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Ras-directed N-glycoproteins are novel early biomarkers for tumorigenesis and malignant transformation, and therapeutic targets of neurofibromatosis type I

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14. ABSTRACT

Neurofibromatosis type I (NF1) is a dominantly inherited disease affecting 3,000 individuals. The hall marker of NF1 is the development of neurofibromas and about 10% of neurofibroma will develop malignant peripheral nerve sheath tumors (MPNSTs). .In this report, we found AXL, c-MET and EGFR are aberrantly expressed in clinical specimens of neurofibromas and MPNSTs, and MPNST cells express high level of glycoproteins. We also found hyperactive Ras unregulated the expression of MGAT5B, one of the glycosyltransferases, to mediate the glycosylation and phosphorylation of kinase receptors in all MPNST cell lines and clinical specimens, and MGT5B shRNA knockdown significantly inhibited the glycosylation and phosphorylation of kinase receptors. We treated cells with 2-Deoxy-D-Glucose (2-DG), a potential glycosylation inhibitor, and found 2-DG inhibited the glycosylation and phosphorylation of AXL, EGFR, and c-MET and impaired receptor-mediated MEK-ERK1/2 and PI3K-AKT signaling in a dose-dependent manner, and inhibited the translocation of receptors from the cytoplasm to the cell surface and retained receptors in the ER and Golgi apparatus, but had no effect on normal human Schwann cells. Furthermore, we found that 2-DG inhibited the tumor development in *NF1+/-;p53+/-* mice. These novel findings suggest MGAT5B is a novel therapeutic target of NF1 to prevent the tumorigenesis and malignant transformation.

15. SUBJECT TERMS

Neurofibromatosis type I, Malignant peripheral nerve sheath tumors, glycosylation and phosphorylation of kinase receptors, Ras,MGAT5B, 2-Deoxy-D-glucose

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Introduction…………………………………………………….………...…..

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Neurofibromatosis type I (NF1) is a dominantly inherited disease affecting 1 in every 2,500 to 3,000 individuals, representing the most common familial cancer predisposition syndrome[1]. It is a progressive condition with variable complications occurring over the time course of the disease. The hallmark of clinical manifestation of NF1 is the development of multiple neurofibromas[2], which are highly heterotypic benign tumors of peripheral nerve sheath mainly composed of immature Schwann cells, fibroblasts, perineurial and inflammatory matrix[3]. NF1 patients are also at high risk for the development of certain malignancies such as pheochromocytomas, childhood myeloid leukemias, neuroblastomas, rhabdomyosarcomas, and malignant peripheral nerve sheath tumors (MPNSTs)[4-6]. Once progressing to MPNSTs, although the resection is possible, most patients will eventually relapse locally or systemically. Because of the potential involvement of underlying nerves and blood vessels, surgical removal of tumors is not always an option[7] Furthermore, once removed, the lesions have a tendency to regrow. There is no effective treatment for NF1, nor effective approaches for predicting or preventing the occurrence of devastating complications. Thus, to discover novel biomarkers to predict and develop agents for preventing or reversing the tumorigenesis and malignant transformation of NF1 are critically needed.

Body…

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Although the specific cell of origin of neurofibromas and MPNSTs is uncertain, the predominant lesion associated with NF1 consists primarily of Schwann cells (60-80%)[8, 9]. Biallelic *Nf1* mutations have been detected in neurofibroma and MPNSTs with NF1[10, 11]. Schwann cells in neurofibroma and MPNST have been shown to possess abnormal properties, including increased invasiveness and induction of angiogenesis[12]. Schwann cells are peripheral nerve glia originating from migrating neural crest stem cells and recent studies have shown that NF1-related malignant astrocytomas originate from neural stem cells, raising the possibility that Schwann cell precursors (progenitors) undergoing *Nf1* loss of heterozygosity (LOH) during embryogenesis might be the neurofibroma-initiating cells[13]. Moreover, several NF1 mouse models have been generated to identify the Schwann cell progenitors as the 'cells of origin' of neurofibromas and MPNSTs[14, 15]. Schwann cell progenitors differentiate by late gestation and do not persist in the adult peripheral nervous system. However, Schwann cell progenitors persist or Schwann cells dedifferentiate into progenitor-like cells in neurofibromas in NF1[16]. Much evidence indicated that the loss of the *Nf1* gene in NF1 plays an important role in the initiation of tumorigenesis of neurofibromas and that all of the known NF1 phenotypes result from the inheritance or appearance of a mutant allele of the *Nf1* gene[17]. The *Nf1* gene product, neurofibromin, functions as a GTPase activation protein (GAP), a negative regulator of the cellular Ras kinase[18]. It has been reported that the levels of activated Ras-GTP due to the loss of neurofibromin in NF1 plexiform neurofibromas and neurogenic sarcomas were approximately 5 and 15 times higher respectively, than the levels present in non-NF1 schwannomas, supporting the hypothesis that an aberrant Ras signaling pathway is the initial event in the tumorigenesis of NF1[19, 20]. However, the molecular mechanisms of the mutational inactivation of *Nf*1 resulting in hyperactive Ras that leads to alterations in uncontrolled growth and dedifferentiation of Schwann cells have not been elucidated in neurofibromas and MPNSTs.

