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Silencing Intersectin 1 Slows Orthotopic Neuroblastoma Growth in Mice

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Abstract

Neuroblastoma accounts for 15% of all pediatric cancer deaths. Intersectin 1(ITSN1), a scaffold protein involved in phosphoinositide 3-kinase(PI3K) signaling, regulates neuroblastoma cells independent of MYCN status. We hypothesize that by silencing ITSN1 in neuroblastoma cells, tumor growth will be decreased in an orthotopic mouse tumor model.

SK-N-AS neuroblastoma cells transfected with empty vector(pSR), vectors expressing scrambled shRNA(pSCR), or shRNAs targeting ITSN1(sh#1 and sh#2) were used to create orthotopic neuroblastoma tumors in mice. Volume was monitored weekly with ultrasound. End-point was tumor volume >1000mm³. Tumor cell lysates were analyzed with anti-ITSN1 antibody by Western blot. Orthotopic tumors were created in all cell lines. Twenty-five days post injection, pSR tumor size was 917.6±247.7mm³, pSCR was 1180±159.9mm³, sh#1 was 526.3±212.8mm³, and sh#2 was 589.2±74.91mm³. sh#1-tumors and sh#2-tumors were smaller than pSCR(p=0.02), no difference between sh#1 and sh#2. Survival was superior in sh#2-tumors(p=0.02), trended towards improved survival in sh#1-tumors(p=0.09), compared to pSCR-tumors, no difference in pSR tumors. Western blot showed decreased ITSN1 expression in sh#1 and sh#2 compared to pSR and pSCR. Silencing ITSN1 in neuroblastoma cells led to decreased tumor growth in an orthotopic mouse model. Orthotopic animal models can provide insight into the role of ITSN1 pathways in neuroblastoma tumorigenesis.

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Keywords

Intersectin; orthotopic tumor; neuroblastoma

INTRODUCTION

Neuroblastoma accounts for 15% of all pediatric cancer deaths and is the most common solid extracranial tumor in the pediatric population. The overall incidence is one in 7,000 to 10,000 live births [1]. The tumor originates from the neural crest and presents most commonly in the adrenal gland [1]. While patients with stage 1 disease have survival rates as high as 97%, those in high risk groups have survival rates as low as 36% [2]. Many different factors have been shown to be associated with worse outcomes, including older age at diagnosis, higher stage disease, as well as certain biologic features of the tumor [1]. A number of molecular changes have been identified to contribute to disease progression and subsequent worse clinical outcome. For example, tumors that have MYC-N amplification are associated with more aggressive subtypes of neuroblastoma, and these patients have a <50% likelihood of having event free survival [1]. Mutations in the anaplastic lymphoma kinase have been identified in familial cases of neuroblastoma, and they can be an important driver in the tumorigenesis [3]. Various phosphatidylinositol 3-kinase (PI3K) isoforms have been implicated in the stabilization of the MYC-N oncoprotein, and inhibiting the PI3K activity has led to decreased growth of neuroblastoma cells [4]

Although much research has focused on understanding these molecular pathways in the hopes of finding a potential therapeutic target, the role of PI3K in neuroblastoma tumorigenesis is less well defined. PI3Ks consist of a family of lipid kinases, and mutations in these kinases have been implicated in different malignancies including colon, breast, gastric, breast, and lung cancers [5]. Recently, PI3K class 2 β (PI3K-C2 β) has been shown to interact with and be regulated by Intersectin 1 (ITSN1) in the N1E-115 mouse neuroblastoma cell line [6]. This ITSN1-PI3K-C2 β pathway was subsequently confirmed to play an important role in neuroblastoma tumorigenesis using an *in vivo* xenograft model [7, 8].

ITSN1 is a multi-domain scaffold protein that consists of two NH₂-terminal Eps15 homology domains, a coiled-coil domain, and five Src homology 3 domains [9]. Through these domains, ITSN1 binds to different proteins to affect signal transduction and endocytosis. ITSN1 overexpression has been shown to transform rodent fibroblasts [10], and ITSN1 regulates activation of the Ras proto-oncogene in a compartmentalized fashion [11]. These findings together with the *in vivo* evidence demonstrating the role of ITSN1 in tumorigenesis [7] provide strong basis for further investigation of ITSN1 tumorigenic capability in a more relevant location for tumor formation. The tumor microenvironment and architecture can simulate the initiation of a tumor that resembles human neuroblastoma in an orthotopic model.

