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# Identification of new long non-coding RNAs associated with medullary thyroid cancer

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## Abstract

Medullary thyroid carcinoma (MTC) represents just 5–10% of all thyroid malignancies. In contrast to the familial MEN2, little is known about the etiology of sporadic MTC. New approaches are required to elucidate the mechanisms underlying the pathogenesis of sMTC. Long noncoding RNAs (lncRNAs), are well-recognized post-transcriptional regulators of genetic expression and recent studies have described multiple aberrantly expressed non-coding RNAs in thyroid cancers. In the current study we have aimed to perform the first screening of multiple lncRNAs in tumoral tissues from MTC patients by qRT-PCR. Our analysis showed the association of 15 lncRNAs from which 6 where new in association with this disease (*RMST*, *SNHG16*, *FTX*, *GAS5*, *IPW*, *MEG3*). The association of these new lncRNAs with overall survival was analyzed by Kaplan-Meier curve.

## Introduction

Medullary thyroid carcinoma (MTC) it is a tumor originated from C-cells and derived from the neural crest which accounts for only 1%–2% of thyroid cancers, although it is responsible for about 13% of all thyroid cancer–related deaths [1,2]. MTC can occur either sporadically (75%) or as the dominant component of the type 2 multiple endocrine neoplasia syndromes (MEN2, 25%). It is considered a rare disease, with an estimated prevalence in the general population of 1/14,300 [http://www.orpha.net; ORPHA Nº: 1332].

The broad term long non-coding RNA (lncRNA) refers to a class of non-coding RNA transcript of minimum 200 nucleotides in length. They have gained widespread attention in recent years as new players in transcriptional, epigenetic, or post-transcriptional regulation of gene expression [3]. To date, only one study has examined the expression of lncRNAs in patients with MTC [4]. Consequently, lncRNAs are attractive and promising targets in cancer prognosis and treatment.

The purpose of this study is to bring insight and deeper understanding into the etiology of sMTC, to a deeper understanding of disease mechanisms, pathogenesis, and searching of new therapeutic targets. To afford this aim, we have analyzed the expression of lncRNAs in this type of tumors.

## Materials and methods

### Experimental subjects

In this study, we have performed lncRNA expression analysis on four sMTC cases (Table 1). All MTC tissues and their corresponding adjacent non-tumor thyroid tissues were obtained from these patients after undergoing surgical resection. The samples were snap frozen in liquid nitrogen and stored at −80°C until use. A written informed consent was obtained from all the participants for clinical and molecular genetic studies. The study was approved by the Ethics Committee for clinical research in the University Hospital Virgen del Rocío (Seville, Spain) and complies with The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18 July 1964).

**Table 1.** Clinicopathological features of included MTC patients

| Characteristics | N |
|---|---|
| Age at diagnosis, years | |
| Median (range) | 46.5 |
| **Gender** | |
| Male | 2 |
| Female | |
| **Inheritance** | |
| Sporadic (absence of any mutation MEN2 related) | 4 |
| **Tumor size, centimeters** | |
| Median (range)<br>Nodal metastasis at diagnosis | 2.75 |
| **Distant metastasis** | |
| Present at initial diagnosis | 3 |

### Screening by lncRNA PCR Array

Total RNA was obtained from tissues of our patients and commercial cells by using RNEasy Purification Kit (Qiagen), according to the manufacturer's instructions. The RNA was quantified by Nanodrop (Invitrogen, USA) and 1 µg of total RNA was reverse transcribed into

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cDNA using PrimeScript RT Reagent Kit (Perfect Real Time; TaKaRa, Osaka, Japan) to determine lncRNA expression levels, using *GAPDH* as internal control. For lncRNA expression analysis, laboratory-verified SYBR®Green qPCR assays (RT² lncRNA PCR Array, Qiagen) were used. Each plate contains 84 lncRNAs already associated with different cancer pathways (Supplementary Table 1). The quantitative real-time PCR (qRT-PCR) was performed at the 7900HT Fast Real-Time PCR System with the 384-Well Block Module (Applied Biosystems). We used the ΔΔCt method for relative quantitation of lncRNAs level expression, where a fold-change of at least two times and a corrected P-value of < 0.05 were used as a criterion of selection.

