Elevated Expression of IRS2 in the Progression from Neurofibroma to Malignant Peripheral Nerve Sheath Tumor

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Abstract. *Background/Aim: Novel drugs to inhibit insulin receptor substrate (IRS) and focal adhesion kinase (FAK) pathways are emerging and will be sarcoma subtype-specific. As a result, defining expression of proteins in these pathways; in select tumors is important in order to formulate therapeutic approaches to malignant peripheral nerve sheath tumors (MPNSTs). Materials and Methods: Fifty-three patients with MPNSTs or neurofibromas (NFs), who were treated at our institution from 1994-2005, were identified. Tumor immonohistochemical staining for multiple key oncogenic proteins was performed and the sections were evaluated in a blinded fashion by a sarcoma pathologist (JDR) and correlated with survival. Results: A total of 88% of MPNSTs expressed IRS2 compared to 48% of NFs. IRS2 expression was significantly higher in MPNSTs than in NFs (p=0.0009). However, IRS1 expression was significantly higher in NFs than MPNSTs (p=0.03). A trend toward an increase in FAK expression in MPNSTs was seen (p=0.11). No difference was seen between MPNSTs and NFs when evaluating the expression of phosphorylated focal adhesions kinase, vascular endothelial growth factor 3, insulin like growth factor receptor 1, neurofibromatosis 1. Univariate analysis of survival indicated that IRS2 and NF1 protein expression, patient age and tumor size were significantly correlated with outcome. Conclusion: MPNSTs have an elevated level of IRS2 and FAK and lower level of IRS1 compared to NFs These data demonstrate for the first time that IRS2 and FAK may be associated with malignant transformation of neurofibromas.*

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The insulin-like growth factor (IGF) signaling system orchestrates a vital role in the initiation and progression of human malignancy. Alterations in the IGF pathway, such as over-activation of insulin-like growth factor-1 receptor (IGF1R) is frequently identified in multiple tumor types (1). Activated IGF1R recruits and phosphorylates adaptor proteins belonging to the insulin receptor substrate (IRS) family. This results in the activation of phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, important triggers of cell proliferation and survival (2). IRS1 and IRS2 represent the initiator molecules in the signal transduction cascade mediated by insulin and other ligands. Several intermediate signaling events including the activation of PI3 Kinase pathway have been implicated in insulin and IGF-1-stimulated mitogenesis (3, 4). However, not only do IRS proteins mediate the insulin-induced mitogenic effects, but they may even contribute to epithelial carcinogenesis.

FAK is a protein tyrosine kinase that is localized to focal adhesions, which are contact points between a cell and its extracellular matrix. FAK has many functions in cells including linking integrin signaling to downstream targets (5). In addition, FAK function promotes tumor cell survival (6) and is important for cell motility (7). FAK is phosphorylated following activation by transmembrane receptors including IGF1R (8, 9). It has been previously shown that FAK and IRS family proteins physically interact and that FAK activity regulates IRS1 mRNA levels (10). Of note, it has also been demonstrated that integrin-mediated phosphorylation of the insulin receptor occurs with the participation of FAK (11).

Definite knowledge of molecular events contributing to peripheral nerve tumor development remains elusive (12). In patients, the mutated *NF1* allele results in loss of neurofibromatosis 1 protein. This leads to reduced suppression of RAS activity since the tumor suppressor function of NF1 is reduced. Cell lines and tumor specimens from patients with malignant peripheral nerve sheath tumor and neurofibromatosis 1 exhibit high Ras activity and loss of *NF1* gene expression (13). This results in the formation of

multiple plexiform neurofibromas in NF1 patients (14). Loss of the second copy of the *NF1* allele is characteristic of MPNST cells and may have a role in malignant transformation of these benign plexiform neurofibromas. However, for malignant transformation of benign neurofibromas to occur, loss of both *NF1* alleles is not sufficient. Additional genetic alterations are requisites for malignant transformation (15-17). Our hypothesis is that FAK and IRS proteins are overexpressed in human MPNST and are associated with its carcinogenesis.

