People have paid much attention to the problem of how, and what antigens should be pulsed to the DCs. Up to now, DCs pulsed with tumor-associated antigens by various means, including whole cell lysate, peptides, proteins, RNA, or DNA, have been studied for antitumor effects in experimental tumor models. These preliminary experiments, immunization with tumor antigens presented by DCs has shown much promise in effectively priming the cellular immune response. Furthermore, some clinical applications using DC-based tumor vaccines have been reported.[4,5] Although anti-tumor cellular immune responses could be induced by DC vaccination in all patients, clinical objective responses were limited in tumor models. Thus, a more effective method of DC-based tumor vaccines is expected to improve the clinical effectiveness of treatment. In this regard, we try to grasp a method of inducing more effective anti-cancer immunoreactions with minimal side effects. Then we exerted glypican3 (GPC3) modifications of DC for presenting tumor antigens to T cells so as to produce specific CTLs against HCC. Glypican3 potentially behaves as an oncofetal liver protein, over expression of GPC3 is detected in fetal liver, whereas, it is absent in normal adult liver, benign hepatocellular nodules, including cases of focal nodular hyperplasia and adenoma.[6] And some researches have reported that GPC3 was over expressed in most types of HCC.[7-11] By comparing AFP and GPC3 immunostaining in the different groups of HCC, Wang et al,[12] could confirm that GPC3 is more sensitive, especially in the group of HCC, which developed from cirrhosis. In general, oncofetal proteins do not seem to play a critical role in tumor progression, but they may be used as tumor markers or as targets for immunotherapy. Komori et al,[13] reported that they had identified the HLA-A2- or HLA-A24 restricted CTL epitopes possibly useful for GPC3 specific immunotherapy of HCC and raised the possibility that some GPC3 peptides may be applicable to cancer therapy. In this study, we made DCs present the whole GPC3 molecules to T cells to generate CTLs against HCC with more epitopes including both major and minor histocompability antigens, regardless of human leukocyte antigen (HLA) haplotypes.

Methods. This study was conducted from September 2007 - February 2008, and the experiments involved in this study were performed in the Pathology Laboratory of China Medical University, Shenyang, China. The study protocol was approved by the Medical Ethical Committee of our institution. We obtained informed consent from all donors. Peripheral bloods were obtained from HLA-A2 positive healthy people. The exclusion criteria were HLA-A2 negative people and people with acute infection, tumor, diseases of immune system, and serologically positive for hepatitis B or C viruses. Hepatocellular carcinoma cell line (HepG2) was maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 100units/mL penicillin, and 100mg/mL streptomycin, referred to as complete medium. Hepatocellular carcinoma cell line is HLA-A2 positive. Recombinant human cytokines granulocyte/macrophage colony-stimulating factor (rhGM-CSF), interleukin-4 (IL-4), tumor necrosis factor-a (rhTNF-a) (all from PEPRO TECH, New Jersey, USA), prostaglandin E2 was purchased from Genzyme Tech, Minnesota, USA. WST-1 was obtained from Shanghai Institute of Biochemistry, Chinese Academy of Science. Enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D, Minnesota, USA. The pEF-hGPC3 plasmids were generous gifts from Dr. Jorge Filmus (Toronto University, Canada). Dendritic cells were generated from peripheral blood mononuclear cells (PBMCs), as described previously,[14] with some modifications. Briefly, PBMCs were isolated from 60 mL of peripheral blood using a Ficoll-pague gradient (Haoyang, China). The cells were resuspended in culture medium using RPMI 1640 supplemented with 2 mM L-glutamine, 100U/mL penicillin, 100 mg/mL streptomycin, and 10% FCS, and allowed to adhere into 6-well plates. After 2 hour of incubation at 37°C, non-adherent cells were transferred to another plate as responder cells and adherent cells were cultured in 2 ml of culture medium supplemented with 100 ng/mL granulocyte-macrophage colony stimulating factor (GM-CSF) and 50 ng/mL IL-4. Fresh medium containing cytokines was substituted every other day. After 5 days, transfections with plasmids of pEF-hGPC3 and empty vector were then performed. Briefly, we remove the culture medium and supplemented with 4 μg of plasmids supernatant with 10 μl Lipofectamine 2000, 100 ng/ml rhGM-CSF and 50 ng/ml rhIL-4. Twenty four hours later, the plasmids were removed and the cells were resuspended with general DC medium including cytokines. Then after more 2d, nonadherent cells can be harvested and used as stimulators. To confirm the

