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ADOPTIVE TRANSFER OF GLYPICAN-3 PEPTIDE-SPECIFIC CD8 T LYMPHOCYTES CAUSES REGRESSION OF TESTICULAR YOLK SAC TUMOR IN MICE

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ABSTRACT:

Background: *Glypican-3 (GPC3) is overexpressed in testicular yolk sac tumor (TYST) which has a poor prognosis and needs novel effective treatment strategies. The potential of GPC3 as a cancer vaccine for TYST was explored in mice in the current study.*

Methods: *Mice were vaccinated with $GPC3_{144-152}$, $CD8^+$ T cells were purified from isolated splenocytes and adoptively transferred to mice with TYST tumor xenograft. Tumor growth was monitored by measuring its volume change; tumors were examined for their cellular GPC3 expression, CD8 T cell infiltration, and cell death by immunohistochemistry and TUNEL staining, respectively.*

Results: *Splenocytes from mice vaccinated with $GPC3_{144-152}$ was able to produce IFN-γ. Adoptive transfer of enriched mouse CD8 T lymphocytes from the splenocytes of these immune mice to the nude mice with xenograft TYST tumor resulted in CD8 T cell infiltration, apoptosis induction and inhibition of GPC3 expression in the tumor tissue, and ultimately caused the regression of the xenograft TYST tumor in a CD8 T cell dependent manner without inducing autoimmunity.*

Conclusions: *To the best of our knowledge, this study is the first demonstrating that adoptive transfer of GPC3 peptide-specific CD8 T cells conferred mice with TYST tumor xenograft the capability to inhibit GPC3 expression and tumor growth. Thus, GPC3 peptide vaccination could be a useful supplementary treatment for TYST.*

Keywords: ***Testicular yolk sac tumor, Immunotherapy, CD8 T cells, Glypican-3.***

INTRODUCTION

As a member of the glypican family of heparan sulfate proteoglycans and able to attach to the cell surface via the glycosylphosphatidylinositol anchor, glypican-3 (GPC3) is physiologically expressed in most of the organs during the fetal and neoinfantile period under 1 year of age, and not expressed in normal tissues except for the placenta and embryonic liver after that time period [1-7]. The human *GPC3* gene encodes a 70-kDa precursor core protein of 580 amino acids, which can be cleaved by furin to generate a 40-kDa amino terminal protein and a 30-kDa membrane-bound carboxyl terminal protein, which has two heparin sulfate glycan chains [5]. In some medical scenarios, the *GPC3* gene is found to be inactivated in Simpson–Golabi–Behmel Syndrome [8] and either overexpressed in 60% of hepatocellular carcinoma, at least in 90% of yolk sac tumor [2, 6, 7, 9], and in some other malignancies to a lesser degree or silenced in some other tumors, as detailed in recent reviews [4, 5].

Besides a cancer diagnostic marker, GPC3 has been used in different phases of several clinical trials as a trial vaccine in treating hepatocellular carcinoma [10-15], melanoma [16], and ovarian clear cell carcinoma [17], as reviewed in a recent report [11].

As the commonest germ cell tumor, testicular yolk sac tumor (TYST) is highly malignant with poor prognosis, and its treatment regime is limited to surgical resection or/and platinum-based combination chemotherapy [6, 7, 9, 18]. Cancer vaccine is an attractive approach because of their low toxicity. Most of the previous studies aiming GPC3 as cancer vaccine candidate have mainly been focused on

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hepatocellular carcinoma, and in the current study we aimed to explore the vaccine potential of GPC3 for treating TYST. We found that splenocytes from mice vaccinated with $GPC3_{144-152}$ peptide is able to produce IFN-γ and adoptive transfer of CD8 T cells purified from the immune splenocytes results in TYST tumor cell apoptosis, infiltration of CD8 T cells in tumor tissue, and ultimately causes regression of the xenograft TYST tumor in nude mice in a CD8 T cell dependent manner and without inducing autoimmunity. Vaccination with short GPC3 peptides could be a supplementary treatment for TYST.

