MicroRNA Profiling of Sporadic and Hereditary Medullary Thyroid Cancer Identifies Predictors of Nodal Metastasis, Prognosis, and Potential Therapeutic Targets

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Abstract

Purpose: While the molecular basis of hereditary medullary thyroid cancer (HMTC) has been well defined, little is known about the molecular pathogenesis of sporadic medullary thyroid cancer (SMTC). In addition, microRNAs (miRNAs) have been shown to be important diagnostic and prognostic markers in cancer but have not been defined in MTC. Our aim was to study the miRNA profile of MTC to identify prognostic biomarkers and potential therapeutic targets.

Experimental Design: MiRNA microarray profiling was carried out in fresh frozen tissues from patients with SMTC (n = 12) and HMTC (n = 7). Differential expression of three miRNAs was confirmed in a validation cohort of SMTC and HMTC samples (n = 45) using quantitative reverse transcriptase-PCR and correlated with clinical outcomes. The functional role of a selected miRNA was investigated in vitro in the human medullary thyroid carcinoma cell line (TT cells) using cell proliferation assays and Western blotting analysis.

Results: MiRs-183 and 375 were overexpressed (P = 0.001; 0.031) and miR-9* was under-expressed (P = 0.011) in SMTC versus HMTC. Overexpression of miRs-183 and 375 in MTC predicted lateral lymph node metastases (P < 0.001; P = 0.001) and was associated with residual disease (P = 0.001; 0.003), distant metastases (P = 0.003; 0.001), and mortality (P = 0.01; 0.011). Knock down of miR-183 expression in the TT cell line induced a significant decrease in the viable cell count and upregulation of the protein LC3B, which is associated with autophagy.

Conclusions: Our data indicate that miRNAs play a pivotal role in the biology of MTC and represent an important class of prognostic biomarkers and therapeutic targets warranting further investigation.

Introduction

Medullary thyroid cancer (MTC) is a neuroendocrine tumor of the thyroid gland derived from parafollicular C-cells (1). It is responsible for a disproportionate number of thyroid cancer deaths because of the lack of adjuvant treatment beyond surgery (2–4).

MTC occurs in a hereditary form (HMTC: 25%) and the more common sporadic form (SMTC: 75%). RET (REarranged during Transfection) gene screening in HMTC has helped identify gene carriers who may then undergo prophylactic thyroidectomy. However, the majority of MTCs are sporadic and typically have a late presentation, nodal metastasis, and a poor prognosis (5, 6). Unlike RET in HMTC, there are no genetic or molecular biomarkers for the sporadic form of disease (7–9).

MicroRNAs (miRNAs) are small non-coding 18–25 nucleotide RNA molecules that negatively regulate protein expression. They have been implicated in the pathogenesis of many cancers and serve as biomarkers of clinical outcome and potential therapeutic targets (10–14). We have previously shown that miRNAs play a role in the pathogenesis of malignant phaeochromocytoma and adrenocortical cancer (15, 16). MiRNA profiling has also been described in papillary thyroid cancer (17), but investigations probing the significance of miRNAs in MTC pathogenesis have not been conducted.

To examine for other drivers of disease in MTC, apart from RET in HMTC, we carried out a miRNA microarray expression profile of a primary cohort of 12 SMTC and 7 HMTC samples where a number of miRNAs were identified to be differentially expressed. A cohort of 45 patients (26 SMTC and 19 HMTC), including the 19 patients from the discovery cohort, were used to validate the miRNA results.
use quantitative reverse transcriptase–polymerase chain reaction (qPCR). Following validation, results were correlated with clinical outcome identifying miRNAs-183 and 375 as predictors of lateral nodal disease and a more aggressive phenotype in HMTC. Finally, the functional role of miR-183 was investigated using the human medullary thyroid carcinoma (TT) cell line, in which, cell death via autophagy induction was demonstrated.

