Bone 52 (2013) 532–539

Contents lists available at SciVerse ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

miR-326 associates with biochemical markers of bone turnover in lung cancer bone metastasis

Karmele Valencia [a,1], Marta Martín-Fernández [b,1], Carolina Zandueta [a], Cristina Ormazábal [a], Susana Martínez-Canarias [a], Eva Bandrés [c,2], Concepción de la Piedra [b], Fernando Lecanda [a,*]

[a] Adhesion and Metastasis Laboratory, Division of Oncology, Center for Applied Biomedical Research (CIMA), University of Navarra, Pamplona, Spain
[b] Bioquímica Investigación, Instituto de Investigación Sanitaria Fundación Jiménez Díaz, Madrid, Spain
[c] Pharmacogenomics Laboratory, Division of Oncology, Center for Applied Biomedical Research (CIMA), University of Navarra, Pamplona, Spain

ARTICLE INFO

Article history:
Received 25 May 2012
Revised 26 October 2012
Accepted 29 October 2012
Available online 7 November 2012

Edited by: Richard Eastell

Keywords:
Biomarker
Zoledronic acid
Osteoclast
Osteolysis
PINP
BGP

ABSTRACT

Recent evidence suggests that miRNAs could be used as serum markers in a variety of normal and pathological conditions. In this study, we aimed to identify novel miRNAs associated with skeletal metastatic disease in a preclinical model of lung cancer bone metastasis. We assessed the validity of these miRNAs as reliable serum biochemical markers to monitor the extent of disease and response to treatment in comparison to imaging techniques and standard biochemical markers of bone turnover. Using a murine model of human lung cancer bone metastasis after zoledronic acid (ZA) treatment, PINP (procollagen I amino-terminal propeptide) was the only marker that exhibited a strong correlation with osteolytic lesions and tumor burden at early and late stages of bone colonization. In contrast, BGP (osteocalcin) and CTX (carboxyterminal telopeptide) demonstrated a strong correlation only at late stages. We performed qPCR based screening of a panel of 380 human miRNAs and quantified bone metastatic burden using micro-CT scans, X-rays and bioluminescence imaging. Interestingly, levels of miR-326 strongly associated with tumor burden and PINP in vehicle-treated animals, whereas no association was found in ZA-treated animals. Only miR-193 was associated with biochemical markers PINP, BGP and CTX in ZA-treated animals. Consistently, miR-326 and PINP demonstrated a strong correlation with tumor burden. Our findings, taken together, indicate that miR-326 could potentially serve as a novel biochemical marker for monitoring bone metastatic progression.

Introduction

Non-small cell lung cancer (NSCLC) accounts for ~75% of all lung cancer cases [1]. Despite recent progress in multimodal management, prognosis remains poor primarily because of the emergence of metastasis to different organs [2]. Metastasis may appear even during early stages of tumor progression [3]. Bone tropism occurs in ~40% of patients with NSCLC and skeletal metastases cause severe pain and skeletal complications (referred to as skeletal related events, SRE) including spinal cord compression and pathological fractures associated with significant morbidity and poor prognosis [4]. These SRE reduce patient quality of life and represent a social burden due to costly treatments [5]. Patients diagnosed with NSCLC benefit from the use of antiresorptive agents such as bisphosphonates or denosumab that delay the onset of SRE [6,7]. Zoledronic acid (ZA) is the most widely used bisphosphonate bone metastases, characterized for its potency. ZA blocks osteoclast activity, decreasing the matrix-released of several growth factors, such as TGF-β or IGF-I, and delays the development of bone metastases.

Patient diagnosis is usually performed using bone scintigraphy screening and is confirmed by radiography and/or computed tomography or magnetic resonance. During the clinical course, the patient's response is assessed using serial radiographs to evaluate bone changes. However, this approach has limitations. This is primarily due to slow detectable changes, and the confounding appearance of lesions containing mixed and/or osteosclerotic areas. Metastatic cells release an array of factors which increase osteoblast and/or osteoclast activities tilting the balance of normal bone remodeling. This process could be assessed through the quantification of circulating levels of biochemical markers of bone formation and resorption. Several serum biochemical markers of bone turnover have been explored to assess skeletal integrity in patients at risk or who exhibit bone lesion progression [8]. A significant correlation has been previously demonstrated between the presence of a marker and extent of metastatic disease [9]. These markers are also useful for the identification of patients most likely to

Abbreviations: NSCLC, Non-small cell lung cancer; TRAP, Tartrate-resistant acid phosphatase; μCT, Micro-computed tomography; i.c., intracardiac; PINP, Procollagen I amino-terminal propeptide; CTX, Carboxyterminal telopeptide; BGP, Bone Gla Protein.

