Increased Nicotinamide Phosphoribosyltransferase in Rhabdomyosarcomas and Leiomyosarcomas Compared to Skeletal and Smooth Muscle Tissue

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Abstract. *Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the rate-limiting step in NAD synthesis and is up-regulated in several human malignancies, including breast, colon, prostate, thyroid, gastric, and several hematopoietic malignancies. In some malignancies, such as gastric, thyroid, and prostate carcinomas, higher NAMPT expression correlates with deeper tumor invasion, increased metastatic potential and chemotherapy resistance. We employed tissue microarray immunohistochemistry to examine NAMPT expression in benign skeletal and smooth muscle, leiomyomas, leiomyosarcomas (graded low-, intermediate-, and high-grade), and spindle, embryonal, pleomorphic, and alveolar rhabdomyosarcomas. We found low to intermediate NAMPT expression in benign tissue, leiomyomas, leiomyosarcomas (low- and intermediate-grades), and spindle cell rhabdomyosarcomas. In contrast, high-grade leiomyosarcomas and embryonal, alveolar, and pleomorphic rhabdomyosarcomas showed high NAMPT expression. Herein we show for the first time that NAMPT is overexpressed in certain sarcoma types and the level of NAMPT expression correlates with tumor behavior.*

Rhabdomyosarcomas (RMS) are rare mesenchymal tumors that are derived from myogenic precursor cells that have histological features of skeletal muscle in different embryological stages of development (1, 2). About 250 RMS occur in the US each year, and these are most often in the pediatric population. Genitourinary system tumors are common in children under 5 years of age, head and neck tumors between the ages of 5-9 years, and RMS of the extremities and trunk are seen in children over 10 years old (1-3). Currently the World health Organization (WHO) employs molecular and histological criteria to classify RMS into three groups or types: embryonal (ERMS, with botryoid and spindle cell variants), pleomorphic (PRMS), and alveolar [ARMS (2)]. Histologically, ERMS are comprised of small, round cells and hyperchromatic nuclei with intermixed polygonal cells with eosinophilic cytoplasm which often contain cross striations, *i.e.* 'strap cells'. ARMS have uniform cells arranged in variably sized nests separated by fibrous tissue septa, which can resemble a pulmonary alveolar pattern (1-3). PRMS are the least common subtype and typically consist of large anaplastic cells with lobate hyperchromatic nuclei and frequent multipolar mitotic figures (1-3). A fourth subtype, sclerosing RMS, occurs in adults and is characterized by hyaline sclerosis and a pseudovascular growth pattern; this entity is not included in the WHO system (4). By immunohistochemistry, RMS are positive for desmin, muscle-specific actin, myogenin, and myogenic differentiation 1 (MyoD1) (1-4).

Leiomyomas (LM) are benign smooth muscle neoplasms commonly occurring in the myometrium and gastrointestinal tract, although they can occur in almost any location (5). Leiomyosarcomas (LMS) are malignant neoplasms arising from smooth muscle cells, or committed precursor mesenchymal stem cells, that can arise anywhere in the body (6). Both neoplasms are spindle cell proliferations forming smooth muscle bundles and fascicles with characteristic elongated and blunt-ended nuclei (5, 6). LMS range from low to intermediate to high-grade based on their histological criteria with varying degrees of nuclear hyperchromasia and pleomorphism, mitotic activity, tumor necrosis, metastatic

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potential, and clinical aggressiveness (5, 6). Immunohistochemically, 70% of LM and LMS are positive for smooth muscle actin, desmin, and h-caldesmon, although none of these immunostains are specific for smooth muscle differentiation (7).