Materials and Methods

Sample collection
Fresh frozen tissue from 19 patients with MTC (12 SMTC and 7 HMTC; 12 females and 7 males) was used to undertake miRNA profiling of sporadic versus hereditary MTC. A further 26 MTC patients (14 SMTC, 12 HMTC) with available paraffin embedded tissue were selected to be utilized as the external validation cohort. Clinical information for these patients was collated from the prospectively maintained University of Sydney, Endocrine Surgical Unit database, medical records, and pathology reports. Ethics approval was obtained from the Northern Sydney Central Coast Area Health Service Human Research Ethics Committee.

MiRNA array profiling
Total RNA was extracted from fresh frozen tumors and paraffin embedded tumors using the miRCURY RNA isolation kit (Exiqon) and the RNeasy FFPE kit (QIAGEN Inc.), respectively following the manufacturer’s instructions. RNA quality was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies Inc.). RNA samples were labeled using the miRCURY Hy3/Hy5 power labeling kit (Exiqon) and miRNA microarray profiling was executed by Exiqon, on the miRCURY LNA Array (version 11.0), which contains capture probes targeting 1084 human miRNA sequences registered in the miRBASE database (available from: http://www.mirbase.org).

qPCR
Differentially expressed miRNAs of proven significance in the literature were selected for validation analysis (18–22). Selected miRNAs were validated in a combined cohort comprising of the original discovery cohort (n = 19) and an external cohort (n = 26) using TaqMan miRNA assays (hsa-miR-9*, Assay ID#:002231; hsa-miR-9, Assay ID#:00583; hsa-miR-183, Assay ID#:002269; hsa-miR-375, Assay ID#:000564; RNU-48, Assay ID#:001006) on the ABI 7900 HT Fast System (Applied Biosystems) following the manufacturer’s instructions. In brief, 10 ng of total RNA was reverse transcribed using a miRNA specific reverse transcriptase primer; 1/15th of the reverse transcriptase reaction was used in a qPCR reaction using the TaqMan 2× Universal PCR mastermix and the following cycle conditions: 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. All samples were run in triplicate. qPCR results were analyzed by the ΔΔCq method relative to the reference gene RNU-48 using DataAssist v1.0 (Applied Biosystems).

Cell culture
Human MTC cells (TT cell line), were obtained from the American Type Culture Collection (ATCC) and cultured in GIBCO F-12K Nutrient Mixture Kgaighn’s Modification (1X) liquid (Invitrogen) supplemented with 5% fetal calf serum (FCS) in a humidified chamber (5% CO2, 37°C). Cells were confirmed mycoplasma free.

Transfection of anti-miR human miRNA inhibitor
Once reaching 80% confluence, TT cells were reverse transfected with anti-miR, miR-183 antagonist (anti-miR-183; Ambion; AM17000) or anti-miR negative control #1 (anti-miR-NC1; Ambion; AM17010) using lipofectamine 2000 (Invitrogen). 50 µl of anti-miR-183 or anti-miR-NC1 in OptiMEM I (Invitrogen), up to a final concentration of 1 μmol/L, were mixed with 50 μl of lipofectamine 2000 (25× dilution in OptiMEM I) per well, incubated at room temperature for 20 minutes, then added to each well of a 24 well plate (100 µl). TT cells (6.25 × 105 cells/ml, 400 µl) were subsequently added to each well. The transfection mixture was incubated (5% CO2, 37°C) for 24 hours. After 24 hours, the cells were either used immediately in assays or the medium was replaced and incubated further. The transfection efficiency was determined as 97% using Cy3-labelled anti-miR-NC1 (Ambion).

Cell proliferation assay
Cell viability was assessed using CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS Assay, Promega). TT cells were cultured as described above. 5000 cells per well in 100 μl medium within a 96-well plate were reverse transfected with anti-miR-183 (100 nmol/L)
or anti-miR-NC1 (100nmol/L). Twenty-four hours after transfection, 20 μl of the CellTiter 96 AQueous One Solution Reagent was added to each of the culture wells, incubated for one hour and then the absorbance at 490 nm was recorded using a 96-well plate reader. The 24-hour time point was selected given previous success with this technique in our laboratory (15).

