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Original Article

Decreased tumorigenic potential of EphA2 over-expression in mouse model of breast cancer following treatment with rAd-T-ephrinA1-caspase3

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Abstract: Objective: To investigate the inhibitory effect of rAd-EphrinA1-Caspase3-T on tumorigenic outcome of EphA2 over-expression in nude mouse model of breast cancer. Method: In situ hybridization histochemistry (ISHH) was used to detect the expression of EphA2 and ephrinA1 mRNA in the human breast cancer tissue samples. The obtained EphA2 over-expressed breast cancer cells were orthotopically implanted into the mammary fat pads of the mouse model. rAd-EphrinA1-Caspase3-T was injected intratumoral into model, and PBS and T lymphocytes were provided to the control groups respectively. The tumor volume was measured, and the tumor growth was analyzed by using immunofluorescence (IF). Result: The positive rates of EphA2 and ephrinA1 mRNA were correlated with the pathological type, tumor volume, lymph node metastasis, clinical staging, and histological grades ($P<0.05$). Furthermore, EphA2 and ephrinA1 mRNA positive stainings were co-localized in similar tumor areas and vascular endothelial cells. In vivo analysis demonstrated that injecting breast cancer cells and touching the subcutaneous tumor showed that the model of breast cancer with over-expression of EphA2 was successfully constructed. Infection with rAd-EphrinA1-Caspase3-T caused increased caspase3 activation while decreased ki67 activation consequently inhibited subsequent tumor growth when compared to matched controls. The results suggest that EphrinA1-Caspas3 targeting of EphA2 with rAd-T secreting EphrinA1-Caspas3 may have therapeutic value. Conclusion: EphrinA1-Caspase3-T is potent and it specifically activates Caspase3 related cell apoptosis, which forms the basis for further development of clinical application of EphA2-targeted cytotoxic effect.

Keywords: EphA2-overexpression of breast cancer model, ephrinAl-caspase3, tumorigenic potential, cytotoxins, nude mouse

Introduction

Tyrosine kinases transmit powerful signals [1, 2] that are essential for diverse biological processes [3, 4] such as migration, adhesion, and angiogenesis. EphA2 signaling transmits powerful signals in the process of tumor development, where forward and reverse signalings have been reported [2, 5, 6]. It also plays an important role in the development of breast cancer. Previous studies have shown that the EphA2 receptor tyrosine kinase is over-expressed in breast cancer [7, 8] and represents a novel, attractive therapeutic target for the treatment of breast tumors [7, 9, 10]. Molecular targeted therapies have the advantage of high specificity and few side effects. It can reverse the malignant biological behavior from the molecular level to the target that may cause cell canceration [8, 11, 12], thereby inhibiting the growth of tumor cells. Under the new biological treatment model, cytotoxic agents inducing apoptosis in cancer cells are used as an anti-cancer chemotherapeutic agent. The cytotoxic T lymphocyte (CTL) targeted treatment of breast cancer provides a new thinking and direction for the current plight [13, 14].

In this study, through ISHH, it was found that EphA2 and ephrinA1 mRNA are highly expressed in breast cancer tissues and are associated

with histological grades, lymph node metastasis, and prognosis of tumors. In previous studies, we have developed an EphA2-targeted agent, EphrinA1-Caspase3-T, a novel cytotoxin composed of ephrinA1, a ligand for EphA2, and caspase3, a marker for efficacy of cancer therapy, and it is a major mediator of apoptosis activated during cellular exposure to cytotoxic drugs, radiotherapy or immunotherapy [15, 16]. Our research found out that, using T-cells as a carrier to culture EphrinA1-Caspase-3-T cells, rAd-EphrinA1-caspase-3-T can act on breast cancer cells in vitro [17]. In short, rAd-EphrinA1-caspase-3-T has targeted killing effect on breast cancer cells in vitro. The present study was undertaken to investigate the effect of rAd-EphrinA1-caspase-3-T on breast tumors in vivo.

We constructed a nude mouse model of EphA2 over-expressing breast cancer, injected rAd-T for treatment, and observed its effect. Furthermore, therapeutic modeling via intratumoral inoculation revealed that rAd-EphrinA1-caspase-3-T significantly inhibited subsequent tumor growth as compared to matched controls. The results indicate that targeting EphA2 with adenoviral vectors may have therapeutic value.

