Published online 2019 May 8.

**Research Article** 

# Prevalence of BRAF<sup>V600E</sup> Mutation and Human Parvovirus B19 Infection in Thyroid Cancer

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Received 2018 September 12; Revised 2018 November 17; Accepted 2018 November 19.

# Abstract

**Background:** Thyroid carcinoma, the most prevalent endocrine malignancy, has increased rapidly in recent decades. A single-base substitution in the BRAF gene is identified as the most common genetic event in thyroid carcinoma. Infections with both DNA and RNA viruses can induce chronic inflammatory diseases and cancer. Human parvovirus B19 (B19V) infection has been implicated in thyroid cancer as the most common inflammation of the endocrine system.

**Objectives:** The study aimed to determine the prevalence of the BRAF<sup>V600E</sup> gene mutation and the presence of B19V infection in malignant and non-malignant thyroid tissue samples.

**Methods:** A cross-sectional study was performed from January 2012 to December 2017 on 108 paraffin-embedded thyroid tissues from patients with thyroid cancer tumor and nodular goiter. After DNA extraction, PCR restriction fragment length polymorphism (PCR-RFLP) and nested PCR assays were used to detect the BRAF<sup>V600E</sup> gene mutation and B19V DNA in tissue specimens, respectively. To confirm PCR-RFLP reliability, the amplified products were subjected to DNA sequencing. Statistical analysis was performed to determine a possible correlation between the occurrence of BRAF<sup>V600E</sup> mutations and clinicopathological characteristics, such as tumor subtypes, gender, age, and B19V presence.

**Results:** Overall, BRAF<sup>V600E</sup> mutation was detected in 77 out of 108 patients (71.3%) using PCR-RFLP, confirmed by DNA sequencing analysis. Using nested PCR, human parvovirus B19 DNA was detected in 14 out of 108 (13%) of the formalin-fixed, paraffin-embedded (FFPE) tissue specimens. Statistical analysis showed a significant difference in the prevalence of the BRAF<sup>V600E</sup> mutation in thyroid cancer patients when compared with the control group ( $P \le 0.001$ ).

**Conclusions:** Since cytological examinations depend on fine needle aspiration of the thyroid (FNAB) cannot be conclusive; hence it might be suggested that detection of the BRAF<sup>V600E</sup> gene mutation can be considered as a feasible assay. However, a low detection rate of the B19V DNA in FFPE tissue samples suggests that B19V infection is not associated with thyroid cancer.

Keywords: BRAF<sup>V600E</sup> Mutation, Human Parvovirus B19, Thyroid Cancer

### 1. Background

Thyroid carcinoma, the most prevalent endocrine malignancy, has increased swiftly in recent decades (1). The disease has several histological forms including papillary, follicular, medullary, and undifferentiated anaplastic thyroid carcinoma (2). Different factors, such as inflammation is possibly involved in tumor progression (3). The genetic modifications involved in developing these disparate subtypes of thyroid cancers and molecular signaling pathways are frequently altered. The proto-oncogene BRAF is one of the Ras/Raf/MEK/ERK/MAP kinase pathway (MAPK pathway) members (4) The BRAF<sup>V600E</sup> mutation, previously defined as BRAFV599E, is a base transversion at nucleotide position 1799 with the replacement of thymine by adenine, which changes a valine to glutamate. In different studies,  $BRAF^{V600E}$  mutation has been reported in 18% - 87% of thyroid cancers (5).

Infection with viruses can induce chronic inflammatory diseases and cancer (6). Both DNA and RNA viruses have been shown to be capable of causing cancer in humans (7). On the other hand, a persistent viral infection might lead to the progression of chronic diseases and ultimately invasive cancer (8). Human parvovirus B19 (B19V), a single-stranded DNA virus, which is pathogenic to humans causes various inflammatory disorders (9). In

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non-erythroid tissues, including liver, heart, synovial, lymphoid, colon, skin, and thyroid cells, expression of B19 viral proteins is associated with specific alterations in genes such as IL-6 and TNF- $\alpha$ , indicating that the persistent B19V virus expression can affect the cellular microenvironment (10). Upregulation of IL-6 is essential in the pathogenesis of the B19V virus infection (11).

Moreover, detecting a higher level of NF- $\kappa$ B in medullary, follicular, and papillary thyroid cancers in comparison with non-cancerous thyroid cells in patients infected with human parvovirus B19V suggests the possible role of B19V in the progression of thyroid cancer (12).

