TPX2 Derived Vaccine to Inhibit Tumorigenesis and Metastasis in Triple Negative Breast Cancer

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Research article

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Abstract

**Background:** triple negative breast cancer (TNBC) lacks of treatment approaches rather than other subtypes of breast cancer. However, it characterizes the highest the level of tumor infiltrating lymphocyte (TIL) than other subtypes, indicating the possibility of immunotherapy.

**Method:** female BALB/c background mice are immunized with a TPX2 derived vaccine 4 consecutive times (once a week) from 6 weeks old, and then 4T1 cells are transplanted to the #4 mammary fatpad. Surface tumor volume and No. of lung metastasis were recorded. TIL and splenocyte was collected for T cell subgroup analysis by methods of flow cytometry and IFN-γ ELISPOT.

**Result:** as a result, TPX2 derived vaccine shows moderate effect, shrinking the tumor volume from 804.4 to 504.5 mm³, and decreasing the number of lung metastasis from 16.6 to 6.0 in control group compared to vaccine group. CD8+ T cell ratio are obviously increased in TIL between vaccine group and control group (5.87% vs 3.37%, P=0.0012). And vaccine could induce strong immune response both in tumor site (87.6 vs 7.0, p=0.0004) and system (28.2 vs 3.8, p<0.0001) through IFN-γ ELISPOT.

**Conclusion:** our result indicated that TPX2 derived vaccine may be an effective approach to inhibit TNBC tumorigenesis and metastasis.

Introduction

Breast cancer is the leading malignant tumor among women over the world(1). The causes are still unclear, although it is suggested that tumor development is related to obesity, hormone disorder, and genetic inheritance such as BRCA1 mutation. As a subtype, triple negative breast cancer (TNBC) accounts for approximately 15–20% of all breast cancer, which is characterized by rare expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2). Meanwhile, since lacking of targeted therapy(anti-Her2 therapy such as trastuzumab) and anti-endocrine therapy, the approaches of TNBC are limited, contributing to the worst prognosis of TNBC in all breast cancer. However, it has been reported that TNBC owns the highest level of tumor infiltrating lymphocyte (TIL) rather than other kinds of breast cancer, indicating the possible strategy of immunotherapy(2).

Fortunately, it has been proven by a trial that immune check point inhibitor (programmed cell death ligand-1, PDL-1) could be beneficial for partial patients (20%-30%) in late stage TNBC in first line, when combined with chemotherapy(3). Therefore, new approaches, especially new immunotherapy method, are required to improve the clinical outcome of TNBC.

We have previously studied that TPX2 protein is highly expressed in TNBC; what’s more, TPX2 level is negatively related to the prognosis of TNBC, while positively related to the lymph node and distant metastasis(4). It is believed that TPX2 over expression could result high chromosome instability (CIN), subsequently causing TNBC tumorigenesis and metastasis(5, 6). Meanwhile, TPX2 inducing high CIN could help TNBC escape from interferon (IFN) dependent T cell anti-tumor effect(5). It has also been reported that over expressed TPX2 inducing high CIN could decrease the expression of main
histocompatibility complex (MHC) and its relating proteins (such as TAP1, B2M)(7). All these indicate that TPX2 may serve an important role in MHC signaling and anti-tumor immune processing.

Herein, we report a vaccine composed of 4 separate peptide epitopes from TPX2 protein, which shows moderate effect on BALB/c mouse model transplanted with 4T1 cells. Our result suggests that TPX2 derived vaccine is effective in prohibition of tumorigenesis and metastasis, based on inducing CD8+ T cell infiltration to tumor site, and activating strong immune response both in tumor site and system.

Material And Method

Mice: female BALB/c background mice were bought from Tongji Medical College and maintained in animal experiment center of Tongji Hospital. All procedures were approved by the animal ethic committee of Tongji Hospital. In our study, 8 weeks-old mice were chosen for vaccination. The detailed vaccine injection time and sequence was detailed as follow:

Cell: 4T1 cell line was also bought from Tongji Medical College and maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). For orthotopically transplanted in mammary, 1 × 10^6 4T1 cells were dissolved in 100 ul Phosphate Buffered Saline (PBS), and then injected to the #4 mammary fat pad of BALB/c female mice.

