RESEARCH ARTICLE

Semaphoring mAb: a New Guide in RIT in Inhibiting the Proliferation of Human Skin Carcinoma

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Abstract

Semaphoring is a transmembrane receptor which participates in many cytokine-mediated signal pathways that are closely related to the angiogenesis, occurrence and development of carcinoma. The present study was designed to access the effect of mono-antibody (mAb) guided radioimmunotherapy (RIT) on skin carcinoma and investigate the potential mechanisms. Semaphoring mAb was acquired from mice (Balb/c), purified with rProtein A column; purity, concentration and activity were tested with SDS-PAGE and indirect ELISA; specificity and expression on the cutanuem carcinoma line and tissue were tested by Western blotting; morphology change was assessed by microscopy. MTT assay and colony inhibition tests were carried out to test the influence on the proliferation of tumor cells; Western blotting was also carried out for expression of apoptosis-associated (caspase-3, Bax, Bcl-2) and proliferation-related (PI3K, p-Akt, Akt, p-ERK1/2, ERK1/2) proteins and analyse the change in signal pathways (PI3K/Akt and MEK/ERK). The purity of purified semaphorin mAb was 96.5% and the titer is about $1×10^6$. Western blotting showed semaphoring mAb to have specifically binding stripes with semaphoring b1b2 protein, B16F10, and A431 cells at 39KDa, 100KDa and 130KDa, respectively. Positive expression was detected both in cutanuem carcinoma line and tissue and it mostly located in cell membranes. MMT assay revealed dose-relate and time-relate inhibitory effect of semaphorin mAb on A431 and B16F10. Colony inhibition tests also showed dose-relate inhibitory effects. Western blotting demonstrated the expression of apoptosis and proliferation-related protein and changes in signal pathway. In conclusion, we demonstrated that semaphorin is highly expressed on the tumor cell-surfaces and RIT with semaphorin mAb has effect in inhibiting proliferation and accelerating apoptosis of tumor cells.

Keywords: Semaphoring mAb - radioimmunotherapy - human cutanuem carcinoma

Introduction

People have a high incidence of skin carcinoma in the areas where suffer from high UV-exposure while traditional treatments such as radiotherapy and chemotherapy have poor prognosis and significant side effect (El-Domyati et al., 2013). Luckily, molecular targeted therapy emerges to be a novel promising treatment for its cancer-cell specific targeting ability with limited adverse effects (Abbasakoor et al., 2013).

Radioimmunotherapy (RIT) is a novel way which uses isotope labeled mono-antibody (mAb) to specifically inhibit proliferation and accelerate apoptosis of tumor cells. Recently, many researches reveal that semaphorin, a transmembrane receptor for many cytokines, participate in many cytokine-mediated signal pathways which are closely related to the angiogenesis, occurrence and development of carcinoma (Cagnoni et al., 2013; Kohno et al., 2014; Suzuki et al., 2014). Many studies have also demonstrated the correlation between semaphorin and skin carcinoma, however, the mechanism is unclear (Cagnoni et al., 2013; Nasarre et al., 2013; Nehil et al., 2013). In this study, we analyse the influence of semaphorin mAb on the survival of human skin carcinoma cell line and its potential mechanism.

Materials and Methods

Cell culture

A431 human skin carcinoma cell line was obtained from Shanghai Institution for Biological Sciences. Cells were subcultured in the incubator with 5% CO_2.

Animal model

All animal experiments were conducted according to protocols approved by the ethics committee of Shanghai (SCXX 2012-0002). 20 male nude mice (BALB/c-nu/nu) used for the experiment were obtained from Shanghai Research Center for Model Organisms with average weight of 20g and age arranged from 4 to 6 weeks. The mice were kept in sterile environment with stable temperature (25~27°C) and moister (40%~50%). The experience conditions meet the requirement of SPF.

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Establishing a mouse model of the human skin carcinoma in situ

The five mice were divided into three groups: Group 1 (one mouse) was injected with 2×10^6 A431 cells each; Group 2 (two mice) were injected with 1×10^6 cells each; Group 3 (two mice) were injected with 0.5×10^6 cells each. All injections were taken with the volume of 100ul and targeted at the bilateral ventral lobes of the nude mice. The in situ skin carcinoma models in nude mice were established, and the changes and the time when the carcinomas grew in those nude mice were observed and recorded.