**Cell death and cell cycle analysis**

Cell death and cell cycle studies were carried out using Annexin V-fluorescein-5-isothiocyanate (FITC) Apoptosis Detection kit I (BD Biosciences) and propidium iodide (PI) assays (Sigma-Aldrich), respectively.

For cell death studies, 24 hours after transfection as described above, TT cells were trypsinised, washed twice with PBS and resuspended in 1× binding buffer at a concentration of 1 × 10⁵ cells/ml. Ten microliters of annexin V-FITC were added to 200 μL of cells and incubated for 30 minutes at 4°C in the dark. After incubation, 300 μL of 1× binding buffer was added to each tube, and the samples were analyzed by flow cytometry within one hour.

For cell cycle studies, 24 hours after transfection as described above, 0.25 × 10⁶ cells were washed with PBS and resuspended in 5% Triton X-100 (75 μl). RNase was added (10mg/ml, 25 μl), followed by PI (0.5 mg/ml, 25 μl). PBS was then added to a final volume of 250 μl. Samples were then analyzed by FACS analysis.

**Western blotting analysis**

Twenty micrograms of total protein extracted from TT cells were separated on a 12% SDS-PAGE gel (Bio-Rad), transferred to nitrocellulose membrane and blocked over-night at 4°C with 5% nonfat dry milk in TBS buffer with 0.1% Tween 20. Membranes were immunoblotted with PDCD4 (clone k4C1, Novus Biologicals) or LC3B (Novus Biologicals) primary antibody for one hour at room temperature. Bound primary antibody was detected using goat antimouse horseradish peroxidase-conjugated secondary antibody (1:1000, Dako) and the ECL Plus Western Blotting Detection System (GE Healthcare). The resulting bands were visualized using the LAS 4000 system (Fujifilm) and analyzed using ImageQuant (GE Healthcare).

**Statistical analysis**

Microarray data was analyzed by using a two-tailed t-test (carried out by Exiqon). Nominal clinical outcome data was assessed with Fisher’s exact test and analyses of non-parametric continuous variables were conducted with a Mann–Whitney U Test using SPSS (V.16, SPSS Inc.) software (P < 0.05 considered significant).

**Results**

**Samples for microarray analysis**

MiRNA microarray profiling was carried out on a cohort of 19 (12 SMTC and 7 HMTC) patients. Of the 12 SMTC samples, three had a known somatic 918 mutation. Specific germline mutations in the 7 HMTC samples are shown in Table 1 (summarizes genetic and clinico-pathologic data).

**Microarray analysis**

Ten miRNAs were found to be significantly differentially expressed between SMTC and HMTC (Fig. 1). Among these miRNAs, four miRNAs (miR-182, miR-183, miR-375, and miR-551b) were overexpressed in SMTC, whereas six (miR-199b-5P, miR-9, miR-9*, miR-223, let-7i, and miR-23a) were underexpressed compared with those in HMTC.

**qPCR validation of selected miRNAs**

MiRNA selection for validation was based on the published significance of these miRNAs in other cancers; for example, hepatocellular (18), lung (19), colorectal (20, 21), and breast cancer (22). Four differentially expressed miRNAs (miR-183, miR-375, miR-9*, and miR-9) were selected for qPCR validation in both the discovery (n = 19) and external cohorts (n = 26).

**MiR-183 and 375 were significantly overexpressed, while miR-9* was underexpressed in SMTC compared with those in HMTC.**

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*Figure 1. Unsupervised hierarchical clustering of miRNAs and samples. Each row indicates a miRNA, each column indicates a sample. The miRNA-clustering tree is on the left and the sample clustering is at the top. The colour scale at the bottom illustrates the relative expression of a miRNA across all samples. Red, expression level above mean; Blue, expression level below mean. The clustering is done on log2 (Hy3/Hy5) ratios, which passed filtering criteria across all samples; SD, < 1.0.*
Table 1. Clinical outcome data

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in HMTC (P < 0.05) when tested in the validation cohort (n = 45; Fig. 2a–2c). MiR-9 was not significantly differentially expressed using the qPCR technique and failed validation.