RMS, LM, and LMS carry several different molecular alterations (2). Amongst RMS, the ARMS are often more aggressive. Eighty percent carry t(2;13) or t(1;13) translocations, resulting in novel malignancy-promoting paired box gene 3-forkhead transcription factor (PAX3-FKHR) and paired box gene 7-forkhead transcription factor (PAX7-FKHR) hybrid transcription factors (2). PRMS typically occur in adults, carry a poor prognosis, and demonstrate complex cytogenetic aberrations (2). In contrast, spindle cell RMS are associated with favorable clinical outcomes and genetically heterogenous features, some involving nuclear receptor coactivator 2 (*NCOA2*) gene rearrangements and *MYOD1* mutations (8-10). Amongst smooth muscle neoplasms, LM growth appears to depend on estrogen and progesterone-initiated signal transduction pathways leading to cell proliferation *via* tyrosine kinase activating growth factors (11). LMS have complex karyotypes with few consistent changes, although the phosphatase and tensin homolog (*PTEN*) and retinoblastoma protein (pRb) loci are often lost at 10q and 13q, respectively (12).

Nicotinamide phosphoribosyltransferase (*NAMPT*) catalyzes the rate-limiting step in the nicotinamide adenine dinucleotide (NAD) salvage pathway and is up-regulated in many different human malignancies, where it promotes cell growth and survival, DNA synthesis, mitochondrial biogenesis, and angiogenesis (13). In several malignancies, higher NAMPT expression correlates with a more aggressive clinical course (14-23). Based on this, we hypothesized that *NAMPT* expression would be increased in LM, LMS, and RMS compared to benign muscle tissue, and NAMPT protein expression would correlate with increased tumor clinical aggressiveness and histological grade. Herein we investigated NAMPT expression in benign muscle and LM, LMS of different grades, and RMS.

Materials and Methods

Tissue microarrays (TMAs), catalog numbers SO2082a and SO281, were purchased from US Biomax, Inc. (Rockville, MD, USA). Together the TMAs contained 23 spindle RMS, 33 embryonal RMS, 36 PRMS, and 27 ARMS, 18 skeletal and 10 smooth muscle samples, 30 LM, 26 LMS (low-, intermediate-, and high-grade), one example of proliferative myositis, and three epithelioid LMS. Each case in the TMAs came in duplicate cores with a 1.0 mm diameter.

NAMPT immunohistochemistry (IHC). The concentration of primary NAMPT antibody was optimized to normal kidney as control tissue. The staining of the TMA was performed in the Tissue Core Histology Lab Facility at the Moffitt Cancer Center. The microarray slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ, USA) as per the manufacturer's protocol with proprietary reagents. Briefly, slides were deparaffinized on the automated system with EZ Prep solution (#950-100; Ventana Medical Systems). The heat-induced antigen retrieval method was used in Cell Conditioning 1 (#950-124; Ventana Medical Systems). Mouse monoclonal antibody to human NAMPT (#ALX-804-717; Enzo life Sciences, Plymouth Meeting, PA, USA) was used at a 1:1000 concentration in Dako antibody diluent (#S0809; Dako, Carpenteria, CA, USA) and incubated for 60 min. The Ventana anti-mouse or rabbit secondary antibodies were used for 16 min. The detection system used was the Ventana OmniMap kit. Slides were then dehydrated and coverslipped as per standard laboratory protocol.

Table I. *Relative nicotinamide phosphoribosyltransferase (NAMPT) staining in the two tissue microarrays comparing benign skeletal and smooth muscle tissue to leiomyomas, different leiomyosarcoma grades, and different rhabdomyosarcoma subtypes.*

Tissue type	Sample number	Average NAMPT IHC score	SEM
Skeletal muscle	18	0.22	0.10
Smooth muscle	10	0.99	0.31
Leiomyoma	30	1.74	0.19
Leiomyosarcoma (total)	26	3.42	0.42
Low-grade	5	2.00	0.63
Intermediate-grade	14	2.50	0.72
High-grade	7	6.29	0.49
Spindle rhabdomyosarcoma	8	2.38	0.75
Alveolar rhabdomyosarcoma	28	8.00	0.69
Pleomorphic rhabdomyosarcoma	26	7.12	0.49
Embryonal rhabdomyosarcoma	30	7.10	0.47

IHC: Immunohistochemistry; SEM: standard error of the mean.