Marking and identification of ^{131}I in anti-semaphorin monoclonal antibody and IgG

The anti-semaphorin monoclonal antibody was obtained from the Sigma Company in American. The marking and identification of ^{131}I in anti-semaphorin monoclonal antibody and IgG were carried in the nuclear institute in Shandong University.

MTT assay

MTT assay was taken to evaluate the difference in proliferation ability of A431 cells after processing with drugs. The control group, ^{131}I-IgG group, semaphorin-mAb group and ^{131}I-semaphorin-mAb group were set with the concentration of drugs of 2ug/ul. Collect the cells when they in the logarithmic growth phase, prepare them for single cell suspension, adjust their concentration to 2×10^3/hole and subculture them in the 96 hole-plate with 3 repeated holes. After cultured for 2 days, drew the growth curve with MTT in the wavelength of 570nm.

The growth inhibition rate=1-the OD value of experiment group/ the OD value of control group

The Apoptosis Kit test

Cells were harvested by trypsinization, washed with ice-cold PBS, suspended in 1×Binding Buffer and transferred to Streaming tube. Cells were mixed with FITC-Annexin V and PI and incubated them in 25 °C in dark for 15min. The sample was mixed with 400μL 1×Binding Buffer analyzed with flow cytometry (BD company) after 1 hour.

Transwell migration assay

The migration test was carried out in the transwell migration room, which was setted in the 24-hole plate. The diameter of the pore in the transwell is 8um. 2×10^4 cells were added in 200ul cell medium (without serum) to the upper room and fetal bovine serum (FBS) were added to the lower room as a chemoattractant. Incubated in 37 °C for 10 hours, the redundant cells in the upper room were removed and the cells which migrated to the lower room were fixed with 4% paraformldehyde and 0.1% crystal violet before counted. Three repeat groups in each experimental group were set.

SPECT Radio-immuno-image (RII) of the nude mice with skin carcinoma

200ug (200ul) ^{131}I-semaphorin-mAb were injected in 3 nude mice model with carcinoma via tail vein.

SPECT static imaging was carried 1h, 4h, 12h, and 24h respectively after the administration. The array of the pinhole collimator was 128 x 128, the Zoom value was 1.33 and the sampling points per frame was 200, 000.

TUNNEL test

The tumor tissues were Paraffin–embed and the apoptosis of the tumor was tested with One Step TUNEL Apoptosis Assay Kit (Beyotime).

Western blot analysis assy

The cells collected after cultivated for 48h was lysed in 4°C for 30min. Cell lysates were centrifuged at 12 000×g for 10 min, and the supernatants were collected into fresh tubes. Lowry assay was taken to measure the concentration of the protein. The 50ug of whole-cell lysates were separated by SDS- PAGE and transferred to PVDF membranes by electro-blotting. After blocked by 5% skim milk, PVDF membranes were incubated with antibody and washed by TBST. The combination of antigen-antibody was detected by Chemiluminescence method.

Statistical analysis

Data were tabulated by Exel 2007 and analyzed by SPSS version 18.0. Results were expressed as the mean±the standard error of the mean (SEM). Group comparisons were performed using Student t test. Differences were considered significant at $p < 0.05$.

Results

The effect of ^{131}I-semaphorin-mAb on the proliferation of A431 in vitro

Colony inhibition tests (Figure 1) showed that compared to the control group, the cells proliferation in three experimental groups was inhibited to different degrees. The max inhibitive effect (54%) was detected in ^{131}I-semaphorin-mAb Group.

The influence of ^{131}I-semaphorin-mAb on the apoptosis of A431 cells

MTT assay (Figure 2) demonstrated that the cells in three experimental groups showed an increase in apoptosis when compared to control group, and the ^{131}I-semaphorin-mAb group showed the max apoptosis rate of 50%.

The influence of ^{131}I-semaphorin-mAb on the migration of A431 cells

Transwell test (Figure 3) exhibited that the migration ability was inhibited in the experimental group, but only ^{131}I-semaphorin-mAb group had a statistic significant difference with control group ($p < 0.01$).

