MicroRNA profile of poorly differentiated thyroid carcinomas: new diagnostic and prognostic insights

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Abstract

The diagnosis of conventional and oncocytic poorly differentiated (oPD) thyroid carcinomas is difficult. The aim of this study is to characterise their largely unknown miRNA expression profile and to compare it with well-differentiated thyroid tumours, as well as to identify miRNAs which could potentially serve as diagnostic and prognostic markers. A total of 14 poorly differentiated (PD), 13 oPD, 72 well-differentiated thyroid carcinomas and eight normal thyroid specimens were studied for the expression of 768 miRNAs using PCR-Microarrays. MiRNA expression was different between PD and oPD thyroid carcinomas, demonstrating individual clusters on the clustering analysis. Both tumour types showed upregulation of miR-125a-5p, -15a-3p, -182, -183-3p, -222, -222-5p, and downregulation of miR-130b, -139-5p, -150, -193a-5p, -219-5p, -23b, -451, -455-3p and of miR-886-3p as compared with normal thyroid tissue. In addition, the oPD thyroid carcinomas demonstrated upregulation of miR-221 and miR-885-5p. The difference in expression was also observed between miRNA expression in PD and well-differentiated tumours. The CHAID algorithm allowed the separation of PD from well-differentiated thyroid carcinomas with 73–79% accuracy using miR-23b and miR-150 as a separator. Kaplan–Meier and multivariate analysis showed a significant association with tumour relapses (for miR-23b) and with tumour-specific death (for miR-150) in PD and oPD thyroid carcinomas. MiRNA expression is different in conventional and oPD thyroid carcinomas in comparison with well-differentiated thyroid cancers and can be used for discrimination between these tumour types. The newly identified deregulated miRNAs (miR-150, miR-23b) bear the potential to be used in a clinical setting, delivering prognostic and diagnostic informations.

Key Words

- thyroid cancer
- poorly differentiated carcinoma
- follicular thyroid carcinoma
- papillary carcinoma
- miRNA profiling
- diagnosis
- prognosis

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Introduction

Poorly differentiated (PD) thyroid carcinomas of conventional type and oncocytic (Hürthle) type (oPD) are biologically situated between well-differentiated papillary

and follicular thyroid carcinomas (PTC and FTC respectively) on the one hand and anaplastic thyroid carcinomas (ATC) on the other. In contrast to the latter, which belongs

to the group of the most lethal human neoplasms, PTC and FTC have an excellent prognosis (DeLellis *et al.* 2004).

These neoplasms are known to be particularly difficult to diagnose, and different diagnostic criteria for these entities have been used in the past, with some focusing more on the pattern of growth (solid, trabecular, insular) and others emphasising high-grade features such as necrosis, atypia or a high mitotic index (Sakamoto *et al.* 1983, Carcangiu *et al.* 1984). The entity was finally recognised by the World Health Organization in 2004, but, the criteria were not well defined (DeLellis *et al.* 2004). A consensus meeting was held in 2007 and a diagnostic algorithm was developed by different experts in thyroid pathology throughout the world (Volante *et al.* 2007). PDs can be admixed with well-differentiated thyroid tumours such as PTCs or FTCs, but even a small PD component determines the patient outcome (Dettmer *et al.* 2011). Oncocytic PDs were originally not included in the consensus proposal; however, it was shown that proposed criteria were also applicable to them, demonstrating an even worse outcome for oncocytic PD compared with conventional PD (Dettmer *et al.* 2012).

The use of immunohistochemical markers such as Galectin-3 or HBME1 are only of limited use because signs of malignancy are easily identifiable (Volante *et al.* 2008, Volante & Papotti 2010, Tallini 2011). Thus so far, this diagnosis relies on haematoxylin-eosin (H&E)-stained slides.

The molecular background of these tumours is only partially understood so far. Specific mutations for this tumour type have not been described. *RET/PTC* rearrangements and *PAX8/PPAR γ* rearrangements appear not to play a role in PD or oPD (Soares *et al.* 2011). Characteristic mutational profiles like *BRAF* and *RAS* in PTC have not been described so far, although at least a subset of these tumours seem to originate from classic PTC, expressing these mutations (Ricarte-Filho *et al.* 2009, Volante *et al.* 2009). *TP53* mutations can be detected more frequently in these tumours than in well-differentiated carcinomas but less frequently than in ATC (Soares *et al.* 2011). To facilitate and to confirm the diagnosis of PD and oPD carcinomas, reliable molecular tests would be beneficial, ideally combined with prognostic information about tumour behaviour.