Materials and Methods

Patients. Under a University of Florida Institutional Review Board approved protocol, we retrospectively reviewed those patients treated for MPNSTs at our institution from 1995-2006. We identified 28 patients undergoing surgical therapy for their disease and evaluated factors associated with outcome in these patients. Demographics, clinicopathologic and treatment-related factors were assessed. Our routine is to base a treatment plan after in house review of the pathologic diagnosis and staging utilizing National Cancer Comprehensive Cancer Network's (NCCN) most current clinical guidelines for the treatment of sarcoma. In addition, we identified 25 patients with neurofibromas undergoing surgical therapy at our institution, in order to compare immunohistochemical staining for focal adhesion kinase (FAK, p-FAK), IGF1R, IRS1, IRS2, and VEGFR3.

Immunohistochemical staining. For FAK staining, paraffin sections were deparaffinized with xylene and blocked with 3% hydrogen peroxide and methanol. Sections were pretreated with Citra-SAR, (DAKO, Carpinteria, CA) 1:10 solution with antigen retrieval for 30 minutes in a steamer. The following primary antibody was used: anti-FAK antibody, diluted 1:200 (cat# 05-537, Upstate Biotechnology, Lake Placid, New York, USA). Subsequently, avidin–biotin–peroxidase procedure with Diaminobenzidine chromogen was used for immunostaining.

To assess IGF-1R expression, avidin–biotin–peroxidase procedure with DAB chromogen was used for immunostaining. Sections were pretreated with a citrate buffer solution 0.01 mol/l citric acid and 0.01 mol/l sodium citrate (pH 6.0) in a steamer for 30 min to ensure antigen retrieval. The following primary antibody was used: anti-IGFIRβ antibody, diluted 1:200 (Abcam, Cambridge, MA, USA). VEGFR3 staining was performed using a similar technique with primary antibody (clone 9D9F9) from Millipore (Billerica, MA, USA) at 1:100 dilution.

NF1 staining was performed following antigen retrieval using Dako solution (S1700) for 30 min at 95˚C. Sections were blocked for 1 hour at room temperature using PBS with 10% normal swine serum. Primary antibody (NF1GRP, #SC-68, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied at 1:200 dilution overnight at 4˚C. Secondary antibody (swine anti-rabbit biotinylated; #P0399, DAKO, Carpinteria, CA) was applied for 2 h at 37˚C after it was diluted 1:200 in blocking buffer. Next, avidin-biotin complex was applied and it was developed with DAB.

IRS1 and IRS2 staining were performed using citra antigen retrieval. IRS-1 (Cat. # 40777) and IRS2 (Cat. # 84906) primary antibodies were obtained from Abcam (Cambridge, MA, USA) and utilized at 1:100 dilution. No Controls were used.

Table I. *Demographics for 28 patients with malignant peripheral nerve sheath tumors*.

Characteristic	Median (SD)	N (%)
Age, years	42 (17.5)	
Tumor size, (cm)	8.5 (5.39)	
History of neurofibroma		8 (32)
Mass on presentation		17 (63)
Pain on presentation		15 (56)
Neuro deficit on presentation		9 (33)
Site		
Trunk		5 (22)
Extremity		12 (52)
Head and Neck		6 (26)
Margins status		
R0		14 (64)
R1/2		8 (36)
Grade		
High		19 (83)
Low		4 (17)
Radiation Therapy		
Preoperative		9 (36)
Postoperative		9 (36)
Any radiation		17 (68)

Pathology review and immunohistochemistry analysis. All samples were re-analyzed by a sarcoma pathologist (JR) and confirmation of MPNST and neurofibroma diagnoses were confirmed with immunohistochemical staining for S100 protein.

Samples were classified on the basis of a positivity score as follows: 0 score for no positive cells, 1+ for <25% of positive cells, 2+ for 25-50% positive, 3+ for 51-75% posity, 4+ for >75% posity.

