Genome-wide Transcriptome Study in Skin Biopsies Reveals an Association of E2F4 With Cadasil and Cognitive Impairment

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Abstract

Background:CADASIL is a small vessel disease caused by mutations in *NOTCH3* that lead to an odd number of cysteines in the receptor, causing protein misfolding and aggregation. The main symptoms are migraine, psychiatric disturbances, recurrent strokes and dementia, as executive function is characteristically impaired.

The molecular pathways altered by this receptor aggregation need to be studied further.

Methods: Agenome-wide transcriptome study(four casespaired with three healthysiblings)was carried out,in addition toa qRT-PCR for validation purposes(ten new cases and eight new controls).Neuropsychological tests were performedto evaluateverbal memory, attention and information processing speed (IPS), motor speed and dexterity, executive function and visuoconstructional skills. The Single-nuclei Brain RNA-seq expression browser (SNBREB) and the GTExPortalwere used to study brain expressionof the significant mRNAs found.

Results: The two most significant differentially expressed mRNAs (*BANP*,p-value=$7.23x10^{-4}$ and *PDCD6IP*, p-value=$8.36x10^{-4}$) were selected for the validation study by qRT-PCR. Additionally, we selectedtwo more mRNAs(*CAMK2G*,p-value=$4.52x10^{-3}$ and *E2F4,* p-value=$4.77x10^{-3}$) due to their association with ischemic neuronal death. *E2F4*showed differential expression in the genome-wide transcriptome study and in the qRT-PCR (p=$1.23x10^{-3}$), and it was upregulated in CADASIL cases. Furthermore, higher *E2F4* expressionwas associated with worseexecutive function (p=$2.04x10^{-2}$) and attention and IPS (p=$8.73x10^{-2}$).*In silico* studies indicated that *E2F4* is expressed in brain endothelial cells.

Conclusions: Skin biopsies of CADASIL patients presented higher mRNA levels of *E2F4*and these higher levels have a significant correlation with worseexecutive function and IPS.

Background

CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, OMIM#125310) is an autosomal dominant inherited small vessel disease (SVD) caused by mutations in the *NOTCH3* gene (19p13). Its real prevalence is unknown, although some studies have shown that up to 4.1/100,000 individuals suffer from CADASIL (1, 2), and as many as 3.2–3.4/1,000 individuals carry a pathogenic mutation in *NOTCH3* (3, 4).

The extracellular domain (ECD) of NOTCH3 is constituted by 34 epidermal growth factor-like repeats (EGFr), each of which is composed of six cysteines. Pathogenic mutations lead to an odd number of these cysteines, disrupting disulfide bridge formation and leading to NOTCH3 protein misfolding, multimerization and aggregation (5–7). This is the main etiopathogenic hypothesis. However, it is not fully understood. It is thought that this protein aggregation could lead to a toxic gain of function, as seems to occur with TIMP3 and vitronectin, proteins associated with vessel extracellular matrix (8, 9), or even interfere with autophagy (10) or cause endoplasmic reticulum stress (11). Likewise, a proteomic

study highlighted the differential protein levels between CADASIL patients and controls regarding extracellular matrix and mitochondrial proteins (12).

Because of its systemic nature, the study of different biopsy tissues, such as brain, muscle and skin, has made it possible to examine the histopathological changes that take place in CADASIL for a better comprehension of the disease, such as the loss of endothelial cells, pericytes and smooth muscle vessel cells, as well as intercellular or cell-matrix adhesions (13, 14). Actually, the hallmark of the disease, granular osmiophilic materials (GOMs) constituted partly by NOTCH3 ECD (15), has a sensitivity of 45–96% and a specificity of 100% for CADASIL diagnosis (16, 17).

The main symptoms of CADASIL are migraine, psychiatric disorders, recurrent small subcortical infarctions and dementia (18). Regarding cognitive impairment, 60% of patients aged > 60 years have dementia, and impaired executive function (EF) was detected globally in 87.5% of individuals (19).

Our aim was to identify mRNA differentially expressed in skin biopsies of CADASIL patients through a genome-wide transcriptome study (GWTS) that could be relevant for understanding the etiopathogenesis of the disease or could be used to identify therapeutic targets for future studies, as well as to study their association with cognitive performance.

