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MicroRNA expression profiles in metastatic and non-metastatic giant cell tumor of bone

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Summary. Giant cell tumor of bone (GCTB) is a skeletal neoplasm, a locally aggressive tumor that occasionally metastasizes to the lungs. To identify novel biomarkers associated with GCTB progression and metastasis, we performed a miRNA microarray on ten primary tumors of GCTB, of which five developed lung metastases and the rest remained metastasis-free. Between metastatic and non-metastatic GCTB, 12 miRNAs were differentially expressed (such as *miR-136, miR-513a-5p, miR-494, miR-224,* and *miR-542-5p*). A decreased level of miR-136 in metastatic versus non-metastatic GCTB was significantly confirmed by the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (p=0.04). To identify potential target genes for the differentially expressed miRNAs, we used three target prediction databases. Then, to functionally validate the potential target genes of the differentially expressed miRNAs, we re-analyzed our previous gene expression data from the same ten patients. Eight genes such as *NFIB*, *TNC*, and *FLRT2* were inversely expressed relative to their predicted miRNA regulators. *NFIB* expression correlated in metastatic GCTB with no or low expression of *miR-136*, and this gene was selected for further verification with qRT-PCR and immunohistochemistry. Verification of NFIB mRNA and protein by qRT-PCR showed elevated expression levels in metastatic GCTBs. Further, the protein expression level of NFIB was tested in an independent validation cohort of 74 primary archival GCTB specimens. In the primary tumors that developed metastases compared to the disease-free group, NFIB protein was moderately to strongly expressed at a higher frequency. Thus, in GCTB, *miR-136* and NFIB may serve as prognostic makers.

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Introduction

Giant cell tumor of bone (GCTB), a primary skeletal neoplasm, shows a strong trend toward local aggressiveness. However, metastasis of the giant cell tumor of bone to the lung is rare, occurring in only 2% of cases. GCTB mostly occurs in young adults (aged 20 to 45 years) with a slight predominance in females (Reid et al., 2002). Histologically, it consists of three major cell types, its tumor pattern including a high number of osteoclast-like giant cells, monocytic round cells, and spindle-shaped mononuclear stromal cells (Wulling et al., 2003).

Probably due to GCTB's rarity, studies concerning it are limited. Yet previous studies suggest as prognostic factors metalloproteinases, the urokinase-type plasminogen activator (uPA) system (Gamberi et al., 2004), c-MYC (Gamberi et al., 1998; Smith et al., 2006), and *TPX2* (Smith et al., 2006). As for our earlier studies on GCTB, they have revealed a set of genes, such as *TNC* and *FLRT2*, (Pazzaglia et al., 2010), and novel proteins associated with an increased risk of aggression (Conti et al., 2011).

MicroRNAs (miRNAs) are small non-coding RNA, containing 19- to 25-nucleotides, which negatively regulate gene expression at transcriptional or post-transcriptional levels (Bartel, 2004; Lim et al., 2005). MiRNA profiles are distinguishable in tumors according to their histopathological, prognostic, and predictive characteristics (Iorio et al., 2005; Mattie et al., 2006; Lebanony et al., 2009). Moreover, miRNA profiling is a more accurate method than mRNA profiling in classifying tumor subtypes (Esquela-Kerscher and Slack, 2006).

The correlation of miRNA expression with cancer progression is clearly demonstrated in several malignancies (Ma et al., 2007; Meng et al., 2007), but in GCTB this issue remains thus far unexplored. Here, we aimed to discover miRNAs related to cancer progression in GCTB by investigating the expression level of miRNAs in ten primary GCTB patients, among whom five developed lung metastases. To determine any correlation between miRNAs and their target gene expression, we reanalyzed mRNA expression data from those same GCTB patients (Pazzaglia et al., 2010).

Materials and methods

Patients and materials

A total of 84 primary tumors of GCTB with complete clinical charts, including 47 females and 37 males, median age 26, from the Rizzoli Orthopaedic Institute, were, prior to any treatment, enrolled in this study. Of 84 patients, 50 were disease-free, and 34 developed lung metastases. Only entities with classic GCT of bone were included. All histological slides were reviewed and diagnosis was confirmed by a pathologist (PP) with expertise in bone tumors. The histological and clinical documentation, based on Enneking's surgical staging system (Wolf and Enneking, 1996), was available for all patients. The minimal follow-up for disease-free patients was set at 60 months. The tissue of all specimens used for the study was vital or solid, without any aneurysmal bone cyst-like areas. After hematoxylin-eosin staining of tissue sections, the percentage of tumor cells was determined for each sample and was equal to or more than 90%.

