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Original Research

SMAR1 promotes immune escape of Tri-negative Breast Cancer through a mechanism involving T-bet/PD-1 Axis

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Abstract: This study was aimed at investigating the specific molecular mechanisms involved in the regulation of immune escape of triple-negative breast cancer (TNBC) by SMRI, so as to provide a new clinical treatment target for the disease. Mouse original 4T1 breast cancer cells were inoculated subcutaneously in BALB/C to establish TNBC mouse model. CD8+T cells with immunological effects were selected from mouse thymus glands for primary culture. The CD8+positive T cells were infected with lentivirus interference vectors, and the proliferation of CD8+ T cells were determined by trypan blue staining and flow cytometry. CD8+T cells and 4T1 cells were cultured together so as to determine the cytotoxic effects of SMAR1-downregulated CD8+ T cells on tumor cells and the expression of cytokines (IFN-γ, TNF-α, IL-2, IL-4 and IL-6). The expressions of SMAR1, T-bet and PD-1 were assayed by Western blot. SMAR1-downregulated CD8+T cells were injected into 4T1 tumor-bearing mice through the caudal vein, and the growth of tumor in mice was monitored. Following the infection of CD8+T cells with SMAR1-downregulated lentiviral system, cell apoptosis level was decreased significantly (control vs. sh-SMAR1: 32.23 ± 12.4 % vs. 18.28 ± 8.93 %, $p < 0.05$). Results from trypan blue staining experiments showed that the proliferation of CD8+ T cells in the SMAR1-downregulated group was significantly increased; SMAR1-downregulated CD8+ T cells promoted the production of IFN-γ, TNF-α, IL-2, IL-4 and IL-6 in 4T1 breast cancer cells ($p < 0.05$). Western blot showed that SMAR1 down-regulation led to significant upregulation of T-bet, while PD-1 was downregulated, when compared to the control group ($p < 0.05$). The downregulation of SMAR1 was associated with significant reduction in tumor size in mice ($p < 0.05$). SMAR1 downregulation enhances the tumor killing effect of CD8+T cells by activating T-bet and down-regulating PD-1

Key words: SMAR1; T-bet; PD-1; Triple negative breast cancer; Immune escape.

Introduction

Breast cancer is the most common malignancy in females worldwide. Its morbidity and mortality in China account for 12.2 and 9.6 %, respectively, and the trend is rapidly growing (1, 2). With peculiar phenotypes, TNBC results in decreases in the effects of endocrine therapy and chemotherapy. Morbidity from TNBC accounts for about 15 % of breast cancers; it is a pathological type with the highest mortality and the worst prognosis in breast cancers (3, 4). In view of the limitations of traditional treatment methods, immunotherapy can improve the survival rate of TNBC patients and clinical prognosis due to its high specificity and special immune memory ability.

Programmed death 1 (PD-1) and programmed death-1 ligand (PD-L1) are important negative immune-regulatory factors (5). The PD-1 receptor on the surface of T cells binds to the PD-L1 ligand expressed on the surface of tumor cells to transmit regulatory signals to the cells, thereby inhibiting T cell activation and proliferation (6). A study has reported that PD-1/PD-L1 can mediate the immune escape of TNBC cells from killer T cells (7). In addition, it has been shown that CD8+T cells massively infiltrate recurrent TNBC tissues, but they do not exert immune killing effects on tumor cells (8). Therefore, it was inferred that their immune activity was restrained. CD8+T cells are immunologically inhibited by some regulation, but the mechanisms involved have not been elucidated. A review of extant literature revealed that CD8+T enhances the functions of related proteins: T-bet activates CD8+T cells, and the ternary complex of SMAR1/SMRT-HDAC1 binds to the T-bet promoter region to suppress its transcription, thereby influencing T cell activation (9).

