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

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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 were based on our preliminary data indicating that human prostate cancer cells produced a group of proteins, called *Slits*, which reduced 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 resulted 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 was that prostate cancer derived *Slits* might inhibit the infiltration of white blood cells into tumors and suppressed immune responses in prostate cancer patients. The goal of this proposal was to examine whether elimination or blockade of *Slits* from prostate cancer cells promoted 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

Slit2 expression in human normal and malignant tissues.

Our initial experiments were to detect the expression of Slit2 in human prostate and other organs. Previous reports in literature showed controversial results with some indicating increased levels of Slit2 expression and the others showing reduction of Slit2 in human cancer samples (1-4). In these previous studies including our preliminary experiments, Slit2 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 Slit2 as well. In order to directly compare the expression of Slit2 in normal and malignant tissues, in situ hybridization technique was applied to detect Slit2 mRNA level.

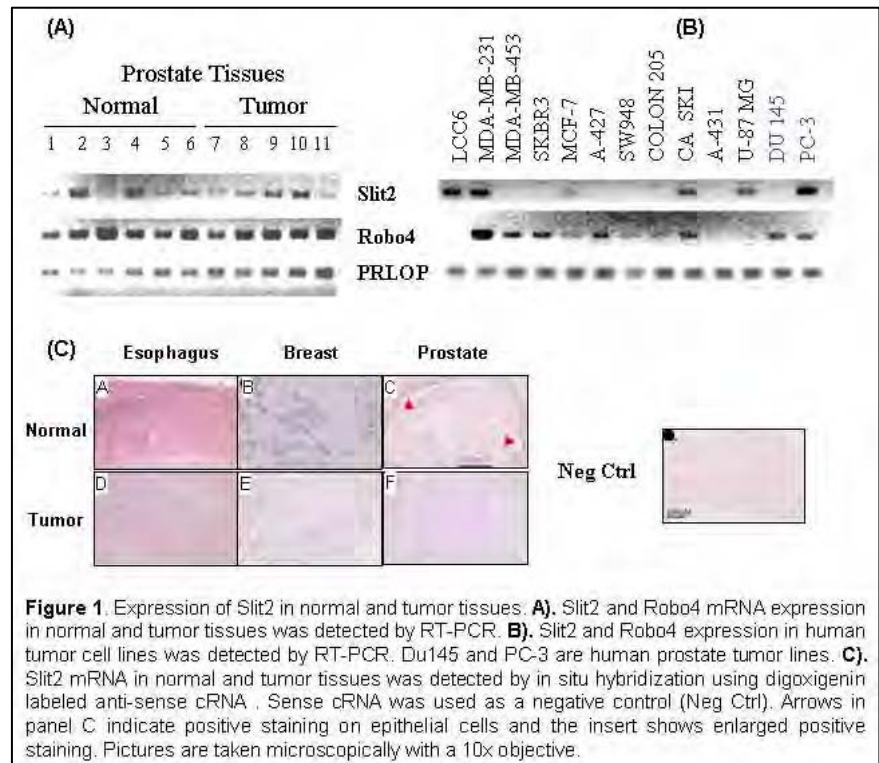
A non-radioactive in situ hybridization was performed as described previously (5). Briefly, Slit2 cDNA fragment between 955 and 1685 bp was cloned into pGEM vector (Promega, Madison, WI). The plasmid was linearized by *ApaI*, and digoxigenin-labeled sense and antisense cRNA were generated by using SP6 polymerase in the presence of digoxigenin-UTP. Human retinoid X receptor- α (RXR- α) was used as a positive control for the intact tissue mRNA. The results indicated that the transcriptional level of Slit2 in human esophagus and breast cancers is decreased compared to normal tissues (Table 1).

Table 1. Expression of Slit-2 mRNA in different tissue specimens using in situ hybridization

Organ Site	% of total (positive cases/total cases)		Difference
	Normal	Tumor	
Esophagus	83.3 (55/66)	43.6 (92/211) SCC: 42.1 (40/95) ACC: 44.8 (52/116)	P=0.03
Mammary gland	100 (6/6)	42.1 (8/19) DCIS: 50 (3/6)	P<0.001
Prostate	64.3 (9/14)	40 (6/15)	P=0.18

SCC: Squamous cell carcinoma, ACC: Adenocarcinoma, DCIS: Ductal carcinoma in situ.

