ORIGINAL ARTICLE

BROX haploinsufficiency in familial nonmedullary thyroid cancer

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Abstract

Background The familial nonmedullary thyroid cancer (FNMTC) is suspected to be a Mendelian condition in up to 3–8% of thyroid cancers. The susceptibility chromosomal loci and genes of 95% of FNMTC cases remain to be characterized. The inheritance of FNMTC appears to be autosomal dominant with incomplete penetrance and variable expressivity. The finding of the causative gene of FNMTC and the identification of patients at risk that need genetic testing were our aim.

Methods We analyzed by whole-exome sequencing patients and non-affected relatives of five families with at least two family members affected by papillary thyroid cancer, selecting for new or extremely rare variants with predicted pathogenic value.

Results A family showed, in all three affected members, a new loss-of-function variant (frameshift deletion) in *BROX* gene at 1q41 that was absent from all internal and external databases. In a second family with three affected relatives, we found an additional new *BROX* variant. The smaller families presented no variants in *BROX* or in the other causative genes studied.

Conclusions *BROX* could be a new causative gene for FNMTC. Variants in *BROX* may result in the haploinsufficiency of a key gene involved in the morphogenesis of MVBs, in the endosomal sorting of cargo proteins, and in EGFR. Functional studies are needed to support this result. The thorough genomic analysis by NGS in all families with three or more affected members should become a routine approach to obtain a comprehensive genetic view and find confirmative second cases.

Keywords *BROX* · FNMTC · Thyroid cancer · NGS · Thyroid · EGFR · *HABP2*

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Introduction

Thyroid cancer occurs in 7–15% of cases presenting thyroid nodules depending on age, sex, radiation exposure, and family history. Approximately 1.1% of people present this condition at some point in their life [1]. In 2015 the number of people with thyroid cancer in the USA was approximately 63,000 with 2000 deaths [2]. In particular, papillary thyroid cancer (PTC) is the commonest histologic sub-type, accounting for at least 90% of all thyroid cancers [2]. PTC remains one of the most treatable cancers with a median 5-year survival rate of 98% [2]. On the contrary, patients with familial nonmedullary thyroid cancer (FNMTC) present PTC at an earlier age, have a more multi-focal and aggressive disease with local invasion, lymph node metastases, and increased risk of recurrence and decreased survival rates [3–7]. FNMTC is a rare disorder, diagnosed when two or more first-degree relatives have thyroid cancer without another familial syndrome. Based on a review of the prevalence of FNMTC from Surveillance Epidemiology and End Results (SEER) database, Mayo clinic and mathematical analysis, if

the definition of having two first-degree relatives with thyroid cancer is used for FNMTC, only 31–38% of members in the family are likely to carry the familial trait, with the rest being sporadic cases. The diagnosis is probable in 96–99% of the cases if at least three family members are affected. This familial condition has been reported in 3–9% of thyroid cancer cases, and in only 5% of the syndromic forms have been identified germline mutations [2]. The advent of next-generation sequencing (NGS) technologies is providing a clear advantage compared to the conventional DNA analysis techniques, such as Sanger sequencing, by allowing a comprehensive overview of larger regions of the genome and offering high sensitivity of mutation detection and quantitative assessment of mutant alleles. Yu et al. designed a panel to capture 31 known cancer susceptive genes possibly related to FNMTC, using NGS [8]. Although they identified some candidate variants, such a targeted approach was best suited for sporadic cases and did not provide any information on all the other genes. The unbiased whole-exome sequencing (WES) approach was indeed used by Gara et al. who identified a susceptibility variant of the *HABP2* gene, not shared by additional families [9–13]. This result supports the genetic heterogeneity of FNMTC and the difficulty for a differential diagnosis between syndromes conditions, sporadic PTC and FNMTC. Many studies that use the FNMTC criterion of two affected members might be analyzing sporadic thyroid cancer cases rather than actual FNMTC cases. We approached the study of families presenting two or more relatives affected by PTC by sequencing the whole exome (WES) with enhanced coverage of the most relevant disease-associated targets.

