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Nicotinamide Phosphoribosyl Transferase Is Increased in Osteosarcomas and Chondrosarcomas Compared to Benign Bone and Cartilage

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Abstract. *Background/Aim: Primary bone neoplasms include osteosarcomas (OS), chondrosarcomas (CS), and giant cell tumors (GCT). Nicotinamide phosphoribosyl transferase (NAMPT) catalyzes the rate-limiting step of nicotinamide adenine dinucleotide synthesis and is increased in multiple tumor types. In malignancies, NAMPT expression often correlates positively with tumor grade, chemotherapy resistance, and metastatic potential. Materials and Methods: Tissue microarray was used to examine NAMPT expression in benign bone and cartilage, GCTs, OS, and different CS grades. Results: For the first time, we showed that NAMPT expression was increased in GCTs and OS compared to benign bone, and in CS compared to benign cartilage. Its expression also increased with higher CS grade. Conclusion: Our data indicate that NAMPT plays a role in bone sarcomas and GCTs, and its higher expression may contribute to increased tumor aggressiveness.*

Malignancies of the bones and joints are rare, comprising 0.2% of all human neoplasms. In the United States, approximately 3,450 were diagnosed in 2017, while in the same year these malignancies caused 1,590 deaths (1, 2). Among these malignancies, osteosarcomas (OS) and chondrosarcomas (CS) constitute 36% and 20-25% of bone and joint malignancies, respectively (1-3). While OS can occur in patients of all ages, it exhibits a bimodal distribution, with disease peaks at 15-19 years and 75-79 years (1-3). Although most cases are sporadic, factors such as radiation exposure, Paget's disease of the bone, and genetic susceptibilities increase OS risk (1-4). They commonly present in the long bones at areas of rapid bone growth, typically adjacent to the metaphyseal growth plate, commonly at the proximal humerus and tibia, and distal femur (1-4). OS is more common in males and confers different survival rates by different ages and anatomic locations (1-4). In adolescents and children, the 5-year survival rate is approximately 55-75%, with older individuals having an approximately 57% 5-year survival rate that falls with increasing age (1-4). Histologically, OS exhibit malignant pleomorphic cells with an osteogenic differentiation that produces osteoid, typically in irregular trabeculae (1-4). While malignant bone is always present, the matrix of conventional OS may have chondroid, fibroblastic, or cartilaginous features (1-4). Less common OS subtypes include telangiectatic, small-cell, periosteal, paraosteal, extraskeletal, and secondary OS (1-4).

CS are adult bone sarcomas and often present as slow-growing heterogenous malignancies in individuals between 30 and 60 years old (2-3, 5). CS are associated with a 5-year survival rate of approximately 70%, are slightly more common in males, and commonly occur in the lower limbs, pelvis, sternum, ribs, and clavicle (2-3, 5). CS is often graded on a I-III scale, with 90% graded I-II and 5-10% grade III, which carry significant metastatic potential (2-3, 5). CS is classified as primary if not associated with a pre-existing chondroid lesion and secondary if they it is, often with an enchondroma or osteochondroma (2-3, 5). Risk factors of CS include Ollier and Maffucci diseases, and radiation exposure (7-9). Histologically, CS typically exhibit a hyaline cartilaginous matrix with admixed malignant

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chondrocytes (2, 5). Grade I CS have a relatively low cellularity and cytonuclear atypia with a mainly chondroid matrix and occasional binucleated cells (2, 5). Grade II-III exhibit increased cellularity, cytonuclear atypia, matrix mucomyxoid degeneration, and increased binucleated cells, mitotic figures, and metastatic potential (2, 5). Less common CS histological types include dedifferentiated CS, mesenchymal CS, clear-cell CS, and extraskeletal myxoid CS (5). Both OS and CS typically present as slow-growing, painful masses that do not resolve over time (2, 4-6).

Giant cell tumors (GCT) are relatively common benign bone tumors and comprise 5% of primary bone tumors (1, 2). They commonly occur in the metaphyseal region of long bones, showing extensive bony destruction by a vascular tumor with numerous osteoclast-like giant cells with many nuclei, mononuclear spindle cells, and often acute hemorrhage with hemosiderin deposition and focal necrosis (1, 2).

