A miRNA signature associated with human metastatic medullary thyroid carcinoma

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Abstract

MicroRNAs (miRNAs) represent a class of small, non-coding RNAs that control gene expression by targeting mRNA and triggering either translational repression or RNA degradation. The objective of our study was to evaluate the involvement of miRNAs in human medullary thyroid carcinoma (MTC) and to identify the markers of metastatic cells and aggressive tumour behaviour. Using matched primary and metastatic tumour samples, we identified a subset of miRNAs aberrantly regulated in metastatic MTC. Deregulated miRNAs were confirmed by quantitative real-time PCR and validated by *in situ* hybridisation on a large independent set of primary and metastatic MTC samples. Our results uncovered ten miRNAs that were significantly expressed and deregulated in metastatic tumours: *miR-10a*, *miR-200b/-200c*, *miR-7* and *miR-29c* were down-regulated and *miR-130a*, *miR-138*, *miR-193a-3p*, *miR-373* and *miR-498* were up-regulated. Bioinformatic approaches revealed potential miRNA targets and signals involved in metastatic MTC pathways. Migration, proliferation and invasion assays were performed in cell lines treated with *miR-200* antagomirs to ascertain a direct role for this miRNA in MTC tumourigenesis. We show that the members of *miR-200* family regulate the expression of E-cadherin by directly targeting *ZEB1* and *ZEB2* mRNA and through the enhanced expression of tumour growth factor β (TGFβ)-2 and TGFβ-1. Overall, the treated cells shifted to a mesenchymal phenotype, thereby acquiring an aggressive phenotype with increased motility and invasion. Our data identify a robust miRNA signature associated with metastatic MTC and distinct biological processes, e.g., TGFβ signalling pathway, providing new potential insights into the mechanisms of MTC metastasis.

Endocrine-Related Cancer

Introduction

Medullary thyroid carcinoma (MTC) is a neuroendocrine tumour thought to originate from neural crest parafollicular C-cells. Approximately 25% of MTC cases occur in the context of autosomal dominant multiple endocrine neoplasia syndrome type 2 (MEN2), MEN2A and MEN2B and familial MTC due to *RET* proto-oncogene germline mutations (Hofstra *et al*. 1994, Santoro *et al*. 1995, Eng *et al*. 1996, American Thyroid Association Guidelines Task Force *et al*. 2009). In hereditary MTC, a genetic diagnosis provides an opportunity for a prophylactic thyroidectomy and cancer cure. However, most cases of MTC are sporadic, presenting with metastatic disease at diagnosis, both in the local and in the regional lymph nodes, and in distant organs, especially in the lungs, liver and bones. While MTC is typically a slow-growing tumour, 40–50% of patients with metastatic disease have a 5-year overall survival rate. Nearly, all patients ultimately succumb to the disease (Hofstra *et al*. 1996). *RET* mutations are necessary but not sufficient for MTC tumourigenesis, and further molecular events (e.g. allelic imbalances between mutant and WT *RET*) are required for the development of MTC (Koch *et al*. 2001). The role of RET in MTC progression is still not well clarified, and in the metastatic context, RET has been demonstrated to cooperate with different signalling transduction pathways (Santarpia & Bottai 2013). However, tumour metastasis is a complex multistep process involving several types of alterations in cell adhesion, migration, survival, lymph/angiogenesis and proteolysis, which can ultimately lead to apoptosis, immune escape and homing to target organs. As a consequence, the expression profiles of specific genes involved in the control of the aforementioned functions and intracellular signal transducers are coordinately modified. The cascade of events that leads to metastasis in thyroid cancer, especially MTC, is still poorly understood. MicroRNAs (miRNAs), a class of non-coding RNA, have been shown to regulate gene expression through specific binding to complementary mRNA target sequences, typically in their 3′-untranslated regulatory regions, which results in the degradation of target mRNAs and/or the inhibition of the translational process (Lujambio & Lowe 2012). A possible role for miRNAs in tumourigenesis may be to function as tumour promoters or tumour suppressors by regulating multiple cellular processes, specifically targeting the genes involved in cancer cell biology (Santarpia *et al*. 2010, Iorio & Croce 2012). Indeed, increasing evidence supports the important role of miRNAs in almost all types of cancers, including thyroid cancer reviewed in Visone *et al*. (2007), Pallante *et al*. (2010), and Iorio & Croce (2012), although there is increasing evidence that supports an important and generalised role for miRNAs in mediating tumour metastasis (Santarpia *et al*. 2010, Valastyan & Weinberg 2011).

In this study, we identified a robust miRNA signature associated with MTC metastases. Analysis of a subset of these miRNAs confirmed their role in the pathobiology and progression of MTC, providing new insights into the complex mechanisms involved in the metastatic process of this tumour. In particular, the down-regulation of the *miR-200* family in MTC cells, which endogenously express high levels of these miRNAs, hinders the epithelial to mesenchymal transition (EMT), by down-regulating E-cadherin expression. *miR-200* down-regulation can cause an increase in tumour growth factor β (TGFβ)-2 and augmented migration and invasion potential of the tumour cells through the direct targeting of *ZEB1* and *ZEB2*. These results strongly suggest an important role for the *miR-200* family in regulating a shift in the MTC cell phenotype during cancer progression.

Subjects and methods

MTC tumour selection and miRNA expression profiling

Paired primary tumour and metastatic lymph node tissues were simultaneously obtained from six different sporadic MTC patients to reduce the potential for unrelated sample variability. A total of 12 samples, six primary and six paired metastatic tumours derived from adjacent metastatic lymph nodes, were used for miRNA expression array profiling (discovery data set). An additional 65 well-characterised MTC tumour samples, 31 primary and 34 metastatic tumours (validation data set), were obtained for confirmation studies (Supplementary Table 1, see section on supplementary data given at the end of this article). Clinical and pathological features of patients and tumours are reported in Supplementary Table 1. All MTC specimens were pathologically confirmed and further examined by immunostaining using at least two of the following neuroendocrine markers: calcitonin, chromogranin, synaptophysin and carcinoembryonic antigen. All patient samples were collected under an approved institutional board protocol at The University of Texas M.D. Anderson Cancer Center. RNA was extracted using miRVANA (Ambion, Foster City, CA, USA) or Trizol reagent

(Invitrogen) as recommended by the manufacturer, with minimal modifications. For array analysis, 720 ng primary/metastasis tumour RNA pairs were labelled with Hy3 and Hy5 fluorescent label using the miRCURY LNA Array power labelling kit (Exiqon, Denmark A/S, Vedback, Denmark), following the procedure described by the manufacturer. The Hy3-labelled samples and a Hy5-labelled reference RNA sample were mixed pair-wise and hybridised to the miRCURY LNA array version 10.0 (Exiqon), which contains more than 1200 capture probes covering all human, mouse and rat miRNA sequences annotated and registered in the miRBASE version 12.0 at the Sanger Institute (http://microrna.sanger.ac.uk/sequences/) and Exiqon miRPlus mature miRNAs. The hybridisation was performed according to the miRCURY LNA array manual using a Tecan HS4800 hybridisation station (Tecan, Grödig, Austria). After hybridisation, the microarray slides were scanned and stored in an ozone-free environment (ozone level below 2.0 ppb) to prevent potential bleaching of the fluorescent dyes. The LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., Santa Clara, CA, USA).