METHODS

Mice: -Female 7-week-old BALB/C mice and male 4-week-old BALB/C nu/nu mice were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences, maintained in individual ventilation cabinets under specific pathogen-free conditions and randomly allocated into groups for each study. All animal experiments were conducted according to international guidelines and regulations for use and care of animals approved by the local animal care ethical committee. All surgery was performed under anesthesia with sodium pentobarbital.

Vaccination of $GPC3_{144-152}$ peptide and isolation of splenocytes and specific CD8 T cells from BALB/C mice: - The immunogenicity of GPC3 is dose-dependent [19] and $GPC3_{144-152}$ can induce more peptide-specific cytotoxic T lymphocytes at a lower dose than that of $GPC3_{298-306}$ [19, 20], and therefore we chose $GPC3_{144-152}$ as the antigen fragment and modified the immunizing strategy accordingly as per literature [13, 21]. Briefly, peptide $GPC3_{144-152}$ (Glu-Tyr-Ile-Leu-Ser-Leu-Glu-Glu-Leu) was purchased from GL Biochem (Shanghai, China), and its purity (>98%) was measured by high-performance liquid chromatography. Mice were primed s.c. in the flank of the abdomen with the mixture of 50 μg of $GPC3_{144-152}$ [22] emulsified in 50 μL of incomplete Freund's adjuvant (IFA) (Sigma) and diluted with 50 μl of saline (GPC3 group) or the same volume of IFA and saline (NS group) and boosted the same way 7 days after.

Enzyme-linked immunospot assay (ELISPOT): - Splenocytes were prepared from mice sacrificed 7 days after the second immunization and resuspended in EZ-CultureTM Serum-free medium (Dakewe) after depletion of RBCs by density gradient centrifugation. ELISPOT kit (U-Cytech) was used to detect antigen-specific IFN-γ producing T cells according to the manufacturer's protocols. In brief, non-cultured lymphocytes (1×10^6 cells /well) were added to plates in the presence of 6 ug/ml $GPC3_{144-152}$ peptide, and incubated for 20 h at 37℃, 5% CO_2 with EZ-CultureTM serum-free medium as control. The spots were counted and analyzed using the Biosys Bioreader 4000 PRO (U-Cytech).

Flow cytometric analysis and cell-sorting with MACS system: - Cell samples (1×10^5 cells) of the two groups were resuspended in 200 μl PBS, mixed with 2 μl FITC-conjugated mouse anti-mouse CD8α monoclonal antibody (Miltenyi), incubated in dark for 1h at 4℃, and then analyzed on a flow cytometer (BD FACS Calibur) to detect the content and purity of $CD8^+$ T cells before and after cell-sorting. $CD8^+$ T cells were purified from bulk splenocytes with the MACS system with anti-mouse CD8a (Ly-2) monoclonal antibody (Miltenyi), and each type of cells was collected separately after cell-sorting and used for adoptive transfer.

Establishment of the TYST tumor, transfer of CD8 T cells to the BALB/C nu/nu mice carrying TYST and monitoring tumor development: - The TYST xenograft tumor was established in our laboratory as described [18]. Briefly, TYST tumor tissues were harvested from mice carrying xenograft tumor, removed excess connective tissues, cleaned up blood in surface, being washed immediately by PBS, sliced into 1 mm^3 pieces, then implanted hypodermically into the unilateral inguinal region of the healthy BALB/C nu/nu mice. Weights of the implanted mice and sizes of the tumors were measured daily from the day when tumors reached 3 mm in diameter. Tumor volume was estimated by using the equation $V = ab^2/2$, where a and b represent the maximal and minimal diameter in millimeters, respectively.

When tumors reached an average volume of 120 mm^3, each mouse of the 5 different groups was injected intravenously via the tail vein with 5×10^6 cells of the designated cell transfer, either saline-immunized $CD8^+$ T cells (NS-$CD8^+$T), GPC3-immunized $CD8^+$ T cells (GPC3-$CD8^+$T), GPC3-immunized splenocytes depleted of $CD8^+$ T cells (GPC3-no-$CD8^+$T), GPC3-immunized T cells without cell sorting (GPC3-mix-T) or only saline (Control group), and boosted with the respective cells one more time on day 5. Each group included five mice, and the mouse weight and the TYST tumor volume was measured every other day and the tumor tissues were harvested on day 21.