Methods

This is an observational case-control study to discover differential mRNA expression through a genome-wide transcriptome study (GWTS) using microarray technology and a real-time quantitative reverse transcription protein chain reaction (qRT-PCR) assay for validation of the results.

1.1. SUBJECTS

Subjects were selected from “CADAGENIA”, a registry in which patients with mutations in *NOTCH3* have been consecutively recorded since 2017 from different parts of Spain, mostly Catalonia (Hospital Vall d’Hebron and Hospital del Mar, Barcelona). For matching purposes, control relatives (such as spouses or siblings) without a known *NOTCH3* mutation were asked to enroll in the registry to avoid any potential bias due to differences between cases and controls, as well as other healthy volunteers.

Epidemiological data, blood analyses, cognition and neuroimaging profiles and skin biopsies were registered.

The inclusion criteria for cases for this differential expression study were: 1) age > 17 years, 2) having a cysteine-affecting *NOTCH3* missense mutation (CNMM), and 3) having a skin biopsy available. The exclusion criteria were: 1) age < 18 years, 2) having a *NOTCH3* mutation other than CNMM, and 3) not having a skin biopsy available.

The inclusion criteria for controls were: 1) age > 17 years, and 2) agreeing to have a skin biopsy. The exclusion criteria were: 1) age < 18 years, 2) having a known *NOTCH3* mutation, and 3) not having a skin

biopsy available.

As additional inclusion criteria for the GWTS, CADASIL patients and controls had to be matched with family members. For the qRT-PCR assay, this criterion was not needed.

1.2. VARIABLES

Detailed clinical-epidemiological data were collected from each patient, including age; sex; vascular risk factors, such as hypertension defined as two measures on different days with blood pressure exceeding 140/90 mmHg or taking antihypertensive treatment; diabetes mellitus (DM), defined as basal glycemia in venous plasma ≥ 126 mg/dl, 2-h post-load plasma glycemia ≥ 200 mg/dl or HbA1c ≥ 6.5% or taking antidiabetic treatment; dyslipidemia; smoking habits; and type of mutation.

The cognitive profile was determined in patients with *NOTCH3* mutations by means of a complete neuropsychological examination. The evaluated cognitive domains included: verbal memory, working memory, executive function, attention and information processing speed, motor speed and dexterity, and visuoconstructional skills.

For global cognition, the Montreal Cognitive Assessment (MOCA) was used as a screening test. Verbal memory was evaluated through the short-term total learning and delayed recall subtests from the Wechsler memory scale-III (WMS-III) word list. Working memory was determined by the forward and backward digits subtests from the Wechsler Adult Intelligence Scale (WAIS-III). EF was assessed by means of: phonetic (letters “P”, “M” and “R”) and semantic category (animals) verbal fluencies, the Stroop Color-Word test —number of words— and the Trail Making Test part B (TMT-B) —execution time—. Attention and IPS were evaluated through the Symbol Digit Modalities Test (SDMT), Stroop Word and Color tests —number of words— and the Trail Making Test part A (TMT-A) —execution time—. Motor speed and dexterity were rated by the Purdue Pegboard test, considering the dominant, non-dominant and both-hand trials. Visuoconstructional skills were evaluated by means of the block designs subtest from the WAIS-III.

Raw scores were adjusted into Z-scores by age and years of education following Spanish normative data (20–22). A higher adjusted Z-score indicates a better performance in all cases. We calculated cognitive domain indices by averaging the adjusted scores within each domain.

1.3. RNA EXTRACTION

A 6-mm skin punch biopsy was obtained for each participant in the study. The homogenization of the tissue was carried out with the *TissueRuptor* (Quiagen) and the RNA was extracted with an RNeasy® Plus Micro Kit (Quiagen), following the manufacturer’s instructions.

1.4. GENOME-WIDE TRANSCRIPTOME STUDY

From each sample, 10 ng of total RNA was used as the starting material. The quality of the isolated RNA was measured previously by capillary electrophoresis using a NanoChip (Bioanalyzer 2100, Agilent).