## 2. Objectives

In the present study, the prevalence of human parvovirus B19 infection in patients with thyroid cancer was investigated. Using PCR restriction fragment length polymorphism (PCR-RFLP), the association between the B19V infection and BRAF<sup>V600E</sup> gene mutation in malignant and non-malignant thyroid tissue samples was also studied.

# 3. Methods

A total of 108 paraffin-embedded thyroid tissues from patients with thyroid cancer tumor and nodular goiter were obtained from the pathology archive of the Khalili Hospital, which is affiliated to the Shiraz University of Medical Sciences (SUMS) 2012 - 2017. The number of specimens with available data for analysis was 108 (Table 1). The study was approved by the local Ethics Committee of SUMS.

## 3.1. DNA Extraction

Genomic DNA from paraffin-embedded blocks was extracted using FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen, Taiwan) according to the manufacturer's instructions. DNA purity and concentration were measured using NanoDrop UV visible spectrophotometers (Biochrom WPA, Inc., England). DNA was also extracted from human thyroid carcinoma (B-CPAP,) and human breast cancer (MCF-7) cell lines, which was used as mutant and wild-type controls of the BRAF gene in this experiment. Extracted DNA was diluted with distilled water to a concentration of approximately 50 - 100 ng/ $\mu$ L, and stored at -80°C for further investigations.

## 3.2. DNA Quality Assessment

To assess the yield and quality of DNA extracted from the tissue samples,  $\beta$ -globin gene was considered as an internal control using the following primer pairs: PC03 (5'-ACACAACTGTGTTCACTAGC-3') and PC04 (5'-CAACTTCATCCACGTTCACC-3') (13) in 25  $\mu$ L PCR mixture containing 50 - 100 ng genomic DNA, 0.5  $\mu$ M of each primer, 50  $\mu$ M of each dNTP, 1.5 mM MgCl<sub>2</sub>, 1× reaction buffer, and 2.0 U of Taq DNA polymerase. Cycling conditions were initial denaturation (94°C for 3 minutes), followed by 35 cycles (denaturation, 94°C for 1 minute; annealing, 55°C for 1 minute; synthesis, 72°C for 1 minute) and a final extension of 5 minutes at 72°C. Agarose gel electrophoresis was used for the separation of amplified DNA fragments.

# 3.3. PCR-RFLP for the Amplification, Detection, and Genotyping of the BRAF Gene

For the mutation analysis of BRAF, a 167 bp DNA fragment in exon 15 of the BRAF gene was amplified using specific primers (14). PCR amplification was carried out in 25  $\mu$ L PCR mixture containing 0.5  $\mu$ M of each primer, 0.2 mM of each dNTP, 100 ng of genomic DNA, and 1.0 U of Taq DNA polymerase (Sigma Co., Ltd.) in 1x reaction buffer supplied by the manufacturer. PCR conditions were: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 40 seconds, elongation at 72°C for 40 seconds, and a final extension at 72°C for 10 minutes. PCR products were electrophoresed on 2% agarose gel, and after visualization of 167 bp PCR fragment, the PCR products were digested with restriction endonuclease TspRI (also known as TscAI) (Thermo Scientific, Germany), according to the manufacturer's instructions, which yielded two major bands of 87 bp and 80 bp. Restriction enzyme TspRI recognizes the ACAGT $\downarrow$ GAAA restriction site and cuts it in the wild-type genotype. T1799A point mutation substitutes T by A so that the enzyme cannot discriminate the new restriction site and the uncut 167 bp PCR products will remain. RFLP products were visualized on 6% low EEO agarose (Cleaver Scientific Ltd, United Kingdom) gel electrophoresis. The V600E mutation does not have the restriction site for this enzyme; hence, the outcomes are a 167 bp band from the mutant allele and two residual bands from the normal allele.

