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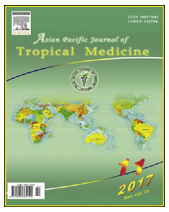

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Glypican-3-specific cytotoxic T lymphocytes induced by human leucocyte antigen-A*0201-restricted peptide effectively kill hepatocellular carcinoma cells *in vitro*

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ABSTRACT

Objective: To investigate potential human leucocyte antigen (HLA)-A2-restricted epitope peptides of glypican-3 (GPC3) and determine the cytotoxicity of peptide-specific cytotoxic T lymphocytes (CTLs) against hepatocellular carcinoma (HCC) cells.
Methods: The potential HLA-A*0201-restricted GPC3 peptides were screened using computer algorithms, T2 cell-binding affinity and stability of peptide/HLA-A*0201 complex assay. The peptide-specific CTLs were generated and their cytotoxicity against GPC3$^+$ SMMC 7721 and HepG2 cells was detected using IFN-γ based enzyme-linked immunospot and lactate dehydrogenase release assays *in vitro*.
Results: A total of six peptides were identified for bindings to HAL-A2 and the GPC3 522–530 and GPC3 229–237 peptides with HLA-A*0201 molecules displayed high binding affinity and stability. The CTLs induced by the GPC3 522–530 or positive control GPC3 144–152 peptide responded to the peptide by producing IFN-γ, which were abrogated by treatment with anti-HLA-A2 antibody. The GPC3 522–530-specific CTLs responded to and killed SMMC 7721 and HepG2 cells, instead of GPC3-silenced SMMC 7721 or HepG2 cells. GPC3 522–530-specific CTLs response to HCC cells was blocked by anti-HLA-A2 antibody.
Conclusions: The GPC3 522–530 peptide contains antigen-determinant and its specific CTLs can effectively kill HCC in a HLA-A2-restricted and peptide-dependent manner. Our findings suggest that this peptide may be valuable for development of therapeutic vaccine.

1. Introduction

Hepatocellular carcinoma (HCC) is a common human cancer with high mortality and its incidence is increasing worldwide [1]. In China, HCC remains the leading cause of malignancy-related mortality in Chinese men younger than 60 years old [2]. Although the advances in surgical techniques and medical treatment have improved the survival of HCC patients, many HCC patients survive less than 5 years [3]. Hence, development of novel therapies for treating of HCC is urgently needed. Antigen-specific immunotherapies, such as antigen-based vaccines, antigen-specific chimeric antigen receptors T cells are promising and some therapeutic strategies have been testing in clinical trials. Antigen-based immunotherapies have several advantages, specifically recognizing and killing tumor cells, immune memory to prevent the recurrence of cancer, and relative less systemic side effects [4]. Accordingly, the key for development of antigen-specific immunotherapies is to discover cellular surface antigen-determinants recognized by CD8$^+$ T cells. However, there is little information on which peptides contain determinants of the tumor-specific surface antigen presented by a specific HLA-I molecule on HCC.

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Glypican-3 (GPC3) is predominantly expressed in HCC cells and some other human cancer tissues, including melanoma, but negatively expressed in normal tissues, except for embryonic liver and the placenta [5,6]. Hence, GPC3 may be an attractive tumor-associated antigen for antigen-specific cancer immunotherapy. Previous studies have shown that GPC3 144–152 peptide contains a HLA-A0201-restricted antigen determinant and can induce antigen-specific cytotoxic T lymphocytes (CTLs) that effectively kill GPC3$^+$ HCC and ovarian cancer in a HLA-A2-restricted fashion [7,8]. A recent study indicates that another 13 peptides may bind to HLA-A2 [9]. However, there is no information on whether these GPC3 peptides contain antigen determinants that induce antigen-specific CTL response *in vitro*. Given that MAGA-A10, EPS8 and nectin-4 peptide-specific CTLs can target antigen-expressing tumor cells [10–12], it is important to validate these peptides.