Among those samples, fresh frozen tissue of 10 samples was available for miRNA profiling, and their mRNA data were available from our previous study by Pazzaglia et al. (2010) for integration analysis. Formalin-fixed, paraffin-embedded (FFPE) sections of these ten samples were used for immunohistochemistry (IHC). Of the ten, five patients developed lung metastases, and the other five developed none. For clinical data see Table 1. IHC of NIFB was also performed and validated for the remaining 74 samples.

The study was approved by the ethics committee of the Rizzoli Institute, Bologna, Italy.

RNA extraction

Total RNA was extracted from 10 frozen tissues (≈150 mg) with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and stored at -80°C in RNA Secure reagent (Ambion, Inc, Austin, TX, USA). Concentration of total RNA was measured with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The Agilent Bioanalyzer served to check the quality of RNA by the RNA 6000 chip, as well as of miRNA by the small RNA chip, according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA, USA)

MiRNA microarray

Labeling and hybridization of RNA samples were performed according to the Agilent protocol version 2.0 (Agilent Technologies) as described previously (Mosakhani et al., 2010). In brief, 100 ng of total RNA was dephosphorylated for 2 hours at 16 °C and labeled with cyanine 3-pCp. Then the samples were vacuum-dried and re-suspended in nuclease-free water. Next, the samples were hybridized on Agilent's miRNA Microarray system (V3) (containing 866 human and 89 human viral miRNAs catalogued in the Sanger miRNA database v12). The microarrays were then washed and scanned with the high resolution Agilent Scanner. The raw data was processed with Agilent's Feature Extraction Software. Further, data were analyzed with

Table 1. Key characteristic of the patients with giant cell tumor of bone.

Sample	Age	Gender	Site	Grade	M/NM	Follow-up*	Outcome	Size
M1	25	F	radius	III (agressive)	M	36	NED2	<5 cm
M2	23	F	radius	II (active)	M	48	NED2	<5 cm
M3	25	M	vertebra	III (agressive)	M	12	NED2	<5 cm
M4	31	F	femur	III (agressive)	M	21	NED2	>5 cm
M5	22	F	tibia	III (agressive)	M	12	NED2	>5 cm
Non-M1	25	F	humerus	I (latent)	NM	60	NED	>5 cm
Non-M2	44	M	tibia	III (agressive)	NM	70	NED	>5 cm
Non-M3	22	F	fibula	II (latent)	NM	156	NED	<5 cm
Non-M4	28	F	radium	II (active)	NM	62	NED	<5 cm
Non-M5	23	M	radium	I (latent)	NM	60	NED	<5 cm

NED: not evidence of disease; NED2: no evidence of disease after treatment of metastatic lesions; M: metastatic; Non-M: non metastatic; *: The time of follow-up was calculated based on the month.

GeneSpring Software Version 11.0.2.

Statistical analysis and pathway information

The statistical analysis of microarray data was performed with the GeneSpring GX Analysis Software Version 11.0.2 (Agilent Technologies). The data were log2-transformed before the analysis and normalized by the 75th percentile method. We removed from the comparisons those miRNAs not detectable in any of the samples; as those undetected were regarded as the miRNAs with a ratio of total gene signal / total gene error <3. Detection calls were defined by Agilent's Feature Extraction Software. A t-test was performed between two groups of samples to find the significance for differentially expressed miRNAs (p-value <0.05 and adjusted p-value [q-value] <0.05 [Benjamini correction for multiple testing]). Any miRNAs detected in only one of the groups or that had an at least two-fold expression change between the groups were considered differentially expressed. In qRT-PCR, miRNA and RNA data are shown as the median of $2^{-\Delta\Delta CT}$ values and at the 25th-75th percentile for their strong non-Gaussian distribution. The statistical significance of any differences in miRNA expression between two groups was calculated with the non-parametric Mann-Whitney and differences in mRNA expression level with a U-test; a p-value less than 0.05 was considered statistically significant.

