




Germline Mutations in Familial Papillary Thyroid Cancer

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Abstract

Thyroid cancer, predominantly of papillary histology (PTC), is a common cancer mostly diagnosed sporadically. Hereditary PTC is encountered in ~5% of cases and may present at an earlier age, with greater risks of metastasis and recurrence, compared with sporadic cases. The molecular basis of hereditary PTC is unknown in most cases. In this study, the genetic basis of hereditary PTC in three Brazilian families was investigated. Whole exome sequencing (WES) was carried out for probands in each family, and validated, pathogenic/likely pathogenic sequence variants (P/LPSVs) were genotyped in additional family members to establish their putative pathogenic role. Overall, seven P/LPSVs in seven novel genes were detected: p.D283N**ANXA3*, p.Y157S**NTN4*, p.G172W**SERPINA1*, p.G188S**FKBP10*, p.R937C**PLEKHG5*, p.L32Q**P2RX5*, and p.Q76**SAPCD1*. These results indicate that these novel genes are seemingly associated with hereditary PTC, but extension and validation in other PTC families are required.

Keywords Hereditary papillary thyroid carcinoma · Whole exome sequencing · Inherited predisposition · Candidate genes

Introduction

Thyroid cancer is the most common endocrine malignancy, and its incidence has continuously increased worldwide, from age standardized rate (ASR) of 4.6/100,000 in 1974–1977 to 15.8/100,000 in 2012–2016 [1]. In Brazil, an estimated 9610 new cases of thyroid cancer (8040 in females) were expected to be diagnosed in 2018 (<http://www1.inca.gov.br/estimativa/2018/sintese-de-resultados-comentarios.asp>; accessed 9th September 2019). The most common histological type is

papillary thyroid carcinoma (PTC), comprising ~85% of all cases, and in most patients, there is no discernible family history of thyroid malignancy. Familial non-medullary thyroid cancer (FNMTc) is reported in ~5% of all thyroid cancers, with only a minority associated with specific cancer susceptibility syndromes, such as Cowden syndrome (MIM No. 158350) and Carney complex (MIM No. 160980) with well-defined driver germline mutations in the *PTEN* and *PRKARIA* genes, respectively [2].

Studies that focused on defining the genetic basis of non-syndromic FNMTc have identified germline mutations in several candidate genes including *TITF-1/NKX2.1* [3], *SRGAPI* [4], *FOXE1* [5], *HABP2* [6–8], and *SRRM2* [9]. Moreover, miRNAs [10] and four chromosomal loci (1q21, 6q22, 8p23.1-p22, 8q24) with no known candidate genes [4, 10, 11] were reportedly associated with FNMTc. Notably, the p.G534E**HABP2* variant (rs7080536) has been detected in some FNMTc families, although co-segregation was incomplete in some families, and this sequence variant is also present in controls with an overall population allele frequency of 2.223e-02 (https://gnomad.broadinstitute.org/variant/10-115348046-G-A?dataset=gnomad_r2_1) [6, 8, 12–14].

Thus, while some FNMTc are accounted for by known genes and chromosomal loci, the molecular basis for most FNMTc remains elusive. In the present study, we applied whole-exome sequencing (WES) methodology to define

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germline pathogenic/likely pathogenic sequence variants (P/LPSVs) in three Brazilian families with autosomal dominant FNMTC.

Materials and Methods

Patients' Identification and Recruitment

The study encompassed individuals from three Brazilian families displaying a likely autosomal dominant inheritance pattern of PTC in at least two generations (Fig. 1). These families were referred to the School of Medicine, Universidade Federal de Minas Gerais. The experimental protocols were approved by the Institutional Review Board at the Universidade Federal de Minas Gerais (ETIC 367/07). All study participants gave written informed consent before participation.

DNA Extraction from Peripheral Blood Leukocytes and Paraffin-Embedded Tissue

Genomic DNA was extracted from peripheral blood samples (II.2, III.1, III.2, and III.3, family A; I.2, II.1, and II.2, family B; I.2, II.1, II.2, and III.1, family C—Fig. 1) using standard protocols. DNA sample (II.1, family C) from paraffin-embedded tissue (FFPE) thyroid tumor tissue was extracted according to manufacturer's instructions (QIAamp DNA FFPE Tissue Kit, Qiagen, Germantown, MD). The quality and quantity of each DNA sample were examined by NanoDrop ND-2000 UV-Vis Spectrophotometer (ThermoFisher, Waltham, MA).