In this study, we employed several online tools to predict the potential GPC3 antigen peptides that bound to HLA-A2 and validated their binding by T2 cell-based binding assays. Furthermore, we generated GPC3 522–530 peptide-specific CTLs and tested their cytotoxicity against peptide-loaded T2 cells and GPC3$^+$ HCC. Our data demonstrated that GPC3 522–530 (FLAELAYDL) bound to HLA-A*0201 molecule and induced CTLs that effectively killed HCC in a HLA-A2-restricted manner. Therefore, GPC3 522–530 peptide may be valuable to develop peptide-based vaccine for intervention of GPC3$^+$ and HLA-A*0201$^+$ HCC.

2. Materials and methods

2.1. Prediction of GPC3-derived HLA-A*0201-binding peptides

The sequence of human GPC3 protein (580-amino acid, P51654.1) was obtained from NCBI and their 9-mer HLA-A2-restricted peptides were predicted using the supermotif algorithm (SYFPEITHI) and the quantitative motif algorithm (BIMAS). The candidate epitope peptides were selected using the NetCTL epitope prediction methods. The peptide GPC3 144–152 (FVGEFFTDV) was designated as a positive control [7]. The GPC3-derived 9-mer peptides and positive control peptide were synthesized and purified by Sangon Biotech, Shanghai, China. Individual peptides with a purity of >95% were used in our experimental system.

2.2. Affinity and stability of candidate peptides

The affinity of individual synthesized GPC3 peptides to HLA-A*0201 molecules was characterized by the T2 cell-binding assay [11,13]. T2 cells were bought from American Type Culture Collection and characterized by HLA-A*0201-positive and TAP-deficient. Briefly, T2 cells were treated with, or without, each peptide (100 μmol/L) in the presence of β2 microglobulin (3 μg/mL, Sigma) in serum-free RPMI 1640 medium with 5% CO_2 at 37 °C for 24 h. After being washed, the cells were treated with anti-HLA-A2 (BB7.2, Biolegend), followed stained with FITC-labeled goat anti-mouse IgG (Cell Signaling Technology). Finally, flow cytometry analysis (FACScan; BD Biosciences) was carried out. The affinity of each peptide was evaluated by fluorescence intensity (FI), as follows:

FI = (mean FI with a peptide − mean FI without a peptide)/mean FI without a peptide.

FI > 1.5, 1.0 < *FI* < 1.5, and *FI* < 1.0 indicated a high, moderate, and low binding affinity to HLA-A*0201 molecules, respectively.

Next, we assessed individual peptides/HLA-A*0201 complex stability using DC50 (dissociation complex 50). We cultured T2 cells for 1 h in the presence of individual peptides (100 μmol/L) and Brefeldin A (10 μg/mL, Sigma). At 0, 2, 4, 6 and 8 h, the cells were washed and the remaining peptide binding to cells was detected by flow cytometry. DC50 of each peptide was calculated as the time needed to dissociate 50% HLA-A*0201/peptide complex.

2.3. Induction of antigen-specific CTLs

Each volunteer signed a written informed consent and the Ethics Committee of the First Affiliated Hospital of Hainan Medical College approved the experimental protocol. Peripheral blood mononuclear cells (PBMCs) were obtained from HLA-A*0201$^+$ young male healthy individuals. PBMCs were separated by Ficoll–Paque density gradient centrifugation and their HLA-A2 expression was identified using flow cytometry after stained by BB7.2. PBMCs (3×10^6 cells/mL) were incubated in RPMI 1640 medium for 90 min at 37 °C with 5% CO_2. After washed out non-adherent cells, the adherent cells were treated with 50 ng/mL of IL-4 and 100 ng/mL of recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) for 5 d to induce monocyte-derived dendritic cells (DCs). The cells were treated with 1 000 U/mL of TNF-α for 2 d to activate DC maturation. The phenotype of activated DCs was analyzed by flow cytometric analysis. The DCs (2×10^4 cells/well) were loaded with each GPC3 peptide (20 μg/mL) and β2 microglobulin (3 μg/mL) for 4 h at 37 °C. Then, we isolated CD8$^+$ T cells from the non-adherent cells using the Dynal CD8$^+$ isolation kit (Invitrogen). To elicit peptide-specific CTLs, GPC3-derived peptide-loaded DCs were co-cultured with CD8$^+$ T cells at a ratio of 10:1 in RPMI 1640 medium supplemented with IL-2 (20 IU/mL) for 7 d. Then the CD8$^+$ T cells were stimulated using the above peptide-loaded DCs for another two cycles. The generated peptide-specific CTLs were harvested for the following experiments.