Single-stranded cDNA suitable for labeling was generated from the total RNA using the GeneChip WT Pico Reagent Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. This kit makes it possible to generate robust expression profiles from as little as 100 pg of total RNA (10 cells). Purified sense-strand cDNA was fragmented, labeled and hybridized to the arrays using the GeneChip Hybridization, Wash and Stain Kit from the same manufacturer. After array scanning, raw data quality control was performed to check the overall performance of the processing.

1.5. qRT-PCR

For this assay, we selected the two most significantly differentially expressed mRNAs from the GWTS (p-value $< 10^{-3}$). As CADASIL is an arteriopathy that leads to brain hypoxemia, we wanted to select the genes from the GWTS that were related to neuronal ischemia in order to show a possible link and that belonged to the top fifteen most significant differentially expressed mRNAs. Therefore, we conducted a bibliographic search in PubMed with the term "(ischemi*[Title/Abstract]) AND *gene*[Title/Abstract]"

As previously reported (23), mRNA levels were measured by qRT-PCR using TaqMan® fluorogenic probes (see Supplemental Table I for those used in this study) on a 7500 Real-Time PCR System (Applied Biosystems, CA, USA). PPIA expression was used to normalize the results, as has been described previously (23).

qRT-PCR was performed using a standard TaqMan® PCR kit protocol consisting of 20 µl of PCR mix, including 5 µl of cDNA, 10 µl of 2x TaqMan® Universal PCR Master Mix (P/N: 4304437, Applied Biosystems, Foster city, CA, USA), 1 µl of TaqMan gene expression assay and 4 µl of water. Reactions were performed in two 384-well plates at 50ºC for two min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for one min. All reactions were run in triplicate and analyzed using the RQ App on Thermo Fisher Connect, following standard quality controls to assess the samples.

The results were a relative quantification (RQ) between the cycles of each sample relative to a single calibrator control sample.

1.6. STATISTICAL METHODS

Statistical and bioinformatics analyses were performed using custom scripts in R language, version 3.6.0 (R Core Team, 2019), with common Bioconductor packages. For the GWTS, after following a standard quality control, the Robust Multi-array Average (RMA) algorithm was used for pre-processing transcriptome data in order to perform background adjustment, normalization and summarization of the probe set expression values. Then, genes whose standard deviation (SD) was below the 65 percentiles of all the SD values, without a known Entrez Gene database identifier and without a valid annotation to the Gene Ontology database, were filtered out from the whole dataset and finally 6485 genes were considered for the statistical analysis. Selection of differentially expressed elements was based on a linear model analysis with empirical Bayes modification for the variance estimates. To deal with the false-discovery rate derived from multiple test comparisons, p-values were adjusted with the Benjamini and Hochberg method (24), considering genes with an adjusted p-value < 0.05 to be statistically significant.

The two most significant differentially expressed mRNAs from the GWTS (p-value < 10^{-3}) were evaluated in the replication cohort by qRT-PCR. Another two significant differentially expressed mRNAs from the top fifteen that were associated with ischemic neuronal death were also analyzed.

As the inclusion of outlying values could lead to erroneous interpretations (25), a box plot was performed for their identification. We used the “ggbetweenstats” function from the “ggstatsplot” package library. To know whether the outliers were statistically significant, and therefore that sample should be excluded, a Dixon's Q test was performed with the “dixon.test” function from the “outliers” package.

A p-value < 0.05 was considered statistically significant, after Bonferroni multivariable test correction, in the validation analysis.

To assess statistical significance, Fisher's Exact Test was used for categorical variables and a Mann-Whitney U test was used for numerical or ordinal variables. Pearson’s test was used to study the correlation between normal numeric variables.

1.7. EXPRESSION PROFILE

Brain expression of the mRNAs replicated in the qRT-PCR was studied using the GTEx Portal (https://gtexportal.org/home/) and expression by brain cell type was studied in the Single-nuclei Brain RNA-seq expression browser (http://ngi.pub/snuclRNA-seq/).

Results

For the GWTS study, we had four CADASIL patients corresponding to three different families, and three sibling controls without CNMM, one per family, in the CADAGENIA registry. These seven patients constituted the discovery cohort. Ten CADASIL patients and eight controls matched by age and sex constituted the validation cohort; blood relatives were not mandatory. No statistically significant differences between cases and controls were observed in age, sex, smoking habits, hypertension, diabetes mellitus, dyslipidemia, migraine, psychiatric disease, stroke or dementia (Table 1).

