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The Use of MicroRNA Biomarkers to Distinguish Enchondroma from Low Grade Chondrosarcoma.

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Abstract

Establishing a definitive diagnosis between benign enchondroma versus low grade chondrosarcoma presents a potential challenge to both clinicians and pathologists. microRNAs (small non-coding RNAs) have proven to be effective biomarkers for the identification of tumors and tumor progression. We present analysis, both array and quantitative PCR, that shows consistently and substantially increased expression of two microRNAs, miRs-181a and -138, in low grade chondrosarcomas compared with enchondromas. The data suggest these microRNAs would provide an analytical distinction between the chondrosarcoma and benign neoplasms that can be performed in formalin fixed paraffin embedded specimens. Together with recent publications, these data indicate that miRs-181a and -138 also play a role in tumor development

and homeostasis and may provide new targets for the development of much needed therapeutic intervention.

Keywords: microRNA chondrosarcoma enchondroma miR-181a miR-138

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Introduction

Despite being the second most common skeletal neoplasm affecting bone, cartilaginous tumors present a diagnostic and treatment dilemma (1). Chondroid lesions run a full spectrum from benign enchondromas to malignant chondrosarcomas. Enchondromas occur in the metaphyseal region of long bones and rarely progress to chondrosarcoma. Discrimination between enchondromas and low grade chondrosarcoma can be difficult and subject to inter-observer variability.

Chondrosarcomas are usually graded into three groups. Grade 1, or low grade lesion, is so similar to enchondromas that the diagnosis is largely dependent on the clinical and radiographic presentation and location. Histologic evidence of invasion of the Haversian canals or of the medullary space with embedded fragments of trabecular bone within the tumor also provides

distinction of chondrosarcoma from benign enchondromas. Although attempts have been made to develop molecular markers to distinguish enchondromas from low grade chondrosarcoma no reliable molecular markers have been developed to date(2).

Higher grade chondrosarcomas are more clearly distinguishable. Grade 2, or intermediate chondrosarcoma, has increased cellularity with increased nuclear size and frequently distinct nucleoli. Focal myxoid change (loss of hyaline cartilage matrix) is a frequent occurrence. Grade 3, or high grade chondrosarcoma, is comparatively rare. They are characterized by marked cellular atypia, hypercellularity, and high mitotic activity. They are generally rapidly growing, aggressive, and frequently metastasize, have an aneuploid pattern on flow cytometry and show complex aberrations on chromosomal analysis. About 10% of chondrosarcomas 'dedifferentiate' and become highly malignant sarcomas (see(3) for review). They have a uniformly poor prognosis and metastasize widely. Because chondrosarcomas are also notoriously resistant to conventional radiation and chemotherapeutic agents, surgical intervention is typically the only treatment strategy.

MicroRNAs are small (~21 nucleotide) non-coding RNA molecules that provide an overarching regulation of gene expression at the post-transcriptional level. They play a critical role in almost all biological processes, including cell division, differentiation, growth and apoptosis. It is not surprising then that changes in microRNA expression have been associated with many diseases, including neurological diseases, cardiovascular disease and particularly cancer(4). MicroRNA activity is highly context dependent. A microRNA can be oncogenic or oncosuppressive in different cell types dependent on the expression level of the target gene(5). MicroRNA

signatures have provided biomarkers for the diagnosis of many tumor types as well as predicting tumor progression(5). Their small size, tight interaction with binding proteins, long half-life in vivo and in fixed tissue specimens together with the development of highly sensitive and relatively simple analytic techniques has facilitated the rapid expansion of their use as biomarkers. MicroRNAs play critical roles in endochondral differentiation(6) but their value as biomarkers or their contribution to chondrosarcoma development remains to be fully established. Recent improvements in RNA isolation have facilitated the analysis of microRNAs in formalin fixed paraffin embedded (FFPE) tissue sections and provide the potential to analyze a wide range of less common tumors, including chondroid lesions, that have been archived over many years(7).

MicroRNA analysis of archived FFPE blocks of chondrosarcomas and enchondromas described here has provided potential new biomarkers for the diagnosis of chondrosarcoma; in particular the distinction between low grade chondrosarcomas and enchondromas.

Methods

Human Chondrosarcoma tissue.