2.4. Silencing of GPC3 gene in HCC cells

Three different GPC3-specific siRNAs and one negative control siRNA were designed, according to the *GPC3* gene sequence (GenBank accession no. NM_004484.3), and synthesized by Shanghai Sangon Biotech. The sequences of siRNAs were GPC3-siRNA-714 (5′-CCU GUU UCC AGU CAU CUA UTT-3′, 5′-AUA GAU GAC UGG AAA CAG GTT-3′); GPC3-siRNA-1633 (5′-CCA GUG GUC AGU CAA AUU ATT-3′, 5′-UAA UUU GAC UGA CCA CUG GTT-3′); GPC3-siRNA-1718 (5′-GCU CUG GUG AUG GAA UGA UTT-3′, 5′-AUC AUU CCA UCA CCA GAG CTT-3′), and non-targeting control (5′-UUC UCC GAA CGU GUC ACG UTT-3′, 5′-ACG UGA CAC GUU CGG AGA ATT-3′). SMMC 7721 and HepG2 cells were bought from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured at a density of 1×10^5 cells and treated with each type of siRNA (50 nmol/L) by

Lipofectamine 2000 (Life Technologies, USA). At 72 h post transfection, the relative levels of GPC3 expression in HCC cells were determined by Western blotting.

2.5. Enzyme-linked immunospot (ELISPOT) assay

The peptide-specific CTLs responses were determined using IFN-γ based ELISPOT assay [14]. CTLs/GPC3 229–237 and CTLs/GPC3 522–530 cells (1×10^4 cells/well) were co-cultured in triplicate with the same number of T2, SMMC 7721, HepG2, SMMC 7721/GPC3-siRNA-1633 and HepG2/GPC3-siRNA-1633 cells in 96-well plates for 24 h. The IFN-γ secreting CTLs were detected by the IFN-γ ELISPOT kit (Dakewe, Shenzhen, China). The effector or target cells alone were used negative controls. The target cells were pre-treated with anti-HAL-A2 (25 μg/mL) at 4 °C for 1 h and used for ELISPOT assay. Then, we counted the number of spot formation cells in each well by the Bioreader 4000 PRO-X (Bio-Sys; Germany) in a blinded manner.

2.6. Lactate dehydrogenase (LDH) release assay

We evaluated the cytotoxic activity of peptide-specific CTLs against target cells by the LDH release assay using the LDH Cytotoxicity Assay Kit (Beyotime, Shanghai, China) [15]. Briefly, the GPC3 522–530 specific CTLs were cultured in triplicate with the target cells (SMMC 7721, HepG2, SMMC 7721/GPC3-siRNA-1633 and HepG2/GPC3-siRNA-1633 cells) at ratios of 10:1, 20:1 and 40:1 for 5 h. The effector or target cells alone were designated as the negative controls while the GPC3 144–152 specific CTLs plus the target cells were served as the positive control. Then, we used a microplate reader to detect the absorbance values of individual wells at 450 nm. The cytotoxicity of effector cells against each type of target cells was determined as:

Cytotoxicity (%) = (Experimental release − Spontaneous release)/(Maximum release − Spontaneous release) × 100%.

2.7. Statistical analysis

Statistical software was the SPSS statistics 19.0 (SPSS Inc., Chicago, USA). One-way ANOVA and post hoc Tukey's multiple comparison test was performed to analyze the difference among the groups. A P value less than 0.05 was considered significant statistical difference.

3. Results

3.1. Selection of candidate GPC3 peptides restricted with HLA-A*0201

Based on the analysis with the three kinds of prediction software including SYFPEITHI, BIMAS and NetCTL-1.2, we selected and synthesized 6 HLA-A*0201-restricted candidate GPC3 peptides. These selected peptides had the highest total scores and a NetCTL-1.2 score of >1.00 (Table 1).

