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Original Articles

Restoration of miR-127-3p and miR-376a-3p counteracts the neoplastic phenotype of giant cell tumor of bone derived stromal cells by targeting COA1, GLE1 and PDIA6

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ABSTRACT

Although generally benign, giant cell tumors of bone (GCTB) display an aggressive behavior associated with significant bone destruction and lung metastasis in rare cases. This and the very high recurrence rate observed after surgical resection ranging from 20 to 55% necessitates the development of more effective treatment strategies. To identify valuable therapeutic targets, we screened a previously identified microRNA signature consisting of 23 microRNAs predominantly down-regulated in GCTB. We preselected eight candidate microRNAs and analyzed the impact of their restored expression on the neoplastic phenotype of GCTB stromal cells (GCTSC). A consistent and significant inhibition of cell proliferation, migration, colony formation and spheroid formation could be induced by transfection of primary GCTSC cell lines with miR-127-3p and miR-376a-3p, respectively. Genome wide expression analysis of miR-127-3p and miR-376a-3p transfected cells revealed four novel target genes for each microRNA. Luciferase reporter assays demonstrated direct interactions of miR-127-3p with *COA1* and direct interaction of miR-376a-3p with *GLE1* and *PDIA6*, suggesting a pivotal role of these genes in the molecular etiology of GTCB. Interestingly, both microRNAs are located within a chromosomal region frequently silenced in GCTB and many other types of cancers, indicating that these microRNAs and their target genes are valuable therapeutic targets for the treatment of GCTB and possibly other tumor entities.

Introduction

Giant cell tumor of bone (GCTB) is a generally benign bone tumor, frequently located at the meta-epiphyseal region of long bones [1,2]. GCTB displays a locally aggressive behavior, associated with expansive osteolytic defects and significant bone destruction [3]. Although rare, GCTB can also manifest a malignant phenotype with metastases in up to 5% of the cases [4,5]. To date, surgical resection of the tumor is the standard therapy, that is, however, associated with a very high recurrence rate, ranging from 20 to 55% depending on the surgical technique and the use of adjuvants [6,7]. The growing knowledge of the tumor biology already led to the development of new treatment options including anti-resorptive bisphosphonates [8] and the monoclonal antibody denosumab directed against RANKL (receptor activator of NF-κB ligand), a key mediator of osteoclast activation [9]. However, these therapies mainly affect the bone destructive osteoclast-like giant cells within the tumor but not the neoplastic cell population that is represented by monocytic, spindle-shaped stromal cells (GCTSC). These cells are characterized by the ability to indefinitely proliferate in culture [10] and by their capacity to form tumors in mice [11]. Subpopulations with stem cell like properties have been identified within GCTSCs characterized by their expression of Stro-1 or c-Met [12,13]. GCTSCs further express osteoblast markers like osteopontin, osteonectin, osterix and RUNX2 (Runt-related transcription factor 2) [14,15] as well as mesenchymal stem cell (MSC) markers including CD73, CD105 and CD166 [10]. These data point to a pre-osteoblastic phenotype of GCTSC and strongly suggest a mesenchymal origin. As GCTSC drive tumor progression by the recruitment of monocytes and the promotion of osteoclast formation, they represent a valuable target for the development of new therapeutic strategies. However, further characterization of this cell population, especially the identification of the factors that drive tumorigenesis, is needed to reveal novel therapeutic targets.

Abbreviations: GCT, giant cell tumor; MSC, mesenchymal stem cell; GCTSC, giant cell tumor-derived stromal cell.