validity of transfections, cytospin preparations of GPC3 transfected DCs were fixed in 4% paraformaldehyde, and incubated overnight with primary mouse monoclonal antibody detecting GPC3 (Santa Cruz, USA), following 5% rabbit serum treatment at 37°C for one hour. After which they were incubated with second antibody and streptavidin-peroxidase complex for 30 minutes. Lastly, they were visualized with diaminobenzidine (DAB) (both from Maixin, China). Hepatocellular carcinoma cells were used as positive controls for GPC3, and negative controls were prepared by non immune rabbit immunoglobin G (IgG) at the same dilution as for the primary antibody. Cells with brown particles appearing in membrane or cytoplasm were regarded as positive cells.

Furthermore, cells were lysed in appropriate amounts of lysing buffer containing 150mM sodium chloride (NaCl), 50 mM Tris, pH 7.4, 1% Nonidet P-40, one mM sodium orthovanadate, one mM ethylenediamine tetraacetic acid, and one mM phenylmethanesulphonylfluoride. Supernatant of the lysate was electrophoresed on 10% sodium dodecyl sulfate -polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene diflouride membrane. After blocking with 5% skimmed milk and 0.2% Tween20 in Tris-buffered saline, the membrane was incubated with rabbit polyclonal anti-GPC3 and anti-β-actin antibody (both from Santa Cruz, USA), washed extensively with 0.2% Tween20 in Tris-buffered saline, and subjected to chemiluminescence detection using goat anti-rabbit IgG (Zhongshan, China), using a DAB kit. The autogeneic mixed lymphocyte reaction (MLR) was performed, and for all assays the responder cells described were adjusted at the concentration of one x 105 cells per well. Dendritic cells which had been matured by culturing with TNF-α (50ng/mL) and prostaglandin E2 (one μg/mL) for 48 hours were treated with 25μg/mL mitomycin C (Sigma-Aldrich, Los Angeles, USA) at 37°C for 30 minutes and subsequently washed 3 times in culture medium before plating. Dendritic cells were mixed with responder cells at the various DC-to-responder ratios. Dendritic cells cultured in the absence of responder cells served as controls for background proliferation. Cells in triplicate wells of a 96-well round bottom plate were cultured for 2d, and subsequently pulsed with 20 μL water-soluble tetrazolium salt -1 (WST-1) during the last 4 hours of culture. The absorbance for measuring wavelength (450 nm) and the reference wavelength (650 nm) were measured. All samples were measured against a background control as a blank. Stimulator index (SI) was calculated following the formula of

SI= optical density (OD) experiment/(OD responder cells+ OD stimulator cells).

And Interferon-γ (IFNγ) assay was performed on the supernatants by a commercially available ELISA kit. Each day, CTL-generating cultures were reefed. The supernatants of cultures were harvested. Each assay was performed according to the manufacturer's instructions. The lower detection limit was 8.0 pg/mL. All samples and standards were run in triplicate.

Moreover, the stimulated responder cells were used as effector cells in the CTL assay using lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche, USA). The HepG2 was used as target cells in the assay. Briefly, target cells and effector cells were resuspended in assay medium (RPMI 1640 with 1% bovine serum albumin), and then target cells (104 cells per well) were cultured with effector cells at different ratios in 96-well at round bottom plate at 37°C. After 5 hours of incubation, the culture plates were centrifuged and the supernatant (100 μl per well) was transferred to another ELISA plate. Hundred microliter per well and LDH detection mixture was then added and incubated in the dark for 30 minute at room temperature. After adding 50 μl stop solution per well, the absorbance of the samples was measured at 490 nm with 650 nm as reference wavelength. The spontaneous release of LDH by target cells or effector cells was assessed by incubation of target cells in the absence of effector cells and vice versa. The maximum release of LDH was determined by incubation of target cells in 1% Triton X-100 in assay medium. The percentage of specific cell mediated cytotoxicity was determined by the following equation:

specific cytotoxicity (%) = [(effector and target mixture - effector spontaneous - target spontaneous)/ (maximum – target spontaneous)] x100.