Methods

NGS procedures

Genomic DNA (at least 2 microg) was extracted from venous blood of affected and unaffected individuals by QIAamp DNA Blood Mini Kit (Qiagen). We performed whole-exome sequencing (WES) by using the Agilent SureSelect QXT Clinical Research Exome that provides a 54Mbase target, including an enhanced coverage of disease-relevant targets from HGMD, OMIM and ClinVar (Agilent Technologies, Santa Clara, CA, USA). Enriched DNA was validated and quantified by microfluidic analysis using the Bioanalyzer High Sensitivity DNA Assay kit (Agilent Technologies) and the 2100 Bioanalyzer with 2100 Expert Software. Libraries were sequenced using the NextSeq500 system performing paired-end runs covering at least 2×150 nt. (Illumina Inc., San Diego, CA, USA). The average exome coverage of the target bases of at least 100X with 90% of the bases covered by at least 40 reads. All possible inheritance mechanisms

were considered, and we focused our attention on variants that were present at a minor allele frequency of ≤ 0.001 in SNP databases (ExAC; gnomAD, 1000 genomes, internal database of ~1500 Italian subjects).

Selection of genomic variants

Sequencing data were processed using in-house software for the execution of the GATK Best Practices pipeline for whole-exome sequencing variant analysis. Reads pre-processing and cleaning was performed with the following tools: TrimGalore, to cut sequence reads with Phred Score less than 20; BWA, to align the reads against the human genome (ver. Hg19); PICARD MarkDuplicates, to remove duplicated reads likely to be sequencing artifacts. GATK (ver.3.8) was used for reading base recalibration prior of variant calling with GATK HaplotypeCaller; variants were genotyped with GATK GenotypeGVCFs and filtered with GATK VariantFiltration. SNVs and INDELs were filtered with different parameters. In particular, we used a frequency threshold of 0.005 compared with ExAC, gnomAD, and internal database of 2700 Italian subjects. Genotype phasing was obtained using GATK PhaseByTransmission. Furthermore, variants were annotated with Annovar to assign frequencies in large-scale variants datasets (1000 Genomes, ExAC, gnomAD) and potential impact on protein function. Finally, a tabular output containing both the variants and their annotation was generated for downstream analyses. All the candidate variants identified by bioinformatic analysis of WES data were confirmed by capillary sequencing of both DNA strands on PCR products. Healthy controls for each family were included in the analysis.

Subjects

We analyzed five families in which two or more relatives received diagnosed with PTC. In particular, three families presented three relatives with PTC (Family A, B and C) (Figs. 1 and 2) and two families with two affected members (Family D and E) (Fig. 2). The families A, C, D and E were referred to our institution for evaluation of affected and nonaffected relatives. Family B had a clinical evaluation by The Division of Endocrinology of University of Siena, Italy. All the patients were affected at the time of the evaluation. Affected individuals, plus A4 as a healthy family member, were studied by whole-exome sequencing. We analyzed three kindred with PTC in family A and two affected relatives in B, C, D and E families. The affected patients were treated according to the ATA guideline [14]. All recruited patients signed an informed consent form. The study was approved by the local ethical committee of the Università della Campania "Luigi Vanvitelli" protocol
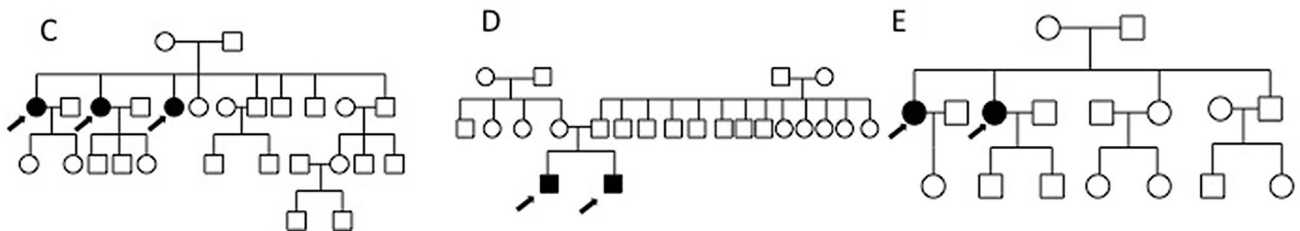
Results

