Experimental and Molecular Pathology 103 (2017) 229–236

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

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

MicroRNA-21 and long non-coding RNA MALAT1 are overexpressed markers in medullary thyroid carcinoma

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ARTICLE INFO

Keywords:
MiR-21
MALAT1
Medullary thyroid carcinoma
siRNA
Proliferation
Invasion

ABSTRACT

Background: Non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), are well-recognized post-transcriptional regulators of gene expression. This study examines the expression of microRNA-21 (miR-21) and lncRNA MALAT1 in medullary thyroid carcinomas (MTCs) and their effects on tumor behavior.
Methods: Tissue microarrays (TMAs) were constructed using normal thyroid (n = 39), primary tumors (N = 39) and metastatic MTCs (N = 18) from a total of 42 MTC cases diagnosed between 1987 and 2016. In situ hybridization with probes for miR-21 and MALAT1 was performed. PCR quantification of expression was performed in a subset of normal thyroid (N = 10) and primary MTCs (N = 32). An MTC-derived cell line (MZ-CRC-1) was transfected with small interfering RNAs (siRNAs) targeting miR-21 and MALAT1 to determine the effects on cell proliferation and invasion.
Results: In situ hybridization (ISH) showed strong (2 + to 3 +) expression of miR-21 in 17 (44%) primary MTCs and strong MALAT1 expression in 37 (95%) primary MTCs. Real-time PCR expression of miR-21 ($P < 0.001$) and MALAT1 ($P = 0.038$) in primary MTCs were significantly higher than in normal thyroid, supporting the ISH findings. Experiments with siRNAs showed inhibition of miR-21 and MALAT1 expression in the MTC-derived cell line, leading to significant decreases in cell proliferation ($P < 0.05$) and invasion ($P < 0.05$).
Conclusion: There is increased expression of miR-21 and MALAT1 in MTCs. This study also showed an in vitro pro-oncogenic effect of MALAT1 and miR-21 in MTCs. The results suggest that overexpression of miR-21 and MALAT1 may regulate MTC progression.

1. Introduction

Medullary thyroid carcinomas (MTCs) are uncommon thyroid malignancy derived from the calcitonin-secreting cells (C-cells) in the thyroid. Most cases of MTCs are sporadic, but there is autosomal dominant inheritance with high penetrance in approximately 25% of cases (Raue et al., 1993). Patients frequently present at an advanced stage and experienced highly variable outcomes (Modigliani et al., 1998). Surgery is considered the mainstay treatment because the tumors are generally insensitive to radiation and chemotherapy, and has led to 5-year and 10-year survival rates of 65–87% and 51–78%, respectively (Chong et al., 1975; Modigliani et al., 1998; Pelizzo et al., 2007; Raue et al., 1993; Scopsi et al., 1996). Most MTCs are slow-growing tumors, but aggressive tumor progression occurs in some cases. Decades of research prompted by the need for prognostic guidance have linked poor outcome to advanced stage, older age, male sex, association with the familial multiple endocrine neoplasm syndrome type 2B (MEN-2B) and histological features including necrosis, high mitotic activity, small cell variant and low calcitonin positivity (Bergholm et al., 1997; Elisei et al., 2008; Franc et al., 1998; Mendelsohn et al., 1980; Modigliani et al., 1998; Scopsi et al., 1996). On the other hand, studies looking into the molecular oncogenesis of MTCs have recognized various RET proto-oncogene mutations in all hereditary MTCs and in 40–60% of sporadic cases (Bugalho et al., 1997; Moura et al., 2009; Romei et al., 1996). Among those with wild-type RET, RAS (HRAS and KRAS) mutations are identified in the majority of cases (68%) (Moura et al., 2011). Despite such intensive effort, the molecular mechanisms underlying MTCs' variable behavior remains poorly understood and patient outcomes are largely unpredictable.

Non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), are well-recognized post-transcriptional regulators of genetic expression. Recent studies have led to the discovery of multiple aberrantly expressed non-coding RNAs in thyroid cancers, as recently reviewed (Chu and Lloyd, 2016; Zhang et al., 2016).

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http://dx.doi.org/10.1016/j.yexmp.2017.10.002
Received 14 July 2017; Received in revised form 24 August 2017; Accepted 25 October 2017
Available online 26 October 2017

Particularly of interest for MTCs is miRNA-21 (miR-21), whose upregulation has been previously associated with lymph node metastasis, more advanced stage and postoperative persistent disease (Pennelli et al., 2015). The pro-oncogenic role of miR-21 has been increasingly elucidated, including its interaction with important RAS-ERK and RAS-PI3K signaling regulators such as PTEN, PDCD4 and SPRY1 (Sharma and Ruppert, 2015). With regard to MTCs, a recent study has found a negative correlation between miR-21 and PDCD4 expression in patient tissues (Pennelli et al., 2015). However, there have not been experimental investigations that demonstrate a causative relation between miR-21 expression and the behavior of malignant cells in MTCs.

MALAT1 is a lncRNA marker of metastasis and poor prognosis in lung and other cancers but previously unexplored in MTCs (Ji et al., 2003; Schmidt et al., 2011). Prior studies have demonstrated that MALAT1 silencing led to attenuated expression of cell-motility-related genes and that MALAT1-deficient lung cancer cells exhibited inferior motility and reduced tumor formation in inoculated mice (Gutschner et al., 2013; Tano et al., 2010). In addition, the pro-oncogenic potential of MALAT1 was supported by its regulation of cell cycle-related transcription factors B-MYB and p53 (Tripathi et al., 2013). Since its characterization in lung cancer, MALAT1 upregulation has also been reported in various other tumor types including follicular cell-derived thyroid tumors (Zhang et al., 2017). Nevertheless, MALAT1 has remained unexplored in medullary thyroid carcinomas.