* Corresponding author at: Division of Oncology, Adhesion and Metastasis Laboratory, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona 31080, Navarra, Spain. Fax: +34 948 194 714.
E-mail address: flecanda@unav.es (F. Lecanda).

[1] These authors have contributed equally to this work.
[2] Present address: Immunology Unit, Hematology Service, Complejo Hospitalario de Navarra, Pamplona, Spain.

8756-3282/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bone.2012.10.033

benefit from antiresorptive therapy [10] and for monitoring response to treatment [11,12]. Current markers of bone formation include bone-specific alkaline phosphatase, the procollagen I carboxyterminal propeptide (PICP), procollagen I amino-terminal propeptide (PINP) and osteocalcin (BGP). PINP and PICP are released into the circulation before synthesis of collagen type I, the most common protein in the skeleton. BGP is a non-collagenous matrix protein of bone synthesized by osteoblasts and secreted directly into circulation. Serum levels of BGP are a reliable index of bone formation, whereas PICP measures the neosynthesis of type I collagen. Bone resorption is assessed by measuring the by-products of collagen degradation, such as cross-linked carboxyterminal telopeptide (CTX), the aminoterminal telopeptide (NTX) of type I collagen, and tartrate-resistant acid phosphatase 5b (TRAP5b), a marker of osteoclast activation. Because biomarkers are relatively inexpensive and noninvasive, novel markers could potentially provide a simple and reliable method for detection; thus, such markers represent an area of great clinical relevance.

A growing body of evidence indicates that miRNAs, small noncoding RNAs that are involved in the regulation of gene expression, are altered during tumorogenesis [13,14] and metastasis [15–18]. More importantly, miRNAs are released to the extracellular milieu into exosomes [19] or microvesicles [20] that are detectable in body fluids [21,22]. Detection of miRNAs in the plasma or serum of lung cancer patients has been suggested as a potential tool with diagnostic, prognostic, and predictive value [23–26].

In this study we screened miRNAs associated with tumor burden in order to investigate, the correlation of biochemical markers of bone turnover in a model of osseous metastasis of lung cancer. Our findings suggest that this strategy is suitable to identify miRNAs as potentially useful biochemical markers for monitoring the metastatic process.

Materials and methods

Cell lines and culture conditions

Lung cancer cell line A549 was obtained from the ATCC (Manassas, VA, USA) and was grown in RPMI 1640 with L-glutamine (Invitrogen, Barcelona, Spain) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin and streptomycin (Invitrogen). Cells were authenticated by sequencing critical KRAS and P53 mutations. A549 cells were retrovirally transduced with a luciferase reporter gene for in vivo image analysis [27]. A549M1 subpopulation was isolated as previously described [28]. This subpopulation displays an overt propensity to form osteolytic bone metastasis in 100% of inoculated mice, whereas no metastases were detected in extra-skeletal sites during the whole experimental period. Animals were treated with zoledronic acid (Zometa®, Novartis) at a single dose of 50 μg/kg or vehicle.

In vivo assays

Female athymic nude mice (Harlan Iberica, Spain) were maintained under specific pathogen-free conditions. All the animals were sacrificed according to the approved protocols of the Local Animal Committee. Four-week old mice were inoculated in the left cardiac ventricle with 2×10^5 cells in 100 μl of PBS as detailed elsewhere [28–30]. For each time point, each treatment group contained 10 animals. A Control (Ctrl) group ($n=2$ for each time point) was sham-inoculated with PBS.