3.2. Binding ability and stability of GPC3 peptides to HLA-A*0201

The affinity of these candidate peptides was evaluated by a T2 cell peptide binding assay. As shown in Table 1, FI values of the peptides of GPC3 169–177, GPC3 229–237, and GPC3 522–530 were more than 1.5 and showed high affinity to HLA-A*0201 molecules, similarly to that of the positive control GPC3 144–152 peptide. However, other three peptides (GPC3 102–110, GPC3 326–334 and GPC3 367–375) displayed low affinity ($FI < 1.5$). Because the stability of antigen peptide/HLA-A2 molecules complex is crucial to CTL immune response, we further examined its stability in T2 cells by measuring the values of DC50. The results indicated that the values of DC50 were >8, 6, and <2 h for the GPC3 522–530, GPC3 144–152 and GPC3 229–237, GPC3 102–1101 and GPC3 169–177, respectively (Table 1). It was notable that while the GPC3 326–334/HLA-A*0201 complex had a higher stability (DC50 = 4 h), its binding affinity was relative lower. Therefore, different HLA-A*0201-restricted GPC3 peptides displayed varying affinities and stabilities.

3.3. Establish of peptide-specific CTLs in vitro

Given that both GPC3 522–530 and GPC3 229–237 peptides had high binding affinity to HLA-A*0201 molecules and these peptides/HLA-A*0201 complexes were relatively stable, we further used these two peptides to test their immunogenicity *in vitro*. Peripheral blood $CD8^+$ T cells from HLA-A*0201 healthy subjects were purified and then stimulated using GPC3-derived peptide-loaded DCs for three cycles of 7 d. The generated CTLs were evaluated for their responses to peptide-loaded T2 cells *in vitro* by IFN-γ-based ELISPOT assay. Similar to the peptide of GPC3 144–152, the GPC3 522–530 peptide

Table 1
Characteristics of the six candidate epitope peptides of GPC3.

Peptide no.	Amino acid sites	Amino acid sequence	SYFPEITHI	BIMAS	TS	COMB[a]	FI	DC50
1	102–110	FLIIQNAAV	26	319.939	8 318.414	1.235 6	0.76	<2
2	169–177	ELFDSLFPV	23	1 055.631	24 279.513	1.403 4	1.93	<2
3	229–237	FLQALNLGI	22	47.991	1 055.802	1.294 4	2.64	6
4	326–334	TIHDSIQYV	23	496.006	11 408.138	1.221 0	0.65	4
5	367–375	FIDKKVLKV	27	40.472	1 092.744	1.276 3	0.82	2
6	522–530	FLAELAYDL	27	402.895	10 878.165	1.345 3	2.33	>8
7	144–152[b]	FVGEFFTDV	18	828.699	14 916.582	1.070 0	2.26	6

TS: SYFPEITHI*BIMAS; FI: Fluorescence intensity; DC50: Dissociation complex 50, which was defined as the time (h) needed to dissociate 50% HLA-A*0201/peptide complex.
[a] COMB prediction score using the web server NetCTL-1.2. [b] 144–152 was used as a positive control.

