CHARACTERIZATION OF GENE EXPRESSION IN DIFFERENTIATED THYROID CANCER STEM CELLS BY RNA-SEQ, AREQUIPA - PERU

Carlos A. Cárdenas-Abarca[2]; Jani Pacheco-Aranibar[1,3]; Cinthia C. Cordova Barrios[1]; Angel S. Mamani-Ruelas[2]; Francis W. Jacobo-Valdivia[1]; Javier L. Valero Quispe[1]; Ivo Palomino Valverde [1]; Jhony R. Rodriguez Mamani[4] y Julio C. Bernabé-Ortiz[1,2]

[1]Catholic University of Santa Maria, Arequipa - Peru.
[2]Carlos Alberto Seguín Escobedo National Hospital - *EsSalud.* Arequipa - Peru.
[3]Institute of DNA Biotechnology Uchumayo, Arequipa - Peru
[4]National University of the Altiplano. Puno - Peru.

Corresponding author: ***Julio C. Bernabe Ortiz, E-mail:*** *jbernabe@ucsm.edu.pe*

ABSTRACT

Advances in next-generation sequencing (NGS) RNA-Seq technology is making it possible to reveal differentiated thyroid cancer (DTC) genotype. Little is known about the genes that are active in this pathology. The expression profile of CDT stem cells in the Arequipa population is described. 11 patients with CDT undergoing thyroidectomy between 2019-2020 from Hospital Nacional Carlos Alberto Seguín Escobedo EsSalud Arequipa were enrolled, and stem cells were isolated from biopsies. Total RNA was isolated from the stem cell cultures and subjected to massive sequencing by RNA-Seq using the Illumina protocol. The most frequent histopathological variants were papillary (81.82%) and follicular (18.18%). Bioinformatic analysis of the massive sequencing (RNA Seq), using IGV software and the GRCh38.p13 genome, showed a prevalence of mutations in BRAF, TERT and P53 genes in all patients. The top 30 genes with the highest frequency of expression were: PCB2, LRMDA, WWOX, RPS29, RBFOX1, FHIT, NTM, RAD51B, PRKN2, MACROD2, ASIC2, TBC1D5, GPC5, EXOC4, IMMP2L, CAMTA1, CDH13, SMYD3, ANKS1B, DAB1, DLG2, FRMDA4A, KCNIP4, CTNNA2, AGBL4, PRKCE, SYN3, ARHGAP1 5, PACRG, C9orf92. This study reports for the first time five new mutations for the BRAF gene and 18 new mutations for the TERT gene.

Keywords: Thyroid cancer, RNA-Seq. Stem cells

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INTRODUCTION

Thyroid cancer currently ranks 9th in frequency worldwide, with an incidence of approximately 7.4 new cases per 100,000 inhabitants. This reality does not escape the country, where it is in eighth place in frequency with an approximate incidence of 7.5 new cases per 100,000 inhabitants, according to data from Globocan (Global Cancer Observatory) for the year 2018[(1)]. It is important to highlight that the incidence of this neoplasm has increased worldwide; however, mortality remains with figures very similar to those of previous decades, the evidence cited in multiple reviews [(2-3)] and current consultation texts [(4)]. In the national literature, there is little information related to epidemiological figures of this disease; however, there is a current review described by Noé Atamary Anahuac, a researcher at the San Ignacio de Loyola University, who in 2018 conducted a national review with data from the Ministry of Health[(5)], concluding that the prevalence of this disease has increased in our country, with the highlands region being the most compromised, but also with mortality rates that have not changed. Even so, there is information on the increase in the presentation of aggressive variants in different world populations, where iodine deficiency seems to play an important role[(6)], often independent of tumor size[(7)]. New

molecular techniques have been developed concerning the new advances in diagnosing and treating thyroid cancer[8]. The isolation and culture of stem cell from tumors and their gene expression analysis greatly help the stratification of this disease's aggressiveness [9] and the search for therapeutic targets [10]. These are of practical importance in situations of aggressive variants of this neoplasm, in which it has been seen that they express mutations of the BRAFV660E gene[11]. State-of-the-art techniques are fulfilling these objectives by providing us with a better understanding of the genetic variables and an understanding of tumor biology with the new generation sequencing tools NGS[12], through the application of mRNA-Seq, which can provide us with information on the prediction of recurrence of this disease[13-17].