Histologic, immunohistochemical analysis and TUNEL staining: - For immunohistochemical analysis, mouse anti-human monoclonal antibodies for Glypican-3 and Ki-67 were from Maxim and used at dilution of 1:200, and rat anti-mouse CD8a monoclonal antibody was from eBioscience. H&E staining was done according to the manufacturer's protocols and immunohistochemical staining was done with Envision method. For terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL), In Situ Cell Death Detection Kit (Roche) was used. All stained samples were subjected to microscopic analysis on a confocal microscope (Olympus) and quantified with image-pro plus 6.0 software.

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STATISTICAL ANALYSIS

Data were analyzed using the SPSS16.0 statistical program, presented as means ± standard deviation, and differences were considered significant if P<0.05 for all statistical tests. Student's t test with equal variance was used for comparison of CD8 T cell immune effect and difference between groups was calculated respectively using Kruskal-Wallis H test, Mann-Whitney U test, Tukey's test, and one-way ANOVA.

RESULTS

(1) $GPC3_{144–152}$ peptide able to stimulate proliferation of IFN-γ producing T cells:

Splenocytes were prepared from the spleen of BALB/C mice vaccinated with $GPC3_{144-152}$ or injected with saline control, and the cytotoxicity potential of its T lymphocytes among the splenocytes was measured by the *ex vivo* IFN-γ ELISPOT assay. The splenocytes from mice vaccinated with $GPC3_{144-152}$ (Fig. 1b & c) released significantly more IFN-γ ($P < 0.001$) judged by the average number of spots ($255.40±15.62×10^6$) than that ($8.40±2.29×10^6$) of the saline injection group (Fig. 1a & c).

Figure 1

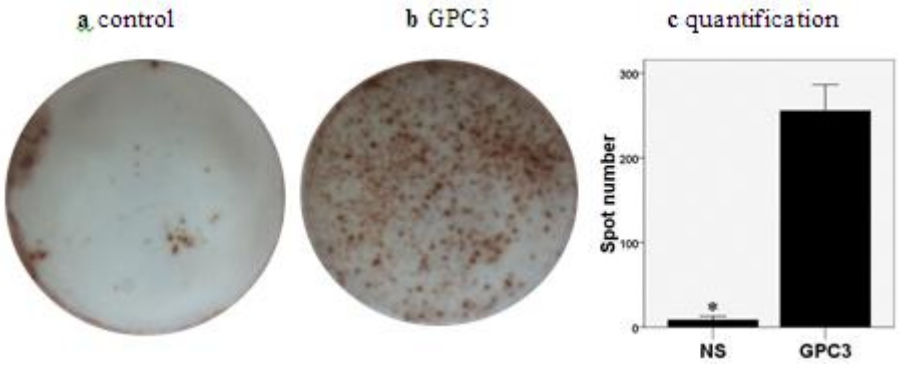

Fig. 1: -Representative results of two *ex vivo* interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays. Splenocytes were prepared 15 days after $GPC3_{144–152}$ vaccination or saline injection and pulsed with the $GPC3_{144–152}$ peptide overnight, and the non-pulsed target was used as the negative control. The ratio of effector cells to target cells (E/T) is 10. **a** control; **b** GPC3; **c** quantification of the spots (*P < 0.001).

(2) Purified CD8 T cells able to inhibit tumor growth in mice without side effect:

$CD8^+$ T cells were isolated with the magnetic beads coated with CD8 antibody and purified with FACS (Fig. 2**a** & **b**), and the content of $CD8^+$ T cells in the splenocytes of mice with GPC3 vaccination (10.18±0.5%, Fig. 2**b**) was significantly more (P<0.05) than that of saline injection control group (6.0±0.2%, Fig. 2**a**).