Materials and methods

Materials and instruments

EphA2 rabbit anti-human poly-clonal antibody (ABCAM, UK), EphrinAl rabbit anti-human poly-clonal antibody (Santa Cruz, USA), Digoxin tag Eph A2 and Ephrin A1 cRNA probe (Fuzhou New Biological Technology Co., LTD.), universal in situ hybridization detection kit III (alkaline phosphatase) (Boster Biological Engineering Co., LTD, Wuhan), T cellsinfected ephrina1-caspase-3 [15], goat serum (Sallebaut), estrogen (98%, E808987, McLane), Matrige gel (Corning), primary antibodies including rabbit anti-Caspase-3 poly-clonal antibody, rabbit anti-Ki67 poly-clonal antibody (1:1000; At 1:500, Biopsydig), and secondary antibody of lamb anti-rabbit Ig (G + M) labeled with FITC (1:200 ABnerve).

Tissue samples

150 cases of archived paraffin specimens were obtained from the radical or modified radical mastectomy of breast cancer in the First Affiliated Hospital of Dali University (Fourth People's Hospital of Yunnan Province) from 2012 to 2015. These specimens never received radiotherapy, chemotherapy or any other special treatment before surgery. The age ranged from 27 to 77 years, with an average of 49.5±2 years. Among them, 41 cases were ≤45 years old, and 109 were >45 years old. 102 patients with tumor diameter of <5 cm and 48 patients with a tumor diameter of ≥5 cm. There were 135 cases of invasive ductal carcinoma and 15 cases of intraductal carcinoma. 87 cases had axillary lymph node metastasis, and 63 cases were without lymph node metastasis. There were 30 cases of TNM stage I, 72 cases with stage II, and 48 cases with stage III-IV. In histological grading, 7 cases were with grade I, 79 cases with grade II, and 64 cases with grade III. All specimens were fixed with 4% paraformaldehyde, embedded in paraffin, and continuously sliced at 4 μm thickness.

Animal models

Animals were housed in a non-barrier animal facility under pathogen-free conditions, with 12-hours light and dark cycle, and access to standard rodent diet and water ad libitum. Experiments were performed in accordance with the NIH guidelines and were approved by the Dali University Medical Center Institutional Animal Care and use Committee. The healthy experimental BALB/c mice were six week old at the onset of the experiment. All mice used in this experiment were provided by Department of Medical Experiment in Chenggong Campus of Kunming Medical University, animal license number: SCXK (Dian) K2015-0002.

Cell culture

T lymphocytes were inoculated into T75 cell culture flasks, followed by a multiplicity of infection of 800 MOI adenovirus (pAd-EphrinAl-Caspase-3). After 3 days, the infected lymphocytes are collected as EphrinAl-Caspase3-T lymphocytes. Monolayer cultured EphrinAl-Caspase-3-T lymphocytes were taken in the logarithmic growth phase, digested with 0.25% trypsin, washed twice with PBS into a cell suspension of 100 μL (containing 5×10^6 lymphocytes).

Breast cancer cells identified as EphA2-positive were isolated from human breast cancer tissue and were cultured to obtain cells in loga-

rithmic growth phase to prepare a cell suspension (1×10^5 cells/mL), and the number of viable cells was calculated using physiological saline. Attenuation was done to the desired concentration (the number of cells was 1×10^6 cells/ml-1×10^7 cells/ml), and were mixed with Matrige gel and estrogen.

Groups and treatment

The obtained mixture of breast cancer cells orthotopically were injected through the nipple into the left mammary fat pads of six week old female mice. After a week, for treatment experiment, 30 mice with average tumor tissue were randomly divided into three groups: ① the treatment group, ② the control group and ③ the T lymphocyte group, with 10 mice in each group respectively. Therapeutic agent were injected intratumoral into group: ① the treatment group = 1×10^6 EphrinA1-Caspase3-T lymphocytes (10 μl); ② the T lymphocyte group = 1×10^6 T lymphocytes (10 μl) and ③ the control group = PBS (10 μl).

