RESEARCH

Circulating miR-375 as a novel prognostic marker for metastatic medullary thyroid cancer patients

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Abstract

This study aimed to identify circulating miRNAs as novel non-invasive biomarkers for prognosis and vandetanib response in advanced medullary thyroid cancer (MTC) patients. We prospectively recruited two independent cohorts of locally advanced/metastatic MTC patients including a subgroup of vandetanib-treated subjects: a discovery cohort ($n=20$), including matched plasma/tissue samples ($n=17/20$), and a validation cohort, yielding only plasma samples ($n=17$). Plasma samples from healthy subjects ($n=36$) and MTC patients in remission ($n=9$) were used as controls. MTC ($n=17$ from 8 patients included in discovery cohort) and non-neoplastic thyroid specimens ($n=3$) were assessed by microarray profiling to identify candidate circulating miRNAs. qRT-PCR and *in situ* hybridization were carried out to validate the expression and localization of a selected miRNA within tissues, and qRT-PCR was also performed to measure miRNA levels in plasma samples. By microarray analysis, we identified 51 miRNAs differentially expressed in MTC. The most overexpressed miR, miR-375, was highly expressed by C cells compared to other thyroid cells, and more expressed in MTC than in reactive C-cell hyperplasia. MTC patients had significantly higher miR-375 plasma levels than healthy controls ($P<0.0001$) and subjects in remission ($P=0.0004$) as demonstrated by qRT-PCR analysis. miR-375 plasma levels were not predictive of vandetanib response, but, notably, high levels were associated with significantly reduced overall survival (HR 10.61, $P<0.0001$) and were a strong prognostic factor of poor prognosis (HR 6.24, $P=0.00025$) in MTC patients. Overall, our results unveil plasma miR-375 as a promising prognostic marker for advanced MTC patients, to be validated in larger cohorts.

Key Words

- medullary thyroid cancer
- prognostic biomarker
- circulating miRNA
- miR-375
- metastases

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Introduction

Medullary thyroid cancer (MTC) is a rare neuroendocrine malignancy with an incidence rate of 0.21 cases per 100,000 person-years (Wells *et al.* 2015, Randle *et al.* 2017). MTC originates from the calcitonin-producing C cells that represent the minor thyroid cell population (less than 0.1% of the glandular mass). About 25% of MTC cases are inherited as familial syndrome (FMTC) or as a component of type 2 multiple endocrine neoplasia syndromes (MEN2A, MEN2B) due to germline-activating mutations in the *RET* proto-oncogene (Leboulleux *et al.* 2004). The remaining 75% of MTC occurs sporadically, with somatic *RET*-activating mutations identified in 50% of cases, and somatic *HRAS* and *KRAS* mutations in 17–68% of *RET*-negative patients (Moura *et al.* 2011, Ciampi *et al.* 2012).

The 10-year overall survival is approximately 95% in patients with intra-thyroid disease, decreasing to 75% and 40% in patients with regional disease and distant metastases, respectively (Roman *et al.* 2006). Local invasiveness and regional lymph node metastases are present in more than 75% of cases at diagnosis, while diffuse distant metastasization, mainly in the liver, lung and bones, is observed in 4–17% of patients (Moley & DeBenedetti 1999, Hadoux *et al.* 2016). The standard surgical treatment, total thyroidectomy and neck dissection, leads to remission almost exclusively in patients with intra-thyroidal disease. Therefore, many patients require repeated interventions for persistent or recurrent MTC. The clinical course of these individuals is heterogeneous and difficult to predict: it varies from metastatic diffusion with rapid progression to death, to indolent/slow-growing disease that remains stable for long time or unexpectedly progresses even a decade after the first treatment.

After unsuccessful surgery, the therapeutic options are limited. Currently, locally advanced and metastatic MTC patients with progressive and/or symptomatic disease are candidates for systemic treatment with multitarget tyrosine kinase inhibitors (TKIs) as vandetanib or cabozantinib.

The monitoring of calcitonin (Ctn) and carcinoembryonic antigen (CEA) serum levels is a central tool in the follow-up of patients. The Ctn and CEA doubling times, when shorter than 6 months, are negative prognostic indicators of progressive disease. However, reliable estimates require repeated measurements over a minimum of 2 years (Wells *et al.* 2015). Furthermore, in patients treated with TKIs, fluctuation in these serum markers have been described regardless of tumor response (Kurzrock *et al.* 2013, Werner *et al.* 2015).

A better patients' prognostic assessment, able to differentiate indolent from aggressive MTC, could significantly improve the clinical management. In patients with progressive disease, the choice of the precise timing to start a systemic treatment, such as vandetanib or other TKIs, is crucial considering the side effects of these drugs and the life-long duration of therapy.

Moreover, there are no clinical neither biological data available to select patients who could benefit from TKI therapy. Thus, there is an urgent need to identify novel non-invasive biomarkers useful for prognosis stratification and to evaluate TKI therapy effectiveness in MTC patients.

In this scenario, circulating cell-free microRNAs (miRNAs) may provide a valuable resource. Nowadays, it is well established that miRNA deregulation is a hallmark of cancer and that distinct tumor types have a unique miRNA profile (Calin & Croce 2006). Tumor-derived miRNAs are present in the blood of cancer patients and may be useful, in patient management, as novel non-invasive biomarkers for diagnosis, prognosis and prediction of therapeutic response (Mitchell *et al.* 2008, Schwarzenbach *et al.* 2014). Nevertheless, miRNAs distinctive of MTC are still poorly characterized (Chu & Lloyd 2016) and, above all, circulating miRNAs have never been analyzed in this tumor type. We hypothesized that miRNAs overexpressed in tumor tissue may be good candidate circulating biomarkers in the plasma of MTC patients, as their levels may be associated with specific clinicopathological characteristics, response to therapy and patient outcome.

The aims of this study were to explore, for the first time, the expression of tumor-specific miRNAs in MTC patients' plasma samples and to investigate their value as biomarker for prognosis and vandetanib response in persistent/recurrent MTC patients. To these purposes, we analyzed a miRNA overexpressed by MTC cells in the plasma of two cohorts of locally advanced or metastatic MTC patients, including a subgroup of patients treated with vandetanib.

Materials and methods

Study subjects and study design

A total of 37 MTC patients with persistent or recurrent metastatic disease, 9 non-metastatic MTC patients in remission and 36 healthy controls were enrolled

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at Fondazione IRCCS Istituto Nazionale Tumori and at Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico between 2011 and 2015. The clinicopathological characteristics of the study population are detailed in Supplementary Tables 2 and 3 (see section on supplementary data given at the end of this article).

All MTC patients underwent total thyroidectomy associated in most cases with neck dissection and received a diagnosis of hereditary or sporadic MTC. Cases included persistent or recurrent MTC patients who had locally advanced/metastatic disease with at least one lesion measurable by standard imaging procedures. Subjects with a history of other malignant tumors were excluded. Systemic treatment and/or radiotherapy had to be discontinued at least 4 weeks prior to study entry. After enrolment, 25 patients with progressive (according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria (Eisenhauer *et al.* 2009)) and/or symptomatic disease received vandetanib. Treatment consisted of once-daily oral vandetanib, administered at approved doses.