Point genetic mutations of BRAF (T1799A) in exon 15 lead to the expression of the BRAFV600E mutant protein, which results in constitutive serine/threonine kinase activation[18,19]. The BRAFV600E mutation is one of the most common genetic alterations in thyroid cancer, occurring in approximately 45% (30 to 70%) of sporadic CPT and 15% of follicular variant CPT [20]. In particular, 80% to 100% of the tall cell variant of TPC harbors the BRAFV600E mutation [20]. The BRAFV600E mutation predicts worse clinical outcomes in the progression of PTCC, including aggressive pathologic features and a higher recurrence rate [21,22]. In addition, it is considered that the BRAFV600E mutation may cause loss of radioactive iodine avidity by thyroid cancer cells and, as a result, refractoriness to radioactive iodine treatment ([21,22]. The RAS point mutation is frequently located in thyroid and other solid cancers [23].

Among the genes of the three RAS isoforms (HRAS, KRAS and NRAS), the NRAS mutation predominates in thyroid tumors [23]. The RAS mutation is relatively rare (0-20%) in the usual form of PTC, whereas almost half of CFT and the follicular variant of PTC harbor the RAS mutation [23, 24]. The RAS mutation is observed in approximately 20% of follicular adenomas, suggesting that the RAS mutation occurs early in tumorigenesis [23, 24]. The RAS mutation dampens GTPase activity, constitutively perpetuating its activity. Likewise, the RAS mutation activates the PI3K-AKT pathway in thyroid tumorigenesis [23, 24]. The tumor suppressor gene PTEN is a negative regulator of the PI3KAKT signaling pathway through the opposite function of PI3K. Mutation or deletion of PTEN leads to follicular thyroid cell tumorigenesis, known as Cowden disease (syndrome) [25]. Cowden syndrome is an autosomal inherited disease caused by germline mutations of PTEN [25]. Cowden disease is a carcinogenic syndrome closely related to a predisposition to an increased risk of thyroid, breast, and endometrial cancers and benign tumors such as hamartomas [25]. PTEN alteration is observed in 40% of CFT [25]. PTEN silencing by hypermethylation of its promoter has also been found in CFT and anaplastic thyroid carcinoma (ATC) [26-28]. Other genes are also mutated in thyroid cancer, such as CTNNB1, TP53, IDH1 and NDUFA13 (GRIM19) [29]. CTNNB1 is involved in the WNT-β-catenin pathway and is often found mutated in CAT [30]. TP53 encodes the p53 tumor suppressor and is involved in many solid cancers. TP53 mutation is frequently observed in CAT (70-80%)[31,32]. CTNNB1 and TP53 mutations have been preferentially observed in CAT or poorly differentiated thyroid carcinoma, suggesting that these genetic alterations may be associated with dedifferentiation or late follicular cell events in cancer progression[31, 32]. Although Hürthle cell thyroid cancer does not carry common genetic alterations such as BRAFV600E, RAS, or RET/PTC[33], 15% harbor mutations of NDUFA13 (GRIM19)[34].

Just as the incidence of this disease has increased[1], so has its aggressiveness [7], in which the usual treatments, such as surgery, radioactive iodine, hormone suppression and chemotherapy, have limitations, even in these clinical situations, in which in other health realities, without going very far, such as Argentina [14], molecular determinations are made in order to detect mutations that explain this aggressiveness, applying molecular therapy directed to these alterations, thus improving the treatment (3) and quality of life of this disease.

The present study applied the RNA-Seq technique to determine the mutational gene expression profile of tumor stem cells from patients diagnosed with differentiated thyroid cancer at the HNCASE EsSalud Arequipa.

METHODOLOGY

Samples

The research protocol was approved by the research committee of the HNCASE, after which samples were obtained from the tissue bank of the pathological anatomy service of this hospital from a total of 11 patients with a histopathological diagnosis of differentiated thyroid cancer (DTC) who underwent surgery at this institution.

Immediately after surgery, the presence of differentiated thyroid cancer was corroborated, and a fresh tissue fragment of approximately 30 mg was placed in an Eppendorf and stored in a cold chain; the sample was transferred to the molecular biology laboratory of the campus of the Catholic University of Santa Maria (UCSM) for processing.

