Journal of
Medical Genetics

A NOVEL TRUNCATING GERMLINE VARIANT REINFORCES TINF2 AS A SUSCEPTIBILITY GENE FOR FAMILIAL NON-MEDULLARY THYROID CANCER.

Journal:	*Journal of Medical Genetics*
Manuscript ID	Draft
Article Type:	Short report
Date Submitted by the Author:	n/a
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Keywords:	Endocrine Gland Neoplasms, Frameshift Mutation, Genetics, Medical, Genetic Testing, Germ-Line Mutation

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TITLE: A NOVEL TRUNCATING GERMLINE VARIANT REINFORCES *TINF2* AS A SUSCEPTIBILITY GENE FOR FAMILIAL NON-MEDULLARY THYROID CANCER.

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Abstract:

Background: It has long been observed that there are families in which non-medullary thyroid cancer (NMTC) occurs, but few syndromes and genes have been described to date. Proteins in shelterin complex have been implied in cancer. Here, we have studied shelterin genes in families affected by NMTC (FNMTC).

Methods: We performed whole-exome sequencing (WES) in 10 affected individuals from four families with at least three affected members. PCR and Sanger sequencing were performed to search for variants in the *TINF2* gene in 40 FNMTC families. TINF2 transcripts and loss of heterozygosity (LOH) were studied in several affected patients of one family.

Results: We found the c.507G>T variant in heterozygosis in the *TINF2* gene in one family, co-segregating in all five affected members. This variant affects the normal splicing. LOH was not observed.

Conclusions: Our results reinforce *TINF2* gene as a susceptibility cause of FNMTC suggesting the importance of location of frameshift variants in *TINF2*. According to our data and previous literature, *TINF2* pathogenic variants appear to be a significant risk factor for development of NMTC and/or melanoma.

Keywords: familial non-medullary thyroid cancer, TINF2, thyroid cancer, melanoma, shelterin, telomere, germline variant.

INTRODUCTION:

Thyroid cancer is the most common endocrine cancer, accounting for 1% of all cancers. Thyroid cancer comprises two entities, medullary thyroid carcinoma (MTC) and non-medullary thyroid carcinoma (NMTC). Familial NMTC (FNMTC) represents 3–9% of all NMTC and is classified as either syndromic or nonsyndromic. Susceptibility genes involved in syndromic FNMTC are well known[1]. On the contrary, no clear causative genes of nonsyndromic FNMTC have been established so far.

Nonsyndromic FNMTC accounts for more than 95% of all FNMTC cases and is defined by the presence of differentiated thyroid cancer of follicular cell origin in two or more first-degree relatives, and in the absence of other predisposition or environmental causes (radiation exposure or iodine deficiency) with no other syndromic features[2]. In spite of the high prevalence of thyroid cancer in the general population, if there are three affected cases, the probability of being truly hereditary increases to 96%[3].

For years the search for genes implied in nonsyndromic FNMTC has been discouraging. Some genes and variants have been proposed to be causative, but results have not been replicated so far in other affected families, perhaps because most of these variations are specific to particular families.

In the last few years, some reports show that telomere abnormalities are related with susceptibility to FNMTC. Many authors have searched for alterations in genes involved in telomeric regulation, both in telomerase and in the shelterin complex genes[4,5,6]. The shelterin complex is a group of six proteins (POT1, TPP1, TINF2, TERF1, TERF2 and RAP1) that play a critical role in protecting the telomeres. This group of genes would be potential candidates involved in the development of FNMTC for several reasons. Firstly, it is well known that somatic pathogenic variants in *TERT* (Telomerase Reverse Transcriptase) gene promoter are involved in the development of NMTC, and, in fact, it is one of the main markers of poor prognosis[7]. Furthermore, the non-proper functioning of telomeres has been shown to be of vital importance in some oncological processes such as melanoma, with which NMTC seems to have ties in common. Patients with papillary thyroid carcinoma (PTC) have more risk of developing cutaneous malignant melanoma (1.8-fold) than healthy subjects, and patients with melanoma have 2.3-fold increased risk of PTC[8]. In addition, other genes such as *BRAF* or the aforementioned *TERT* play key roles in both diseases[9].