Patients in remission and healthy subjects were also enrolled as controls. Remission was defined as no structural evidence of disease (NED) and undetectable Ctn and CEA within the reference range during the follow-up (median follow-up from surgery: 65 months). The study was approved by the Independent Ethical Committees of Istituto Nazionale Tumori and Ca' Granda Ospedale Maggiore Policlinico; each subject provided written informed consent upon recruitment.

Patients flow through the study is schematically represented in Fig. 1, whereas the number of samples used in each analysis is summarized in Supplementary Table 1. MTC patients were prospectively recruited in two independent cohorts: we first sequentially recruited 20 MTC patients in the 'discovery cohort' (MTC1), and subsequently, 17 MTC patients in the 'validation' cohort (MTC2) following the same inclusion and exclusion criteria. Healthy control subjects were selected to match MTC patients for gender and age distribution (HC1 ($n=19$) and HC2 ($n=17$)).

Formalin-fixed paraffin-embedded (FFPE) surgical specimens were obtained from 17/20 MTC patients of the discovery cohort (MTC1 FFPE). Five non-neoplastic thyroid and four thyroid with diagnosis of C-cell hyperplasya (CCH) FFPE were collected for comparison.

Baseline blood samples were obtained from all the subjects at study entry (T0). One patient of the discovery cohort was subsequently excluded from the study of

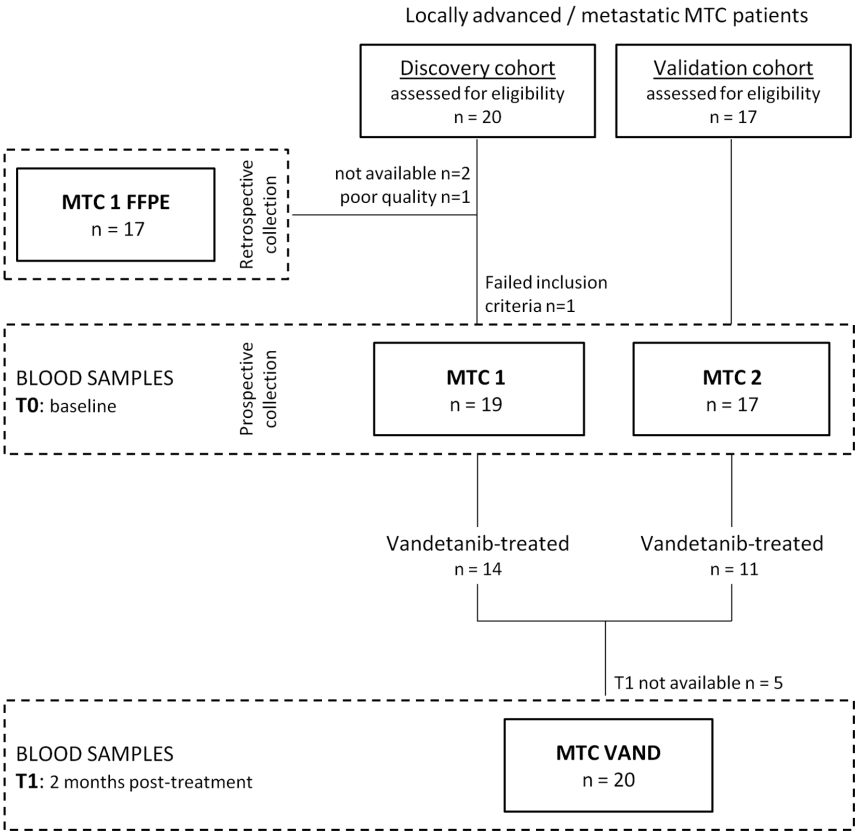

Figure 1
Flowchart of patients recruitment and samples collection.

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circulating miRNAs for a concurrent pancreatic tumor diagnosed after the enrolment. For the subgroup of patients treated with vandetanib, paired blood samples were collected at study entry (T0) and 2 months after the start of vandetanib treatment (T1). To investigate the variations of candidate circulating miRNAs after treatment, we considered only 20 patients (MTC VAND), out of the 25 treated with vandetanib, as 5 cases were excluded due to the lack of the post-therapy blood sample.

Patients were followed for tumor progression with radiological imaging at regular intervals as for clinical practice. The clinical response to vandetanib was determined based on the RECIST 1.1 criteria (Eisenhauer *et al*. 2009) and the first evaluation of objective response was performed at a median time of 3 months (range 2–4 months) after the start of vandetanib. Overall survival (OS) was calculated from the date of blood withdrawal to the date of death or of the last follow-up.

Tumor burden assessment

Metastatic burden was estimated by a semi-quantitative method, and a score ranging from 0 to 100 was assigned to each persistent/recurrent MTC patient. An experienced radiologist (GC) reviewed images performed at study entry (total body TC and MRI for bone lesions) and calculated the total burden score for each patient. Five sites of disease were identified: bone, mediastinal nodes, liver and lung, that are the most common sites for distant metastases, plus one additional site called 'other' including neck and less frequent distant metastasis sites (e.g. kidney, brain, adrenal gland). According to the definition of 'target lesion' described in RECIST 1.1 (Eisenhauer *et al*. 2009), all target and non-target lesions were analyzed in each site, and a score ranging from 0 to 20 was assigned: 0 for no lesions, 5 for non-target lesions only, 10 for up to two target lesions, 15 for up to four target lesions and 20 for ≥ five target lesions. The tumor burden score was calculated as the sum of the scores for each site.

Biological sample collection and nucleic acid extraction

Twenty-six FFPE specimens, comprising primary tumors and/or nodal metastases, were obtained from 17 MTC patients. Five non-neoplastic thyroid FFPE specimens were obtained from subjects undergone thyroidectomy for benign thyroid nodular hyperplasia. Four thyroid FFPE specimens with diagnosis of CCH were obtained from patients operated for benign nodular goiter. CCHs were classified as 'reactive', namely 'reactive C-cell proliferation not associated with MTC' (Perry *et al*. 1996). Prior to nucleic acids extraction, hematoxylin- and eosin-stained and calcitonin-immunostained tissue sections were reviewed by an experienced pathologist (MLC), and the areas of interest were manually microdissected. DNA was extracted by proteinase K digestion in PCR buffer at 56°C overnight; total RNA was extracted using the miRNeasy FFPE kit (Qiagen) and its quality was assessed by Bioanalyzer (Agilent).

A total of 101 blood samples from cases and controls were analyzed. From each subject, 10 mL of blood were collected in an EDTA vacuum tube and processed within 2 h by centrifugation at 2000 ***g*** for 10 min at room temperature. Plasma fraction was transferred to RNase-free tubes and stored at −80°C until RNA extraction. A lipemia-independent hemolysis score (HS) was calculated for each plasma samples as previously described (Appierto *et al*. 2014). Total RNA, was extracted from plasma using the miRNeasy Kit (Qiagen) essentially as previously reported (Callari *et al*. 2013). Briefly, plasma samples were thawed on ice and centrifuged at 2000 ***g*** for 10 min at 4°C. Subsequently, 350 µL of plasma were mixed with 1300 µL of Qiazol Lysis Reagent (Qiagen) plus 1.25 µg/mL of MS2 bacteriophage RNA carrier (Roche) and 0.15 fmol of UniSp6 exogenous synthetic RNA (Exiqon).