Statistical and bioinformatic analysis of microarray data

The quantified signals were normalised using the global locally weighted scatterplot smoothing (Lowess) regression algorithm on the background-subtracted data (background corrected Normexp with offset value 10) (Ritchie et al. 2007). Student's t-tests or Mann–Whitney U tests were performed to identify various levels of miRNA expression in the primary vs metastatic tumours. The miRNAs with $P < 0.05$ were considered to be significant. The miRNA hierarchical clustering analysis was performed using GeneSpring GX version 4.0 (Agilent Technologies, Inc. and Strand Life Sciences Pvt. Ltd., San Francisco, CA, USA). Single channel median corrected signals were scaled to the median values of all known miRNAs, with the threshold set to 1. The clustering algorithm used a Pearson uncentered distance metric and a complete linkage rule.

Validation of microarray data

MiRNA quantitative real-time PCR Expression of mature miRNAs was analysed by the TaqMan miRNA Assay (Applied Biosystems) using conditions defined by the manufacturer. Briefly, single-stranded cDNA was synthesised from 5.5 ng total RNA in a 15 μl reaction volume using the TaqMan MicroRNA Reverse

Transcription kit (Applied Biosystems). The reactions were first incubated at 16 °C for 35 min and then at 42 °C for 30 min, followed by an inactivation step (85 °C for 5 min). Quantitative PCR amplification of the cDNAs was performed using sequence-specific primers from the TaqMan MicroRNA Assay Human Panel (Applied Biosystems). The 20 μl PCR included 10 μl 2× Universal PCR Master Mix (no AmpErase UNG), 2 μl of each 10× TaqMan MicroRNA Assay Mix and 1.5 μl RT product with an amplification program of 95 °C for 9 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative expression level was computed using the $2^{-\Delta Ct}$ analysis method. To correct for variability among different samples, expression level of a target miRNA was normalized to the expression levels of two different reference genes (as genes found stably expressed across most of the samples), RNU6B (RNU6-6P) and miR-16. The Wilcoxon test was used for statistical analysis. Statistical significance between miRNA expression in the primary and metastatic tumours was set as 0.05. We performed a further comparison T-test analysis analysing the miRNA expressions of sporadic and hereditary tumours. Statistical significance was set at 0.05.

Tissue microarray and miRNA expression by in situ hybridisation Standard formalin-fixed paraffin-embedded (FFPE) MTC specimens were obtained from archived patient specimens representing the same tumours analysed by qRT-PCR. To generate the tissue microarray, 4 μm sections were H&E stained to identify representative regions for a core biopsy. Three core tissue biopsies were obtained from each tumour specimen representing different areas of the tumours. The presence of tumour tissue within the final arrayed samples was verified by H&E-staining. For the in situ detection of miRNA expression, 4 μm sections of the tissue microarray were deparaffinized in a xylene series and rehydrated through an ethanol series (100–25%). After performing a proteinase K digestion (30 μg/ml; Roche Applied Science) for 10 min and fixation in 4% paraformaldehyde, slides were prehybridised in hybridisation solution (50% formamide, 5× SSC, 500 μg/ml yeast tRNA, 1× Denhardt's solution) for 1 h and hybridised overnight with double digoxigenin (DIG)-labelled miRNA-locked nucleic acid probes (Exiqon). After stringent washes (50% formamide, 2× SSC) at a specific hybridisation temperature, chromogenic detection of signals was performed using an anti-DIG antibody (Roche, 1:400 dilution) and alkaline phosphatase-conjugated secondary antibody (Ventana) using NBT-BCIP as the substrate according to the manufacturer's instructions. We also used a double-DIG-labelled U6

snRNA probe as a positive control (Exiqon). Only the presence of cytoplasmic staining (mature miRNA) in tumour cells was recorded and classified as positive or negative. The sections were evaluated independently by two blinded and experienced pathologists according to the percentage of stained cells, with <20% of the cells stained was designated as low expression of miRNA, and more than 20% of the cells stained was designated as high expression of miRNA. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score.

Determination of labelling efficiency was performed for each single probe at different serial concentrations. Scramble probes were used as negative controls. MiRNA sequences probes are reported in Supplementary Table 2, see section on supplementary data given at the end of this article.

MTC cell culture

MTC TT and MZ-CRC-1 (MZ) cell lines, previously characterised by immunocytochemistry, RET gene mutation (Supplementary Figure 1, see section on supplementary data given at the end of this article) and Affymetrix Array SNP6.0 analysis, were grown in F-12K medium (TT) and in DMEM (MZ) supplemented with 10% foetal bovine serum using standard cell culture methods. Adenoviral vector infection and antagomir transfection efficiencies were tested using a Luciferase assay as described previously (Takahashi et al. 1985). Antagomir probes for miR-200b and miR-200c (Exiqon) were transfected using 10 μl Lipofectamine 2000 (Invitrogen, Inc.) and 20 nM mercury LNA probes according to the manufacturer's instructions. Total RNA and protein were isolated at 48–72 h.

Protein expression analysis

Western blot and confocal immunofluorescence

Western blot analysis: protein lysates (40 μg) were separated on 10–12% gradient polyacrylamide gels (Bio-Rad) and transferred to PVDF membranes for standard western blot analysis. Antibodies against epithelial and mesenchymal markers included the following: anti-E-cadherin (Cell Signaling Technology, Danvers, MA, USA), anti-N-cadherin (BD, Franklin Lakes, NJ, USA), anti-β-catenin (Millipore, Billerica, MA, USA), anti-vimentin (Abcam, Cambridge, UK), anti-ZEB1/2 (Cell Signaling Technology, Inc.; and Novus Biologicals, Littleton, CO, USA), anti-TGFβ-1 (Abcam), anti-TGFβ-2 (Abcam), anti-TWIST1 (Santa Cruz, Dallas, TX, USA) and anti-SNAI1 and 2 (Abcam).

Antibody interactions were detected by an IR-conjugated anti-mouse antibody using an Odyssey Imaging System and Analysis Software (Li-Cor Bioscience, Lincoln, NE, USA). Normalisations were performed to β-actin. Confocal immunofluorescence: MTC cells were plated onto Lab-Tek 8-chamber slides (Thermo Scientific, Nunc, NY, USA). Following transfection (48–72 h), immunofluorescence was performed according to Cell Signaling Technology's protocol. Antibodies for β-catenin (L54E2, Cell Signaling), E-cadherin (Cell Signaling), anti-N-cadherin (Cell Signaling) and fluorochrome-conjugated secondary antibodies were used. Confocal microscopy was performed with an Olympus FV 500 laser confocal IX-71 microscope.

Cell proliferation and matrigel invasion assays

The effect of miR-200 expression on cell proliferation was examined using an Aqueous One Solution Cell Proliferation Assay (Promega), which employs the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). Stably transfected MZ and TT cells were cultured in 96-well plates with the addition of MTS at the indicated periods, followed by absorbance readings at 490 nm to determine the relative cell viability. The invasion properties of stably transfected MZ and TT cells were examined as described previously using the standard Matrigel trans-well assay (Molina et al. 2010).

Immunohistochemistry assays

Immunohistochemistry was performed on 4 μm FFPE tumour tissues for TGFβ-1 (Ab28381), TGFβ-2 (Ab36495), E-cadherin (Ab1416) and vimentin (Ab8069). Positivity for the markers was defined based on staining intensity and tumour cellularity.