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. 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. The difference between normal and malignant tissues is statistically significant. Sixty-four percent (9/14) of non-malignant prostate tissues expressed Slit2 whereas 40% (6/15) of prostate tumors were positive although the difference is not statistically significant.



We may have to add more samples for the analysis. These results are different from what were observed in some literature and in our preliminary studies. In our hand, Slit2 mRNA was detected in all prostate tumor samples by RT-PCR (Fig. 1a). Moreover, a human prostate tumor cell line PC-3 expressed Slit2 whereas another line Du145 was negative (Fig. 1b). A similar pattern was also observed with other tumor cell lines including breast cancer LCC6 MDA-MB-231, MDA-MB-453, SKBR3 and MCF-7), colon cancer (SW948 and Colon 205), and epidermoid cancer (CA SKI and A431). However, it is to note that all normal or tumor tissues and most of tumor cell lines expressed Robo4, a receptor for Slit2. There is an apparent discrepancy between results obtained from RT-PCR and in situ hybridization assays. In situ hybridization shows that in some tumor samples, adjacent normal tissues surrounding tumors expressed Slit2 while the expression of Slit2 in tumors was decreased or undetectable (Fig. 1c). It suggests that positive signals of Slit2 from tumor samples in RT-PCR or Western blot assays may come from the part of adjacent normal tissues. In situ detection of Slit2 mRNA dissects the

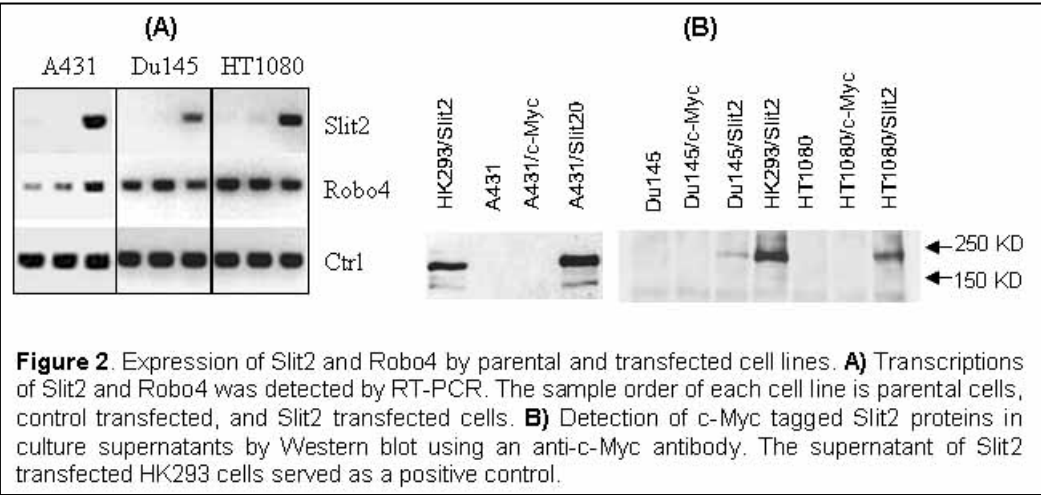
expression of Slit2 in normal and malignant tissues of clinical samples and demonstrates that Slit2 is decreased in tumor cells.

In order to detect the protein level of Slit2, we tried to stain tumor samples with several commercially available anti-human anti-Slit2 antibodies from Santa Cruz. Unfortunately, none of the antibodies showed a positive staining. We also contracted a company to generate two chicken anti-human Slit2 peptide antibodies. Again, they could not stain Slit2 on sections by immunohistochemical technique. One reason may be that these antibodies are generated in animals immunized with synthesized Slit2 peptides and are unable to bind to native Slit2 proteins. Administration of whole c-Myc tagged Slit2 protein as antigens may result in antibodies capable of detecting Slit2 proteins. However, it is challenging to purify enough amounts of Slit2 proteins from cultures of transfected HK293 cells due to a low yield.