At the molecular level, OS often carry *p53* and retinoblastoma gene mutations (2, 6, 10-13). Additionally, underexpression of dedicator of cytokinesis 5, cyclin-dependent kinase inhibitor 1, limbic system-associated membrane protein, and tumor necrosis factor receptor superfamily member 10A/D, and overexpression of runt-related transcription factor 2, ATP-dependent DNA helicase Q4, secreted phosphoprotein, and integrin binding sialoprotein bone sialoprotein is common (12-13). OS also often exhibit either increased telomerase expression or increased alternative lengthening of telomeres, contributing to their malignant growth (14). CS often carry isocitrate dehydrogenase mutations resulting in the production of the oncometabolite δ-2-hydroxyglutarate (2, 5, 13). Additionally, alterations in retinoblastoma tumor pathway signaling, *p53* mutations, and phosphatidylinositol-4,5-bisphosphate 3-kinase/mammalian target of rapamycin kinase activation are common in CS (2, 5, 15-18). Similarly, to OS, CS frequently overexpress telomerase and carry telomerase promoter mutations that promote metastasis (19). GCTs commonly exhibit mutations of H3 histone family member 3A and receptor activator of nuclear factor kappa-light ligand, accompanied by telomeric association, where chromosomal ends form dicentric or multicentric ring chromosomes (20).

Nicotinamide phosphoribosyl transferase (NAMPT) catalyzes the rate-limiting step of nicotinamide adenine dinucleotide synthesis and exhibits increased expression in several malignancies, including gastric, thyroid, urothelial, renal, cervical, oral and cervical squamous cell carcinomas, rhabdomyosarcomas, and leiomyosarcomas (21-26). NAMPT expression also increases with increasing grade of cervical dysplasia (26). NAMPT expression has not been previously examined in OS or CS to our knowledge. Here we used microarray technology to examine NAMPT protein levels in osteoblastic, chondroblastic, and telangiectatic OS, grade I-III CS, and GCTs of the bone.

Table I. *Relative nicotinamide phosphoribosyltransferase staining in the two tissue microarrays comparing benign bone and cartilage to osteosarcomas and different grades of chondrosarcomas and giant cell tumors of the bone.*

Tissue	Number of type	Average IHC samples	SEM score
Benign			
Bone	25	0.54	0.25
Cartilage	7	0	0
Osteosarcoma			
Telangiectatic	8	5.63[a]	0.61
Osteoblastic	52	6.73[a]	1.23
Chondroblastic	4	5.0[a]	0.58
Chondrosarcoma			
Well-differentiated	43	1.79[b]	0.84
Intermediately differentiated	10	3.90[bc]	0.84
Poorly differentiated	6	9.0b[c]	0
Giant cell tumor	36	7.81[a]	0.36

Significantly different from: [a]Bone, [b]cartilage, [c]well-differentiated chondrosarcoma at $p<0.05$.

Materials and Methods

Tissue microarray (TMA). Two TMAs, OS208 and T261, were purchased from US Biomax, Inc. (Rockville, MD, USA). Both TMAs were interrogated by an antibody to NAMPT. Together the two TMAs contained 25 samples of benign bone, 7 of benign cartilage, eight of telangiectatic OS, 53 of OC, four of chromoblastic OS, 36 of GCT, and 43, 10, and six samples of well-, moderately, and poorly differentiated CS, respectively. All tissue samples in the TMAs were 1.5 mm in diameter.

NAMPT immunohistochemistry (IHC). The concentration of the primary antibody to NAMPT was optimized to normal kidney as control tissue, the where concentrations of the primary and secondary antibodies were titrated until optimal immunohistochemical NAMPT protein identification was achieved. The staining of the TMAs was performed at 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, the slides were deparaffinized on the automated system with EZ Prep solution (Ventana Medical Systems). The heat-induced antigen retrieval method was used in Cell Conditioning 1 (Ventana Medical Systems). Mouse monoclonal antibody to human NAMPT (Enzo Life Sciences, Plymouth Meeting, PA, USA) was used at a 1:1,000 concentration in Dako antibody diluent (Dako, Carpentaria, CA, USA) and incubated with TMAs for 60 min. Ventana anti-mouse secondary antibodies were used for 16 min. The detection system used was the Ventana OmniMap kit. Slides were then dehydrated and cover-slipped per standard laboratory protocol.