**Clinical outcome data**

Clinical outcome data was available in 44 of 45 patients in the combined cohort (Table 1). Thirty-eight patients had a total thyroidectomy and bilateral central neck dissection. Five patients underwent a total thyroidectomy without lymphadenectomy and one patient had a hemithyroidectomy alone. Twenty-four of 44 had an additional lateral lymph node dissection, with histologic nodal disease in 21 cases.

Following surgery, 25 patients suffered from "residual" disease; 20 with persistent disease (high calcitonin 3 months after initial surgery) and 5 with recurrent disease (clinical and biochemical cure at 3 months but disease recurrence thereafter). Over a mean follow up of 6.9 years (median: 4 years; range: 0.5–35.6), 25% (11 patients) were found to have distant metastases and overall mortality due to disease was 18% (8 patients).

Analysis of clinical outcome variables between SMTC and HMTC, while indicative of worse outcomes for SMTC patients, were not significantly different for lateral node metastases (SMTC: 60% vs. HMTC: 38%; P = 0.14), residual disease (SMTC: 60% vs. HMTC: 53%; P = 0.43) distant metastases (SMTC: 32% vs. HMTC: 16%, P = 0.19), or mortality (SMTC: 28% vs. HMTC: 5%; P = 0.06).

**MiR-183 and miR-375 predict lateral lymph node metastases in MTC**

We examined for whether our 3 validated miRNAs could predict for lateral lymph nodal disease. Three of 44 patients had a total thyroidectomy and central neck dissection but possessed residual disease by postoperative calcitonin definition (patients #19, 22, and 26). These patients were excluded from analysis given biochemical evidence of disseminated (potentially lateral neck node) disease but without histologic proof; thus minimizing the possibility of confounding through misclassification.

Seventeen of the remaining 41 patients did not have a lateral node dissection and had normal postoperative basal serum calcitonin levels. Given a recent study showing an absence of lymph node metastases in patients with a basal calcitonin under 20 pg/ml undergoing bilateral neck dissection (23), these 17 patients were considered to be lateral lymph node negative. We do however recognize that occult disease may not universally manifest with elevated calcitonin (5). Twenty-four patients underwent an additional lateral lymph node dissection, of whom 21 had confirmed histologic lateral lymph node metastases. Three patients did not show evidence of lymph node metastases on histology and were therefore analyzed within the node negative group.

Log expression values of miR-183 and miR-375 were compared between groups. Higher expression
of these miRNAs was associated with lateral lymph node metastases (Fig. 3a, b; \( P < 0.001 \) and \( P = 0.001 \), respectively).

**MiR-183 and miR-375 prediction of high-risk mutations in HMTC**

Nineteen HMTC patients in the validation cohort were divided into high risk RET mutations (\( n = 4; \) ATA risk C and D) and low risk RET mutations (\( n = 15; \) ATA risk A and B) based on the American Thyroid Association (ATA) risk levels (24). MiR-183 and 375 mean log expression values
were higher in high versus low risk HMTC mutations but these differences were not significant ($P = 0.09; 0.2$).

**MiR-183 and miR-375 predict residual disease, metastases, and mortality**

Further analysis revealed significant associations between miRNA expression and other clinical outcome measures. MiR-183 and 375 expression log values were significantly higher in patients with residual disease ($P = 0.001; 0.003$) and distant metastases ($P = 0.003; 0.001$). Expression also correlated strongly with death due to disease ($P = 0.01; 0.011$). These results associate miRNAs-183 and 375 with clinically aggressive disease.

**Comparison of log expression values of miR-183 in TT cells to mean of SMTC and HMTC**

The expression level of miR-183 in the TT cell line was measured using qPCR and compared with the expression levels seen in SMTC and HMTC tumor samples ($n = 45$). The log expression value in the TT cell line was higher than the mean of all sporadic and hereditary samples (Supplementary Figure S1). The high expression of miR-183 facilitated assays using the miR-183 antagonist in the TT cell line.