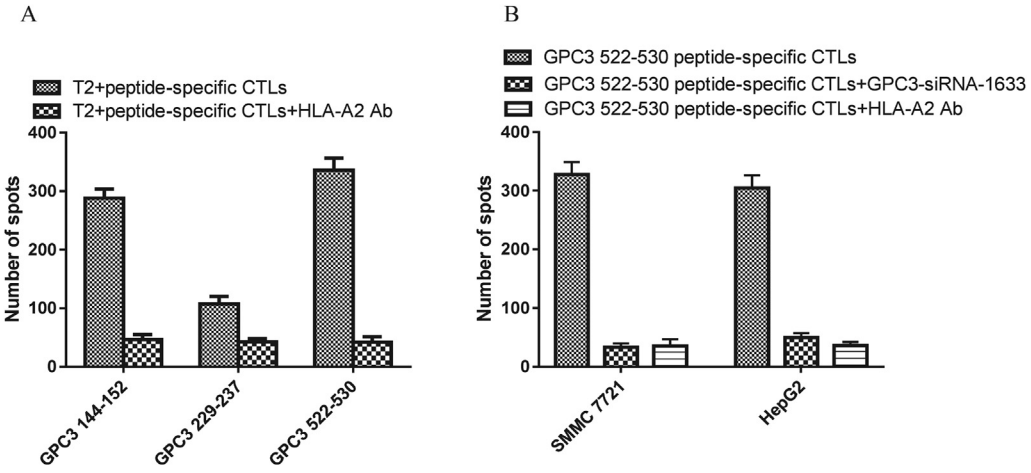

Figure 1. ELISPOT analysis of the IFN-γ responses of GPC3 peptide-specific CTLs *in vitro*.
$CD8^+$ T cells were separated from healthy subjects ($n = 2$) and stimulated with autonomous DCs that had been loaded with the indicated GPC3 peptides to induce antigen-specific CTLs for three cycles *in vitro*. The generated peptide-specific CTLs responding to peptide-loaded T2 cells or T2 cells that had been pre-treated with anti-HLA-A2 antibody and loaded with the peptide were determined in triplicate by IFN-γ based ELISPOT. The GPC3 522–530 peptide-specific CTLs responding to SMMC 7721, GPC3-silenced SMMC 7721 or SMMC 7721 had been pre-treated with anti-HLA-A2 antibody were determined in triplicate by IFN-γ based ELISPOT. Similarly, HepG2 cells were tested.

induced strong CTL responses while the peptide GPC3 229–237 induced moderate levels of CTL responses (Figure 1A). Evidentially, the GPC3 522–530 loaded T2 cells, similar to the GPC3 144–152 peptide, stimulated a great number of IFN-γ-secreting CTLs while the GPC3 229–237 loaded T2 cells only stimulated much less numbers of IFN-γ-secreting CTLs. More interestingly, pre-treatment of T2 cells with BB7.2 dramatically reduced the numbers of spot forming cells. Hence, GPC3 522–530 peptide had potent immunogenicity and the generated CTLs responded to the peptide in a HLA-A2-restricted manner.

