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Evaluation of Downstream Regulatory Element Antagonistic Modulator Gene in Human Multinodular Goiter

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Background: DREAM (Downstream Regulatory Element Antagonistic Modulator) is a neuronal calcium sensor that was suggested to modulate TSH receptor activity and whose overexpression provokes an enlargement of the thyroid gland in transgenic mice. The aim of this study was to investigate somatic mutations and *DREAM* gene expression in human multinodular goiter (MNG).

Material/Methods: DNA and RNA samples were obtained from hyperplastic thyroid glands of 60 patients (54 females) with benign MNG. *DREAM* mutations were evaluated by PCR and direct automatic sequencing, whereas relative quantification of mRNA was performed by real-time PCR. Over- and under-expression were defined as a 2-fold increase and decrease in comparison to normal thyroid tissue, respectively. RQ M (relative quantification mean); SD (standard deviation).

Results: DREAM expression was detected in all nodules evaluated. DREAM mRNA was overexpressed in 31.7% of MNG (RQ *M*=6.26; SD=5.08), whereas 53.3% and 15% had either normal (RQ *M*=1.16; SD=0.46) or underexpression (RQ *M*=0.30; SD=0.10), respectively. Regarding *DREAM* mutations analysis, only previously described intronic polymorphisms were observed.

Conclusions: We report *DREAM* gene expression in the hyperplastic thyroid gland of MNG patients. However, DREAM expression did not vary significantly, and was somewhat underexpressed in most patients, suggesting that DREAM upregulation does not significantly affect nodular development in human goiter.

MeSH Keywords: DNA Mutational Analysis • Gene Expression • Goiter, Nodular • Kv Channel-Interacting Proteins

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Background

Multinodular goiter (MNG) is a very common disease that is defined as an enlargement of the thyroid gland, characterized by heterogeneity in growth and function of thyroid follicular cells [1]. Iodine deficiency is the main environmental etiology for goiter [2]. In areas with sufficient iodine intake, the incidence of MNG has been documented in more than in 4% of the general population; single and multiple thyroid nodules were found in 0.8% of men and 5.3% of women, with an increased frequency in women over 45 years of age [3]. A strong genetic predisposition to goiter has been suggested by family and twin studies: family studies have shown that children of parents with MNG have a noticeably higher risk of goiter compared with the risk for children of healthy parents, and several twin studies have shown a higher concordance rate of goiter for monozygotic than dizygotic twins [4].

DREAM (downstream regulatory element antagonist modulator), also known as potassium channel interacting protein (KChIP-3) or calsenilin, is a Ca^{2+}-dependent protein that binds specifically to DRE (downstream regulatory element) in DNA, acting as a transcriptional repressor [5]. Binding of DREAM to DREs is regulated by nuclear Ca^{2+}, by the interaction with other nucleoproteins like αCREM and CREB (cAMP response element-binding protein), and by the PI3K (phosphatidylinositol 3-kinase) pathway [5–7]. DREAM is highly expressed in the central nervous system, testes, thymus, and the thyroid gland [5,8–10]. In thyroid follicular cells, DREAM modulates the transcriptional activity of thyroid transcription factor-1 (TTF-1), paired box 8 (Pax8), and forkhead box E1 (Foxe1/TTF-2), and represses thyroglobulin (Tg) gene expression [10,11].

Transgenic mice with thyroid tissue-specific up-regulation of *DREAM* were found to express higher levels of TSHR (thyroid-stimulating hormone receptor) and cAMP, with induction of *NIS* (sodium/iodide symporter) and *TPO* (thyroperoxidase) genes, in thyroid gland than in wild type; hyperproliferation of thyroid gland and goiter development were also observed in transgenic mice with *DREAM* overexpression [12]. In humans, expression of DREAM MRNA and protein levels were found to be more than twice as high in patients with multinodular goiters [12]. Together, these data suggest that DREAM is able to modulate TSHR levels in the human thyroid gland, and up-regulation of endogenous DREAM may contribute to the development of thyroid nodules [12].

Thus, the aim of this study was to evaluate somatic mutations and *DREAM* gene expression in patients with multinodular goiter.

Material and Methods

Patients

We evaluated 60 patients with benign MNG (54 females and 6 males; mean age, 53 years; range, 24–85 years) recruited from the Endocrinology Clinics, Clinical Hospital of Faculty of Medicine, University of Sao Paulo, Brazil. None of the patients presented family history of goiter. A multinodular goiter was defined as a thyroid gland with 2 or more nodules larger than 1 cm, determined by ultrasound. The exclusion criteria were: toxic goiter, thyroid cancer, history of irradiation, and thyroiditis. Samples of hyperplastic thyroid gland were collected from all patients after total or partial thyroidectomy and immediately frozen in liquid nitrogen.

