Does a Common Genetic Event Exist for Familial Thyroid Cancer? Results from a Large Family with fnmtc

Cantara Silvia*, Baldassarri Margherita1, Marzocchi Carlotta1, Capitani Katia1, Alfonso Sagnella1, Valerio Laura1, Anna Cantore1, Meloni Ilaria1, Renieri Alessandra2,5 and Capezzone Marco4

Abstract

Background: Despite several efforts, the genetic susceptibility of familial non medullary thyroid cancer (FNMTc), has remained still elusive.

Methods: We performed Whole Exome Sequencing (WES) in a large family with 9 available members, 6/9 (67%) affected by FNMTc.

Results: We found two missense variants, with CADD score >20: the c.C1519A (p.Pro507Thr, rs773271544) in PRKCε gene and the c.G1019A (p.R340Q) in CCZ1B gene. These alterations were absent in healthy subjects (n=40) and in 30 sporadic thyroid cancer patients. The p.P507T was possibly pathogenetic by SIFT and PRKCε is implicated with MAPK activation by STRING. When we searched for this mutation in other families, we failed to confirm this genetic event as causative of cancer in other 20 FNMTc patients belonging to 8 kindred.

Conclusions: We concluded that the PRKCε p.Pro507Thr possibly represents a private mutation even if other studies including large FNMTc family are needed to define the percentage of familial thyroid cancer cases due this alteration.

Keywords: Familial non medullary thyroid cancer; PRKCε; Whole exome sequencing; Private mutation

Introduction

Familial Non-Medullary Thyroid Cancer (FNMTc), defined as the occurrence of the disease in three or more first-degree relatives of the patient, constitutes about 5–15% of all NMTC cases, including syndromic and non-syndromic forms [1]. Among FNMTc’s, papillary thyroid carcinoma (PTC), a malignant epithelial tumor showing follicular differentiation with concrete nuclear characteristics, represents the most common histological subtype [2].

The syndromic FNMTc forms (Cowden, Gardner, Carney complex, Werner, and DICER1 syndromes) are characterized by a preponderance of non-thyroidal tumors associated with Mendelian cancer syndromes, and genetic alterations have been defined. On the contrary, for the non-syndromic form, which represents the majority of all FNMTc’s (95%), the genetic inheritance remains unclear, although it is believed to be either an autosomal dominant condition with incomplete penetrance and variable expressivity or a polygenic disorder likely associated with low-penetration alleles [3-7].

In recent years, many susceptibility genes have been identified through linkage analysis, genome-wide association studies (GWAS), whole exome (WES), and next-generation sequencing [8-12]. Additionally, several...
low-penetrance risk variants have been identified [13-15], and it has been estimated that the five best-characterized SNPs identified in GWAS may contribute to 11% of PTC familiarity [1]. However, none of these mutations has been confirmed in families other than those in which they were initially identified.

The identification of predisposing genes for FNMTC is crucial to identify individuals at high risk of developing the disease because, although still debated, FNMTC is more aggressive than the sporadic counterpart, presenting a higher recurrence rate, an early age of onset, and decreased disease-free survival compared to sporadic forms [16].

Protein kinase Cε (PRKCε) belongs to the family of protein kinase C. It is overexpressed in several solid tumors and plays critical roles in pathways that lead to cancer development, such as MAPK signaling. PRKCε has been implicated in epithelial-to-mesenchymal transition (EMT) [17], cell migration, invasion, and tumor metastasis development [18-22]. Somatic rearrangements of PRKCε have been described in papillary thyroid carcinoma cell lines [23], and modulation of PRKCε by microRNA 146a results in papillary thyroid cancer development [24].

To investigate whether a common genetic event is responsible for FNMTC occurrence, we analyzed a large family with nine members using Whole Exome Sequencing (WES), with six of them being affected by FNMTC.