Paraffin blocks of FFPE human enchondromas and chondrosarcoma were obtained from the Henry Ford Hospital tumor archive. The tumor grade was confirmed in fresh hematoxylin-eosin stained sections of all paraffin blocks by an experienced musculoskeletal pathologist. Normal articular cartilage from a large series of 7 donors, ages 26 to 82 was obtained from the National

Disease Research Interchange (Philadelphia, PA) and from patients at limb amputation (Henry Ford Hospital). All tissue procurement was conducted under supervision of the Henry Ford Health System Institutional Review Board. Freshly dissected articular cartilage or new curls of approximately 10 paraffin sections (after discarding the first approximately 10 section to avoid oxidized tissue) were used for RNA extraction.

Rat chondrosarcoma cells and tumor tissue.

Swarm rat chondrosarcoma cells (RCS) are a widely used chondrocyte cell line. They have a stable phenotype that closely resembles articular chondrocytes and have been used extensively for the analysis of chondrocyte protein and molecular biology and as a model to investigate the pathology and treatment of chondrosarcoma(8). When injected subcutaneously in rats or immune compromised mice a tumor resembling grade 1 chondrosarcoma grows within 5 to 10 weeks. RCS (10^7 cells in 0.5 ml of Dulbecco's Modified Eagle's Medium) were injected subcutaneously between the scapulae of 4 week old Sprague Dawley rats. Animals were sacrificed 10 weeks later by CO_2 inhalation. The chondroid lesion was subsequently dissected and tissue obtained for histologic analysis and RNA isolation. Freshly isolated tumor tissue and rat tibial articular and rib cartilage from 10 week old rats (used as control tissue) was dissected and pulverized in liquid nitrogen in preparation for RNA isolation. All animal experimentation was performed in accordance with the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, and was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee.

Chondrogenesis from bone marrow mesenchymal stem cells.

Human bone marrow mesenchymal stem cells (BMSC) were cultured in pellets in chondrogenic medium and cells collected prior to differentiation (0 days), at early chondrogenic (3 days), full chondrogenic (7 days) and hypertrophic stages (14 days)(9). RNA samples extracted from human BMSCs at the four stages of chondrogenic differentiation. Stages of differentiation were assessed from analysis of chondrogenic markers, type II collagen and aggrecan, and hypertrophic marker type X collagen using qPCR(9, 10).

RNA isolation

Total RNA was isolated from freshly dissected tissue using the miRNeasy minikit (Qiagen, CA). For RNA isolation from archived paraffin blocks FFPE sections were first dewaxed using xylene and RNA extracted by proteinase K digestion followed by Qiazol phenol/chloroform extraction and silica column purification (miRNeasy minikit, Qiagen, Valencia, CA) within 24 hrs. of cutting. The concentration and quality of RNA was measured using Nanodrop 2000 spectrophotomer. This protocol provided approximately 1-2 ug high quality RNA from five 10 um paraffin sections.

MicroRNA array analysis

MicroRNA expression was profiled using Low Density TaqMan microRNA arrays on a 7900HT real time PCR System as described previously(11). TaqMan Human miRNA array A and array B (Megaplex RT human pool A and pool B, part No. 4444913) were used following the

manufacture's recommended protocol. The RNA was reverse transcribed using the TaqMan microRNA Reverse Transcription Kit and the TaqMan microRNA multiplex RT assays. cDNA from multiplex RT reactions was combined with TaqMan PCR mix and quantitative PCR performed on an Applied Biosystems 7900 HT thermocycler. Raw cycle threshold (CT) values were calculated using SDS 2.3 and RQ manager 1.2 software (Applied Biosystems) applying automatic baselines and threshold settings. The CT values were imported into StatMiner 4.2 (Integromics Inc., Philadelphia, PA) for global normalization of each sample.