It has been proposed that germline pathogenic variants in telomere-related genes, such as *POT1*, described in familial melanoma, might also predispose individuals to thyroid cancer[10]. We ourselves searched for pathogenic variants in *POT1* in our NMTC families but found no evidence[11]. No differences have been observed in the number of copies or in the expression of various genes of the shelterin complex in six families with FNMTC either[4].

Although it seems that genes of the shelterin complex could be somehow involved in the development of FNMTC, until now results in the literature are scarce. Our aim was to assess if our FNMTC families presented variants in the genes of the shelterin complex that could explain predisposition to develop NMTC.

MATERIAL AND METHODS

Study Subjects

We designed a multicentric study in Spain to collect blood specimens, and clinical data from families with at least two members with NMTC, confirmed by histology, without history of other malignancies, and without clinical characteristics suggestive of syndromic FNMTC. We recruited 40 FNMTC families (33 with two affected members and 7 with three or more affected members) from 17 hospitals in Spain.

We performed whole-exome sequencing (WES) in 10 affected individuals from four families with at least three affected members. These data were previously published by our group[12] along with the methods described in detail. Nowadays, we have reviewed our WES data, but focussing on the shelterin complex genes (*POT1, TPP1, TINF2, TERF1, TERF2,* and *RAP1*). Moreover, we studied the *TINF2* gene in the index cases of 40 FNMTC-recruited families.

This project was approved by the Ethics Committee of the Hospital Clínic of Barcelona, Spain (Reg. HCB/2016/0200) and was conducted in accordance with the Declaration of Helsinki. Patients gave written informed consent before undergoing evaluation and testing.

DNA Extraction and *TINF2* Sanger gene Sequencing.

Genomic DNA was extracted from peripheral blood samples. The whole (5′UTR region, exons from 1 to 6, and intronic regions) *TINF2* gene (NM_012461.3) was studied in lymphocyte DNA from the index cases of our 40 families. PCR, followed by Sanger sequencing, was performed using the following primers (Sigma–Aldrich, Saint Louis, MO, USA): 5'UTR and exons 1-2, forward 5'TTAAAGCTGAGCGACCCAGT3' and reverse 5'AATCCACAGGAGCCTCTGAC3', exons 3-5: forward 5'TTCCGCGAGTACTGGAGTTT3', and reverse 5'CGGAGCCCATGGAA CTATT3', and exon 6 forward 5'CTGGGGCAAAACATGTAAGG3' and reverse 5'CTGTTGAT CCAATCCTGACTCA3'. Touchdown PCR conditions were as follows: denaturation at 95°C for 5 min, 10 cycles (95°C for 1 min, 65–60°C for 1 min, 72°C for 1 min), followed by 25 cycles (95°C for 1 min, 55 °C for 1 min, 72°C for 1 min). PCR products were purified using Pure-IT ExoZAP® PCR Cleanup (Ampliqon) and bidirectionally sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequenced products were run in an ABI3500xl Genetic Analyzer (Applied Biosystems) and analyzed using Sequencing Analysis v6.0 software (Applied Biosystems).

Transcript Analysis:

Total RNA from affected individuals II.3 and II.4 (figure 1A) and 5 healthy controls were isolated from 10ml of peripheral blood using Trisure TM reagent (Meridian Bioscience) and chloroform extraction. RNA was cleaned-up using RNeasy Mini Kit (QIAGEN), following the manufacturer's protocol with an additional step of DNase digestion using RNase-Free DNase Set (QIAGEN). A total of 300–500ng of RNA was retrotranscribed using PrimeScript RT reagent kit (Takara), combining random and oligo-dT primers. PCR primers located on exon 2 forward 5'TTGGGCCCAAGTCCTGAAAG3' and exon 6 reverse 5'ATCGCATGTTCTTCCTTGCT3' were used to amplify a whole exon upstream and downstream from the exon containing the variant c.507G>T of interest. PCR assays were performed in 25 ul reaction volume containing 100 ng of cDNA as template, using TaKaRa Taq DNA Polymerase (Takara). Samples were denatured at 95ºC for 5 min, followed by 35 cycles consisting of 98ºC for 10sec, 60ºC for 30sec, and 72ºC for 1.5 min; and a final extension step at 72ºC for 10 min. PCR products were sequenced and analyzed as described above. Reference transcript NM_012461.3 was used for sequence alignment and transcript annotation.