Evaluation of NAMPT staining. Relative NAMPT protein expression was determined as immunostain intensity scored on a 0 to 3 scale as follows: no staining: 0, light staining: 1, moderate staining: 2, and heavy staining: 3. The percentage of cells stained

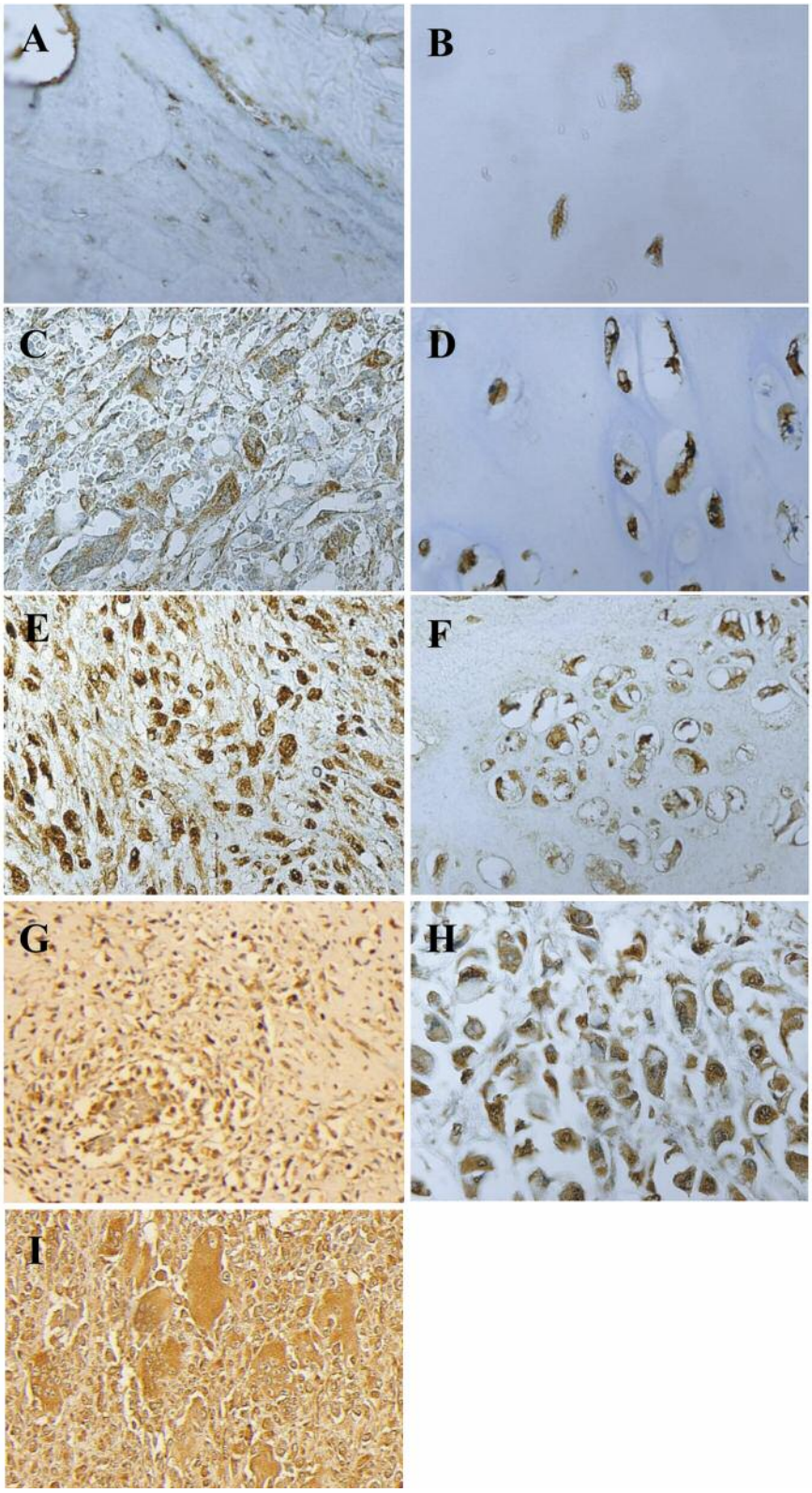

Figure 1. *Representative immunostaining of nicotinamide phosphoribosyl transferase in benign bone (A), benign cartilage (B), telangiectatic osteosarcoma (C), grade I chondrosarcoma (D), osteoblastic osteosarcoma (E), grade II chondrosarcoma (F), chondroblastic osteosarcoma (G), grade III chondrosarcoma (H) and giant cell tumor of the bone (I). Original magnification ×400.*

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 minimal score of 0 and a maximal score of 9. Nuclear and cytoplasmic NAMPT staining was seen in all tissue samples examined. Therefore, NAMPT staining in both the nuclear and cytoplasmic compartments were measured and quantified as one.

Statistical analysis of TMA results. The standard error of the mean (SEM) IHC score was calculated using the standard deviation for the staining scores of each tumor type and dividing this number by the square root of the sample size (22, 26). The significance of differences in all the experiments was calculated using prism software version 5.02 (Graph Pad Inc., San Diego, CA, USA).

Results

Following IHC processing none of the tissue samples on the TMAs were lost. The number of cases examined, the IHC scoring result, and the SEM of each data set are given in Table I. Examples of benign tissues and different OS types, CS grades, and giant cell tumor of the bone are shown in Figure 1. NAMPT was significantly increased in each OS type compared to benign bone tissue and was also significantly increased in CS compared to benign cartilage, with significant differences being found between benign bone and all three OS types and between benign cartilage and all three CS grades. Significant differences were also found between well-differentiated and intermediate and poorly differentiated CS. Lastly, differences in NAMPT expression between benign bone and GCTs of the bone were also significant (Figure 1).

Discussion