Table 1
Clinical-epidemiological features of the discovery (GWTS) and replication (qRT-PCR assay) cohorts.

	GWTS cases (n = 4)	GWTS controls (n = 3)	p-value	qRT-PCR cases (n = 10)	qRT-PCR controls (n = 8)	p-value
Age (mean ± SD, years)	52 ± 15	58 ± 16	0.21	49 ± 15	42 ± 10	0.31
Female	75% (3/4)	67% (2/3)	1	50% (5/10)	50% (4/8)	1
Smoker	25% (1/4)	33% (1/3)	1	10% (1/10)	25% (2/8)	0.56
HTN	0%	33% (1/3)	0.43	36% (3/10)	13% (1/8)	0.59
DM	0%	0%	1	10% (1/10)	0%	1
Dyslipidemia	25% (1/4)	0%	1	30% (3/10)	25% (2/8)	1
Migraine	50% (2/4)	67% (2/3)	1	30% (3/10)	25% (2/8)	0.19
Psychiatric disease	25% (1/4)	33% (1/3)	1	60% (6/10)	25% (2/8)	0.61
Stroke	0%	0%	1	40% (4/10)	0%	0.25
Dementia	0%	0%	1	0%	0%	1

HTN: hypertension; DM: diabetes mellitus.

For the distribution of the mutations in the cases of the Discovery/Validation analyses, see Fig. 1.

The GWTS did not show statistically significant differential expression after Benjamini and Hochberg correction (Table 2). Nevertheless, the two most significant differentially expressed mRNAs, with p-value $< 10^{-3}$ (Table 1): *BANP*, p-value = 7.23×10^{-4} and *PDCD6IP*, p-value = 8.36×10^{-4}, both downregulated in cases; and the two most relevant mRNAs associated with ischemic neuronal death from the top fifteen most significant differentially expressed mRNAs: *CAMK2G*, downregulated in cases (p-value = 4.52×10^{-3}), and *E2F4*, upregulated in cases (p-value = 4.77×10^{-3}), were selected for a second study using qRT-PCR in a new cohort of ten CADASIL patients and eight controls.

Table 2
Top fifteen most differentially expressed mRNAs from the Genome-Wide Transcriptome study.

Gene symbol	Gene name	logFC	p-value
BANP	BTG3 associated nuclear protein	-1.40	7.23×10^{-4}
PDCD6IP	Programmed cell death 6 interacting protein	-1.20	8.36×10^{-4}
TMEM176A	Transmembrane protein 176A	-1.23	1.87×10^{-3}
C16orf70	Chromosome 16 open reading frame 70	-1.10	2.37×10^{-3}
JCHAIN	Joining chain of multimeric IgA and IgM	-1.46	2.87×10^{-3}
CCDC149	Coiled-coil domain containing 149	-1.00	3.57×10^{-3}
GLT8D2	Glycosyltransferase 8 domain containing 2	-1.76	3.95×10^{-3}
PPIE	Peptidylprolyl isomerase E	0.94	3.97×10^{-3}
ZFP64	ZFP64 zinc finger protein	-0.99	4.40×10^{-3}
CAMK2G	Calcium/calmodulin dependent protein kinase II gamma	-0.85	4.52×10^{-3}
CPA3	Carboxypeptidase A3	-1.10	4.66×10^{-3}
E2F4	E2F transcription factor 4	0.89	4.77×10^{-3}
ZBTB24	Zinc finger and BTB domain containing 24	-0.91	4.95×10^{-3}
AP3M2	Adaptor related protein complex 3 subunit mu 2	-1.26	4.96×10^{-3}
SFMBT2	Scm-like with four mbt domains 2	-0.98	5.37×10^{-3}

logFC: log fold change.

In the qRT-PCR assay (Table 3), *E2F4* mRNA levels were significantly higher in CADASIL patients compared to controls after Bonferroni correction (relative quantification of 1.84 ± 1.09 in cases and 0.66 ± 0.11 in controls, p-value = 1.23×10^{-3}).