Isolation and culture of stem cells

Biopsies were washed with 1x phosphate buffer solution (DPBS, GIBCO, Cod: 14190144), digested with collagenase type II at 0.15% (Sigma-Aldrich, Cod: C6885) for 60 min at 37 ºC and neutralized with culture medium (DMEM, GIBCO, Cod: 11995) 10% fetal bovine serum (FBS, GIBCO, Cod: 10270), 1% antibiotic and antifungal (Antibiotic-Antimycotic 100X, GIBCO, Cod: 15240); then centrifuged for 10 min at 1200g. The *pellet* obtained was resuspended in a culture medium maintained under normoxic conditions (21%O_2 -5%CO_2) until clusters of adherent cells formed on the surface of the flask, and continued incubation until adequate expansion was achieved (50% confluence).

Total RNA and RNA-seq extraction

For the isolation of total RNA, the Direct-zol™ RNA MiniPrep kit from ZYMO RESEARCH was used, following the company's instructions. The RNA that passed quality control was labeled and stored at a temperature of -20°C and then shipped, with a dry ice transport system, to the laboratory of Admera Health Biopharma Services in the USA. For sequencing by Next Generation sequencing RNA-seq.

Bioinformatics Analysis

The number of reads was 40 to 60 million per sample during sequencing. ***Raw data***: The previously constructed sequencing data is processed by FastQC and MultiQC v1.8 software, and a FastQ file is generated.

The IGV (integrative genomics viewer) software was used to visualize the alignments found, comparing them against the GRCh38.p13 genomic base (version 13 of the consortium 38 of the human reference genome) to detect mutations.

RESULTS

Figure 1, on the left, shows the frequencies of expression of the first 24 common genes in the 11 cases of patients with CDT studied. For example, the PCB2 gene is the most highly expressed in the 11 patients with differentiated thyroid cancer, with the KCNIP4 gene ranking 24th. On the right of the figure are the transcript frequencies of the 25th to 48th most commonly expressed genes in the 11 cases of patients with CDT studied. For example, the CTNNA2 gene ranks 25, while the SPAG16 gene ranks 48 in the 11 cases of the population studied.

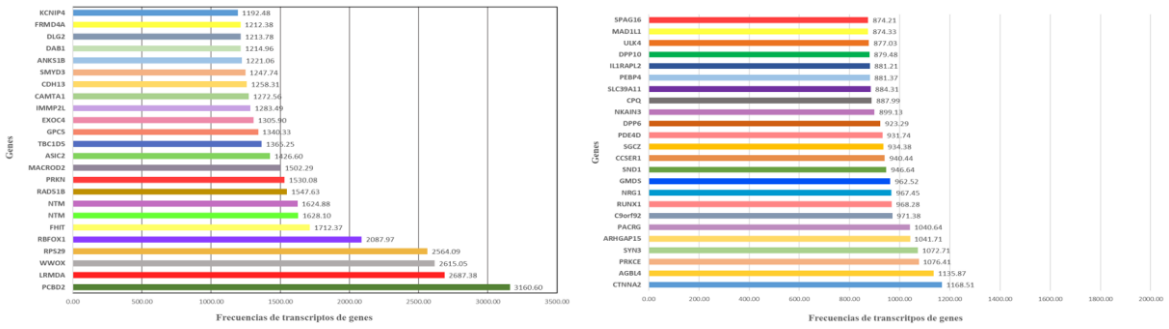

Figure 1. Genes with the highest frequency of expression common to the 11 cases of Differentiated Thyroid Cancer studied.

The sequences were normalized and were listed based on expression intensity. The first 24 tops are shown on the left and 25 to 48 in the figure on the right, out of a total of 19365 transcript types.

Table 1. Genes with the highest frequency of expression common to the 11 cases of Differentiated Thyroid Cancer.

