MicroRNAs miR-146-5p and let-7f as prognostic tools for aggressive papillary thyroid carcinoma: a case report

SUMMARY
Papillary thyroid cancer (PTC) is the most incident histotype of thyroid cancer. A certain fraction of PTC cases (5%) are irresponsive to conventional treatment, and refractory to radioiodine therapy. The current prognostic factors for aggressiveness are mainly based on tumor size, the presence of lymph node metastasis, extrathyroidal invasion and, more recently, the presence of the BRAFV600E mutation. MicroRNAs (miRNAs) have been described as promising molecular markers for cancer as their deregulation is observed in a wide range of tumors. Recent studies indicate that the over-expression of miR-146b-5p is associated with aggressiveness and BRAFV600E mutation. Furthermore, down-regulation of let-7f is observed in several types of tumors, including PTC. In this study, we evaluated the miR146b-5p and let-7f status in a young male patient with aggressive, BRAFV600E-positive papillary thyroid carcinoma, with extensive lymph node metastases and short-time recurrence. The analysis of miR-146b-5p and let-7f expression revealed a distinct pattern from a cohort of PTC patients, suggesting caution in evaluating miRNA expression data as molecular markers of PTC diagnosis and prognosis.

SUMÁRIO
O carcinoma papilífero (PTC) é o histotipo mais prevalente de câncer de tiroide. Cerca de 5% dos casos são refratários ao tratamento convencional e à radioiodoterapia. Os fatores prognósticos para agressividade mais utilizados atualmente são o tamanho do tumor, a presença de metástases linfonodais ao diagnóstico, a presença de invasão extratiroideana e, mais recentemente, a presença da mutação BRAFV600E. A análise de perfil de expressão de microRNAs (miRNA) mostra que esses pequenos RNAs são marcadores moleculares promissores para o câncer, por apresentarem desregulação de sua expressão em uma ampla gama de tumores, incluindo o PTC. Estudos recentes revelam a associação entre o aumento da expressão do miRNA e miR-146b-5p e a presença da mutação BRAFV600E como um fator de pior prognóstico no PTC. Além disso, observa-se a diminuição da expressão de let-7f em diversos tipos de tumores, incluindo tumores tiroideanos. Neste relato de caso, realizamos a quantificação da expressão de miR-146b-5p e let-7f em um paciente jovem, de sexo masculino, apresentando PTC positivo para a mutação BRAFV600E com extensas metástases linfonodais ao diagnóstico e recidiva precoce. A análise da expressão de miR-146b-5p e let-7f mostrou um padrão diferente do observado em um grupo de pacientes PTC, sugerindo a necessidade de cautela na interpretação da expressão de miRNAs como marcador molecular no diagnóstico e prognóstico de PTC.
miRNA expression in an aggressive papillary thyroid cancer

**INTRODUCTION**

Papillary thyroid cancer (PTC) is the most incident histotype of thyroid cancer, accounting for more than 80% of the 35,000 annual cases in the USA. To date, prediction of PTC outcome is mainly based on tumor size, gender, age of patient at time of diagnosis, presence lymph node metastasis, capsular invasion, and extra-thyroidal extension. Although most PTCs cases display overall good prognosis, a certain fraction of PTC cases (5%) are irresponsive to conventional treatment and refractory to radioiodine therapy (1). Thus, a number of cases diagnosed as low-risk PTCs eventually recur locally and as lymph node metastases, leading to death (2), indicating the lack of current reliable outcome predictors. Indeed, no consensus has been achieved regarding the clinical application of molecular markers to predict the outcome of PTC patients (3).

More recently, several studies suggest that the presence of mutation in BRAF oncogene is associated with a poor outcome due to frequent extra-thyroidal invasion, recurrence, and lower survival rate (4-6). In this context, microRNAs (miRNAs) arise as interesting markers for cancer, as their deregulation affects numerous cell processes, such as proliferation and apoptosis (7). MiRNAs negatively regulate protein levels by binding to 3’UTR of target mRNA and impairing protein translation (8). The potential of miRNAs as PTC molecular markers is highlighted, once their expression may be detected from formalin-fixed paraffin-embedded (FFPE) tissue, fine needle aspiration cytology (FNAC), as well as serum samples (9).