Here we have shown for the first time that NAMPT is increased in OS, CS, and GCTs. In the CS, NAMPT expression increased with CS grade and was greatest in grade III (poorly differentiated) CS. High-grade CS has an increased metastatic potential and confer a worse prognosis (27, 28). Similarly, higher NAMPT expression is seen with increasing grade of cervical dysplasia, leiomyosarcoma, oral and cervical squamous cell carcinoma, and Fuhrman grade in renal cell carcinoma (22-24, 26). Additionally, increased NAMPT expression was shown to confer a worse prognosis in endometrial adenocarcinoma and to confer resistance to chemotherapeutic agents (21, 29, 30). Lastly, higher NAMPT expression positively correlated with more aggressive rhabdomyosarcoma subtypes, compared to less aggressive subtypes (26). Our data that NAMPT expression increases with higher grade of CS add support to the hypothesis that NAMPT overexpression in a malignancy confers a more aggressive clinical course and a worse prognosis.

Interestingly, NAMPT expression was high in GCT of the bone (Table I, Figure 1). The great majority of these tumors are benign and only exert locally destructive effects (2, 4-6, 20). High NAMPT expression was seen in renal oncocytoma, which is also usually a benign lesion (23). Based on this, it appears likely that increased NAMPT expression is a reflection of an increased cellular demand for nicotinamide adenine dinucleotide and is not a specific marker for malignant transformation. Further work will be needed to clarify this hypothesis.

Here we have shown for the first time that NAMPT is increased in GCT of the bone and in two bone sarcoma types. We also show for the first time that NAMPT expression increases with higher CS grades. Taken together our data help confirm that high NAMPT expression is seen in a wide variety of malignancies, including sarcomas.

Conflicts of Interest

The Authors have no conflict of interest to report in regard to this study.

Authors' Contributions

All Authors assisted in the writing of the manuscript. Liurka Lopez was also consulted on the histology and pathology of OS and CS. James Cotelingam also took the photographs and Domenico Coppola oversaw the process of making the IHC staining. Lastly, Rodney Shackelford assembled the final manuscript and performed the statistical amylases.

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