SMAR1 gene coding, identified from mouse T cell library, is a matrix-associated region binding protein (10). It has been reported that SMAR1 can recruit chromatin-modified complexes such as HDAC 1/SIN3, SMRT and HADC 6, and regulate the expression of target genes (11). In addition, it can activate p54 and p53, and ultimately give rise to G2/M phase block of cells, thereby acting a potent tumor suppression factor (12). Several reports indicate that SMAR1 is significantly down-regulated in some human tumor cells, but positively expressed in TNBC. Apart from its tumor suppression function, SMAR1 controls T cell development by regulating TCRβ transcription and Eβ enhancer as well as TCRβ gene rearrangement (13). It interacts with MARBP and Cux/Cdp which cooperate and regulate the

transcription of TCRβ gene.

It has been confirmed that up-regulated SMAR1 expression in TNBC is closely related to poor prognosis in patients, but negatively correlated with T-bet expression (14). *In vitro* experiments have revealed that SMAR1 gene silencing can promote the secretion of cytotoxic factors by CD8+ T cells. Therefore, this study was aimed at investigating if suppression of SMAR1 can advance T-bet transcription to enhance the killing effect of CD8+T cells on tumors. This was carried out through *in vivo* and *in vitro* experiments, and functional trials.

Materials and Methods

Establishment of animal models and preparation of single cell suspension

Six BALB/C mice were acclimatized in SPF-class barrier for 2 days, and then subcutaneously injected with 1.0 x 10^6 4T1 TNBC cells, to produce an Xeno-transplanted tumor mice model. Thereafter, the mice were returned back to a warm cage, and allowed free access to feed and water for 14 days prior to the commencement of the study.

Preparation of single cell suspension

The mice were anesthetized by intraperitoneal injection of 1 % sodium pentobarbital (0.02 mL/g). Their skins were cleaned with cotton ball moistened in alcohol. Then, the mice were placed on an ultra-clean platform and their thoracic cavities were opened. Thymus tissues were excised, wrapped with sterilized gauze, and placed in a container containing 2 mL of RPMI-1640 media. The tissues were slightly crushed with a pincett, and then the cell suspension was aspirated with a 1-ml sample injector through the gauze and placed in a 50-mL centrifuge tube. The suspension was centrifuged at 1000 rpm for 10 min at room temperature. With supernatants discarded, it was mixed with 5 ml of re-suspended cells in 1640 medium.

Selection of CD8+positive cells by CD8+T cell flow cytometry

Single cell suspension separated from mouse thymus was centrifuged, and the supernatant was discarded. FITC-labeled CD4 and CD8 cloned antibodies were added to the single cell suspension and incubated at room temperature. The suspension with antibodies was subjected to flow cytometry, and the cell mass with positive signal was selected. The number of cells used met the demands of the trial. Adhesions were eliminated by FSC-W/FSC-H and SSC-W/SSC-H, while dead cells were eliminated by DAPI/SSC. Lymphocyte population and T cells were selected by FSC/SSC. Finally, CD4/CD8 was used to select CD8 and T cells for analysis.

Determination of apoptosis by flow cytometry

Apoptosis in each group was determined by apoptosis reagent kit (BD Science, New Jersey, USA). The cells were infected with lentivirus for 48 h, and the cell culture medium was aspirated and retained. The digested cells were centrifuged at 1000 rpm for 5 min, washed with PBS, and centrifuged twice. Then, the mixture was re-suspended in 100 μl of 1X Blinding buffer, and 5 μl of PI and 5 μl of Annexin V were added, followed by incubation at room temperature in a dark place for 15 min.

Trial of tumor killing effect

The killing effect of CD8+ cells was determined using MTT assay. The ratios of target cells to effector cells were set as 1:2, 3:2, 5:1, and 15:1. The target control group contained 4T1 cells + culture medium; the effector cell group had CD8+ T cells + culture medium, while the experimental group consisted of SMAR1 down-regulated CD8+ T cells +4T1 cells. Each group had three duplicate wells. Determinations were made at a wavelength of 570 nm. The percentage tumor killing effect was calculated as shown below:

Tumor killing effect (%) = {[1 - (experimental group - effector control group)]/target control group - blank group)} × 100

SMAR1 lentivirus infection

SMAR1 down-regulated lentiviral vector and AgeI/AgeI enzyme digestion were purchased from Shanghai Jima Pharmaceutical Co., Ltd. The titer of SMAR1 virus was set at 2E+8. The cells were inoculated 24 h in advance, and their degrees of fusion reached 50 – 70 %. The virus solution and the medium were mixed in a volume ratio of 1:1000, and then directly added to the cells to be transfected, and polybrene was added to a final concentration of 5 - 10 μg/mL. After 24 h of infection, a medium containing appropriate concentration of puromycin was used as replacement for clonal screening until all cells in the uninfected group died, and the viable cells in the infected group were successfully infected. Proteins were extracted from the cells selected, and the degree of transfection was determined by western blot.