In this study, we hypothesized that an orthotopic neuroblastoma mouse model would demonstrate reduced tumor initiation when human neuroblastoma cells depleted of ITSN1 were implanted into the adrenal gland. Since the presence of ITSN1 is required for human

neuroblastoma cells to achieve optimal growth, this orthotopic neuroblastoma mouse model could serve as a phenotypic “read out” in a physiologically relevant microenvironment and as a tool to understand the ITSN1-mediated tumorigenesis by molecularly manipulating other targets in the pathway.

MATERIALS AND METHODS

Cell culture and stable silencing of ITSN1

Stable SK-N-AS neuroblastoma cell lines silencing ITSN1 were cultured in RPMI with 10% fetal bovine serum and 1 µg/ml of puromycin (Gibco, Waltham, MA) at 37°C in 5% CO₂. SK-N-AS cell lines expressing the vector alone (pSUPER.retro.puro, pSR), a scrambled shRNA (pSCR) or pSR expressing shRNAs for ITSN1 (sh#1 or sh#2) have been previously described [7]. Briefly, Phoenix-GP cells were transiently transfected with 20 µg of the above-mentioned vectors along with 5 µg of a plasmid encoding the VSV-G envelope to generate viral particles. The following day, media in Phoenix-GP cells was replaced by host cells complete media (RPMI + 10% FBS) and SK-N-AS cells were seeded for future infection. 48h–72h post-transfection, conditioned media from Phoenix-GP cells was collected, filtered, and used to infect SK-N-AS cells seeded the day before. Infection was followed by selection with 2 µg/ml puromycin to obtain stable lines.

Animal studies

Approval for all animal protocols was granted by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. Female NCr nude mice age 7 weeks (Harlan, Indianapolis, IN), were used for experimentations. Procedures and ultrasound measurements were performed under general anesthesia using isoflurane inhalation.

Generation of orthotopic neuroblastoma tumors

Tumors were created as described in Chiu et al [12]. Briefly, a transverse incision on the left flank was made to expose the left adrenal glands. Then 2 µL of phosphate buffered saline (PBS) containing 10⁶ cells of different vectors such as sh#1, sh#2, pSCR, or pSR were injected into the left adrenal gland before the incision was closed in two layers. There were seven animals in the control group, pSCR and pSR, and eight animals in the experimental groups, sh#1 and sh#2. Tumor growth was monitored weekly using a VisualSonics Vevo 2100 sonographic probe (Toronto, Ontario, Canada). The animal was euthanized and the tumor harvested when tumor volume reached >1000 mm³.

High frequency ultrasound

After securing the mouse in a prone position, a VisualSonics Vevo 2100 Sonographic probe (Toronto, Ontario, Canada) was applied to the left flank to locate the left adrenal gland and the tumor. Serial cross-sectional images (0.076 mm between images) were taken. The tumor volume was measured using the 3-D reconstruction tool (Vevo Software v1.6.0, Toronto, Ontario, Canada).

Tumor harvest

Once tumor volume reached $>1000\text{mm}^3$, the animal was sacrificed using inhaled CO_2 . 1/3 of the tumor was harvested and snap frozen for Western blot analysis. The remaining tumor was fixed in 10% buffered formalin.

Histologic evaluation

Formalin-preserved tumors were sectioned, serially dehydrated and embedded in paraffin. Five-micron thick sections were placed on glass slides, stained with hematoxylin and eosin (H&E) and examined with light microscopy.

Western-blot of tumor specimen

Mouse tumor was homogenized in 0.5–1.5 ml of PLC lysis buffer (50 mM HEPES, pH 7.5, 150 mM) supplemented with phosphatase and protease inhibitors (sodium vanadate, PMSF, aprotinin, leupeptin and benzamidine) using a homogenizer. Cell lysates were collected in 0.5ml of PLC lysis buffer. 10 μg of total protein were loaded onto a NuPAGE™ Novex™ 4–12% Bis-Tris Protein Gel (ThermoFisher, Waltham, MA) and transferred to a 0.45 μm pore size PVDF membrane (Millipore, Billerica, MA). The polyclonal rabbit anti-ITSN1 antibody has been previously described [13] and antibody to β -actin was purchased from Sigma (St. Louis, MO). Total protein (100 μg) of the homogenized samples were loaded onto the gel and blotted as described.