Quantitative PCR

RNA was reverse transcribed using the TaqMan miRNA reverse transcription kit (Invitrogen, USA, Part No.4366596) utilizing pooled individual miRNA-specific stem-loop RT primers (Applied Biosystems, Foster City, CA). qPCR was performed in triplicate using the TaqMan qPCR assay. Each PCR application reaction was performed in a final volume of 20 ul containing 1.2 ul of cDNA, 10 ul TaqMan 2x universal PCR Master mix, 1 ul miRNA-specific primer/probe mix, and 7.8 ul of pure water. RT-PCR was carried out on an Applied Biosystems7500 thermocycler, using the following condition: 95^{o}C for10 min, followed by 40 cycles of 95^{o}C for 15 s and 60^{o}C for 1 min. Raw data were analyzed with SDS relative Quantification Software version 2.2.3 (Applied Biosystems, Inc.). The data were normalized to miR-16 (shown to be expressed with the least variation among microRNAs in a series of 9 cartilage samples, data not shown) and to the average expression level in 7 normal cartilage samples from the age range matching that of the donors for chondrosarcoma samples (55 to 82 years, average age 67)(11).

Results

Array analysis of microRNAs suggests possible biomarkers that distinguish enchondromas from low grade chondrosarcoma.

The primary objective of these studies was the identification of microRNA biomarkers that would distinguish benign enchondromas from low grade chondrosarcomas. MicroRNA array analysis of normal tissue, enchondromas and chondrosarcomas classified as grade one or higher grade (2 or 3) identified a series of microRNAs with expression levels in chondrosarcomas distinct from that seen in normal tissue or enchondromas (Table 1). In most cases microRNA expression was similar in normal articular cartilage and enchondromas.

miRs 181a and 138 distinguish enchondromas from chondrosarcoma.

MicroRNAs selected from array analysis were verified in an expanded series of normal tissue, enchondromas and chondrosarcomas. MicroRNAs that showed decreased expression with chondrosarcoma development in array analysis had variable expression levels in the expanded tissue samples such that none showed significantly lower levels of expression in chondrosarcoma compared with enchondroma when analyzed by qPCR. However; the microRNAs that showed the largest increase in expression comparing normal cartilage and enchondromas with chondrosarcoma were verified using qPCR. miRs-181a and -138 had an approximately 100 fold

greater expression in low grade chondrosarcomas than normal cartilage and approximately 7 fold greater expression than in enchondromas (Figure 1). miR-221 although not showing an increase in expression in array analysis was included in the qPCR analysis based on our previous studies demonstrating that this microRNA stimulated cell division in chondrocytes (12). qPCR analysis showed that miR-221 expression was significantly greater in low grade chondrosarcomas than enchondromas. miRs-143 and -21 showed significantly greater expression in chondrosarcoma compared with normal tissue and a trend toward greater expression levels in chondrosarcoma than enchondroma but this was not significant. The analysis suggests two patterns of expression of microRNAs. The first includes miRs-181a, -143 and -21 and shows increased expression in all chondrosarcoma grades compared with normal tissue or enchondromas. The second group includes miRs-138 and -221 and shows increased expression in low grade chondrosarcoma compared with normal tissue or enchondromas but lower levels of expression in high grade chondrosarcomas compared with low grade chondrosarcomas (Figure 1).

Expression of miRs-181a and -138 is increased in rat chondrosarcoma tissue and cells compared with normal cartilage.

Analysis of microRNA expression in RCS cells and tumors generated by subcutaneous injection of these cells showed expression levels consistent with that seen in human chondrosarcomas. Both the cells and tumor tissue developed have significantly greater expression of miRs -181a and -138 than normal cartilage tissues. miRs-143 and -21 showed a trend to increased expression in the chondrosarcoma cells and tumors but this was not significant (Figure 2). Expression of

miR-221 was well above the detection limit in chondrosarcoma cells and tumor but below the level of detection in normal rat cartilages.

miR-181a shows strong expression associated with chondrogenesis.

The chondrogenic differentiation of mesenchymal stem cells in pellet culture provides a well-defined, convenient model system to identify genes or ncRNAs associated with cartilage development(10). miR-140 has been considered cartilage-specific(13). It is expressed in other cell types but has been shown to be a critical regulator of cartilage development(14). As seen in these experiments it is highly expressed in chondrocytes, is up-regulated during early chondrogenic differentiation and continues to have high expression during further differentiation in this model (Figure 3). miR-181a, although expressed at a lower level, shows a very similar expression profile to miR-140. miR-221 shows an inverse relationship with chondrogenesis. Expression levels of the other microRNAs studied showed no relation to chondrogenesis (Figure 3).