Recent studies have explored the use of several miRNAs as cancer diagnostic tools (10). In the last 4 years, several studies have demonstrated the potential of miRNAs in PTC diagnosis. Although miR-146b-5p is down-regulated in other types of cancer (11-13), this miRNA is highly overexpressed in PTCs (14,15), reliably distinguishing this type of cancer from follicular carcinoma (FTC), and from benign lesions (16,17). Moreover, Schwertheim and cols. have shown that a set of miRNAs that includes miR-146b-5p distinguished well-differentiated from undifferentiated thyroid tumors (18). Recent studies suggested that miRNA expression may be used as a thyroid cancer prognostic tool. The expression levels of miR-146b-5p, along with other two miRNAs, miR-221 and -222, were significantly higher in high-risk PTC patients who showed extra-thyroidal invasion (19). Moreover, among BRAFV600E-positive PTCs, the aggressive subset presents high miR-146b-5p expression levels (20). Let-7f down-regulation was firstly described by Takamizawa and cols. (21) in lung cancer with poor prognosis, and rapidly arose as a promising cancer molecular marker (22). Although let-7f down-regulation has been described in PTC, its usefulness as a diagnostic and prognostic marker for PTC is uncertain.

In this study, we present a case report of a young male patient presenting an aggressive, BRAFV600E-positive papillary thyroid carcinoma, displaying lymph node metastases at diagnosis and short-term recurrence after surgery, in whom we evaluated the expression pattern of the miRNAs miR-146b-5p and let-7f.

**SUBJECT AND METHODS**

**Case report**

A 23-year-old caucasian male (named here ABC) with parents bearing Hashimoto’s thyroiditis, was submitted to a health checkup, and was diagnosed with hypothyroidism, positive for anti-TPO and anti-TG antibodies. At the ultrasound examination, calcified thyroid nodules of 12 mm and 8 mm were found at the isthmus and right lobe, respectively. The patient ABC also had lymphadenopathy of 33 mm, with calcification at level III of right side, and 9 mm at level III of left side. Fine needle aspiration citology (FNAC) pattern was compatible with PTC in the thyroid and in the right lateral lymph nodes, but not in the left side. The patient underwent total thyroidectomy, and central compartment and right lateral lymph node dissection (levels II to V). Histopathological examination of the primary tumor confirmed the diagnosis of PTC (Figure 1A) with lymphatic invasion. Metastases were found in right lymph nodes at levels III (2 out of 7), IV (6 out of 6) (Figure 1B), V (2 out of 3), and at the central compartment (3 out of 4). Neither vascular nor peri-neural invasion were detected. According to the American Joint Committee on Cancer Staging System, the tumor was classified as T3N1bM0 and MACIS score was 3.37 (23,24).

Three months after surgery, the patient received 200 mCi of 131I under Thyrogen® (Genzyme, Cambridge, MA, USA) stimulation (TSH 140 μIU/mL). Thyroglobulin and anti-Tg antibodies levels were 0.15 ng/mL and > 3,000 IU/mL, respectively. Eight months later, ultrasound examination showed lymphadenopathy at levels II, III and IV of the left side, the
largest one with 19 mm, and elevated anti-Tg antibody levels (11,580 IU/mL). FNAC revealed PTC and PET-CT examination showed FDG uptake at level II. At the second surgery, lateral lymph node dissection was performed on the left side (levels II to V). Histopathological examination of lymph nodes revealed metastases at levels II (2 out 4), III (3 out 17), IV (2 out of 8) and V (9 out of 11). The patient received 200 mCi of 131I under Thyrogen® stimulation. Three months after the second surgery, radioiodine uptake was negative.

**Patient population**

Human PTC and non-tumor paired thyroid tissue samples were collected from patients, including ABC, after the informed consent was signed. This sample belongs to the thyroid tumor tissue bank, approved by the ethical committee of the Institute of Biomedical Sciences (no. 1030/CEP), Universidade de Sao Paulo. Thyroid carcinomas were classified according to the last WHO classification (25).