Gen	Name	Synonyms	Location
PCBD2	Pterin-4 alpha-carbinolamine dehydratase 2	PHS2; DCOH2; DCOHM.	5q31.1
LRMDA	Associated with differentiation of leucine-rich melanocytes.	CDA017; C10orf11.	10q22.2-q22.3
WWOX	WW domain containing oxidoreductase	FOR; WOX1; DEE28; EIEE28; FRA16D; SCAR12; HHCMA56; PRO0128; SDR41C1; D16S432E.	16q23.1-q23.2
RPS29	Ribosomal protein S29	S29; uS14; DBA13.	14q21.3
RBFOX1	Fox homologue 1-binding RNA	2BP1; FOX1; A2BP1; FOX-1; HRNBP1.	16p13.3
FHIT	histidine triad fragile diadenosine triphosphatase.	FRA3B; AP3Aase.	3p14.2
NTM	Neurotrimine	HNT; NTRI; CEPU-1; IGLON2; HNT; NTRI; CEPU-1; IGLON2	11q25
RAD51B	RAD51 paralog B	REC2; R51H2; RAD51L1.	14q24.1
PRKN	Parkin RBR E3 ubiquitin protein ligase	PDJ; AR-JP; LPRS2; PARK2.	6q26
MACROD2	Mono-ADP ribosyl hydrolase 2	C2orf133; C20orf133.	20p12.1
ASIC2	Acid-sensing ion channel subunit 2	ACCN; BNC1; MDEG; ACCN1; BNaC1; ASIC2a; hBNaC1.	17q11.2-q12
TBC1D5	TBC1 domain family member 5	KIAA0210.	3p24.3
GPC5	Glypicane 5	GLYPICAN 5	13q31.3
EXOC4	Exocyst complex component 4	SEC8; Sec8p; SEC8L1.	7q33
IMMP2L	Inner mitochondrial membrane peptidase subunit 2	IMP2; IMP2-LIKE; IMMP2L-IT1.	7q31.1
CAMTA1	Calmodulin-binding transcription activator 1	CANPMR.	1p36.31-p36.23
CDH13	Cadherina 13	HRC; P105.	16q23.3
SMYD3	SET and MYND domain containing 3.	KMT3E; ZMYND1; ZNFN3A1; bA74P14.1.	1q44
ANKS1B	Ankyrin repeat and sterile 1B-containing alpha motif domain.	EB1; AIDA; EB-1; ANKS2; AIDA-1; cajalin-2.	12q23.1

DAB1	DAB adaptor protein 1	SCA37.	1p32.2-p32.1
DLG2	MAGUK 2 large scaffold protein discs	PSD93; PSD-93; PPP1R58; chapsyn-110.	11q14.1
FRMD4A	FERM domain containing 4A	FRMD4; CCAFCA; bA295P9.4.	10p13
KCNIP4	Potassium voltage-dependent channel-interacting protein 4	CALP; KCHIP4.	4p15.31-p15.2
CTNNA2	alpha 2 catenin	CAPR; CTNR; CAP-R; CT114; CDCBM9.	2p12
AGBL4	AGBL carboxypeptidase 4	CCP6.	1p33
PRKCE	Protein kinase C epsilon	PKCE; nPKC-epsilon.	2p21
SYN3	Synapsin III	Synapsin - 3, CN28H9.2.	22q12.3
ARHGAP15	Rho GTPase activator protein 15	BM046.	2q22.2-q22.3
PACRG	Parkin coregulated	GLUP; PACRG2.1; PARK2CRG; HAK005771.	6q26
C9orf92	Chromosome 9 open reading frame 92	Em:AL513424.1.	9p22.3

Mutation analysis. For the mutation search, the sequences of specific genes were aligned to the reference genome using the IGV software described in the methodology. Table 2 shows the BRAF mutations found in the 11 patients of the study, taking as reference the human gene bank DNA sequence "Human Build 38 Genome Reference Consortium" (GRCh38.p13). Of the six mutations found, the BRAF rs113488022 missense variant mutation is already described in the literature; the remaining five mutations are de novo.

TERT mutations found in the 11 patients in the study, referenced to the human gene bank DNA sequence "Genome Reference Consortium Human Build 38" (GRCh38.p13). Of the 23 mutations found, the TERT mutations "rs1751161082 missense variant", "rs1749094617 missense variant," and "rs33954691 synonymous variant" were already described in the literature; the remaining 20 mutations are de novo.

Table 2. Location of common BRAF point mutations in the 11 cases of Differentiated Thyroid Cancer.

BRAF GENE	
Mutation	**Bibliographic reference**
First name: rs889134706 Type of variant: SNV Alleles: C > T, G Chromosome: 7:140924572 (GRCh38) Exon 1 BRAF: Missense variant Functional consequence: Not reported	https://www.ncbi.nlm.nih.gov/snp/?term=BRAF+7%3A+140924572
Type of variant: SNV Alleles: T > A Chromosome: 7: 140850164 (GRCh38) Exon 2	De novo mutation

Type of variant: SNV Alleles: T > A Chromosome: 7: 140800452 (GRCh38) Exon 7	De novo mutation
Type of variant: SNV Alleles: T > A, C, G Chromosome: 7: 140794401 (GRCh38) Exon 8	De novo mutation
Type of variant: SNV Alleles: A > T Chromosome: 7: 140777028 (GRCh38) Exon 14	De novo mutation
Type of variant: SNV Alleles: A > T Chromosome: 7: 140754195 (GRCh38) Exon 15	De novo mutation