Tumor volume

Tumor dimensions were measured by digital caliper at a given time point on every alternate day, and volume was calculated using the following formula: volume = length × width^2 × 0.52. All mice were sacrificed 15 days after treatment, and tumors were resected for analysis.

In situ hybridization histochemistry (ISHH)

Following the instructions of "Universal In Situ Hybridization Detection Kit III (Alkaline Phosphatase)", BCIP/NBT color development, nuclear fast red counter-staining, neutral gum sealing, and light microscope observation were performed. The label-free probe hybridization solution was used as the negative control instead of the probe, and the positive film was used as the positive control. The positive signal of EphA2 and ephrinA1 mRNA is defined that the cytoplasm and membrane are stained purple or purple-blue. High-power microscope fields (5 times) were randomly selected for each slice, and more than 500 cells were counted, and (+) and (-) were distinguished according to the proportion of stained cells in the number of tumor cells. EphA2 mRNA was considered positive with every high-power field (5 times) >20%, otherwise it was regarded as negative. Regarding ephrinA1 mRNA, it was considered positive with every high-power field (5 times) >10%. The results judgment was approved by two senior pathologists.

ELISA

The tumor tissues of the models were obtained at 2nd, 5th, 8th, 11th, and 14th day after the treatment. After homogenization, the operation was performed according to the operating instructions of the ELISA test kit. The microplate reader was used to measure the optical density (OD value) of each well at 450 nm, in order to detect the content of EphrinAl-Caspase-3.

Immunofluorescence (IF)

The obtained tumor tissue was placed in 4% paraformaldehyde, fixed for 48 hours and transferred to 15% and 30% sucrose solution for dehydration. After completion, OCT was embedded and sliced in a -20°C incubator. The fluorescence microscope was used to observe the expression of green fluorescence. The distribution of protein T lymphocytes was studied by immunofluorescence staining to detect the expression of Caspase-3 and Ki-67.

Statistical analysis

The SPSS 20.0 statistical software was used for data analysis. The χ^2 chi-square method was used to analyze the comparison and correlation of sample rates. T-test was used to analyze the animal treatment groups between the two groups, and $P<0.05$ was considered statistically significant. All graphing was performed by GraphPad Prism 5.0.

Results

Expression of EphA2 and ephrinA1 mRNA in breast cancer

The study found out that EphA2 and ephrinA1 were mainly expressed in the cytoplasm and membrane of tumor cells and vascular endothelial cells, while a small numbers were located in the cytoplasm of inflammatory cells. The positive staining was shown as brown or dark brown with uniform staining (**Figure 1**). The positive expression rate of EphA2 and ephrinA1

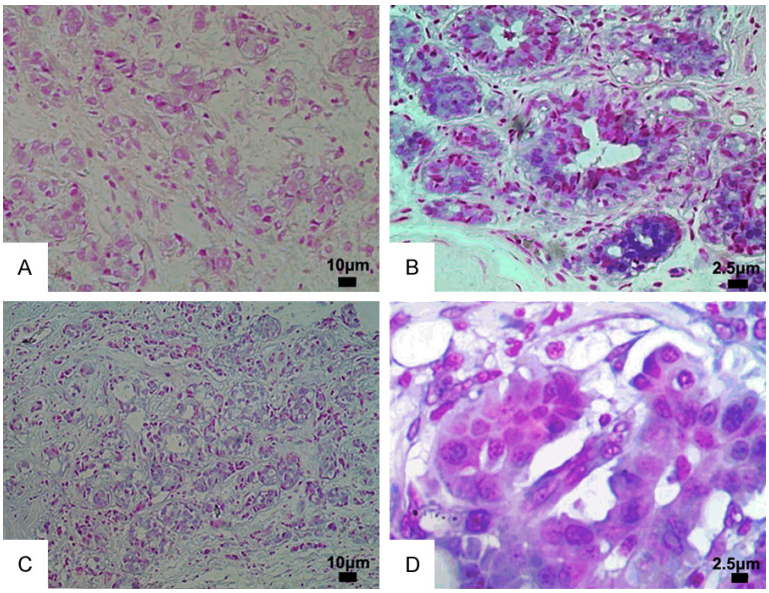

Figure 1. Positive and negative expression of EphA2 and EphrinA1 in invasive ductal breast cancer (ISHH). (A) Negative expression of EphrinA1 Mrna (10×10). (B) Positive expression of EphrinA1 mRNA (10×40). (C) Negative expression of EphA2 mRNA (10×10). (D) Positive expression of EphA2 mRNA (10×40). Bar = 10 μm (A, C), 2.5 μm (B, D).

