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ORIGINAL ARTICLE

Expression and clinicopathological role of *miR146a* in thyroid follicular carcinoma

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Abstract

Purpose Dysregulation of microRNA expression has been involved in the development and progression of follicular thyroid carcinoma (FTC). The aim of this work was to study the expression of *miRNA146a* in FTC and the association with clinicopathological features of the disease.

Methods Thirty-eight patients affected by FTC were included in the study. Twenty patients carrying follicular thyroid adenoma (FA) were also enroled as the benign counterpart of FTC. Total RNA including *miRNA146a* was extracted from formalin-fixed paraffin-embedded (FFPE) pairs of affected/unaffected tissue and its expression was assessed by real-time PCR. Two selected target genes, *TRAF6* (tumour necrosis factor receptor-associated factor 6) and *IRAK1* (Il-1 receptor-associated kinase 1/2), were also analysed.

Results *miR146a* expression in FTC tissue was overall not downregulated in malignant versus unaffected tissue, but its expression was inversely correlated with clinicopathological features of FTCs at diagnosis. A decreased expression of *miR146a* became apparent in FTC thyroid tissue of widely compared to minimally invasive tumours. However, *miR146a* expression differences between contralateral unaffected tissue (extra-FTC) and FTC were not observed regardless of clinicopathological features. *IRAK1*, a known target for *miR146a*, was upregulated in FTC and the increase was mainly appreciable in Hurtle FTC variant. Unexpectedly, *miR146a* did not correlate with *TRAF6* showing an inverse trend compared to *IRAK1* although both genes regulate the activity of nuclear factor- kB (NF-kB).

Conclusion The results of this study indicate that downregulation of *miR146a*, inversely correlated with clinicopathological features of FTCs at diagnosis and suggest a possible involvement of *miR146a* in FTC development. *IRAK1* over-expression in FTC may be related to tumour development/progression. In vitro experiments are needed to support this hypothesis.

Keywords Follicular thyroid carcinoma · *miR146a* · *IRAK1* · *TRAF6*

Introduction

More than 95% of thyroid carcinomas derive from follicular thyroid cells and are classified into well-differentiated papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC; conventional or oncocytic type) [1]. FTC is the second most common thyroid carcinoma after PTC and its incidence peak is between the ages of 40 and 60

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years [1, 2]. The prognostic factors of FTC include age, sex, stage (based on pTNM parameters) and tumour characteristics. Minimally invasive follicular cancer has been associated with good survival and is considered a low-risk neoplasia when compared with widely invasive follicular cancer [2]. Vice versa, some variants of FTC, such as Hurtle cells carcinoma and insular cancer are associated to a worst prognosis [2]. On the contrary, follicular adenomas (FA) are benign tumours (conventional or oncocytic type).

A non-invasive diagnostic tool is required to distinguish between benign and malignant follicular lesions because neither ultrasound nor needle aspiration is decisive. So far, the diagnosis of FTC of a thyroid nodule can only be obtained by complete removal of the thyroid mass and subsequent histological examination confirming capsular invasion.

In most malignant thyroid carcinomas several genetic alterations in oncogenes involved in the activation of cell signalling pathways have been observed [3]. Many authors studied the expression of non-coding candidate genes, such as microRNAs (miRNAs, miRs) in cancer tissues [4]. Changes in the expression of multiple regulatory RNA genes is an intriguingly hypothesis to explain thyroid carcinogenesis process [5, 6]. miRNAs represent a class of gene regulators constituted by small (about 22 nucleotide lengths), non-coding single-stranded RNAs [7, 8]. Several independent studies described miRNA expression deregulation in different types of thyroid tumours, compared to their unaffected counterparts. Moreover, a significant variability in miRNA expression profile between different kinds of thyroid cancers is observed, even if they originate from the same type of thyroid cells [6].

One of the most studied miR in PTC is *miR146a*, a member of the *miR146* miRNA family along with *miR146b*, two evolutionary conserved miRNA genes. Considering that only limited information is available for miRs, and in particular for *miR146*, and FTC [9–13], in this paper we evaluated the expression of *miR146a* in FTC.