Genomic DNA was extracted from peripheral blood by the commercial QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer's instructions.

RET and *RAS* mutational status analysis

Germline and somatic *RET* gene status was assessed for the occurrence of mutations in exons 8, 10, 11, 13, 14, 15 and 16, while somatic *RAS* gene mutations were evaluated in exons 1, 2 and 3 (*H-RAS*) and exons 2, 3 and 4 (*K-RAS*). 200 ng of DNA were amplified and directly sequenced as previously reported (Hofstra *et al*. 1996, Moura *et al*. 2011, Boichard *et al*. 2012).

miRNA microarray profiling

miRNA microarray profiling was performed on 8 MTC cases, selected as representative of the entire discovery cohort for stages (TNM) and genetic lesions, among those that had both primary tumor and lymph node metastasis specimens available. Three non-neoplastic thyroid specimens, with the highest RNA quality, were included for comparison. RNA was labeled and processed according to the manufacturer's recommended protocol,

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and miRNA expression was analyzed using SurePrint G3 Human miRNA 8x60K microarrays (Agilent Technologies), as previously described (De Cecco *et al.* 2017). Primary data were collected using Agilent's Feature Extraction software, v10.7 (Agilent Technologies). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and are accessible through GEO series accession number GSE97070 (https://ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97070).

miRNA bright field *in situ* hybridization

miRNA bright field *in situ* hybridization (ISH) studies were performed on 4 CCH and 5 MTC FFPE samples (from the discovery cohort), as previously described (Gualeni *et al.* 2015) by using double-DIG-LNA probes (Exiqon). Hsa-miR-21 and scramble-miR probes were used respectively as positive and negative controls to ensure adequate sample quality and specificity of staining. Positive signals appeared as brown dots usually localized in the cytoplasm. Signals were quantified using a scoring system from 0 to 16 obtained by multiplying the number of dots/cell (0=no signal, 1=1–3 dots/cell, 2=4–6 dots/cell, 3=7–9 dots/cell and 4=>10 dots/cell) with the percentage of positive cells (1=0–24%, 2=25–49%, 3=50–75% and 4=>75%).

Real-time quantitative reverse transcription PCR

miRNA expression was quantified by two-step real-time quantitative reverse transcription-PCR (qRT-PCR). For tissues miRNA, 50 ng of RNA was reverse transcribed by the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Quantification was performed by TaqMan miRNA assays (Applied Biosystems). RNU6B small nucleolar RNA, already reported as endogenous control in MTC (Abraham *et al.* 2011, Mian *et al.* 2012), was used for data normalization. For plasmatic miRNAs, qRT-PCR was performed by the miRCURY LNA Universal RT microRNA PCR system (Exiqon) according to the manufacturer's instruction. Synthetic RNA UniSp6 was used for normalization. All qRT-PCR were performed in triplicate on the ABI PRISM 7900HT Real-Time PCR System. Data were analyzed with the Sequence Detection System 2.4 and the RQ Manager 1.2.1 software, using the $2^{-\Delta\Delta Ct}$ method with a confidence interval (CI) set at 95%.

Statistical analysis

For microarray analysis, miRNAs differentially expressed between MTC and non-neoplastic thyroid samples were identified using the limma R package (Phipson *et al.* 2016). Multiple-testing correction was performed using the Benjamini–Hochberg false discovery rate (FDR). miRNAs with FDR <0.05 and absolute fold-change ≥2 were considered differentially expressed.

Statistical differences between qRT-PCR expression values and differences in patients clinicopathologic characteristics between the two case lists were evaluated by the Mann–Whitney *U* test, Wilcoxon test or χ^2 test as appropriate. Correlation between miRNA expression and tumor burden or HS was evaluated using the Spearman correlation coefficient. Receiver-operating characteristic (ROC) curves and area under the curve (AUC) were used to assess miR-375 ability to discriminate MTC patients from healthy control subjects or remission patients. The Youden index method (Ruopp *et al.* 2008) was applied to identify the optimal miRNA expression cut-off value. Survival analyses were performed by the Kaplan–Meier method and the differences in OS were assessed by the log-rank test. The Cox proportional hazard model was applied for univariate and multivariate analysis. For multivariate analysis, only significant variables from univariate analysis were considered. Association between categorical variables was assessed by Fisher's exact test.

All *P* values were two sided, and $P<0.05$ was considered statistically significant. Statistical analyses were performed using Graphpad Prism version 5 and R software with related Bioconductor packages.

Results

Patient characteristics

Patient clinicopathological features, including *RET/RAS* mutational status, are detailed in Supplementary Table 2 and summarized in Table 1. According to germline *RET* genotyping, 33 patients had sporadic MTC, 2 patients had MEN2B and 2 patients had isolated familial MTC. Among patients with sporadic MTC, M918T was the most common *RET* mutation, being present in 16 out of the 24 cases with known *RET/RAS* mutational status. The clinicopathological characteristics did not differ significantly between the two cohorts of MTC patients analyzed for circulating miRNAs (MTC1 $n=19$ and MTC2 $n=17$). MTC patients were matched for sex and age with the respective group of healthy controls (HC1 $n=19$ and HC2 $n=17$). For both cases and controls, the mean age at study entry was 55 years (range: 19–82 years for MTC and 14–86 years for HC), and 58% of the subjects were males (21 males and 15 females for both MTC and HC).

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Table 1 Clinicopathological characteristics of the medullary thyroid cancer (MTC) patients.

Patients characteristics	MTC1 ($n=19$)	MTC2 ($n=17$)	*P*-value[a]
Sex, *n* (%)			0.47
Male	10 (53)	11 (65)	
Female	9 (47)	6 (35)	
Age, mean (range) years			
Age at diagnosis	50 (28–68)	48 (8–82)	0.96
Age at T0	57 (32–77)	54 (19–82)	0.80
Primary tumor classification, *n* (%)			0.96
T1/T2	5 (28)	4 (29)	
T3/T4	13 (72)	10 (71)	
Na	1	3	
Lymph node status at diagnosis, *n* (%)			0.30
NX	1 (6)	2 (14)	
N0	0 (0)	1 (7)	
N1a/b	17 (94)	11 (79)	
Na	1	3	
Sporadic/genetic forms			0.08
Sporadic	19 (100)	13 (76)	
MEN2B	0 (0)	2 (12)	
Familial MTC	0 (0)	2 (12)	
Metas.tatic disease at T0, *n* (%)			0.59
1 metastatic site	6 (32)	4 (24)	
≥2 metastatic sites	13 (68)	13 (76)	
Extent of disease, *n* (%)			0.12
Only locoregional disease	1 (5)	4 (24)	
Distant metastatic disease	18 (95)	13 (76)	
Site of disease, *n* (%)			0.74
Bone	12 (63)	9 (53)	
Liver	10 (53)	7 (41)	
Lung	6 (32)	9 (53)	
Mediastinum	11 (58)	11 (65)	
Other	10 (53)	13 (76)	
Follow-up, median (range) months			
FU from surgery	80 (22–243)	75 (18–203)	0.72
FU from T0	30 (3–59)	18 (3–63)	0.20
Status at last follow-up, *n* (%)			0.09
DWD	11 (58)	5 (29)	
AED	8 (42)	12 (71)	