Discussion

Our microRNA analysis of archived paraffin blocks shows the expression levels of miR-181a and miR-138 is able to distinguish low grade chondrosarcoma from enchondroma. Both microRNAs are strongly expressed in low grade chondrosarcomas at levels approximately 100 fold greater than in normal articular cartilage and 7 fold greater than expressed in enchondromas. A number of studies have previously examined microRNA expression in chondrosarcomas and

chondrosarcoma cell lines(15-17), however; there has been no description of microRNAs ability to distinguish enchondromas from chondrosarcomas. Elevated expression of miR-181a in chondrosarcomas is consistent with previously published data comparing microRNA expression in chondrosarcomas with that seen in articular cartilage(16) or in chondrocytes derived from normal articular cartilage (15, 16). The studies by Sun et al showed a significantly higher expression in low grade chondrosarcomas than normal cartilage and substantially higher expression in high grade chondrosarcoma. Our observations are similar with the exception that the difference in miR-181a expression between low and high grade chondrosarcomas is not as great as that observed by Sun et al. The microRNA array analysis published by Yoshitaka et al suggested, in contrast to the studies presented here, that both miR-138 and -221 were downregulated in chondrosarcoma compared with chondrocytes isolated from normal cartilage. This can be explained by the choice of chondrocytes subjected to multiple rounds of subculture rather than cartilage for comparison. We have shown that expression of miR-221 is stimulated several fold by the isolation of chondrocytes and enhanced further by their subsequent subculture(12). Similarly it has been found that miR-138(18) (GG unpublished data) is highly upregulated under culture conditions similar to those used by Yoskitaka et al. In addition Yoshitaka et al presented averaged expression of microRNA in chondrosarcomas of all grades. Our data suggests that the expression level of miRs-138 and -221 is lower in higher grade chondrosarcomas than low grade chondrosarcoma (Figure 1) which would also aggravate their failure to observe the stimulation of these microRNA in pooled chondrosarcomas compared with cultured chondrocytes.

The capacity of expression levels of miRs-181a and -138 to distinguish chondrosarcomas is further supported by the observation that that these microRNAs also showed significantly increased expression in the rat chondrosarcoma tumor and cell line compared with normal cartilage. The similarities of this model to human chondrosarcomas also support the potential of this model for defining the function of these microRNAs in chondrocytes and chondrosarcomas and their value as targets for chondrosarcoma therapy.

The association of miR-181a expression with the chondrogenic differentiation of mesenchymal stem cells suggests it plays an important role in the normal processes of chondrocyte and cartilage development. Recent studies also suggest that in addition to their value as biomarkers miRs -181a and -138 play roles in the development of chondrosarcomas. Sun et al (16, 19) have shown that miR-181a is up regulated by hypoxic environments like that associated with tumor growth and stimulates VEGF expression and angiogenesis. They have also shown that inhibition of miR-181a decreased VEGF expression, MMP1 activity, angiogenesis and substantially reduced tumor growth and metastasis in a mouse xenograft model. Additional studies of leukemia development also suggest miR-181a may be an effective therapeutic target(20). The studies have shown that although the members of the miR-181 family have identical seed sequences they are able to affect specific pathways due to regulatory functions of other regions of these small RNAs, specifically the pre-miRNA loop nucleotides. mir-181a-1, but not mir-181a-2b-2 and mir-181-c/d controls the development of leukemia cells. Consequently miR-181a can be specifically targeted to inhibit leukemia cell development without significantly affecting normal development. A similar association might be present in chondrosarcomas and normal cartilage. We have detected a similar profile of expression of miR-181c expression in normal

cartilage, enchondroma and low and high grades of chondrosarcoma to that of miR-181a, however; the expression levels was approximately 20 fold lower than that of miR-181a and frequently below the limits of detection in normal cartilage and enchondroma (data not shown).