mRNA was not related to the patient's age (P>0.05) but was related to pathological type, tumor size, lymph node metastasis, clinical staging and histological grades (P<0.05). Whereas the positive expression rate of EphA2 and ephrinA1 in invasive ductal carcinoma is higher than that of intraductal carcinoma. And the positive expression rates of EphA2 and ephrinA1 in the larger tumor group, lymph node metastasis group, late clinical stage, and higher histological grade groups were higher than those of other groups (**Tables 1**, **2**). According to statistical analysis, the positive expression rate of EphA2 and ephrinA1 mRNA shows correlation (P<0.05) (**Table 3**). It was also found that EphA2 and ephrinA1 proteins in breast cancer tissues are co-localized in resembling tumor areas and vascular endothelial cells, with a similar distribution.

The effect of rAd-Ephrina1-Caspase-T in breast cancer tumors

Subcutaneous tumors can be palpated one week after cell inoculation. After successful modeling of breast cancer cells in tumor-bearing animals, the effect on tumor volume can be observed at various time points after group treatment (**Figure 2A**). On day 0 and day 3, there was no statistically significant difference in tumor volume between the groups (P>0.05). On day 6th, a significant difference in tumor volume could be found, and the difference in tumor volume increased over time. Compared with the control group or T lymphocyte group, the treatment group had statistically significant differences (P=0.002, P=0.036). Although the tumor volume of the T lymphocyte group increased more slowly than that of the control group, there was no statistically significant difference between the two groups (P>0.05). After the experiment, the tumors of each group were stripped and removed (**Figure 2B**). It can be found that the tumor volume of the treatment group was significantly reduced in comparison with that of the control group and T lymphocyte group.

EphrinA1-caspase3 secretion in tumor tissue

The tumor tissues of the killed mice were collected for frozen section, and the treatment group tumor tissue was observed under a fluorescence microscope. Scattered green fluorescent protein labeled EphrinAl-Caspase-3-T lymphocytes could be seen. The control group and T lymphocyte group did not detect the presence of green fluorescent protein (**Figure 3A**). ELSIA detects the secretion of EphrinA1-Caspase-3 in the treatment group (**Figure 3B**). The EphrinAl-Caspase-3 expression in the treatment group can be detected on the 3rd day, reaching a peak on the 6th day, and then the secretion amount gradually decreased. The expression of EphrinA1-Caspase-3 was not detected in the control group and T lymphocyte group.

Detection of caspase-3 and KI-67 levels in tumor tissues

Caspase3 antibody was used to perform immunofluorescence detection on tumor tissues (**Figure 4A**). Caspase3 positive cells in the treatment group showed a higher expression than T lymphocytes group and control group. The Ki67 antibody was used for immunofluorescence detection on tumor tissues (**Figure**

Table 1. Expression of EphA2 mRNA in relation to clinicopathological parameters in the breast

Clinicopathological parameters	n	EphA2mRNA		χ^2	*P* (2-sides)
		+ (%)	- (%)		
Age					
≤45	41	28 (68.3)	13 (31.7)	1.375	0.241
>45	109	63 (57.8)	46 (42.2)		
Pathological type					
Intraductal carcinoma	15	5 (33.3)	10 (66.7)	5.218	0.022
Invasive ductal carcinoma	135	86 (63.7)	49 (36.3)		
Tumor size					
<5 cm	102	52 (51.0)	50 (49.0)	12.533	0.0008
≥5 cm	48	39 (81.2)	9 (18.8)		
Metastasis of lymph node					
+	87	59 (67.8)	28 (32.2)	4.437	0.035
-	63	32 (50.8)	31 (49.2)		
TNM staging					
I	30	12 (40.0)	18 (60.0)	5.215	0.036
II	72	45 (62.5)	27 (37.5)		
III-IV	48	34 (70.8)	14 (29.2)		
Histological grade					
I	7	2 (28.6)	5 (71.4)	7.201	0.027
II	79	45 (57.0)	34 (43.0)		
III	64	44 (68.8)	20 (31.2)		