Cell adhesion

Electron microscopy MTC cell line samples were fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 h. After fixation, the cells were washed and treated with 0.1% Millipore-filtered cacodylate-buffered tannic acid, post-fixed with 1% buffered osmium tetroxide for 30 min and stained en bloc with 1% Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated and embedded in LX-112 medium (polymerised at 70 °C for 2 days). Ultrathin sections were cut on a Leica Ultracut microtome (Leica, Deerfield, IL, USA), stained with uranyl acetate and lead citrate in a Leica EM Stainer and examined with a JEM 1010 transmission

electron microscope (JEOL, USA, Inc., Peabody, MA, USA) at an accelerating voltage of 80 kV. Digital images were obtained using an AMT Imaging System (Advanced Microscopy Techniques Corp., Danvers, MA, USA).

Bioinformatics

miRNA targets, gene ontology and pathway analysis Initially, three programs, PicTar (http://pictar. mdc-berlin.de/), miRanda (http://www.microrna.org/micro-rna/home.do) and TargetScan (http://www.targetscan. org/), were used to predict the mRNA targets of the ten miRNAs. To strengthen our search, we used an additional bioinformatic approach employing the intersections of two or more databases with GOmir software (Roubelakis et al. 2009). GOmir target names were then cross-referenced using DAVID and Entrez Gene (http://www. ncbi.nlm.nih.gov/sites/entrez?db=gene) to obtain Entrez Gene ID numbers. Entrez Gene ID lists were used to perform pathway analysis (DAVID and Ingenuity Pathway analysis). GO analysis was performed using the GO annotations downloaded from http:// geneontology.org/.
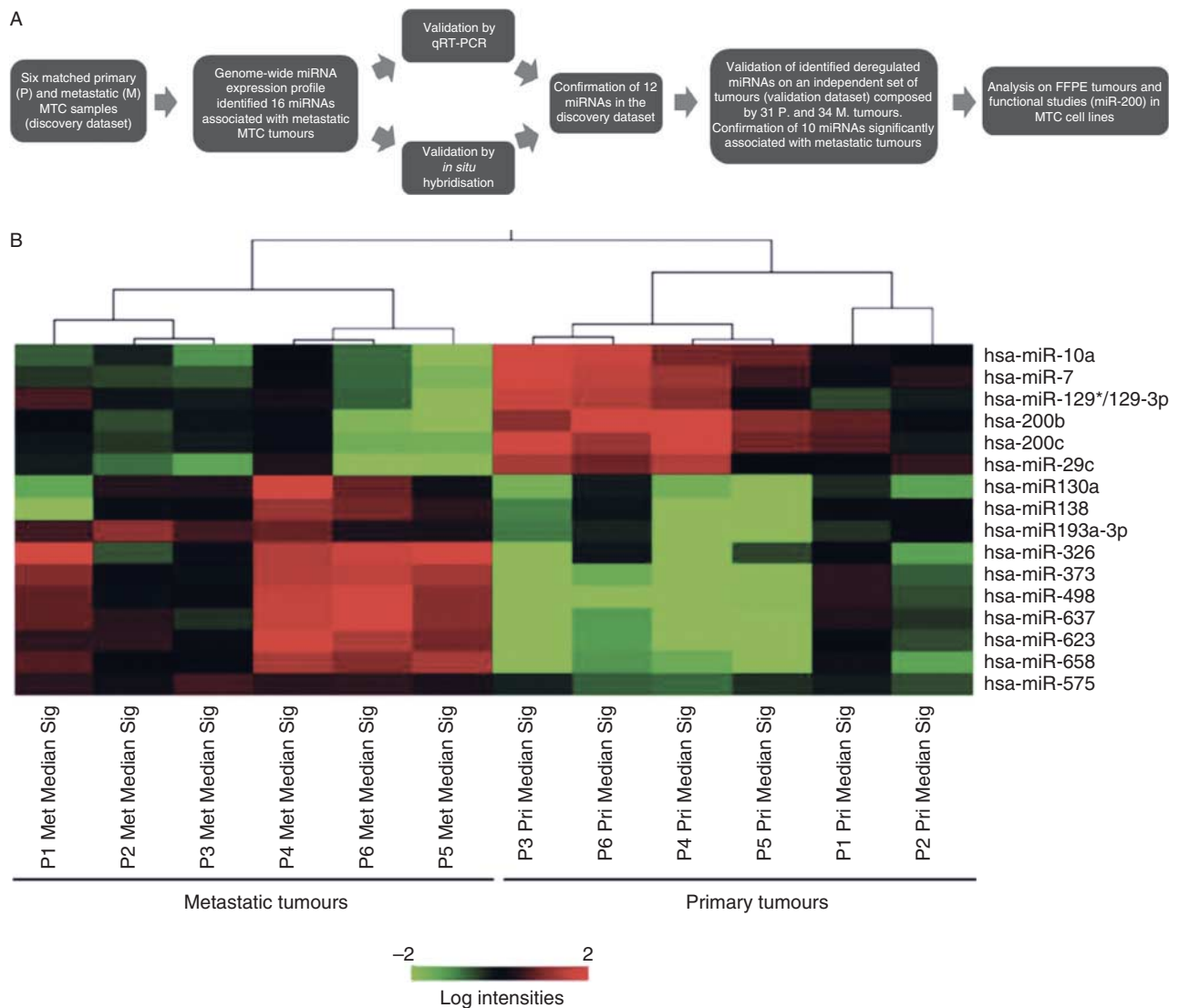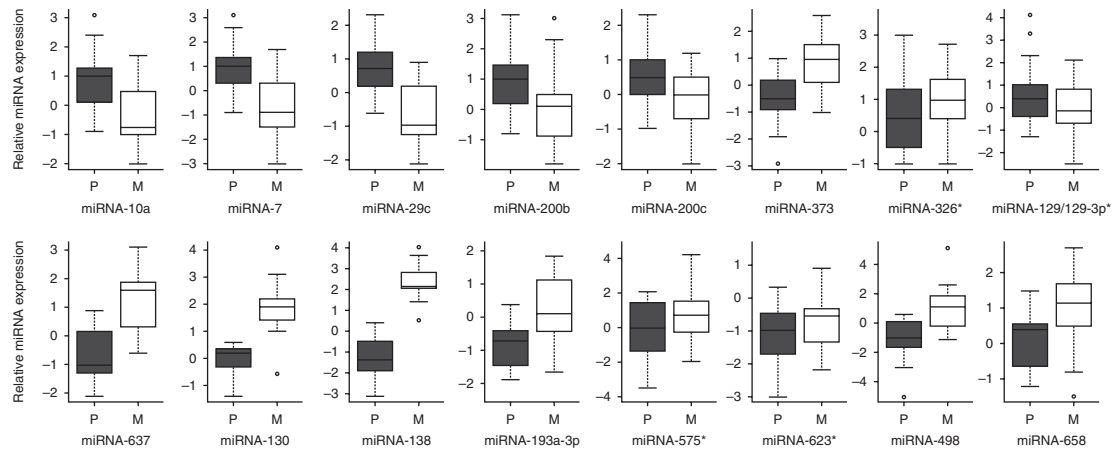

Figure 1

Experimental design and flow chart showing the identification of a metastatic miRNA signature in medullary thyroid carcinoma (A). MiRNA expression profiling of matched primary and metastatic tumour samples (discovery data set). Supervised hierarchical cluster one-minus correlation, average for linkage of class prediction, identified 16 significant deregulated miRNAs in metastatic samples (B).

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Figure 2

Quantitative real-time PCR analyses on an independent set (validation data set) of primary (P; grey box plots) and metastatic tumours (M; white box plots) demonstrated and confirmed 12 significant miRNA genes associated with metastatic tumours. *MiR-129/129-3p*, *miR-326*, *miR-575* and *miR-623* were not significant differentially expressed between the P and M groups (asterisks indicate the four miRNAs not statistically significant).