## Statistical analysis

Overall survival rates were calculated by the Kaplan-Meier method with the long-rank test applied for comparison. P-value < 0.05 was considered as statistically significant.

## Results

The expression profiles of 84 lncRNAs, already associated with different cancer pathways, in 4 tumoral and non-tumoral paired tissues were determined by SYBR®Green qPCR assays. Fifteen differentially expressed lncRNAs were detected in our samples (all adjusted $P \leq$

**Table 2.** Aberrant LncRNAs in MTC tissues: All significant lncRNAs obtained by qRT-PCR (7900HT Taqman system) through the RT2 lncRNA PCR Arrays. RQ represents the fold-change. From the normalized value of 1.00 of non-tumoral tissues, we represent those lncRNAs downregulated (< 2.00) and those ones upregulated (< 2.00) in our study. The significant p-value was < 0.05. All available information about their implication in other type of cancers is also compiled on the last column of the table, with special mention when they have been linked with thyroid cancer.

| Sample | Detector | Avg Ct | Avg Delta Ct | Delta Delta Ct SD | RQ | Described in cancer (is it described into thyroid cancer)? |
|---|---|---|---|---|---|---|
| Non-tumoral | *ZFAS1* | 29.275 | 3.833 | 0.000 | 1.000 | Not associated with thyroid cancer but it is related with colorectal, gastric, ovarian, prostate, hepatic, bladder, esophagus and breast cancers. |
| Tumoral | *ZFAS1* | 23.903 | 2.997 | -0.836 | 1.785 | |
| Non-tumoral | *RMST* | 30.659 | 5.217 | 0.000 | 1.000 | Not associated with thyroid cancer but it is related with breast cancer. |
| Tumoral | *RMST* | 24.888 | 3.982 | -1.235 | 2.353 | |
| Non-tumoral | *SNHG16* | 31.328 | 5.885 | 0.000 | 1.000 | Not associated with thyroid cancer but it is related with esophageal squamous cell carcinoma, gastric, lung, glioma, bladder, breast, colorectal and cervical cancers. |
| Tumoral | *SNHG16* | 25.200 | 4.294 | -1.591 | 3.014 | |
| Non-tumoral | *FTX* | 30.498 | 5.056 | 0.000 | 1.000 | Not associated with thyroid cancer but it is related with hepatocellular, colorectal, renal, breast cancers as well as in leukemia and melanoma. |
| Tumoral | *FTX* | 23.914 | 3.008 | -2.048 | 4.135 | |
| Non-tumoral | *GAS5* | 30.056 | 4.613 | 0.000 | 1.000 | Associated with thyroid cancer, among other tumors (Low expression of long non-coding RNA GAS5 is associated with poor prognosis of patients with thyroid cancer. |
| Tumoral | *GAS5* | 23.297 | 2.390 | -2.223 | 4.668 | |
| Non-tumoral | *IPW* | 30.182 | 4.739 | 0.000 | 1.000 | Not associated with thyroid cancer. |
| Tumoral | *IPW* | 23.908 | 3.002 | -1.738 | 3.335 | |
| Non-tumoral | *MALAT1* | 31.932 | 6.489 | 0.000 | 1.000 | Associated with different cancers and other pathologies, and with thyroid cancer (Upregulation of long noncoding RNA MALAT1 in papillary thyroid cancer and its diagnostic value. Liu J et al. Future Oncol. 2018 Jul 10; MicroRNA-21 and long non-coding RNA MALAT1 are overexpressed markers in medullary thyroid carcinoma. |
| Tumoral | *MALAT1* | 26.359 | 5.453 | -1.036 | 2.051 | |
| Non-tumoral | *MEG3* | 31.919 | 6.477 | 0.000 | 1.000 | Associated with different cancers and other pathologies, and with thyroid cancer (Long noncoding RNAs: emerging players in thyroid cancer pathogenesis. |
| Tumoral | *MEG3* | 22.835 | 1.928 | -4.548 | 23.397 | |
| Non-tumoral | *PTCSC1* | 28.910 | 3.467 | 0.000 | 1.000 | Associated with thyroid cancer Long noncoding RNAs: emerging players in thyroid cancer pathogenesis. |
| Tumoral | *PTCSC1* | 26.362 | 5.455 | 1.988 | 0.252 | |
| Non-tumoral | *PTCSC3* | 29.431 | 3.989 | 0.000 | 1.000 | The polymorphism rs944289 predisposes to papillary thyroid carcinoma through a large intergenic noncoding RNA gene of tumor suppressor type. |
| Tumoral | *PTCSC3* | 26.283 | 5.377 | 1.388 | 0.382 | |
| Non-tumoral | *TUG1* | 29.886 | 4.443 | 0.000 | 1.000 | LncRNA TUG1 influences papillary thyroid cancer cell proliferation, migration and EMT formation through targeting miR-145. |
| Tumoral | *TUG1* | 24.500 | 3.593 | -0.849 | 1.802 | |
| Non-tumoral | *ADAMTS9-AS2* | 31.702 | 6.259 | 0.000 | 1.000 | Not associated with thyroid cancer but it is related with lung and glioma cancers. |
| Tumoral | *ADAMTS9-AS2* | 27.908 | 7.002 | 0.743 | 0.598 | |
| Non-tumoral | *PRNCR1* | 31.918 | 6.475 | 0.000 | 1.000 | Not associated with thyroid cancer but it is related with gastric, colorectal and prostate cancers. |
| Tumoral | *PRNCR1* | 28.308 | 7.402 | 0.927 | 0.526 | |
| Non-tumoral | *RMRP* | 22.623 | -2.820 | 0.000 | 1.000 | Not associated with thyroid cancer but it is related with breast, lung, gastric and colon cancers. |
| Tumoral | *RMRP* | 18.382 | -2.524 | 0.295 | 0.815 | |
| Non-tumoral | *H19* | 31.640 | 6.197 | 0.000 | 1.000 | Associated with thyroid cancer, among other tumors (Epigenetic Modifications in Thyroid Cancer Cells Restore NIS and Radio-Iodine Uptake and Promote Cell Death. |
| Tumoral | *H19* | 27.891 | 6.984 | 0.787 | 0.580 | |