Recent data also suggests miR-138 might play a role in chondrosarcoma development. Chondrosarcomas have been shown to harbor a variety of mutations in genes necessary for normal cartilage development. One very commonly mutated (approximately 50% of chondrosarcomas analyzed) cartilage-specific gene is Col2a1. In addition mutations in several genes essential for normal type II collagen fibrillogenesis have been identified consistent with abnormal type II collagen staining observed in 75% of a panel of 43 human chondrosarcomas(21). Mutations in ADAMTS 3, the type II procollagen N propeptidase, has been observed in both the human tumor and rat chondrosarcoma cell line and has been shown to generate abnormal fibrillogenesis in rat chondrosarcoma cells(22). It has been suggested that the aberrant fibril matrix affects the normal differentiation program and allows unchecked tumor growth(21). It has recently been shown that miR-138 suppresses Col2a1 expression by direct inhibition of two transcription factors essential for Col2a1 expression(18). These observations suggest the vastly increased expression of miR-138 might also play a role in chondrosarcoma tumor development by suppressing type II collagen synthesis.

In summary these data suggest that analysis of miRs-181a and -138 in either FFPE sections or biopsy samples may have the ability to provide a clear distinction between benign enchondroma and low grade chondrosarcomas which would be of significant clinical value. Additionally

accumulating data also suggest these microRNAs play a role in tumor development and would provide new targets for much needed therapeutic intervention.

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Declaration of Interest

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Figure 1. Q PCR analysis of selected microRNAs in normal cartilage, enchondroma, low grade chondrosarcoma and higher grade chondrosarcoma. Ct values were normalized to miR-16 and the average value in 7 normal cartilage samples from donors age 55 to 82. Shown is the average and standard error of fold change. Normal, indicates normal cartilage samples, chsa low, indicates grade 1 chondrosarcoma, chsa high, indicates chondrosarcoma of grade 2 or3, all chsa, indicates pooled chondrosarcomas. N=the number of patient samples analyzed. * and ** indicate significant difference with enchondroma (P< 0.05 or 0.005 respectively). + and ++ indicate significant difference with normal cartilage samples (P< 0.05 or 0.005 respectively).

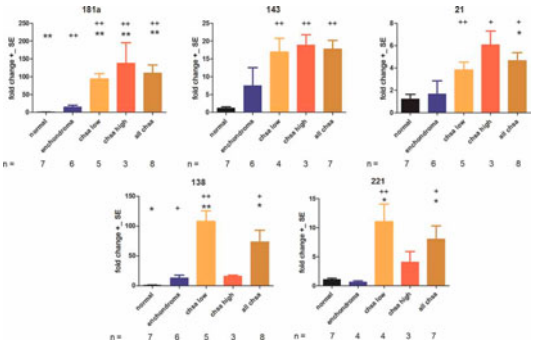

Figure 2. Expression of microRNAs in rat chondrosarcoma. MicroRNA expression was analyzed in rat chondrosarcoma cells, tissue developed from subcutaneous injection of RCS cells (duplicate samples) and rat articular and rib cartilages (duplicate samples of each). microRNA expression is expressed as the average fold change and standard error after normalizing CT values to miR-16 and the average expression in normal cartilage with standard error. Chsa indicates chondrosarcoma. + and ++ indicate significant difference to normal cartilage ($P<0.05$ and <0.005 respectively).

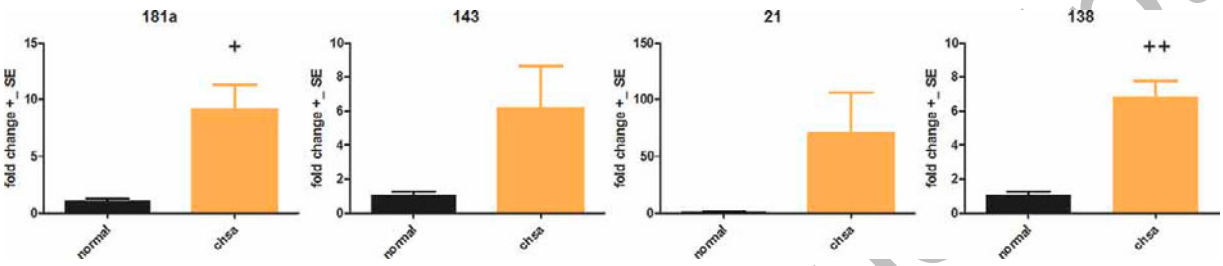

Figure 3. MicroRNA expression during mesenchymal stem cell chondrogenesis. RNA was extracted from duplicate samples of mesenchymal stem cells in pellet culture under chondrogenic conditions. MicroRNAs were profiled using Low Density TaqMan microRNA arrays. Average fold change in expression of duplicate samples is shown after normalizing to the expression level at day 0. Early chondrogenesis, full chondrogenesis and hypertrophic differentiation occur at days 3, 7 and 14 respectively.