Determination of the expression of inflammatory cytokines by ELISA

The levels of IFN-γ, TNF-α, IL-2, IL-4 and IL-6 secreted by cells were determined using a horseradish peroxidase-labeled immunological antibody sandwich assay. Antibodies of IL-6, IL-2, IL-4, TNF-α, and IFN-γ were coated in a 96-well ELISA plate and incubated with appropriate amount of serum. The amount of enzyme and enzyme-bound cytokines were determined using tetramethyl benzidine as substrate. Absorbance values were read at 450 nm and 600 nm in a microplate reader and fitted into a standard curve which was used to calculate the sample concentration.

Determination of protein expression by western blot

The cells were digested, centrifuged, and collected. Appropriate amount of RIPA lysate was added, and the digest was allowed to stand for 30 min on ice, centrifuged at 4 ℃, and the supernatant was discarded. The concentration of proteins was determined using bicinchoninic acid assay. After separation with 12 % polyacrylamide gel electrophoresis, the proteins were transferred to the PVDF membrane by a semi-dry transfer method, and blocked with 5 % skim milk powder for 1 h at room temperature. The protein bands were incubated with the primary antibodies SMAR1 (Abcam, 1:2000 dilution), T-bet (Abcam, 1:1000 dilution), and PD-1 (Abcam, 1:2000 dilution) at 4 ℃ overnight. Thereafter, the bands were washed three times with TBST (10 min per wash). Subsequently, the secondary anti-

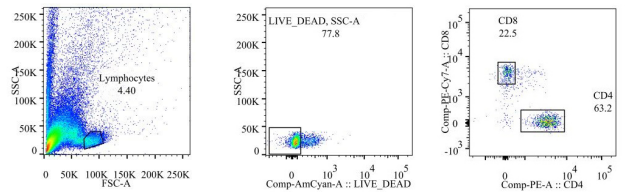

Figure 1. Screening of primary cultured CD8+ T cells by flow cytometry.

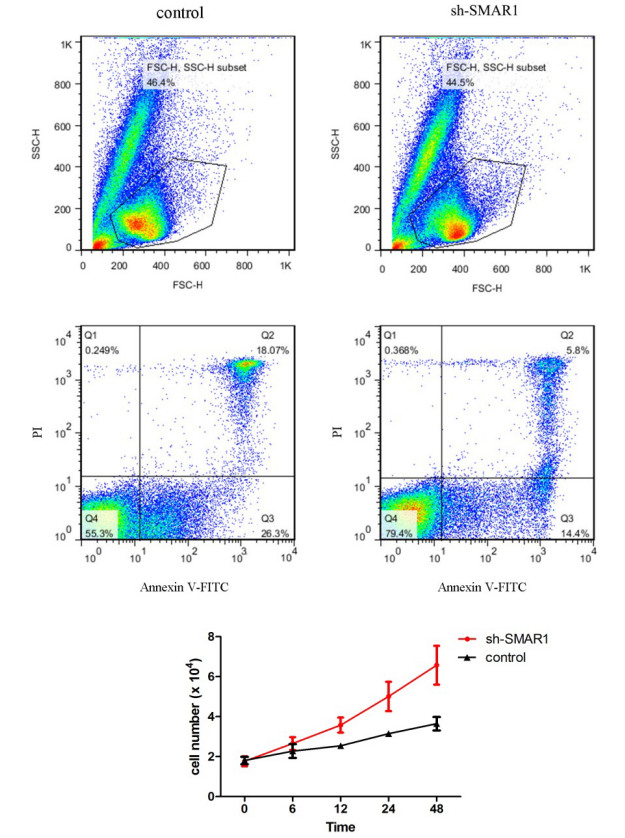

Figure 2. Effect of SMAR1 on proliferation and apoptosis of primary cultured CD8+ T cells. After knocking out SMAR1, the apoptosis level of CD8+ T cells was significantly decreased (control vs. sh-SMAR1, 32.23 ± 12.4 % vs. 18.28 ± 8.93 %, $p < 0.05$). Cell proliferation assay with Trypan blue staining showed that the proliferation of CD8+T in the down-regulated SMAR1 group was significantly improved.