MicroRNAs (miRNAs) are a class of non-coding RNAs that were discovered about 20 years ago (Lee *et al.* 1993). However, it took 10 more years until the scientific community recognised their important role in practically all cell processes (Bartel 2004). It is known today that they are also involved in human cancer (Bartel 2004, Nikiforova

et al. 2008, Keutgen *et al.* 2012, Leone *et al.* 2012). These tiny regulators can function as oncogenes or tumour suppressor genes by regulating the expression of target genes through loss or gain of miRNA functions (Galasso *et al.* 2012). miRNA expression signatures have been identified in various human solid malignancies and in thyroid carcinomas (He *et al.* 2005, Nikiforova *et al.* 2008, Galasso *et al.* 2012, Dettmer *et al.* 2013a). A range of miRNAs (miR-155, miR-21, miR-31, miR-146b, miR-221, miR-222) are known to be deregulated in PTC (Tetzlaff *et al.* 2007, Chen *et al.* 2008, Nikiforova *et al.* 2008, Schwertheim *et al.* 2009, Yip *et al.* 2011). They have been proven to be a valuable diagnostic tool in fine-needle aspiration biopsies (FNAB) and surgical specimens and are also able to predict patient outcome (Chen *et al.* 2008, Menon & Khan 2009, Nikiforova *et al.* 2009, Yip *et al.* 2011). However, the information in the literature on PD is very limited and is absent for oPD (Nikiforova *et al.* 2008, Schwertheim *et al.* 2009).

The aim of this study is to analyse a large set of PD and oPD carcinomas and to establish the miRNA profile of PD and oPD on a large scale, covering the expression of almost 800 different miRNAs, and compare it with well-differentiated thyroid carcinomas. Further, we investigated whether PD and oPD had distinct miRNA signatures, as was demonstrated recently for FTC, oncocytic FTC (oFTC) and follicular variant of papillary thyroid carcinoma (FVPTC) (Dettmer *et al.* 2013a,b). Finally, we evaluated the clinical relevance of deregulated novel candidate miRNAs and assessed their prognostic value.

Materials and methods

Thyroid samples

The study population was enriched with patients having an adverse clinical outcome (ACO), as described elsewhere (Dettmer *et al.* 2011). This approach tremendously increases the statistical power if one wants to assess the factors which may be responsible for an adverse outcome. Nevertheless, one has to bear in mind that this patient collective does not reflect the normal population in a pathology department. ACO was defined when a patient had a least one of the following features: local relapse after first radioiodine therapy, distant metastases or tumour-associated death.

In total, we identified 99 thyroid carcinomas with an ACO and used 128 age-, stage- and gender-matched cases as controls. Of those 227 tumours, 64 with an ACO and 35 controls underwent miRNA expression analysis. In total, 107 thyroid neoplastic and non-neoplastic samples were

analysed, including 27 PD thyroid carcinomas (14 PD and 13 oPD), 27 PTC and 17 FVPTC (follicular variant of PTC), 16 follicular thyroid tumours (FTC) and 12 oFTC and eight normal thyroid tissues. Patient characteristics are summarised in Table 1. All samples of this retrospective study were formalin-fixed, paraffin-embedded (FFPE) tissues. FFPE tissues were received from the University Hospital Zurich and surrounding pathology institutes, approved by the Cantonal Research Ethics Board (STV 28-2006). The study was conducted according to the REMARK guidelines (McShane *et al.* 2005).

All tumours were classified according to widely accepted histologic criteria used for diagnosis (DeLellis *et al.* 2004, Volante *et al.* 2007). Six 15 µm thick FFPE tissue samples per case were microdissected before molecular analysis and it was ensured that representative tumour material was used for RNA extraction. The examiner was blinded to the clinical outcome and the histological diagnosis.