Evaluation of NAMPT staining. Relative NAMPT protein expression was determined as immunostain intensity scored on a 0-3 scale as follows: no staining as 0, light staining as 1, moderate staining as 2, and heavy staining as 3. The percentage of cells stained was measured, with no detectable staining as 0, 1-33% as 1, 34-66% as 2, and 67-100% as 3. The final IHC score was the product of the percentage of cells stained multiplied by the intensity score, allowing for a maximal score of 9 and a minimal score of 0. Nuclear and cytoplasmic NAMPT staining was seen in all tissue samples examined, although at low levels in benign skeletal and smooth muscle. We therefore measured and quantified NAMPT staining in the nuclear and cytoplasmic compartments. In all cases, the levels of staining were the same for the duplicate tissue cores.

Statistical analysis. The standard error of the mean (SEM) IHC score was calculated by using the standard deviation for the staining scores of each tumor type and dividing this number by the square root of the sample size.

Results

Following IHC processing, we were left with eight spindle RMS, 30 embryonal RMS, 26 PRMS and 27 ARMS, 18 skeletal and 10 smooth muscle samples, 30 LM, 26 LMS (five

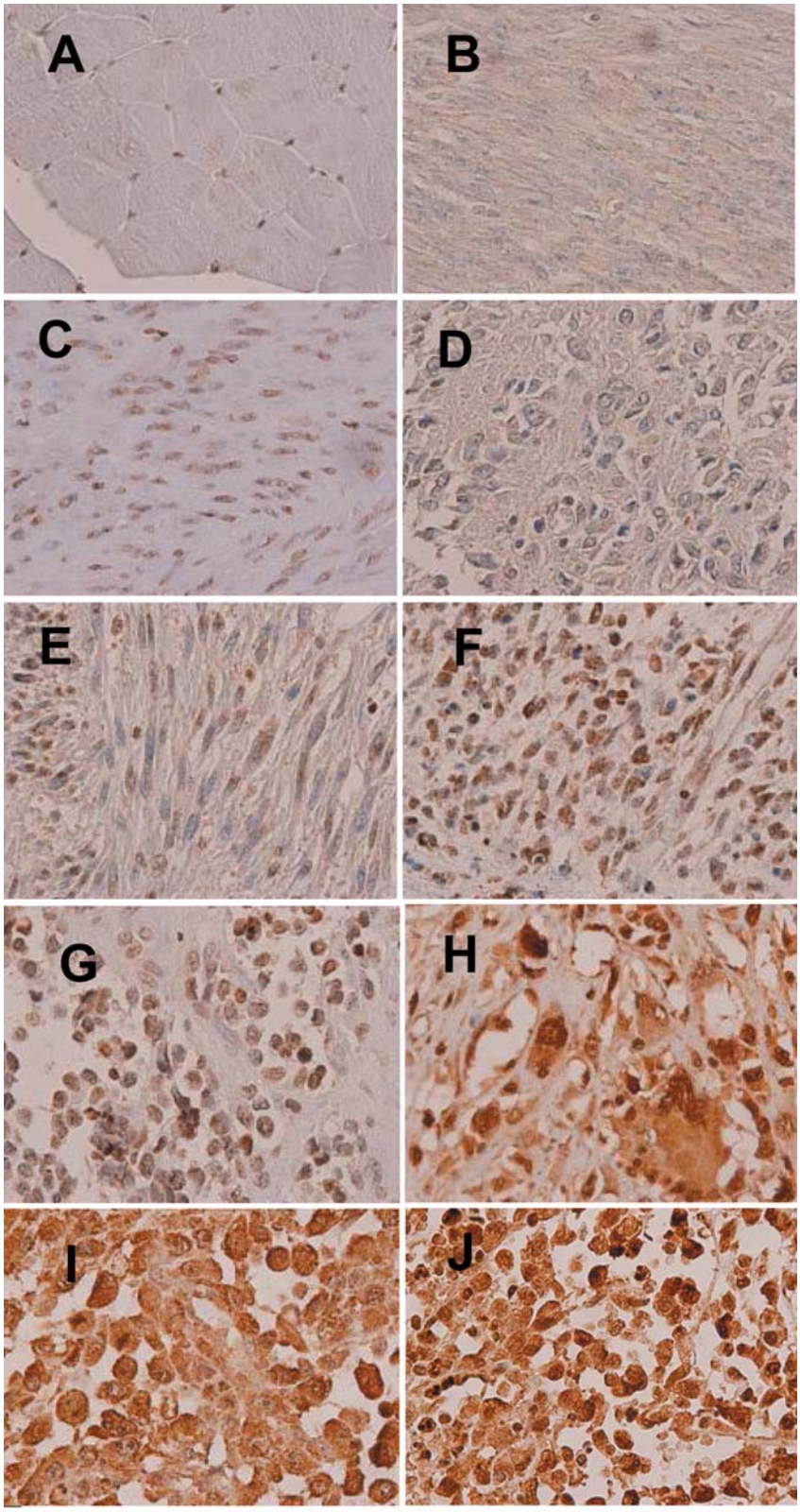

Figure 1. *Representative nicotinamide phosphoribosyltransferase (NAMPT) immunostaining of skeletal muscle (A); smooth muscle (B); leiomyoma (C); low-grade leiomyosarcoma (D); intermediate-grade leiomyosarcoma (E); high-grade leiomyosarcoma (F); epithelioid leiomyosarcoma (G); and pleomorphic (H), alveolar (I) and embryonal rhabdomyosarcomas (J). All photos are high-power views (×400).*

low-, 14 intermediate-, and seven high-grade), and three epithelioid LMS. Some cases were lost in IHC processing; hence we analyzed a fewer number of cases than were on the TMAs. The cases of proliferative myositis and epithelioid LMS were not analyzed due to the low number of samples provided. Examples of NAMPT IHC of sample tissues are shown in Figure 1. The number of cases examined and the quantified IHC results are given in Table I.

Discussion

LM, LMS, and RMS exhibit increased hypoxia-inducible factor-1-alpha and -2-alpha expression and 27% of RMS have increased signal transducer and activator of transcription 3 (STAT3) activity – signal transduction cascades known to increase NAMPT expression (13, 24-30). In gastric, lung, endometrial and breast cancer, astrocytomas, and melanoma, increased NAMPT expression correlates