**Cell proliferation assay**

To investigate the effect of forced repression of miR-183 in TT cells, the CellTiter 96AQueous One Solution Cell Proliferation Assay (Promega) was used. This is a colorimetric method for determining the number of viable cells in proliferation or cytotoxic assays.

TT cells were transfected with miR-183 antagonist (100 nmol/L) for 24 hours and then treated with CellTiter 96AQueous One Solution reagent (20 µl; Promega) for 1 hour. There was a significant decrease in the number of viable TT cells at 24 hours (pooled data from 3 independent experiments carried out in triplicate) compared with the scrambled control, anti-miR-NC1 (Fig. 4).

**Effect of miR-183 antagonist on cell proliferation and cell death in TT cell line**

To further characterize the effect of miR-183 antagonist on cell viability and to determine any functional effect, Annexin V cell surface expression was investigated to examine if the decrease in number of viable cells was due to induction of apoptosis. There was no significant increase in Annexin V cell surface expression at 16 and 24 hours after transfection with the anti-miR-183 compared with the scrambled control, anti-miR-NC1 (Supplementary Figure S2a).

To determine if anti-miR-183 transfection in the TT cell line would affect cellular proliferation, cell-cycle analysis was carried out. DNA synthesis was not reduced in miR-183 antagonist transfected cells compared with scrambled control anti-miR-183, at 16 and 24 hours, as showed by propidium iodide staining (Supplementary Figure S2b).

**Knock-down of miR-183 expression increases LC3B protein expression**

We hypothesized that cell death was occurring via induction of autophagy in the miR-183 antagonist treated TT cells as there was no significant increase in Annexin V cell surface expression, a marker for apoptosis. To investigate for this, changes in the expression of programmed cell death 4 protein (PDCD4-pro-apoptotic protein) and microtubule associated protein 1 light chain 3B (LC3B1) were investigated by western blotting and densitometry.

After anti-miR-183 transfection for 24 hours, there was a significant increase in LC3B1 expression but no change in expression of PDCD4, when compared with the scrambled negative control, anti-miR-NC1 (Figure 5: representative blot from 3 independent experiments, carried out in triplicate; Figure 6: densitometry results). α-tubulin was used as a loading control.
MTC accounts for 5% to 10% of all thyroid cancers and occurs in two forms: HMTC and SMTC. Mutations in the RET protooncogene are responsible for HMTC and have been well characterized to date. Less is known about drivers of tumorigenesis in the more common form of disease, sporadic MTC.

Somatic RET mutations, principally M918T, have been detected in 23% to 85% of SMTC patients (25–27). However, this has not been consistently seen across studies (28, 29). SMTC, with somatic M918T mutation, correlates with the presence of positive lymph nodes at diagnosis, reduced survival, and a higher probability of persistent disease after surgery (26). To date, there are no identified molecular or genetic biomarkers to diagnose SMTC at an early stage to facilitate a preclinical diagnosis, predict clinical outcome, and identify possible therapeutic targets.

With this in mind, we conducted a miRNA microarray analysis in a cohort of SMTC and HMTC tumor samples. These analyses typically compare malignant tissue with either benign or normal tissue of the same organ to detect miRNAs implicated in the disease process. This is not feasible for C-cells of the thyroid from which MTC arises and we therefore chose to compare SMTC against HMTC.

To our knowledge, this study is the first of its kind and identified 10 differentially expressed miRNAs (4 overexpressed and 6 underexpressed in SMTC vs. HMTC) in MTC. Four of ten (2 overexpressed and 2 underexpressed in SMTC vs. HMTC) were selected for further validation using qPCR as outlined previously in Materials and Methods.

We found miR-183 to be overexpressed in SMTC compared with HMTC and the result was validated in the larger cohort (n = 45) using qPCR. MiR-183 was also overexpressed in the TT cell line compared with our cohort of HMTC.