Table 2. Expression of ephrinA1 mRNA in relation to clinicopathological parameters in the breast

Clinicopathological parameters	n	ephrinA1mRNA		χ^2	*P* (2-sides)
		+ (%)	- (%)		
Age					
≤45	41	27 (65.9)	14 (34.1)	0.007	0.935
>45	109	71 (65.1)	38 (34.9)		
Pathological type					
Intraductal carcinoma	15	6 (40.0)	9 (60.0)	4.723	0.030
Invasive ductal carcinoma	135	92 (68.1)	43 (31.9)		
Tumor size					
<5 cm	102	61 (59.8)	41 (40.2)	4.303	0.038
≥5 cm	48	37 (77.1)	11 (22.9)		
Metastasis of lymph node					
+	87	64 (73.6)	23 (26.4)	6.195	0.013
-	63	34 (54.0)	29 (46.0)		
TNM staging					
I	30	15 (50.0)	15 (50.0)	6.516	0.041
II	72	48 (66.7)	24 (33.3)		
III-IV	48	35 (72.9)	13 (27.1)		
Histological grade					
I	7	2 (28.6)	5 (71.4)		
II	79	50 (63.3)	29 (36.7)	6.345	0.042
III	64	46 (71.9)	18 (28.1)		

Table 3. Correlation between the expression of EphA2 mRNA and that of ephrinA1 mRNA in the breast cancer

EphrinAlmRNA	EphA2mRNA		χ^2	*P* (2-sides)
	+	-		
+	67	31	7.025	0.008
-	24	28		

4B). It was found that the expression of Ki67 in the treatment group was significantly lower than that of the T lymphocyte group and the control group.

Discussion

The Eph receptor tyrosine kinases (RTK) and their ephrin ligands have been intensely studied for the role they play during embryonic development, particularly within the central nervous system. Some of these proteins, particularly EphA2 and ephrinA1, are of increasing interest in recent years due to their documented or suspected involvement in mediating processes leading to the formation and progression of malignancy. Moreover, expression of EphA2 is associated with poor prognosis, increased metastasis, and decreased survival. The EphA2 receptor tyrosine kinase is frequently overexpressed in many cancers, including 40% of breast cancers. Importantly, this protein is highly over-expressed with regard to percentage of patient tumor and percentage of cells within a tumor, and in a plasma membrane-localized receptor that can internalize on ligand binding. EphA2 ligand ephrinA1 induces EphA2 phosphorylation, intracellular internalization and degradation, thus inhibiting tumor progression. The finding points towards the possible existence of a feedback loop mechanism where EphA2 acts to suppress expression of ephrinA1 and vice versa [7]. Increased expression of EphA2 following knockdown of ligand ephrinA1 expression is not due to activation of the MAPK pathway since levels of phospho-ERK do not change following ephrin knockdown [18]. EphA2 overexpression is common in advanced gastric tumors, and investigation has also linked EphA2 with mutant p53 [19]. C1GALT1 is associated with promoting soluble EphrinA1 mediated cell migration through activation of EphA2 in gastric cancer [20]. Kinch found that the expression of EphA2 in lung cancer has nothing to do with histological type and degree of differentiation, but is related to its clinical stages [21]. However, Ishikawa et al. found that higher expression of EphA2 and ephrinA1 is related to favorable clinicopathological features in stage I non-small cell lung carcinoma [22]. Brantley-Sieders et al. pointed out that the expression of EphA2 and ephrinA1 in breast cancer gradually increased with the increase of tumor malignancy, and was related to survival rate [23]. In short, the results of these studies have shown that the relationships between EphA2 and ephrinA1 and clinicopathological factors in different tumors, even in the same tumor, are not completely consistent. In the present work, we found out that the mRNAs of EphA2 and ephrinA1 were also highly expressed in cancer tissues, and their positive rates were 60.67% and 65.33%, respectively, which suggests that the two may be related to the occurrence and development of breast cancer, but regarding the up-regulation of EphA2 and ephrinA1 expression in breast cancer, the exact mechanism has not yet been proven. The expression of EphA2 and ephrinA1 mRNA was correlated with pathological type, tumor size, lymph node metastasis, clinical stages and histological grades ($P< 0.05$). It can be seen that the positive expression of the respective proteins of EphA2 and ephrinA1 and the positive expression of their respective mRNAs is related to the malignant behavior of breast cancer to a certain extent, suggesting that the two may be related to the development, metastasis and degree of malignancy of breast cancer. EphA2 and ephrinA1 are the most widely studied with respect to tumorigenesis, angiogenesis, and metastasis and represent sought-after therapeutic targets because of their expression and diverse functions in several different types of cancers [24, 25].