Whole-Exome Sequencing

DNA was subjected to whole exome capturing and sequencing using the Roche NimbleGen V2 chip (Madison, WI) or

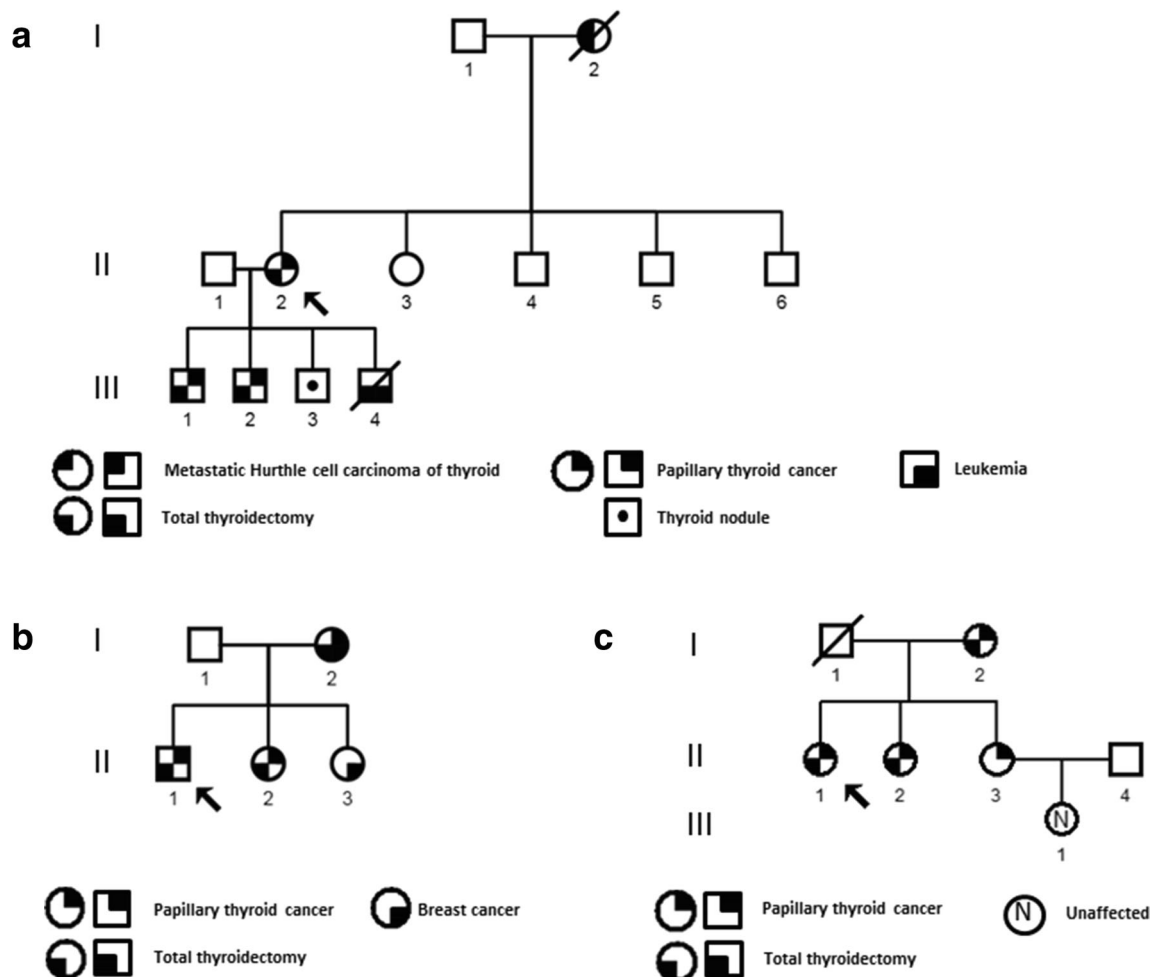


Fig. 1 Pedigree status from families A, B, and C with respect to papillary thyroid cancer and the genotypes for each heterozygous mutation identified. Circles and squares represent female and male members,

respectively. The proband is indicated by a black arrow. Deceased members are represented by diagonal lines. **a** Pedigree from family A; **b** pedigree from family B; **c** pedigree from family C

Nextera (San Diego, CA) with the Illumina HiSeq2000 sequencing platform (San Diego, CA). Sanger sequencing was used to validate the candidate variants identified by WES in these affected family members.