The primary aim of this study was to assess the expression of *miR146a* in thyroid tumour tissue of patients who underwent thyroidectomy with histological diagnosis of FTC and to describe any possible association with main tumour clinicopathological features at diagnosis.

In addition, we analysed the expression of two selected target genes, tumour necrosis factor receptor-associated factor 6 (*TRAF6*) and Il-1 receptor-associated kinase 1/2 (*IRAK1*), key adaptor proteins of the NF-kB signalling cascade, resulting in inhibition of NF-kB activation [14, 15]. Both genes are targets of *miR146a* and proposed to be part of the NF-kB -induced negative feedback loop [16, 17]. NF-kB is involved in the immune response, inflammation, apoptosis and other biological processes [18].

Materials and methods

Subjects

A retrospective case–control study on 38 cases of FTC was performed. The study was approved by the local Ethics Committee (file No. 122/08). Patients' inclusion criteria were: (i) confirmed histological diagnosis of FTC, (ii) availability of complete medical records on oncologic history, and (iii) possibility to analyse both affected and unaffected thyroid tissues. All patients were of Caucasian origin. Tumours were classified according to the thyroid malignancy World Health Organisation classification and staged according to the eighth edition of the AJCC/TNM cancer staging system published by the American Joint Committee on Cancer [19]. Twenty patients with diagnosis of FA were also taken into account, considering adenoma as the benign counterpart of follicular carcinoma. No control group of healthy subjects was enroled for ethical reasons.

Tissue samples

Thirty-eight formalin-fixed paraffin-embedded (FFPE) pairs of FTC and contralateral normal tissue (extra-FTC) tissue blocks and 20 FFPE pairs of FA and contralateral unaffected tissue (extra-FA) blocks were obtained. The extra-tumour tissues were obtained at a distance of at least 2 cm from FTC nodule. Secondly, a confirmation of histopathologic diagnosis was performed by a second pathologist. FFPE tumour tissues were macrodissected to retrieve pure tumour tissue. Two 10 µm thick sections were cut from each FFPE block.

RNA isolation

Total RNA including miRNA was extracted from FFPE by using the RecoverAll kit (Ambion, Thermo Fisher Scientific Inc., Wilmington, Delaware, USA), according to the manufacturer's instructions.

RNA concentration and quality were determined by NanoDrop scanning (NanoDrop 1000, Thermo Fisher Scientific Inc., Wilmington, Delaware, USA).

miR real-time RT-PCR analysis

To evaluate hsa-miR-146a-5p expression (TaqMan MIRNA Assays; assay ID 000468) Taqman reverse transcription was performed according to Jazdzewski et al. [20], using U6 -small nuclear RNA (U6-snRNA) as an endogenous loading control (TaqMan MIRNA Assays; assay ID 001973). Primers for U6snRNA and *miR146a* were part of Taqman MicroRNA Assay, (Life Technologies, Thermo Fisher

Scientific Inc., Wilmington, Delaware). In brief, TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, Thermo Fisher Scientific Inc., Wilmington, Delaware, USA) was used to retrotranscribe total RNA. Real-time PCR analysis was performed with TaqMan Universal Master Mix II (Life Technologies, Thermo Fisher Scientific Inc., Wilmington, Delaware, USA) on the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, California, USA).

Relative expression levels were determined by normalising *miR146a* in each sample to U6-snRNA and calculated with the formula $2^{-\Delta\Delta Ct}$ [21]. Experiments were conducted in triplicate.

miR146a target gene selection

Systematic literature review was made to strengthen the *miR146a* targets selection using the electronic database PubMed (http://www.ncbi.nlm.nih.gov/pubmed). The search focused on *IRAK1* and *TRAF6* genes and was confirmed by scores from different softwares used to look for relevant mRNA target for *miR146a*: TargetScan 5.0, PicTar, miRDB, mirtarbase and TargetRank [22–24].