Results

A miRNA expression profile signature of metastatic MTC

Among several cancer subtypes, MTC represents 2–8% of thyroid cancer cases. Despite this low frequency, there is a disproportionate number of deaths from MTC compared with other thyroid cancers. Indeed, 50% of MTC patients with metastatic or recurrent disease have a survival rate of 4 years. To assess the potential role of miRNAs in MTC tumour progression and metastasis, we applied a multistep approach that began with an array expression analysis of paired patient samples (Fig. 1A). We validated the expression of deregulated miRNAs in an independent set of MTC tumours (Supplementary Table 1) and identified a robust miRNA signature associated with metastasis. Furthermore, we confirmed the reliability of our genes by *in vitro* analysis. It is important to note that unlike many tumours, normal tissue in MTC is not easily available for direct comparison because of the rarity of normal thyroid C-cells. Comparative analysis of the miRNA expression arrays between primary and metastatic MTC samples identified 16 miRNAs with significant ($P<0.05$) differences greater than twofold (Fig. 1B). Within this group, we found six miRNAs that were selectively down-regulated in metastatic tumours: *miR-7*, *miR-10a*, *miR-29c*, *miR-129/129-3p* and *miR-200b/-200c*. Ten miRNAs were found to be up-regulated: *miR-130a*, *miR-138*, *miR-193a-3p*, *miR-326*, *miR-373*, *miR-498*, *miR-575*, *miR-637*, *miR-623* and *miR-658*. To validate this miRNA signature, we performed qRT-PCR on an independent sample set (validation data set) (Fig. 2). While miRNA expression almost invariably correlated with the array results, qRT-PCR analysis showed a statistically significant deregulation of only 12 miRNAs (Fig. 2). Four miRNAs (*miR-129/129-3p*, *miR-326*, *miR-575* and *miR-623*) were found to be non-significant in the two cohorts of tumour samples (primary and metastatic tumours). Moreover, sporadic and hereditary MTC tumours in the validation data set showed not significant differences in terms of miRNA expressions. *In situ* hybridisation analysis was performed to confirm cancer cell-specific expression of miRNAs (Fig. 3). An important aspect of our study is that it shows how selected miRNAs are expressed in specific tumour regions or cell types (Fig. 3). The *in situ* hybridisation confirmed the specificity of only 12 miRNAs for the MTC cancer cells (Fig. 3). Four miRNAs (*miR-326*, *miR-623*, *miR-637* and *miR-658*) were not tumour specific. When these four miRNAs are taken together with *miR-129/129-3p* and *miR-575* (excluded by qRT-PCR), six of the original 16 miRNAs were excluded from further analysis. It is noteworthy that specific miRNAs analysed (e.g. *miR-7*, and *miR-200b/c*) were also expressed in C-cell hyperplasia (Fig. 3).

The miRNA metastatic signature identifies gene targets involved in metastatic functions

The miRNA metastatic signature of ten miRNAs identified candidate target genes coupled to cell-to-cell communication, cell adhesion, the EMT, tight gap junctions and extracellular matrix (ECM) interactions (see Supplementary Figure 2A and B, see section on supplementary data
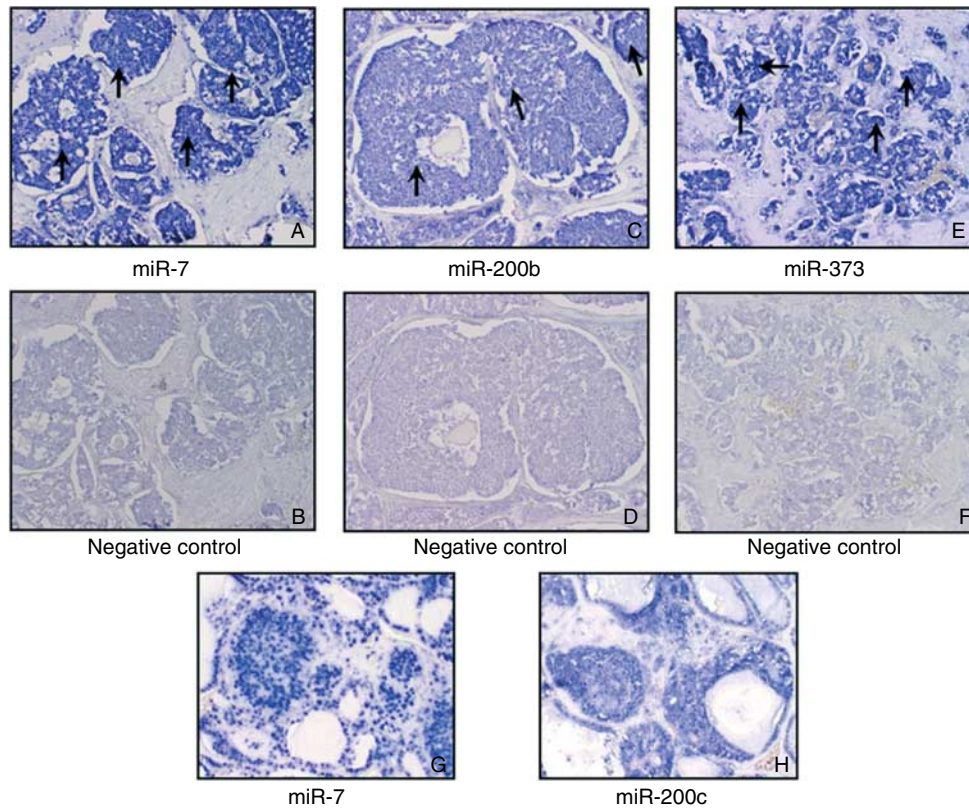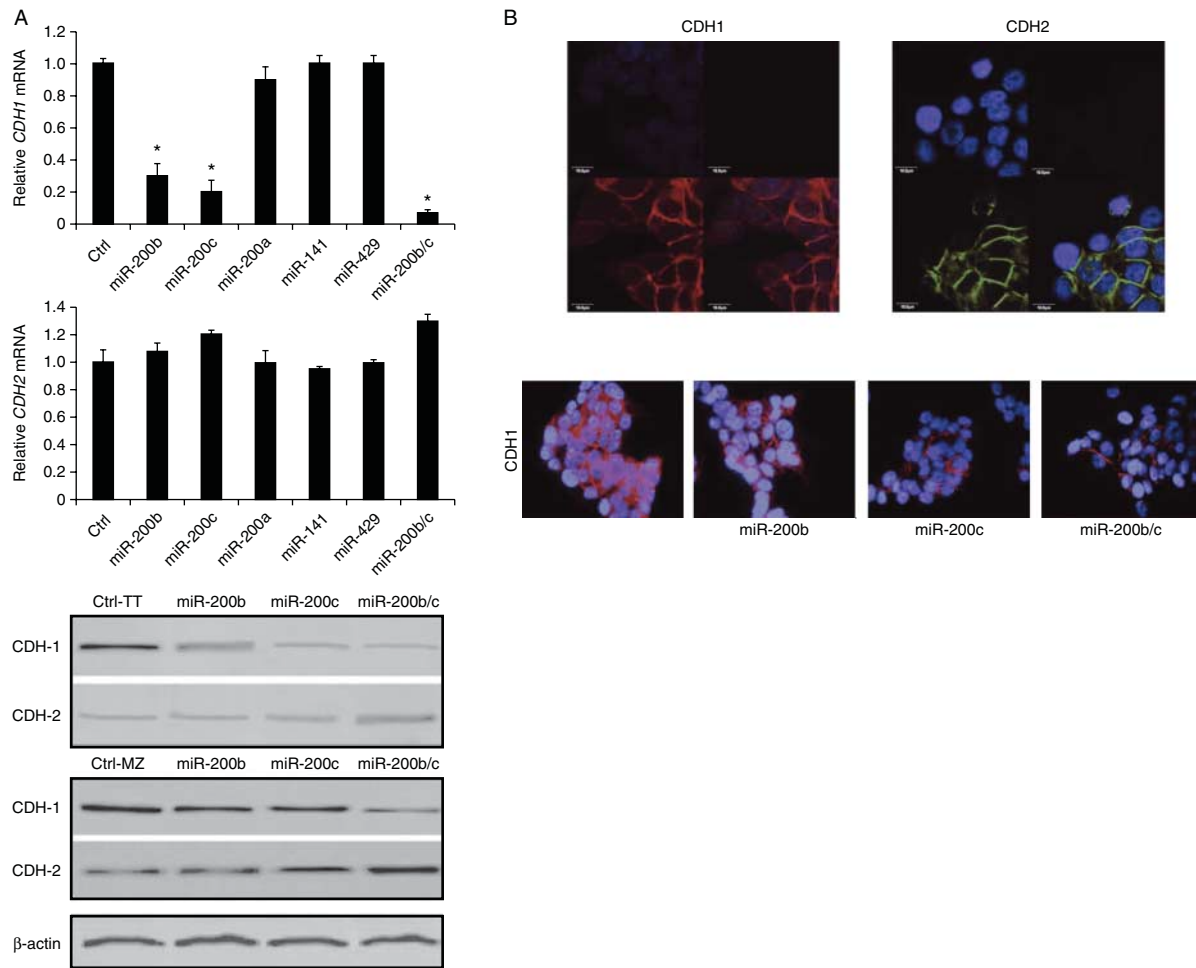
Figure 3