Tumors were classified and staged according to the thyroid malignancy World Health Organization classification Organization classification (Hedinger *et al*. 1988) and the 6th edition of TNM staging (Compton *et al*. 2012). T0, time corresponding to baseline blood sample collection.
[a]Clinicopathologic characteristics of the two case lists of MTC patients were compared by χ^2 test or Mann–Whitney *U* test, as appropriate.
AED, alive with evidence of disease; DWD, dead with disease; FU, follow-up; MTC, medullary thyroid cancer; na, data not available.

According to the TNM classification, 23 out of the 32 patients with available clinical information (72%) had advanced tumors (T3–T4) and 28/32 (88%) had lymph node metastases at the initial surgery. At study entry, 5/36 patients (14%) had only locoregional disease, whereas 31/36 (86%) had distant metastases. Twenty-six/36 MTC patients (72%) had multiple metastatic lesions involving different organs. The most common distant sites of metastases were mediastinum (61%), bone (58%), liver (47%) and lung (42%). At data cut-off (September 2016), the median duration of follow-up was 25 months, 16/36 patients (44%) had died of disease and 20/36 (56%) were alive with evidence of disease.

miRNA expression in MTC tissue samples

To identify candidate circulating miRNAs in MTC patients, we first performed a miRNA microarray analysis of 8 primary tumors and 9 paired nodal metastases (6 synchronous and 3 metachronous), as cases representative of the entire discovery cohort, in comparison with 3 non-neoplastic thyroid tissues. We identified 51 miRNAs differentially expressed in MTCs compared to non-neoplastic thyroids (Fig. 2A). In MTCs, 27 miRNAs were overexpressed (the top ranking three were miR-375, miR-10a-5p and miR-4465) and 24 were underexpressed (the top ranking three were miR-30a-5p, miR-130a-3p and let-7i-5p). Almost all primary and metastatic lesions from the same patients clustered together, with the exception of two metachronous metastases. Moreover, we did not find any miRNA significantly differentially expressed between nodal metastases and primary tumors, showing they share a common miRNA expression profile. miR-375 was the most overexpressed miRNA: its expression was 22.49-fold higher in MTC specimens than that in non-neoplastic tissues, with similar levels between primary tumors and nodal metastases, either synchronous or metachronous (Fig. 2B). Expression analysis in all the available MTCs and non-neoplastic specimens by qRT-PCR validated the microarray results, as shown in Fig. 2C. miR-375 relative expression was consistently higher in all the analyzed tumors than that in non-neoplastic thyroid specimens ($P=0.001$). The proper normal control for MTC is difficult to obtain since, in non-neoplastic thyroid, C cells are poorly represented and interspersed throughout the gland. To overcome this problem, we analyzed the miR-375-specific expression and localization by ISH analysis in reactive CCH samples, as enriched source of non-neoplastic C cells, and in MTC cases. We observed that miR-375 signal was markedly prevalent in the cytoplasm of the Ctn-positive hyperplastic C cells and MTC cells (Fig. 2D). Its expression was consistently higher in tumor (mean score: 15) than in hyperplastic C cells (mean score: 8). Conversely, stromal and non-neoplastic follicular cells

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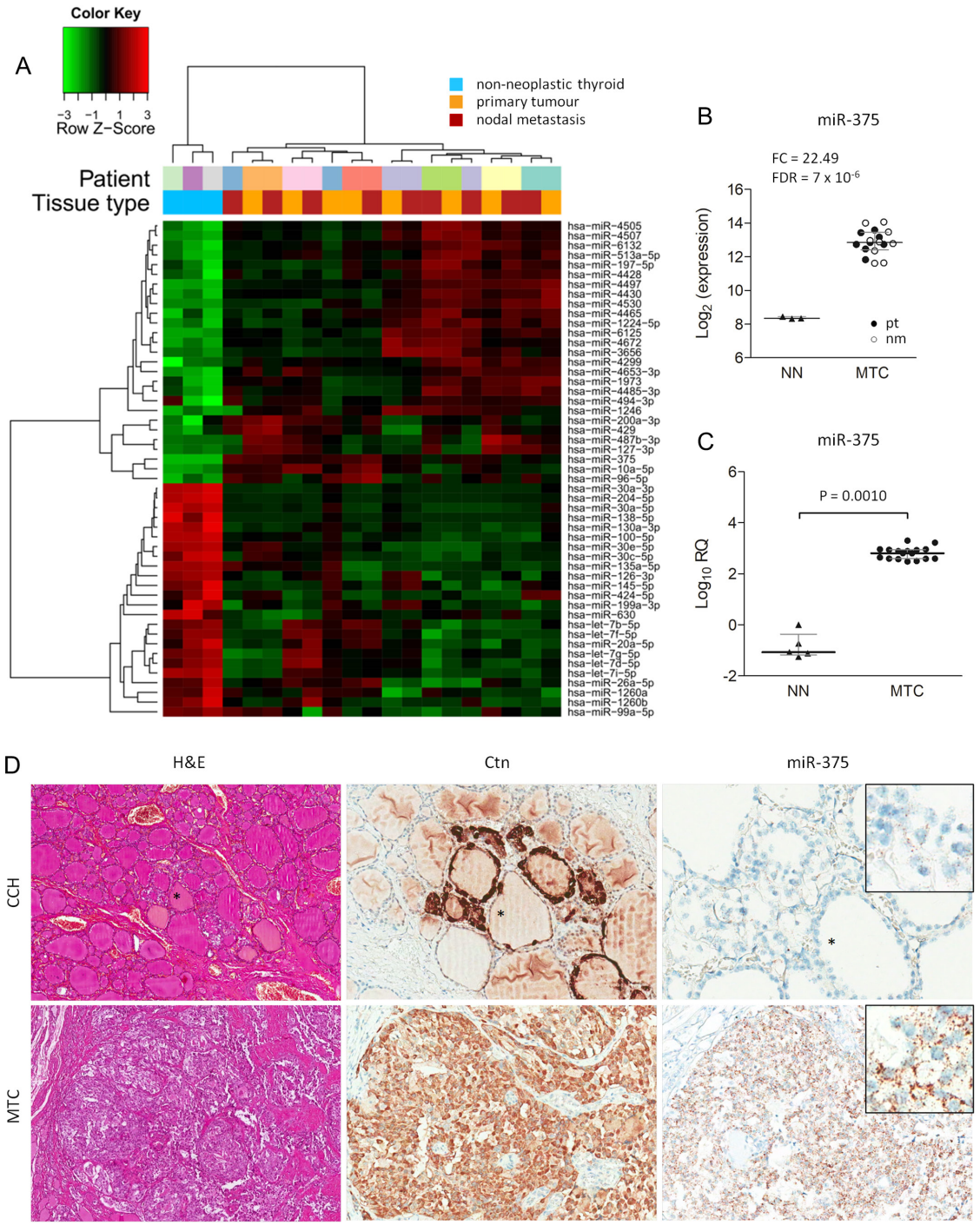