Figure 2

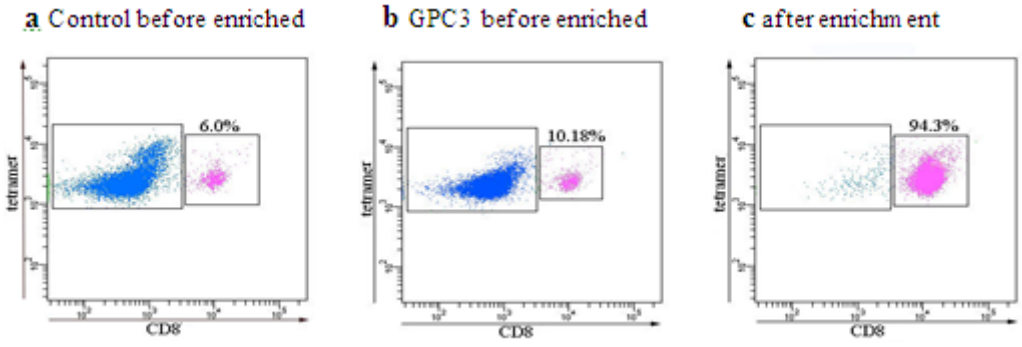

Fig. 2: -The population of $CD8^+$ T cells was quantified at single cell level and enriched by cell sorting with a flow cytometer. The numbers in each small quadrant box represent the percentage of CD8 cells in the sample, and GPC3-vaccination (**b** 10.18%) generated significantly more CD8 T cells (P<0.05) than saline injection (**a** 6.0%). **a** $CD8^+$ cells from the control mice; **b** $CD8^+$ cells from the GPC3-vaccinated mice; **c** FACS-sorting enrichment significantly increased the percentage of $CD8^+$ cells in the cell population (P <0. 05).

Those $CD8^+$ T cells from control mice and vaccinated mice were further purified by FACS-sorting, reaching a purity of 94.3% (Fig. 2**c**). The tumor size in mice having received GPC3-specific CD8 T cells (GPC3-CD8T group)

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significantly decreased (P<0.01) even at day 3 post adoptive transfer as compared to that of the control groups (control group receiving no transfer, and NS-CD8T group having received transfer of CD8 T cells from mice treated with saline as mock immunization) (Fig. 3**a**). The size difference of the tumor between the above-mentioned groups became more and more pronounced along the observation period until Day 21, the endpoint of the experiment (Fig. 3**a**,**b** & **c**, Table 1). Transfer of GPC3-immune splenocytes (without CD8 T cell purification) had similar TYST inhibition effect as transfer with the purified GPC3-immune CD8 T cells whereas transfer of GPC3-immune splenocytes after CD8 lymphocyte depletion (without CD8 T cells) had no TYST inhibition (data not shown), demonstrating that CD8 T cells were the main causal factor in TYST growth inhibition. The weights of all the 3 groups of mice with xenograft TYST tumor increased slowly without weight loss after adoptive transfer and with no significant difference between each group, suggesting that adoptive transfer of $GPC3_{144–152}$ peptide-specific CD8 T cells had no obvious toxic and side-effect.