Statistical analysis. Differences among mean values were analyzed using a two-sided Student's T-test. Correlations were analyzed by Spearman's correlation test. Univariate tests of significance were performed using Wilcoxon rank sum and Kruskal-Wallis tests. Survival analysis was performed utilizing the Cox proportional hazards model. Two-sided p-values <0.05 were considered statistically significant. All analyses were performed using STATA 10 (College Station, Texas, USA).

Results

Demographics of the patients and MPNSTs utilized in the study are shown in Table I. The median age of patients was 42 years. The median tumor size was 8.5 cm. A history of neurofibromatosis was identified in 8 (32%) patients. On initial presentation, 17 patients (63%) had a mass and 15 patients (56%) had pain. Tumor location was in the extremity in 52% of patients, with the rest in the trunk or head and neck. An R0 resection was achieved in 64% of patients. The majority of tumors (84%) were classified as being high grade. Adjuvant or neoadjuvant radiation therapy was delivered to 68% of patients. The Median follow up time was 36 months.

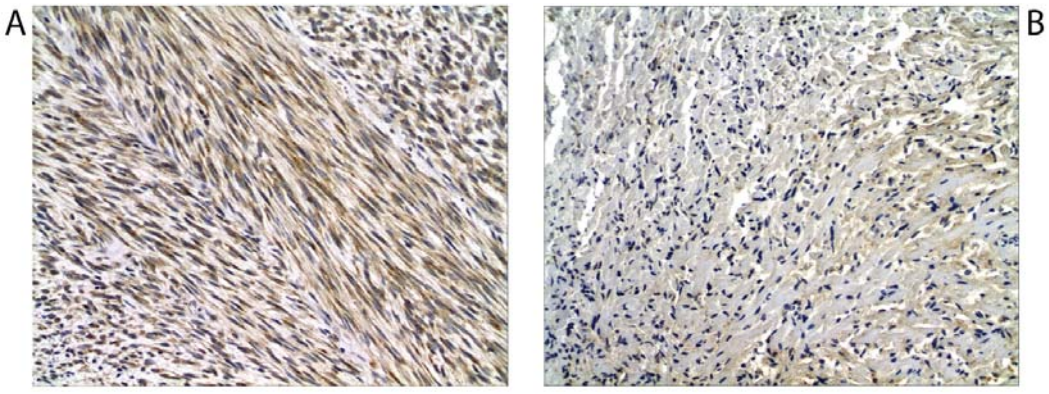

Figure 1. *Immunohistochemical staining for IRS2 in malignant peripheral nerve sheath tumor (A) compared to that in neurofibromas (B).*

Table II. *Mean immunohistochemical (IHC) score and percentage of tumors positive for staining in (MPNSTs) and neurofibromas (NF).*

Protein stained	MPNST IHC score, (% tumors +)	Mean NF IHC score, (% tumors +)	*p*-value
FAK: Focal adhesion kinase	2.63 (89)	2.16 (96)	0.114
IRS1: Insulin receptor substrate 1	0.370 (30)	1.46 (50)	0.028
IRS2: Insulin receptor substrate 2	2.04 (88)	0.870 (48)	0.0009
pFAK: Phospho- focal adhesion kinase	3.11 (96)	3.46 (87)	0.392
VEGFR3: Vascular endothelial growth factor receptor 3	1.65 (65)	1.48 (48)	0.579
IGF1R: Insulin like growth factor receptor 1	0.630 (26)	0.565 (30)	0.844
NF1: Neurofibromatosis 1	2.88 (85)	2.72 (96)	0.247

Immunohistochemical staining results are shown in Table II. The majority of MPNST and neurofibromas stained positively for FAK, p-FAK and NF1. There was a trend towards an increased mean immunohistochemical score for FAK expression in MPNST compared to neurofibromas (p=0.11). For p-FAK and NF1 there was no significant difference in immunohistochemical staining score or frequency between benign and malignant tumors. While the frequency of positive staining for benign and malignant tumors was lower for IGF1R and VEGFR3 compared to FAK and NF1, there was no significant difference in staining score or frequency for these two markers between MPNSTs and neurofibromas. Of note, there was a significant increase in positive staining score and frequency for IRS1 in neurofibromas compared to MPNSTs (p=0.028). On the contrary, there was a significant increase in staining score and frequency for IRS2 in MPNSTs compared to neurofibromas (p=0.0009). An example of increased IRS2 expression in MPNSTs compared to neurofibromas is shown in Figure 1.