Student's t test was used to compare differences in responder cells proliferation, IFNγ secretion, and CTL reactivity against HepG2. Statistical significance was defined as $p<0.05$.

Results. Under the phase contrast microscope, we found similar morphological characters in the DCs transfected with pEF-hGPC3 plasmid, and we carried out immunocyte-chemical staining to confirm that DCs transfected with pEF-hGPC3 expressed the target antigen of GPC3 (**Figures 1a & 1b**). Western blot showed a successful transfection with a high efficiency. The GPC3 expression was detected by the polyclonal antibody in DCs transfected with pEF-hGPC3 (**Figure 2**). The proliferation of responder cells was measured by WST-1 assay, and the SI values were compared statistically among the respective groups. The proliferation of effector cells stimulated by GPC3 modifying DCs was not significantly different in comparison with other groups at any ratios of DC/R ($p>0.05$, **Table 1**).

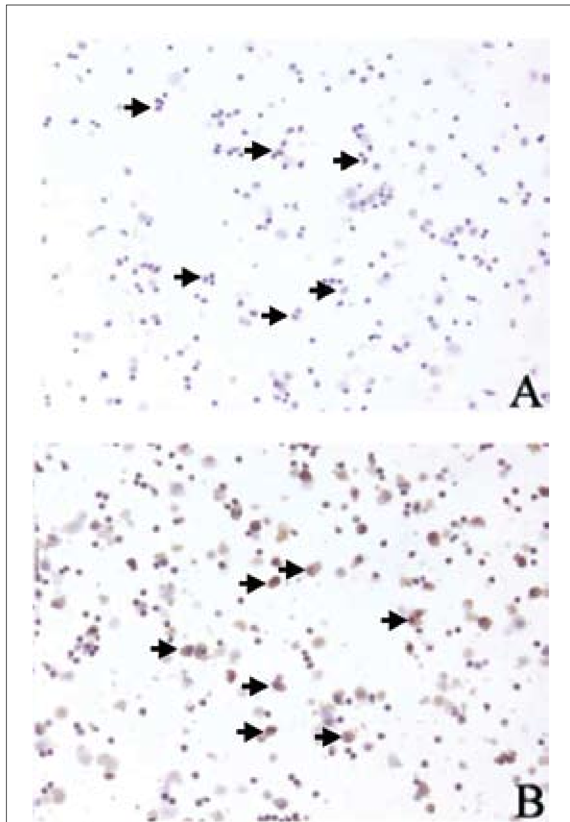

Figure 1 - After 6 days culture of adherent peripheral blood mononuclear cells with granulocyte-macrophage colony stimulating factor and interferon-γ, immature dendritic cells were transfected with pEF-GPC3 plasmid for 48 hours. Cells were stained with anti-glypican3 antibody. The intense cytoplasma or membrane staining were detected in pEF-hGPC3 transfected immature dendritic cells (DCs), which was indicated with arrowheads. While no immunoreactivities were observed in the vector transfected DCs, which was indicated with arrows. a) Empty vector transfected DCs, b) pEF-hGPC3 transfected DCs. Original magnification, x400.

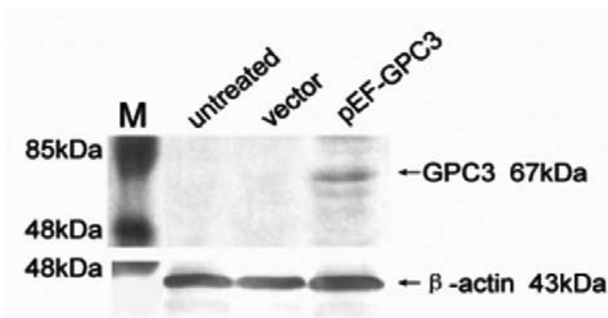

Figure 2 - Western blot analysis of glypican-3 (GPC3) expression in dendritic cells (DCs) transfected with empty vector and pEF-GPC3 (lanes 2-3). The untreated DCs was included as a normal control (lane 1). The data shown are representative of 3 independent experiments for each cell population. M - marker.