Materials and Methods

Patients

An Italian family with NMTC aggregation, represented by 9 members (Figure 1A) (F:6) was recruited at the Section of Endocrinology of the University of Siena, Italy. Mean age at diagnosis was 40.6±6.4 years (range 33-52 years). Eight out of 9 (88.8%) patients were submitted to total thyroidectomy. Histological examination showed the presence of papillary thyroid cancer (PTC) in 3 cases, PTC with tall cells in 1 case, PTC with tall and oxyphil cells in 2 cases and follicular adenoma in 2 cases. Lymphnode metastases were present at diagnosis in 2/6 (34%) patients. Two/6 female members (34%) had also breast cancer, in all cases diagnosed after thyroid cancer (3 and 8 years later) (Table 1). All patients are in disease remission. A second group composed by 20 FNMT patients belonging to 8 families (17 fe-males) was analyzed to confirm the results. Among these patients, 15/20 (75%) had the classic variant of papillary thyroid cancer (PTC), 2/20 (10%) had follicular thyroid cancer (FTC) and 3/20 (15%) had the diffuse sclerosing variant of papillary thyroid carcinoma. As control groups, 40 healthy subjects and 30 sporadic PTCs (F: 21) were also included. For sporadic cases, 25/30 (84%) had PTC, 2/30 (6.7%) had PTC follicular variant and 3/30 (10%) had FTC. All procedures performed in this study were in accordance with the local ethical committee (Ethical Committee of Region Toscana, Area Vasta Sud Est, AOUS. Protocol ID: 10167). Patients signed an informed consent for FNAC, genetic analysis and surgery procedures as part of their treatment plan.

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes with QIAamp® DNA blood mini kit (Qiagen, Milan, Italy) according to manufacturer's instruction. DNA quality and concentration were assessed with Nanodrop One (Thermo Scientific, Milan, Italy).

Whole Exome Sequencing

Whole exome sequencing (WES) was performed using the Illumina platform in 9 family members (6 affected and 3 unaffected siblings). All genomic DNA samples went through quality controls for DNA concentration and quality (PicoGreen and 1% agarose gel). Library and exome capture were performed using the TrueSeq Exome Illumina kit (Illumina, Inc. San Diego, CA). Post-Library Quality

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age at diagnosis (yrs)</th>
<th>Histotype</th>
<th>Histological variant</th>
<th>TNM</th>
<th>Second primary tumor</th>
<th>Site of second tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient I-1</td>
<td>M</td>
<td>67</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
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<tr>
<td>Patient I-2</td>
<td>F</td>
<td>63</td>
<td>PTC</td>
<td>classical</td>
<td>T3N0Mx</td>
<td>no</td>
</tr>
<tr>
<td>Patient II-1</td>
<td>F</td>
<td>42</td>
<td>PTC</td>
<td>classical</td>
<td>T1N0Mx</td>
<td>yes</td>
</tr>
<tr>
<td>Patient II-2</td>
<td>M</td>
<td>44</td>
<td>PTC</td>
<td>tall and oxyphil cells</td>
<td>T1N0Mx</td>
<td>no</td>
</tr>
<tr>
<td>Patient II-3</td>
<td>F</td>
<td>45</td>
<td>PTC</td>
<td>tall and oxyphil cells</td>
<td>T3N0Mx</td>
<td>no</td>
</tr>
<tr>
<td>Patient II-4</td>
<td>F</td>
<td>39</td>
<td>Follicular adenoma</td>
<td>n.a.</td>
<td>n.a.</td>
<td>yes</td>
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<tr>
<td>Patient II-5</td>
<td>M</td>
<td>35</td>
<td>PTC</td>
<td>classical</td>
<td>T3N1Mx</td>
<td>no</td>
</tr>
<tr>
<td>Patient II-6</td>
<td>F</td>
<td>35</td>
<td>Follicular adenoma</td>
<td>n.a.</td>
<td>n.a.</td>
<td>no</td>
</tr>
<tr>
<td>Patient II-7</td>
<td>F</td>
<td>33</td>
<td>PTC</td>
<td>tall cells</td>
<td>T1N1Mx</td>
<td>yes</td>
</tr>
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Table 1: Histological characteristics of the FNMT patients.
Controls were performed by Agilent 2100 Bioanalyzer. The clonal clusters were created on version 3 flowcells and the pair-end 2X100bp sequencing was performed on HiSeq2000, following the standard Illumina protocol. Data were filtered against dbSNP132 and control populations (1000 Genomes Project Consortium; http://www.1000genomes.org/data) using eVai software (enGenome v2.3). All variants were screened according to their frequency, location, mutation category, literature, and mutation database (ClinVar database, LOVD database, HGMD database). Polymorphisms (minor allele frequency, MAF > 0.01) and synonymous variants were excluded. Missense variants were predicted to be damaging by CADD-Phred prediction tools for functional effect prediction. Frameshift, stop-gain, and splice site variants were prioritized as pathogenic. Variants with a minor allele frequency (MAF) less than 0.01 % in 1000 genome and ExAC-nonTCGA were excluded. Missense variants were predicted to be damaging by CADD-Phred prediction tools for functional effect prediction. Frameshift, stop-gain, and splice site variants were prioritized as pathogenic. Variants with a minor allele frequency (MAF) less than 0.01 % in 1000 genome and ExAC-nonTCGA were selected for bioinformatics evaluation.