Caspase-3 is one of the most important apoptosis performers in Caspase family. Fernandez-Alnemri first cloned Caspas-3 from the cDNA library of Jurkat T lymphocytes, and it encodes a cysteine protease with a molecular weight of 32 kD. The Caspase-3 is not only the most important terminal shear enzyme in the procedure of apoptosis but also an important component of cytotoxic mechanism of cytotoxic T cell. Caspase3 discovered activation of Caspase in some cancer cell apoptosis. It may be

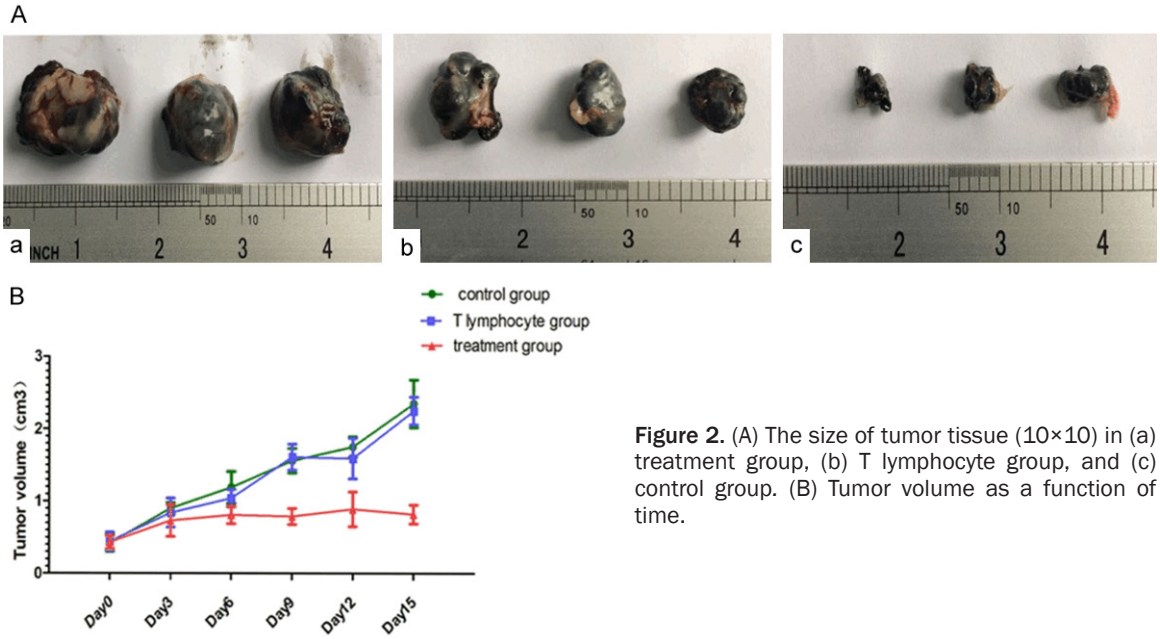

Figure 2. (A) The size of tumor tissue (10×10) in (a) treatment group, (b) T lymphocyte group, and (c) control group. (B) Tumor volume as a function of time.