*The influence of ^{131}I-semaphorin-mAb on the A431 cells in vivo*

TUNEL Apoptosis Assay Kit (Figure 4) demonstrated that after processed with ^{131}I-semaphorin-mAb, the A431 cells *in vivo* showed an increased apoptosis potency, the T/NT ratio also increased.
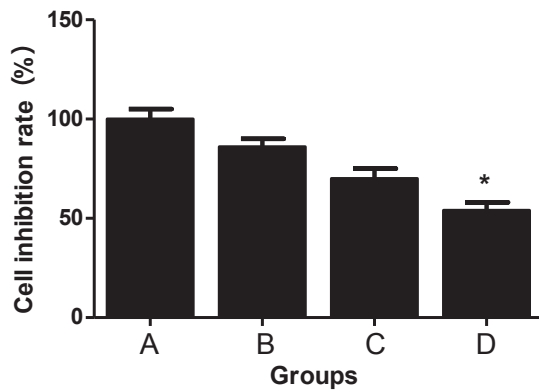
Figure 1. The Inhibition on A431 Cells after Administrated Drugs. A: the control group; **B:** the ^{131}I-IgG group; **C:** the semaphorin-mAb group; **D:** the ^{131}I-semaphorin-mAb group
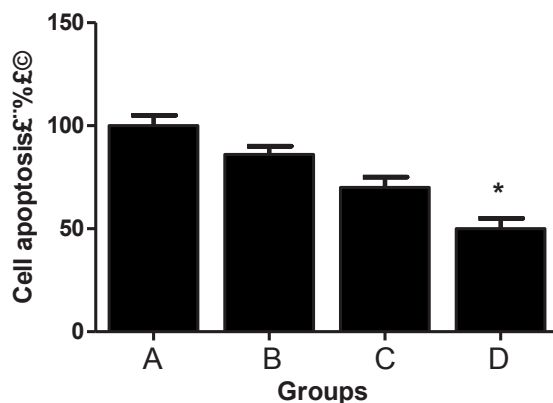
Figure 2. The Inhibition on A431 Cells after Administrated Drugs. A: the control group; **B:** the ^{131}I-IgG group; **C:** the semaphorin-mAb group; **D:** the ^{131}I-semaphorin-mAb group
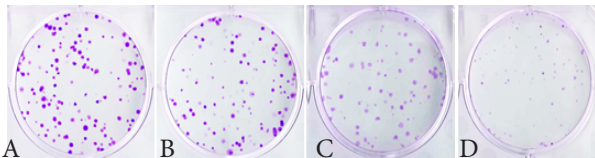

Figure 3. The Inhibition of Migration Ability after Administration of Drug. A: the control group; **B:** the ^{131}I-IgG group; **C:** the semaphorin-mAb group; **D:** the ^{131}I-semaphorin-mAb group

The apoptosis-related protein and signal channel detected by Westernblot analysis

Western blot (Figure 5) displayed that semaphorin mAb could enhance the expression of apoptosis-related protein (Caspase-3, Bax, Bcl-2) that induce the apoptosis of utanuem carcinoma, and decrease the expression of proliferate-related protein (PI3K, *p*-Akt, Akt, *p*-ERK1/2, ERK1/2). It illustrated that semaphorin mAb plays a role in the PI3K/Akt and MEK/ERK signal channel and had inhibitory effect to skin carcinoma.