RNA isolation

Total RNA was extracted from FFPE tissue samples with the RecoverAll kit (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quantity was assessed with a spectrophotometer (NanoDrop 1000; Thermo Scientific, Waltham, MA, USA). MiRNA quality was assessed by amplification of a small nucleolar RNA, RNU44.

miRNA expression analysis

Quantitation of mature miRNA expression levels in thyroid tumours and normal thyroid tissue was performed using TaqMan Human Microarray Assays v3 (Applied Biosystems, Life Technologies), which is designed to detect 768 human miRNAs. The array was investigated on an ABI 7900 platform (Applied Biosystems, Life Technologies). Briefly, 150 ng of total RNA was reverse transcribed using a high-capacity cDNA archive kit (Applied Biosystems, Life Technologies) followed by a preamplification and amplification on ABI 7900 Real-Time PCR System (Applied Biosystems, Life Technologies). Endogenous controls RNU44 and U6 snRNA (Applied Biosystems, Life Technologies) were used for the normalisation of RNA input and non-human miRNA ath-miR159a was used as a negative control.

miRNA expression levels were calculated by relative quantitation using DataAssist v3.0 software (Applied Biosystems, Life Technologies) and the fold-expression changes were determined by $2^{-\Delta\Delta C_t}$ method as compared with normal thyroid tissue (Livak & Schmittgen 2001).

Table 1 Patient characteristics

Tumour type	PTC		P value	FVPTC		P value	FTC		P value	oFTC		P value	PD		P value	oPD	
	ACO	CG		ACO	CG		ACO	CG		ACO	CG		ACO	CG		ACO	CG
Gender																	
Male	7	2	0.75	4	3	0.77	0	2	0.27	1	5	0.21	4	6	0.34		
Female	13	5		5	5		4	10		3	3		10	7			
Age (mean in years $\pm$ S.E.M.)	47.4 $\pm$ 3.6	48.9 $\pm$ 5.5		49.2 $\pm$ 5.9	52.8 $\pm$ 6.7		42.5 $\pm$ 4.6	42.7 $\pm$ 5.1		76.5 $\pm$ 0.9	60.9 $\pm$ 2.7		65.4 $\pm$ 10.4	71.5 $\pm$ 9.2			
Tumour stage																	
pT1	2	2	0.5	0	1	0.18	0	0	0.007	0	0	0.59	0	0	0.098		
pT2	3	0		0	0		3	0		1	1		0	3			
pT3	15	5		8	7		1	12		3	7		14	10			
pT4	0	0		1	0		0	0		0	0		0	0			
Overall survival (OS) (months $\pm$ S.E.M.)	76.9 $\pm$ 11.7	67.9 $\pm$ 10.6		73.7 $\pm$ 15.7	89.0 $\pm$ 17.5		83.4 $\pm$ 22.1	114.9 $\pm$ 16.3		114.8 $\pm$ 35.9	116.6 $\pm$ 19.7		70.0 $\pm$ 39.6	63.4 $\pm$ 57.5			
Tumour specific survival (TSS) (months $\pm$ S.E.M.)	76.9 $\pm$ 11.7	67.9 $\pm$ 10.6		73.7 $\pm$ 15.7	89.0 $\pm$ 17.5		83.4 $\pm$ 22.1	114.9 $\pm$ 16.3		114.8 $\pm$ 35.9	119.8 $\pm$ 22.4		70.0 $\pm$ 39.6	63.4 $\pm$ 57.5			
Relapse-free survival (RFS) (months $\pm$ S.E.M.)	11.0 $\pm$ 1.8	67.9 $\pm$ 10.6		26.3 $\pm$ 13.3	89.1 $\pm$ 17.6		6.5 $\pm$ 1.3	114.9 $\pm$ 16.3		52 $\pm$ 34.6	119.9 $\pm$ 22.4		15.8 $\pm$ 18.2	20.3 $\pm$ 22.7			

PTC, papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; FTC min. inv., minimally invasive thyroid carcinoma; PD, poorly differentiated thyroid carcinoma; oFTC min. inv., oncocytic minimally invasive thyroid carcinoma; oPD, oncocytic poorly differentiated thyroid carcinoma; ACO, adverse clinical outcome; CG, control group.