Table 1 depicted the clinical-pathological information of the FNMTC patients of families A and B. FNMTC cases received
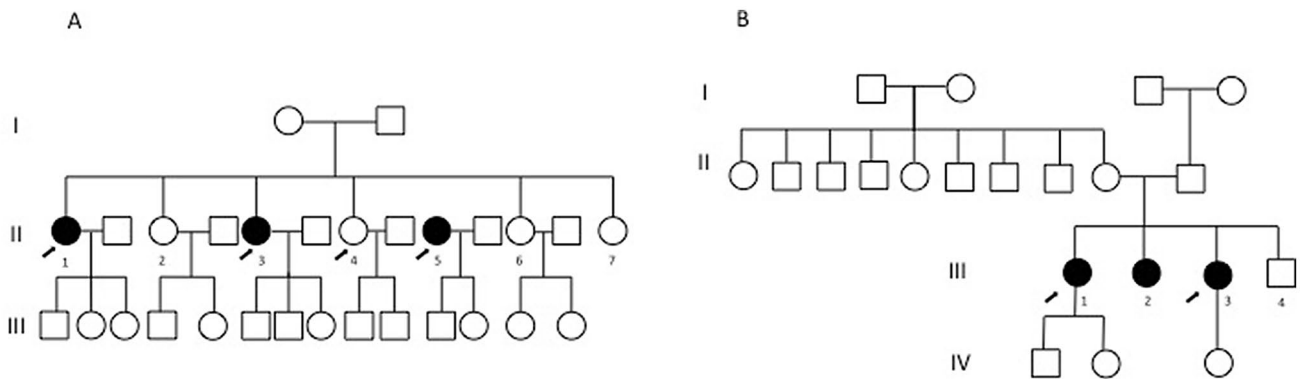
Fig. 1 Pedigree of families A and B. Arrow indicates patients and normal relatives studied by whole-exome sequencing

Fig. 2 Pedigree of families C, D, and E. Arrow indicates patients and normal relatives studied by whole-exome sequencing

Table 1 Clinicopathological features of six patients of families A and B

Patient	A1	A2	A3	B1	B2	B3	Ref. value
Age at diagnosis (years)	45	37	37	31	29	35	NA
Finding at presentation	Thyroid nodule on ultrasound screening	Thyroid nodule on ultrasound screening	Thyroid mass	Thyroid nodule on ultrasound screening	Thyroid nodule on ultrasound screening	Thyroid mass	NA
Histologic type of cancer	Papillary thyroid micro carcinoma	Multifocal Papillary thyroid cancer	Papillary thyroid cancer, follicular variant	Papillary thyroid cancer	Papillary thyroid cancer	Papillary thyroid cancer	NA
Tumor diameter (cm)							
Right lobe	ND	<1	3.5	No	No	2,3	NA
Left lobe	ND	4.0×3.5×2.5	ND	0.5	1.0	No	NA
Isthmus	ND	ND	ND	0.3	No	No	NA
Stage of cancer (TNM)a	T1N0M0	T2N0M0	T1N0 M0	I T3N1aM0	I T3N1aM0	T2N0M0	NA
TSH (µIU/ml)	0,612	0.05	0,545	0.52	0.859	No	0.27–4.20
Free T4 (pg/ml)	14,2	14.4	12,3	16.9	15.3	No	9.39–17.1
Free T3 (pg/ml)	3,7	3.8	2,8	3.61	3.90	No	1.82–4.62

total thyroidectomy with prophylactic central neck node dissection. PTC was confirmed by surgical pathology in all FNMTC patients. Post operatory adjuvant RAI ablation up to 100 mCI was performed and all patients were on thyroid hormone treatment. Median follow up period was 78 ± 8 months. The patients showed an excellent response, an undetectable serum thyroglobulin level and negative cervical US.

Whole exome sequencing identified FNMTC susceptibility gene candidates

We first searched for possible gene variants in genes involved in sporadic cases. Molecular analysis by WES of the ThyroSeq v2 and v3 panel genes, comprising the most commonly detected alterations SNVs/indels in the *BRAF, RAS, EIF1AX, TERT, RET, DICER1, TP53, PTEN,* and *PIK3CA* genes, did not reveal rare or pathogenic variants in any of these genes [15–17]. Subsequently, we excluded the presence of significant variants of genes so far identified for familial FNMTC: *HABP2*, *FOXE1* and *MAP2K5*. The complete sequencing of these genes confirmed that no variants were present in analyzed families. We thus extended the analysis to all the other variants in both known and unknown genes. In particular, we performed multiple evaluations using unbiased combinations of filters. First, we searched for loss-of-function autosomal recessive variants shared by affected family members. No shared variant was found, as expected, considering most cancer susceptibility genes so far identified recognize an autosomal dominant model. Considering FNMTC a rare disease, the causative mutation should be very rare in the general population. Moreover, since the incidences of thyroid cancer in different countries range from 1 to 12.5 per 100,000, and that FNMTC only accounts for less than 5% of thyroid cancer cases, SNPs and Indels with allele frequencies higher than 0.001 (either in the ExAC or dbSNP databases) were excluded [18–21]. We selected several shared rare heterozygous variants in genes that are also highly expressed in thyroid tissue.