Table 3

Mean, standard deviation and p-values of the mRNAs from the qRT-PCR assay. Ten cases and eight controls were evaluated. The table shows the final size after qRT-PCR quality controls and removal of significant outliers.

	Cases RQ (mean ± SD)	Controls RQ (mean ± SD)	p-value
BANP (10 cases, 7 controls)	1.09 ± 0.36	1.05 ± 0.39	1
PDCD6IP (9 cases, 8 controls)	1.72 ± 0.81	1.64 ± 0.61	0.884
CAMK2G (10 cases, 8 controls)	1.03 ± 0.48	0.87 ± 0.17	0.12
E2F4 (10 cases, 7 controls)	1.84 ± 1.09	0.66 ± 0.11	1.23×10^{-3}
RQ: relative quantification; SD: standard deviation.			

To study the relationship between *E2F4* and neuropsychological performance, five CADASIL patients with qRT-PCR data were analyzed. Each neuropsychological domain (ND) was altered in > 50% of the patients, except visuoconstructional function. See Supplemental Table II, Supplemental Figure I.

A significant association was observed with EF, correlation= -0.93, p-value = 2.04×10^{-2}, and a tendency was observed in attention and information processing speed (IPS), correlation= -0.82, p = 8.73×10^{-2}, adjusted by age and educational level (Table 4). Higher *E2F4* expression was associated with a worse score in the EF and IPS tests. The other cognitive domains were not associated with *E2F4*.

Table 4
p-value of the association between E2F4 levels and domain alteration, adjusted by age and educational level.

Neuropsychological Domain	Cor	p-value
Executive function	-0.93	2.04×10^{-2}
Attention and information processing speed	-0.82	8.73×10^{-2}
Motor speed	-0.21	7.40×10^{-1}
Visuoconstructional skills	-0.49	4.05×10^{-1}
Verbal memory	-0.16	7.93×10^{-1}
Working memory	0.16	7.97×10^{-1}
Cor: correlation		

The GTEx Portal showed *E2F4* is expressed in the brain, as well as in other tissues (Supplemental Figure II), and the SNBREB showed that it is also expressed in brain endothelial cells (Supplemental Figure III).

Discussion

E2F4 mRNA was associated with CADASIL patients and was upregulated in the skin biopsies of cases vs controls in the GWTS and in the qRT-PCR study. Whether the overexpression of *E2F4* is due to a compensatory mechanism for a lack of protein production or whether the protein is actually elevated remains to be determined.

Its levels were additionally inversely correlated with EF and attention and IPS, which are the principal cognitive functions that are altered in CADASIL (26). Therefore, it might be useful as a biomarker of early detection of cognitive impairment and monitoring of the evolution of the disease. More studies focusing on this topic are needed.

Interestingly, we observed that *E2F4* was expressed in endothelial cells in the brain in addition to the skin. These cells have been found to be altered in CADASIL histopathological studies (13, 27, 28) and they are involved in blood flow regulation, a mechanism that has been seen to be altered in numerous studies focusing on this disease (29–31).

The E2F4 protein belongs to the E2F family of transcription factors and plays a crucial role in controlling the cell cycle. Previous studies observed that primary cerebellar granule neurons (CGNs) infected with an adenovirus expressing E2F4 vs controls had higher survival after an ischemic insult (32). Nevertheless, E2F4 levels decreased after hypoxia in non-infected CGNs. They also showed that overexpression of E2F4 had no effect on neuronal viability in the absence of ischemia. Therefore, E2F4 is related to neuronal survival in ischemic situations.

E2F4 is also part of a complex containing Smad3, which acts as a transducer of transforming growth factor-β (TGFβ) signals (33). TGFβ is a protein related to hereditary SVDs (34) such as CARASIL, caused by *HTRA1* gene mutations. HTRA1 is also associated with LTBP-1 and they regulate bioavailability of TGFβ (35). Importantly, these two proteins have been associated with NOTCH3 ECD deposits (36) and HTRA1 has shown less activity in CADASIL patients (37).

Therefore, E2F4 could indicate an overactivation of the TGFβ pathway in CADASIL patients.