0.05). From all the differentially expressed lncRNAs, 8 downregulated and 7 upregulated lncRNAs had not been published yet in association with any thyroid carcinoma (Table 2).

In addition, analysis of overall survival was performed by using Kaplan-Meier curve although it is not significant (available under request).

## Discussion

Many efforts are being made to establish the biological and clinical relationships between lncRNAs and cancer. They are involved in a variety of biological processes through the regulation of gene expression [5,6]. In this manner, lncRNAs regulate transcription and epigenetic events, leading cells adapting to a changing environment.

It is important to highlight that one of the upregulated lncRNAs that we have obtained in this study was *MALAT1*, which has been already associated with MTC [4]. This fact reinforces the validity of our approach. In this study, we have evaluated 84 different lncRNAs, already associated with cancer pathways, in 4 MTC patients through qRT-PCR, showing the significant association of 3 downregulated and 4 upregulated new lncRNAs that had not been published yet in association with neither MTC nor any thyroid carcinoma.

This study is not devoid of limitations. We have compared by qRT-PCR the expression levels of different lncRNAs in a group of MTC patients and normalizing to the levels detected in normal adjacent thyroid tissues (with mostly follicular cells). Although normal C-Cells would be our perfect control tissue, there is very little number of them in the normal thyroid. Thus, we decided to use thyroid follicular cells because they are very close to the MTCs and they express the thyroid transcription factor 1, as well as C-Cells do. Then, we consider that this comparison approach was a good alternative, as some previous studies also confirmed [4,7,8].

## Conclusions

We describe here six new lncRNAs (*RMST*, *SNHG16*, *FTX*, *GAS5*, *IPW*, *MEG3*) which could play an interesting role in this rare tumor, that to date has any effective therapy or prognosis. Further studies with larger sample sizes would be needed to confirm the role of these new lncRNAs in MTC that maybe can serve as predictive cancer biomarkers or targets for preventive drugs.

## Data availability

The expression data from the qPCR assays used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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## Supplementary material

Supplementary table 1: The 84 lncRNAs from the RT$^2$ lncRNA PCR Arrays.

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