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In a previous study, we analyzed the microRNA expression profile of GCTSCs and compared it to the expression pattern seen in MSCs, their putative progenitor cells. We identified a signature consisting of 23 microRNAs predominantly down-regulated in the tumor cells [16]. MicroRNAs are small (~20 nt) non-coding, single stranded RNA molecules that negatively regulate their target genes on a post-transcriptional level [17]. Growing evidence exists that microRNAs play critical roles in tumor development and progression and thus represent, together with their target genes, promising targets for the development of new treatment strategies. Therefore, the aim of this study was the identification of those microRNAs that are functionally involved in the development and maintenance of the neoplastic phenotype of GCTSCs. Eight candidate microRNAs were selected from the identified signature on the basis of their expression level, the degree of down-regulation in the tumor cells and their known functions. The functional impact on the neoplastic properties of GCTSCs was evaluated after mimic-mediated re-expression of these microRNAs. MiR-127-3p, miR-376a-3p and their novel target genes *COA1*, *GLE1* and *PDIA6* could be identified as promising targets for therapeutic interventions in GCTB and probably other tumors.

Materials and methods

The use of cell lines derived from patient tissue was approved by the Ethics Committee of the University of Heidelberg and informed consent to analyze tumor tissue and to publish clinical details was obtained from all individuals included in the study.

Sample preparation and cell culture

Primary GCTSCs were isolated from tissue samples derived from tumor resections in our clinic. The tissue was mechanically cut in small pieces and digested with 1.5 mg/ml collagenase B (Roche Diagnostics, Mannheim, Germany) for 3 h at 37 °C in Dulbecco's modified Eagle medium (DMEM) (Lonza GmbH, Köln, Germany) containing 4.5 g/l glucose and supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), and 100 U/ml penicillin/streptomycin (Lonza). Cells were collected by centrifugation, washed twice in PBS and cultured in DMEM. Twenty-four hours after plating, cells were carefully treated with Trypsin/EDTA (Lonza) leaving the giant cells attached in the culture flask. Detached cells were cultured for further 3 passages eliminating any remaining giant cells and histiocytes.

MSCs were isolated from fresh bone marrow samples derived from the iliac crest. Cells were fractionated on a Ficoll-Paque Plus density gradient (Amersham Pharmacia, Uppsala, Sweden), and the low-density MSC-enriched fraction was washed and seeded in culture flasks. MSC culture medium consisted of DMEM high glucose (Lonza) 12.5% FCS, 1× NEAA (non-essential amino acids) (Life Technologies, Darmstadt, Germany), 50 µM 2-mercaptoethanol (Life Technologies) and 4 ng/ml bFGF (basic fibroblast growth factor) (Merck Chemicals GmbH, Schwalbach, Germany). After 24–48 h, cultures were washed with PBS to remove non-adherent material. During expansion, medium was replaced twice a week.

Transfection of primary cell lines

For restoration of candidate microRNAs, GCTSCs derived from GCTB patients were transfected with microRNA mimics (Life Technologies, Darmstadt, Germany) by electroporation. Control cells were transfected with a microRNA mimic negative control representing a random sequence molecule that has been extensively tested and validated to not produce identifiable effects on known microRNA function. Transfection was carried out with the electroporation unit MP-100 (Life Technologies). Cells were cultured until they reached ~80% confluence, trypsinized and washed twice in PBS. For transfection, 10^6 cells were resuspended in 100 µl R-buffer containing 5 µM microRNA mimic. After electroporation with two pulses at 1200 V for 15 ms, cells were plated in DMEM and cultured for 48 h before they were used for the different assays. Transfection efficiencies were quantified using a negative control siRNA labeled with Alexa Fluor 488 (Qiagen, Hilden, Germany). The percentage of fluorescent cells was analyzed 48 h after transfection by flow cytometry.

RNA extraction

Total RNA was extracted using mirVana miRNA isolation kit (Invitrogen, Darmstadt, Germany). RNA concentrations and purity were determined with a NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). Extracted RNA was used for both miRNA expression and RT-qPCR gene expression analyses.