Variant Calling and Annotation

For each of the studied samples, raw sequence files were prepared using the Genome Analysis Tool Kit (GATK). Each fastq file was aligned against the human hg19/GRCh37 reference genome and a variant call format (VCF) file generated for each sample. PCR duplicates were removed using Picard (<http://picard.sourceforge.net/>); reads around known and detected indels were realigned, and base quality was recalibrated using GATK.

All generated VCF files were analyzed using HIPAA-compliant Variantx Genomic Intelligence platform and Genomic Intelligence diagnostic console (<https://www.variantx.com/>) (Variantx Inc., Framingham, MA, 01701, USA), Genome AnalysisTK 2.3-9 (Theragen Etx Bioinformatics Team), Mendel, MD (<https://mendelmd.org/>), and Ingenuity Variant Analysis

Sequence variants associated with gain or loss of function, compound heterozygotes, heterozygous ambiguous, haploinsufficient, homozygous or hemizygous, located at a microRNA binding site; frameshift, in-frame indel, or stop codon changes and also assigned “pathogenic/likely pathogenic” by PROVEAN [15], SIFT, Polyphen-2, and CADD [16] algorithms were selected for further analyses.

Genes that harbored P/LPSVs were queried for potential relevance to thyroid cancer tumorigenesis by applying the following criteria: (i) predicted functional consequences using the Ensembl database (<https://www.ensembl.org/index.html>); (ii) publications relating each gene to cancer, based on PUBMED search of the gene name; (iii) pathway annotation, which includes all pathways in which a given gene product has been involved at GeneCards (<http://www.genecards.org/>), OMIM (<https://www.omim.org/>), and UniProt (<http://www.uniprot.org/>) databases; (iv) the occurrence of deleterious or possible deleterious somatic mutations in the COSMIC database (<http://cancer.sanger.ac.uk/cosmic>); (v) mouse models (when available), gathered from KOMP (<https://www.mousephenotype.org/aboutikmc/about-komp>); Mouse Genome Informatics (MGI; <http://www.informatics.jax.org/>) and HomoloGene (<http://www.ncbi.nlm.nih.gov/homologene>); and (vi) minor allele frequency of less than 1% in gnomAD database (<http://gnomad.broadinstitute.org>; ac). Following these filtering steps, data were manually reviewed to generate final candidate gene list.

WES Sequence Data Validation and Mutation Analysis

Validation of the candidate P/LPSVs on DNA extracted from all consenting individuals was carried out using Sanger

sequencing. The same variants were also sequenced in an independent series of 90 ethnically matched elderly controls (≥ 65 years of age) who consented to participate in the study using a previously approved ethics protocol (ETIC 367/07).). In addition, allele frequencies of all pathogenic variants were queried with data derived from the 1000 Genomes Project (1000G), Exome Variant Server (ESP), 10,000 UK Genome (UK10K), The Genome Aggregation Database (gnomAD; <https://gnomad.broadinstitute.org/>; accessed 6th January 2020) as well as the Brazilian Initiative on Precision Medicine database (<http://bipmed.iqm.unicamp.br/genes>; accessed 6th January 2020).

Results

Clinical Cases Study

The study included 11 individuals from three Brazilian families (at least three affected members from each family) displaying a likely autosomal dominant inheritance pattern of PTC in at least two successive generations (Fig. 1). Of relevance is the fact that all studied affected patients presented with classical and/or follicular variants of PTC. Table 1 summarizes clinical, histological, and molecular data.

The proband of family A (II.2) was diagnosed with PTC at age 32 years. Her family history (Fig. 1a) included a mother (I.2) diagnosed with metastatic Hurthle Cell carcinoma at age 38 years, two sons (III.1 and III.2) diagnosed with PTC at age 39 and 36 years, respectively, a son who died of leukemia at age 14 years (III.4), and another son aged 36 (III.3) with thyroid nodules who refused biopsy. Pathology report of tumor from II.2 showed infiltrating papillary carcinoma (follicular variant). Immunohistochemistry studies demonstrated positivity for Cytokeratin-7, TTF-1 and thyroglobulin in tumor cells.