Loss of Heterozygosity Analysis:

To explore a possible loss of heterozygosity (LOH), we studied four paraffin-embedded tumor samples from patients II.3, II.4, II.5 and III.1 (figure 1A). We isolated DNA from tumors as previously described[13] and thereafter, we amplified the region where the c.507G>T variant is present, using primers forward 5'CCTGGCCACTAACCCACTT3' and reverse 5'CCCACACTCTGCCCTTACAT3' to obtain a product of 189bp. We then sequenced the fragment by Sanger sequencing.

RESULTS:

We re-evaluated genetic variants in exomes obtained in 10 affected individuals from four families with at least three affected members, focusing on all the genes implied in the shelterin complex. In one family (figure 1A), with five NMTC-affected members, we found a heterozygous variant c.507G>T in *TINF2*. This variant was confirmed by PCR and Sanger sequencing in all five affected members of the family. This change is not described in GnomAD or cancer databases. After this finding, we analyzed the whole *TINF2* gene in the index cases of the rest of our 40 families, without detecting other suspected *TINF2* pathogenic variants in any of them. Neither have we found variants suspected of pathogenicity in the rest of genes of the shelterin complex.

Therefore, to test if the c.507G>T variant could affect the splicing, we evaluated this variant in SpliceAI (0.66) and Pangolin (0.81), both predicting a deleterious effect (donor loss). Afterwards, we analyzed RNA obtained from II.3 and II.4 affected patients. After sequencing, we observed three transcripts: a) the full-length transcript, b) the minor alternative transcript variant lacking exons 4 and 5 (Δ4&5) previously described[14], and c) a new alternative transcript lacking exon 4 (Δ4) (figure 1B). The minor alternative transcript was not detected in any of the controls.

Deletion of exons 4&5 variant predicts a p.(Glu134SerfsTer8) truncated protein and the skipping of exon 4 predicts the preservation of the reading frame p.(Glu134_Gln169del) but loss all 36 amino acids of exon 4 (figure 1C). As shown in figure 1B, all these three transcripts seem to be present in approximately equal proportions.

LOH was not observed in any of four tumor samples from four different patients.

DISCUSSION

Our RNA data show c.507G>T variant increases the amount of the Δ4&5 alternative transcript, predicting a truncated protein p.(Glu134SerfsTer8). Additionally, variant c.507G>T produces a new in-frame deletion, predicting the p.(Glu134_Gln169del) change in the TINF2 protein. Schmutz I. et al.[14] reported the c.604G>C variant in the *TINF2* gene in three families affected by NMTC and/or melanoma. Curiously, both changes (c.507G>T and c.604G>C) are located in the last nucleotide of the exon, c.507G>T in exon 4 and c.604G>C in exon 5, both producing splicing effects (table 1).

At present, there are five different frameshift/nonsense variants described in the *TINF2* gene, found in twelve families (table 1). Twelve patients are affected only by NMTC, nine only by melanoma, four by both; one is affected by cancer but not NMTC/melanoma and one not affected by cancer. According to the current data, only NMTC and melanoma appear recurrently among these families. Other malignancies, such as breast or colon cancer are difficult to categorize as part of the syndrome or only phenocopies.

On the other hand, loss-of-function (mainly missense but also nonsense and frameshift) variants in *TINF2* are widely known to cause dyskeratosis congenita (DC)[15,16]. All these loss-of-function variants appear exclusively in a short stretch called DC patch (codons 269 to 298) towards the C-terminal part of the protein. By contrast, the *TINF2* variants described until now in families with NMTC and/or melanoma, including c.507G>T (table 1), are all located before codon 269 (figure 1D). Localization of TINF2 in telomeres is mainly determined by its interaction with TERF1[17]. Schmutz I. et al[14]. and He H. et al[18]. showed truncating TINF2 protein variants found in FNMTC had lost the ability to bind to TERF1. If TERF1 interaction is lost, telomere protection is not compromised, but TINF2 is not accumulated at telomeres, giving rise to long telomeres[19], impairing the telomere tumor suppressor pathway[14, 18, 20,]. TINF2 as well as TERF1 and POT1 are negative regulators of telomere length.