Figure 3

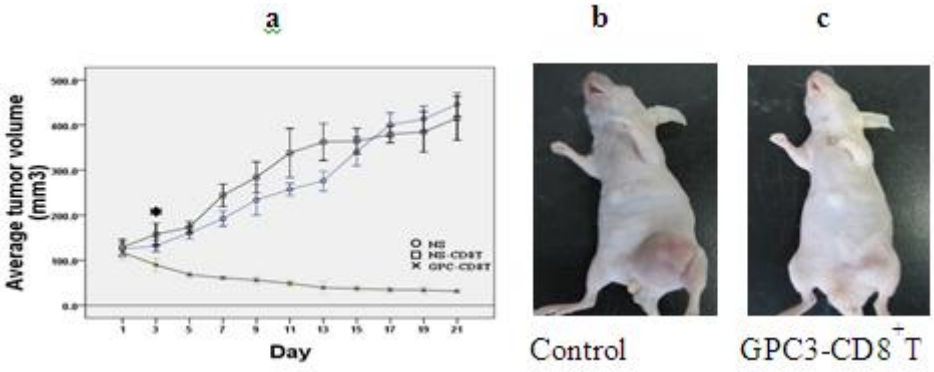

Fig. 3: -Adoptive transfer of GPC3–specific CD8 T cells into BALB/C mice inhibited TYST tumor growth. Data points were the mean (n=5) with standard deviations and representative of two independent and reproducible experiments. There were significant volume differences in the group of mice having received CD8 T cells from GPC3–vaccinated mice from day 3 (P <0.01) and thereafter from the rest groups. **a** detail changes of the tumor volume of the groups of mice every other day during the 21-day period. * P <0.01. **b** & **c** grossly view of the representative TYST tumor in mice having received adoptive transfer of CD8 lymphocytes from mice vaccinated with saline (**b** NS-CD8 transfer group) or $GPC3_{144-152}$ peptide (**c** GPC3-CD8 transfer group).

TABLE 1: Mouse weight and tumor size at Day 21 after adoptive transfer of $CD8^+$ T cells were measured and the tumor volume was calculated as in Methods (* P<0.01)

Groups	Weights（g）		Tumor volumes（mm^3）	
	pretransfer	posttransfer	pretransfer	posttransfer
Control	22.46 ± 0.49	24.16 ± 0.49	125.04±7.86	445.90±12.91
NS-$CD8^+$T	23.46 ± 0.50	25.76 ± 0.62	129.06±9.00	415.12±24.03
GPC3-$CD8^+$T	23.14 ± 0.57	23.72 ± 0.57	117.63±7.77	31.56 ± 1.60 *

(3) Adoptive transfer of $GPC3_{144-152}$ specific CD8 T cells inhibited GPC3 expression in TYST tumor:

GPC3 was highly expressed histologically in patients and mice with yolk sac tumors [2, 6, 7, 9] but it is unknown whether adoptive transfer of immune CD8 T cells generated from $GPC3_{144-152}$ vaccination will affect GPC3 expression in TYST tumor. To this end, tumor sections were stained with GPC3 antibody and it was found that the GPC3 protein expression was significantly (P<0.01) inhibited in the TYST tumor tissue of mice having received the GPC3-immune CD8 T cells (Fig. 4) as compared with that of the control group or the NS-CD8T group having received transfer of non-specific CD8 T cells (CD8 T cells from mice mock immunized with saline).

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Figure 4

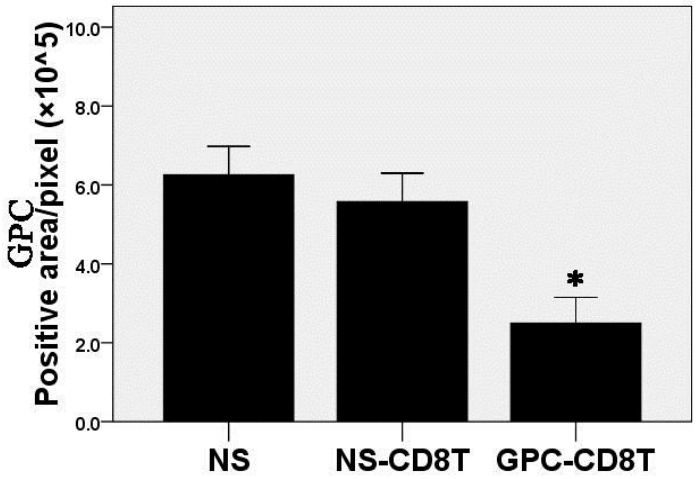

Fig. 4: -Inhibition of GPC3 expression in TYST tumor after CD8 T cell transfer. TYST tumor sections were stained with GPC3 antibody, and ten random fields of cells were examined, positive cells counted and graphed. There were significantly less GPC3 expression (* P<0.01) in TYST tumor section of mice having received adoptive transfer of CD8 lymphocytes from mice vaccinated with $GPC3_{144-152}$ peptide (GPC3-CD8 transfer group) than those having received adoptive transfer of CD8 lymphocytes from mice vaccinated with saline (NS-CD8 transfer group) or having received no transfer at all (control group).