Verification of microarray results by quantitative reverse transcriptase polymerase chain reaction (qRT- PCR)

Microarray expression profiles of the selected *miRNAs, i.e. miR-1, miR-494, miR-136,* and *miR-513a-5p*, were verified by qRT-PCR on the same 10 fresh frozen GCTBs. These miRNAs were selected based on the presence of their potential target genes in the list of differentially expressed genes between metastatic and non-metastatic tumors or on their q-value <0.05. Reverse transcription and RT-PCR were carried out with TaqMan MicroRNA Assays (PE Applied Biosystems, Foster City, CA, USA), according to manufacturer's guidelines. The snRNA RNU44 primer assay (Applied Biosystems) served as a control for normalization. The relative quantification for each miRNA was calculated by the equation $2^{-\Delta\Delta Ct}$. A pool of healthy lymphocytes served as a relative calibrator.

mRNA gene expression profiling

The mRNA expression data in this study were analyzed by Pazzaglia et al. (2010). The patient material was the same as in the present study.

We reanalyzed the available data using GeneSpring (v.11.0.2) software. Fold changes and a t-test were calculated for each of the >50000 transcripts in the GCTB samples.

MiRNA target prediction and relation to gene expression profile

Target prediction was performed mostly with TargetScan (http://www.targetscan.org/) (Lewis et al., 2005), as it is the principally used target prediction database in the GeneSpring software. Other web-based computational approaches, including Sanger miRBase (www.microrna.sanger.ac.uk/targets/v5) (Griffiths-Jones et al., 2008) and miRanda (www.microRNA.org) (Betel et al., 2008) also helped us to find the target. The inverse relation between miRNA and mRNA we analyzed with GeneSpring (v.11.02), and we tested the significantly differentially expressed genes obtained in group comparisons whose expression is up-regulated in response to a down-regulated miRNA, or vice versa. The list of predicted target genes of differentially expressed miRNAs was translated to the gene expression experiment, creating a new list from which the gene expression levels of the deregulated miRNA target genes could be determined.

Verification of NFIB expression by qRT- PCR

Using qRT-PCR, we verified expression of *NFIB*, a gene significantly over-expressed in metastatic tumor samples within the microarray analysis. This gene was selected as being one of the putative target genes of the significantly validated miRNA, *miR-136*. Reverse transcription of RNA was performed with the High Capacity cDNA Archive kit (Applied Biosystems) and quantification by the TaqMan Expression Assays (Applied Biosystems) according to manufacturer's instructions. Cycling conditions for RT-PCR consisted of an initial incubation at 50°C for 2 min, followed by 45 cycles of 95°C for 10 min, 95°C for 5 sec, and 60°C for 1 min. The *ACTB* gene (Applied Biosystems) served as a housekeeping gene for normalization. The relative quantification (RQ) was calculated by the $2^{-\Delta\Delta CT}$ comparative method. A pool of healthy lymphocytes served as a relative calibrator.

Immunohistochemistry (IHC)

We further assessed expression of NFIB at protein level by IHC on paraffin-embedded sections of all 10 GCTBs. Then we validated the clinical impact of NFIB protein expression on a larger series, including 74 paraffin-embedded samples of primary GCTB lesions from 45 disease-free and 29 metastatic patients. We used mouse monoclonal anti-NFIB antibody (Abnova, Littleton, CO, USA, dil 1:1000) and Streptavidin-biotin peroxidase DAB (Dako, Glostrup, Denmark) as detection systems. Tissue sections of liver served as positive controls, and negative controls were performed by omitting the primary antibody.

According to the percentage of NFIB positivity in cells, samples were classified as negative with less than

10% positive cells, as weakly positive with ≤25% positive cells, as moderately positive with positive cells between 25% and 49%, and as strongly positive with more than 50% positive cells. Protein was considered over-expressed when a moderate to strong immune-reactivity occurred. Results were reported by two independent pathologists with expertise in bone tumors. Moreover, to see the correlation of NFIB expression at mRNA level with its corresponding level of protein, the samples were divided into three groups based on their NFIB positivity (first group: ≤25%, second: 25% to 49%, and third: >50%), and then an arbitrary number from 1 to 3 was assigned to each sample and the Pearson coefficient (r) was calculated.