The maximum allowed Ct value for calculations was 37. Outliers among replicates were excluded and *P* values were adjusted using Benjamini–Hochberg false discovery rate. The data are presented as the fold change of miRNA expression in tumours relatively to normal thyroid tissues after normalisation to endogenous controls RNU44 and U6 snRNA.

Patient follow-up

Complete follow-up data were collected using chart reports and the cancer registry of the canton Zuerich and recorded as overall survival (OS), tumour-specific survival (TSS) and relapse-free survival (RFS) as described previously (Dettmer *et al.* 2011).

Statistical analysis

DataAssist v3.0 software (Applied Biosystems, Life Technologies) was used to calculate agglomerative hierarchical clustering and RQ Plots between thyroid specimens. Assessment of the sample distribution (Kolmogorov–Smirnov test), descriptive statistics, Chi-squared Automatic Interaction Detection (CHAID), Kaplan–Meier survival analysis (log rank test) and Cox regression analysis were performed with SPSS 21.

Results

miRNA expression profiles of PD and oPD

Seventeen miRNAs showed significant deregulation in PD and oPD as compared with normal thyroid tissue. Both PD and oPDs showed upregulation of miR-125a-5p, -15a-3p, -182, -183-3p, -222 and miR-222-5p, and downregulation of miR-130b, -139-5p, -150, -193a-5p, -219-5p, -23b, -451, -455-3p and miR-886-3p (Table 2). The most upregulated miRNAs were miR-183-3p (sevenfold) in PD and miR-221 and miR-885-5p in oPD. The most downregulated miRNA in both tumour types was miR-219-5p, demonstrating 30-fold downregulation in PD and 160-fold in oPD as compared with normal tissue (Table 2). The unsupervised hierarchical clustering analysis of miRNA expression showed separate clusters for PD, oPD and for normal thyroid tissue (Fig. 1).

Progression of deregulated miRNAs from well-differentiated to PD tumours

Next, we compared expression of these 17 deregulated miRNAs in PD tumours with their expression in