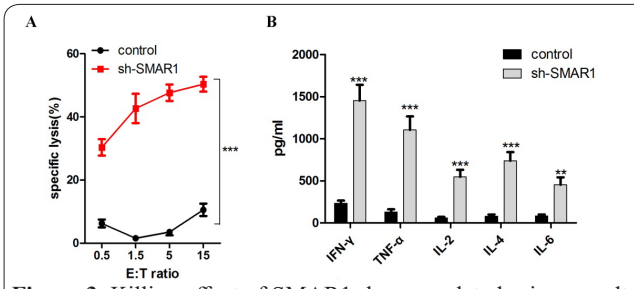

Figure 3. Killing effect of SMAR1-downregulated primary cultured CD8+ T cells on tumor cells. SMAR1-downregulated CD8+T cells induced 4T1 to generate more IFN-γ, TNF-α, IL-2, IL-4 and IL-6, and stronger tumor cell killing effect. The killing effect was much stronger in the SMAR1 down-regulated group than in the control group for each E:T ratio. $^{**}p < 0.05$, ***, $p < 0.01$, compared with the control group.

body (1:2000 dilution) was added, and the mixture was incubated for 2 h at room temperature, and then washed three times with TBST (10 min/wash). The bands were analyzed with chemiluminescence method, and photographed with a gel imaging system. The gray value of each band was counted.

Statistical analysis

All data were processed by software MedCalc (Maria Kelke, Belgium). Measurement data were expressed as mean±SD. Student's *t*-test was used for comparison between groups. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

Results

Screening of primary cultured CD8+ T cells by flow cytometry

Adhesions were eliminated by FSC-W/FSC-H and SSC-W/SSC-H, and then dead cells were removed by DAPI/SSC. The lymphocyte population was selected by FSC/SSC, and CD8+T cells were selected for analysis, as shown in Figure 1.

Effect of SMAR1 on proliferation and apoptosis of primary cultured CD8+ T cells

As shown in Figure 2, after downregulation of SMAR1, CD8+ T cells produced a significant decrease in cell apoptosis (control vs. sh-SMAR1: 32.23 ± 12.4% vs. 18.28 ± 8.93%, $p < 0.05$). Results from cell proliferation assay with trypan blue staining showed that the proliferation of CD8+T cells in the down-regulated SMAR1 group was significantly increased. These results suggest that down-regulated SMAR1 inhibits CD8+ T cell apoptosis, and promotes cell proliferation.

Tumor cell killing effect of SMAR1-downregulated primary cultured CD8+ T cells

Infection of CD8+T cells with SMAR1 down-regulated lentivirus resulted in enhancement of the killing effect of T cells and the secretion of cytokines. The CD8+T cells were infected with SMAR1-down-egulated lentivirus and co-cultured with target 4T1 breast cancer cells. It was found that CD8+T cells induced 4T1 breast cancer cells to generate more IFN-γ, TNF-α, IL-2, IL-4 and IL-6 (Figure 3B), and stronger tumor killing effect. At the same ratios of effector cells to target cells (E:T ratios), the killing effect was much stronger in the SMAR1 down-regulated group than in the control group (Figure 3A).