Figure 2

miRNA expression in MTC tissue samples. (A) heatmap representing color-coded expression levels of the 51 miRNAs differentially expressed in MTC primary tumours ($n=8$) and matched nodal metastases ($n=9$) compared to non-neoplastic thyroids ($n=3$). (B) Scatter dot plots represent miR-375 microarray intensity expression values in tumor (MTC; pt, primary tumor; nm, nodal metastasis) and non-neoplastic thyroid samples (NN). (C) Scatter dot plots represent miR-375 qRT-PCR relative quantification values (RQ) in MTCs (MTC, $n=17$) and non-neoplastic thyroid samples (NN, $n=5$). MTC and NN samples used in microarray profiling were also included in the qRT-PCR analysis. Data represent the mean values from two independent PCR experiments. In scatter dot plots horizontal lines indicate median values and error bars indicate the interquartile range. Statistical significance was evaluated by Mann–Whitney *U* test. (D) Hematoxylin and eosin stain (H&E), immunohistochemistry for calcitonin (Ctn) and bright field ISH for miR-375 (miR-375) in one example of CCH and MTC FFPE specimens. In the cases shown, miR-375 expression is strong in neoplastic cells (score 16) and moderate in hyperplastic C cells (score 6). Original magnification: H&E, 4x; Ctn, 10x; miR-375 ISH, 20x for CCH, 10x for MTC; inset 40x. Images are acquired by Aperio Scanscope whole-slide imaging.

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displayed negative or very low miR-375 expression (Supplementary Fig. 1A and B).

These results indicate that miR-375 represents a promising candidate to be investigated in the blood of MTC patients.

miR-375 expression in MTC plasma samples

miR-375 levels were analyzed in the plasma samples obtained at baseline from 19 MTC patients of the discovery cohort (MTC1) and in 19 sex- and age-matched healthy controls (HC1) by qRT-PCR. miR-375 was detected in all samples. No significant correlation was observed between the plasma levels of miR-375 and the degree of hemolysis of the specimens, ensuring that miR-375 levels were not influenced by possible blood cell disruption occurring during sample preparation (Supplementary Fig. 2A and B). Interestingly, miR-375 mean relative expression level was significantly higher in MTC patients than in the control group ($P=0.0003$; Fig. 3A). This result was validated in an independent cohort of 17 MTC patients (MTC2), compared with 17 sex- and age-matched healthy controls (HC2, $P=0.0119$; Fig. 3B). The ROC curve analyses showed that miR-375 levels were moderately accurate in discriminating patients from controls, with AUC values of 0.85 (95% CI: 0.72–0.98) and 0.75 (95% CI: 0.58–0.93) for the discovery and the validation cohort, respectively (Fig. 3C and D).

Despite plasma miR-375 levels being very low in the control subjects, we hypothesized that normal C cells of the healthy thyroid could concur in miR-375 secretion. Therefore, we examined the differences in miR-375 expression levels between the whole group of metastatic MTC patients (MTC1 and MTC2 analyzed together) and a group of MTC patients in remission (MTC NED), both groups without C cells from normal thyroid as possible source of circulating miR-375. The latter had slightly, though not statistically significant, lower levels of miR-375 than control subjects, and significantly lower levels of miR-375 than persistent/recurrent MTC patients ($P=0.0004$; Fig. 3E). Importantly, miR-375 levels were able to discriminate patients in remission from those with persistent/recurrent MTC, with an AUC value of 0.88 (95% CI: 0.78–0.99). According to the optimal cut-off value of 0.04 persistent/recurrent MTC patients were identified with sensitivity and specificity of 86.1% and 88.9%, respectively (Fig. 3F).

These results demonstrate that plasma miR-375 levels are significantly higher in MTC patients than in healthy subjects and patients in remission.

Association of plasma miR-375 levels with clinicopathological characteristics and overall survival in MTC patients

We investigated the possible association between miR-375 levels and patients' clinicopathological features. Notably miR-375 levels were significantly higher in individuals with distant metastases than in those with locoregional disease only ($P=0.0019$; Fig. 4A). Moreover, after subgrouping patients by the tumor burden score (<50 and ≥50), we observed that those with a widespread disease had the highest levels of miR-375 ($P=0.0197$; Fig. 4B) and that its expression was significantly correlated with the tumor burden ($r=0.6341$, $P<0.0001$; Fig. 4C). Male patients displayed higher miR-375 expression than females ($P=0.0061$; Fig. 4D), while no difference was observed by sex among healthy controls (Supplementary Fig. 3A). No significant association was found with patients' age at diagnosis or mutational status (Supplementary Fig. 3B and C).

As the parameters associated with higher miR-375 levels may influence the disease course, we investigated whether plasma levels of miR-375 were associated with the patients' outcome. The prognostic significance of miR-375 was determined, within the subgroup of patients with distant metastasis, by Kaplan–Meier survival analysis (Fig. 4E). The relative miR-375 expression levels were categorized, according to the median, into high (≥ median) and low (< median). This analysis revealed that patients with higher levels of miR-375 had a striking and significantly worse OS ($P<0.0001$; HR = 10.61; 95% CI: 3.809–29.53). Median follow-up duration was 36 months (14–63 months) and 19 months (3–43 months) for the group of patients with low and high levels of plasma miR-375, respectively. In addition, univariate survival analysis showed that poor prognosis was associated only with male sex ($P=0.02715$), tumor burden ($P=0.0749$) and high plasmatic levels of miR-375 ($P=0.00004$; Table 2). Patient outcome was not associated with age at diagnosis, *RET/RAS* mutational status or vandetanib treatment. Notably, by multivariate survival analysis, only high levels of miR-375, but not male sex nor tumor burden, maintained the prognostic significance of worse outcome (HR = 5.52; 95% CI: 1.98–15.41; $P=0.00108$; Table 2).

