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TITLE: Enhancement of Tumor Immunotherapy by Blockade of a Prostate Tumor
Derived Immunosuppressive Factor

PRINCIPAL INVESTIGATOR: Xu Hui, Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, Alabama 35294-0109

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14. ABSTRACT Slit2 is a soluble protein that has been demonstrated to regulate cell migration and inhibit inflammatory reactions. Recent studies suggest that Slit2 may play a role in tumor development. However, conflict results have been reported about the expression level of Slit2 in normal and tumor tissues and the effect of Slit2 on development. The current studies in this report have for the first time demonstrated that forced expression of Slit2 in tumors suppresses the growth of human prostate tumor Du145, fibrosarcoma HT1080 and epidermoid tumor A431 cells in an anchorage independent way. Further experiments indicate that Slit2 inhibits tumor growth and reduces metastasis of HT1080 tumors in lungs of nude mice. Additionally, in situ detection of transcriptional level indicates that Slit2 is down regulated in human tumor samples compared to normal tissues that mostly express Slit2 mRNA. Since all three tumor cell lines in the current studies express Robo4, a receptor for Slit2, the suppressive effect of Slit2 on tumors is likely mediated by the interaction of Slit2 with the receptor. These data imply that Slit2 is a tumor suppressor which is down regulated during tumor development. The effect of Slit2 on tumorigenesis is largely unexplored and further studies are required to define the mechanism for Slit2 mediated suppression of tumors.					
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INTRODUCTION

The proposed studies for this award is based on our preliminary data indicating that human prostate cancer cells produce a group of proteins, called *Slits*, which reduce the mobilization of white blood cells, an important physiological process for the development of immune responses. *Slits*-mediated reduction in the mobilization of white blood cells results in suppression of immune responses. Strong clinical evidence indicates that the infiltration of white blood cells in tumors is related to the prognosis of patients. The hypothesis is that prostate cancer derived *Slits* may inhibit the infiltration of white blood cells into tumors and suppress immune responses in prostate cancer patients. The goal of this proposal is to examine whether elimination or blockade of *Slits* from prostate cancer cells promotes immune responses to tumors.

The original proposal applied for three years funding. According to the comments from reviewers, the funding was cut to 1.5 years. The proposal and time line for proposed studies was revised.

Task 1. To detect the expression and function of Slits by human tumors

- a) Detect the expression of Slits expression in human normal and malignant prostate tissues (Month 1-3).
- b) Examine the leukocyte infiltration in human prostate tumor tissues (Month 1-3).
- c) Examine the effect of tumor derived Slits on the migration of T cells and dendritic cells. (Month 3-6).
- d) Develop RNA interference technique to silence Slit genes in tumor cells. (Month 3-6)

Task 2. To examine the role of Slits in the induction of anti-tumor immunity

- a) Examine the effect of *Slits* on the tumorigenicity of tumor cells (Month 7-12)
- b) Examine the role of T cells in *Slit* mediated effects on tumor development (Month 10-14).
- c) Examine the effect of *Slit2* on tumor evasion from immunity (Month 10-16)
- d) Examine the effect of *Slits* in regression of established tumors mediated by activated T cells (Month 12-18).