With this criterion, five genes: *SPOC2, G3PB1, FAM122C, AIF1L,* and *BROX* were selected among two dozen as the best susceptibility gene candidates for FNMTC. The most interesting was the loss of function variant in *BROX,* while the others were missense variants. We then performed segregation analysis by genotyping.

We found a positive segregation results for *BROX* only, while the other genes were excluded by further genotyping in nonaffected family members.

BROX haploinsufficiency in familial nonmedullary thyroid cancer

A new variant of *BROX* gene consisting in a frameshift deletion chr1:222892283 (NM_001288579:c.119delG:p.R40fs)

was found in three members of family A and confirmed by Sanger sequencing. The predicted protein of 46 kDa should be truncated after ∼50 residues, comprising its Bro1 domain. We confirmed the absence of this null variant in two unaffected members of the family. Interestingly, we screened this gene in the other families and found a new variant in 5′UTR region of *BROX* gene (chr1: 222886144 NM_144695:c.-2898C > T) in two sisters of family B (Fig. 1). We have no direct evidence of a functional effect of this second variant.

Discussion

The susceptibility chromosomal loci and genes of 95% of FNMTC cases remain to be characterized. To elucidate the molecular basis of FNMTC, we performed a high coverage whole-exome sequencing on five FNMTC families. For the first time, we describe a frameshift mutation of *BROX* gene. The predicted protein of 46 kDa will result truncated after ∼50 residues, deleting its Bro1 domain. This conceivably reduces the likelihood of a gain-of-function effect. This variant should generate a null allele, considering the very small size of the protein product whenever it is expressed. Thus, a haploinsufficiency model is predicted, with the reduction of *BROX* expression that predisposes to disease or, alternatively, with the occurrence in the thyroid of a somatic hit that inactivates the remaining allele. This is the first report showing a possible association between *BROX* variants and cancer susceptibility. *BROX* is a protein-coding gene expressed in several tissues and with its highest mRNA levels in the thyroid gland, as demonstrated by the transcriptome analysis of 27 different human organs and tissues from 95 individuals [22]. *BROX* encodes for human Brox, a 46-kDa protein that has a Bro1 domain-like sequence and a C-terminal thioester-linkage site of isoprenoid lipid. Bro1 domain is necessary for the morphogenesis of multivesicular bodies (MVBs) and is involved in the endosomal sorting of cargo proteins, including integrin and EGFR degradation in lysosomes [23–25]. EGFR is one of the key regulators of cell survival and growth. Its aberrant expression or uncontrolled activity is directly implicated in a variety of tumours. Ultimately, *BROX* loss of function could induce EGFR aberrant activity and tumor growth. Our results suggest the need to address new research on the genetic basis of FNMTC, looking to different pathways, comprising the overall working mechanism of *BROX* regulated EGFR trafficking, which is of great interest for its direct association with cell survival and cancer signaling. A hypothesis of the possible pathogenetic mechanism of *BROX* haploinsufficiency in FNMTC is suggested in Fig. 3.

The *BROX* gene in ExAC shows a lower number of loss-of-function variants (and no homozygous subject) in comparison with the prediction. This may indicate an important

role, but heterozygous individuals could be considered as healthy until the occurrence of cancer. The final demonstration of the role of *BROX* in FNMTC necessarily passes through the demonstration of a second family with a very similar allele. By analyzing the remaining four families, we identified a new variant (chr1: 222886144 NM_144695:c.-2898C > T) in 5′UTR region of *BROX* gene. This is a chromatin-rich region. The sequences of the untranslated regions (UTRs) of mRNAs play important roles in post-transcriptional regulation [26]. The regulation of gene expression can also occur through a post-transcriptional modulation of the amount of gene product and that this modulation can be mediated by 5′ untranslated exon 1, suggesting a role in dysregulation of *BROX* function. Although this mutation may be suggestive, data from additional families are necessary.