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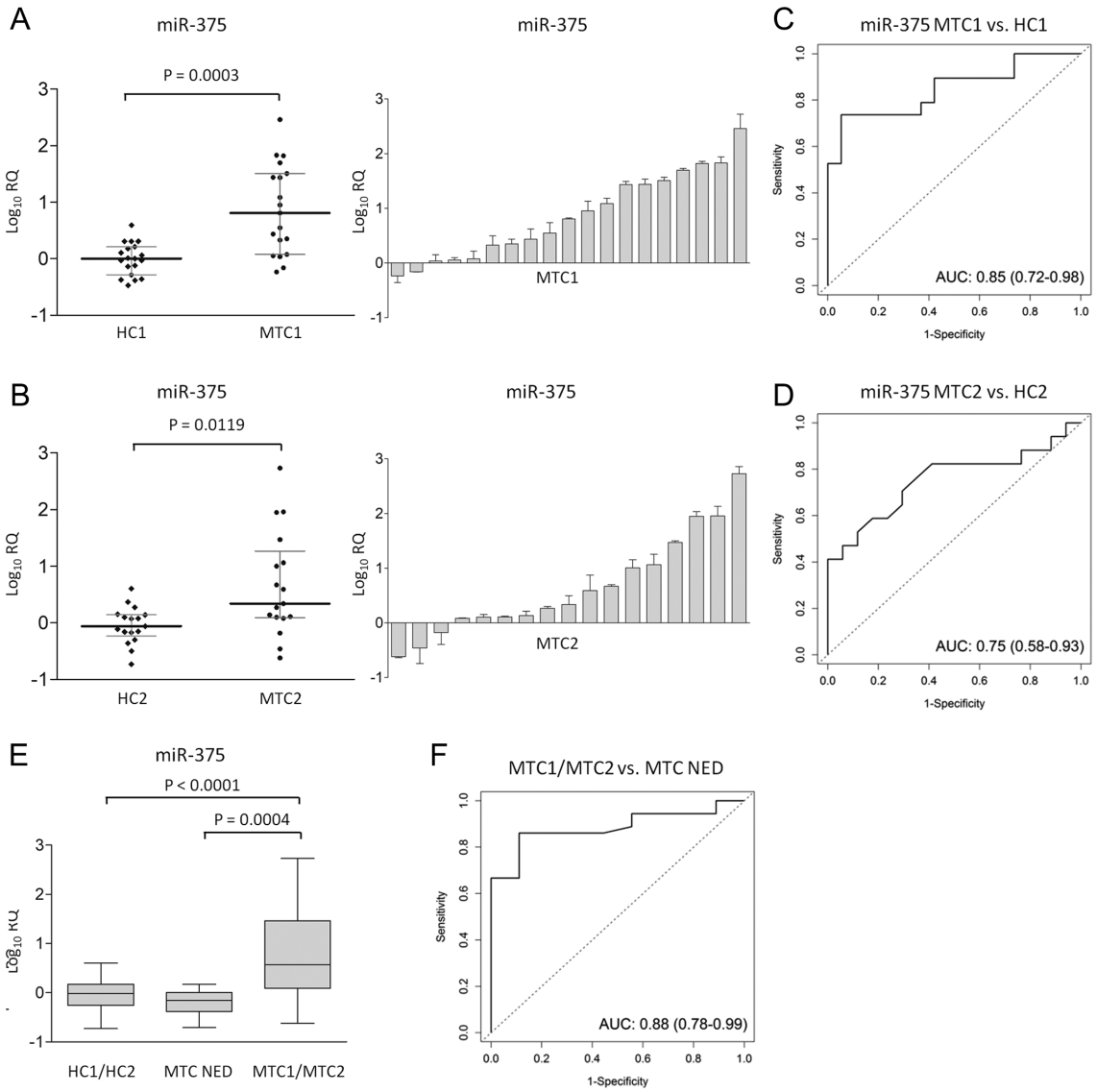

Figure 3

miR-375 expression in plasma samples. (A and B) Scatter dot plots (left panels) represent miR-375 qRT-PCR relative quantification values (RQ) in plasma samples of MTC patients in the discovery and in the validation cohorts (MTC1, $n=19$; MTC2, $n=17$) and matched healthy controls (HC1, $n=19$; HC2 $n=17$). Horizontal lines indicate median RQ values, error bars indicate the interquartile range. Histograms (right panels) show the distribution of miR-375 RQ in MTC1 and MTC2 plasma samples. Data represent mean values+S.D. from two independent PCR experiments. (C and D) ROC curve analysis for plasma miR-375 plotted to discriminate MTC patients from HC subjects in the two cohorts. (E) Box plots represent plasma miR-375 RQ values in HC1-HC2 ($n=36$) healthy controls, MTC patients in remission (MTC NED $n=9$) and in MTC1-MTC2 combined cohorts ($n=36$). Boxes represent the interquartile range, horizontal lines indicate median values and error bars indicate maximum and minimum values. Statistical significance was evaluated by Mann–Whitney *U* test. (F) ROC curve analysis for plasma miR-375 plotted to discriminate MTC patients in persistence/recurrence (MTC) from MTC patients in remission (MTC NED).

Plasma miR-375 analysis in patients treated with vandetanib

Variations of plasma miR-375 levels were investigated in a subset of 20 patients treated with vandetanib (Supplementary Table 4). At the first objective response evaluation after the start of vandetanib, 9 patients (45%) achieved a partial response (PR), 10 patients (50%) had stable disease (SD) and 1 patient (5%) was in progression (PD). *RETM918T*-positive patients had a statistically significant ($P=0.016$) higher response rate to vandetanib (PR in 66.7%) compared with

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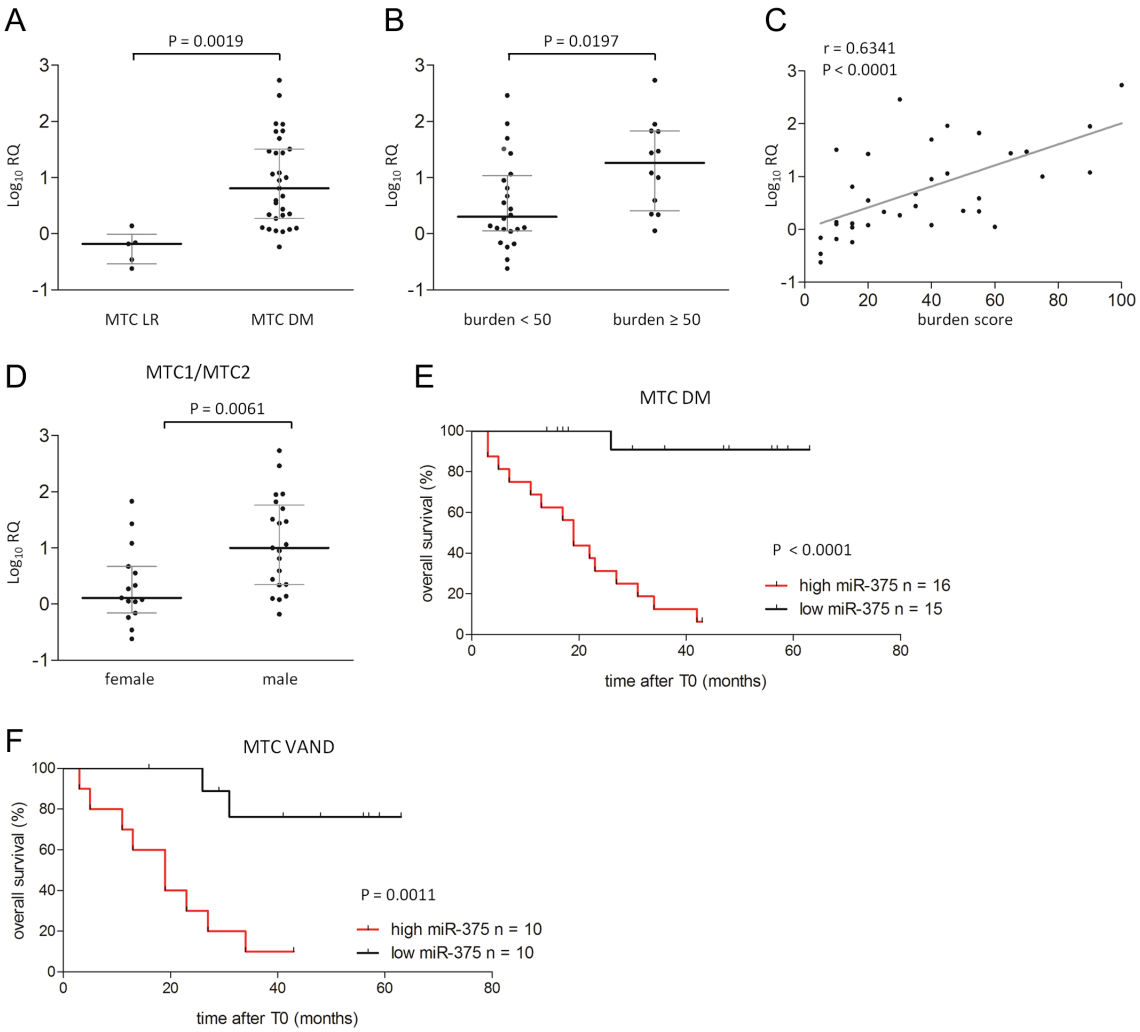