The proband (II.1) of family B (Fig. 1b) presented a multifocal PTC at age 43 years. His sister (II.2) and his mother (I.2) were also diagnosed with PTC at age 22 and 70 years, respectively. In addition, his mother (I.2) and another’s sister (II.3) were diagnosed with breast cancer at 73 and 52 years, respectively. Pathology reports of tumors from the proband (II.1) and II.2 showed differentiated infiltrating (classical variant) papillary carcinoma.

The proband (II.1) of Family C (Fig. 1c) was diagnosed with the classical variant of PTC at age 18 years. Her family history included a mother (I.2) and two sisters (II.2 and II.3) who were also diagnosed with PTC at 65, 47, and 45 years of age, respectively. Her niece (III.1) is unaffected. Patients II.2 and II.3 had follicular and classical variants of PTC, respectively.

All patients affected by PTC underwent total thyroidectomy and radioactive iodine treatment. None of these patients had a past history of ionizing radiation exposure.

Table 1 Clinical, histological, and molecular data

Patient	Age at diagnosis	Sex	Histological type	TNM staging (AJCC, 2018)	Candidate genes in each family
Family A					
II.2	32	Female	Follicular	T2N0M0	<i>ANXA3, NTN4</i>
I.2	38	Female	Hurthle	T4bN1M1	
III.1	39	Male	Classical and follicular	T1aN0M0	
III.2	36	Male	Classical	T1N0M0	
Family B					
II.1	43	Male	Classical	T3N1aM0	<i>SERPINA1</i>
I.2	70	Female	Classical	T3N1M0	
II.2	22	Female	Classical	T2N1M0	
Family C					
II.1	18	Female	Classical	T1aN0M0	<i>FKBP10, PLEKHG5, P2RX5, SAPCD1</i>
I.2	67	Female	Follicular	T1aN0M0	
II.2	45	Female	Follicular	T1aN0M0	
II.3	47	Female	Classical	T1aN0M0	

AJCC, American Joint Committee on Cancer

WES Analysis

Family A

Exome sequencing of the proband (II.2) yielded a total of 49,402 variants in 17,359 genes, with an average read depth of 138× per base and a mean base call quality of 1379. After excluding common SNPs with minor allele frequency (MAF) of $\geq 1\%$, a total of 488 genes with mutations predicted to have a potentially damaging effect were identified within the coding regions by *Ingenuity Variant Analysis*. Similar analysis using *Mendel, MD* yielded a list of 160 genes. Overall, 39 candidate genes were identified by both analyses tools. All 39 genes were queried for any supportive evidence to their putative involvement in thyroid carcinogenesis. Following this selection step, four variants in four genes were selected for validation. Only two mutations in two genes were validated: p.D283N**ANXA3* (MIM No. 106490) and p.Y157S**NTN4* (MIM No. 610401). These two germline variants were also identified in the two other PTC-affected individuals in family A (III.1 and III.2) and in the individual with thyroid nodules (III.3) (Fig. 1a); p.D283N**ANXA3* has been reported in the gnomAD ($3.99\text{e}-6$; $8.59\text{e}-4$) database. Y157S**NTN4* was not present in any of the 90 healthy ethnically matched controls as well as in the gnomAD and Brazilian Initiative on Precision Medicine databases.

Family B

Exome sequencing of patient II.1 resulted in 49,338 variants in 17,478 genes, with an average read depth of X130/base and mean call quality of 1472. *Ingenuity Variant Analysis* identified 112 P/LPSVs (in 91 genes) most likely to be

implicated in PTC pathogenesis. Similar analysis using *Mendel, MD* yielded 227 P/LPSVs in 158 genes. Only two P/LPSVs were shared by both pipelines. Sanger sequencing validated only the c.1087G>T;p.G172W**SERPINA1* (MIM No 107400) gene variant in the proband and all genotyped affected family members (I.2, II.1, and II.2) with PTC. None of the Brazilian population controls harbored the c.1087G>T;p.G172W**SERPINA1* mutation which was not reported in the Brazilian Initiative on Precision Medicine database. The rate of this variant in the gnomAD database was $8.59\text{e}-4$.