Limitations

This study has several limitations. Firstly, the small sample size due to the low frequency of the disease in question. Matching the patients by age and sex and by family in the GWTS allowed us to minimize biases in the interpretation of the results.

Secondly, the target organ in CADASIL is the brain, but samples were obtained from skin tissue. Nevertheless, histopathological studies of skin biopsies have shown the typical hallmarks of the disease and have been used to understand its etiopathogenesis (13, 27, 28).

And thirdly, the GWTS study did not present significant differentially expressed mRNAs associated with CADASIL after adjusting for multiple comparisons. Massive data studies in low prevalence diseases such as CADASIL, which include different types of cells, may have the inconvenience of presenting less power to detect statistical significance when small differences exist, even if they are determinant. Choosing the mRNAs most significantly associated with CADASIL in the GWTS for evaluation by the qRT-PCR technique may be a useful and valid approach. Genetic studies in CADASIL have also had to resort to the use of non-significant data to deal with this lack of power, subsequently obtaining data of scientific interest. For example, the creation of a polygenic score from the most significant SNPs associated with WMH volume in CADASIL patients, suggesting that multiple SNPs with small effects modify the total WMH load in patients with CADASIL, rather than SNPs with larger effects (38).

Conclusions

Our results showed higher levels of *E2F4* mRNA in CADASIL skin biopsies and the highest levels of expression were associated with the worst EF and attention and IPS. E2F4 is a protein that is related to neuronal survival in ischemic conditions and the TGFβ pathway. *E2F4* might serve to monitor the disease and cognitive status, making it important for future clinical trials or even for therapeutic targeting. However, further studies are needed to elucidate the role of *E2F4* in CADASIL.

Abbreviations

CADASIL
cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy.
SVD

small vessel disease.
ECD
extracellular domain.
EGFr
epidermal growth factor-like repeats.
GOMs
granular osmiophilic materials.
EF
executive function.
GWTS
genome-wide transcriptome study.
qRT-PCR
real-time quantitative reverse transcription polymerase chain reaction.
CNMM
cysteine-affecting *NOTCH3* missense mutation.
MOCA
Montreal Cognitive Assessment.
WMS-III
Wechsler memory scale-III.
WAIS-III
Wechsler Adult Intelligence Scale.
TMT-B
Trail Making Test part B.
TMT-A
Trail Making Test part A.
RQ
relative quantification.
RMA
Robust Multi-array Average algorithm.
SD
standard deviation.
IPS
information processing speed.
TGFβ
transforming growth factor-β.

Declarations

Ethics approval and consent to participate:

This study was approved by the local ethics committee. A written informed consent document was provided before any study procedure was performed and it was signed by the patient or representative.

Consent for publication:

not applicable.

Availability of data and materials:

The datasets used and analyzed in the present study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contributions:

conception and design of the work: EM, OM, JJB, JM, JR and IFC. Data acquisition, analysis and interpretation: EM, JJB, NC, CC, NPTA, JCM, CGF, FOA, EE, AM, RP, ARC, GR, JK, JMF, JM, JR and IFC. All authors have substantively revised this work.

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Figures

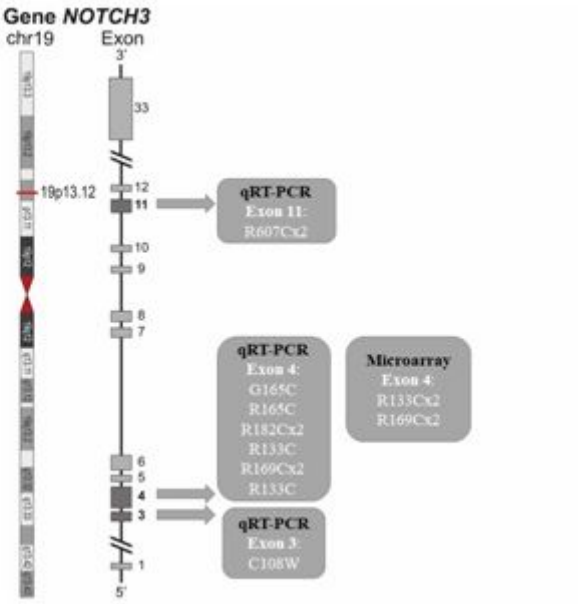

Figure 1

Distribution of mutations from the cases of the GWTS and qRT-PCR studies. Chr: chromosome.