One of the most important characteristics of transformed cells is an increase in N-glycosylation of cell surface proteins, known as the 'Warren phenomenon'[21]. The appropriate and accurate modification of sugar or glycan to proteins mainly depends on the action of highly

specific and precisely located enzymes known as glycosyltransferases and glycosidases in different tissue and cells[22]. It has been reported that activated Ras directs N-glycosylation in transformed cells and that these changes are most readily monitored by the analysis of complex-type N-glycosylation. For example, stable transfectants of NIH3T3 cells with activated *c-H-ras, c-K-ras or N-ras* are tumorigenic in nude mice and display the alterations in size-distribution of cell surface glycopeptides patterns which are highly correlated with invasiveness and metastasis[23]. Transient expression of activated *Ras* or overexpression of wild type *Ras* in NIH3T3 cells resulted in the significant differences in cell surface glycoproteins shortly after transfection and was independent of morphological transformation. It has been further reported that Ras differentially activates some glycosyltransferases to modify specific molecules involved in the malignant transformation. This is supported by the facts that the activities of the branching N-acetylglucosaminyltransferase III and V were elevated 2- to 2.5- fold whereas N-acetylglucosaminyltransferase I and II were unaltered, suggesting the formation of increased amounts of bisected glycans and structures carrying a Gal β1-GlcNAc β1-6Man-branch. The activities of the elongating β4-galactosyltransferase and β3-N-acetylglucosaminyltransferase were increased 5- to 7-fold in transformed cells, indicating a strongly enhanced capacity to synthesize polylactosaminoglycan chains[24, 25]. Moreover, Ras-directed N-linked carbohydrate modification on cell surface components and subsequent acquisition of invasiveness apparently precedes the morphological transformation[26]. Cancer-specific oligosaccharides in the serum of patients with pancreatic cancer have been determined to be useful biomarkers for pancreatic cancer, suggesting the possibility Ras-directed glycans present on the tumor cell surface may release and circulate in peripheral blood stream of patients[27], which may be the early diagnostic biomarkers for tumorigenesis and transformation of NF1.
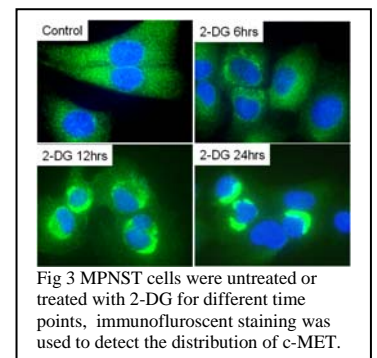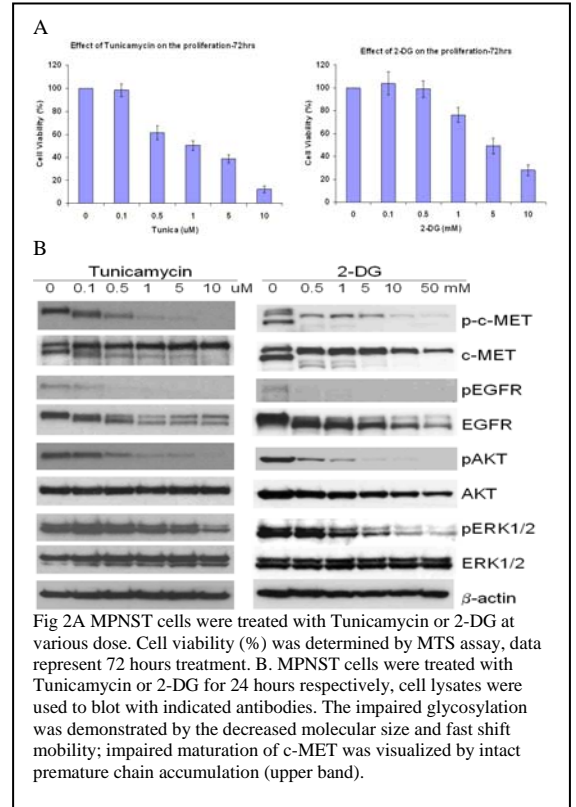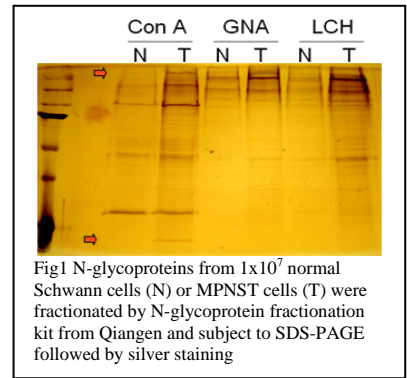
Schwann cell progenitors are tumor-initiating cells of neurofibromas and MPNSTs and growth factor signaling is broadly implicated in the maintenance of the progenitor population[9, 28]. Growth factor receptors depend mainly on the glycosylation for stabilization, maturation, transportation to the cell surface, phosphorylation and activation. Neurofibromas and MPNSTs contain basic fibroblast growth factors, platelet-derived growth factors, insulin-like growth factor 2, neurogulin, as well as unidentified heparin-binding growth factors[29]. Mature Schwann cells normally lack the expression of EGFR and c-MET, however, Schwann cells from human neurofibromas and MPNST cells with NF1 express high levels of EGFR and c-MET[30]. Schwann cell progenitors from *Nf1+/-* and *Nf1-/-* mouse embryos expressed high level of EGFR and c-MET, as well as other growth factor receptors such as erbB2 and erbB3[31, 32]. In addition, both EGFR and c-MET have been specifically implicated in central or peripheral nervous system progenitor expansion and associated with the tumorigenesis in *Nf1+/-:p53+/-* mouse tumor model[33]. Thus, we hypothesize that these mitogenic cytokines that are not present in normal peripheral nervous system would act on *Nf*1+/- and *Nf*1-/- Schwann cells in NF1 to maintain elevated Ras signaling and to directly mediate downstream intracellular signaling through their cognate receptors, which synergistically promote population of Schwann cell progenitors that have lost the inhibitory signaling normally provided by axons in neurofibromas and MPNSTs in NF1.