Table 2 Fold changes of different miRNAs in thyroid tumours

Assay	PD origin PTC		oPD		FTC min inv ACO		FTC min inv CG		oFTC min inv ACO		oFTC min inv CG		FVPTC ACO		FVPTC CG		Classic PTC ACO		Classic PTC CG	
	RQ	P value	RQ	P value	RQ	P value	RQ	P value	RQ	P value	RQ	P value	RQ	P value	RQ	P value	RQ	P value	RQ	P value
hsa-miR-125a-5p	1.5102	0.4881	2.4358	0.0187	1.2707	0.4421	1.6433	0.2316	4.0741	0.3049	4.7852	0.0075	2.5696	0.2219	1.7166	0.066	1.4341	0.3937	2.1557	0.5633
hsa-miR-130b	0.8801	0.9389	0.1602	0.0187	1.783	0.6458	2.5355	0.2316	1.731	0.7234	0.3676	0.0926	0.4902	0.2219	2.3146	0.3188	0.6463	0.3937	0.7208	0.6894
hsa-miR-139-5p	0.1707	0.0201	0.3858	0.1434	0.677	0.4421	0.388	0.0238	0.536	0.3049	0.7897	0.507	0.6616	0.2219	0.3725	0.066	0.2414	0.0066	0.405	0.0453
hsa-miR-150	0.1761	0.0504	0.2722	0.0947	0.354	0.2963	0.1426	0.103	0.4136	0.3049	0.2988	0.134	0.6563	0.4591	0.2031	0.1116	0.7581	0.5937	1.3036	0.6661
hsa-miR-15a-3p	5.1826	0.0504	4.6616	0.1309	5.1007	0.2963	3.3332	0.1252	8.4616	0.4806	4.7142	0.1786	5.0306	0.1008	3.7938	0.1657	4.9066	0.0628	8.3654	0.5639
hsa-miR-182	4.1321	0.3134	15.7878	0.0102	3.0254	0.4586	5.8046	0.103	10.0218	0.3049	17.0327	0.1449	4.4518	0.2219	1.7252	0.5809	2.4388	0.0383	3.2818	0.6282
hsa-miR-183-3p	7.529	0.099	13.6166	0.0102	3.388	0.4118	10.2013	0.0027	10.4589	0.3049	29.1113	0.0667	2.4758	0.2219	8.1253	0.1401	1.5139	0.5937	3.393	0.5639
hsa-miR-193a-5p	0.1959	0.0504	0.2206	0.0105	0.3138	0.2352	0.2039	0.0053	0.4504	0.3049	0.288	0.0075	0.5907	0.2125	0.4214	0.066	0.2925	0.0066	0.4258	0.0639
hsa-miR-219-5p	0.0301	0.0504	0.006	0.009	0.0132	0.2963	0.0415	0.231	0.1188	0.3049	0.0102	0.1786	0.1312	0.2219	0.0422	0.178	0.0093	—	0.1217	0.4679
hsa-miR-221	0.9105	0.9788	12.2539	0.0081	4.3356	0.4118	5.1579	0.1045	17.4066	0.3049	29.5232	0.0075	13.3133	0.0649	19.4575	0.066	8.8605	0.0692	8.4463	0.0453
hsa-miR-222	1.6391	0.8249	9.7331	0.0081	4.8395	0.2963	4.9973	0.2857	7.3821	0.3049	7.0607	0.0084	27.2159	0.1829	11.7024	0.0853	13.9184	0.001	19.3691	0.4679
hsa-miR-222-5p	1.2917	0.9389	7.2611	0.0491	1.2929	0.6677	1.5184	0.5663	4.754	0.4118	5.5375	0.0192	2.8774	0.1599	11.8716	0.264	1.4406	0.5676	1.4444	0.6379
hsa-miR-23b	0.7987	0.8369	0.1962	0.0466	0.9723	0.9397	1.4223	0.3509	1.0668	0.9409	0.745	0.5174	1.3934	0.4802	2.2949	0.1401	0.7094	0.5228	0.8915	0.804
hsa-miR-451	0.2865	0.0504	0.4047	0.2194	0.3729	0.2963	0.4661	0.1612	0.4923	0.3049	0.2964	0.0447	0.3157	0.1008	0.3498	0.089	0.1935	0.0292	0.2783	0.1313
hsa-miR-455-3p	0.406	0.4881	0.1474	0.0454	0.2969	0.2963	0.141	0.103	1.2864	0.8904	0.0566	0.0447	0.4076	0.2219	0.2612	0.1116	0.3434	0.1663	0.7686	0.6661
hsa-miR-885-5p	1.917	0.7069	42.8892	0.0204	0.2762	0.2963	1.1838	0.739	105.7278	0.575	520.8887	0.3223	0.7637	0.5749	0.8291	0.6616	15.8382	0.4348	4.2795	0.6155
hsa-miR-886-3p	0.1247	0.0504	0.2513	0.1482	0.3488	0.3146	0.4431	0.2978	0.3415	0.3049	0.779	0.6609	0.5886	0.4591	0.1852	0.1401	0.3833	0.3051	0.5471	0.5639

PTC, papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; FTC min. inv., minimally invasive thyroid carcinoma; PD, poorly differentiated thyroid carcinoma; oPD, oncocytic minimally invasive thyroid carcinoma; ACO, adverse clinical outcome; CG, control group. *P* values are indicated in relation to normal thyroid tissue. Significant *P* values given in bold.

Figure 1

Unsupervised hierarchical clustering analysis (Pearson correlation, complete linkage) of poorly differentiated (PD) and oncocytic poorly differentiated (oPD) thyroid carcinomas and normal thyroid tissue based on

their miRNA expression. PD (green), oPD (pink), and normal thyroid tissue (blue) form three distinct clusters.

well-differentiated thyroid carcinomas (Table 2). Some miRNAs (miR-221 and miR-222) that were upregulated in PTC and FVPTC showed loss of expression in PD ($P < 0.05$). Other miRNAs (miR-15a-3p and miR-183-3p ($P < 0.05$)) were upregulated in PD tumours as compared with well-differentiated tumours.

When oPD cancers were compared with well-differentiated oncocytic tumours, a number of miRNAs (miR-125a-5p, -183-3p, -219-5p, -221 and miR-885-5p) showed loss of expression (Table 2). In contrast, miR-222 was twice as highly expressed in oPD as compared with oFTC.