Table 1 - Autologous responder cells proliferation stimulated by mature Dcs.

Groups	Stimulator index			
	GPC3 modified	Empty vector	DC alone	*P*-value
1:10 (DC : R)	3.25 ± 0.33	3.11 ± 0.19	3.09 ± 0.20	0.702
1:50 (DC : R)	2.08 ± 0.13	2.09 ± 0.43	1.94 ± 0.78	0.924
1:100 (DC : R)	1.20 ± 0.20	1.12 ± 0.20	1.37 ± 0.50	0.650

DC: R means DC - responder cell. The results are represented as mean±SD of 3 wells. The proliferations of responder cells exposed to vector and pEF-GPC3 transfected DCs were examined by WST-1 assay. The DCs untreated was included as a normal control. GPC3 - glypican3, DCs - dendritic cells

Table 2 - Interferon-γ secretion in MLR supernatants after different cell population stimulation.

Groups	IFN-γ concentrations in supernatant (pg/mL)			
	GPC3 modified	Empty vector	DC alone	*P*-value
1:10 (DC:R)	516.78±25.86	484.31±22.84	505.22±27.08	0.348
1:50 (DC:R)	259.04±52.33	227.12±26.87	238.24±8.01	0.640
1:100 (DC:R)	183.62±40.27	197.69±35.84	190.69±12.04	0.867

DC: R means DC: responder cell. The results are represented as mean ± SD of 3 wells. GPC3 -glypican3, DC - dendritic cell, IFN-γ - interferon-γ, MLR - mixed lymphocyte reaction

Table 3 - The cytolytic reactivity of effector cells exposed to different groups of DCs were assessed by LDH assay.

Group	Cytotoxicity against HepG2 (%)			
	GPC3 modified	Empty vector	DC alone	*P*-value
10:1 (E:T)	20.02 ± 4.00*	8.45 ± 2.25	7.10 ± 3.24	0.005
50: 1 (E:T)	35.36 ± 5.34*	12.12 ± 3.44	13.84 ± 2.84	0.001
100: 1 (E:T)	37.51 ± 1.77*	11.72 ± 2.15	14.71 ± 4.38	0.000

E:T means effector cells - target cells *$p<0.001$. The cytolytic reactivity of effector cells exposed to vector and pEF-GPC3 transfected DCs were assessed by LDH assay. Cytotoxicity of mature DCs-derived CTLs against HepG2 cells. The results are represented as mean ± SD of 3 wells. The DCs untreated was considered as a normal control. HepG2 - hepatocellular carcinoma cell line, GPC3 - glypican3, DC - dendritic cell, LDH - lactate dehydrogenase,

Moreover, due to IFN-γ is a Th1-associated cytokine critically involved in the development of cell-mediated immune response, we also analyzed the IFN-γ production in an in vitro recall response to DCs from the responder cells. The IFN-γ secretion was measured by ELISA assay, and the concentrations of IFN-γ from supernatants were compared statistically among the respective groups ($p>0.05$, Table 2). The concentrations of IFN-γ in group of GPC3 modifying DCs were not significantly different in comparison with that of other groups at any ratios of DC/R. We selected HCC

cell line HepG2 as targets for GPC3-specific CTLs, which exhibits expression of GPC3. The cytotoxicity of effector cells against HepG2 cells was measured by LDH assay, and the specific cytotoxicity in groups of HepG2 lysate pulsed and GPC3 transfected DCs were showed in **Table 3**. And the results demonstrated that effector cells primed by DCs transfected with GPC3 could lyse GPC3 expressing HepG2 cells dramatically ($p<0.001$).