Results altogether suggest that if TERF1-binding site is somehow maintained, patients will only present DC features, but the weaker the ability of TINF2 and TERF1 to interact, the greater the likelihood that patients will develop some type of cancer, especially NMTC and/or melanoma.

LOH was not observed in our studies in consistency to others[14] suggesting a mechanism of haploinsufficiency.

Though we have not performed a functional study of the altered protein, given that the change is very similar to the one previously described and the alteration by RNA splicing is confirmed, we consider that protein functionality resulting from c.507G>T variant is highly impaired.

We cannot completely exclude the possibility that some other structural, copy number, non-coding gene regulative variants, etc., might also explain the phenotype in the family investigated.

CONCLUSION

According to our data and in agreement with what was observed in other reported families, we suggest that frameshift/nonsense variants in *TINF2* before codon 269 predispose to two types of cancer: differentiated thyroid cancer and melanoma in an autosomal dominant manner. *TINF2* variants do not seem frequent, but as they have been found in non-related families, we encourage the study of *TINF2* gene in all cases of NMTC and/or melanoma with a suspected familial background. Results will improve our knowledge of its penetrance and our understanding of the genotype-phenotype relationship.

Acknowledgements

We are grateful to the patients and their families for their participation, to their physicians for providing samples and clinical histories and to Doina Bird for the revision of the English version of the Manuscript.

Author disclosure statement

No competing financial interests exist.

Funding statement(s)

This work was funded by

The Catalan Society of Endocrinology and Nutrition (Fellowship 2015/2016),

Hospital Clínic de Barcelona (End-of-Residence Award 2016/2017), and

The Spanish Society of Endocrinology and Nutrition (Beca Morreale-Escobar 2017/2018).

Author contribution statement

J.O.: Conceptualization (lead); formal analysis (lead); investigation (lead); resources acquired (equal), writing – original draft (supporting); writing – review and editing (equal). **O.D.:** formal analysis (supportive); writing – review and editing (equal). **M.M.:** Resources acquired (equal); writing – review and editing (equal). **I.H.:** Resources acquired (equal); writing – review and editing (equal). **A.B.:** Methodology (equal); writing – review and editing (equal). **M.M.:** Methodology (equal); writing – review and editing (equal). **A.T.:** Methodology (equal); writing – review and editing (equal). **S.M.:** Investigation (supporting); writing – review and editing (equal). **R.D.G.:** Investigation (supporting); writing – review and editing (equal). **A.O.:** Conceptualization (supporting); formal analysis (supporting); investigation (supporting); resources acquired (equal), writing – original draft (lead); writing – review and editing (equal).