Figure 4

Association of plasma miR-375 levels with clinicopathological characteristics and overall survival in MTC patients. Scatter dot plots represent plasma miR-375 relative quantification values (RQ) (A) in MTC patients with locoregional disease only (MTC LR $n=5$) vs MTC patients with distant metastatic disease (MTC DM $n=31$) and (B) in MTC patients divided on the basis of tumor burden score (burden score <50 $n=24$ and ≥50 $n=12$). (C) Scatter plot show the positive correlation between miR-375 RQ values and patients' tumor burden score defined by Spearman correlation analysis. (D) Plasma miR-375 levels in female ($n=15$) and male ($n=21$) MTC patients. In all scatter dot plots, horizontal lines indicate median RQ values and error bars indicate the interquartile range. Statistical significance was evaluated by Mann–Whitney *U* test. (E and F) Kaplan–Meier overall survival analysis in MTC patients with distant metastasis (MTC DM in E, $n=31$) and in the subgroup of patients treated with vandetanib (MTC VAND in F, $n=20$) categorized, according to the median, into patients with high and low miR-375 plasma levels. Survival curves were compared using log-rank test.

other patients (PR in 0%). A match-paired analysis revealed that the levels of miR-375 were not significantly different before and 2 months after the start of vandetanib, even when the analysis was performed separately in the group of patients that achieved a PR or had SD (Supplementary Fig. 4A and B). Although not statistically significant, patients with PR to vandetanib displayed a higher miR-375 level reduction from pre-therapy to post-therapy than patients with SD. Indeed, mean RQ values were unchanged from T0 to T1 in patients with SD and were 3.4-fold reduced after treatment in patients that achieved a PR.

Remarkably, high levels of miR-375 were significantly associated with reduced OS also in the subgroup of vandetanib-treated patients (HR=8.309; 95% CI: 2.341–29.50; $P=0.0011$; Fig. 4F).

Discussion

In this study, by using microarray profiling of tumor tissues to drive the discovery of disease-specific circulating miRNA, we identified miR-375 as a candidate prognostic biomarker for metastatic MTC patients.

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Table 2 Univariate and multivariate Cox proportional hazard analysis for outcome in MTC patients with distant metastasis.

Variables	Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Plasmatic miR-375 levels	7.03 (2.79–17.75)	0.00004	5.52 (1.98–15.41)	0.00108
Age at diagnosis	1.01 (0.97–1.05)	0.56277	–	–
Tumor burden score	1.03 (1.01–1.06)	0.00749	1.009 (0.98–1.034)	0.48271
Sex (male vs female)	5.49 (1.21–24.84)	0.02715	2.82 (0.56–14.09)	0.20644
Mutational status (*RETM918T* vs other)	0.41 (0.14–1.20)	0.10448	–	–
Vandetanib treatment	1.29 (0.40–4.13)	0.66538	–	–

Univariate and multivariate analyses were performed for the 26 MTC patients with distant metastases that have all the considered clinicopathological data available. miR-375, age at diagnosis and tumor burden score were considered as continuous variables, whereas, sex, mutational status and vandetanib treatment as categorical variables. Only the variables that were statistically significant in univariate were considered for the multivariate analysis.
CI, confidence interval; HR, hazard ratio.

There are few studies on miRNA expression in MTC, mostly focused on a limited group of miRNAs, analyzed by qRT-PCR (Mian *et al.* 2012, Duan *et al.* 2014, Gundara *et al.* 2014, Pennelli *et al.* 2015, Spitschak *et al.* 2017). Array-based miRNA profiling in MTC was firstly performed in 2008 (Nikiforova *et al.* 2008) in different thyroid tumors, including 2 MTC cases, and only recently in larger cohorts of MTCs (Hudson *et al.* 2013, Lassalle *et al.* 2016). In this study, we performed a miRNA microarray profiling to identify miRNAs overexpressed in both primary tumors and metastases in a cohort of advanced patients (stage III and IV) to be challenged as circulating biomarkers in plasma derived from the same patients. We defined a signature of miRNAs differentially expressed in MTC compared to non-neoplastic tissue, that included miRNAs already recognized in MTC as miR-375, mir-10a, miR-127-3p, miR-138-5p and miR-130a-3p, together with a novel set of miRNAs previously unreported for this disease.

We demonstrated that nodal metastases and primary tumors had the same global miRNA profile, in agreement with previous data (Gundara *et al.* 2014). Although another recent study (Santarpia *et al.* 2013) identified a list of ten miRNA associated with metastatic MTC, we did not validate their signature in our caselist; we hypothesize that this can be either due to the use of different array platforms or due to the different patient selection criteria (all stage III and IV in our caselist).

Microarray data showed that miR-375 was the most upregulated miRNA in MTC primary tumors and nodal metastases, either synchronous or metachronous. While the overexpression of this miRNA has already been reported in MTC (Mian *et al.* 2012, Hudson *et al.* 2013, Lassalle *et al.* 2016), we provide, for the first time, more insights regarding the specific thyroidal miR-375 expression at the single-cell level by ISH analysis. This analysis allowed us to perform the more appropriate comparison with the normal counterpart of MTC, the benign C cells derived from reactive CCH. We have coherently demonstrated that miR-375 is poorly expressed in normal follicular cells and, of notice, also in stromal cells of the tumor microenvironment. At variance, hyperplastic C cells express miR-375, though at lower levels than neoplastic C cells. Our results indicate that the high expression of miR-375 is associated with the C-cell lineage within the thyroid gland and suggest that its deregulation could be involved in MTC tumourigenesis. Indeed, miR-375 was already identified as aberrantly expressed in multiple types of cancer including other neuroendocrine tumors (Nishikawa *et al.* 2011, Miller *et al.* 2016), and overexpressed, in particular, in breast and prostate cancer (Yan *et al.* 2014).