Detection of miRNA transcripts by *in situ* hybridisation in medullary thyroid neoplastic cells. *In situ* hybridisation analyses using double DIG-conjugated, LNA-modified DNA probes complementary to miRNAs were performed on 4 μm formalin-fixed paraffin-embedded sections of MTC specimens confirming the expression of miRNAs in neoplastic cells for only ten miRNAs. *In situ* hybridisation shows the expression of *miR-7*, *miR-200b* and *miR-373*, confined to tumour cells (arrows) in three different MTC cases (panels A-C-E). Negative controls for each tumour are also reported (panels B-D-F). The *in situ* hybridisation is a quantitative but also an essential qualitative technique, which allows the exact cellular localization for each miRNA identified. Figure 3 also shows the expression of *miR-7* and *miR-200c* in two different cases of C-cell hyperplasia (panels G and H).

given at the end of this article). These capabilities are specifically involved in the metastatic process. Using the intersections of two or more miRNA target databases, we obtained 1159 mRNA targets as potential downstream effectors of this subset of miRNAs. Many of these mRNAs have been well recognised as molecular linkers in the maintenance of cytomorphology, cell adhesion, cell movement and ECM remodelling. As a result, it is conceivable that this ten miRNA gene signature may contribute to the regulation of important aspects of the MTC metastatic process through the regulation of genes and biological functions implicated in cancer progression. Gene Set Enrichment and Pathway Analysis identified potential target mRNAs involved in several and important gene pathways that may be linked to tumour invasion, metastasis and progression (Supplementary Figure 2A and B). TGFβ pathway is one of the most important signals regulated by the miRNA target population.

miR-200 and cell phenotype of MTC

Members of the *miR-200* family have been previously identified as playing a key role in tumour progression through the regulation of the EMT process (Mongroo & Rustgi 2010). This gene family, which exists as two genome clusters (Supplementary Figure 3, see section on supplementary data given at the end of this article), was among those miRNAs significantly repressed in MTC metastasis, suggesting an equivalent role in this tumour type. In order to address this possibility, we showed that members of the *miR-200* family prevent EMT by regulating E-cadherin expression (Fig. 4A and B) through the direct targeting of ZEB1 and ZEB2 (Fig. 5A and C), but not that of other EMT markers such as SNAI1, SNAI2 or TWIST (TWIST1) (data not shown). Furthermore, down-regulation of the *miR-200* family in MTC cell lines, which express endogenous levels of these miRNAs, induced EMT.

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Figure 4

Functional analyses with the antagomirs for miR-200b, showing that members of this family are able to significantly regulate the expression of E-cadherin and slightly affect the expression of N-cadherin in MTC cell lines (A). Quantitative real-time PCR and western blotting analyses showed that E-cadherin (CDH1) was significantly regulated by *miR-200b* and *-200c* (A). There was an additive effect of the two identified *miR-200b* and *-200c* on the regulation of E-cadherin expression (A). Asterisks indicate statistical significance. Confocal microscopy confirmed the presence of both E-cadherin (CDH1) and N-cadherin (CDH2) in MTC cell lines and the regulation of E-cadherin expression by *miR-200* (B) with an additive effect by *miR-200* family members (B).

In fact, we observed concurrent down-regulation of E-cadherin expression with up-regulation of vimentin, TGFβ-1 and TGFβ-2 (Fig. 5B and C), thus increasing the invasion potential of MTC tumour cells. Interestingly, *ZEB1* and *ZEB2* expression is similarly regulated in transfected MTC and anaplastic thyroid carcinoma cell lines, suggesting the important role of these genes in tumour aggressiveness (Supplementary Figure 4, see section on supplementary data given at the end of this article). These results strongly suggest an essential role for the *miR-200* family in repressing EMT and cancer progression in MTC. Changes in mRNA abundance were confirmed by qRT-PCR analysis, and western blot analysis clearly demonstrated the effect of *miR-200* on the protein expression of these tumour markers (Figs 4A and 5A, B and