X-ray radiography was performed for all mice, with mice placed on the prone position on sensitive radiographic film (MIN-R, Eastman Kodak). The percentage of osteolytic area of femur and tibia for each animal reference to the total bone area of femur and tibia was assessed with computerized image analysis system, AnalySIS® (soft imaging system GmbH, Münster, Germany). High resolution X-ray film scans with 2× magnification were captured at 1200 ppi using a Epson Expression 1680 Pro scanner (Long Beach, CA, USA). Quantification of metastatic area was performed twice after calibration by two independent observers.

Only whole tibiae were analyzed by a μCT system (micro CAT ™ Preclinical Solutions, Knoxville, TN, USA) at 75.0 kVp and 250.0 μA. The scans were performed at 10 μm resolution. 2D CT images were reconstructed using a standard convolution-back projection procedure with a Shepp–Logan filter. Images were stored in 3D arrays with a voxel size of 19 μm × 19 μm × 23 μm.

For bioluminescence imaging and analysis, mice were anaesthetized and injected intraperitoneally with 1.5 mg of D-luciferin in 100 μl of PBS. Imaging was completed at 2 min exactly for each group of mice with a Xenogen IVIS system coupled to Living Image acquisition and analysis software (Xenogen Inc.). Photon flux was calculated for each mouse by using a circular region of interest for each hind limb. Background value (from luciferin-injected mouse with no tumor cells) was subtracted from each measurement.

The perimeter occupied by TRAP+ cells at tumor–bone interphase referenced to the total perimeter of tumor–bone interphase (expressed as %) was measured in 4 different sections for each animal after TRAP staining (Sigma, St. Louis, MO).

RNA isolation and quantitative real-time RT-PCR analysis of miRNA

Total RNA was isolated from serum by using TRIzol-SL® reagent (Invitrogen) according to the manufacturer's instructions. cel-miR-39, cel-miR-54 and cel-miR-238 were spiked-in as internal controls for RNA isolation. miRNA profiling was performed using pre-aliquoted microRNA PCR primer sets in 384-well PCR plates (Exiqon) to study miRNAs expression profiling in the serum. A pool of RNA of mice from the same day was used to reach 40 ng total RNA. Duplicates of each time point were performed: control mice (non-injected mice), and mice injected with M1cells and sacrificed at days 7, 14, 21 and 28 postinjection. No pre-amplification was necessary.

microRNA validation was performed by sequence-specific quantitative reverse transcriptase-PCR (qRT-PCR) primers for miR-184, 33a, 326, 497, 193b and endogenous control miR-16 was purchased from Applied Biosystems. A pre-amplification reaction was performed to increase the amount of cDNA in the samples. qRT-PCR analysis was measured using Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). The PCR Master Mix containing Taqman® 2× Universal PCR Master Mix (No Amperase UNG), 10× Taqman® assay, and reverse transcription products in 20 μl volume were processed as follows: 95 °C for 10 min and then 95 °C for 15 s, 60 °C for 60 s for up to 40 cycles. Signal was collected at the end point of every cycle. Gene expression was considered as the ΔCT values of the specific miRNAs after normalizing with the internal controls miR-16 together with an average of the three pike-in miRNAs and relative quantification values were plotted.

Biochemical markers of bone turnover

For each mouse, blood was obtained under anesthesia by intracardiac puncture into an EDTA containing tube (Microvette®, Sarstedt, Nümbrecht, Germany). Serum was obtained after centrifugation at 400 *g* for 15 min at 4 °C, and was further aliquoted and stored at −80 °C until subsequent analysis. Serum Bone Gla Protein (BGP) was determined by ELISA for the specific quantitative determination of mouse osteocalcin levels (Osteocalcin mouse, DRG, Germany). Sensitivity of this assay was 1 ng/ml. Intra- and inter-assay coefficients of variation of the method were <6% and <8% respectively. Serum aminoterminal propeptide of collagen I (PINP) was assayed by an ELISA specific for rat and mouse PINP (Rat/Mouse PINP EIA, IDS, UK). Sensitivity of the assay was 0.7 ng/ml. Intra- and inter-assay variation coefficients of the method were <5.0% and <8.2% respectively. Serum 5b isoenzyme of tartrate-resistant acid phosphatase (TRAP5b) was measured by an ELISA specific for mouse TRAP (Mouse TRAP Assay,