GEN TERT	
Mutation	**Bibliographic reference**
Name: rs1751161082 Type of variant: SNV Alleles: T > C Chromosome: 5:1293763 (GRCh38) Exon 2 TERT: Missense variant Functional consequence: Not reported	https://www.ncbi.nlm.nih.gov/snp/?term=TERT+5%3A+1293763.
Type of variant: SNV Alleles: C > G Chromosome: 5: 1293765 (GRCh38) Exon 2	De novo mutation
Type of variant: SNV Alleles: C > G Chromosome: 5: 1293766 (GRCh38) Exon 2	De novo mutation
Type of variant: SNV Alleles: C > A Chromosome: 5:1293910 (GRCh38) Exon 2	De novo mutation
Type of variant: SNV Alleles: A > G Chromosome: 5:1293913 (GRCh38) Exon 2	De novo mutation
Type of variant: SNV Alleles: C > A Chromosome: 5: 1293914 (GRCh37) Exon 2	De novo mutation
Type of variant: SNV Alleles: G > T Chromosome: 5:1294136 (GRCh38) Exon 2	De novo mutation
Type of variant: SNV Alleles: A > C Chromosome: 5:1294238 (GRCh38) Exon 2	De novo mutation
Type of variant: SNV Alleles: A > C Chromosome: 5:1294313 (GRCh38) Exon 2	De novo mutation
Type of variant: SNV Alleles: T > G Chromosome: 5: 1294315 (GRCh38) Exon 2	De novo mutation

Type of variant: SNV Alleles: C > T Chromosome: 5:1294430 (GRCh38) Exon 2	De novo mutation
Type of variant: SNV Alleles: G > A Chromosome: 5: 1294479 (GRCh38) Exon 2	De novo mutation
Type of variant: SNV Alleles: T > C Chromosome: 5: 1280318 (GRCh38) Exon 4	De novo mutation
Type of variant: SNV Alleles: T > A Chromosome: 5:1278733 (GRCh38) Exon 6	De novo mutation
Name: rs1749094617 Type of variant: SNV Alleles: T > C Chromosome: 5:1272217 (GRCh38) Exon 7 TERT: Missense variant Functional consequence: Not reported	https://www.ncbi.nlm.nih.gov/snp/?term=TERT+5%3A+1272217.
Type of variant: SNV Alleles: G > T Chromosome: 5: 1272218 (GRCh38) Exon 7	De novo mutation
Type of variant: SNV Alleles: T > C Chromosome: 5: 1272221 (GRCh38) Exon 7	De novo mutation
Type of variant: SNV Alleles: T > C Chromosome: 5: 1264568 (GRCh38) Exon 11	De novo mutation
Type of variant: SNV Alleles: A > C Chromosome: 5: 1264493 (GRCh38) Exon 11	De novo mutation
Type of variant: SNV Alleles: A > G Chromosome: 5: 1264446 (GRCh38) Exon 11	De novo mutation
First name: rs33954691 Type of variant: SNV Alleles: G > A Chromosome: 5:1255405 (GRCh38) Exon 13 TERT: Synonymous variant Functional consequence: Not reported	https://www.ncbi.nlm.nih.gov/snp/?term=TERT+5%3A+1255405.

DISCUSSION

This study aimed to determine the gene expression profile by the RNA-Seq technique of tumor stem cells from patients diagnosed with differentiated thyroid cancer at the HNCASE EsSalud Arequipa.

This research was carried out due to the increase in the incidence of new cases of differentiated thyroid cancer worldwide[35] and in the country[36]; as one of the hypotheses, the increased presence of mutations, such as the BRAF mutation^{V600E}, in the molecular biology of this pathology[37].

Figure 1 shows the 48 genes with the highest frequency of expression, common to the 11 cases of TDC in the study population. Table 1 shows the first 30 of these genes mentioned above, all already described in the literature, expressed in

healthy thyroid tissue and whose pathogenic alterations participate in the oncogenesis of TDC.