Expressions of SMAR1, T-bet, PD-1

In order to find out the specific molecular mechanisms involved in the SMAR1 regulation of T cell, the

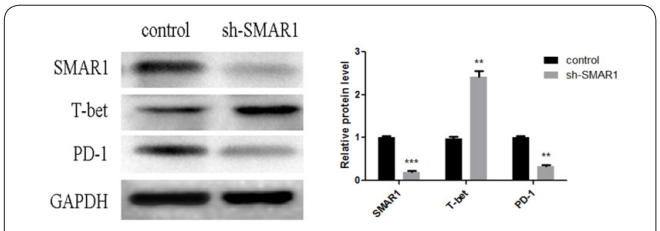

Figure 4. Expressions of SMAR1, T-bet and PD-1. The expressions of SMAR1, T-bet and PD-1 were determined using western blot. Following down-regulation of SMAR1, T-bet was significantly upregulated while PD-1 was downregulated, when compared with the control group ($p < 0.05$). $^{**}p < 0.05$, compared with the control group; $^{***}p < 0.01$, compared with the control group.

expressions of SMAR1, T-bet and PD-1 were determined by western blot. The results revealed that after down-regulation of SMAR1, T-bet was significantly up-regulated while PD-1 was down-regulated, relative to the control group ($p < 0.05$) (Fig 4).

Effect of SMAR1 knockout on the killing effect of CD8+T cells on breast cancer cells in vivo

The CD8+T cells of the SMAR1 knockout group and the control group were injected into 4T1 tumor bearing mice through the caudal vein so as to observe the growth of tumor in mice. The finding revealed that the size of tumor in mice in the SMAR1 down-regulated group was lessened, when compared with the control group ($p < 0.05$) (Fig 5).

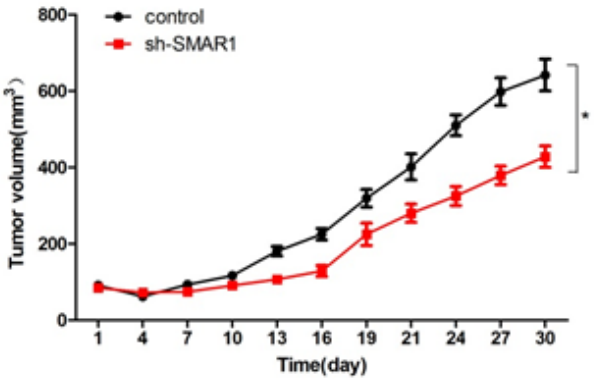

Figure 5. Effect of SMAR1 knockout on the killing effect of CD8+T cells on breast cancer cells *in vivo*. *$p < 0.05$, tumor size in mice in the SMAR1 down-regulated group compared with the control group.

Discussion

The main components of the immune system (T lymphocytes or T cells) perform specific cellular immunity and immuno-regulatory functions. Mature T cells can be divided into two sub-populations i.e. CD4+ T cells and CD8+ T cells, based on differences in function and MHC molecular restriction (15). Anti-tumor immunity is mediated by T cells, in which type I adaptive immune responses mediated by CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ type I helper T cells (Th1 cells) form the bases of anti-tumor immunity. CD8+ T cells are important immune effector cells that induce cells to produce various cytokines (IL-2, IL-4, IL-6, TNF-α and IFN-γ) by releasing perforin and granzymes. In addition, CD8+ T cells can rapidly proliferate and expand in quantity after re-contacting with antigen. The CD8+ T cells play an important role against infection, tumor immunity, transplant immunity and certain autoimmune responses (16). Tumor immune escape is an important mechanism involved in the onset and progression of tumor. In recent years, studies have shown that CD8+ regulatory T cells, a group of important T cell subsets, may play a key role in tumor immune escape.

First discovered by Szabo *et al.* in 2000, T-bet is a new transcription factor belonging to the T-box gene family. It is expressed in Th cells, and it promotes the differentiation of Th1 cells. Studies have shown that T-bet promotes the expression of IL-33 receptor on the surface of LCMV-infected Th1 cells, thereby promoting the amplification of antigen-specific CD4+ T cells (17). Recent studies have revealed that T-bet is an important transcription factor that regulates the differentiation and functions of type I effector T cells such as CD4+Th1 and CD8+ CTLs. Moreover, T-bet plays an important regulatory role in the anti-tumor immune response mediated by CD8+ T cells. A recent report revealed that T-bet directly inhibits the transcriptional activity of PD-1, thereby resisting tumors (18).