with cellular de-differentiation, greater depth of invasion, increased tumor growth, lymph node metastases, and a higher clinical TMN stage (13). Additionally, NAMPT expression has been correlated with increased resistance to chemotherapeutic agents (fluorouracil, doxorubicin, paclitaxel, etoposide, and phenylethyl isothiocyanate), reduced patient survival, and a worse prognosis (14-23).

Herein we showed that compared to benign smooth and skeletal muscle, there are progressively higher levels of NAMPT expression in LM, low- and intermediate-grade LMS, and high-grade LMS (Table I). LMS are graded based on tumor cellularity, degree of differentiation, presence of tissue necrosis, degree of nuclear atypia and the number of mitotic figures per 10 high-powered fields, with high-grade LMS having a worse prognosis than low- and intermediate-grade LMS (31, 32). LMS have been classified by two, three, and four-tier systems from well-differentiated to highly anaplastic, with each system having advantages and disadvantages with regard to predictive ability and reproducibility among pathologists (5, 6, 31-37). We found only a small difference in NAMPT expression between low- and intermediate-grade LMS, but noted a markedly higher expression in high-grade LMS. Although the clinical significance of this finding will require further study, the correlation of increased tumor dedifferentiation and histological grade with higher protein expression suggests that up-regulation of *NAMPT* might have prognostic significance. Generally, LMS, ERMS, PRMS and ARMS are clinically aggressive tumors, while the spindle cell variant of ERMS is associated with a better clinical outcome (8-10). In the present study, we showed that the spindle cell variant of ERMS exhibits a lower level of NAMPT expression, which correlates with less aggressive clinical behavior.

We showed for the first time that NAMPT is elevated in two sarcoma types, demonstrating that increased oncogenic NAMPT is not unique to carcinomas. Additionally, in LMS and RMS, the levels of NAMPT expression showed some correlation with known clinical outcomes for these sarcomas. Our data further suggest the NAMPT inhibitor KF866, which has had some success in treating human cancer, might have value in treating LMS and RMS, particularly those with poorer prognoses and higher NAMPT expression levels (38, 39).

Conflicts of Interest

The Authors report no conflicts of interest.

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