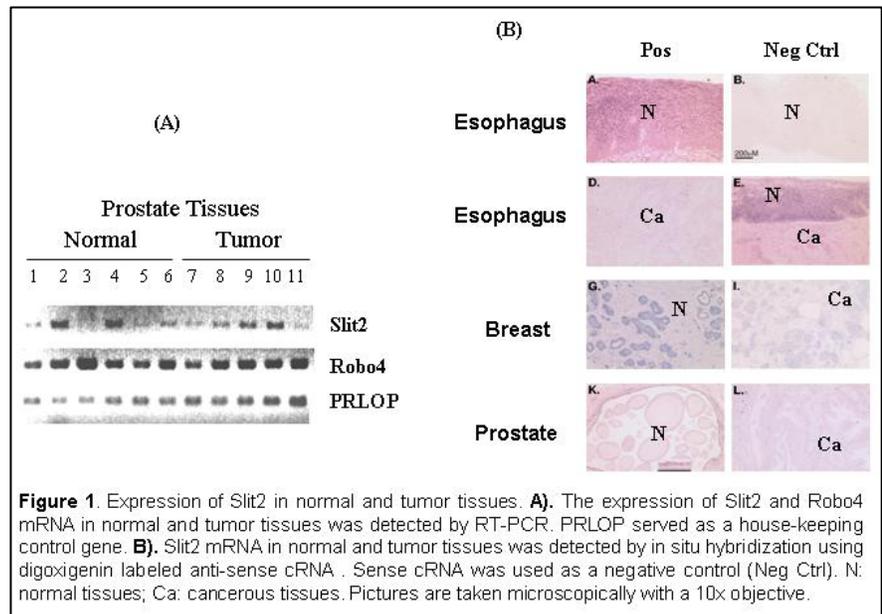
BODY

1. Detection of Slit expression in human normal and malignant prostate tissues.

Our initial experiments were to detect the expression of Slits in human prostate tumors. Previous reports in literature showed controversial results with some indicating increased levels of Slit expression and the others demonstrating reduction of Slits in human cancer samples. In these previous studies including our preliminary experiments, Slit transcriptional levels in tumor samples were measured by RT-PCR or real time PCR and protein levels were detected by Western blot. However, most of tumor samples have various portions of normal tissues that may express Slits. In order to directly compare the expression level of Slits in normal and malignant prostate tissues, we applied in situ hybridization technique to detect the mRNA level of Slit2. Digoxigenin labeled Slit2 anti-sense cRNA was used as a probe and sense cRNA was used as a negative control. The method for in situ hybridization was published in our previous studies (1).

The results indicated that the transcriptional level of Slit2 in human prostate cancer is decreased compared to normal tissues (Fig. 1). Sixty-three percent (9/14) of non-malignant prostate tissues expressed Slit2 whereas 40% (6/15) of prostate tumors were positive. In some tumor samples, adjacent

normal tissues surrounding tumors expressed Slit2 while the expression of Slit2 in tumors was decreased or undetectable. This result is different from our preliminary data which showed almost 100% of prostate tumor samples positive for Slit2 as



detected by RT-PCR. Since most non-malignant prostate tissue samples were not really normal (enlarged or inflamed), we included normal and malignant tissue samples from breast and esophagus in our studies in order to verify the expression of Slit2 in other types of tumors. In

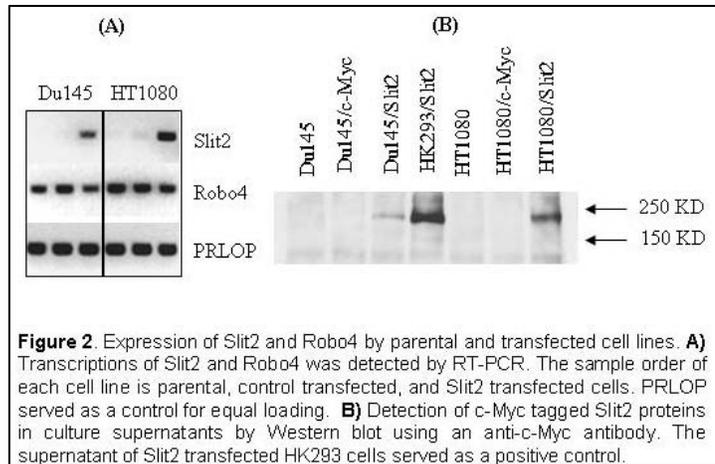
these tissues, the result was even more striking. All normal mammary glands and 83% of normal esophageal mucosa expressed Slit2 whereas 42% of breast cancer and 43% of esophagus tumors were positive for Slit2, respectively. In situ technique dissected the expression of Slit2 in normal and malignant tissues of clinical samples and demonstrated that Slit2 was decreased in tumor cells (Fig. 1).

In attempt to detect the protein level of Slit2, we tried to stain tumor samples with all commercially available anti-human anti-Slit2 antibodies from Santa Cruz. Unfortunately, none of the antibodies showed positive staining. We also contracted a company to generate two chicken anti-human Slit2 peptide antibodies. Again, they could stain Slit2 on sections by immunohistochemical technique. We are in the process generating antibodies to whole c-Myc tagged Slit2 protein and hope to use antibodies to verify protein expression levels of Slit2 in tumors.