Glycosylation is a common synthetic step for many transmembrane receptor families that are targets for cancer therapy including EGFR, c-MET, IGF-RI, c-KIT, RET and VEGFR. However, recent studies have demonstrated cancer cell resistance to targeted therapies for single or multiple tyrosine kinase receptors, which is caused by the activation of parallel and compensatory receptor-mediated intracellular signaling cascades[34]. Thus, inhibiting most, if not all, of growth factor receptors that are deregulated in Schwann cell progenitors of NF1 may be of therapeutic benefit. We propose to selectively inhibit the glycosylation of growth factor receptors that are deregulated by hyperactive Ras in Schwann cell progenitors to open a new preventive and therapeutic window for NF1.

Key Research Accomplishments and reportable outcomes.

N-Glycoproteins are highly expressed in MPNST cells and 2-DG is a potential glycosylation inhibitor. It has been reported that loss of *Nf1* leads to the activation of Ras which upregulates the expression of glycoproteins[18, 35]. We detected the expression levels of N-glycoproteins in MPNST cell lines via Qproteome Mannose Glycoprotein Kit (Qiagen). Compared with human normal Schwann cells (NSC, N), MPNST cells (T) express high level of N-glycoproteins and display different N-glycoprotein signatures (Fig 1). We found a few bands in which the molecular sizes are at 150kD and 180KD, these bands may be c-Met and EGFR in MPNST cells. We are subjecting these bands to Mass Spectrometry analysis to identify their characteristics. To investigate whether the glycosylation status of kinase receptors affects phosphorylation status kinase receptors and their kinase activity, we selected Tunicamycin and 2-Deoxy-D-glucose (2-DG) as two potential glycosylation inhibitors. Because proteins travelling to the Golgi apparatus for the consequent steps of glycosylation must be bound by mannose-6-phosphate in order to attach itself to the mannose-6-phosphate receptor and because of the structural similarity between mannose and 2-DG, we hypothesized that 2-DG may be a competitor of mannose for inhibiting the glycosylation of proteins. Thus, we treated MPNST cell lines with these two inhibitors respectively in different doses from 24 hours to 72 hours, we found that both Tunicamycin and 2-DG inhibited the proliferation of MPNST cell lines (Fig 2A) but had no effect on NSCs (data not shown); inhibited the glycosylation (faster shift) and phosphorylation of EGFR and c-MET and impaired MEK-ERK1/2 and PI3K-AKT intracellular signaling in a dose dependent manner (Fig 2B). Moreover, 2-DG inhibited the maturation of c-MET as demonstrated by the poorly glycosylated premature chain (small c-MET band with fast shifting) and failed to cleave into one mature α-chain (Fig 2B) and another mature β-chain, and poorly glycosylated EGFR (small EGFR band with fast shifting) (Fig 2B). In addition, 2-DG inhibited the transportation of receptors from the cytoplasm to the cell surface and retained receptors in the ER and Golgi apparatus (Fig 3). To date, this is the first time for us to detect 2-DG inhibiting the glycosylation and phosphorylation of kinase receptors. These data suggest that inhibiting glycosylation of kinase receptors attenuates their phosphorylation and activation, and blocks their intracellular signaling.