**Bioinformatics analysis**

Combined Annotation Dependent Depletion (CADD) software was used and, when the score was >20, Predict SNP, a consensus classifiers for prediction of disease-related mutations, was applied (https://oschmidt.chemi.muni.cz/predictsnp/). Predict SNP elaborates a total score derived from six different software: Multivariate Analysis of Protein Polymorphism (MAPP), Sorting Intolerant from Tolerant (SIFT), Predictor of human Deleterious Single Nucleotide Polymorphisms (PhD-SNP), Polymorphism Phenotyping v1 (PolyPhen-1), Polymorphism Phenotyping v2 (PolyPhen-2) and Screening for Non-Acceptable Polymorphisms (SNAP). Swiss-Model and PyMOL software were utilized to build the three-dimensional protein structure of wild-type and mutant PRKCɛ protein.

**PCR and Sanger sequencing**

To validate the results obtained by WES and to explore the genetic variants in sporadic PTC cases, healthy subjects and a validation cohort of FNMTc patients, PCR and Sanger sequencing were performed. Primers used for CCZ1B and PRKCɛ are reported in Table 2. For all amplicons, PCR conditions were 1.5 mM MgCl2 with an annealing temperature of 60°C (35 cycles). For all other Sanger sequencing (i.e. the whole coding sequence of PRKCɛ, APC p.R414C; LRKK2 p.R340Q; PRKCɛ p.P507T, LRRK2 p.G2019S; AIRE p.T441M; TERC, hTERT etc.) primers are available upon request to cantara@unisi.it.

DNA sequencing was carried out using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and BigDye Xterminator Purification Kit on automated DNA capillary sequencer (Applied Biosystems 3130xl Genetic Analyzer).

**Measurements of Relative Telomere Length**

Q-PCR assay was performed as previously described [25] on 50 ng/µl of genomic DNA. Telomere length quantification involved determining the relative ratio of telomere (T) repeat copy number to a single copy gene (S) copy number (T/S ratio) in experimental samples using standard curves. This ratio is proportional to the average telomere length. 36B4, encoding acidic ribosomal phosphoprotein P0, has been used as the single copy gene. Primers and PCR conditions have been as reported [25].

**Statistical analysis**

All statistical analyses were carried out by using the software GraphPad Prism version 5. A value of p< 0.05 was considered statistically significant. Statistical differences in RTL were verified by One-way Anova, followed by Bonferroni’s test, to compare two or more independent groups.

**Results**

Identification of CCZ1B and PRKCɛ as candidate susceptibility genes We performed Whole-Exome Sequencing using peripheral-blood DNA from 6 affected family members and 3 unaffected siblings. We initially identify approximately 800 variants (classified as pathogenic, likely pathogenic or uncertain) per patient. Using filtering criteria (SNVs/Indel ≤ 1% in HapMap18 and 1000 Genome database, CADD-phred >20, exonic variants), we restricted to 4 variants in 4 different genes reported in Table 3: the p.R340Q in CCZ1B, the p.P507T in PRKCɛ, the p.R414C in APC, the p.G2019S in LRRK2 genes.