We also evaluated the possibility of discriminating PD and oPD from well-differentiated thyroid tumours, using the CHAID algorithm. It revealed a 73.3% overall accuracy for the separation between FTC and PD and a 75% accuracy for oFTC and oPD using miR-23b as a separator with a cut-off of 0.5-fold. MiR-150 (cut-off of 0.2-fold) was able to separate PTC from PD with an accuracy of 79.3% using CHAID.

Deregulated miRNAs and patient survival

One significant deregulated miRNA in PD and oPD was able to predict a decreased RFS in these tumours: miR-23b (Fig. 2). This miRNA has been confirmed as the only independent predictor of tumour relapse in a multivariate

Cox regression analysis including patient age, tumour stage and gender-matched tumours. The hazard ratio was $\text{Exp}(B) = 2.62$; 95% CI: 1.01–6.77). MiR-150 demonstrates a significantly decreased TSS in a Kaplan–Meier analysis (Fig. 2), which could also be confirmed in a multivariate Cox regression analysis including age, stage and gender-matched tumours for TSS with patients having a 5.03-fold increased (95% CI: 1.29–19.69) risk of a fatal outcome (Table 3). Of note, tumour necrosis and/or an increased mitotic index was not able to identify further patients with an adverse outcome in PD patients.

Discussion

We analysed a large series of PD thyroid carcinomas for the complete miRNA profile of the Sanger Database v16 generating more than 80000 TaqMan PCR-based data points and compared it with the miRNA profile of well-differentiated tumours. All PD tumours were diagnosed according to widely accepted criteria and the Turin proposal (DeLellis *et al.* 2004, Volante *et al.* 2007), ensuring diagnostic accuracy and comparability of these data with other studies.

MiRNAs have drawn increasing attention in recent years. Their deregulation has been shown in many different human tumours including thyroid neoplasms and they

Figure 2

Kaplan-Meier survival analysis including poorly differentiated (PD) and oncocytic poorly differentiated (oPD) thyroid carcinomas: relapse-free survival is significantly reduced for miR-23b. Tumour-specific survival is reduced for miR-150. Tests are log-rank tests.

have been increasingly used as diagnostic and prognostic markers by our group and others (Lu *et al.* 2005, Nikiforova *et al.* 2008, Sheu *et al.* 2010, Dettmer *et al.* 2013a).

miRNA expression profiles of PD and oPD

So far, there is only very limited information available on miRNA profiles in PD carcinomas and to our knowledge no information on oPD (Nikiforova *et al.* 2008, Schwertheim

et al. 2009). The first study on miRNA profiles in PD was done by our group and out of ten reported miRNAs at that time, we were able to confirm seven in this study including miR-183, -221 and -222 (Nikiforova *et al.* 2008). Four more miRNAs were concordantly expressed in our present study as well (miR-129, -146b, -181a and -339). However, they were not found to be significantly deregulated in this work and therefore were excluded from further analysis. Out of the remaining three miRNAs, miR-181b was not included in the present array, miR-187 was not amplified, while miR-213 was the only one not being concordantly expressed. The other study on PD miRNA profiles was carried out by Schwertheim *et al.* (2009). They reported ten miRNAs and we are currently able to confirm eight of them; miR-222 is discordantly expressed and does not reach statistical significance in their study and one (miR-181b) was not in our array.

As facts accumulate, we know that tumours originating from the same cell of origin can present with distinct miRNA profiles (Nikiforova *et al.* 2008, Dettmer *et al.* 2013a,b). Although there were a few outliers, the unsupervised hierarchical clustering analysis including significant deregulated miRNAs in PD and oPDs separates these tumours into two distinct groups, suggesting that this also might be true for these types of thyroid carcinomas.

Progression of deregulated miRNAs from well-differentiated to PD tumours

Several miRNAs are increasingly deregulated between well-differentiated and PD tumours. Among them, miR-221 and miR-222 have been well described in PTC for some time (Nikiforova *et al.* 2008). They are both known to negatively regulate *P27* and an absent *P27* expression can also be observed in various aggressive human neoplasms (Visone *et al.* 2007). Interestingly, the latter two miRNAs are less deregulated in PD compared with PTC, suggesting that this pathway is not driving tumour progression in PD. We were unable to find significant correlations when looking into survival data between miR-221 and miR-222 and PD or oPD, which is consistent with this observation. In contrast, miR-183-3p is upregulated in PD as compared with well-differentiated PTC or FVPTC and is also involved in decreased patient survival, supporting the progression model from well-differentiated to PD carcinomas. Interestingly, miR-183 has been shown to be also upregulated in aggressive prostate cancer where it targets *SMAD4* and *DKK3* in the WNT/ β -catenin pathway (Ueno *et al.* 2013).