The immunosuppressive receptor PD-1 is expressed by long-term stimulated CD4+ and CD8+ T cells after T cell activation, and it is a member of immunoglobulin superfamily (19). Although CAR-modified adoptive transmission T cell therapy usually yields very good results in the treatment of hematological malignancies in immunotherapy, its clinical application in solid tumors faces many challenges. One of the major obstacles is the immunosuppressive microenvironment of tumors. These include the inhibition of tumor infiltrating lymphocytes (TIL) and genetically engineered T cells by up-regulation of inhibitory receptors, particularly the up-regulation of immunosuppressive receptor PD-1 (20). It has been reported that the expression level of PD-L1 on the surface of various tumor cell membranes is elevated, and that tumor cells skillfully utilize PD-L1 to bind to the PD-1 receptor on the surface of T cells, so as to transmit an inhibitory signal which results in immune escape. Several preclinical studies and clinical trials have indicated that CAR-T cell therapy combined with anti-PD-1 antibodies can significantly enhance the anti-tumor effect of CAR-T cells (21, 22). Therefore, the use of PD-L1 or PD-1 blocking antibodies to enhance the anti-tumor immune response of cancer patients can be considered as a new tumor immunotherapy for obtaining a sustained clinical response.

Low expression of SMAR1 in tumors is associated with poor prognosis of multiple cancers, but not much is known about the expression of SMAR1, its phonotypes, and its function on tumor infiltrating T lymphocytes. It is known from previous trials and extant literature that poor prognosis of TNBC is negatively related to the overexpression of SMAR1. The induction and expression of PD-1 on antigen-specific T cells is considered as the immune response that regulates foreign and auto-antigens. Although tumor antigen-specific T cells infiltrate the tumor stroma, many tumors still continue to grow. The underlying mechanisms of tumor-specific T cell dysfunction are not yet known. Consequently, it is supposed that cytokines in tumor microenvironment may weaken anti-tumor immune response. Based on previous studies, it can be scientifically assumed that SMAR1 gene silence down-regulates PD-1 and enhances the tumor-killing function of CD8+T cells, which is regulated by initiating T-bet transcription.

The present study attempted to mimic the actual situation of tumor immunity. The target cells used in the study were the same type of breast cancer cell line. Mouse 4T1 breast cancer cell lines were inoculated subcutaneously in BALB/C mice to establish a TNBC mouse model. The primary cultured CD8+ T cells with immune effect were screened from the thymus, and a "T cell-tumor cell reaction" was performed in target cells. After SMAR1 down-regulation, they were co-cultured with 4T1 cells inactivated with mitomycin to observe

any changes in their effector cells. First, flow cytometry was used to sort CD8+ T cells from the thymus tissues of the tumor-bearing mice. Then, the CD8+ T cells were infected with SMAR1 down-regulated lentivirus so as to study changes in cell proliferation and apoptosis. The findings suggest that down-regulation of SMAR1 led to significant decreases in apoptosis in CD8+T cells, and significant increases in their proliferation ability. From these results, it can be hypothesized that up-regulation of CD8+ T cells enhance the killing effect of T cells. Subsequently, through co-culturing of T cells and 4T1 tumor cells, it was found that SMAR1 knockout CD8+ T cells induced 4T1 cells to produce more IFN-γ, TNF-α, IL-2, IL-4 and IL-6, and stronger tumor-killing effect: at the same E:T ratio, the killing effect of the cells was significantly higher in the SMAR1 down-regulated group than in the control group. Furthermore, it was found that suppression of SMART1 mediated the up-regulation of T-bet and the down-regulation of PD-1.

The results obtained in this study reveal that SMAR1 inhibition negatively activates PD-1 by promoting the activation of T-bet, thereby enhancing the tumor-killing effect of CD8+T cells. These findings provide a new and exciting lead on the mechanism involved in tumor immune escape, as well as its treatment.

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Interest conflict
There is no conflict of interest to be declared by the author.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Zhu Yichao, Chen Cheng; Deng Rong, Wu Pingping, Huang Xing collected and analysed the data; Wang Xiaohua, Xu Hongxia wrote the text and all authors have read and approved the text prior to publication.

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