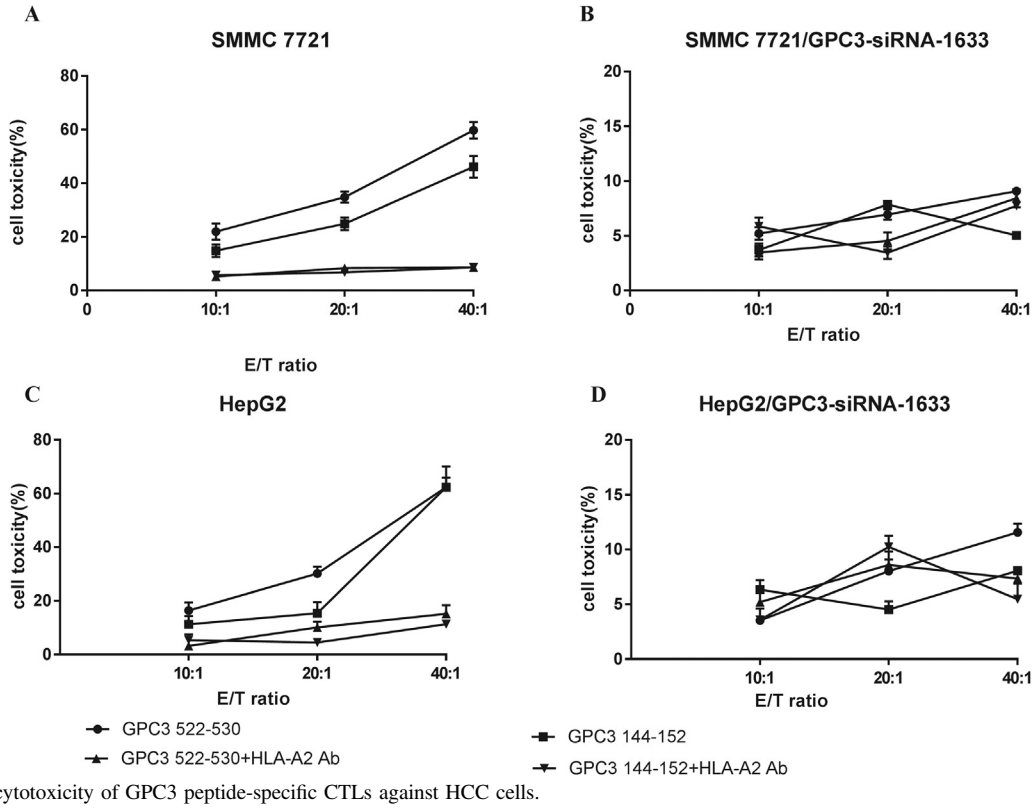

Figure 2. The cytotoxicity of GPC3 peptide-specific CTLs against HCC cells.
(A and C) The cytotoxicity of GPC3 522–530 peptide-specific CTLs against SMMC 7721 and HepG2 cells was detected in triplicate using LDH release assay and GPC3 144–152 peptide-specific CTLs were taken as positive controls.
(B and D) The cytotoxicity of GPC3 522–530 peptide-specific CTLs against SMMC 7721 or HepG2/GPC3-siRNA-1633 cells was detected in triplicate by the measurement of LDH release.

3.4. The specific cytotoxicity of CTLs induced by GPC3 522–530 peptide

Previous studies have shown that SMMC 7721 and HepG2 cells expressed HLA-A02 and GPC3 [7,14,16]. To determine the specificity of CTLs, we treated SMMC 7721 and HepG2 cells with the indicated GPC-3 specific siRNAs and their GPC-3 expression were determined by Western blot. We found that transfection with GPC3-siRNA-1633, but not with GPC3-siRNA-714 or GPC3-siRNA-1718, dramatically reduced GPC3 expression level in these HCC cells. Furthermore, we found both cells stimulated a great number of IFN-γ secreting CTLs induced by GPC3 522–530 peptide (Figure 1B). In contrast, the GPC3-silencing SMMC 7721 or HepG2 cells failed to induce strong IFN-γ responses. Similarly, pre-treatment of SMMC 7721 or HepG2 cells with anti-HLA-A2 antibody almost prevented SMMC 7721 or HepG2 cell-induced IFN-γ responses in our experimental condition. Thus, SMMC 7721 and HepG2 cells can effectively present GPC3 522–530 peptide, which stimulates potent IFN-γ responses by peptide-specific CTLs in a HLA-A*0201-restricted fashion.

Finally, we tested the cytotoxicity of GPC3 522–530 peptide-specific CTLs by LDH assays. The GPC3 522–530 or GPC3 144–152 specific CTLs were co-cultured with SMMC 7721, GPC3-silencing SMMC 7721 or SMMC 7721 and GPC3-silencing SMMC 7721 that had been pre-treated with anti-HLA-A2 antibody at the indicated ratios for 5 h. The levels of LDH in the supernatants of cultured cells were measured and the specific cytotoxicity of CTLs was calculated in Figure 2A. Both GPC3 522–530 and GPC3 144–152 specific CTLs killed SMMC 7721 cells in a dose-dependent fashion. The cytotoxicity of CTLs against SMCC 7721 cells were obviously reduced by pre-treatment with anti-HLA-A2 antibody. However, both GPC3 522–530 and GPC3 144–152 specific CTLs had little cytotoxicity against GPC3-silenced SMMC 7721 cells regardless of pre-treatment with antibody (Figure 2B). Similar patterns of results were achieved in HepG2 cells (Figure 2C and D). Therefore, the cytotoxicity of GPC3 522–530 and GPC3 144–152 specific CTLs is antigen-specific and HLA-A2-restricted.