Fig1 N-glycoproteins from 1×10^7 normal Schwann cells (N) or MPNST cells (T) were fractionated by N-glycoprotein fractionation kit from Qiangen and subject to SDS-PAGE followed by silver staining

Fig 2A MPNST cells were treated with Tunicamycin or 2-DG at various dose. Cell viability (%) was determined by MTS assay, data represent 72 hours treatment. B. MPNST cells were treated with Tunicamycin or 2-DG for 24 hours respectively, cell lysates were used to blot with indicated antibodies. The impaired glycosylation was demonstrated by the decreased molecular size and fast shift mobility; impaired maturation of c-MET was visualized by intact premature chain accumulation (upper band).

Because 85% of *NF1+/-; p53+/-* mice will develop tumors, most of them are MPNSTs in the age of 15 weeks[36]. When mice grow to 6 weeks, we randomly separate these mice into two groups, control group containing 10 *NF1+/-;p53+/-* mice was treated with 250mg/kg of glucose per day and treatment group containing 10 *NF1+/-;p53+/-* mice was treated with 250mg/kg of 2-DG per day. Mice have been treated for 7 weeks so far, 3 mice in control group were detected the tumor formation, no tumors were found in 2-DG treatment group, suggesting 2-DG has a potential to prevent the tumor development in *NF1+/-;p53+/-* mice. We are keeping breeding *NF1+/-;p53+/-* mice expend our investigation in this field.

Fig 3 MPNST cells were untreated or treated with 2-DG for different time points, immunofluroscent staining was used to detect the distribution of c-MET.

The Expression of MGAT5B correlated with hyperactive Ras in MPNST and Ras-related malignancies: People with NF1 have a 10% lifetime risk of developing MPNSTs and loss of *Nf1[37]* gene leading to the hyperactive Ras is the key step to develop these malignancies. It has been reported that Ras upregulates the expression of glycoproteins but molecular mechanism has not been elucidated. The appropriate and accurate modification of sugar or glycan to proteins mainly depends on the action of highly specific and precisely located enzymes known as glycosyltransferases and glycosidases in different tissue and cells. To detect the expression of glycosyltransferases and glycosides in MPNST cell lines, the Human Glycosylation RT² Profiler™ PCR Array (Qiagen) has been used to profile the expression of 84 key genes encoding enzymes that add glycans to proteins or remove glycans from glycoproteins. Compared with NSC, we did not find any change in glycosidase mRNA expression levels. However, multiple glycosyltransferases (FUT8, MGAT3, MGAT4A, MGAT5, MGAT5B, GALNT13, GALNT14, POMT1 and ST8SIA2) were overexpressed in different MPNST cell lines, especially MGAT5B was highly expressed in all MPNST cell lines (Fig 4A), MGAT5B protein expression was confirmed by western blot in MPNST cell lines (Fig 4B left), and colon and pancreatic cancer lines (Fig 4B right). The overexpression of MGAT5B mRNA was also confirmed by RT-
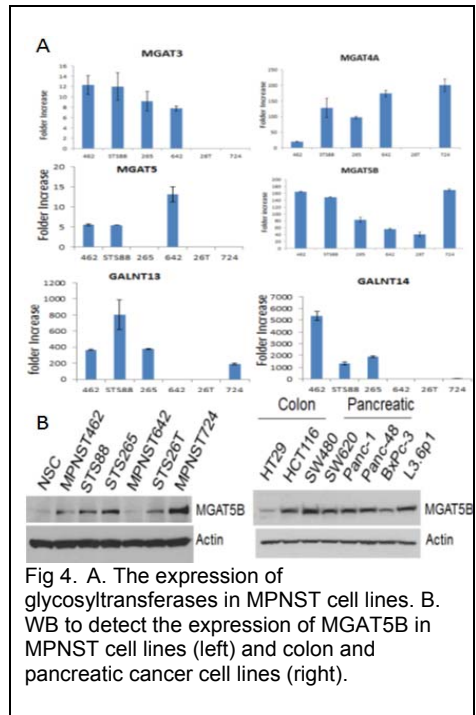

Fig 4. A. The expression of glycosyltransferases in MPNST cell lines. B. WB to detect the expression of MGAT5B in MPNST cell lines (left) and colon and pancreatic cancer cell lines (right).

PCR in MPNST cell lines (Fig 5A, left) and colon cancer cell lines (HCT116, SW489 and SW620) and pancreatic cancer cell lines (Panc-1, Panc-48 and L3.6p1) with *K-ras* mutations, but no or lower expression in HT29 and BxPC-3 without *K-ras* mutations (Fig 5A,right). Real time PCR and RT-PCR also detected the high levels of MGAT5B mRNA expression in clinical MPNST specimens (Fig 5B) The expression levels of MGAT5B correlated with RAS kinase activity in MPNST cell lines (Fig 5C, left) and the expression of mutated *K-ras* ^{G12D} upregulated the expression of MGAT5B in STS26T cells (Fig 5C, right). These data suggest that RAS regulates the expression of MGAT5B in MPNST and Ras-related malignancies.