This study was approved by the Ethical Committee of Hospital das Clinicas, Sao Paulo University and written informed consent was obtained from all patients.

Extraction of nucleic acids

Both DNA and RNA were extracted from the largest nodule using the AllPrep DNA/RNA Mini Kit (Qiagen) following the manufacturer's protocol. DNA and RNA were used for genetic mutations and gene expression analysis, respectively.

PCR amplification and sequencing

The 9 coding exons of *DREAM* were amplified by PCR using the following primers: 1F – 5'AGGGGTGGAGCGATAGAAG 3', 1R – 5'caaaggaaagtggaacaaGAG 3'; 2F – 5' GTTCAGGCTGGCCTCATCTA 3', 2R – 5' caatagagacaggggcgatg 3'; 3F – 5' GTTCCTCCACCTGCTAT TTTG 3', 3R – 5' Taaaggcccctgggatatt 3'; 4-5F – 5' CAAGGGGGT GGAGAGAGG 3', 4-5R – 5' cccagggtgactcacaagat 3'; 6F – 5' ATGGATGCCGTCAGTCTCTT 3', 6R – 5' cagtctctggatggacagc 3'; 7F – 5' CTTCTCTCTCCAGCTCGTC 3', 7R – 5' gagtagggaggctcagagg 3'; 8F – 5' CCAGAGTAGTCACAGGGGCA 3', 8R – 5' agacaagagggcaagtggag 3'; 9F – 5' CTCCCTGCACCAATAAGAC3', 9R - 5' CTGGCAGGATGGAGGTTTCT 3', designed using *Primer3 web* version 4.0.0 [13].

All PCR products were pretreated with an enzymatic combination of exonuclease I and shrimp alkaline phosphatase (U.S. Biochemical Corp., Cleveland, OH) and directly sequenced using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) in an ABI 3100 XL automatic sequencer (Applied Biosystems, Foster City, CA). Electropherograms obtained were compared with the sequences of *DREAM* gene (ENST00000295225) present in the Ensembl database (*http://www.ensembl.org/*) using Sequencher commercial software (Gene Codes).

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Quantitative real time PCR

cDNA was generated using 1 ug of DNA-free RNA samples by *QuantiTect Reverse Transcription* (Qiagen) according to the manufacturer's instructions. Quantitative real-time PCR measuring of DREAM mRNA was performed with commercially available assay primers (Hs_KCNIP3_1_SG QuantiTect primer assay, NM_001034914, NM_013434, Qiagen) in a PCR assay buffer that contained SYBR Green as fluorescent dye (QuantiTect SYBR Green PCR kit, Qiagen) per the manufacturer's instructions. Fluorescence was detected using the Step One Plus™ Real-Time PCR (Applied Biosystems) system. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs_GAPDH_1_SG QuantiTect primer assay, NM_001256799, NM_002046, Qiagen) was used as the endogenous normalizing gene. A commercial pool of RNA from 47 normal human thyroid tissues (ages, 8–78 years) was used for comparisons (CLONTECH, BioChain, and Ambion). Relative quantification was performed by $2^{-\Delta\Delta CT}$ method [14]. Over- and under-expression were defined as a 2-fold increase and decrease in comparison to normal thyroid tissue, respectively.

Statistical methods

Data are presented as mean ±SD or median and interquartile range as appropriate, whereas proportions and frequencies were used for categorical variables.

We used one-way ANOVA test or Kruskal-Wallis ANOVA on ranks, as appropriate, to compare clinical and hormonal patient characteristics between groups with low, high, and normal levels of *DREAM* gene expression. Nominal data were analyzed by χ^2 test. All statistical analyses were performed using SigmaStat™ for Windows (version 3.5, Systat Software, Inc.) and considering $p<0.05$ as significant.

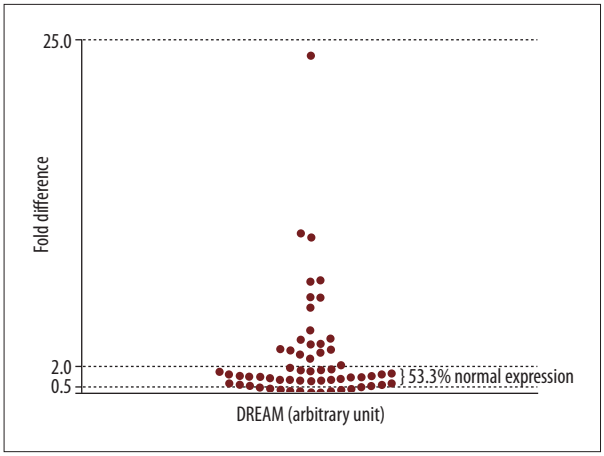

Figure 1. Graphical distribution of mRNA *DREAM* expression levels of thyroid nodular tissue from 60 patients with MNG.