(4) CD8 T cell infiltration into TYST tumor tissue and tumor cells die by apoptosis:

We wanted to know the underlying mechanism(s) how the tumor size decreased. At the endpoint of the adoptive transfer experiment at day 21, we removed the tumors and immunohistochemically stained the sections of them with anti-CD8 antibody or the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method. While there were less CD8-positive T cells infiltrating the tumor of the NS-CD8T group mice having received CD8 T cells from mice mock immunized with saline (Fig. 5**a**), there were significantly more (P<0.05) $CD8^+$ T cells in the tumor of the GPC3-CD8T mice having received adoptive transfer of the immune CD8 T cells (Fig. 5**b**). Significantly more apoptosis of tumor cells (P=0.001) were observed in tumor tissue of mice having been adoptively transferred with the GPC3-specific CD8 T cells (Fig. 6**c**) than in the control (Fig. 6**a**) or NS-CD8T mice (Fig. 6**b**, Table 2). The proliferation index of the TYST tumor cells was examined with Ki67 staining (Table 2).

Figure 5

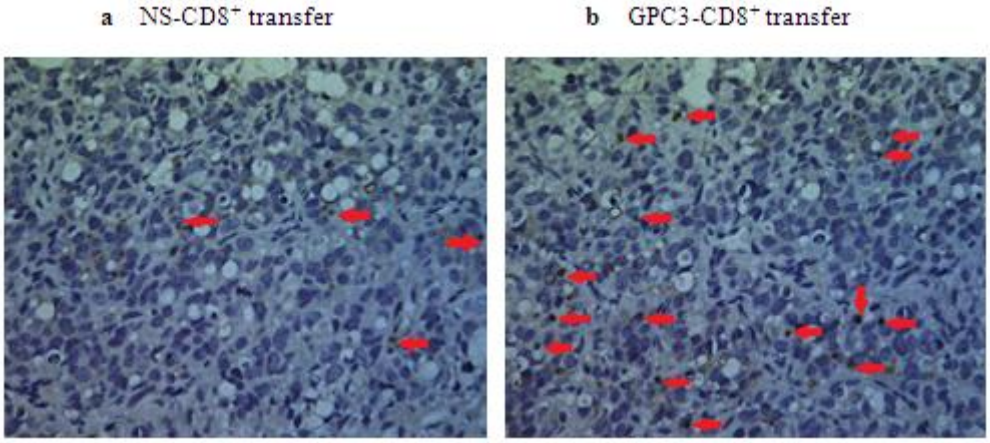

Fig. 5: -Immunohistochemical staining of tumor sections from specimen collected 21 days after adoptive CD8 cell transfer showing infiltration of CD8 T lymphocytes. Significantly more CD8 T cells (P<0.05) in TYST tissue of mice (**b**) having received adoptive transfer of CD8 lymphocytes from mice vaccinated with $GPC3_{144-152}$ peptide (GPC3-CD8 transfer group) than those (**a**) having received adoptive transfer of CD8 lymphocytes from mice vaccinated with saline (NS-CD8 transfer group). **a** NS-CD8; **b** GPC3. Magnification, ×400.

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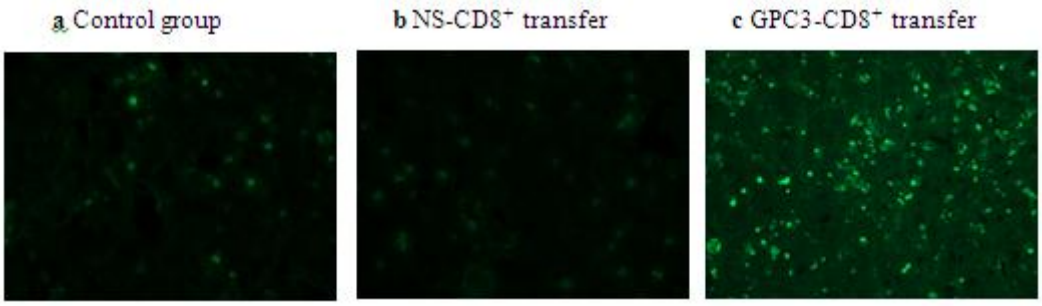