MAGT5B is a potential AKT phosphorylation protein: Since MGAT5B is preferentially located in the Golgi apparatus, suggesting that posttranslational modification such as phosphorylaton promote MGAT5B transportation from the cytoplasm into Golgi apparatus. Motif Scan (www.scansite.mit.edu) graphic results indicated Ser192 (RARWTSD) in MGAT5B is the potential AKT phosphorylation site (Fig 6A). Because AKT is highly activated in MPNSTs, we hypothesize that AKT may phosphorylates ser192 in MGAT5B to promote its transportation from the cytoplasm into the Golgi apparatus to promote its glycosyltransferase activity. To investigate this, MGAT5B full length cDNA was
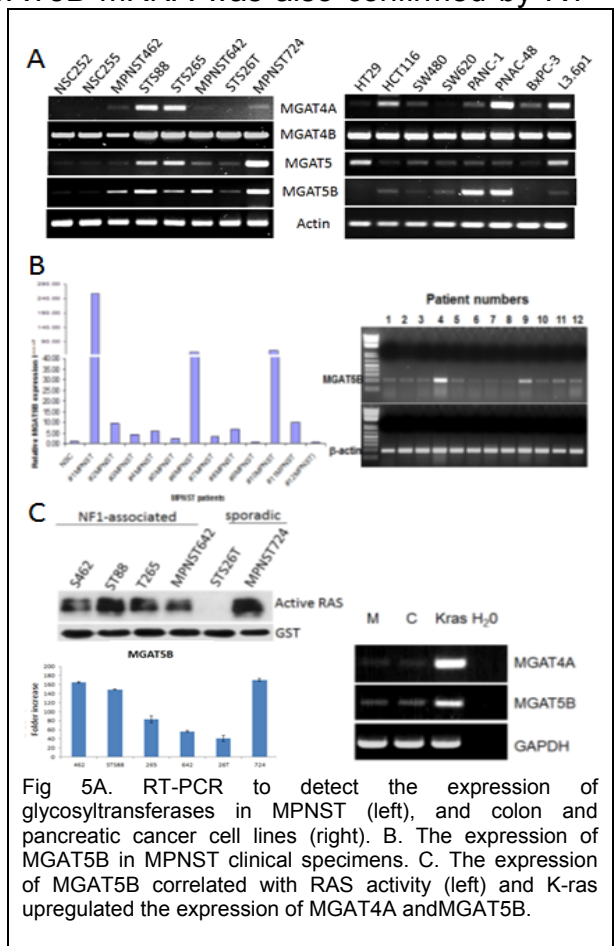

Fig 5A. RT-PCR to detect the expression of glycosyltransferases in MPNST (left), and colon and pancreatic cancer cell lines (right). B. The expression of MGAT5B in MPNST clinical specimens. C. The expression of MGAT5B correlated with RAS activity (left) and K-ras upregulated the expression of MGAT4A andMGAT5B.

cloned into pEGFPN1 to generate pEGFPN1-MAGT5B, and site-directed mutagenesis (Stratagene) was used to mutate Ser 192 into Alan 192 (S192A) to generate pEGFPN1-MGAT5BS192A (mutated). Two plasmid constructs were

transfected into MPNST724 cells, the localization of GFP-tagged MGAT5B or GFP-tagged MGAT5BS192A was observed under the fluorescence microscope, we found wild type MGAT5B was preferentially located in Golgi apparatus while MGAT5BS192A (mutated) was distributed in the ER and cytoplasm (Fig 6B left), suggesting the phosphorylation of ser192 at MGAT5B is involved in MGAT5B transportation. Because commercial available anti-MGAT5B antibody can nonspecifically recognize multiple protein bands by western blot analysis, we used Myc-tagged MGAT5B protein to detect the interaction between AKT and MGAT5B. pcDNA3.1-MGAT5B-Myc plasmid was transfected into MPNST724 cells and the cell lysates were immunoprecipitated by either anti-AKT or anti-Myc antibody respectively, we found MGAT5B-Myc fusion protein was detected in the AKT immunocomplexes and AKT protein in the MGAT5B-Myc immunocomplexes. (Fig 6B right). These data indicated that AKT associates with MAGT5B and has a potential to phosphorylate MGAT5B in MPNST cell lines. We are working on the in vitro AKT kinase assay to identify whether ser192 in MGAT5B is the AKT phosphorylation