Pathway information

Only the target genes, which were differentially expressed between metastatic and non-metastatic tumors and inversely correlated with miRNAs detected in our study, were screened to find their significant interaction with and involvement in biological networks. We found the significant pathways by the hyper-geometric test in the ConsensusPathDB (CPDB), using the Chipster Software Version 1.4.7 (http://chipster.csc.fi/). The CPBD database integrates pathway and other functional interaction resources and provides a list of significant pathways (p<0.05) as an output.

Results

MiRNA expression profile of metastatic and non-metastatic GCTBs

Comparison of miRNA profiles between metastatic and non-metastatic tumors revealed 12 differentially expressed miRNAs. Of these 12, 6 miRNAs were exclusively detectable in metastatic tumors but in none of the non-metastatic tumors; *miR-513a-5p and miR-let-7a** were in 60%, while *miR-224, miR-10b*, miR-934, and miR-876-5p* were in 40% of the metastatic tumors. In contrast, 5 other miRNAs of the 12 were expressed exclusively in non-metastatic tumors, and in none of the metastatic tumors; *miR-136 in 60%, and miR-542-5p, miR-505*, miR-542-3p, and miR-1* in 40% of the non-metastatic tumors. The one remaining miRNA, *miR-494,* showed expression in both non-metastatic and metastatic tumors but, significantly, it showed a 3.2-fold increase in metastatic samples (q=0.007).

Validation of miRNA expression by qRT-PCR

The validation of the four selected miRNAs (*miR-1, miR-494, miR-136.* and *miR-513a-5p*) by qRT-PCR showed a similar expression trend with the microarray analysis. However, miRNAs undetectable by the microarray analysis in the metastatic or non-metastatic group showed only a slight expression with qRT-PCR, which may have been due to technical differences in the sensitivity and specificity of the different assays. Using a pool of healthy lymphocytes as a relative calibrator, we observed a lower expression of *miR-1* and *miR-136* and higher expression of *miR-494* and *miR-513a-5p* in metastatic tumors than in non-metastatic tumors (Table 2). However, only for *miR-136* the difference in expression between non-metastatic and metastatic tumors was significant (p=0.04) (Fig. 1A).

Relationship between miRNA and mRNA expression

MicroRNAs regulate gene expression negatively at the level of mRNA transcription or at protein level