Discussions. The use of DC vaccines is becoming increasingly widespread and currently in the forefront for cancer treatment. Improved understanding of the requirements for T cell activation and the special roles of DC in antigen presentation and evolution of new vaccine technologies, however, has begun yielding more encouraging outcomes.[15,16] Dendritic cell precursors can be readily isolated from the peripheral blood and expanded in vitro to large numbers with the appropriate cytokines.[17,18] Such DCs have been loaded with tumor antigens by a variety of means, including transfection, infection with recombinant viruses, incubation with the tumor protein or peptide. The optimal way of making DCs stimulate and activate T cell to induce effective antitumor response with minimal side effect remains to be clarified. Considering effective immune target for tumor immunotherapy, it is vital to choose a tumor antigen that could not be lost by tumor cells through immuno editing.[19] Some researchers reported that GPC3 is involved in the carcinogenesis of HCC via control of noncanonical A name of cell signal transduction Wnt signals.[20] Moreover, Komori et al[13] reported that almost all HCC cells expressed HLA-class I molecules as far as they could examine. For these reasons, we think that GPC3 is a very useful as a target tumor antigen for the immunotherapy of HCC. Glypican-3 belongs to the family of heparan sulfate proteoglycans that are linked to the exocytoplasmic surface of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. The size of the core proteins of glypican is similar (60 to 70kDa), and, as expected, they all contain an N-terminal secretory signal peptide and a hydrophobic domain in the C-terminal region required for the insertion of the GPI anchor.[21] And it is a new sensitive and specific immunohistochemical marker of HCC,[12, 22, 23] and may be regarded as a target for immunotherapy.[24] Several studies have revealed that DCs pulsed with weakly immunogenic tumor associated antigen (TAA) are capable of stimulating TAA-specific CTL proliferation and cytotoxicity in vitro and in vivo and thus it represent new means for designing tumor-specific vaccines.[25-27] Moreover, some studies have shown that DCs that ingest apoptotic tumor cells or pulsed with tumor cell lysate could also effectively generate tumor-specific T-cell response in vitro when mature.[1,28] However, there are few reports about the differences among those ways of manipulating DCs.

In this article, we regarded GPC3 as the target for immune attack. To investigate the potential efficacy of this modification of DC in vitro, we used GPC3, the HCC specific antigen, to modify DC and tested the ability of stimulating and activating T cells, and inducing specific CTL against HepG2. The data show that there were no significant differences among respective groups in stimulating and activating T cell in vitro. And the results revealed that DCs transfected with GPC3 gene could induce potent and relatively more specific cytotoxicity against HepG2 than those of DCs alone, empty vector transduced DCs. Based in these findings, we speculated that although mature DCs have the ability of inducing effective antitumor immunity, its effectiveness is based on the existence of tumor antigen, and through full-length GPC3 gene modifying DCs, multiple GPC3 peptides are presented on HLA class I molecules and recognized by autologous CTLs, which can overcome the limitations of peptide-based vaccines,[13] such as a priori knowledge of the patient's HLA haplotype to select appropriate peptides compatible with that particular haplotype. Loading DC with the entire protein has the advantage that peptides binding to a broad set of HLA molecules can be processed from the protein. However, the protein is delivered in limited amounts, resulting in a transient presentation of antigenic peptides, mostly presented on HLA class II molecules. Genetic modification of the DC ex vivo, using an entire Ag-coding sequence could avoid the drawbacks of pulsing tumor lysate, which was consistent with other reports.[29,30] Therefore, it may permit a more potent immune response involving the presentation of epitopes from all possible restriction elements.

We considered that more HCC and normal liver cell lines should be included in this study besides HepG2 to confirm the specificity and availability of cytotoxicity promoted by GPC3-DCs on HCC cells. However, it was a preliminary study to investigate the effects of GPC3 transfected human peripheral blood-derived DCs on GPC3 overexpressing HepG2 cells, which has been demonstrated a specific cytotoxicity. Furthermore, animal experiments should also be performed to confirm and provide a new strategy of the use of GPC3-DCs in vivo. Therefore, the cytotoxicity induced by GPC3-DCs is required further investigations, and we will engage in this field further. Taken together, our study indicate that monocyte-derived human dendritic cells modified with full-length cDNA of GPC3 gene can generate GPC3-specific CTLs whose lytic activity against GPC3 overexpressing target cell, HepG2 cell line. Our data offer a new and potentially valuable option of GPC3-based immunotherapy for HCC.

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