Vaccine: 4 separate epitope peptides derived from TPX2 protein were combined as our vaccine; their peptides sequences were RGTRGCTI, PVPHFDTI, GAYKAEMW, RTPNRYHL, respectively; which were synthesized by Ontoresinc Cor. (http://www.ontoresinc.cn/). Each 50ug of 4 epitopes were combined and dissolved in 50 ul PBS, and then 50 ul adjuvant were added for vaccination (complete Freund’s adjuvant at first time and incomplete Freund's adjuvant for the following 3 times). The vaccination place was subcutaneously near the scapula. For mice in control group, 50 ul PBS plus 50 ul adjuvant were combined and then injected in the same place.

IFN-γ ELISPOT: TIL was extracted by tumor dissociation kit (Nwbio tec, Beijing), following the company’s guide. Spleen was crushed and then filtered through a 70um cell strainer (Absin) and lysed by ACK lysis buffer for eliminating red blood cell, then supernant was collected as splenocyte. 2.0 × 10^5 cells (TIL or splenocyte, seperately) were plated into each well of a 96 well plate coated with anti IFN-γ antibody (MabTech, OH) and vaccine(all 4 peptides) or control. Then after 72 h, plates were washed and a secondary biotinylated anti-mouse antibody (MabTech, OH) was added and incubated over night at 4°C. Then plate was washed by PBS and HRP streptavidin was added to each well and incubated for 1 h. Then AEC substrate was added, incubated until the color change. Then the plate was washed and dry, and read by automated plate reader system (CTL Technologies, Shaker Heights, OH).

Flow cytometry: single TIL or splenocyte was detected using flow cytometry for expression of surface markers such as CD4, CD8, CD25 (eBioscience), and intracellular marker of FoxP3 by the instrument of company (BD). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).
Statistic: tumor was measured by vernier caliper when nodule palpable and parameter was recorded by maximum diameter (A) and minimum diameter (B) and calculated by the formula \( \text{volume} = \frac{1}{2}AB^2 \) (8). Data were analyzed by t-test.

Result

**Anti-TPX2 vaccine inhibited tumorigenesis and lung metastasis in TNBC**

Surface tumor at mice fat pad was recorded when nodule palpable. After 8 days of 4T1 tumor cell injection, most of the tumor nodules could be touched in fat pad of control group (adjuvant group). After 4 weeks, ulcer occurs at the surface of some tumor nodules, then mice were sacrificed. The mean tumor volume was 504.5 mm\(^3\) in vaccine group (n = 5) compared to 804.4 mm\(^3\) in adjuvant group (n = 5) with a p-value of less than 0.0001 on day 29 after 4T1 tumor cell injection (Fig. 1A). It has been demonstrated that 4T1 cell transplanted to BALB/c female mice could mimic the metastasis behavior of human TNBC, such as lung metastasis(9). So that the number of tumor nodule metastasized to lung surface was also calculated in our study. the number of lung metastasis in control group was 16.6, compared to 6.0 in vaccine group with a p value of 0.0003 (Fig. 1B). The representative pathology image of primary tumor site and lung metastasis were shown in Fig. 1C and D. As a conclusion, our result indicated that TPX2 derived vaccine could inhibit TNBC tumorigenesis and lung metastasis in 4T1 cell transplanted to BALB/c mouse model.

**Anti-TPX2 vaccine increased CD8 + T cell infiltrating in tumor site**

We then examined T cell subgroup and ratio in TIL by using tumor dissociation kit. As expected, CD8 + T cell ratio in all CD3 + T cell was obviously increased in vaccine group (5.87%) rather than control group (3.38%) with a p value of 0.0012 (Fig. 2A). Interestingly, CD4 + T cell ratio in all CD3 + T cell was also increased in vaccine group (24.55%) than in control group (18.44%) with a p value of 0.0003 (Fig. 2B). However, CD4 + regulatory T cell (Treg, CD4 + FOXP3+) in all CD4 + was unexpectedly elevated in vaccine group (51.29%) than in control group (37.09%), with a p value of less than 0.0001(Fig. 2C), indicating that TPX2 vaccine could to some extent induce tumor tolerance.

**Anti-TPX2 vaccine inducing strong immune response both in tumor site and system**

IFN-\( \gamma \) ELISPOT was performed to detect the immune response of vaccine. As expected on TIL, it revealed that TPX2 derived vaccine could induce obvious immune response in vaccine group (87.6 spot/well) compared to control group (7.0 spot/well, p = 0.0004) (Fig. 3A). Meanwhile, TPX2 derived vaccine could
also induce high immune response in system; IFN-γ ELISPOT of splenocyte indicated 28.2 spot/well in vaccine group compared to 3.8 spot/well in control group (p<0.0001) (Fig. 3B).