Forced expression of Slit2 suppresses tumor growth

Since in situ hybridization assays indicated that Slit2 expression was decreased in tumors, we decided first to examine whether restore or forced expression of Slit2 had any effect on tumor development. Human fibrosarcoma HT1080, prostate tumor Du145, and epidermoid tumor line A431 do not

express Slit2 mRNA but are positive for a Slit receptor Robo4 (Figure 2a). These cell lines were



transfected with a vectors encoding human Slit2 tagged with c-Myc or a control empty vector encoding only the c-Myc tag. Stable transfectants were established under selection with G418 (500 – 1200 µg/ml), an antibiotics which kills untransfected cells at the concentrations. The expression and production of Slit2 was verified with RT-PCR by specific primers (Fig. 2a) and with Western blot by using an anti-c-Myc antibody (Fig. 2b). HT1080 is a widely used tumor

cell line and can develop metastatic tumors in lungs when intravenously administered in athymic nude mice. Therefore, this cell line was used in animal experiments of the current studies.

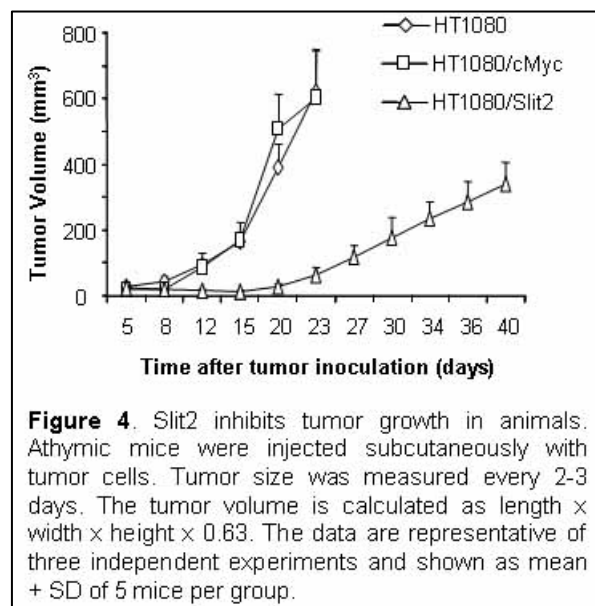
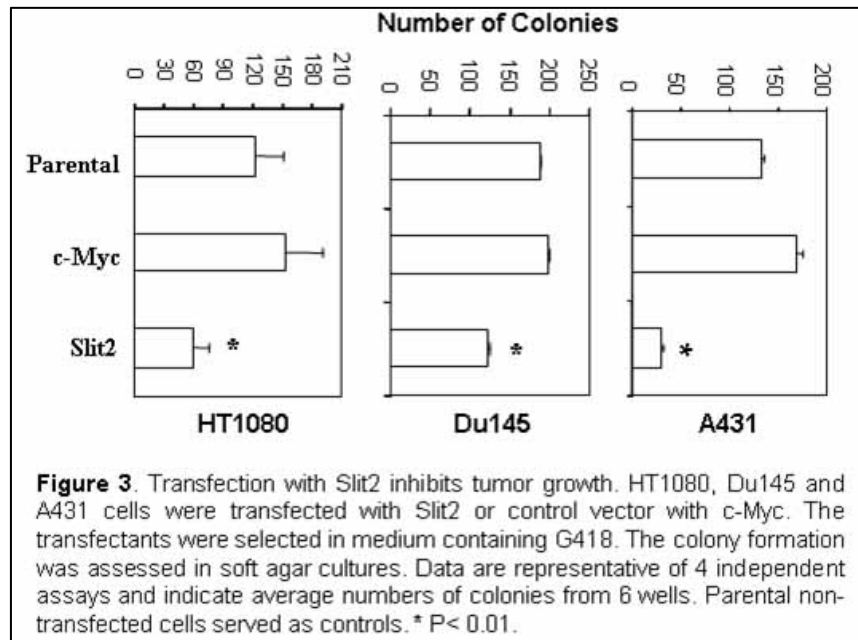
Forced expression of Slit2 suppressed the growth of Slit2 transfected Du145, HT1080 and A431 tumor

cells in an anchorage independent manner. In soft agar cultures, colony formation of Slit2 transfected cells was significantly reduced compared to parental tumor cells and controls transfected with an empty vector (Fig. 3). The effect of Slit2 on tumor growth in tissue culture plates was, however, marginal and not statistically significant (data not shown). It suggests that Slit2 mediated inhibition of tumor growth is anchorage independent.