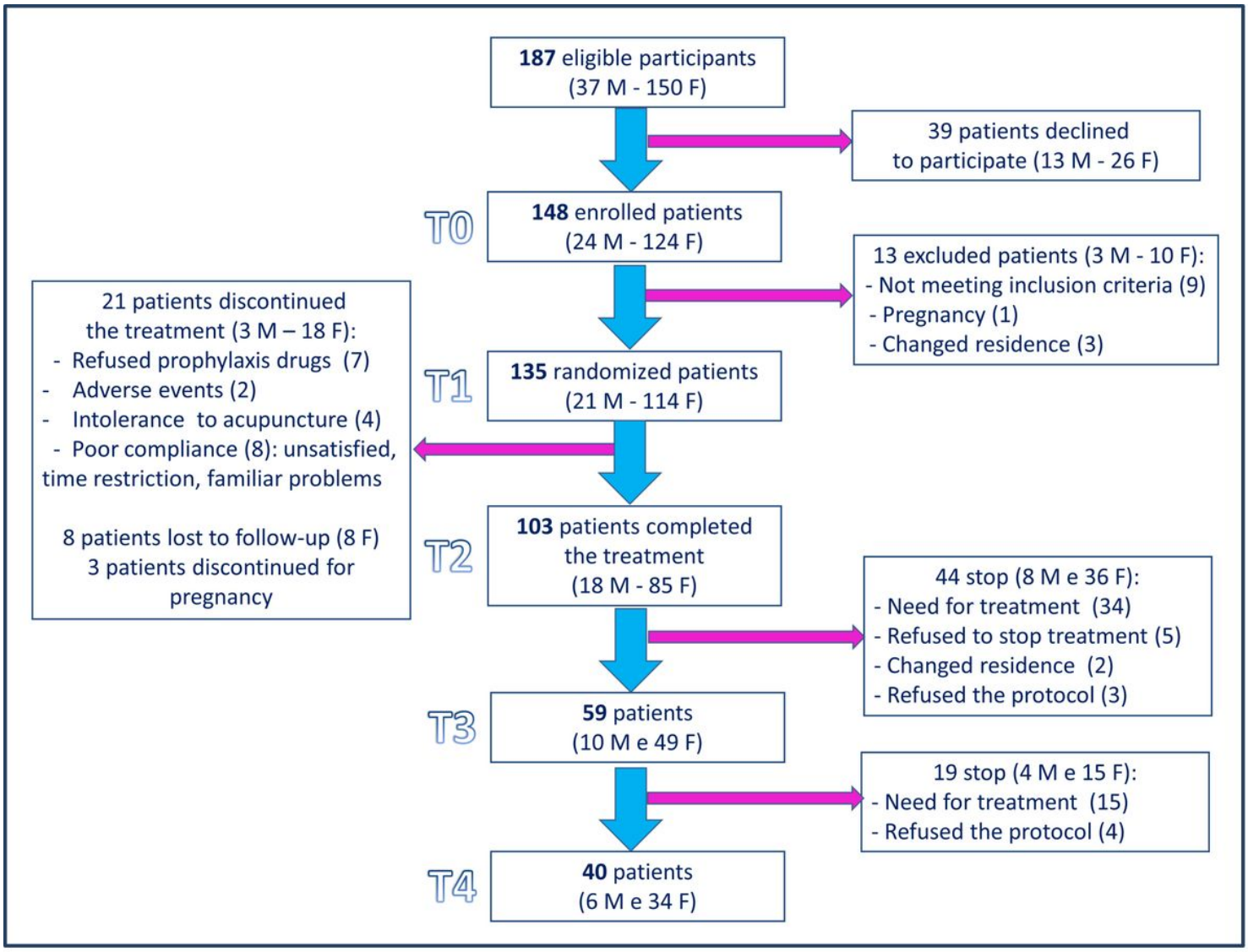

Figure 2

E2F4 relative quantification box plot for cases and controlsfrom the qRT-PCR experiment.

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