miR target gene real-time RT-PCR analysis

To evaluate *IRAK1* (Assay ID Hs.PT.58.2789622, IDT, Tema Ricerca, Milan, Italy) and *TRAF6* (Assay ID Hs.PT. 58.4313477, IDT) expression, human *TBP* (Tata Binding Protein) (Assay ID Hs.PT. 58v.39858774, IDT) and *GUSB* (Glucoronidase beta) (Assay ID Hs.PT. 58v.27737538, IDT) were used as endogenous loading controls [25, 26]. Reverse transcription was performed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA) according to manufacturer's instructions. The iQ multiplex Powermix (Bio-Rad Laboratories, Hercules, California, USA) was used to simplify real-time detection of multiple targets in a single tube. The expression level of each target gene was determined relative to the geometric averaging of the two internal control genes (*TBP* and *GUSB*) [27] and calculated using the $2^{-\Delta\Delta Ct}$ method. One sample among FA, chosen as internal calibrator, was used in all the real-time PCR reactions. Experiments were conducted in triplicate. RT-PCR, real-time conditions and primers sequence are reported in Supplementary Table 1.

Statistical analysis

Statistical analysis was performed by using Prism 5 software (Graph-Pad, San Diego, CA). Data were presented reporting bar plots, median values and standard deviation (SD). Parameters distribution was calculated by D'Agostino and Pearson test. Continuous variables were compared using one-way analysis of variance (ANOVA) test when variables were normally distributed or Mann–Whitney test or Kruskal–Wallis test when not-normally distributed. Correlations between *miR146a* expression and clinicopathological parameters were analysed using Spearman's rank correlation coefficient. Analyses in relation to age were performed considering the entire group of patients enroled. The relationship among continuous variables was evaluated performing simple linear regression analysis. A p value <0.05 was considered statistically significant.

Table 1 Clinicopathological features of patients affected by follicular thyroid carcinoma ($n = 38$)

	Number	%
Age (mean 54.9 years; range 18–85 years)		
Gender		
Male	18	47.36%
Female	20	52.63%
Histotype		
Follicular classical variant	26	68.42%
Oncocytic Hurtle variant	12	31.58%
Invasiveness		
Minimally invasive	31	81.57%
Widely invasive	7	18.43%
Primary tumour extension		
T1	7	18.42%
T2	14	36.84%
T3	14	36.84%
T4	3	7.9%
Lymph Node metastases		
N0	36	94.73%
N1	2	5.26%
Distant Metastases		
M0	34	89.48%
M1	4	10.52%
pTNM Stage		
Stage I	26	68.42%
Stage II	8	21.05%
Stage III	0	0%
Stage IV	4	10.53%

Primary tumour extension, lymph node and distant metastases refer to the eighth edition of the AJCC/TNM cancer staging system published by the American Joint Committee on Cancer [19]: T1: tumour < 2 cm in greatest dimension, limited to the thyroid; T2: tumour between 2 and 4 cm, limited to the thyroid; T3: tumour > 4 cm limited to the thyroid or gross extrathyroidal extension invading only strap muscle; T4: gross extrathyroidal extension into major neck structures; N0: no evidence of regional lymph nodes metastasis; N1: metastasis to regional nodes; M0: no evidence of distant metastasis: M1: distant metastasis

Results

Subjects

Demographics and clinical data of cases included in this study are summarised in Table 1. The mean age at the time of surgery was 54.9 years (range 18–85; SD 19.2). Among the 38 patients (18 males and 20 females), 26 presented the follicular classical variant and the others the oncocytic Hurtle variant. Most of the subjects had minimally invasive tumours, without lymph node or distant metastases (Table 1). Lesions were classified according to the AJCC/TNM cancer staging system [19]: most have been categorised in stage I ($n = 26$), followed by stage II ($n = 8$) and stage IV ($n = 4$).

Twenty patients (two males, 10%, and 18 females, 90%) with histological diagnosis of FA functioned as controls, considering adenoma as the benign counterpart of follicular carcinoma. Nine FA were classified as classical (45%) and 11 (55%) as oncocytic variant. Age at diagnosis was between 20 and 77 years (mean 47.9 ± 15.8).