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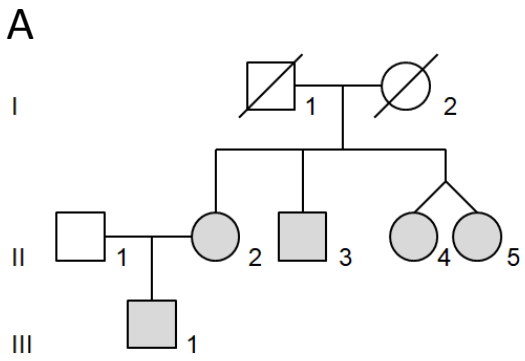
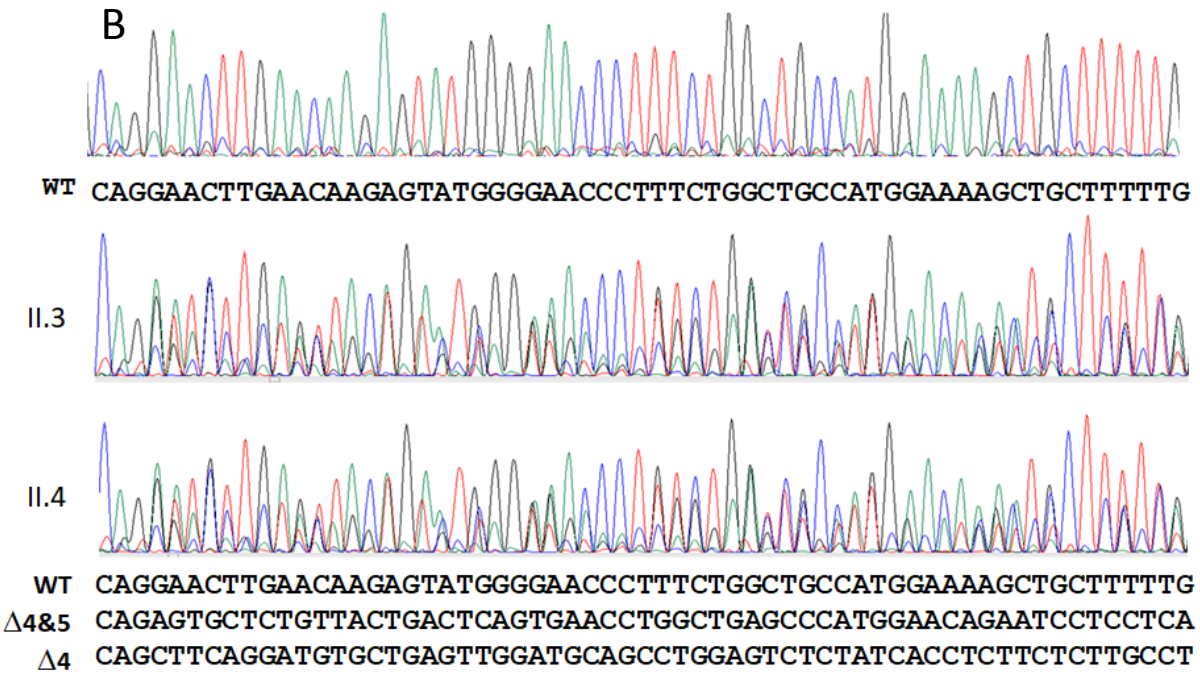
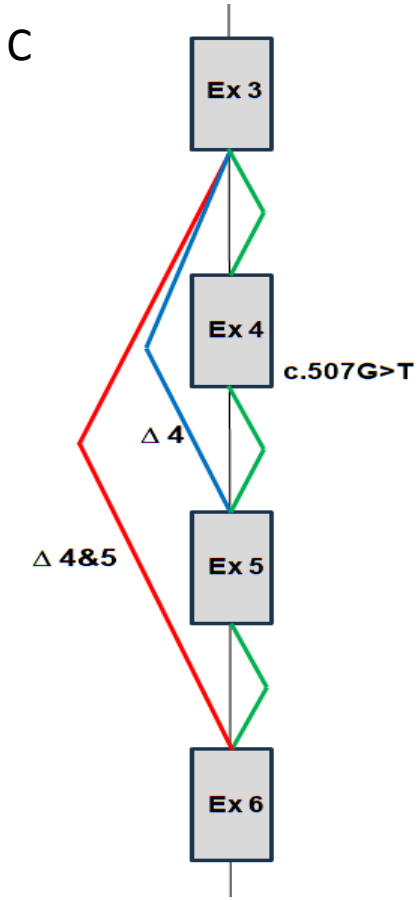
Legends:

Figure 1. (A) Family with five NMTC affected members (in grey). All affected members carry the c.507G>T variant in *TINF2* gene. (B) Electropherograms of cDNA from control and patient's samples II.3 and II.4. WT: full-length transcript. Δ4&5: the alternative transcript variant lacking exons 4 and 5, and Δ4: a new alternative transcript, lacking exon 4. (C) Diagram of the *TINF2* gene from exon 3 to exon 6. Location of the c.507G>T variant at the end of exon 4. Full-length transcript (lines in green). Alternative transcript variant lacking exons 4 and 5, enhanced by the c.507G>T variant (lines in red) and the new alternative transcript lacking exon 4 also produced by the c.507G>T variant (lines in blue). (D) Location of frameshift and nonsense variants in *TINF2* described in families with differentiated thyroid cancer and/or melanoma. DC: Dyskeratosis Congenita patch. The numbers in parenthesis indicate codons.

Table1: *TINF2* frameshift and nonsense variants described in families with thyroid cancer and/or melanoma.