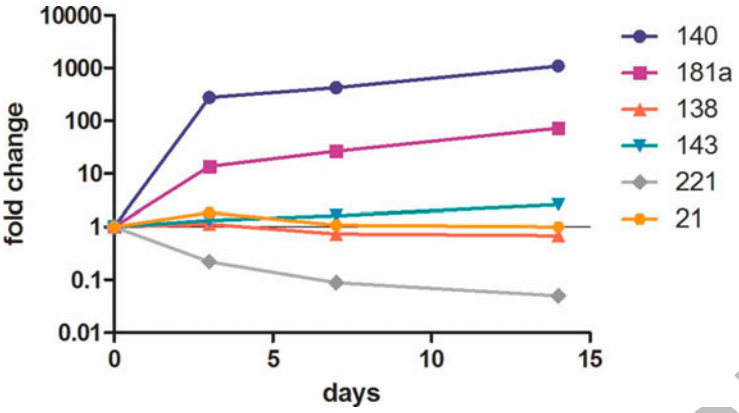

Table 1. MicroRNA array comparison of normal cartilage, enchondroma and chondrosarcoma.

											Norm&Enc h - Chsa G1[1]
	Normal		**Enchondroma**		**Chse grade 1**		**Chse grade 2/3**		**Normal and Enchond**		
microRNA	avg	SD	avg	SD	avg	SD	avg	SD	avg	SD	ΔCt
miR-138	38.54	1.65	29.74	0.54	27.65	1.74	31.00	0.02	35.61	4.60	7.96
miR-143	36.10	2.12	34.43	2.01	31.55	0.48	31.09	0.12	35.54	2.13	3.99
miR-181a	36.63	0.30	34.86	0.54	32.38	1.50	31.61	0.59	36.04	0.95	3.66

miR-145	34.14	2.18	32.06	1.93	30.29	1.45	29.48	0.73	33.44	2.23	3.15
miR-21	30.79	1.19	30.57	0.98	28.44	1.18	27.76	0.11	30.72	1.07	2.28
miR-218	35.72	1.39	31.20	1.44	32.77	1.32	32.03	1.31	34.22	2.61	1.45
miR-34a	33.98	0.84	29.87	0.71	31.36	0.96	29.96	0.04	32.61	2.19	1.25
miR-376a	32.83	0.98	33.39	0.81	31.83	1.71	33.83	0.03	33.02	0.92	1.19
miR-210	29.25	0.71	30.82	2.37	28.59	1.37	28.17	0.57	29.77	1.53	1.18
miR-320	25.62	0.87	24.82	0.57	26.49	0.90	26.53	0.34	25.36	0.84	-1.13
miR-193b	27.1	0.60	27.03	0.23	28.4	1.1	27.0	0.9	27.1	0.4	-1.28

	7				1	2	4	6	3	9	
miR-539	32.51	0.75	33.50	1.56	34.22	2.20	35.78	1.50	32.84	1.10	-1.39
miR-582-5p	37.58	1.75	38.99	0.20	39.75	0.50	39.11	0.12	38.05	1.56	-1.70
miR-628-5p	30.78	0.89	29.98	1.03	32.29	1.74	31.76	1.37	30.51	0.96	-1.78
miR-196b	29.85	0.68	29.33	0.49	31.74	1.05	28.71	0.06	29.68	0.65	-2.07
let-7c	30.81	0.84	33.23	1.38	33.86	1.99	32.76	0.03	31.62	1.55	-2.24
miR-29c	29.43	1.54	30.92	0.78	32.41	1.16	31.75	1.40	29.93	1.48	-2.48
N^2	6		3		3		2		9		

[1] The average and standard deviation of normalized Ct values for the microRNAs showing more than one Ct difference between pooled normal tissue and enchondroma compared with low grade chondrosarcoma (Norm & Ench-Chsa G1). [2]number of samples analyzed.