Expression of miR146a

The expression of *miR146a*, normalised to the endogenous control U6snRNA, was not significantly different between adenoma and cancer and their unaffected counterpart: extra-FA (0.93 ± 1.73), FA (0.36 ± 0.40), extra-FTC (0.94 ± 1.61) and FTC (0.57 ± 0.69) ($p = 0.15$) (Fig. 1). There was no truly significance of altered *miR146a* expression in affected compared to unaffected tissues both in FA and FTC carriers ($p = 0.72$ and $p = 0.11$ respectively).

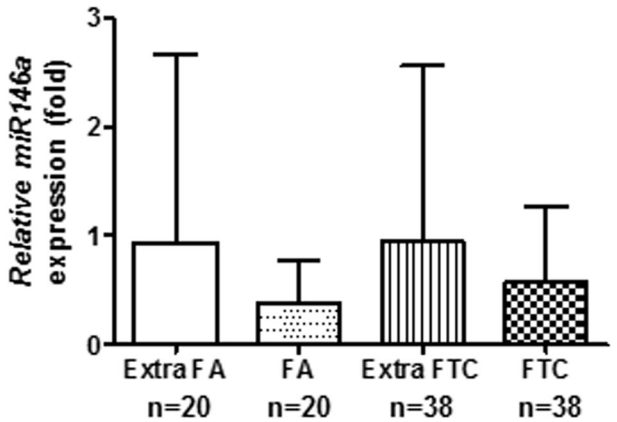

Fig. 1 miR146a expression levels in follicular thyroid adenoma (FA) and follicular thyroid carcinoma (FTC) tissue pairs. Relative expression of *miR146a* in Extra-FA, FA, Extra-FTC and FTC tissues; $p = 0.15$, Kruskal–Wallis test. The error bars represent SD

Expression of TRAF6 and IRAK1

TRAF6 expression (normalised to two endogenous control genes, *GUSB* and *TBP*) was significantly lower in FTC compared to FA tissue, both in affected and unaffected, surrounding tissues (Fig. 2a). However, no correlation was found between *miR146a* and *TRAF6* expression in extra-FTC ($r = 0.16$, $p = 0.32$) and FTC ($r = 0.04$, $p = 0.77$). On the contrary, *IRAK1* expression was upregulated in FTC compared to all the other tissues (Fig. 2b). In particular, *IRAK1* expression was positively correlated to *miR146a* expression both in extra-FTC and in FTC tissues ($r = 0.69$, $p < 0.01$ and $r = 0.35$, $p = 0.04$, respectively). These results suggest that, despite no differences in *miR146a* expression, *TRAF6* was significantly downregulated and *IRAK1* was significantly upregulated in FTC.

Expression of miR146a and FTC clinicopathological features

Then, *miR146a*, *IRAK1* and *TRAF6* expressions were analysed in relation to primary FTC extension: TNM stage, invasiveness and histotype at diagnosis. When tumour dimension was evaluated, *miR146a* expression was significantly higher only in extra-FTC tissue of subjects presenting small (T1) tumours compared to FTC and to all the other extra-FTC tissues of different dimensions.

The expression of *miR146a* did not differ in FTC tissues with different dimensions (Fig. 3a). Increasing the dimension of the tumour, also extra-FTC tissue could be altered since the expression of *miRNA146a* is reduced. *IRAK1* expression significantly increased in FTC with the highest tumour size (T4) compared to all the other tumour extension. No statistically significant differences were seen between extra-FTC tissues, nor when comparing FTC to extra-FTC tissues for any tumour dimension (Fig. 3b). No *TRAF6* differences were observed when considering the primary tumour extension (data not shown).

Considering TNM stages, *miR146a* (Fig. 3c) and *TRAF6* expression (data not shown) did not significantly differ neither in FTC nor in extra-FTC tissues at any stage. Moreover, their expression was similar at any stages in FTC compared to the relative extra-FTC. Instead, *IRAK1* expression was significantly higher in FTC compared to extra-FTC at stage I (Fig. 3d). In the more severe stages, *IRAK1* expression was similar, suggesting that no differences in expression occur with the increase of the tumour severity.