**BRAF mutation detection**

Genomic DNA was extracted from thyroid tissue using the salting-out protocol. Briefly, after digestion with proteinase K, protein was precipitated with 5 M NaCl, and DNA was isolated with isopropanol. Exon 15 of BRAF, containing the hotspot T1799A mutation, was amplified by PCR using specific primers (FW: 5’- AAACCTCTTATACATTGCTTCGTGTTCCGC-3’; RV: 5’-GGCCAAAATATTTAAATCATTGGA-3’), and the product was purified in QIAquick® PCR purification kit (Qiagen, Valencia, CA, USA). For direct sequencing reaction, the purified product was amplified using BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies), forward primer, and injected in multiparilla sequencer MegaBace1000 (GE Healthcare, Little Chalfont, UK) (26). The similarity of the resulting sequence was analyzed and confirmed using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/).

**MicroRNA expression**

Excised tumor tissues were immediately stored in RNAlater®. Total RNA was extracted by phenol-chloroform using TRIzol® reagent (LifeTechnologies, Carlsbad, CA, USA), according to the manufacturer’s instructions, and stored at -80°C. Detection of miRNAs miR-146b-5p, let-7f and RNU6B was performed using commercial kits (Life Technologies) according to the manufacturer’s specifications. Briefly, cDNA was synthesized from 10 ng of total RNA using specific primers in a stem-loop based technology with TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). Expression was analyzed by qPCR using specific Taqman miRNA Assay (Life Technologies) and TaqMan® Master Mix no AmpErase® UNG (Life Technologies).

**Statistical analysis**

Quantification of miRNA expression is presented as mean ± S.E., and data were submitted to Student’s t-test to compare results between the two groups. Differences were considered significant at p < 0.05.
RESULTS

Analysis of genomic DNA from a set of PTC samples and paired contra-lateral thyroid tissue revealed \( \text{BRAF}^{T1799A} \) mutation in 41% of patients, including the patient ABC (7/17).

\( \text{miR-146b-5p} \) is substantially up-regulated in the cohort of PTCs from our tissue bank when compared with their matched contra-lateral samples, irrespective of their \( \text{BRAF}^{T1799A} \) status (Figure 2A). Interestingly, both the primary tumor and metastatic tissue of ABC exhibited lower \( \text{miR-146b-5p} \) fold-change levels compared with the cohort of PTC samples (Figure 2A), and no statistical differences compared with its paired normal thyroid tissue \((P = 0.773)\).

On the other hand, the \( \text{BRAF}^{T1799A} \) tumors showed no significant changes in \( \text{let}-7\)f expression levels, while BRAF-wild type tumors exhibited increased \( \text{let}-7\)f levels in comparison with matched contra-lateral thyroid tissue (Figure 2B). ABC primary tumor, which was \( \text{BRAF}^{T1799A} \)-positive, exhibited a slight increase in \( \text{let}-7\)f levels \((31\%, P < 0.01)\) (Figure 2B). However, the metastatic tissues, also positive for \( \text{BRAF}^{T1799A} \), displayed decreased \( \text{let}-7\)f levels (Fig 3B).

![Figure 2. \( \text{miR-146b} \) and \( \text{let}-7\)f levels in PTC samples. Expression of \( \text{miR-146b-5p} \) (A) and \( \text{let}-7\)f (B) in PTC samples in comparison with contra-lateral thyroid tissue. Values are represented as mean ± SE of expression fold-change matched to contra-lateral thyroid tissue. RNU6B gene expression was used as an internal control. ABC, patient ABC; Meta IV, lymph node metastasis at level IV; Meta VI, lymph node metastasis at level VI. (*) P < 0.01, against matched thyroid contra-lateral tissue.](image)

DISCUSSION

MiRNAs have been described as markers for diagnosis and prognosis, once their expression pattern may classify tumor types and aggressiveness accurately (10). Recent studies showed that molecular screening of miRNA expression in thyroid tumors is important in distinguishing malignant from benign tumors, and that different thyroid cancer histotypes display distinct miRNA expression (17, 27). However, in patient ABC, the expression pattern of \( \text{miR-146b-5p} \) and \( \text{let}-7\)f levels could not be associated with aggressiveness and poor prognosis, as previously described in the literature.