Discussion

At present, the first-line treatments like surgery, radiotherapy and chemotherapy have limited effect for cancer while their adverse effects are significant. Targeting carcinomas with therapeutic mAbs has proven
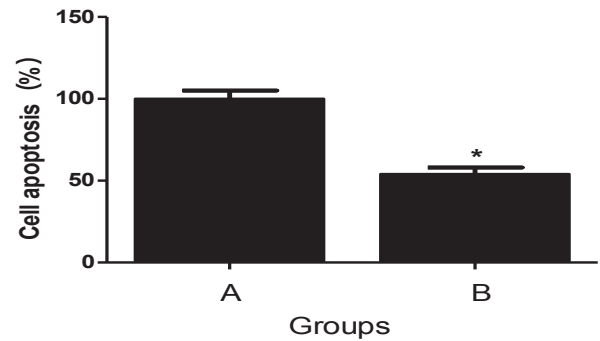
Figure 4. The Influence of ^{131}I-semaphorin-mAb on the A431 Cells *in vivo*. A: the control group; **B:** the ^{131}I-IgG group
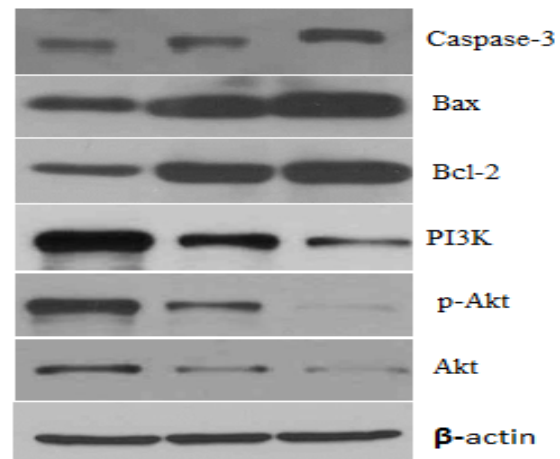
Figure 5. The Influence on the Apoptosis-Related Protein and Signal Channel after Administration of ^{131}I-semaphorin-mAb. A: the control group; **B:** the ^{131}I-IgG group

to be an effective approach to treat cancers (Staudacher et al., 2014). Safe and feasible, RIT leads to satisfactory response rates with acceptable toxicity (Hohloch et al., 2014). The active principle of RIT in carcinoma is the targeting of tumor cells by therapeutic mAb labeled with radioactive isotopes, acting primarily by killing tumor cells via the emission of radioactive ray (Staudacher et al., 2014). After administrated, the mAb specifically bind to the receptor in tumor cells, and the radioactive isotope emit radioactive ray. The ray could damage the DNA and then inhibit the proliferation and promote the apoptosis of the tumor cells. Since its introduction in 1993 to treat B-cell lymphoma, RIT has provided an advanced and practical treatment choice and it is currently approved for follicular lymphoma as consolidation after first-line treatment and for refractory or relapsed disease (Trotta et al., 2003; Kovtun et al., 2006; Sommer et al., 2011; Song et al., 2011). At present, RIT has been successfully applied to the treatment of ovarian cancer (Frost et al., 2013; Grunberg et al., 2014), colorectal cancer (Guo et al., 2013; Sharkey et al., 2013), and lung cancer (Bouilhol et al., 2013; Fujiwara et al., 2014; Staudacher et al., 2014).

In various human tumors, the expression of semaphorin is found to be changed comparing to normal tissues, and semaphoring receptors, that is plexins and neuropilin, are widely expressed. SemaphorinN is a trans-membrane glycoprotein and semaphorinN is the co-receptor for many cytokines such as semaphorin 3 (Sema 3),

hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) (Cagnoni et al., 2013; Kohno et al., 2014; Suzuki et al., 2014). SemaphorinN have been found linked to diverse receptor tyrosine kinases (RTKs), such as Met, ErbB2 and VEGFR2 (Cagnoni et al., 2013). Growing number of studies has revealed the association of semaphorins and their receptors to multiple cancer hallmarks, such as metastatic properties, angiogenesis, regulation of proliferation and apoptosis (Rehman et al., 2013). Semaphorins also play a role in the recruitment and activity of carcinoma-associated immune cells, which have demonstrated to be involved in almost all steps of tumor progression (Muratori et al., 2012). However, the receptor complexes are found in a cell-specific manner and can mediate distinctive signaling cascades, which can either promote or inhibit tumor progression, depending on the cancer type and cellular context (Tamagnone, 2012). Thus besides their primary function of wiring the neuronal network, semaphorins are involved in many aspects of tumor biology, and notably, they are emerging to be the modifiers in tumor progression and can be considered to be a promising therapeutic targets in cancer (Cagnoni et al., 2013).