This study aimed to characterize the expression of miR-21 and MALAT1 in MTCs. The effects of MALAT1 and miR-21on tumor behavior were also experimentally examined using an MTC-derived cell line.

2. Material and methods

2.1. Tissue microarrays

Tissue microarrays (TMAs) were constructed with formalin-fixed paraffin-embedded (FFPE) tissues of normal thyroid (N = 39), primary tumors (N = 39) and metastases (N = 18) from a total of 42 MTC cases diagnosed between 1987 and 2016. Each sample was represented by triplicate 0.6-mm cores made using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI). Clinical features of the patients are summarized in Table 1. The study was approved by the Institutional Review Board at the University of Wisconsin-Madison.

2.2. Immunohistochemistry

Immunohistochemistry staining was performed using the Ventana BenchMark Ultra system (Ventana Medical Systems, Inc., Tucson, AZ) according to the manufacturer's automatic protocols. Primary antibodies were used to label Ki-67 (1:50 dilution with Van Gogh Yellow; Biocare, Pacheco, CA), CD15 (clone MMA, prediluted; Ventana Medical Systems, Inc., Tucson, AZ), chromogranin A (clone LK2H10, prediluted; Ventana Medical Systems, Inc., Tucson, AZ), calcitonin (polyclonal, prediluted; Ventana Medical Systems, Inc., Tucson, AZ) and synaptophysin (polyclonal, prediluted; Cell Marque, Rocklin, CA). Marker expression was visualized with DAB. The percentage of tumor cells with positive staining were scored as rare (< 5%), focal (5–25%) and diffuse (> 25%). The intensity of staining was graded as negative (0), weak (1 +), moderate (2 +) and strong (3 +).

2.3. In situ hybridization (ISH)

ISH was conducted on TMAs to investigate the clinical and biologic relevance of miR-21 and MALAT1 regulation in thyroid carcinogenesis. The staining for miR-21 was conducted using miRCURY LNA™ microRNA detection (FFPE) kit (Exiqon, USA) with 5′-DIG and 3′-DIG labeled miRCURY LNA™ detection probes for miR-21 according to the manufacturer's instructions, as previously reported (Guo et al., 2015). The slides were deparaffinized and rehydrated to PBS. Proteinase-K treatment (15 μg/ml) at 37 °C for 30 min was performed. Sections were washed, dehydrated and air-dried. Double-DIG-labeled LNA probes were then denatured by heating to 94 °C for 4 min. Slides were hybridized with probes for 1 h at 55 °C. Sections were blocked using the DIG wash and Block Buffer Set (Roche, Mannheim, Germany) at room temperature for 15 min according to the manufacturer's recommendations. Alkaline phosphatase (AP)-conjugated anti-DIG (Cat #11093274910, Roche) was diluted 1:800 in the blocking solution and incubated for 60 min at room temperature. NBT-BCIP substrate (Cat #SK-5400, Vector Laboratories, USA) was developed for 2 h at 30 °C according to the manufacturer's instructions.

TMAs were probed for MALAT1 expression using the RNAscope 2.0 HD-Brown Manual Assay (Advanced Cell Diagnostics, Newark, CA) as per manufacturer's recommendations with the following modifications: antigen retrieval for 15 min, protease digestion for 30 min and probe incubation overnight at 40 °C. The probes used are hs-Malat1 (400811), hs-ACTB (Actin, positive control, 310,141) and dapB (negative control, 310,043) (Advanced Cell Diagnostics). MALAT1 expression levels were visualized with DAB. Under light microscopy, staining intensity was manually scored each for MALAT1 and miR-21 as negative (0), weak (1 +), moderate (2 +) and strong (3 +).

2.4. RNA isolation and quantitative polymerase chain reaction

Total RNA was extracted from FFPE tissues from a subset of the TMA cohort (10 normal thyroid and 32 MTCs) and cell culture samples. The extraction was performed with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions, and RNA quality and concentrations were assessed with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). One microgram of total RNA was reverse-transcribed using the All-in-One miRNA RT-qPCR detection kit (GeneCopoeia, Rockville, MD). Quantitative reverse transcription PCR (RT-qPCR) was performed on a CFX96 PCR detection system (Bio-Rad Laboratories, Hercules, CA) using Bullseye EvaGreen qPCR master mix (MIDSCI, St. Louis, MO), normalized to RN5-8S6 or 18S rRNA; relative fold change was

Table 1
Clinical features of included patients (N = 42).

Characteristics	N (%)
Age at diagnosis, years	
Median (range)	46.5 (4 to 88)
Gender	
Male	22 (52)
Female	20 (48)
Inheritance	
Multiple endocrine neoplasia type 2A	14 (33)
Multiple endocrine neoplasia type 2B	1 (2)
Multiple endocrine neoplasia type 2, unspecified	3 (7)
Family history of MTCs, not genotyped	2 (5)
Sporadic	22 (52)
Tumor size, centimeters	
Median (range)	1.5 (0.1 to 6.8)
Nodal metastasis at diagnosis (N = 36[a])	18 (50)
Postoperative biochemical (calcitonin) persistence (N = 38[a])	23 (61)
Distant metastasis (N = 35[a])	
Present at initial diagnosis	2 (6)
Present by follow-up endpoint	7 (20)
Mediastinum	3 (9)
Lung	1 (3)
Breast	1 (3)
Axilla	1 (3)
Liver	3 (9)
Kidney	1 (3)
Retroperitoneal lymph nodes/soft tissue	1 (3)
Pelvic lymph nodes/soft tissue	1 (3)
Bone	4 (11)

[a] Cases with available clinical information.