# 3.4. Amplification and Detection of Human Parvovirus B19 DNA by Nested-PCR

Nested PCR was used to amplify a 106 bp DNA fragment of the B19V VP1 gene. A plasmid containing the gene for capsid protein VP1 was used as the positive control. The final PCR product was obtained by two rounds of PCR amplification of DNA extracted from formalin-fixed, paraffin-embedded (FFPE) samples with the use of primers (5'-AACGCCTCAGAAAAATACCC-3' and 5'-TAAGTGCTGAAACTCTAAWGG-3') for the first round

Histology Type	Gender		Age Range, y				Total
	Female	Male	0-20	21-40	41 - 60	61 - 85	
СУРТС	38	17	3	29	15	8	55
FVPTC	12	4	3	4	6	3	16
Oncocytic PTC	1	0	0	1	0	0	1
Micropappilary carcinoma	5	1	0	2	4	0	6
тсртс	0	1	0	1	0	0	1
МТС	3	5	0	5	3	0	8
FTC	2	1	1	2	0	0	3
Hurthle cell carcinoma	2	0	0	0	2	0	2
ATC	1	0	0	0	1	0	1
Nodular goiter	10	5	1	6	8	0	15
Total	74	34	8	50	39	11	108

Table 1. The Clinicopathological Characteristics of Patients with Thyroid Disorders Enrolled in this Study

Abbreviations: ATC, anaplastic thyroid cancer; CVPTC, classic variant of papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid cancer; FTC, follicular thyroid carcinoma; MTC, medullary thyroid carcinoma; PTC, papillary thyroid carcinoma; TCPTC, tall cell papillary thyroid carcinoma.

and primers 5'- CAAAAGCATGTGGAGTGAGG-3' and 5'-CACYTTATAATGGTGCTCTGG-3', for the second round of the assay.

The reaction mixture for the primary PCR assay consisted of 5  $\mu$ L of extracted DNA in a total volume of 50  $\mu$ L with final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs, 2.0 U AmpliTaq DNA polymerase, and a 0.7  $\mu$ M of each primer of the outer primer sets. The reaction was performed under the following conditions: denaturation at 94°C for 3 minutes; amplification for 35 cycles at 94°C for 1 minute, 53°C for 45 seconds, and 72°C for 45 seconds; and extension at 72°C for 5 minutes. After the first-round of PCR assay, 1  $\mu$ L of the first-run PCR product was used as a template for the second round of PCR assay, which yielded a 106 bp product. The 106 bp nested-PCR amplicons were visualized using a UV transilluminator after running on 2% agarose gel electrophoresis. All the reactions were performed in duplicate and parallel with positive and negative controls.

## 3.5. DNA Sequencing

To confirm the reliability of the PCR-RFLP results, the amplified products were purified with the PCR purification kit (Qiagen, Germany), according to manufacturer's instruction and sequenced using ABI 3730XL DNA analyzer (Bioneer Sequencing Service, South Korea).

#### 3.6. Statistical Evaluation

Statistical analysis was performed using SPSS22 (SPSS Inc., Chicago, IL, USA). Categorical variables were plotted into contingency tables and evaluated using Fisher's exact test and Mann-Whitney U test. All reported P values are twosided and P < 0.05 was considered to be statistically significant.

# 4. Results

We examined 108 FFPE tissue samples from patients with thyroid disorders. Samples were collected from 74 (68.5%) women and 34 (31.5%) men. Their mean age was 41 years (range, 11 - 84 years). Fifty-five (51%) of the patients were classified as histological subtype classic PTC and 16 out of 108 (14.8%) patients were diagnosed as follicular PTC variants, respectively, which clearly formed the majority of the cases (Table 2). Overall, the BRAF<sup>V600E</sup> mutation was observed in 77 out of 93 thyroid cancer patients (82.8%) using PCR-RFLP and confirmed by DNA sequencing analysis. Of those, 46 (49.4%) were patients with classic PTC and 13 (14%) with follicular PTC, respectively. The remaining were diagnosed with other thyroid disorders (Table 2). No BRAF<sup>V600E</sup> mutation was detected in 15 nodular goiter tissue samples (Table 2). Mann-Whitney U test analysis showed a significant difference in the prevalence of BRAF<sup>V600E</sup> mutation in thyroid cancer patients in comparison with the control group (P < 0.001).

Using nested-PCR, human parvovirus B19 DNA was detected in 14 out of 108 (13%) of FFPE tissue samples (Table 2). However, there was no statistically significant difference in the frequency of B19V infection amongst patients and the control group (P = 0.4).