Correlations between immunohistochemical staining results for oncogenic proteins is shown in Table III. Utilizing a Spearman's correlation coefficient cut off of 0.6, there was no meaningful correlations between staining for any two proteins. The closest correlation of significance was –0.5853 between VEGFR3 and IRS1. This represents an increase in staining for IRS1 with decreased staining for VEGFR3.

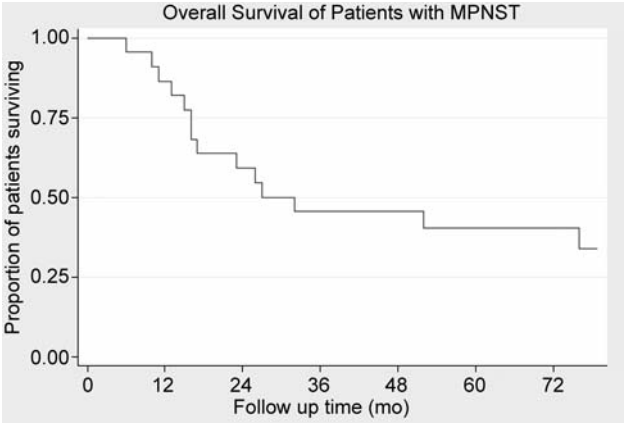

Figure 2. *Overall survival for all patients with malignant peripheral nerve sheath tumor.*

The median survival was 18 months for patients with MPNST (Figure 2). A Cox proportional hazards model was used to identify correlations between immunohistochemical staining in patients with MPNST and survival (Table IV). Factors associated with reduced survival on univariate analysis included lowerd NF1 and IRS2 expression, $p<0.05$; increasing patient age, $p<0.001$; and increasing tumor size, $p<0.001$. A multivariate analysis was performed but not considered valid due to the small sample size.

Table III. *Correlations between immunohistochemical staining utilizing Spearman's correlation test.*

	S100	Phospho-focal adhesion kinase	Focal adhesion kinase	Insulin receptor substrate 1	Insulin receptor substrate 2	Vascular endothelial growth factor receptor 3	Insulin like growth factor receptor 1	Neurofibro-matosis 1
S100	1.0000							
Phospho- focal adhesion kinase	0.2598	1.0000						
Focal adhesion kinase	–0.2936	0.2931	1.0000					
Insulin receptor substrate 1	–0.1531	0.1102	0.1095	1.0000				
Insulin receptor substrate 2	0	–0.0516	–0.0503	–0.0812	1.0000			
Vascular endothelial growth factor receptor 3	0.0137	0.1606	0.0306	–0.5853	–0.1191	1.0000		
Insulin like growth factor receptor 1	–0.0307	–0.0443	–0.1962	–0.0759	0.1366	–0.1526	1.0000	
Neurofibromatosis 1	–0.4579	–0.0683	0.2773	0.0011	0.0382	–0.0233	0.0575	1.0000

Discussion

Molecular events contributing to peripheral nerve tumor development are largely unknown. In NF1 patients, loss of the NF1 protein product is believed to be the earliest event as patients inherit a mutated *NF1* allele and have reduced suppression of RAS activity (12). This contributes to the formation of multiple benign plexiform neurofibromas in these patients (12). Loss of the second copy of the *NF1* allele is found in MPNST cells and may contribute to malignant transformation of these peripheral nerve tumors (12). However, loss of both *NF1* alleles is not sufficient for malignant transformation of benign neurofibromas (12). Additional genetic alterations are required for malignancy and remain to be determined (15-18).