Statistical analysis

All statistical analysis was performed using GraphPad Prism Version 4.00 for Windows (GraphPad Software, San Diego California USA). One-way ANOVA testing was done to determine the difference in tumor volume between groups, with post hoc analysis using Tukey's Multiple Comparison test. Overall survival was calculated using Kaplan-Meier survival curves. A p value of <0.05 was considered statistically significant.

RESULTS

in vitro Testing

Our previous studies demonstrated that the SK-N-AS cell line expresses ITSN1 [7]. To confirm that the ITSN1 expression has been decreased with short hairpin RNA's, Western blots were performed on the molecularly altered SK-N-AS cell lines pSR (empty vector), pSCR (scrambled shRNA), sh#1 and sh#2. There was marked reduction in ITSN1 expression in sh#1 and sh#2 compared to that of controls pSR and pSCR (Figure 1A).

in vivo Testing

All four cell lines (SK-N-AS cells containing pSR, pSCR, sh#1, or sh#2 vectors) were individually injected into the mouse adrenal gland (Figure 2A), and all animals injected developed tumors (Figure 2C). A total of 8 animals in each experimental arm, sh#1 and sh#2 were used, and 7 animals in each control group, pSCR and pSR. All tumors were clearly identified and monitored using high frequency ultrasonography (Figure 2B). Tumors appeared heterogeneous on ultrasound, with some areas of calcification. Tumor formation in

the pSR and pSCR control groups was detected as early as 11 days post injection, while the earliest tumor in sh#1 and sh#2 groups was not detected until day 18-post injection.

Tumor volume was analyzed at day 25-post injection across all groups: tumor volume for the sh#1 group was $526.3 \pm 212.8 \text{ mm}^3$, sh#2 was $589.2 \pm 74.91 \text{ mm}^3$, pSCR was $1180 \pm 159.9 \text{ mm}^3$, and pSR was $917.6 \pm 247.7 \text{ mm}^3$. When compared to the tumor volume in the pSCR group, the tumor volume for sh#1 and sh#2 was significantly smaller ($p=0.02$). There was no difference in growth between the two intervention groups sh#1 and sh#2 (Figure 1B). Probably due to the variability within the pSR group tumor size at day 25, there was no difference between pSR and the two intervention groups.

The mice in the sh#1 group on average survived 29.6 ± 4.4 days, sh#2 survived 29.8 ± 1.4 days, pSCR 26 ± 2.65 days, and pSR 27.7 ± 2.75 days post injection. Animals in the sh#2 group survived significantly longer than those in the pSCR group ($p=0.02$). There was a trend for longer survival in the sh#1 group compared to pSCR ($p=0.09$). There was no difference in survival between the sh#1 and sh#2 groups.

Gross Examination at Necropsy

At necropsy, there was evidence of local metastatic disease within each group. There were two out of eight (25%) animals in the sh#1 group with evidence of intraperitoneal metastasis. The sh#2 group had five out of eight (63%) mice with local metastasis. The control animals also demonstrated intraperitoneal metastasis: pSCR had 4/7 (57%), and pSR had 3/7 (43%). The metastatic lesions were found on the peritoneum in close proximity to the primary tumor (Figure 3C). The metastatic lesions were also seen on ultrasound prior to necropsy (Figure 3D). There was no evidence of gross metastatic disease outside of the abdominal cavity in any group.

Histologic Examination of Tumors

Representative H&E slides were created from each experiment group. All groups demonstrated small round blue cells that were consistent with neuroblastoma (Figure 4). Both ITSN1 silenced tumors as well as the control tumors had varying degrees of tumor necrosis throughout the examined sections. Mitotic activity was seen within all specimens. All groups had similar proportion of cells positive for ki67 (Figure 5).

Western Blot

Western blot analysis of the harvested tumor samples showed a significant reduction of ITSN1 expression in the sh#1 and sh#2 groups compared to that in the pSR and pSCR groups. This reduction of ITSN1 expression *in vivo* confirmed the prior *in vitro* manipulation (Figure 1C).

DISCUSSION

Here, we demonstrated that silencing ITSN1 resulted in slower tumor growth in an orthotopic neuroblastoma model. Reduction of ITSN1 expression was associated with a slower rate of tumor growth (Figure 1B) and longer animal survival before humane end

points were met. Additionally, post necropsy analysis of the tumor samples confirmed that ITSN1 expression was indeed significantly decreased in the groups expressing the ITSN1 shRNAs. There was no discernible histologic change between tumors with or without decreased ITSN1 expression.