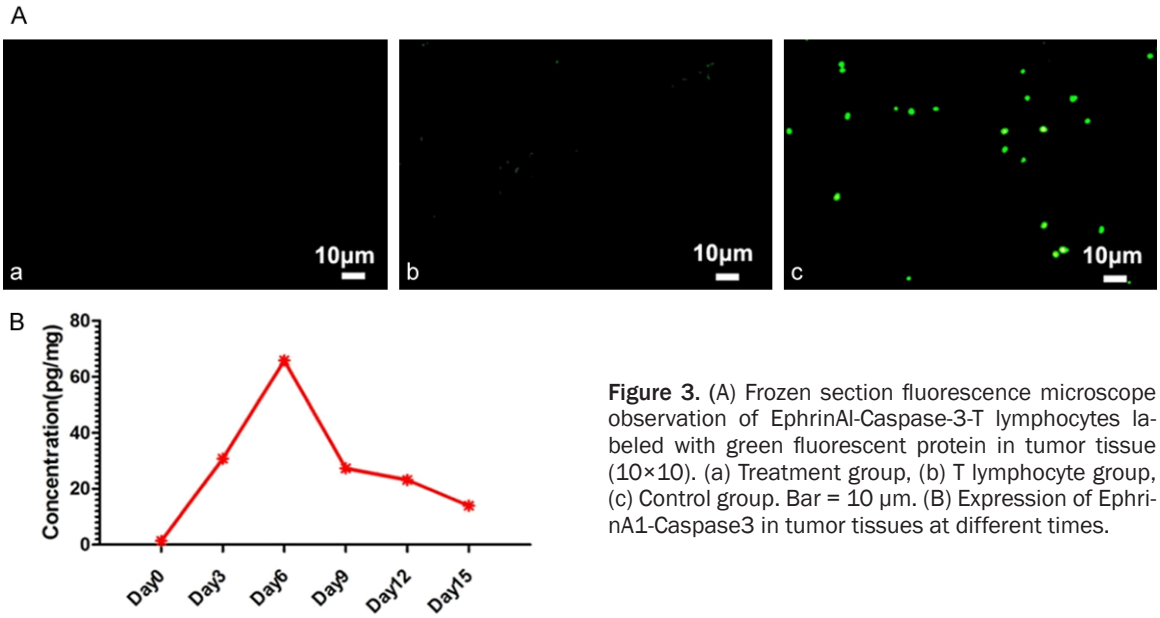

Figure 3. (A) Frozen section fluorescence microscope observation of EphrinAl-Caspase-3-T lymphocytes labeled with green fluorescent protein in tumor tissue (10×10). (a) Treatment group, (b) T lymphocyte group, (c) Control group. Bar = 10 μm. (B) Expression of EphrinA1-Caspase3 in tumor tissues at different times.

related to CTL-mediated granzyme B delivery and subsequent caspase3 activation [26]. Studies by Eifel et al. have shown that down-regulation or loss of caspase expression is associated with breast cancer [27]. Nakopoulou et al. found that the high expression of Caspase-3 protein existed in about 75.2% (103 samples in 137 samples) breast invasive cancer samples [28]. The high expression of Caspase-3 protein is also related to breast cancer specific survival rate in patients with breast cancer [29]. In Recent years, a new series of Caspase related apoptosis-inducing treatment is adopted to cure cancer. Ki67 is a nuclear antigen closely related to mitosis, and it can only be detected in the nucleus of proliferating cells. This can be used to judge cell proliferation activity. Ki67 is very closely related to the occurrence and development of breast cancer [30]. The positive expression rate of Ki67 in normal breast tissue is very low, about 3%. Results of some researchers have shown that