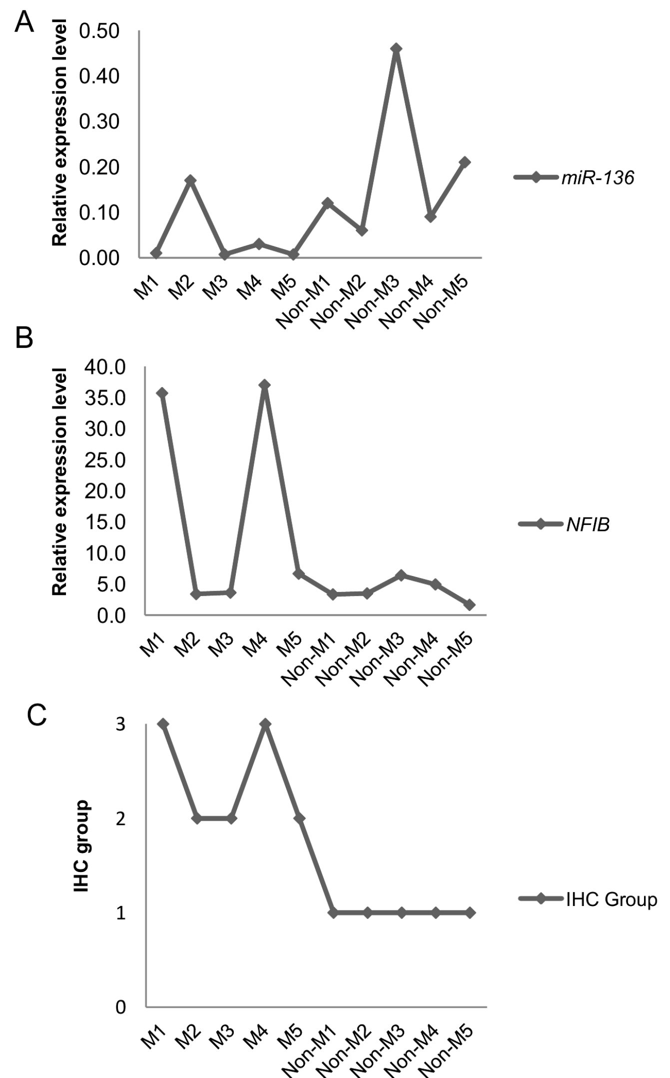

Fig. 1. Expression levels of miR-136 **(A)**, NFIB mRNA **(B)**, and NFIB protein **(C)** in 10 GCTBs. For NFIB protein expression, the cases were classified into three groups by immunostaining (IHC), group 1 (<25% positive cells), group 2 (25%-49% positive cells) and group 3 (>50% positive cells). M: Metastatic, Non-M: Non-metastatic.

(Whichard et al., 2011). We therefore evaluated the putative target genes of miRNAs by using gene expression profiling on the same 10 GCTB patients (Pazzaglia et al., 2010) to see the inverse relation between differentially expressed miRNAs and their target genes. The genes targeted by exclusively or over-expressed miRNAs in metastatic tumors were expected to show under-expression compared to that of non-metastatic tumors, and vice versa.

The integrated analysis of miRNA and mRNA data indicated an inverse correlation between miRNA and mRNA expressions. We found three under-expressed target genes, *PDPN* (p=0.02), *BAALC* (p=0.04), and *NR2F1* (p=0.04), correlating with the exclusively expressed miRNAs in metastatic tumors, and five over-expressed target genes, *TNC* (p=0.03), *NET1* (p=0.009), *SETBP1* (p=0.01), *NFIB* (p=0.02), and *FLRT2* (p=0.001), correlating with miRNAs absent from metastatic samples (Table 3).

Table 2. MiR-1, miR-494, miR-513a-5p, and miR-136 median expression level.

miRNA	M/Non-M	Median $2^{-\Delta\Delta CT}$	25	75	p-value
miR-1	M	7.38	6.84	13.17	0.6
	Non-M	8.05	0.88	12.59	
miR-494	M	42.96	29.75	392.07	0.11
	Non-M	23.67	0.65	29.24	
miR-513a-5p	M	3.04	2.54	3.19	0.07
	Non-M	0.73	0.29	0.78	
miR-136	M	0.03	0.02	0.09	0.04
	Non-M	0.35	0.29	0.64	

M: metastatic; Non-M: non-metastatic.

mRNA level of NFIB expression

Consistent with the microarray result, the qRT-PCR analysis revealed a higher level of the *NFIB,* a *miR-136* target gene, in metastatic tumors (median (RQ)=6.65, 25th=3.59, 75th=35.75) compared to non-metastatic ones

Table 3. Target genes of differentially expressed miRNAs in metastatic compared with non-metastatic.

Over-expressed gene	Detected miRNA	Undetected miRNA	Under-expressed gene
NET1	hsa-miR-1	NR2F1	hsa-miR-513a-5p
NFIB	hsa-miR-136	BAALC	hsa-miR-513a-5p
SETBP1	hsa-miR-1	PDPN	hsa-miR-934
TNC	hsa-miR-1, miR-542-5p		
FLRT2	hsa-miR-136		

Table 4. qRT-PCR and immunohistochemistry data of NFIB in patients with giant cell tumor of bone.

Sample	RQ±SD	IHC
M1	35.75±0	>50%
M2	3.37±1.07	25-49%
M3	3.59±0.66	25-49%
M4	37.01±1.32	>50%
M5	6.65±1.18	25-49%
Non-M1	3.31±0.77	<25%
Non-M2	3.47±0.75	<25%
Non-M3	6.38±0.05	<25%
Non-M4	4.92±0	<25%
Non-M5	1.63±0.16	<25%

RQ: Relative quantity; M: metastatic; Non-M: non-metastatic.