Results

Hormonal data

Mean of TSH (thyroid-stimulating hormone), T3 (triiodothyronine), and free T4 (thyroxine) levels in MNG patients were 1.46 mIU/L (±0.94), 2.1 nmol/L (±0.8), and 14.2 pmol/L (±2.6), respectively.

Molecular analysis

Genetic analysis showed no mutations or rare variants in DNA sequence of *DREAM* gene in our cohort of MNG patients. Only 2 previously described intronic polymorphisms, rs2248415 and rs117109173, were observed in 91.6% and 8.3% of patients, respectively. These SNP frequencies did not significantly differ from that described in the dbSNPs databank.

Table 1. Clinical and hormonal data of MNG patients categorized according to their *DREAM* mRNA expression.

DREAM expression	Low	Normal	High	P
Fold change	0.3 (0.15–0.4)	1.1 (0.5–1.9)	6.0 (2.0–23.8)	–
Sex (F: M)	8: 1	28: 3	18: 2	0.992
Age (years)	60.0 (54.5–67.2)	57.0 (42.2–65.0)	49.5 (44.5–57.5)	0.095
TSH (mIU/L)	1.34 (0.98–2.43)	1.00 (0.72–1.69)	1.33 (1.04–2.23)	0.241
T3 (nmol/L)	1.6 (1.5– 1.7)	1.9 (1.7–2.2)	1.9 (1.6–2.7)	0.262
free T4 (pmol/L)	12.5±1.7	14.4±2.7	14.0±3.1	0.171
Nodular size* (cm)	2.0±0.3	3.1±2.7	3.2±1.4	0.620
Thyroid weight (g)	62.3 (42.2–161.4)	37.0 (16.7–74.0)	58.0 (22.3–116.3)	0.164

Reference values: TSH: 0.40–4.50 mIU/L; T3: 1.0–3.0 nmol/L; free T4: 9.0–19 pmol/L. * Nodular size was defined by the largest diameter.

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Quantitative real-time PCR revealed normal expression DREAM in 32 MNG patients (RQ *M*=1.16; SD=0.46), whereas 19 and 9 cases had over- (RQ *M*=6.26; SD=5.08) and under-expression (RQ *M*=0.30; SD=0.10), respectively (Figure 1). No correlation between *DREAM* mRNA expression and patient characteristics was observed (Table 1).

Discussion

The pathogenetic mechanisms of human goiter are still unclear. Recently, upregulation of *DREAM* has been proposed as an important contributor to the development of thyroid nodules and goiter in mice and humans [12].

In this study, we evaluated mRNA *DREAM* expression in 60 patients with MNG, using quantitative real-time PCR. However, *DREAM* was overexpressed in no more than 31.7% of cases, whereas 53.3% and 15% of patients had normal and lower *DREAM* expression, respectively. Although a key assumption in studying mRNA expression is that it is informative in the prediction of protein expression, an important limitation of our study was that only DREAM mRNA levels were measured. Nevertheless, some authors have demonstrated that DREAM mRNA and protein levels were correlated in different tissues in human and rats [11,15].

With respect to thyroid gland, only Rivas et al. [12] reported the evaluation of DREAM expression in a very small number of patients with MNG [12]. In this earlier work, measurement of DREAM protein levels was performed in 16 nodular samples using Western blot. In contrast to our results, a higher than 2-fold increase in DREAM expression was reported in 10 cases (62.5%), suggesting an etiopathological role of elevated levels of this protein in human multinodular goiter [12].

Activating mutations in the cAMP signal transduction TSH pathway have been shown to be involved in development of toxic multinodular goiter [16]. Curiously, DREAM transgenic mice maintained a euthyroid state [12]. Thus, we also investigated the occurrence of putative activating DREAM mutations in our cohort of MNG patients. However, only intronic polymorphic variants were found. Based on the *gene* mutation information maintained in the Sanger Institute *COSMIC database* (*http://cancer.sanger.ac.uk/cosmic/*), *DREAM* mutations have been found at a very low frequency (<1%) in a wide variety of human cancers, including cancers of the breast, endometrium, kidney, and intestines.

Conclusions

The present study involving *DREAM* genetic and expression analysis in the hyperplastic thyroid gland of and expressive cohort of MNG patients found no activating *DREAM* mutations. DREAM expression did not vary significantly, and was underexpressed in most cases, suggesting that DREAM upregulation does not significantly affect nodular development in human goiter. The published data are very limited and additional studies are required to elucidate the role of DREAM in the pathological process of human goitrogenesis.

Conflict of interest statement

The authors had not conflicts of interest to disclose.

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