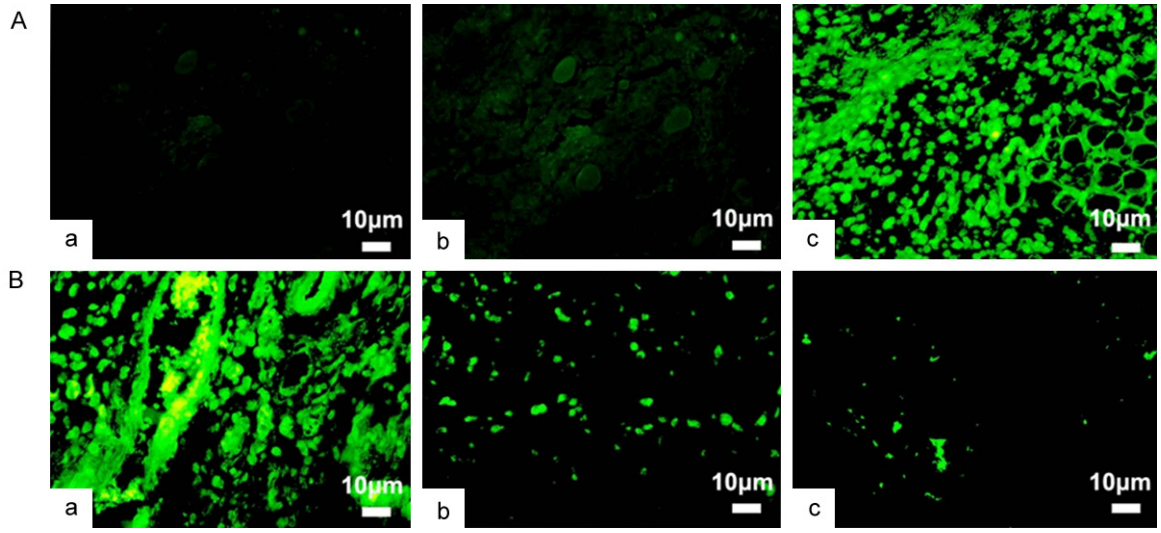

Figure 4. (A) Immunofluorescence detection of caspase3 expression in tumor tissue (10×10). (a) Treatment group, (b) T lymphocyte group, (c) Control group. Bar = 10 μm. (B) Immunofluorescence detection of ki67 expression in tumor tissue (10×10). (a) Treatment group, (b) T lymphocyte group, (c) Control group. Bar = 10 μm.

the positive expression rate of Ki67 in breast cancer tissue is as high as 76.1% [31]. In addition, breast cancer patients with a higher Ki67 positive expression rate have higher cancer cell proliferation activity, higher histological grades, often accompanied by lymphatic metastasis, and poor prognosis [32]. Ki67 is currently one of the most reliable indicators for judging the prognosis of malignant tumors. In the current study, the contents of caspase-3 and Ki67 in tumor tissues were detected at the same time. In this study, based on the characteristics of EphA2 and its ligand ephrinA1 involved in the occurrence and development of breast cancer, and using the related effects of caspase3 and T lymphocytes, we designed the rAd-EphrinA1-Caspase3-T adenovirus vector based on cytotoxicity, and observed its effects on tumorigenicity through a mouse model of breast cancer. As expected, the positive rate of caspase-3 in the treatment group was found to be significantly increased, while the positive rate of Ki67 was significantly decreased, which indicates that EphrinA1-Caspase-3-T cells can inhibit breast cancer cells growth activity in vivo.

Recent studies have shown that those tumors that over-express EphA2 including metastatic melanoma and other breast, prostate, colon, lung, ovarian, and esophageal cancers can be targeted using monoclonal antibodies [33] and peptides [34]. The adenoviral approach offers the advantages over purified protein-based approaches mainly due to the ability of adenoviral vectors to express high levels of ephrinA1 continuously and over longer periods of time [35]. It is particularly notable that we were able to achieve a powerful anti-tumor response with a single intratumoral inoculation at 1×10^6/mL of ephrinA1-caspase3-T (10 mL), and this was sufficient to achieve tumor shrinkage. This indicates that this method is sufficient to inhibit the potential of tumor. However, we still have a lot of work to do. In this study, we did not relate the efficacy of targeting strategy to EphA2 activation and degradation. If the present work can be extended to analogous situations in human, then adenoviral-based targeting of EphA2 could provide therapeutic benefit for such conditions. Moreover, this trial only involved the treatment of cancer in situ and did not involve the treatment of metastatic and recurrent cancer. However, it is worth noticing that total toxicity was not observed in the xenograft model. Future studies will be necessary to evaluate the toxicity issues associated with adenoviral-based ephrinA1-caspase3-T targeting. In conclusion, the major finding of the current study is that the targeting of EphA2 over-expressing breast malignant cells with adenoviral vector expressing ephrinA1-caspase3 is sufficient to decrease tumorigenic potential in vivo.

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Disclosure of conflict of interest

None.

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