2. Forced expression of Slit2 suppresses tumor growth

Based on the result indicating that Slit2 expression is decreased in tumors, the effect of Slit2 on tumor cells was examined. Instead of knocking down Slit2 in some Slit2 positive tumor cell lines, human Slit2 gene tagged with c-Myc was introduced into human prostate cancer cell line Du145 that did not express a detectable level of any Slit transcripts but expressed a membrane receptor Robo4 for Slit. Stable transfectants were selected in cultures containing G418 (500 – 800mg/ml), an antibiotics which kills non-transfected cells. The expression and production of Slit2 was verified with RT-PCR by specific primers and with Western blot by using an anti-c-Myc antibody (Fig. 2). The effect of Slit2 transfection on tumor cells was

examined in vitro and in nude mice. With similar strategy, we also developed a stable Slit2 transfectant of human fibrosarcoma cell line HT1080 which, like Du145, originally did not express Slit2 but the receptor Robo4 (Fig. 2). HT1080 is a widely used tumor cell line and can develop metastatic tumors in lungs when



intravenously administered in nude mice. Therefore, addition of this cell line not only provides supporting evidence for Slit2 mediated effects on prostate Du145 tumor but also serves as a model to investigate metastasis.

Forced expression of Slit2 did not significantly affect the growth of tumor cells in tissue culture plates (data not shown). In soft agar cultures, however, colony formation of Slit2 transfected cells were significantly reduced compared to parental tumor cells and controls transfected with an empty vector (Fig. 3). This results implicates that Slit2 inhibits tumor growth in an anchorage independent way.

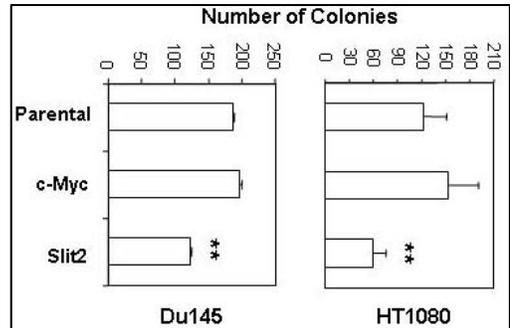


Figure 3. Transfection with Slit2 inhibits tumor growth. HT1080 and Du145 cells were transfected with Slit2 or control vector with c-Myc tag. Stable transfectants were selected in medium containing G418. The colony formation was assessed in soft agar cultures. Data are representative of 4 independent assays and shown as Mean±SEM of 6 wells. Non-transfected parental cells served as controls. ** P< 0.01.

In further studies determining the effect of Slit2 on tumor growth, tumor cells were inoculated in nude

mice that were defect in thymus and could not develop T cell mediated anti-tumor immunity. Tumor cells were suspended in 50% Matrigel and five millions cells were inoculated subcutaneously. Tumor size was measured every 2-3 days. Compared to parental and control transfected tumor cells, the growth of Slit2 transfected tumors was significantly suppressed (Fig. 4). It is to note that control vector encoding c-Myc tag somehow had inhibitory effects on Du145 growth in mice. The inhibition of Slit2 on Du145 tumor growth, although significant, was not as

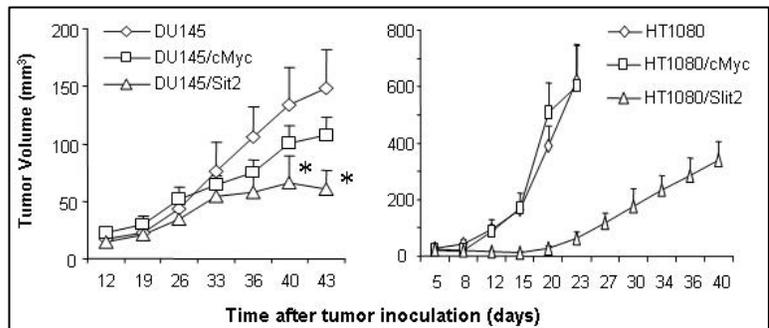


Figure 4. Slit2 inhibits tumor growth in animals. Athymic mice were injected subcutaneously with tumor cells. Tumor size was measured every 2-3 days. The tumor volume is calculated as length x width x height x 0.63. The data are representative of three independent experiments and shown as mean ± SD of 5 mice per group. * p<0.05

dramatic as on HT1080 tumors. This experiment was repeated three times and similar results were observed.