Fig. 6: -Analysis of sections from TYST tumor specimen collected 21 days after adoptive CD8 cell transfer with terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL). **a** TUNEL staining of tumor section of the control mice with mock transfer (control group); **b** TUNEL staining of tumor section from mouse having received CD8 T cells from saline-injected mice (NS-CD8 transfer group); **c** TUNEL staining of tumor section from mouse having received CD8 T cells from GPC3-immuned mice (GPC3-CD8 transfer group). Quantification of apoptotic cells was listed in Table 2.

TABLE 2: Quantification of TUNEL positive rate (* P=0.001)

Group	Ki-67 (%)	TUNEL (%)
Control	40.40 ± 1.96	1.92 ± 0.57
NS-$CD8^+$T	42.12 ± 2.10	3.24 ± 0.57
GPC-$CD8^+$T	28.12 ± 1.81	36.88 ± 3.33 *

(5) No evidence of autoimmune reactions in GPC3 vaccinated mice or mice receiving CD8 T cell transfer:

To evaluate the risk of autoimmunity from $GPC3_{144-152}$ immunization or CD8 T cell transfer, the tissues (brain, liver, lungs, and heart) of mice were pathologically examined and critically scrutinized, and compared with those in control mice. All tissues examined had normal structures and cellularity (data not shown). Moreover, these mice were apparently healthy without abnormality and autoimmunity, such as arthritis, dermatitis, or neurologic disorder. These results indicate that $GPC3_{144-152}$ peptide immunization or $CD8^+$ T cell transfer did not recognize normal self-cells and attack the normal tissue that could possibly express GPC3 at physiologically low levels, which is consistent with previous results from other laboratory [20].

DISCUSSION

Tumor antigens are useful in diagnosis and some of them that are not expressed in normal tissues except those immune privileged ones can be ideal candidates for cancer immunotherapy, and GPC3 is one of them [5, 7]. Several different peptides of 8–11 residues from GPC3 (580 amino acids in total) have been studied in detail [19, 21-24] and peptide with the highest binding score *in silico* and the highest binding affinity to $H2\text{-}K^b$ or $H2\text{-}D^b$ in vitro may not be the most efficient one for inducing cytotoxic T lymphocytes [21]. The vaccine potential of $GPC3_{144-152}$ peptide with a HLA-A2 binding score of 828 *in silico*, ranking fourth among the 9 different peptides tested [22], has already been examined in a melanoma mouse model [16], a preclinical mouse study [19] and several clinical trials for HLA-A2-positive hepatocellular carcinoma patients [10-15] and clear cell carcinoma patients [17].

The therapeutic potential of GPC3 peptide vaccines has so far been studied in 4 types of cancer, i.e., hepatocellular carcinoma [10-15], melanoma [16], ovarian clear cell carcinoma [17], and TYST (this study). Besides being a different tumor, our detail experimental strategy and results from this study are different and contrast remarkably with those previous ones in several ways. $CD8^+$ T cells (1×10^7) generated from vaccination of HLA-A*24:02-restricted $GPC3_{298-306}$ [19] were directly injected into Colon26/GPC3 tumors (mouse colorectal cancer expressing GPC3, C26G) with a diameter of 5 mm [20] and tumors became smaller 7 days after injection, whereas we adoptively transferred $CD8^+$ T cells (5×10^6 cells) generated with HLA-A*02:01-restricted $GPC3_{144-152}$ [19] via the tail vein into mice with TYST of a diameter of 3 mm and the tumor volumes were significantly reduced even 3 days after the transfer and thereafter (Fig. 3 & Table 1). Transferring $CD8^+$ T cells to sublethally irradiated lymphopenic mice also had tumor growth inhibition for established C26G tumors [20]. They also utilized i.p. injection of the $GPC3_{298-306}$ peptide-pulsed BM-DC, which worked to prevent the initiation of tumor growth planted via the s.c. route but had no efficacy against the established C26G tumor [20]. Although the outcome (inhibition of tumor growth) might be similar, the