After applying other filters to identify candidate single-nucleotide variants and/or insertions/deletions that segregated with all affected members but were not present in unaffected siblings, we confirmed only the p.R340Q in CCZ1B and the p.P507T in PRKCɛ genes with a CADD-phred of 23.2 and 23.3, respectively. All patients were heterozygous for these mutations. The presence of these variants was confirmed by Sanger sequencing in all subjects (Figure 1B). We then applied in silico tools to investigate the pathogenic role of these mutations and found that the p.R340Q in CCZ1B resulted as a neutral variant with all applied programmes, whereas the p.P507T in PRKCɛ in which a proline is substituted by

<table>
<thead>
<tr>
<th>Table 2: Primer sequences</th>
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<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>CCZ1B</td>
</tr>
<tr>
<td>PRKCɛ</td>
</tr>
</tbody>
</table>

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PRKCɛ p.P507T is not found in other FNMTC patients

Due to the possible implication of the PRKCɛ p.P507T mutation with the MAPK pathway hyper-activation, we hypothesized that it could be causative of the familial form of PTC. We then analyzed by Sanger sequencing the PRKCɛ p.P507T together with the whole coding sequence of the gene in 20 FNMTC patients belonging to 8 families. These patients were already screened for some of the alterations that have been described in the literature [1] to be associated with FNMTC: the p.G534E in HABP2, TERC and hTERT mutations and the p.A339V in TITF-1/NKX2.1 gene. All patients were negative for these variants. Similarly, all subjects were wild type for the PRKCɛ p.P507T variant.

We also searched for this variant in 40 healthy subjects and 30 sporadic cases by Sanger sequencing. All individuals analyzed were negative for the mutation.

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addition, no other potentially impactful mutations were found in the PRKCɛ gene. Only 2/20 (10%) of the FNMTC patients displayed the rs1228790462 associated with a synonymous variant (p.Gln719=).

**Does a susceptibility background predispose to FNMTC?**

We first described that telomere length and telomere instability constitute a genetic background that predisposes to the development of familial thyroid cancer [25, 28-30]. We measured the relative telomere length (RTL) in our family and found a particularly short telomere compared to the other groups (Figure 3A). The RTL was shorter for both affected and unaffected siblings, even compared to the RTL of the FNMTC control group, indicating a progressive erosion potentially implicated in the oncogenic transformation of somatic cells. In addition, 4 members displaying thyroid diseases (both thyroid cancer or adenomas) were heterozygous for the p.R414C mutation (MAF <0.01) in the APC gene. This variant is reported to be associated with the hereditary cancer-predisposing syndrome (ClinVar: https://www.ncbi.nlm.nih.gov/snp/rs137854567/) and is deleterious by PredictSNP (87%). PRKCɛ and APC are both MAPK activators, as shown in Figure 3B. Similarly, although with a CAD of 13.22, all family members were heterozygous for the p.T441M mutation in the AIRE gene. This mutation is located in the zinc finger region, has a MAF <0.01, and, according to the in silico prediction tool that we have applied, is potentially deleterious. Using PROMO [31, 32], a virtual laboratory for the identification of putative transcription factor binding sites, we found that both Ets and Elk1 (activated by ERK1/2) are potential transcription factors for AIRE.

**Discussion**

Somatic rearrangements of PRKCɛ have been described in papillary thyroid carcinoma cell lines and PRKCɛ itself is a positive modulator of MAPK pathway. Therefore, PRKCɛ seems a natural candidate for cancer susceptibility particularly familial form of thyroid cancer [18-24]. FNMTC constitutes about 5–15% of all NMTC cases, including syndromic and nonsyndromic forms, and this high familial risk can be attributed to both rare, high-penetrance mutations and common, low-penetrance variants [1]. Scientific and technological advancements in genomics have allowed whole genome and exome screening to become the state-of-the-art tool for the identification of driver mutations in tumors. So far, only a few NMTC susceptible gene(s) and low-penetrance variant(s) contributing to NMTC have been described [1]. We performed WES on a large family composed of nine members, six of them affected with FNMTC. In this family...
we identified, for the first time, one potentially disease-causing germline variant in PRKCɛ gene that, in humans, encodes PRKCɛ protein. PRKCɛ, a member of the protein kinase C (PKC) family proteins, is overexpressed in most solid tumors and plays critical roles in different processes that lead to cancer development [33-37]. At the systemic level, PRKCɛ activation has protective roles in cardiac and brain ischemia and heat shock response while uncontrolled PRKCɛ activation is associated with cancer development [33-37]. The Raf-1 kinase acts downstream PRKCɛ regulating important intracellular signaling pathways involved with proliferation, differentiation and apoptosis. The oncogenic function of PKCɛ potentially occurs via activation of the Ras/Raf pathway which results in the transcription of genes involved in cell proliferation and growth [38]. Functionally PRKCɛ plays crucial roles in almost all aspects of tumor development, namely cell transformation, proliferation, cancer cell survival, epithelial mesenchimal transition (EMT), migration and invasion [33-37].