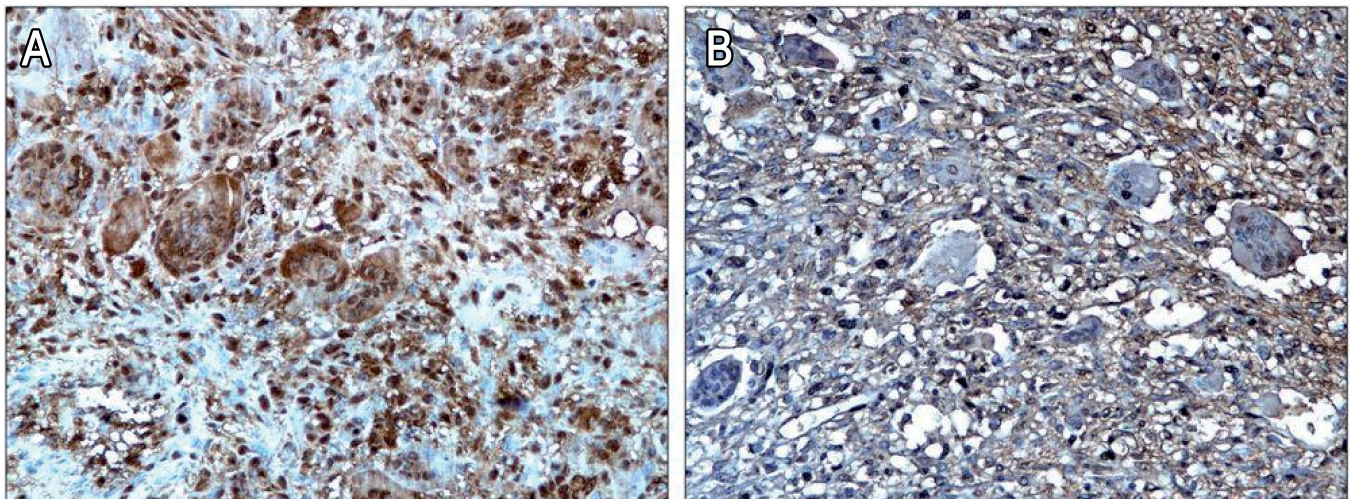

Fig. 2. NFIB protein expression in GCTB patients. **A.** Higher expression of NFIB in mononuclear cells of metastatic GCTB. **B.** Negative or weakly positive immune-reactivity in non-metastatic samples. IHC, x 40

(median (RQ)=3.47, 25th=3.31, 75th=4.92) (p=0.09) (Fig. 1B, Table 4).

NFIB expression at protein level

IHC for NFIB was carried out on the 10 GCTB specimens. IHC showed differing immunoreactivity between metastatic and non-metastatic GCTBs. Primary tumors of metastatic GCTBs showed moderate to strong immunostaining (more than 25% of positive cells), whereas non-metastatic GCTBs showed focal and weak nuclear positivity (Fig. 1C, Table 4). Subsequent validation of the clinical impact of NFIB protein on a larger series of primary GCTBs showed a moderate to strong nuclear protein expression in 20 of 45 disease-free patients (44%) and in 24 of 29 (84%) metastatic GCTBs. Specifically, the majority of metastatic tumors showed strong and diffused immunostaining in both multinuclear and mononuclear cells (≥50% positive cells), whereas in non-metastatic GCTBs, the protein was expressed predominantly in the mononuclear component of the nuclear level (Fig. 2A,B).

The Chi square with a Fisher's exact test revealed a statistically significant correlation between expression level of NFIB and metastasis occurrence (χ^2=15.0, p=0.03). None of the other variables, such as age, sex, size of tumor, or outcome was significantly associated with expression of NFIB at protein, mRNA, or *miR-136* level. Pearson coefficient calculation revealed a significant correlation between mRNA and protein level of NFIB expression within individual cases (r=0.83, p=0.002).

Biological networks

We examined the inversely correlating target genes with the identified miRNAs between metastatic and non-metastatic tumors to find significantly operating networks or pathways. Based on the analysis of these target genes, the FOXA1 transcription factor network appeared with the hyper-geometric test in the ConsensusPathDB (p<0.001). Of note, the two components of this pathway, *NFIB* and *NR2F1*, differentially expressed between metastatic and non-metastatic tumors, are target genes of *miR-136* and *miR-513a-5p*, respectively.

Discussion

We studied miRNA expression in ten primary GCTBs, in which five developed lung metastasis and five remained disease-free at the minimum follow-up of 60 months, to see the differences in miRNA expression patterns between non-metastatic and metastatic GCTBs. Using the microarray analysis we could find, between these two groups, 12 differentially expressed miRNAs.

Four selected miRNAs, including *miR-1, miR-136, miR-494,* and *miR-513a-5p*, were further verified with qRT-PCR. This provided us with results similar to the microarray analysis, except *miR-136* (p=0.04), however, other miRNAs, showed only a trend (not significant) in expression between metastatic and non-metastatic tumors, which was probably due to the limited sample size.