MiR-183 is a member of a family of three miRNAs, the others being miR-182 and miR-96, and is located on the 7q31–34 locus (30). These miRNAs have been found to be overexpressed in colorectal cancer (31) and melanoma (32), showing its oncogenic potential in a variety of neoplastic subtypes. Recently Li and colleagues (18) have shown that miR-183 inhibits apoptosis in human hepato-cellular cells by repressing the expression of the proapoptotic gene (PDCD4). This miRNA may thus play an important role in the development of hepatocellular carcinoma in humans. However, miR-183 has also been shown to have a tumor suppressor effect in lung cancer cells (19). This opposing dual function may be explained by the target tissue-type and miRNA targets expressed in that tissue but is an area requiring further investigation of mechanistic pathways.

Following validation of selected miRNAs, correlation with clinical data was undertaken to identify miRNA biomarkers associated with aggressive tumor biology and related clinical outcome, such as lateral compartment lymph node involvement, residual disease, metastases, and death. Primary tumor expression values of miR-183 and 375 directly correlated with all examined clinical outcomes.

It could be argued that this is merely reinforcing evidence of a more aggressive disease phenotype in SMTC, particularly given higher expression levels in SMTC tumors. However, we failed to show any significant differences in clinical outcomes when we examined for this in our cohort of SMTC versus HMTC; although we acknowledge that there was a trend toward a higher mortality in our SMTC group.

The finding that miRNAs-183 and 375 predict for lateral node metastases requires validation in a larger cohort before they can become clinically useful, however this observation raises the possibility of using miRNA biomarkers to guide surgical decision-making. With regard to lymphadenectomy in MTC for example, the current ATA clinical practice guidelines for patients with clinically and radiologically negative lateral lymph nodes recommend total thyroidectomy and bilateral central neck dissection (24). In contrast, certain groups advocate prophylactic lateral neck dissection in MTC based on primary tumor size, basal calcitonin, and the status of central lymph nodes (6, 23, 33). The miRNA expression status of miR-183 and miR-375 in the primary tumor, once validated in a larger clinical cohort, may act to prompt prophylactic node dissection or at the very least, guide clinicians to more intensive postoperative surveillance of the lateral neck compartment for evidence of recurrent disease.

While suggestive, significant miRNA differences between high- and low-risk HMTC genotypes were not significant. This may be a result of our relatively small cohort of patients, with only 4 patients being defined as possessing a high-risk genotype. An extended validation cohort will be necessary to investigate for an association in future.

Given that miR-183 has been shown to have a functional role in tumorigenesis in other cancer types (18, 19), we investigated its functional role in the TT cell line. Knock down of miR-183 expression in the TT cell line was found.
to induce a significant decrease in the number of viable cells. Our results suggest that the mechanism of cell demise may be autophagy, an alternate pathway of cell death. A previous study in hepatocellular carcinoma showed miR-183 knockdown resulted in apoptosis (18). This difference in the mechanism of action could be explained by the difference in tissue type but requires further studies with a focus on the miRNA-autophagy interaction.

Conclusion

miRNAs have been shown to be important diagnostic and prognostic markers in cancer. Little is known about the biological drivers of malignancy in SMTC compared with HMTC. We report for the first time 10 miRNAs deregulated in SMTC versus HMTC. Overexpression of miRs-183 and 375 in MTC are predictors of lateral lymph node metastases, residual disease, distant metastases, and mortality. The utilization of these miRNAs as biomarkers of more aggressive disease has the potential to change current management guidelines, where an additional prophylactic lateral neck dissection may be considered appropriate in clinically and radiologically node negative MTC patients.

Functional experiments in the TT cell line have shown that forceful repression of miR-183 induces cell death, potentially via autophagy. Further work is required to investigate the role of these miRNAs as a therapeutic intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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MicroRNA Profiling of Sporadic and Hereditary Medullary Thyroid Cancer Identifies Predictors of Nodal Metastasis, Prognosis, and Potential Therapeutic Targets

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