C). Furthermore, transfected TT (Fig. 5D) and MZ-CRC-1 (Fig. 5G) MTC cells with *miR-200* showed a clear difference in cell phenotype, with cells acquiring a typical spindle shape (Fig. 5D and G) and a loss of cell adhesion (Supplementary Figure 5). The EMT phenomenon with cells with spindle-mesenchymal phenotypes is often visible in metastatic MTCs showing an aggressive behaviour (Supplementary Figure 6). Using an *in vitro* invasion assay, the *miRNA-200* transfected MTC cells showed a significant increase in invasion compared with the controls (Fig. 5E and H), with no evident effect on cell proliferation (Fig. 5F and I). To support our cellular data, by IHC assay, we stained our discovery data set and several primary and metastatic tumours (validation data set), confirming the higher expression of vimentin (Fig. 6A and B),
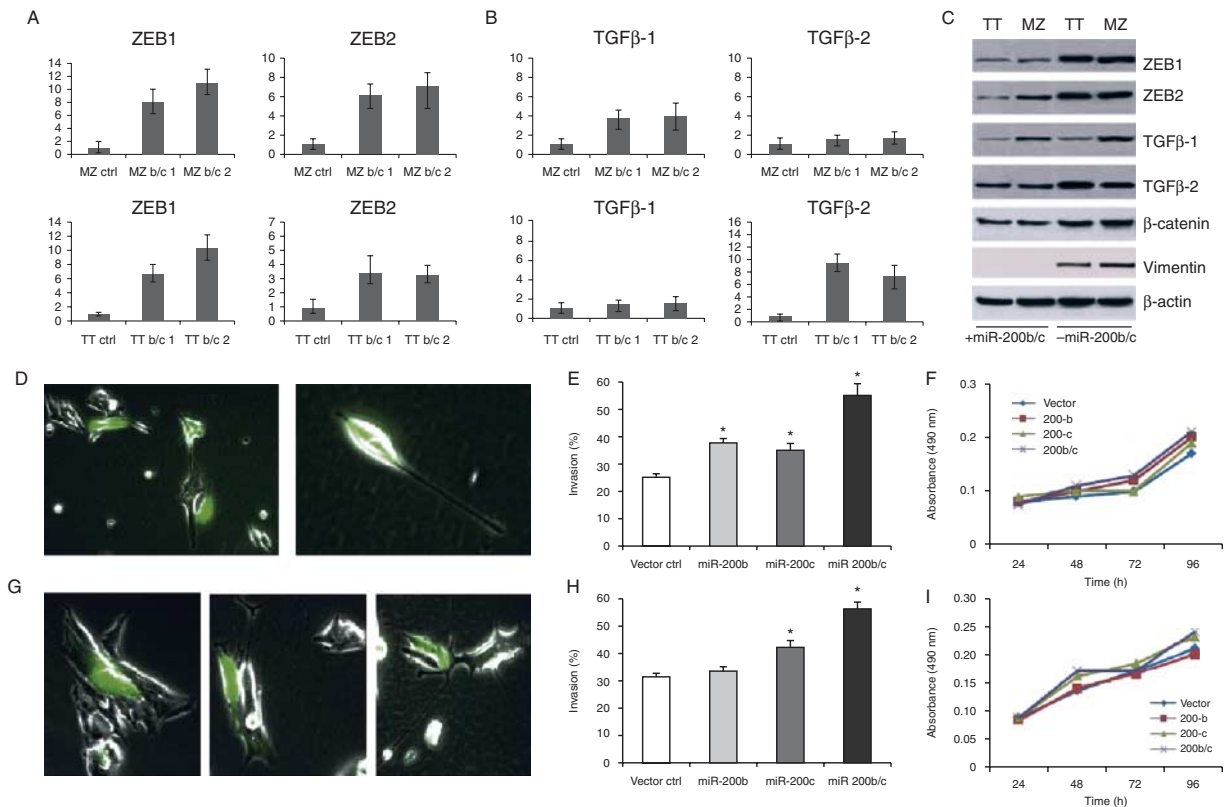
Figure 5

MiR-200 regulates the epithelial to mesenchymal transition and the metastatic features of medullary thyroid carcinoma cells. *MiR-200* modulates E-cadherin transcriptional repressors *ZEB1* and *ZEB2* in MTC cell lines (A and C). *MiR-200* significantly affects the expression of TGFβ-1 and TGFβ-2 (B and C). Moreover, down-regulation of *miR-200* in MTC cells led to an increase in vimentin, a well-established mesenchymal marker (C), whereas β-catenin expression was not affected (C). *MiR-200* affects cell shape (D and G), migration and invasion (E and H) but not proliferation (F and I) in MTC TT and MZ-CRC-1 cell lines, respectively. Neoplastic cells undergo a shift from an epithelial to a mesenchymal phenotype through the regulation of important EMT signals.

lower expression of E-cadherin (Fig. 6C and D) and higher expression of TGFβ-1 (Fig. 6E) and TGFβ-2 (Fig. 6F) in metastatic tumours. Of note, the significant increase in the expression of TGFβ-2 in metastatic tumour samples ($P=0.0062$, Table 1) and its inverse correlation with *miR-200* expression ($P=0.005665$, Supplementary Figure 7), which provides a strong support for a crucial role played by the miR-200/TGFβ axis during MTC progression.

Discussion

For nearly all tumour types, metastases correlate with poor prognosis and outcome in cancer patients, and they are ultimately responsible for most cancer-related deaths (Larimore & Wells 1997). Metastases arise following the spread of cancer cells to distant organs; therefore, the identification of mechanisms that control the progression of this process is critical for discovering a cancer cure. Genomic studies on thyroid cancers of follicular origin

have identified several deregulated miRNA genes that are gaining potential utility as biomarkers, but their specific role in metastasis is less clear (Nikiforova *et al.* 2008, Pallante *et al.* 2010, de la Chapelle & Jazdzewski 2011, Dettmer *et al.* 2013, Jacques *et al.* 2013, Swierniak *et al.* 2013). To date, few studies have reported deregulation of few miRNAs in thyroid cancer of parafollicular origin associated with prognosis (Abraham *et al.* 2011). Different reports demonstrated the role of *miR-200* in thyroid tumours of follicular origin and in undifferentiated histotypes (Braun *et al.* 2010). Thus, the role of miRNAs in thyroid cancer metastases remains largely unknown. To identify potential new biomarkers involved in the pathobiology and metastasis of MTC, we performed miRNA profiling in paired primary and metastatic tumour samples. Our combined data set, which included array-based identification followed by qRT-PCR and *in situ* confirmation, uncovered ten miRNAs with significant deregulation associated with metastatic MTC tumours.
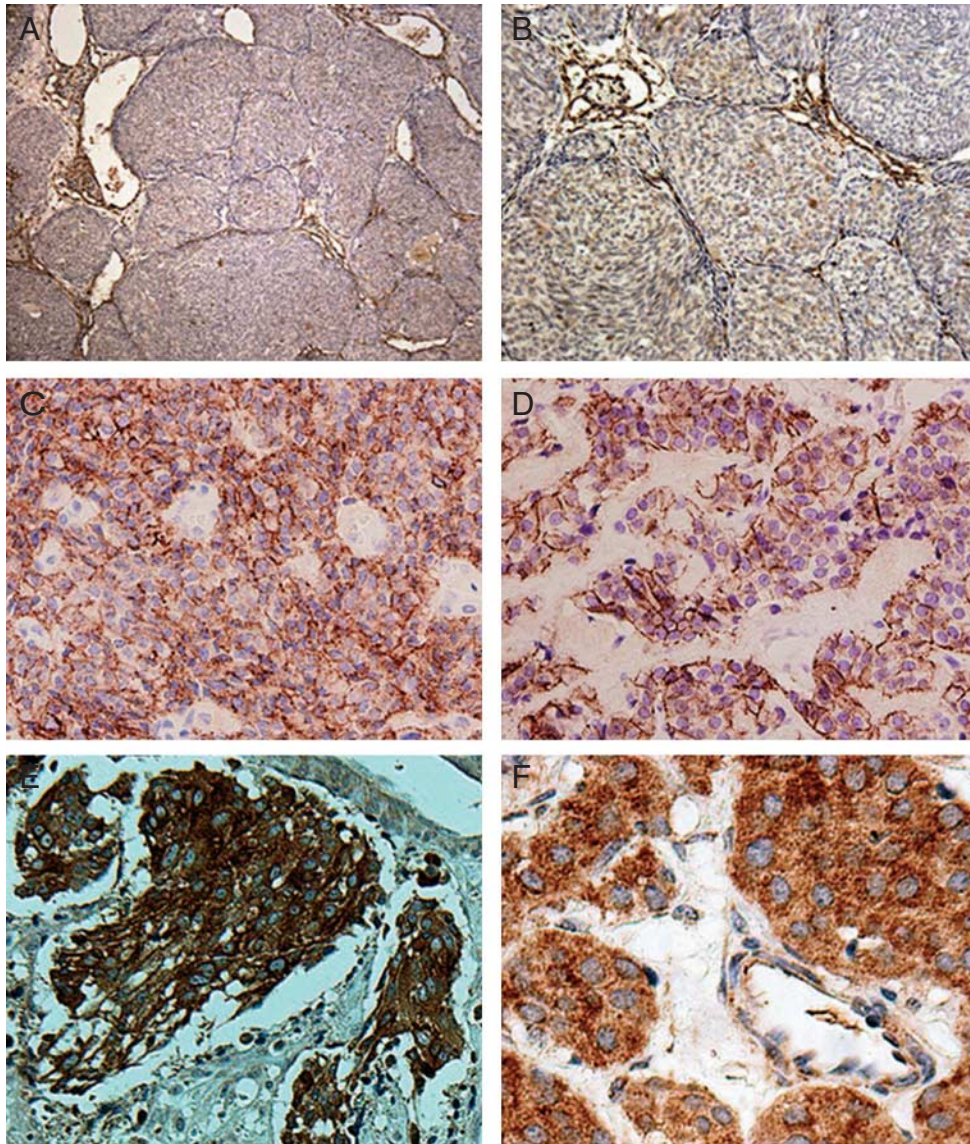
Figure 6