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underlying mechanisms involved are different, as discussed below. Depletion of specific cell subsets in mice with specific antibody found that NK cells, $CD4^+$ T cells, and $CD8^+$ T cells contribute to the anti-melanoma effect elicited by ES-DC-GPC3 [16], which is not the case for the C26G tumor case [20]. For C26G, $CD4^+$ T cells were found not necessary for the anticancer property of $GPC3_{298–306}$ immunization after more than 90% depletion of $CD4^+$ and $CD8^+$ T cells [20], which is in sharp contrast with results from our study and those of Nakatsura et al [20]. We didn't see any therapeutic effect by transferring the $GPC3_{144-152}$ immunized T cells of non-CD8 origin (Table 1 and data not shown).

The mechanism(s) why CD8 T cells generated by GPC3 peptide can suppress cancer growth remains to be investigated. CD8 T cells generated by $GPC3_{144–152}$ peptide can kill cultured ovarian CCC cells by mild, but clear, specific cytotoxicity [24]. In this study we found that mice having received adoptive transfer of $GPC3_{144–152}$-immune CD8 T cells had significantly more CD8 T cell infiltration and more apoptotic cells in their tumor tissue of reduced size, suggesting that adoptive transfer treatment somehow, at least in a part, either directly or by triggering signaling mechanism(s) indirectly killed some cancer cells by apoptosis, and ultimately caused the regression of the tumor, and the fine detail of the underlying mechanism(s) warrants further research.

The implication of our result that adoptive transfer of CD8 T cells suppressed GPC3 expression in the TYST tumor is elusive and remains to be explored. Either a tumor suppressor or an oncofetal protein, GPC3 can stimulate or inhibit cell proliferation depending on the signaling pathways (Hedgehogs, Wnts, etc) involved [4]. Since there is not much work published on TYST about the expression, variation and changes of its GPC3, we can only infer from work of other types of cancer, which also express GPC3. RNA interference has been used in several studies to examine the molecular mechanism and biological effects of GPC3 suppression in human ovarian cancer cells [25] and hepatocellular carcinoma cells *in vitro* and *in vivo* [26, 27]. GPC3 knockdown sensitizes ovarian cancer cell line to paclitaxel by activation of apoptosis pathway [25], promotes the tumorigenicity, tumor weights and volumes of the human ovarian cancer cells in athymic nude mice [25], by downregulating the expression of tissue inhibitor of metalloproteinase-1 and upregulating the expression of transforming growth factor-β2 protein, matrix metalloproteinase (MMP)-2, and MMP-9. Results from hepatoma research become more complicated. Two studies report that silencing glypican-3 expression induces apoptosis in human hepatoma cells [27, 28] by inhibition of Huh7 proliferation [28] and stimulating the Bax/Bcl-2/cytochrome *c*/caspase-3 apoptotic pathway in HepG2 cells [27]. Down-regulation of GPC3 significantly inhibit the proliferation by enhancing TGF-β2 expression and signaling [26], invasion and tumorigenicity in nude mice of human hepatocellular carcinoma cell lines [29], which is in sharp contrast from another study to its cancer growth promotion result in the Hep G2 and Hep 3B hepatoma cells by blocking the endogenous GPC3 expression with an antisense transcript [30].

Our study is the first to show that adoptive transfer of GPC3-specific CD8 T cells is able to inhibit GPC3 expression in the tumor tissue and suppress TYST tumor growth in mice, therefore GPC3 is useful not only for diagnosis but also for possible immunotherapy or prevention of TYST. Considering the 95% homology at the amino acid level between the human and mouse GPC3 sequence [5], GPC3 could be an ideal target for immunotherapy for human TYST and this possibility remains to be further studied.

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