MGAT5B promoted tumor lung metastasis: We transfected pEGFPN1, pEGFPN1-MGAT5B or pEGFPN1-MGAT5BS192A plasmid respectively into STS26T cells to establish three stable cell lines. We injected these three cell lines separately into the tail veins of hairless SCID mice to observe the experimental tumor lung metastasis. After 8 weeks of breeding, mice were sacrificed, lung weights were measured and tumor micrometastasis in lung was measured under the microscope. We found 4 of 6 mice in GFP group, 5 of 6 mice in MGAT5B group showed experimental tumor lung metastasis while only I of 6 in MGAT5BS192A mice showed experimental tumor lung metastasis (Fig 6C), suggesting MGAT5BS192A significantly inhibited *in vivo* experimental tumor lung metastasis. Furthermore, MPNST cells expressing GFP-tagged MGAT5B were treated with control (DMSO) or PI3 kinase inhibitor Ly294002 for 24 hours, the localization of GFP-MAGT5B protein was observed under the fluorescence microscope, we found the expression of MGAT5B protein in control group (DMSO) was localized in the Golgi apparatus while AKT kinase inhibition retained MGAT5B in the ER and cytoplasm (treatment group) (Fig 6D). These data suggested that the phosphorylation of ser192 in MGAT5B plays a critical role in promoting MGAT5B transportation from the cytoplasm into the Golgi apparatus and mediating experimental tumor lung metastasis.

Transient knockdown of MGAT5B (siRNA) blocked the phosphorylation of c-Met and EGFR (Fig 7A), impaired migration and invasion (Fig 7B), inhibited cell proliferation (Fig 7C), arrested cells in G2 phase (Fig 7D) and induced apoptosis of MPNST cell lines (Fig 7E). Stable knockdown of MGAT5B (shRNA) significantly inhibited MGAT5B mRNA and protein expression levels (Fig 8A); arrested cells in G1 phase (Fig 8B); blocked the phosphorylation of kinase receptors
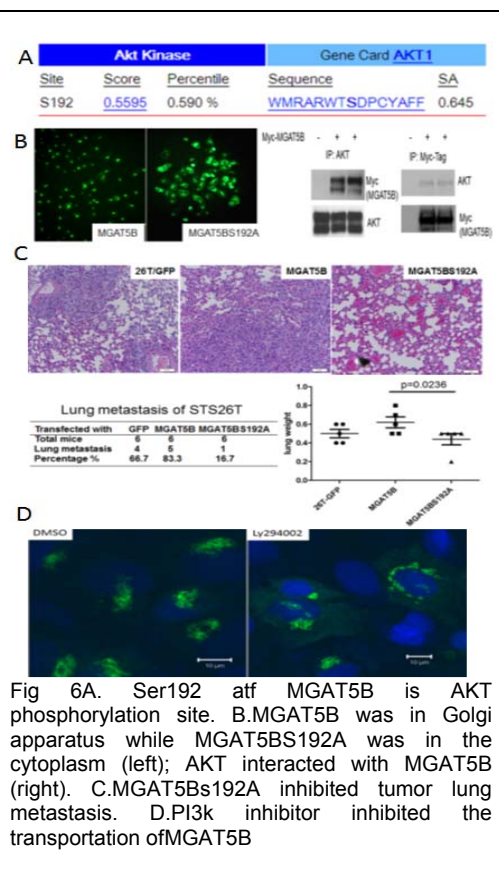