Immunostaining for vimentin in a metastatic MTC tumour at two different magnifications (panels A and B). In a consecutive series of primary and metastatic MTC samples (the validation data set), the expression of vimentin was significantly higher in the metastatic tumour sites ($P=0.004567$). The expression of E-cadherin in a paired primary and metastatic MTC tumour (panels C and D respectively); this marker was expressed in both tumour components but with a trend of loss of expression in the metastatic component. The same trend for E-cadherin was seen in the validation MTC data set. TGFβ-1 (panel E) and TGFβ-2 (panel F) protein expression in the validation data set analysed showed a higher expression of both markers in metastatic tumour sites, particularly the TGFβ-2 protein ($P=0.0062$). Of note, the inverse correlation between the expression of miR-200 and TGFβ-2 in the discovery ($P=0.046788$) and in the validation MTC tumour data sets ($P=0.005665$, supplementary file) indirectly confirming the regulation of TGFβ pathway by miR-200.

Therefore, by different molecular approaches that include cell-specific in situ hybridisation, we have demonstrated the MTC-specific expression of miRNAs, suggesting a role for this class of molecules in tumour progression. Noteworthy, we demonstrated the expression of specific miRNAs also in c-cell hyperplasia, suggesting a potential role of these miRNAs during MTC tumourigenesis as well.

Among the miRNAs identified, we were drawn to miR-200 because of its previously reported role in EMT in a variety of tumours (Burk et al. 2008, Korpal & Kang 2008), including thyroid tumours of follicular origin (Braun & Hüttelmaier 2011). By examining specific target gene expressions and functions related to metastasis through genetic assays on human tumour samples and in vitro

Table 1 Tumour growth factor β-1 and -2 protein expression analysed by immunohistochemistry in primary and metastatic medullary thyroid carcinomas demonstrated a significant association between high expression of TGFβ-2 and metastatic tumours. The same subset of tumours with high expression of TGFβ-2 showed a significant lower expression of *miR-200b/c* (supplementary files)

	Primary tumours N	Metastatic tumours N	*P* value
TGFβ-1[a]			
IHC (low)	14	12	0.2975
IHC (high)	13	21	
TGFβ-2[b]			
IHC (low)	19	9	0.0062
IHC (high)	12	25	

TGFβ protein 1 and 2 immunostainings on two data sets of medullary thyroid carcinomas. For pair-wise comparisons, immunostaining data were collapsed in low (0–2+) and high scores (3+) based on staining intensity and tumour cellularity.

[a]For TGFβ-1 protein expression analysis, five tumours were not available for IHC.

[b]TGFβ-2 staining intensity in primary and metastatic medullary thyroid carcinomas was significantly inversely correlated with *miRNA-200b* expression ($P=0.005665$, ANOVA test on linear regression models).

experiments on MTC cell lines, we were able to confirm the important role that those genes have in tumour progression. Although almost all members of the *miR-200* family (*miR-200a-b-c*, *miR-141*, and *miR-429*) have been shown to play a variety of crucial roles in metastatic processes (Sossey-Alaoui *et al.* 2009), *miR-200b* and *miR-200c* have been directly correlated with the regulation of E-cadherin and vimentin expression in epithelial cells (Ahmad *et al.* 2011), EMT and cell adhesion, while controlling stem cell-like properties in various types of tumours (Bracken *et al.* 2008, Gregory *et al.* 2008). Moreover, these two miRNAs have been shown to be markers of aggressiveness and chemoresistance in several tumours. The connection between *miR-200* family members and EMT has revealed a complex gene-regulatory network involved in tumour progression, involving E-box binding transcription factors such as *ZEB1/EF1*, *ZEB2/SIP1*, *SNAI1*, *SNAI2* and *TWIST1* (Korpal *et al.* 2008, Park *et al.* 2008, Kong *et al.* 2009). A common mechanism for these transcription factors is to repress E-cadherin expression through their direct binding to the E-box motifs within the promoter of E-cadherin, converting epithelial cells into mesenchymal or mesenchymal-like cells that are capable of acquiring an invasive phenotype (Peinado *et al.* 2007, Thiery *et al.* 2009). An EMT-like process occurs during the course of tumour progression (Gröger *et al.* 2012) when adherens junctions become partially dissociated and when epithelial cells switch from a collective invasion pattern to a detached and disseminated cell migration method (Wells *et al.* 2011). By array analysis, we found *miR-200b* and *miR-200c* to be significantly down-regulated in MTC metastases, consistent with reports showing a deregulation of this miRNA class during the metastatic process. Deregulation of other members of the *miR-200* family, even if not statistically significant (data not shown), was also associated with metastatic tumours, thus suggesting a potential common regulatory network controlled by this miRNA family.

To derive mechanistic insight into the role of *miR-200* in MTC, we examined the available cell culture models. Both human TT and MZ-CRC-1 MTC cell lines endogenously express *miR-200*, suggesting that these cancer cells, despite their embryogenetic origin, preserve an epithelial phenotype. Therefore, we treated the MTC cell lines with antagomirs for miR-200b and miR-200c and found that these cells progressively lost the ability to express E-cadherin, acquired the ability to express vimentin and showed a slight increase in N-cadherin levels. Loss of E-cadherin has been widely demonstrated to be associated with metastasis in several neoplastic conditions (Rodriguez *et al.* 2012), although the reduction of E-cadherin by itself seems not to confer full metastatic capabilities, at least in differentiated follicular thyroid cancers (Calì *et al.* 2007). Previous studies have linked *miR-200* expression with an epithelial phenotype and the ZEB family. Accordingly, expression of the *miR-200* family, particularly *miR-200b* and *miR-200c*, has been found in epithelial tissues (Kong *et al.* 2009, De Craene & Berx 2013) and is negatively correlated with *ZEB1* and *ZEB2* expression during embryonic development and metastasis (Brabletz & Brabletz 2010). ZEB family proteins are zinc finger and homeobox domains containing transcription factors. While ZEB proteins mainly function as transcriptional suppressors, they are able to activate transcription, dependent on the DNA context and cell type (Postigo 2003). One of the targets suppressed by ZEB proteins is E-cadherin. Down-regulation of E-cadherin is one of the hallmarks of the EMT, a critical feature of normal embryonic development, which is also utilised by malignant epithelial tumours to spread beyond their origin (Sánchez-Tilló *et al.* 2012). We demonstrated the functional involvement of *miR-200* in EMT and tumour invasion in MTC as well as direct regulation of ZEB1 and ZEB2 by *miR-200*. In our study, we identified the *miR-200* family as suppressors of EMT through direct targeting of *ZEB1* and *ZEB2*, which are well-known transcriptional repressors of E-cadherin. A noteworthy knockdown of