**Discussion**

In our manuscript, we have demonstrated that TPX2 derived vaccine is effective in BALB/c background mice transplanted with 4T1 cell line, through leading CD8 + T cell infiltration to tumor and inducing strong immune response in tumor site and system.

Our experiment indicates that TPX2 derived vaccine could induce CD8 + T cell infiltration in tumor site. It has been demonstrated that the prognosis of TNBC is positively associated with TIL and its subpopulation CD8 + T cell; on the contrary, the proliferation of TNBC is related to decreasing of CD8 + T cell in TIL (10–12). So it is reasonable to suggest that elevating CD8 + T cell infiltration in tumor site could benefit the prognosis of TNBC. In our study, TPX2 derived vaccine successfully induced CD8 + T cell infiltrating in tumor site and stimulated strong immune response and thus showed moderate effect in prohibition of tumorigenesis. However, it has also been suggested that CD8 + T cell with functional T cell receptor (TCR) only take few parts in all CD8 + T cells of TIL, even though in some “hot carcinoma” (13). That means using vaccine could stimulate the formation of antigen specific TCR, subsequently increase TCR specific CD8 + T cells. Our data also suggest that TPX2 derived vaccine could induce strong immune response in system, and then decrease lung metastasis. It is easy to understand that vaccine works systematically, and to some extent modified the tumor environment in system and then inhibit the metastasis of TNBC.

There is no obvious side effect observed in our study. The body weight of mice in vaccine group showed no decreasing after vaccine injection (data not shown). Meanwhile, no autoimmune response occurred in our vaccine group. However, there are also some limitations in our study. Firstly, our study and TPX2 derived vaccine was only suitable for BALB/c background mice; since human has totally different MHC background compared to mice, it is easy to understand that our vaccine is only fit for mice, rather than for human kind. Secondly, in our study, it demonstrated that Treg in TIL seems to be unexpectedly evoked, indicating that our vaccine works in an immune-suppressed microenvironment, which could inhibit the effect of vaccine.

**Conclusion**

In conclusion, our work has supplied a TPX2 derived vaccine which showed moderate effect in a TNBC mouse model. Our vaccine could induce high immune response in tumor site and system, and increase the infiltration of CD8 + in tumor site. Further work should be done to overcome the unexpected evocation of Treg, and deliver it to human kind.

**Declarations**
Ethical Approval and Consent to participate: the animal procedures were approved by the animal ethic committee of Tongji Hospital.

Consent for publication: all authors agree to publication.

Availability of supporting data: Not applicable.

Competing interests: Not applicable.

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Authors' contributions: Y.J. for designing, writing, and part of experiment; Y. L. & L.C. for part of experiment; X. T. for data processing; J.L. & S.Y. for guiding.

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Figures

A. **tumor growing curve**

|        | Control | Vaccine |
|--------|---------|---------|
| 0      | 200     | 200     |
| 10     | 500     | 800     |
| 20     | 1000    | 1500    |
| 30     | 1500    | 2000    |
| * ***  |         |         |

B. **No. of lung metastasis**

|        | control | vaccine |
|--------|---------|---------|
| No. in lung surface | 5 | 10 |
| No. in lung surface | 15 | 20 |
| ***       |         |         |

C. Figure 1

D. Figure 1
vaccine inhibits tumorigenesis and lung metastasis in TNBC. A, tumor growing curve between vaccine and control group; B, No. of metastasis in the surface of lung between vaccine and control group; C-D, representative pathologic image of primary TNBC and lung metastasis. ***, P<0.001.

Figure 1

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Figure 2

Vaccine induces CD8+ T cell infiltration in tumor site. A, CD8+ T cell ratio between vaccine and control group; B, CD4+ T cell ratio between vaccine and control group; C, Treg ratio between vaccine and control group. ** $p < 0.05$, *** $p < 0.001$. 
vaccine induces strong IFN-γ secretion in tumor and system. A, IFN-γ secretion in TIL between vaccine and control group; B, IFN-γ secretion in splenocyte between vaccine and control group. *** $p<0.001$. 
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