Family C

Exome sequencing of PTC-affected patients I.2 (mother) and II.1 (daughter) in family C resulted in 57,436 variants in 15,060 genes. The mean base call quality was 1022 and average read depth was X130/base. Using the above outlined filtering steps, *Ingenuity Variant Analysis* identified 252 P/LPSVs in 194 genes that were shared by both patients. Similar analysis using *Mendel, MD* led to a list of 198 variants in 132 genes. Twelve seemingly P/LPSVs were commonly shared by both pipelines. Of these, four heterozygous P/LPSVs were validated by Sanger sequencing: c.666G>A;p.G188S**FKBP10* (MIM No. 607063), c.2815C>T;p.R937C**PLEKHG5* (MIM No. 611101), c.494T>A;p.L32Q**P2RX5* (MIM No. 602836), and c.285C>T;p.Q76**SAPCD1*. These four PLSVs were present in all three affected genotyped family members (Fig. 1) as well as in a heterozygous state in a tumor sample from patient II.1. This latter finding implies that there was no allelic loss of the mutant allele in the tumor sample, at least in the region that was genotyped.

None of 90 ethnically matched controls nor did the unaffected case (III.1, family C) carry any of these validated P/LPSVs in these four genes. The rates of these variants in the gnomAD database are $1.77e-5$ (*FKBP10*), $4.12e-03$ (*P2RX5*), and $8.42e-4$ (*SAPCD1*). None were reported in the Brazilian Initiative on Precision Medicine database or in mouse databases.

Discussion

In this study, P/LPSVs in the *ANXA3*, *NTN4*, *SERPINA1*, *FKBP10*, *PLEKHG5*, *P2RX5*, and *SAPCD1* genes emerged as candidates for being FNMTTC susceptibility genes. Notably, the previously reported FNMTTC-associated predisposition genes (*AARS*, *AGK*, *ALB*, *ATP13A2*, *CDH11*, *CDS2*, *CIS*, *CTDSP1*, *DR6*, *EDC4*, *EFCAB8*, *EIF3A*, *FGD6*, *FGFR4*, *FOXA3*, *FOXE1*, *GPR187*, *HABP2*, *IDE*, *ITGGAD*, *KDSR*, *KLHL3*, *LZTR1*, *MAPKAK3*, *NAPB*, *NFRKB*, *NHLH1*, *NSMF*, *PARP4*, *PDPR*, *RNF213*, *SALL4*, *SRGAP1*, *SMARCD3*, *SRRM2*, *SVIL*, *TERT*, *THBS4*, *TINF2*, *TITF-1/NKX2.1*, *ZNF17*) [6, 8, 13, 14, 17] were not present in any of the families reported herein and therefore excluded as contributing to PTC phenotype. Moreover, despite the association of breast cancer and PTC in family B, raising the possibility of Cowden syndrome, the *PTEN* gene as well as the *SDH* family of genes were not mutated in that family, despite adequate coverage ($> 100\times$).

The *ANXA3* gene, located at 4q21.21, encodes calcium-dependent phospholipid-binding proteins—Annexin A3. Abnormal expression of *ANXA3* plays a role in different cellular processes, associated with tumorigenesis: cell proliferation, apoptosis, invasion, metastasis, and drug resistance [18]. In vitro and in vivo assays demonstrated increased of thyroid cancer cells proliferation concomitant with reduced *ANXA3* expression [18, 19].

Netrin-4 (*NTN4*) gene, located at 12q22, encodes for NTN4 proteins—basement membrane components [20]. NTN4 may play a role in development and progression of several cancer types such as breast [21], gastric [22], and melanoma [23]. Low NTN4 expression may promote tumor growth, cellular proliferation, and angiogenesis inhibition [21, 24]. NTN4 is markedly downregulated in prostate [25], breast [21], melanoma [23], and cervical cancers [26]. The involvement of *NTN4* gene in PTC has not been reported.