In further studies determining the effect of Slit2 on tumor growth in vivo, tumor cells

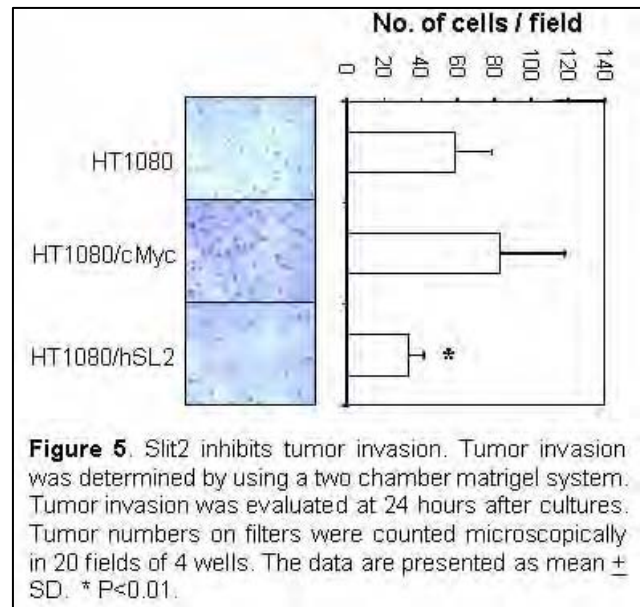
were inoculated subcutaneously in nude mice that were defect in thymus and could not develop T cell mediated anti-tumor immunity. Tumor growth was monitored. Compared to parental tumor cells, the growth of Slit2 transfected tumors was significantly suppressed (Fig. 4). The growth of tumors that were transfected with the control vector was similar to parental controls.

The data from in vitro and in vivo experiments indicate that forced expression of Slit2 in tumor cells inhibit tumor growth, implying that Slit2 has tumor suppressive effects.



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

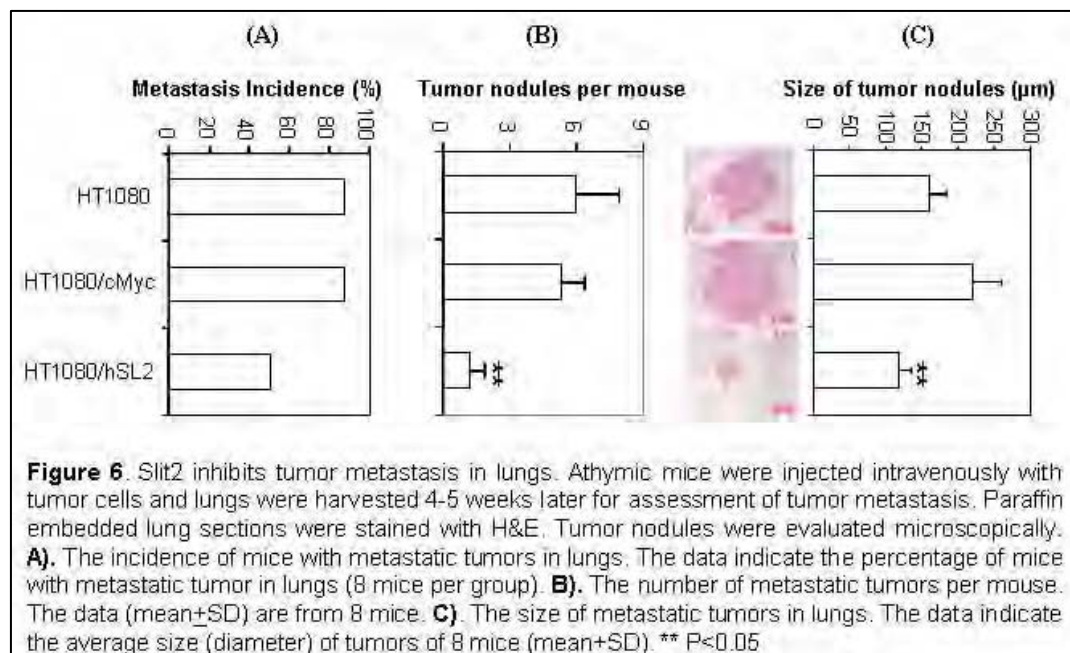
In order to address the effect of Slit2 on tumor metastasis, HT1080 tumor cells were used. In the initial experiments, we examined tumor invasion in a two chamber system with Matrigel coated membrane (BD, Biosciences). Matrigel resembles matrix structures in tissues. In the assay, cells were placed in the upper chamber which was separated from the lower chamber by a membrane (pore size 0.8 μ M) coated with



Matrigel. The invaded tumor cells that were attached on the bottom of the membrane were counted 24 hours after incubation. Results indicated that Slit2 transfection significantly inhibited the invasion of the tumor cells compared to parental and control vector transfected cells (Fig. 5).