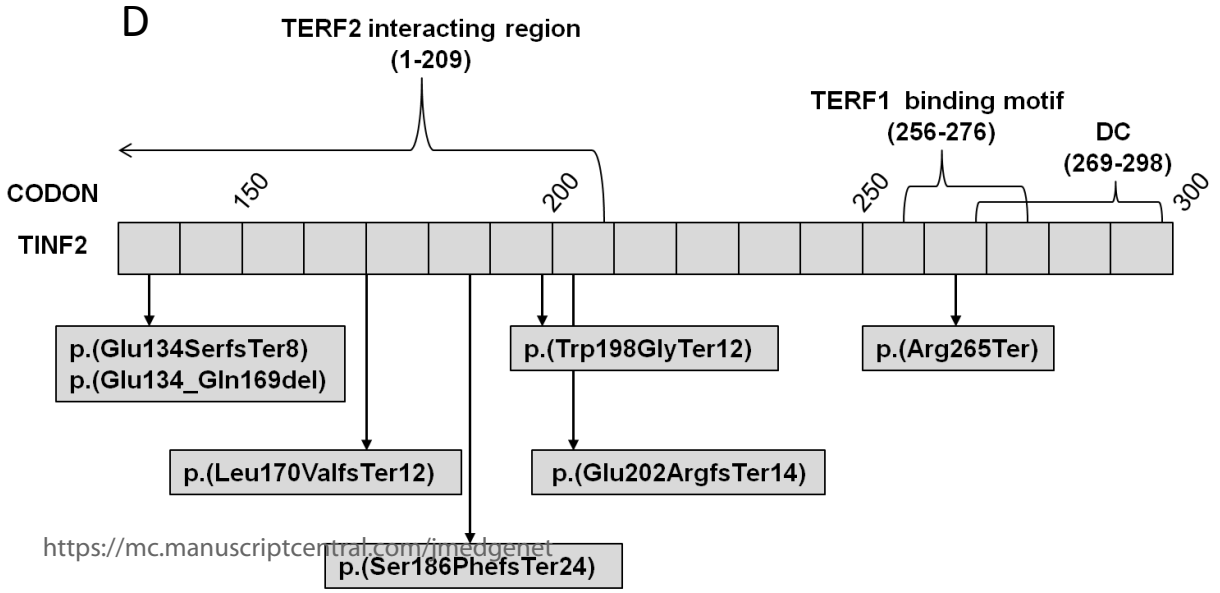


Table 1

germline variant	predicted effect	observed effect	phenotype	reference
c.507G>T	p.(Gln169His)	p.(Glu134SerfsTer8) and p.(Glu134_Gln169del)	Five affected members. Only papillary thyroid carcinoma (PTC)	this report
c.557del	p.(Ser186PhefsTer24)	p.(Ser186PhefsTer24)	Family 2: one patient affected by follicular thyroid cancer + melanoma+ breast cancer	Schmutz I. et al. 2020
c.591del	p.(Trp198GlyfsTer12)	p.(Trp198GlyfsTer12)	Eight affected members: only PTC:n=4; PTC+ melanoma:n=2;only melanoma: n=2)	He H. et al. 2020
c.604G>C	p.(Glu202Gln)	p.(Leu170ValfsTer12) and p.(Glu202ArgfsTer14)	Family 1: two affected patients by PTC (proband and his mother). His mother also with colon cancer	Schmutz I. et al. 2020
c.604G>C	p.(Glu202Gln)	p.(Leu170ValfsTer12) and p.(Glu202ArgfsTer14)	Family 3: one patient with melanoma (13y) + diffuse astrocytoma (17y) and one person with no cancer (adult)	Schmutz I. et al. 2020
c.604G>C	p.(Glu202Gln)	p.(Leu170ValfsTer12) and p.(Glu202ArgfsTer14)	Family 4: one patient with rectal (65y)and breast (65y) cancer. One patient with thyroid cancer but not tested for the familial variant.	Schmutz I. et al. 2020
c.793C>T	p.(Arg265Ter)	Not tested	6 kindreds: six patients with melanoma, one patient with PTC and one patient with both.	Jensen M.R. et al. 2023