SERPINA1, also known as $\alpha 1$ -AntiTrypsin (AAT), located at 14q32.13, is highly expressed in colorectal cancer [27], cutaneous squamous cell carcinoma [28], and papillary thyroid carcinoma [29]. Although the mechanistic role of *SERPINA1* in thyroid tumorigenesis is not fully understood, cell invasion and migration in colorectal cancer have been associated to *SERPINA1* upregulation of fibronectin [27]. Furthermore, the C-terminal 26-residue peptide of

SERPINA1 presents a mitogenic action, stimulating malignant cell proliferation [28].

The suppressor APC domain containing 1 (*SAPCD1*) gene, located to 6p21.33, is associated with DNA repair [30]. Expression of *SAPCD1* is somatically decreased in breast cancer, and a deleterious variant (p.Q76*) has been reported to be associated with lung cancer predisposition [31].

The *PLEKHG5* (also known as *TECH* or *Syx*) gene localizes to 1p36.31, a region frequently associated with somatic tumor gene rearrangements [32]. *PLEKHG5* encodes for a guanine nucleotide exchange factor that selectively couples RhoA activation at leading cell edges with Dia1 signaling and ROCK suppression. *PLEKHG5* leads to migration of human cells, a feature suggestive of its involvement in malignant transformation [33]. Indeed, p.R937C**PLEKHG5* somatic mutation has been detected in urinary tract carcinoma (COSMIC database; <http://cancer.sanger.ac.uk/cosmic>).

The *FKBP10* gene, located at 17q21.2 encodes for the FK506 binding protein 10 [34]. Ge et al. [35] reported that *FKBP10* is present at higher levels in renal cancer cells compared with non-tumoral cells, inducing cell cycle progression, cell proliferation, migration, and invasion. *FKBP10* expression is decreased in epithelial ovarian carcinomas [36]. Notably, 191 non-synonymous somatic *FKBP10* point mutations are noted in the COSMIC database but not the p.G188S mutation.

The *P2RX5* gene localizes to 17p13.2 and encodes an ion channel receptor for adenosine 5'-triphosphate (ATP) and adenosine, which acts as extracellular purinergic signaling molecules [37]. Overexpression of *P2RX5* is reported in high grade bladder cancer⁵⁶, basal cell and squamous carcinomas [38], and colon cancer [39]. It was suggested that activated *P2RX5* receptors may be associated with growth inhibition of cancer cells, switching the cell cycle from proliferation into a state of differentiation [37]. Presently, mutations in this gene have not been shown to contribute to cancer formation and/or predisposition.

The association of papillary carcinoma and Hurthle Cell carcinoma in only one patient from one single family (I:2, family A) may represent a rare co-occurrence only of a sporadic Hurthle Cell case in a FNMTTC.

Although germline mutations in more than one gene were detected in each family, it seems unlikely that the inheritance pattern is multigenic, as these families were selected based on the unusual clustering of thyroid cancer in more than one generation a pattern suggestive of autosomal dominant FNMTTC. The inherent inability to single out one gene variant stems in part from the size of the families and the lack of unaffected individuals who could be genotyped. Furthermore, there have been no published functional analyses or animal models that examined the putative role of these genes in FNMTTC predisposition.

The list of genes described in this study should be considered tentative at best, as there are no functional studies or animal models that prove the direct involvement of these mutations in thyroid tumorigenesis. Moreover, the list for genes that have been reported in FNMTC is long and exhaustive [3–14, 17, 19, 29]. No single gene seems to account for more than one or a handful of families. One can speculate as to the reasons for this genetic reality in FNMTC predisposition: inclusion of phenocopies in the analyses, oligogenic inheritance, population genetic-based differences, to name a few [40–43]. This feature of multiple genes in inherited cancer syndrome is similar to inherited prostate cancer where no single gene accounts for more than 5% of all inherited cases [42].

Conclusion

In conclusion, our results suggest that *FKBP10*, *PLEKHG5*, *P2RX5*, *SAPCD1*, *ANXA3*, *NTN4*, and *SERPINA1* may contribute to susceptibility to non-medullary thyroid cancer. The validation of these results and affirming the possible contribution of the genes reported herein to other FNMTC families as well as the exact pathogenic mechanism await further studies.

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Compliance with ethical standards

Ethics Statement The experimental protocols were approved by the Institutional Review Board at the Universidade Federal de Minas Gerais (ETIC 367/07).

Disclosure Statement The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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