In addition, no significant association between the prevalence of B19V infection and  $BRAF^{V600E}$  gene mutation

Histology Type		B19V Infection					
	Muta	nt BRAF	Wild-T	_			
	Positive	Negative	Positive	Negative	-		
СVРТС	6	40	1	8	55		
FVPTC	2	11	1	2	16		
Oncocytic PTC	0	1	0	0	1		
Micropappilary carcinoma	2	2	0	2	6		
тсртс	0	1	0	0	1		
МТС	0	7	0	1	8		
FTC	0	2	0	1	3		
Hurthle cell carcinoma	0	2	0	0	2		
ATC	0	1	0	0	1		
Nodular goiter	0	0	2	13	15		
Total	10	67	4	27	108		

Table 2. The Molecular Frequencies of BRAF<sup>V600E</sup> Mutation and B19V Infection in Various Subtypes of Thyroid Disorders

Abbreviations: ATC, anaplastic thyroid cancer; CVPTC, classic variant of papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid cancer; FTC, follicular thyroid carcinoma; MTC, medullary thyroid carcinoma; PTC, papillary thyroid carcinoma; TCPTC, tall cell papillary thyroid carcinoma.

was detected.

#### 5. Discussion

A number of studies have shown that viruses play a significant role in the development of human cancers (15, 16). However, there is ongoing research on a number of viruses, known to have a persistent infection, such as B19V to understand its role in the development of human cancers (17-22). Recently, the B19V infection was implicated in thyroid cancer, which is the most common inflammation of the endocrine system (23). The presence of specific receptors for B19V and the development of persistent infection by the virus in thyroid tissue cells might increase the likelihood of the virus involvement. In the present study, we investigated the prevalence of human parvovirus B19 DNA with nested PCR in patients with thyroid cancers.

Initially, by using PCR-RLFP, the prevalence of BRAF<sup>V600E</sup> mutation amongst 108 patients with thyroid cancers was determined. BRAF<sup>V600E</sup> mutation was detected in 77 out of 93 (82.8%) of the patients, and the results were confirmed by DNA sequencing. All the mutant cases were considered heterozygous for BRAF<sup>V600E</sup>. Overall, the rate of BRAF<sup>V600E</sup> mutation in PTC patients was significantly higher in comparison with other types of thyroid cancers. BRAF<sup>V600E</sup> mutant was not detected in FFPE thyroid tissue samples from patients with goiter disease. In another study, by using direct DNA sequencing, the prevalence of BRAF<sup>V600E</sup> mutations in papillary thyroid cancer was reported at 77.8% in Iranian patients (24).

In contrast with other reports on the high prevalence of human parvovirus B19 DNA in thyroid tissues from patients with thyroid cancer (25), in our study, by using nested PCR, parvovirus B19 DNA was detected in only 12 out of 93 (12.9%) of FFPE cancerous tissue specimens. This inconsistency between our study and other reports can be due to the DNA extraction procedure, the sensitivity of the primers used, or the age of FFPE samples. False positive results by PCR products contamination could be another explanation.

Interestingly, B19V DNA was also detected in two out of 15 (13.3%) patients with goiter disease. These results indicate that there are no significant differences between the presence of B19V DNA in malignant and non-malignant tissues samples.

We were also not able to find any correlation between infection with human parvovirus and thyroid cancer. Our results also indicated no association between the B19V infection and papillary thyroid carcinoma with the BRAF<sup>V600E</sup> mutation.

In conclusion, due to high prevalence of the BRAF<sup>V600E</sup> gene mutation amongst Iranians with thyroid cancer, it might be suggested that molecular approaches, to detect the BRAF<sup>V600E</sup> mutation, can be considered as a feasible assay when cytological examinations, based on fine needle aspiration biopsy of the thyroid (FNAB), cannot be definitive either due to insufficient material or indefinite morphological criteria for preoperative diagnosis of thyroid nodules. However, the low detection of B19V DNA in FFPE tissue samples by PCR assay suggests that B19V infection is

not associated with thyroid cancer. Therefore, to further confirm this result, more experiments such as immunohis-tochemistry assay and real-time PCR are recommended.

# Acknowledgments

The authors would like to thank Mr. H. Argasi at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his invaluable assistance in editing this manuscript.

### Footnotes

**Conflict of Interests:** The authors declare no conflict of interest.

**Ethical Approval:** The study was approved by the local Ethics Committee of SUMS.

**Funding/Support:** The project was financially supported by the Shiraz University of Medical Sciences, Shiraz, Iran under the agreement No. 94-01-45-9326.

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