Some studies evaluated the role of PRKCɛ in thyroid cancer. Nearly 20 years ago, Knauf et al reported an isozyme-specific reduction of PRKCɛ, which occurs through a post-transcriptional mechanism in 8/11 PTC tissues, affecting translation or stability of the PRKCɛ protein. The authors concluded that the decreased abundance of PRKCɛ may promote tumor progression by prolonging cellular life span [23]. The same authors found a rearrangement of PRKCɛ in a thyroid follicular carcinoma cell line (PCCL3) suggesting that this protein may play a role in thyroid tumorigenesis. In the same cell line, the induction of RET/PTC1 or RET/PTC3 expression, a key event in the pathogenesis of PTCs, resulted in PRKCɛ activation. As supposed by the authors, this selective downregulation of PRKCɛ following prolonged RET/PTC activation promotes cell survival and clonal expansion [39]. Another study showed that the microRNA-146a, significantly overexpressed in PTC, targets PRKCɛ to modulate papillary thyroid tumour development through suppression of PRKCɛ expression and deregulation of the Ras/Raf-1 signaling pathway.
pathway [24]. In agreement with these authors, we might suppose that PI3K/Akt, Stat3 and MAPK/ERK pathways are the likely mediators of PRKCe-induced transformation. Interestingly, when we search for the p.P507T mutation in other FNMTCs, all patients were wild type again indicating that FNMTC mutations represent private mutations that occur on a fragile genetic background. This genetic instability may be linked to particularly short telomeres has already demonstrated [25, 28-30] and confirmed in this family. In addition, some of the family members studied in this paper, are heterozygous for two important pathogenetic alterations: the APC p.R414C linked to the hereditary cancer-predisposing syndrome (present in 4 affected members) and the p.T441M in AIRE gene (in all affected members). AIRE, the autoimmune regulator, plays a key role in shaping central immunological tolerance by facilitating the negative selection of T cells. AIRE has been associated with the polyglandular syndrome type 1 (APS1) [40] and with cancer [41-43]. According with the literature [44] and with PROMO, a virtual laboratory for the identification of putative transcription factor binding sites, ERK1/2, which is linked to PRKCe, activates Ets and Elk1 potential TF for AIRE. We can speculate that all alterations found in this family contribute to the MAPK activation thus sustaining cell proliferation and contribute to cancer development (Figure 3C).

Limitations: Although some of the data presented in our paper seem to support that FNMTCs are characterized by a complex genetic instability associated with private mutation(s), limitations are present in the study and are linked to the absence of confirmatory functional studies for the AIRE and APC mutations and the lack of demonstration of the non-random segregation of mutations in the family. Family members analyzed, while representative of different Italian regions are all Italians and thus expanding the sample size to other countries could be beneficial to eliminate potential environmental influences. In addition, confirming these genetic alterations in tissues would be important, but unfortunately, the patients underwent surgery many years ago in different clinics, and therefore, it was not possible to retrieve the histological specimens.

Taken together our results contribute to highlight that the p.P507T in PRKCe is a potential pathogenetic mutation causative of familial thyroid cancer but, the absence of this mutation in other families lead us to conclude that FNMTCs patients have a genetic instability on which, a private mutation(s) acts to induce hereditary cancer. Future work including more functional studies at protein level will improve our knowledge on the potential role of PRKCe mutation on the development of FNMTC.

Conflict of Interest: None

References


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