In further experiments examining the effect of Slit2 on tumor metastasis, tumor cells were injected intravenously into athymic nude mice and tumor metastasis in lungs was determined five weeks after tumor cell inoculation. HT1080 tumor cell line developed metastatic tumors in lungs in more than 80% of nude mice. Slit2 transfected tumors, however, developed

metastatic tumors in about 50% of mice. The incidence of tumor metastasis of control vector transfected tumor cells



was similar to parental controls (Fig. 6a). In further analysis, the number of tumor nodules was counted. The mice that were inoculated with parental or control vector transfected tumor cells had significantly more tumor nodules in lungs than mice that were injected with Slit2 transfected cells (Fig. 6b). Moreover, the tumor nodules in mice injected with Slit2 transfected cells were significantly smaller than those in mice injected with parental and control vector transfected cells (Fig. 6c). Collectively, these data indicate that Slit2 inhibits tumor invasion and metastasis.

KEY RESEARCH ACOMPLISHMENTS

In situ hybridization has demonstrated that Slit2 transcription in tumor cells is decreased compared to adjacent normal and normal control 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. Certainly, detection of Slit2 protein in normal and tumor tissues will provide further confirmation. However, lack of antibodies and difficulties to detect soluble proteins on tissue sections are obstacles for the experiments. Since the result from the experiments proposed in Task 1 indicated a decrease of Slit2 in tumors, further experiments were focused on the effect of Slit2 on tumor cells.

Three stable human tumor cell lines transfected with human Slit2 gene have been established, which represent different tumor types. Slit2 inhibits the growth of all three tumor lines. These cell lines provide useful models for future mechanistic studies in vitro and in vivo.

Our data for the first time proven that Slit2 inhibits tumor growth in animals. Furthermore, the data also for the first time demonstrate that Slit2 inhibits tumor invasion and suppresses lung metastasis in animal models.

REPROTABLE OUTCOMES

Three stable Slit2 transfectants of human tumor cell lines, HT1080, Du145 and A431 have been established.

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

An abstract was accepted for poster presentation in 2006 SID Annual Meeting, Philadelphia, PA. Dr. Hee Kyung Kim, a postdoctoral fellow in my lab, received Career Enhancement Award from UAB for the presentation.

A manuscript is in the final version of preparation.

CONCLUSION

Our studies, for the first time, demonstrate that forced expression of Slit2 in tumors suppresses tumor growth and metastasis in vivo. Additionally, in situ detection of gene transcription indicates that Slit2 is down regulated in tumors compared to normal tissues that mostly express Slit2 mRNA. Since most of tumor cell lines, and more importantly, all of our three Slit2 transfected tumor lines 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 largely unknown. Slit2 is a neuronal factor that regulates cell migration. Intensive studies have been carried out to determine mechanisms for Slit2 mediated regulation of cell migration. Since Slit2 mediated effects on tumorigenesis are largely unexplored, further studies are required to define the mechanism for Slit2 mediated suppression of tumors.

Most recently, we used an antibody array from Sigma (Panorama[®] Ab Microarray - Cell Signaling Kit) to screen molecular changes in Slit2 transfected tumors compared to controls. This antibody array includes antibodies against 224 molecules related to tumor suppressor, oncogene, apoptosis, cell cycle and other molecules that have been reported to have effects on tumor development. The initial analysis reveals interesting patterns although it is premature to present the data here.

Collectively, the award, even though cut to half, helped us develop cell lines, animal models and initial studies on molecular mechanisms for Slit2 mediated effects on tumors. Our plan is to apply for more funding to continue the project. On the basis of current findings, further studies will be focused on the role of Robo4 in Slit2 mediated effects on tumors and molecular mechanisms for Slit2 mediated effects on tumor growth and metastasis.

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APPENDICES

None