Although the origin of circulating miRNAs is still controversial, previous reports demonstrate that miRNAs overexpressed in tumor tissues may be detected in plasma and correlate with patient clinicopathological characteristics and prognosis (Schwarzenbach *et al.* 2014). Circulating miRNAs display many advantages as biomarkers: they can be obtained with minimally invasive procedures, are easily quantified and have remarkable stability in the blood (Mitchell *et al.* 2008). We identified miR-375 as the best candidate to be quantified in the plasma of metastatic patients: (i) it was the most overexpressed miRNA in MTC and it was also abundantly expressed in metastases, supposed to be the source of circulating miRNAs in persistent/recurrent patients after thyroidectomy; (ii) it was poorly expressed in stromal cells within the tumor, a potential confounding source of circulating miRNAs; (iii) the circulating levels of miR-375 were not affected by the degree of hemolysis of the sample, and it was not mentioned among the miRNAs profiled in different peripheral blood cells (Duttagupta *et al.* 2011, Pritchard *et al.* 2012). In addition, miR-375

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expression levels in tumor have been recently correlated with clinical-pathological features and outcome of MTC patients (Galuppini *et al.* 2017), higher miR-375 expression being linked to a worst patient' outcome.

Importantly, we demonstrate that plasmatic miR-375 levels can discriminate with good sensitivity and specificity MTC patients from age- and gender-matched healthy controls and from MTC patients in remission. This latter group, being individual without thyroid as advanced MTC patients of our cohorts are, represents a further control group useful to investigate if normal C cells of the thyroid gland may in part contribute to the levels of plasma miR-375. We observed that patients in remission had slightly lower miR-375 levels than healthy controls, but the difference is not statistically significant suggesting that the contribution of normal C cells to the levels of plasma miR-375 is minimum.

We performed plasma miR-375 analysis by qRT-PCR using a synthetic spike-in RNA as normalizer. Although a general consensus about an optimal normalization strategy for circulating miRNA is still lacking, exogenous spike-in RNAs have already been exploited as normalisers in absence of a defined optimal endogenous miRNA control (Toiyama *et al.* 2013, Schwarzenbach *et al.* 2015).

We showed, in two independent cohorts, that circulating miR-375 levels are higher in metastatic MTC patients compared to controls and that a significant increase is observed in patients with advanced disease. Indeed, higher plasma miR-375 levels are associated with the presence of distant metastases and are directly correlated with the tumor burden, confirming the tumor origin of this miRNA. Circulating miR-375 levels were higher in male compared to female patients. Since this difference was not observed in matched healthy controls, we speculated that it is probably linked to a worse clinical course or to a more advanced disease in male patients, as previously reported (Saad *et al.* 1984, Pazaitou-Panayiotou *et al.* 2014).

Ctn and CEA doubling time are considered strong prognostic indicators of MTC progression and death (Barbet *et al.* 2005, Laure *et al.* 2008), though they display some critical issues. Indeed, the determination of Ctn and CEA doubling time is obtained by serial measurements to be performed over an extended period of time. Moreover, nearly all patients treated with *RET* inhibitors experienced an initial decline in serum Ctn or CEA and transient fluctuations that did not reflect only responsiveness to treatment, being due to the direct effect on Ctn and CEA transcription and secretion (Keno-Stuart *et al.* 2007, Kurzrock *et al.* 2013, Postel-Vinay *et al.* 2013, Werner *et al.* 2015).

Our data indicate that plasmatic miR-375 levels are significantly associated with patient outcome. In particular, among subjects with distant metastases, a strikingly reduced OS was found in those with higher miR-375 levels (HR=10.61; 95% CI: 3.809–29.53; $P<0.0001$). Although tumor burden results are significantly associated to prognosis of advanced MTC patients (HR 1.03; 95% CI 1.01–1.06; $P=0.00749$), plasmatic miR-375 shows a far better prognostic value (HR 7.03; 95% CI: 2.79–17.75; $P=0.00004$) and is also significant in multivariate analysis.

Notably, 15/16 patients deceased at the time of data cut-off had miR-375 levels above the threshold fixed on the median value, and these high miR-375 levels were detected in plasma even 42 months before the patient's death. In contrast, only 1/15 patients still alive at the last follow-up had high miR-375 levels. Importantly, multivariate survival analysis showed that high miR-375 levels are a strong prognostic marker of poor outcome in patients with distant metastases (HR=5.52; 95% CI: 1.98–15.41; $P=0.00108$).

Moreover, we demonstrate that the prognostic value of miR-375 is also maintained in the subgroup of patients treated with vandetanib. In addition, a higher miR-375 reduction from pre- to post-therapy was observed in patients who achieved PR vs those with SD. Although the statistical significance has not been reached, likely due to the limited number of patients analyzed, the trend observed suggests to consider miR-375 also as a possible marker of response to treatments in future studies. To assess this point, further studies involving a larger cohort of patients and blood samples at multiple time-points are needed. In the future, it would be also interesting to investigate the possible application of miR-375 in MTC diagnosis and follow-up, by testing its levels in patients before thyroidectomy and during the course of the disease, or to distinguish aggressiveness at presentation, e.g. metastatic vs non-metastatic MTC at diagnosis; novel cohorts of MTC patients need to be recruited for these purposes.

Since no predictive markers are currently available to select which patients with advanced MTC could benefit from systemic TKIs, radiological progression of disease according to RECIST 1.1 criteria remains the only factor of selection. We could speculate that in those patients with a low tumor burden along with a low miR-375 levels, systemic treatments could be delayed, favoring the employment of locoregional treatments. On the contrary, in those patients with high levels of miR-375, TKIs therapy should be promptly started in light of the worse prognosis.

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Our exploratory biomarker study points, for the first time, at circulating miR-375 as a novel independent prognostic marker for metastatic MTC patients. The main limitation of this study is the limited number of patients involved, which is mostly due to the low frequency of MTC.

Although validation of these results in a larger cohort is necessary, plasma miR-375 analysis seems a promising prognostic tool, as it may be evaluated, also in patients undergoing TKIs, by a minimally invasive, easy and cost-effective procedure thus ultimately leading to better clinical decision on the management of metastatic MTC patients.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/ERC-17-0389.

Declaration of interest

L Licitra is a consultant/advisory board member for AstraZeneca, Bayer, Boehringer, Bristol-Myers Squibb, Debiopharm, Eisai, Merck-Serono, MSD, Novartis, Roche and Sobi; reports receiving research funds, through her institution, from AstraZeneca, Boehringer, Eisai, Merck-Serono, MSD, Novartis and Roche; and reports receiving travel reimbursement from Bayer, Debiopharm, Merck-Serono, and Sobi.; L D Locati received honoraria from EISAI. P Romeo received 1 year fellowship from Astra Zeneca. No potential conflicts of interest were disclosed by the other authors.

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