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miR-200 with antagomirs in the MTC cell lines resulted in ZEB1 expression similar to that found in anaplastic thyroid cancer cell lines. Importantly, we found that *miR-200* regulates the expression of additional 'metastatic' markers, such as TGFβ-1 and TGFβ-2, which have been previously shown to be associated with metastatic features in several tumours (Jakowlew 2006, Katsuno *et al.* 2013). The TGFβ pathway is involved in several processes, including cell proliferation, differentiation, migration and apoptosis (Elliott & Blobe 2005). Importantly, recent studies addressed an important role played by TGFβ in mediating stromal–epithelial interactions during cancer progression (Stover *et al.* 2007, Tait *et al.* 2007, Bierie *et al.* 2008). Interestingly, two recent papers reported that the abnormal activation of TGFβ signalling induces cancer progression in BRAF-mutant thyroid carcinomas (Eloy *et al.* 2012) and higher levels of TGFβ-2 expression in the sera of metastatic MTC patients (Broutin *et al.* 2011).

In our study, we also found of particular interest the concurrent presence of the two cadherins (E and N) in both MTC cell lines, which in some ways resemble the neurocrest origin and epithelial differentiation of these tumour cells. Metastasis in MTC may be caused by the reversible shifting status of these cells that undergo a change from an epithelial to a mesenchymal 'status,' perhaps acquiring stem cell-like features due to the presence of cancer stem cells in MTC, as recently reported (Zhu *et al.* 2010), which could be regulated in part by *miR-200* expression.

Several tumour cells, including MTC, are known to reactivate embryonic programs, such as EMT, to obtain a selective advantage, such as enhanced invasiveness (Sánchez-Tilló *et al.* 2012). As EMT is tightly linked to invasive carcinomas, molecular regulators of this process may serve as key points for therapeutic intervention. Furthermore, in this study, we demonstrated a robust and reliable miRNA signature and target-regulated genes associated with human metastatic MTCs.

miRNAs down-regulated in metastatic tumours

We found three additional miRNAs, *miR-7*, *miR-10a* and *miR-29c*, which were down-regulated in metastatic tumours compared with the parental primaries. While we did not perform more detailed mechanistic studies on these miRNAs, all have previously documented roles in tumourigenesis. *miR-7* has been recently demonstrated to regulate the expression of epidermal growth factor receptor (EGFR) in several cell systems (Webster *et al.* 2009, Giles *et al.* 2011). Notably, EGFR has been found to be

overexpressed in various types of thyroid carcinomas, including MTC, and to partially contribute to the regulation of the *RET* proto-oncogene (Gujral *et al.* 2008, Santarpia *et al.* 2009), a driver of thyroid tumourigenesis (Koch *et al.* 2001). The second down-regulated gene, *miR-10a*, which is located between the *HOXB4* and *HOXB5* genes on chromosome 17q21, has been demonstrated to be involved in the regulation of *HOXD4* in breast cancer cells (Tan *et al.* 2009) and in acute and chronic myeloid leukaemia (Agirre *et al.* 2008, Garzon *et al.* 2008). Moreover, this miRNA shows high-sequence homology with *miR-10b*, which has been shown to be an important regulator of breast cancer progression. Deregulation of *miR-10a* has been associated with metastases in other types of carcinomas (Veerla *et al.* 2009). Finally, *miR-29c*, together with other *miR-29* family members (i.e. *miR-29a* and *miR-29b*), up-regulates p53 levels and induces apoptosis in a p53-dependent manner. The *miR-29* family members also directly suppress p85 alpha (the regulatory subunit of PI3 kinase) and CDC42 (a Rho family GTPase), both of which negatively regulate p53 (Park *et al.* 2009) that has been shown to play an important role in cell proliferation and apoptosis in MTC. *MiR-29* targets expression of diverse proteins like collagens, and transcription factors, which may take part in abnormal migration, invasion or proliferation of cells and may favour development of cancer (Sengupta *et al.* 2008, Schmitt *et al.* 2013). Furthermore, members of the *miR-29* family can modulate the ECM by targeting tenascins and can be activated by interferon signalling, which suggests a role in the immune system and in the tumour microenvironment (Schmitt *et al.* 2013).

miRNAs up-regulated in metastatic MTC tumours

Five miRNAs – including *miR-130a*, *miR-138*, *miR-193a*, *miR-373* and *miR-498* – were found to be up-regulated in metastatic tumours. Therefore, the expectation is that there would be a coordinated down-regulation of their mRNA targets, which could include tumour suppressor genes as well as genes that promote apoptosis or cell migration or inhibit angiogenesis. *MiR-130a* was found to regulate angiogenesis by modulating the expression of the anti-angiogenic homeobox genes *GAX* (*MEOX2*) and *HOXA5* (Chen & Gorski 2008); *miR-130a* was also found to be deregulated in prostate tumours (Boll *et al.* 2013). Deregulation of *miR-138* has been shown to be associated with the overexpression of human telomerase RT protein in human anaplastic thyroid carcinoma cell lines (Mitomo *et al.* 2008) and to suppress invasion, promoting apoptosis

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in head and neck squamous cell carcinoma cell lines (Liu *et al.* 2009). *MiR-193* affects the activation of the caspase cascade. Putative targets of this miRNA include genes encoding death receptors, caspases and other apoptosis-related genes (Santarpia *et al.* 2010). Deregulation of *miR-373* has been shown to promote invasion and metastases in human breast cancer cell lines (Huang *et al.* 2008) and to behave as a tumour suppressor in oestrogen receptor-negative breast tumours (Keklikoglou *et al.* 2012). It has also associated with other types of tumours, such as prostate and ovarian cancers (Santarpia *et al.* 2010). *MiR-498* has been demonstrated to correlate with disease-free survival in patients with stage II colon cancer (Schepeler *et al.* 2008).

Of note, our data identify mRNA targets which are involved in important signalling pathways coupled to metastasis in MTC (Supplementary Figure 2A and B), such as the TGF-β, PI3K/Akt-mTOR, Wnt/β-catenin and NOTCH pathways. Some of these signalling pathways have already been shown to be involved in MTC progression (Cook *et al.* 2010), while others certainly deserve further investigation. Taken together, our results identify a group of metastasis-related gene targets and signals whose expression could be regulated by this subset of miRNAs. Several of these miRNAs have been shown to be associated with many aspects of the metastatic process in several types of cancers, thereby supporting an important regulatory effect of this miRNA signature in MTC metastasis and progression. Our data provide potential mechanistic insight into the role of miRNAs in regulating metastatic capabilities, such as cell adhesion, migration and invasion, maintenance of differentiation, ECM modelling and interactions, and the modulation of other important oncogenic signals, some of which have been previously reported to be involved in MTC progression. Because the *miR-200* family functions as putative tumour suppressors and represents biomarkers for aggressive thyroid cancers, the restoration of *miR-200* expression may have therapeutic implications for the treatment of metastatic and drug-resistant MTC tumours.

In conclusion, this study provides a leap forward in our understanding of metastatic MTC because it connects the expression levels of ten miRNAs and their associated biological functions and pathways with metastatic MTC. Further studies of this miRNA signature are required to fully understand the role of each miRNA in MTC metastasis. In the near future, some of these miRNAs might be useful as prognostic markers of MTC, providing an important avenue for developing new diagnostic and therapeutic targets for the treatment of metastatic MTC.

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