The computer analysis of the sequences shows 6 SNV (single nucleotide variant) mutations of BRAF taking as reference the human genome assembly database GRCh38.p7 (version 7 of the Human Genome Research Consortium 38), mentioned below: SNV C>T, G on Chromosome 7:140924572 in exon 1, which was already described in the literature[40], the remaining de novo ones not found in the literature as SNV T>A chromosome7: 140850164 in exon 2, SNV T>A chromosome 7: 140800452 in exon 7, SNV T>A, C, G chromosome 7: 140794401 in exon 8, SNV A>T chromosome 7: 140777028 in exon 14 and SNV A>T chromosome 7: 140754195 in exon 15; the locations of the BRAF mutation^{V600E} (chromosome 7: 140753335-140753336) could not be identified. As mentioned above, the most common mutational hotspot in BRAF is T1799A in exon 15, which confers glutamate to valine substitution at amino acid 600 (V600E) in the BRAF protein. BRAF^{V600E} is the most common genetic alteration in PTC, it has a high prevalence in classical PTC and the tall cell variant, although it is generally rare in the follicular variant[38], and its genomic localization according to the GRCh38 database is on chromosome 7: 140753335-140753336; the presence of BRAF^{V600E} has shown increased aggressiveness and worse prognosis in thyroid cancer, one of the mechanisms being activation of downstream genes of the MAPK pathway, which may lead to a slight down-regulation of the sodium iodide symporter (NIS), resulting in inaccurate localization of NIS at the cell membrane[41], leading to a decrease in the ability of tumor cells to uptake iodine so that during adjuvant therapy with I-131 that relies on radioactive iodine uptake by tumor cells, the presence of BRAF^{V600E} may lead to a decrease in a therapeutic effect, resulting in a poor prognosis[42]. The findings correlate the failure to find aggressive BRAF mutations with the patients' initial stages I and II in our investigation (localized disease stages). It is important to measure the clinical impact of de novo mutations in the follow-up of the evolution of this oncologic disease.

In the TERT point mutations common in the 11 patients in our study population carrying differentiated thyroid cancer, there are 21 SNV (single nucleotide variant) mutations of TERT taking as reference the human genome assembly database GRCh38.p13 (Human Genome Research Consortium version 13 38), listed below: SNV T>C on chromosome 5:1293763 in exon 2, SNV T>C on chromosome 5: 1272217 in exon 7 and SNV G>A of chromosome 5:1255405 in exon 13 , which were already described in the literature, the remaining de novo not found in the literature as SNV C>G of chromosome 5: 1293765 in exon 2, SNV C>G of chromosome 5: 1293766 in exon 2, SNV C>A of chromosome 5: 1293910 in exon 2, SNV A>G of chromosome 5: 1293913 in exon 2, SNV C>A of chromosome 5: 1293914 in exon 2, SNV G>T of chromosome 5: 1294136 in exon 2, SNV A>C of chromosome 5: 1294238 in exon 2, SNV A>C of chromosome 5: 1294313 in exon 2, SNV T>G of chromosome 5: 1294315 in exon 2, SNV C>T of chromosome 5: 1294430 in exon 2, SNV G>A of chromosome 5: 1294479 in exon 2, SNV T>C of chromosome: 5:1280318 in exon 4, SNV T>A of chromosome 5: 1278733 in exon 6, SNV G>T of chromosome 5: 1272218 in exon 7, SNV T>C of chromosome 5: 1272221 in exon 7, SNV T>C of chromosome 5: 1264568 in exon 11, SNV A>C of chromosome 5: 1264493 in exon 11 and SNV A>G of chromosome 5: 1264446 in exon 11. In our study, mutations 1295228 C>T and 1295250 C>T (denoted C228T and C250T respectively), corresponding to -124 C>T and -146 C>T of the translation start site in the telomerase reverse transcriptase (TERT) promoter[43], which are the most studied in thyroid cancer and are related to the aggressiveness and prognosis of the disease, these findings could be related to the well-differentiated histopathological variants and a recent diagnosis of the population of our work, even so, close clinical follow-up for the impact of the de novo mutations found in response to treatment and evolution of our patients would be of great value.

Regarding the coexistence of P53 with TERT and BRAF, no significant correlations have been observed between the BRAF mutation^{V600E} or the overexpression of p53 protein and the clinicopathological characteristics of CDT patients[39]. Likewise, it is noteworthy that a papillary carcinoma harboring a TERT promoter mutation has an increased risk of anaplastic transformation independent of P53 or other mutations[39].

Finally, knowledge of the set of thyroid cancer genes and their correlation with clinical subgroups of CDT patients provides information on thyroid cancer tumorigenesis and opens avenues to develop prognostic biomarkers and therapies targeting driver genes in thyroid cancer, evidence already demonstrated in multiple publications[44].

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