3. Forced expression of Slit2 inhibits tumor metastasis in lungs.

In order to address the effect of Slit2 on tumor metastasis, HT1080 tumor cells were used. This tumor cell line developed metastatic tumors in lungs of nude mice at 5 weeks after

intravenous administration. Forced expression of Slit2 significantly inhibited the development of metastatic tumors in lungs. Four of eight inoculated mice developed metastatic tumors in lungs compared to 6 and 7 of 8 mice that were inoculated with parental or control vector transfected tumor cells, respectively (Fig. 5). In further studies, step sections of tumor positive lungs were cut and the number of tumor nodules was counted microscopically. The results indicated that the number of tumor nodules in lungs with Slit2 transfected tumors was significantly lower than that in controls ($P < 0.01$).

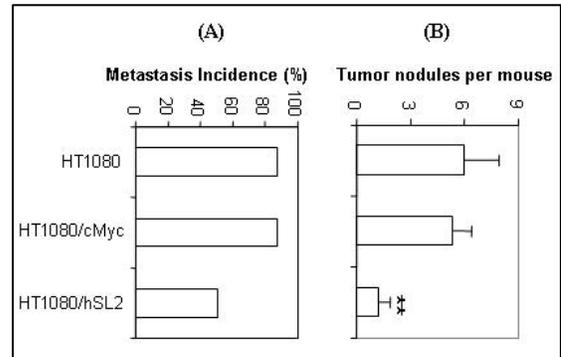


Figure 6. Slit2 inhibits tumor metastasis in lungs. Athymic mice were injected intravenously with tumor cells and lungs were harvested 4-5 weeks later. Paraffin embedded lung sections were stained with H&E. Tumor nodules were evaluated microscopically. **A)** The incidence of mice with metastatic tumors in lungs. The data indicate the percentage of mice with metastatic tumor in lungs (8 mice per group). **B)** The number of metastatic tumors per mouse (mean±SD). ** $P < 0.05$

KEY RESEARCH ACOMPLISHMENTS

In situ hybridization has demonstrated that Slit2 transcription in tumor cells is decreased compared to normal tissues. Unlike previous studies including our preliminary experiments, which examined Slit2 levels in tumor tissue samples by RT-PCR or Western blot, the current studies directly compare the expression level of Slit2 in tumor samples that contained cancerous and adjacent normal tissues and provide strong evidence supporting the down regulation of Slit2 in tumors.

Two stable human tumor cell lines transfected with human Slit2 gene have been established, which includes human prostate tumor cell line Du145. Transfection with Slit2 gene inhibits the growth of tumor cells in agarose cultures. These cell lines provide useful models for future mechanistic studies in vitro and in vivo.

Slit2 inhibits tumor growth and suppresses lung metastasis in animal models.

REPROTABLE OUTCOMES

Two stable Slit2 transfectants of human tumor cell lines, HT1080 and Du145 have been established.

An abstract was accepted for poster presentation in 2004 AACR Annual Meeting in Anaheim, CA.

CONCLUSIONS

Our studies have for the first time demonstrated that forced expression of Slit2 in tumors suppresses tumor growth and metastasis in animal models. Additionally, in situ detection of transcriptional level indicates that Slit2 is down regulated in tumors compared to normal tissues that mostly express Slit2 mRNA. Our ongoing efforts are to generate anti-Slit2 antibody to verify protein levels and further confirm the down regulation of Slit2 in tumors. Since both Du145 and HT1080 tumor cells express Robo4, a receptor for Slit2, the suppressive effect of Slit2 on tumors is likely mediated by the interaction of Slit2 with the receptor. Robo4 is one of four receptors (Robo1-4) for Slit2 and the function of the receptor in the regulation of cell growth is unknown. The effect of Slit2 on tumorigenesis is largely unexplored and further studies are required to define the mechanism for Slit2 mediated suppression of tumors.

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APPENDICES

None