Considering tumour invasiveness, significantly lower *miR146a* expression was observed only in widely invasive tumours when compared to minimally invasive FTC. In fact, *miR146a* expression did not significantly change

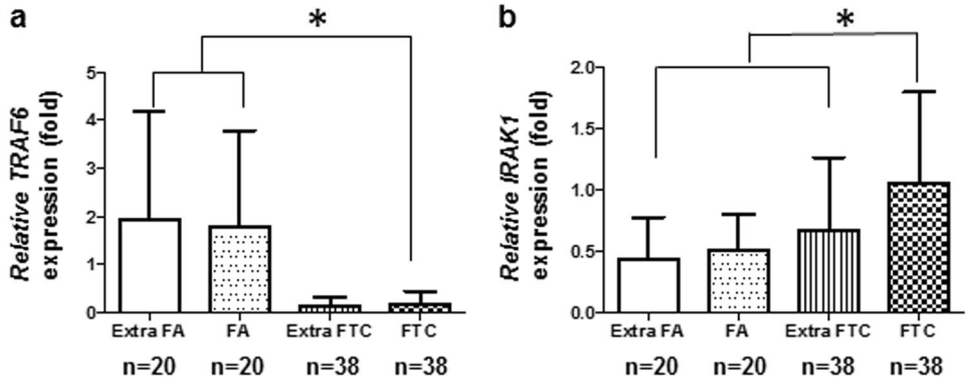

Fig. 2 *TRAF6* and *IRAK1* expression levels in follicular thyroid adenoma (FA) and follicular thyroid carcinoma (FTC) tissue pairs. **a** Relative expression of *TRAF6* in Extra-FA, FA, Extra-FTC and FTC tissues. $*p \leq 0.01$; Kruskal–Wallis test. **b** Relative expression of *IRAK1* in Extra-FA, FA, Extra-FTC and FTC tissues. $*p < 0.01$; Kruskal–Wallis test. The error bars represent SD