It is not clear whether the overexpression of \( \text{miR-146b-5p} \) is associated with \( \text{BRAF}^{T1799A} \) status. While initial studies showed no correlation between this miRNA and oncogenic activation (9), recent studies have demonstrated a positive correlation between the overexpression of \( \text{miR-146b-5p} \) and aggressive \( \text{BRAF}^{T1799A} \) tumors (19). Moreover, in the literature, high expression of \( \text{miR-146b-5p} \) is associated with BRAF-mutated tumors that display more aggressive phenotypes (20). Conversely, our cohort of patients did not show any correlation regarding \( \text{BRAF} \) status and \( \text{miR-146b-5p} \) expression. \( \text{MiR-146b-5p} \) is associated with the deregulation of \( \text{NFκB} \) and \( \text{TGFB} \) pathways, while the blockage of \( \text{MiR-146b-5p} \) action restores \( \text{TGFβ} \) signal transduction in thyroid cells in vitro (28). Non-thyroid tumors frequently exhibit reduced levels of \( \text{miR-146b-5p} \) (11, 29). We have previously shown that activation of MAPK kinase pathway in normal thyroid cells increases \( \text{miR-146b-5p} \) levels in vitro (28). Therefore, the overexpression of \( \text{miR-146b-5p} \), only observed in thyroid tumors, could be related with a thyroid-specific oncogenic activation, which might include the MAPK pathway.

The association between \( \text{BRAF}^{T1799A} \) and \( \text{let}-7\)f expression also remains poorly understood. Classically, the main target of \( \text{let}-7\)f is \( \text{RAS} \) oncogene, an important transducer for the MAPK pathway. Interestingly, a functional study revealed that the reinforced expression of \( \text{let}-7\)f in a PTC cell line blocks ERK phosphorylation without affecting \( \text{RAS} \) protein levels (30). On the other hand, we have previously shown that activation of RET/PTC rearrangement in rat normal thyroid cell line, but not of \( \text{BRAF} \) mutation, decreases \( \text{let}-7\)f levels. In our cohort of patients, \( \text{BRAF}^{T1799A} \)-positive tumors showed no change in \( \text{let}-7\)f levels, in comparison with contra-lateral thyroid tissue, while \( \text{BRAF} \)-wild type tumors displayed increased levels of this miRNA. Interestingly, the patient ABC, which was \( \text{BRAF}^{T1799A} \)-positive, showed slightly increased \( \text{let}-7\)f expression, even though his metastatic tissue, also \( \text{BRAF}^{T1799A} \)-positive, showed decreased levels of \( \text{let}-7\)f. Little is known about the targets regulated by \( \text{let}-7\)f in PTC. Different mRNA targets might be regulated by \( \text{let}-7\)f to promote matrix remodeling, vascular infiltration, and the colonization...
of the metastatic site. Functional studies are needed to elucidate whether the loss of let-7f by primary tumor is implicated in thyroid tumor progression and what is the clinical significance of this data.

To date, clinical and histological features, such as age, gender, extra-thyroidal invasion, and, vascular invasion, histotype, and more recently, \( \text{BRAF}^{V600E} \) status, are still the indicated parameters for accurate PTC outcome prediction. Furthermore, recent lines of evidence have shown that the number of lymph node metastases is also important to predict the patient’s outcome, and may be used as an independent prognostic factor for aggressiveness (31), which corroborate the clinical characteristics of patient ABC.

This case showed to be interesting because of the discordance of two widely studied PTC miRNA markers in this index patient and our cohort of patients. Our data indicate that the expression pattern of \( \text{miR-146b-5p} \) and let-7f genes may not reproduce the clinical features of the tumor, clearly illustrating that outcome prediction based on the analysis of the expression of a single or few miRNA genes should be viewed with caution.

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REFERENCES

19. Jou CS, Chen RF, Chou FF, Chang HW, Chen YJ, Lee YF, et al. miR-146b is highly expressed in adult papillary thyroid carcinomas with high risk features including extrathyroidal invasion and the \( \text{BRAF}(V600E) \) mutation. Thyroid. 2010;20(5):489-94.