FNMTC is a rare manifestation of a common condition and it is characterized by a more aggressive biological behavior. Earlier age at disease onset, a higher rate of nodal metastases, extrathyroidal tumor extension and increased severity in successive generations have been described [7, 27–29]. A recent meta-analysis of Wang et al. reporting 12 studies with a total of 12,741 patients confirmed these findings [30]. Researches to identify candidate cancer predisposition genes in non-syndromic FNMTC have brought mainly low-to-moderate penetrance genes [31]. The finding of *HABP2* G534E polymorphism, claimed to be a new susceptibility gene of FNMTC. G534E, is reported in gnomAD (https://gnomad.broadinstitute.org/) 6224 times with 32 homozygous subjects (frequency = 0.022): these numbers may be important to indicate either a very incomplete penetrance or the co-segregation by chance. Further studies support this view with the lack of an increased risk [9–12]. In a recent study from Ye et al., *MAP2K5* mutation was identified as a novel susceptibility gene for FNMTC [32]. Cirello et al. did not confirm this data, and we found an intronic, non-significant, variant of *MAP2K5* in affected members of two out of five studied families [33]. Despite the availability of modern NGS techniques and of huge genomic databases of hundred thousand individuals, no genetic test is yet available for FNMTC so far. Even if rare variants have been recently evidenced, the lack of a specific genetic test for FNMTC has resulted in the development of a clinical definition based on family history [34]. The most stringent definition of FNMTC requires two first-degree relatives with NMTC at the time of diagnosis of the patient in question, in the absence of a known familial syndrome. However, a clinical definition presents a number of problems. Clearly, the first family member (the index case) diagnosed with NMTC cannot be properly known as harboring familial illness, nor will the second till three cases are known.

In conclusion, we identified new variants in the *BROX* gene, involved in EGFR degradation pathway, in association with familial cases of FNMTC in which three relatives were diagnosed with PTC. We believe that this result may suggest new insights on the genetic basis of FNMTC. The involvement of EGFR trafficking in thyroid cancer growth will be a rational basis for future investigations to unravel the overall working mechanism of its key role in cell survival and cancer signaling. Only families where three first-degree relatives are affected can be considered to represent true familial

Fig. 3 Hypothesis for the pathogenetic mechanism of FNMTC: *BROX* haploinsufficiency induces altered EGFR degradation pathway in follicular thyroid cells, with EGFR accumulation and aberrant cell growth
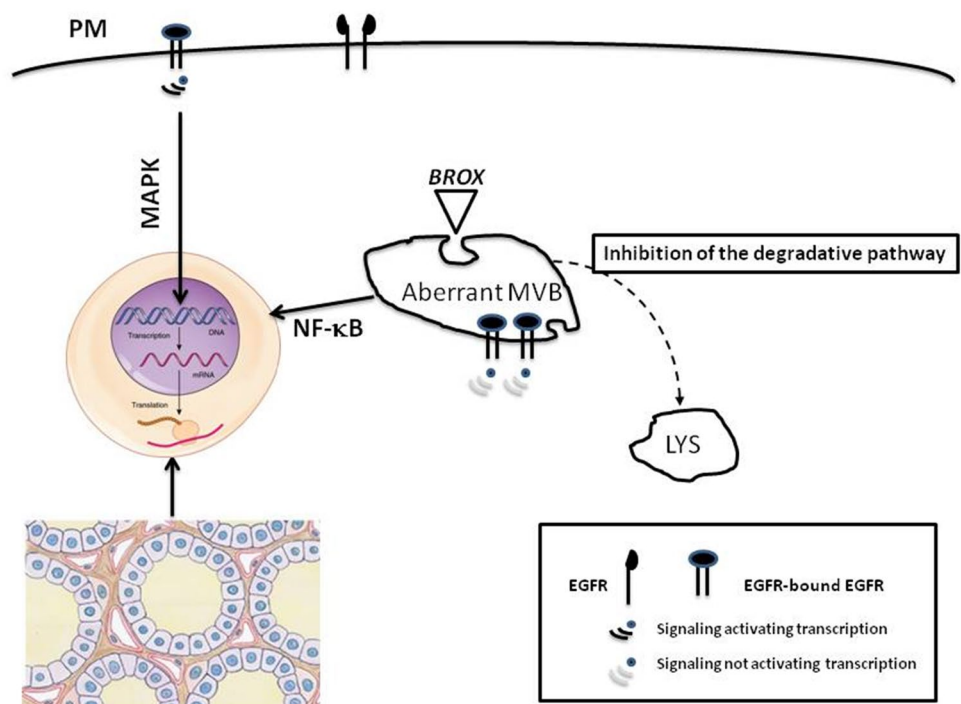
FNMTC. Moreover, our finding can add information for the management FNMTC. The NGS results are deeply influencing both our understandings of diseases and their clinical management. This revolutionary approach needs extreme caution and expertise in the interpretation of the results, and underlines the need for data sharing in rare disease research.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors have nothing to disclose.

Ethics approval The study was approved by the local ethical committee of the Università della Campania "Luigi Vanvitelli" protocol n°54 del 2/5/2019 on Horizon 2020 Solve the Unsolved project.

Informed consent The informed consent was obtained from the patients included in the study.

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