Within each group of animals, there were local tumor metastases found at necropsy. The metastatic lesions were confined to the abdominal cavity, and they appeared to be in close proximity to the primary tumor. This observation of local metastases after the primary tumor developed into a large size was unique to the SK-N-AS cell line, as it was rare to have local metastases for another human neuroblastoma cell line KELLY even at a large primary tumor size [12]. Changes in the ITSN1 expression did not alter this tumor growth property of SK-N-AS, and other pathways might contribute to local metastasis. This local metastasis phenomenon was only made possible using an orthotopic tumor model, which is more relevant for studying metastasis in the future.

Our orthotopic neuroblastoma model combined with non-invasive longitudinal tumor size measurements also offered other advantages in cancer research. The model can serve as an authentic “read out” of the molecular phenotype as modified *in vitro*. In the controlled condition of cell culture, molecular changes can often be predicted and replicated. However, in an actual tumor microenvironment within a live organism, other pathways also come into play, and manipulations *in vitro* do not always translate on a macro scale [14]. In this study, we have decreased ITSN1 expression *in vitro* and produced the predicted change in an orthotopic tumor *in vivo*. Our orthotopic model can be further utilized to conditionally express certain proteins such as anaplastic lymphoma kinase *in vivo* at pre-selected stages of tumor development using inducible systems such as the tetracycline-controlled transcriptional activation system [15]. It can also be used as a model for surgical resection to test novel treatment protocols [11, 16].

ITSN1 is highly expressed in the central nervous system and consists of an ITSN1-short (ITSN1-S) and an ITSN1-long (ITSN1-L) form [9]. Neuroblastoma tumors predominantly express ITSN1-S, but overexpression of either form is associated with poor prognosis [14, 16]. Anchorage-independent growth of neuroblastoma has been shown to require ITSN1, and when ITSN1 was silenced in IMR-5 neuroblastoma cells, the anchorage-independent growth phenotype was rescued by overexpression of PI3K-C2 β [7]. This result suggested that PI3K-C2 β signaling was downstream of ITSN1 in the process of tumor formation. Indeed the association of the ITSN1 signaling pathway with receptor tyrosine kinases signaling points to the importance of this pathway in neuroblastoma tumorigenesis [13]. Inhibitors of the PI3K pathway in combination with other inhibitors have demonstrated increased rates of apoptosis in neuroblastoma, again demonstrating that this pathway is involved in tumorigenesis [17]. This involvement of kinases in neuroblastoma was further substantiated by Eleveld *et al.*, who reported that the recurrent human neuroblastoma tumors frequently harbor mutations in the Ras-MAP kinase pathway [18]. Growing evidence points to the involvement of ITSN1 in the various signaling pathways in neuroblastoma, and further studies are needed to clarify how this molecular scaffold regulates other known pathways in tumorigenesis.

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Figure 1.

in vitro: A. sh#1 and sh#2 groups demonstrated decreased expression of ITSN1 as compared to control groups, pSR and pSCR, which maintained ITSN expression. Actin was used as a loading control. *in vivo*: B. The mean tumor volume with standard error of the mean for each treatment group at day 25-post injection. When compared to pSCR (control), both sh#1 and sh#2 had significantly smaller tumor volumes ($p < 0.05$). C. Western blot analysis of tumor lysates confirmed decreased expression of ITSN1 in the experimental groups, sh#1 and sh#2, compared to the control groups, pSR and pSCR. Actin served as loading control.

Figure 2.

A. Orthotopic tumor was created by injecting cells into the left adrenal gland (*) through a left flank incision. B: Tumors were monitored using ultrasound, where the left kidney (KID) with adjacent neuroblastoma tumor (TUM) could be seen arising from the adrenal gland. C. Necropsy confirmed the tumor location (TUM) in relationship to other organs as seen on ultrasound.

Figure 3. Gross image (A) and corresponding ultrasound image prior to necropsy (B) of solitary tumor (TUM) without evidence of metastatic disease. Gross image (C) and corresponding ultrasound image prior to necropsy (D) of the primary neuroblastoma tumor (TUM) and the intraperitoneal metastatic lesion (M). KID: kidney.

Figure 4.
H&E staining of paraffin-embedded tumor sections. A. sh#1 tumor B. sh#2 tumor C. pSCR tumor D. pSR tumor all demonstrate small round blue cells. Scale bar equals 100µm.

Figure 5. Ki67 staining of paraffin-embedded tumor sections. A. sh#2 tumor B. pSCR tumor C. pSR tumor all demonstrate similar proportion of cells positive for ki67 (20×).