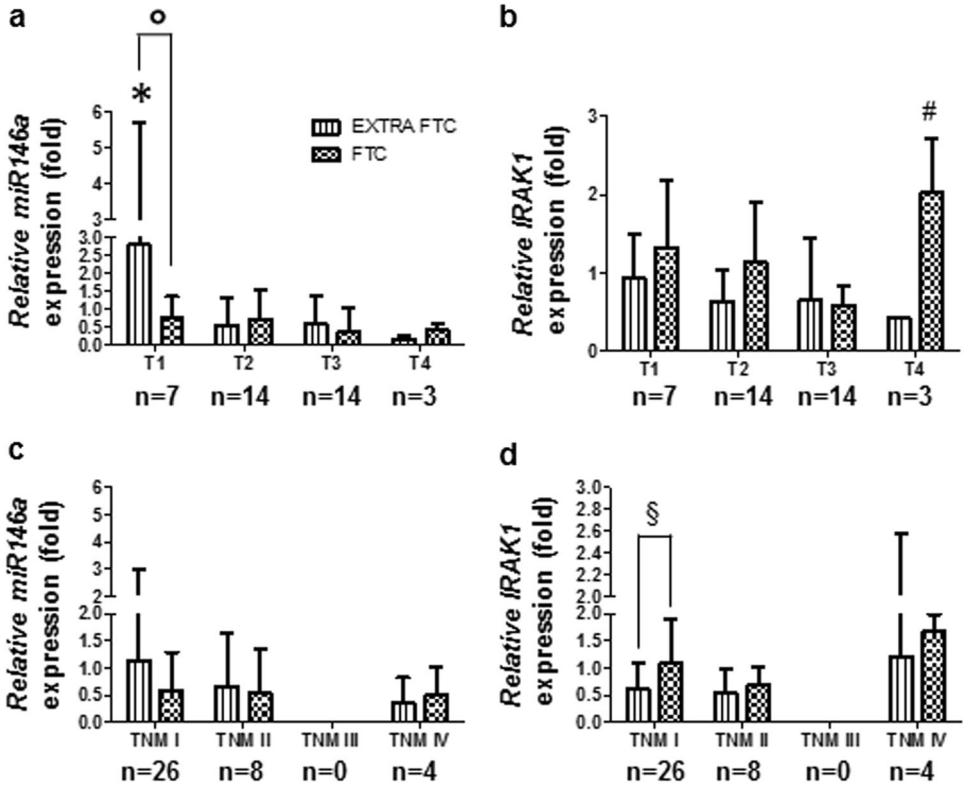

Fig. 3 Relative expression of *miR146a* and target genes related to primary tumour extension and stage. **a** *miR146a* relative expression in Extra-follicular thyroid carcinoma (FTC) compared to FTC considering the tumour dimension; statistically significant differences were found only for T1 tumour. $°p = 0.03$ Mann–Whitney test. Differences were present when comparing Extra-FTC tissues of different dimension (T1 to T4). $*p = 0.03$ Kruskal–Wallis test. **b** *IRAK1* relative expression in Extra-FTC compared to FTC considering the dimension; statistically significant differences were not found for any tumour dimension; Mann–Whitney test. Differences were present when comparing FTC tissues of different dimension (T1 to T4). $\#p = 0.01$ Kruskal–Wallis test. **c** *miR146a* relative expression in Extra-FTC compared to FTC considering the stage; statistically significant differences were not found at any TNM stage. **d** *IRAK1* relative expression in Extra-FTC compared to FTC considering the stage; statistically significant differences were found only in TNMI. $§p = 0.04$; Mann–Whitney test. The error bars represent SD

between extra-FTC tissues, nor in FTC compared to the relative extra-FTC (Fig. 4).

No significant differences were found for *TRAF6* and *IRAK1* expression according to invasiveness (Table 2). Again, this suggests that in the most invasive cancers, *miR146a* was present at the lowest level.

Considering histotype, no significant differences in *miR146a* expression between extra-FTC and FTC samples were found, neither for the follicular classical variant ($n = 26$) (data not shown) nor for the oncocytic variant (Fig. 5a). In this latter histotype, *IRAK1* expression was significantly higher in FTC tissue compared to the unaffected counterpart

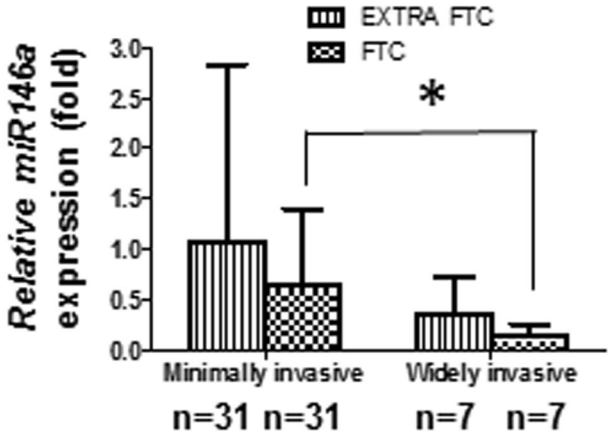

Fig. 4 Relative expression of *miR146a* related to tumour invasiveness. *miR146a* relative expression differences were not found in Extra-follicular thyroid carcinoma (FTC) compared to FTC nor in minimally invasive tumour, $p = 0.81$, nor in widely invasive tumours. $p = 0.12$. Mann–Whitney test. Differences between minimally and widely invasive tumour where seen only when considering FTC tissue. *$p = 0.02$; Mann–Whitney test. The error bars represent SD

Table 2 *TRAF6* and *IRAK1* expression related to FTC invasiveness ($n = 38$)