Altered expression of several important proteins including endothelial growth factor receptor have been reported in MPNSTs (12, 17-18). EGFR protein is detected in primary MPNSTs, MPNST cell lines and some neurofibroma Schwann cells, but not in normal human Schwann cells (17). Later events in the malignant transformation of Schwann cells although not observed in neurofibromas, include genetic mutations targeting regulators of the retinoblastoma protein (pRb) and p53 tumor suppressor pathways (12).

There are limited studies that have studied the importance of IGF1R and associated signaling proteins in MPNSTs. In one study, investigators demonstrated evidence for the expression of IGF1R in nerve sheath tumors in NF1 (19). The expression pattern varied between the tumor types, the cell types, and between tumors of the same type (19). These authors concluded that IGF and IGF- 1R are a prerequisite to maintain Schwann cell stability in the postnatal period and to prevent Schwann cell apoptosis (19).

Others have found that IRS1 is constitutively activated in a variety of solid tumors, including rhabdomyosarcoma, liposarcoma, and leiomyosarcoma (3, 20). In addition, blocking the constitutively activated IRS1 signaling in breast cancer cells with a dominant-negative IRS1, an IRS1 with all 18 potential tyrosine-phosphorylation sites replaced by phenylalanines (F18), dramatically reduced cancer cell growth (3). In contrast to these findings, we observed reduced staining for total IRS1 protein in MPNSTs compared to neurofibromas. Therefore, it appears that increased expression of IRS1 does not contribute to the malignant transformation in MPNST.

There have been no previous studies that have demonstrated increased expression of IRS2 in MPNSTs compared to neurofibromas. Recently, it was shown that IRS1 and IRS2 mediate different functions in cells (21-22). Although their exact contribution to the malignant phenotype has not been

Table IV. *Univariate analysis of factors impacting on survival in patients with malignant peripheral nerve sheath tumor.*

Variable	*p*-value
Protein Staining	
Phospho- focal adhesion kinase	0.8526
Focal adhesion kinase	0.8564
Insulin receptor substrate 1	0.9525
Insulin receptor substrate 2	0.0308
Vascular endothelial growth factor receptor 3	0.9583
Insulin like growth factor receptor 1	0.5668
Neurofibromatosis 1	0.0470
S-100	0.9879
Patient age	<0.0001
Patient presentation	
Mass	0.8948
Pain	0.7080
Neurologic symptoms	0.3363
Tumor characteristics	
Size	0.0001
Grade	0.3376
Location	0.4965
Treatment	
R status of resection	0.1355
Pre-op RT	0.4902
Post-op RT	0.3167
Any RT	0.2137

elucidated, in certain cell types, IRS1 has been shown to be associated with the control of cell growth and proliferation, while IRS2 has been demonstrated to be associated with cell migration and invasion (23). Of interest, in the present study, although increased IRS2 expression was identified in MPNSTs compared to neurofibromas, lower IRS2 levels were identified in those patients with reduced survival. The factors associated with this finding require further study.

There are several limitations to our study. Our sample size may not have sufficient power to detect true associations that exist among certain variables. Point estimates for certain variables, including FAK staining and R0 resection, suggest that there might be trends that would be uncovered if more samples were available for analysis.

There are differing opinions concerning the importance of high IGF signaling protein expression in promoting poor survival in sarcoma. In Ewing's sarcoma, investigators reported that IGF1R and insulin receptor are expressed in virtually all Ewing's sarcoma tumors (20). Usually both of the receptors are present in the same tumor but when one receptor is lacking, the other one is always present. They identified that high expression of IGF1R, insulin receptor and IGF-Is, mRNA is significantly associated with more favorable clinical outcomes. Overall, their clinical data are in contrast with the assumption that higher amounts of IGF/IGF1R are a surrogate for higher aggressiveness and indicate that in some lines of cancers the transition to frank malignancy and poor treatment responsiveness seem to be associated with a reduction of IGF system activity (20).

We have demonstrated that IRS2 expression is elevated in the progression of neurofibromas to MPNSTs but that increased IRS2 expression does not necessarily correlate with poor survival.

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