In metastatic GCTB, *miR-136* had no or a very low level of expression in both microarray and qRT-PCR analyses. Thus far, no evidence exists regarding the role of *miR-136* in any type of cancer. Instead, numerous other miRNAs identified in the present study have previously been implicated as cancer-related. *MiR-1* and *miR-542-5p*, miRNAs absent from metastatic GCTBs, act as tumor-suppressor miRNAs in various cancers (Bray et al., 2011; Leone et al., 2011; Nohata et al., 2011). Similarly, no expression of *miR-542-5p* has been detectable in either unfavorable neuroblastoma or osteosarcoma when compared to expression in favorable neuroblastoma and normal osteoblasts (Schulte et al., 2010; Lulla et al., 2011).

As for *miR-542-3p*, which was undetectable in our metastatic GCTBs, it is a cell cycle regulator and inhibits cell proliferation (Yoon et al., 2010). One of the exclusively expressed miRNAs in our metastatic group of GCTB was *miR-224*, the over-expression of which is associated with progression of colorectal cancer (Arndt et al., 2009). These miRNA may thus be associated with metastases in GCTB and may elevate risk for metastasis development. Between metastatic and non-metastatic groups, several differentially expressed genes, such as *NFIB, TNC, NET1,* and *PDP*, emerging from the reanalysis of mRNA expression profiles, negatively correlated with the miRNAs detectable in our microarray analysis. Of note, over-expressed NFIB in metastatic tumors correlated with *miR-136*, a validated miRNA in our study. However, more functional studies are necessary to show whether loss of *miR-136* expression alone is sufficient to stabilize expression of *NFIB* transcripts in metastatic patients. *NFIB* was selected for further verification at mRNA and protein level, by use of RT-PCR and immunohistochemistry. NFIB showed higher expression in metastatic GCTB at both mRNA and protein level (an increase in mRNA by RT-PCR was not statistically significant). An independent validation cohort with a larger series of cases (74 samples) confirmed the pattern of NFIB expression by IHC.

Several studies have shown the role of *NFIB* in the development and progression of various tumors. NFIB regulates cell viability and proliferation during transformation and functions as an oncogene in small-cell lung cancer (SCLC) (Dooley et al., 2011). An increased level of Nfib is also very frequently detectable in lymph node and liver metastases of SCLC in the mouse model (Dooley et al., 2011). Moreover, development and progression of triple-negative breast cancer demonstrate the role of NFIB; there, silencing of the NFIB gene reduces proliferation, while it enhances the apoptotic signaling pathway (Moon et al., 2011).

The other two over-expressed target genes in metastatic tumors if compared with non-metastatic

GCTB were TNC and NET1. Their over-expression correlated with the absence of expression of the respective genes *miR-542-5p* and *miR-1*.

As for our earlier study related to GCTB, it found over-expression of TNC to be a biological risk factor for metastases and to be associated with poor prognosis (Pazzaglia et al., 2010). NET1 has, similarly, been implicated in gastric cancer, where it is over-expressed and participates in gastric cell proliferation and invasion (Leyden et al., 2006). Moreover, genes involved in regulation of apoptosis, cell death, and DNA repair are down-regulated in the presence of high levels of NET1 (Bennett et al., 2011). PDPN was one of the under-expressed genes in our metastatic group of GCTB and correlated with exclusively expressed *miR-934* in metastatic GCTB. One study shows that PDPN distinguishes primary tumors of the adamantinoma of long bones from adenocarcinoma metastatic to bone, and distinguishes osteo-fibrous dysplasia from fibrous dysplasia; and it is expressed neither in metastatic adenocarcinoma nor in fibrous dysplasia (Kashima et al., 2011).

We identified differentially expressed miRNAs and their altered target genes between primary tumors of metastatic and non-metastatic GCTB. Identification of novel biomarkers such as *miR-136* and its target, *NFIB*, may aid in distinguishing primary tumors of GCTBs with a higher risk for metastasis. It also may aid in choosing suitable therapeutic and follow-up strategies for GCTB patients. It should, however, be evident that the data presented here are based on a relatively small sample size, and further studies are warranted to support our findings.

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