Invasiveness	Tissue	Target gene expression	*p*-value
TRAF6			
Minimally invasive ($n = 31$)	Extra-FTC	0.15 ± 0.2	0.79
	FTC	0.14 ± 0.18	
Widely invasive ($n = 7$)	Extra-FTC	0.08 ± 0.03	0.45
	FTC	0.28 ± 0.52	
IRAK1			
Minimally invasive ($n = 31$)	Extra-FTC	0.71 ± 0.64	0.07
	FTC	1.01 ± 0.72	
Widely invasive ($n = 7$)	Extra-FTC	0.51 ± 0.44	0.09
	FTC	1.14 ± 0.88	

Mann–Whitney test

(Fig. 5b). No differences were found for *TRAF6* expression (data not shown). Therefore, the increased *IRAK1* expression seems to be related to histotype, being significant only in the oncocytic variant.

Expression of miR146a and patients' age and sex

Linear regression analysis was performed and *miR146a* resulted not significantly related to patient age, neither in FTC ($F = 0.19$, $p = 0.90$) nor in FA ($F = 1.27$, $p = 0.30$). Similarly, *TRAF6* and *IRAK1* expressions did not change in relation to age in FA ($F = 1.27$, $p = 0.30$), or FTC ($F = 0.59$, $p = 0.45$).

Considering gender, *miR146a* expression was significantly higher in females (1.54 ± 2.05) compared to males (0.27 ± 0.26) in extra-FTC ($p < 0.01$, Mann–Whitney test) but not in FTC (females 0.74 ± 0.79, males 0.36 ± 0.51) ($p = 0.06$), even if trend was maintained. There were no differences between males and females in *IRAK1* expression, neither in extra-FTC nor in FTC tissues ($p = 0.11$ and $p = 0.81$, respectively). *IRAK1* expression was higher in FTC (1.01 ± 0.70) compared to the unaffected tissue (0.60 ± 0.67) only in males ($p < 0.01$).

Finally, gender did not influence *TRAF6* expression.

Gender study was not possible in FA samples because only 2 out of 20 controls were males.

Discussion

In the present study, the analysis of a considerable case series of follicular lesions demonstrated a lack of significant *miR146a* expression dysregulation in FTC. Moreover, its expression does not vary with tumour size or stage, histotype or age of the carrier. *miR146a* is not overall downregulated in malignant versus unaffected tissue. However, a significant downregulation was found in thyroids affected by invasive FTC, only in neoplastic tissue. Finally, higher expression levels of *miR146a* were found in female FTC

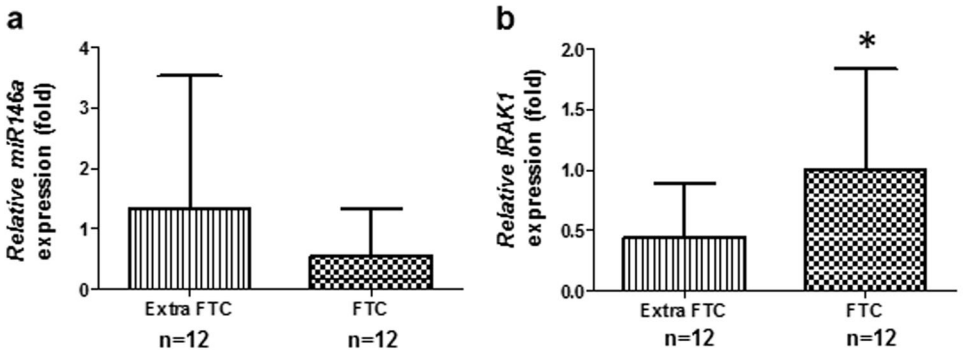

Fig. 5 Relative expression of *miR146a* and *IRAK1* related to the tumour histotype. **a** *miR146a* relative expression in Extra/follicular thyroid carcinoma (FTC) tissues of Hurtle/oncocytic variant. $p = 0.4$; Mann–Whitney test. **b** *IRAK1* relative expression in Extra/FTC tissues of Hurtle/oncocytic variant tumours. *$p = 0.03$; Mann–Whitney test. The error bars represent SD

and extra-FTC tissues, compared to males. This gender-related result is concordant with previous data in hepatocellular carcinoma tissues [28].