RT-qPCR

First strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using 1 µl Omniscript (Qiagen), 10 µM oligo-dT primer, 5 mM dNTPs and 10 U RNaseOut (Invitrogen, Karlsruhe, Germany) for 1.5 h at 37 °C in a total volume of 20 µl. After synthesis cDNA was further diluted 1:10 with 10 mM Tricine. RT-qPCR was performed in the real-time thermal cycler Mx3000p (Agilent Technologies, Waldbronn, Germany) in a total volume of 20 µl using Absolute QPCR SYBR Green mix (Thermo Scientific, Dreieich, Germany) and 2 µl of diluted cDNA as template. Samples were heated to 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 20 s and extension at 72 °C for 30 s. After the last cycle, a melting curve analysis was performed to verify the specificity of the amplified PCR products. Calculated gene expression was normalized on the basis of the expression of *RPS13* (ribosomal protein S13) in the corresponding sample. The primers are listed in the supplemental Table S1.

RT-qPCR of microRNAs

Quantification of microRNA expression was done using the TaqMan MicroRNA reverse transcription kit from Applied Biosystems (Darmstadt, Germany) according to the manufacturer's instructions. In brief, 10 ng of total RNA was subjected to cDNA synthesis using microRNA specific stem-loop primer. For RT-qPCR, 1.5 µl of cDNA was used in a total volume of 20 µl containing microRNA specific primers and TaqMan probes. Samples were heated to 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and a combined annealing/extension step at 60 °C for 60 s. The reaction was carried out in the real-time thermal cycler Mx3000p from Agilent Technologies. Calculated microRNA expression levels were normalized on the basis of the RNU6B expression in the corresponding sample. RNU6B is a small nuclear RNA frequently used as reference RNA for microRNA quantification.

Proliferation assay

For the analysis of cell proliferation 5×10^3 cells were seeded in a 96-well plate 48 h after transfection, cultured in DMEM containing 10% FCS and counted after 0, 24, 48, 72, 96 and 120 h. For counting, cells were washed in PBS and trypsinized for 10 min at 37 °C. Trypsinization was stopped by the addition of DMEM/10% FCS and cells were counted directly in the 96-well plate using a MACSQuant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). All analyses were done in triplicate.

Migration assay

The *in vitro* cell migration was measured using the Oris™ 96-well cell migration assay kit (Platypus Technologies, Madison, USA) following the manufacturer's instructions. In brief, 3×10^4 cells were seeded in 96-well plates in which cell-seeding stoppers were inserted. These stoppers prevent the attachment of cells within a defined detection zone of 2 mm in diameter in the center of each well. After 24 h the cell seeding stoppers were removed and the cells were washed with PBS. Cells were cultured in DMEM for further 24 h to allow migration, washed with PBS, stained with PBS supplemented with 1 µM calcein-AM and photographed. The area of migrated cells within the detection zone was quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Colony formation assay

Cells were trypsinized, counted and 3×10^4 cells were resuspended in DMEM containing 0.3% agar, 10% FCS and 100 U/ml penicillin/streptomycin. The cell suspension was layered on top of 0.5% agar in 35 mm culture plates and incubated at 37 °C in a humidified incubator for 28 days. Colonies containing >20 cells were counted under a microscope. Experiments were done in triplicate with six different GCTSC cell lines.

Spheroid assay

Cells were seeded at low densities (1×10^3 cells/ml) in 24-well low-adhesion plates in NeuroCult NS-A basal serum-free medium (human) (StemCell Technologies, Vancouver, Canada) supplemented with 2 µg/ml heparin (StemCell Technologies), 20 ng/ml hEGF (R&D Systems, Wiesbaden-Nordenstadt, Germany), 10 ng/ml hFGF-b (PeproTech, Hamburg, Germany) and NeuroCult NS-A proliferation supplements (StemCell Technologies). Per sample five wells were plated in parallel. The total number of spheroids was counted under the microscope after incubation at 37 °C in a humidified incubator for 7 days.

Genome-wide gene expression profiling

Expression profiling was done at the "Genomics and Proteomics Core Facility" at the "Deutsche Krebsforschungszentrum" (DKFZ) in Heidelberg, Germany. HumanHT-12 v4 Expression BeadChip (Illumina) was used to detect the expression of 31000 annotated genes in four GCTSC cell lines. Data were normalized across all samples before gene expression in wild-type cells and cells transfected with a negative control microRNA were compared to gene expression in miR-127-3p and 376a-3p transfected cells, respectively.