Table 3 Clinicopathological characteristics and expression of miR-150 and miR-23b in PD and oPD

MiRNA (fold change)	miR-150 <0.2	miR-150 ≥0.2	P value	miR-23b <0.5	miR-23b ≥0.5	P value
Age (years)	68.8±9.7	67.1±11.2	0.774	69.6±9.6	64.7±12.5	0.615
pT						
pT 1–2	1	2	0.446	2	1	
pT 3–4	14	9		12	8	0.584
Gender						
Male	7	3	0.082	6	2	0.024
Female	8	8		8	7	

Although there were initially 27 PD and oPD, the assay for miR-150 failed in one sample and for miR-23b in four samples, reflecting the difficulties with downregulated markers.

MiR-222 is more highly expressed in oPD than in oFTC, while other miRNAs are lost in the more aggressive tumours such as miR-139-5p, -219-5p, and -23b, further supporting the progression model. MiR-139-5p is lost in aggressive breast carcinomas, targeting Ras and PI3K members as well as NFκB (Krishnan *et al.* 2013), while miR-23b is known to be suppressed by c-Myc (Gao *et al.* 2009). All those pathways and genes are known to be involved in thyroid tumourigenesis for a long time (Yamashita *et al.* 1986, Nikiforov *et al.* 2009).

Diagnostic use

Several miRNAs may be used diagnostically to distinguish between PTC/FVTC/FTC and PD. The most promising candidates would be the ones which are significantly deregulated between the entities such as miR-150, -183-3p, -222 and miR-222. The separation between FTC and PD and between oFTC and oPD can be particularly difficult. While feeding the CHAID-algorithm with these four biomarkers, an overall accuracy of about 75% could be achieved for both clinically relevant questions with the use of only one miRNA: miR-23b. The accuracy increased to almost 80% when using miR-150 as a separator between PTC and PD. These miRNAs may help to diagnose difficult cases in the future and to stratify patients appropriately. One limitation of the study is the fact that most of these markers are downregulated. If one tests for their expression and obtains a negative result, it is difficult to know whether the tested marker is strongly downregulated and therefore not detectable by PCR or whether the assay did not work properly.

Biological implications of reported miRNAs and patient survival

A very important aspect of the present work is not only to show the actual miRNA profile of the tumours but also to

provide information about the clinical consequences of their deregulation. A subset of the significantly deregulated miRNAs has an impact on patient survival and can predict tumour relapse and survival even in tumours with such an adverse outcome. These miRNAs are known to play important roles in other malignancies, such as oesophageal squamous cell carcinoma (Yokobori *et al.* 2013) for miR-150 or prostate cancer for miR-23b (Gao *et al.* 2009). Tumour necrosis, an increased mitotic index or convoluted nuclei, normally very strong indicators of an adverse outcome, were not able to further sub-stratify patients in the PD groups, which is not surprising, because these features are part of the Turin criteria which define these tumours (Volante *et al.* 2007).

So far, miRNAs in thyroid cancer are used as diagnostic and predictive tools only. Nevertheless, miRNA-based therapies have been shown to be an effective instrument in hepatocellular carcinoma and hopefully may one day also be available in thyroid cancer (Kota *et al.* 2009).

Conclusions

This is the first comprehensive miRNA profile for PD and oPD in the literature. We compared the expression of the most deregulated miRNAs in PD and oPD to their expression in the most common thyroid carcinomas in PTC, FVPTC, FTC and oFTC, aggressive and non-aggressive. A subset of 17 miRNAs is able to separate PD from oPD and normal thyroid tissue, suggesting that these are in fact two distinct entities. Two of those markers, miR-150 and miR-23b, bear the potential to provide prognostic and diagnostic evidence at the same time.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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