In our study, we set a control group and three experimental groups: the ^{131}I-IgG group, the semaphorin-mAb group and the ^{131}I-semaphorin-mAb group. Vitro experiments such as MTT assay, the apoptosis kit test and the transwell migration assay were carried out to evaluate the influence of different drugs on the growing, apoptosis, invading and migration of tumor cells. The results are demonstrated in Figure 1-3. We found that both ^{131}I-IgG and semaphorin-mAb have some positive effects in inhibiting the growing, invading and migration while promoting the apoptosis of the tumor cells. However, the effects are not so significant as ^{131}I-semaphorin-mAb.

As we demonstrated previously, RIT uses radiolabeled antibodies, in which mAb have interaction with specific antigen in target tumor cells while radioactive isotopes release tumoricidal dosed of radiation to target cells (Jandl et al., 2013). In the ^{131}I-IgG group, although ^{131}I emit radioactive ray to kill the tumor cells, however, IgG did't specifically bind to the tumor cells. If administrate the drug to human boy, the drug would distribute widely, the doses in the target cells would diminished and couldn't reach the tumoricidal doses of radiation while the normal tissues would be damaged. In the semaphorin-mAb group, although semaphorin-mAb could specifically binds to antigens which is widely presents on the tumor cells, but the effect is also limited. Without the radiation, the principle of the tumoricidal effect may relay on the reaction between the antigen-antibody. The binding occupies thesemaphorin receptor and blocks the signal pathway regulating almost all steps in tumor progression, thus the cancer hallmarks such as metastatic properties, angiogenesis, proliferation and apoptosis are changed (Muratori et al., 2012; Rehman et al., 2013). As for the ^{131}I-semaphorin-mAb group, it takes advantage of both the antigen-antibody interaction and the tumoricidal radiation to kill the tumor cells specifically and effectively (Jandl et al., 2013).

In our vivo studies, ^{131}I-semaphorin-mAb also showed its stable anti-tumor effect. The result of TUNEL apoptosis assay kit (Figure 4) demonstrated that the tumor cells showed an increased apoptosis potency after processed with ^{131}I-semaphorin-mAb. The increased T/NT ratio also indicating that the drug had little adverse effect on other normal tissues. Western blot (Figure 5) identified that semaphoring mAb can enhance the expression of apoptosis-related protein (Caspase-3, Bax, Bcl-2) that induce the apoptosis of utanuem carcinoma, and decrease the expression of proliferate-related protein (PI3K, *p*-Akt, Akt, *p*-ERK1/2, ERK1/2). The result illustrated that the mAb plays a role in the PI3K/Akt and MEK/ERK signal channel which are closely related to many aspect of tumor biology such as the metabolism, proliferate and apoptosis.

In general, our study demonstrated that the RIT with ^{131}I-semaphorin-mAb is highly specialized targeting at the target cell and has notable effect on tumor cells while have little adverse effect on other normal tissues. These merits make RIT a promising treatment to carcinoma.

However, several limitations should be mentioned. Firstly, although the specific antigen is highly expressed on the tumor cell-surface, some normal cells also express the same antigen (Staudacher et al., 2014). Altering the radionuclide or combing RIT with radio-sensitising drugs or small molecule enzyme inhibitors are the good ways the improve the safety and efficacy of RIT (Al-Ejeh F, 2011). Secondly, carcinomas are easy to evolve due to the defective DNA damage responses (Bartkova et al., 2005) which would diminish the effects of the treatment. F. Al-Ejeh (Al-Ejeh et al., 2010) suggested that DNA repair inhibitors would be an attractive target to solve this problem. Thirdly, RIT has been successfully used in the treatment of lymphoma (Trotta et al., 2003; Kovtun et al., 2006; Sommer et al., 2011; Song et al., 2011) ovarian cancer (Frost et al., 2013; Grunberg et al., 2014), colorectal cancer (Guo et al., 2013; Sharkey et al., 2013), and lung cancer (Bouilhol et al., 2013; Fujiwara et al., 2014; Staudacher et al., 2014) but thus far has not demonstrated significant efficacy in humans beyond disease stabilization in solid tumors (Guo et al., 2013). Further researches need be carried out to find out the mechanism and improve the method to amplify the extent of application.

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