Fig 6A. Ser192 atf MGAT5B is AKT phosphorylation site. B.MGAT5B was in Golgi apparatus while MGAT5BS192A was in the cytoplasm (left); AKT interacted with MGAT5B (right). C.MGAT5Bs192A inhibited tumor lung metastasis. D.PI3k inhibitor inhibited the transportation ofMGAT5B
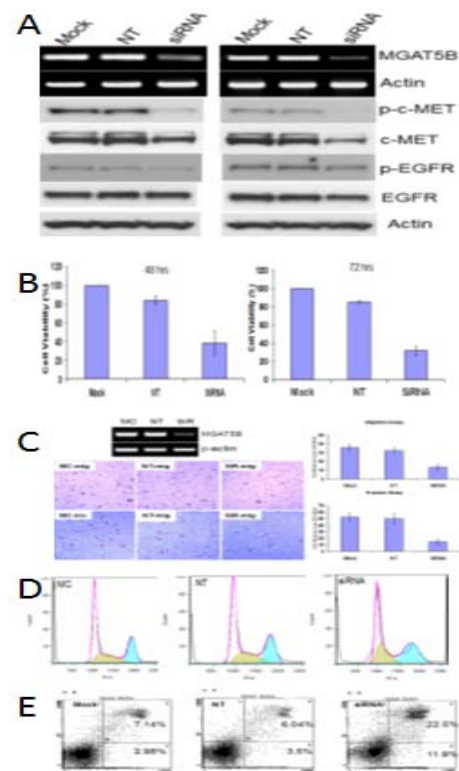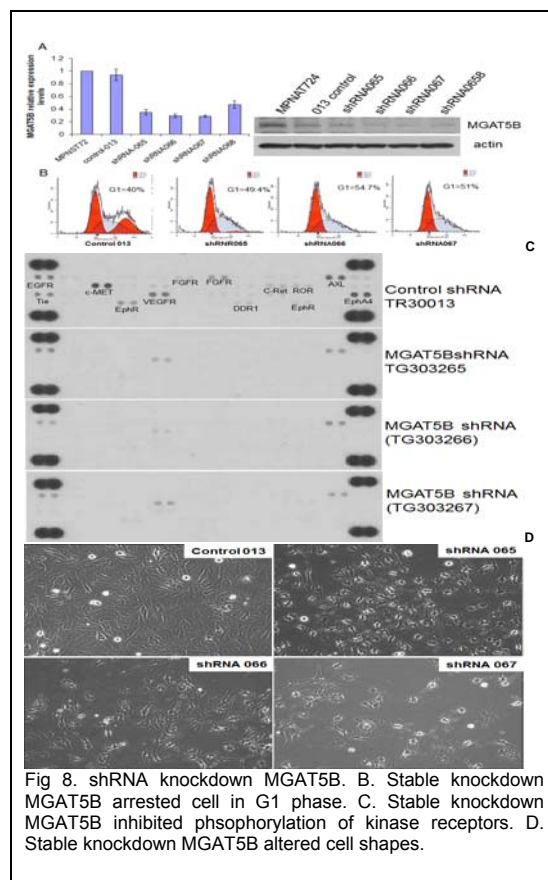

Fig 7..A. Transient knockdown MGAT5B inhibited phosphorylation of c-MET and EGFR. B. Knockdown MGAT5B inhibited cell proliferation. C. Knockdown MGAT5B inhibited migration and invasion. D. Knockdown MGAT5B arrested cells in G2 phase. E. knockdown MGAT5B induced apoptosis

such as c-MET, c-Ret, FGFR, Tie, EGFR, AXL, EphR, EphA4, ROR, PDGFR, et al (Fig 8C). In addition, the morphology of MPNST cells normally are round or polygonal, with few cells showing elongations. However, when MGAT5B is knocked down, filamentous protrusions began to appear and cell shapes shrunk, suggesting shRNA knockdown of MGAT5B is associated with a marked alterations in cell morphology (Fig 8D). These data suggest that downregulation of MGAT5B significantly attenuate the phosphorylation of kinase receptors and their intracellular signaling.

2 We have produced about 13 wild type and 12 NF1+/-; p53+/- mice. After 6 weeks of growth, 50ul of peripheral blood samples from wild type and NF1+/-; p53+/- mice were collected and total glycoproteins were isolated via Qproteome Total Glycoprotein Kit and separated by 10% SDS-PAGE, multiple glycoprotein bands were observed in *NF1+/-; p53+/-* mice compared to wild type mice, these glycoproteins are subject to Mass Spectrometry analysis to discover the early diagnostic glycoprotein markers in the blood stream. We are working on the profiling of N-glycan structures by Lectin microarray: The LecChip™ Ver 1.0 lectin microarrays and reagents were purchased

Fig 8. shRNA knockdown MGAT5B. B. Stable knockdown MGAT5B arrested cell in G1 phase. C. Stable knockdown MGAT5B inhibited phsophorylation of kinase receptors. D. Stable knockdown MGAT5B altered cell shapes.

from GP Bio Sciences (San Diego, CA). As a high-throughput platform for glycan analysis, this Lectin microarray system are being used to qualitatively and quantitatively profile glycoprotein glycan patterns of both cell surface and cytosolic glycans from normal cells and tumor cells. This array-based assay has been demonstrated for rapid analysis of glycosylation profiles of multiple glycoproteins. The N-glycoproteins will be fractionated and purified respectively from cells or serum described above with Qiagen Qproteome N-glycoprotein kit. Purified glycoproteins will be labeled with Cy3 monoreactive dye (GE Healthcare). The labeled glycoprotein concentrations will be adjusted to 50ug/ml. We'll apply 100ul of Cy3-labeled glycoprotein solution in probing buffer (TBS containing 0.05% Tween 20) to each well on the glass slide and incubate the slides at $25^{o}C$ until the binding reaction reaches equilibrium. After the binding of the Cy3-labeled glycoprotein probes with immobilized lectins, the fluorescent images of the arrays will be acquired by through service from the Genomics and Proteomics Core Laboratory at Texas Children's Hospital by using evanescent-field fluorescence scanner GTMASScan III (Nippom Laser & Electronics Lab). The data will be analyzed with the Array Pro Analyzer version 4.5 (Media Cybernetics, Inc). The display of lectins in a microarray format enables a fast, sensitive and high-throughput profiling of sugar structures of surface glycoproteins (Fuc, Sia, Asialo, Man, Gal, poly-LacNac, N-glycan, O-glycan) with subtle differences. We'll define the glycans that specifically present in tumor cells with NF1 and discover the distribution size patterns specific to tumor cells.