4. Discussion

Antigen-based immunotherapies are attractive, because it can induce antigen-specific CTLs that specifically kill antigen-expressing tumor cells. Actually, antigen peptide-based immunotherapy has the following advantages: the potential antigen determinant peptides can be predicted for their affinity to a specific type of HLA class I molecules; these peptides can be easily synthesized; and this approach is highly safe without application of any viral vector [17]. CTL determinant peptides have been considered to be one of the promising methods for the development of immunotherapy for cancers [18]. Hence, identification of tumor-specific surface-expressing antigens and their antigen determinants will be of great interest. Previous studies have shown that GPC3, alpha-fetoprotein, SSX-2, NY-ESO-1 and MAGE-A10 are preferably expressed in HCC [19–21]. GPC-3 is exclusively expressed in 70%–81% of HCC tumors [22,23]. GPC3 regulates many pathways in HCC pathogenesis, including the Yap and Wnt signaling and may be HCC potential treatment of molecular target [24,25]. In this study, we screened GPC3-specific antigen peptides using the SYFPEITHI, BIMAS and NetCTL-1.2 [26,27]. We found that six potential peptides had varying affinities and stabilities to HLA-A*0201 molecules on T2 cells. Among them, the GPC3 522–530 and GPC3 229–237 displayed a high affinity and stability. Both peptides may be valuable for inducing CTLs.

Antigen-specific CTLs are crucial for erasing HCC. In this study, we found that the GPC3 522–530, GPC3 229–237 and GPC3 144–152 peptides effectively generated CTLs *in vitro*. Furthermore, we found that GPC3 229–237-specific CTLs only had moderate levels of IFN-γ responses to the corresponding peptide-loaded T2 cells, suggesting that GPC3 229–237 may have a relatively lower level of immunogenicity in our experimental system. Hence, high affinity and stability of antigen peptide to HLA class I molecules are not consistent with its immunogenicity. In contrast, the GPC3 522–530-specific CTLs, like the GPC3 144–152 specific CTLs, responded to peptide-loaded T2 cells and $GPC3^+$ SMMC 7721 and HepG2 cells in a HLA-A*0201-restricted and tumor antigen specific manner. Evidentially, treatment with anti-HLA-A2 antibody completely blocked the IFN-γ responses and cytotoxicity of the GPC3 522–530 and GPC3 144–152 specific CTLs. In addition, the GPC3 522–530 and GPC3 144–152 specific CTLs did not respond to GPC3-silenced SMMC 7721 and HepG2 cells. Although high affinity and stability for antigen peptides to HLA molecules do not indicate these peptides can be spontaneously presented, our data indicated that the GPC3 522–530 did not contain a cryptic determinant, rather than a dominant determinant that was naturally presented by HCC cells. Our data also extended previous findings that the GPC3 144–152 specific CTLs had a high avidity and recognized $GPC3^+$ HCC and ovarian cancer cells in a HLA-A2-restricted fashion [7,8,28]. Given that the majority of Asians has HLA-A2 expression, the strong cytotoxicity of the GPC3 522–530 peptide-specific CTLs suggests that the GPC3 522–530 may be valuable to develop of new vaccines for treating HCC [29]. Most patients with HCC have high levels of serum GPC3, which may affect the function of GPC3 peptide-specific CTLs. Previous studies have shown that tumor antigen releasing into blood can suppress CTLs-mediated antitumor effect by tolerogenic liver sinusoidal endothelial cells, which eliminated and cross-presented carcinoembryonic antigen to carcinoembryonic antigen-specific CTLs [30,31]. However, these liver sinusoidal endothelial cells-stimulated CTLs had no immunoreactivity due to the expression of programmed death-ligand 1. Blockage of programmed death-1 and programmed death-ligand 1 interactions can enhance the cytotoxicity of peptide-specific CTLs [32]. Further researches of the effect of tumor antigen shedding into the circulation or the extracellular spaces on immune function will help in design of better antigen-specific CTLs for antitumor therapies.

In conclusion, our data indicated that GPC3 522–530 (FLAELAYDL) contained CD8 dominant determinant and effectively induced CTLs *in vitro* with potent cytotoxicity against HCC in a HLA-A2-restricted and antigen-specific manner. Our findings may aid in design of new antigen-based immunotherapies for human HCC. Future researches will be necessary to validate the immunogenicity of GPC3 522–530 in HCC patients by testing their CTL responses.

Conflict of interest statement

The authors declare they have no conflicts of interest.

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