Considering tissue expression of two target genes, *TRAF6* and *IRAK1*, different results were found. *TRAF6* was less expressed in the presence of FTC, compared to FA; but its expression was neither related to *miR146a* nor to any clinicopathological characteristics of tumours or subjects. *IRAK1* was significantly upregulated in thyroids affected by FTC, only in neoplastic tissue, and mainly in oncocytic variant. Such upregulation increases with the size of carcinoma but not with the worsening of TNM or invasion. Surprisingly, a positive correlation with *miR146a* expression emerged.

Previous studies demonstrated that *miR146a* expression was upregulated in PTC compared to unaffected thyroid tissue [29], in anaplastic thyroid cancer [30], and strongly increased in PTC compared to FA [31]. Considering FTC, Ma et al. [29] suggested *miR146a* upregulation, demonstrating its role, together with *miR146b*, in inducing tumour progression via inhibition of ST8SIA4, an enzyme involved in polysialic acid synthesis. Despite the research of Ma and colleagues was based on a larger cohort of patients, significant experimental differences compared to our study are present: (i) only thyroid cancer tissue was collected, disregarding extra-tumour counterpart, (ii) normal thyroid tissue samples, from patients diagnosed with thyroid nodules, served as control, (iii) total RNA was extracted from snap-frozen and not FFPE tissues, (iv) no further characterisation of miR expression was carried out considering clinicopathological characteristics of FTC and (v) miRNA expression by qPCR was determined by using Sybr Green and not specific probes.

Conversely, *miR146a* expression was found reduced in FTC respect to PTC tissues [30] and recently, a down-regulation of *miR146a* in cancer was suggested [32, 33], proposing that *miR146a* could negatively regulate NF-kB signalling pathway and thus affect the process of tumour formation due to the presence of the binding site of NF-kB in *miR146a* promoter region.

Beyond the expression of *miR146a*, our results suggest that *TRAF6* and *IRAK1* should be mainly regulated by other mechanisms. Both proteins encoded by these genes are key adaptors downstream of Toll-like and IL-1 receptors signalling pathway and are known to be subjected to a negative feedback by *miR146a* [34]. In breast cancer cells, *miR146a* inhibits the expression of *IRAK1* and *TRAF6*, contributing to reduce receptor signalling, impairing NF-kB activity, and suppressing the expression of NF-kB target genes [14].

In this study, we observed *IRAK1* upregulation in FTC compared to other tissues, suggesting that other factors different or in addition to *miR146a* are involved in the progression of FTC. This could explain the absence of *IRAK1* upregulation in FA, in spite of low levels of *miR146a*.

TRAF6 is downregulated in thyroids of patients with FTC compared to patients with FA but our data suggest that other genes and/or molecular factors, different from *miR146a*, must be involved in its regulation.

Even if this is a descriptive study, without ambition to provide pathophysiological explanations, and limited by the absence of a healthy control group, results provide important information to better define genetic alterations of thyroid follicular lesions. Moreover, this is one of the largest FTC cohorts collected so far, with the added value of having studied *miRNA146a* expression in conjunction with clinicopathological features of tumours and carriers. However, in vitro experiments are needed to support the cause-and-effect relationship between *miRNA146a* and the development and/or progression of FTC.

In conclusion, *miR146a* expression is reduced only in widely invasive FTC compared with minimally invasive tissues, whereas the expression of *IRAK1* is increased, and mainly appreciable in Hurtle FTC variant, while the expression of *TRAF6* is unchanged.

Although the utility of the identification of these markers in a patient sample is still undetermined, taken together all these results provide new insights into the role of some genetic patterns (*miR146a, IRAK1, TRAF6)* in both follicular cancer and the surrounding unaffected tissue, thus tracing new lines for leading future researches on the genetics and the cause-effect relationship in the process of tumorigenesis of thyroid follicular cancer.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. "Research involving human participants and/or animals: This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all the subjects included in the study. Informed consent was obtained from all individual participants included in the study.

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