4 For 2-DG experiments: Animal studies will be conducted following the guideline of the Institutional Animal Care and Use Committee (AUCUC-07-95-06336). We have produced another 20 *NF1+/-/p53+/-* mice, we divided them into two groups 10 mice in control group have been treated with 250mg/kg per day of glucose and 10 mice have been treated with 250mg/kg/per day of 2-DG for 7 weeks. So far, two mice developed tumors in control group, no tumors were found in treated group, we keep treating these mice till mice in control group

develop tumors. We will generate up to 100 *NF1+/-;p53+/-* mice. 62 baby mice were born in recent days, we expected to get 30 *NF1+/-/p53+/-* mice to continue this experiments. Mouse neural crest cells develop into Schwann cell precursors between E11 and E13 in mouse sciatic nerve, develop immature Schwann cells by E15 and Schwann cells by E18 (Jessen and Mirsky,2005). Collaborated with Dr. Luis F. Parada at Southwest medical Center, we have generated *Nf1:p53* animal models in which *Nf1+/-;p53+/-* mice developed tumors in postnatal 15 weeks, most of them are MPNSTs. Because *Nf1-/-* mice are embryonic lethality in 12 day gestation. We will dissociate E12.5 dorsal root ganglion (DRG) from *Nf1-/-,p53-/-* embryos (dead embryos) to make single cell suspension and culture in the stem cell media. Flow cytometry will be used to sort the Schwann cell progenitors containing EGFR$^+$; P75$^+$ cells. These cells are neurofibroma-initiating cells and form tumors in nude mice. We will utilize Flow cytometry to sort EGFR$^+$:P75$^+$ cells from postnatal sciatic nerve and culture in the stem cell media. These mouse MPNST models provide means to test the cancer prevention and therapeutic strategies. Schwann cell progenitors will be plated directly on ammoniated collagen-coated, 35 mm tissue culture dishes (15 DRG explants per dish) and cultured in stem cell medium. After 3 days, Schwann cell progenitors will be divided into two groups. Control group will be treated with 5mM of glucose and treatment group will be treated with 5mM of 2-DG separately for 48 hours. We will evaluate the effects of 2-DG on the proliferation, clonogenicity, migration/invasion, and apoptosis of cells. *In vitro* sphere formation assay will be conducted to assess the effects of 2-DG on the self-renewal capability of Schwann cell progenitors. Furthermore, we will determine whether 2-DG inhibits tumor establishment of these Schwann progenitors in nude mice. 1x10^5 viable Schwann cell progenitors from each group will be injected aseptically s.c. into the flank of Nod-SCID mice by hand-held injection using 25G needle on a 1ml syringe. 10 mice will be injected for each group. Keep treating Nod-SCID mice with 2-DG (250mg/kg per day) and glucose (250mg/kg per day) separately for four weeks, the tumor formation will be monitored every other day. Once s.c. tumor size in control group reaches to 15mm, mice will be sacrificed, they will undergo necropy for the harvest of tumors and tissues for isolating protein and total RNA for further molecular experiments:

6　We will identify tyrosine kinase receptors (TKRs) that are disrupted by 2-DG using Proteome Profiler Array (R&D) as we did in MGAT5B knockdown experiments.
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Conclusion…………………………………………………………………………

1. 1 N-glycosylation signatures indicated that MPNST cells express high level of N-glycosylation proteins compared with normal Schwann cells.

2. 2-DG, a competitor of mannose, is a novel glycosylation inhibitor, inhibiting the glycosylation and phosphorylation of tyrosine kinase receptors, and blocking the transportation of kinase receptors from Golgi apparatus to cell surface.

3. 2-DG prevents the tumorigenesis in *NF1+/-;p53+/-* mice.

4. MGAT5B,one of the glycosyltransferses, is highly expressed in MPNST cell lines and MPNST clinical specimens, which is correlated with hyperactive Ras kinase activity, and *Ras* upregulates the expression of MGAT5B in MPNST cell lines.

5. Knockdown of MGAT5B significantly inhibits the glycosylation and phosphorylation of general kinase receptors and receptor-mediated intracellular signaling, inhibits the migration and invasion of MPNST cell lines and phosphorylation of c-MET.

6. Ser192 in MGAT5B is phosphorylated by AKT and mutation of Ser192 in MGAT5B (MGAT5BS192A) blocked its transportation from the cytoplasm to Golgi apparatus and significantly inhibited experimental lung tumor metastasis.

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