

# Differential expression of two activating transcription factor 5 isoforms in papillary thyroid carcinoma

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**Background:** Activating transcription factor 5 (*ATF5*) is a member of the activating transcription/cAMP response element-binding protein family of basic leucine zipper proteins that plays an important role in cell survival, differentiation, proliferation, and apoptosis. The *ATF5* gene generates two transcripts: *ATF5* isoform 1 and *ATF5* isoform 2. A number of studies indicate that *ATF5* could be an attractive target for therapeutic intervention in several tumor types; however, so far, the role of *ATF5* has not been investigated in papillary thyroid carcinoma (PTC).

**Methods:** Quantitative real-time reverse transcription polymerase chain reaction and immunohistochemical staining were used to study *ATF5* mRNA and protein expression in PTC.

**Results:** We report here that *ATF5* is expressed more in PTC tissue than in normal thyroid tissue. Furthermore, this is the first study that describes the presence of both *ATF5* isoforms in PTC.

**Conclusion:** These findings could provide potential applications in PTC cancer treatment.

**Keywords:** papillary thyroid carcinoma, *ATF5*, therapeutic target, qRT-PCR, IHC

## Background

Thyroid cancer is the most common type of endocrine malignancy. Four thyroid tumor types have been identified based on their histopathological features: papillary (PTC), follicular (FTC), anaplastic (ATC), and medullary thyroid carcinomas (MTC).

PTC and FTC are well-differentiated cancers. Among them, PTC is the most common histotype and accounts for more than 80%–85% of all thyroid malignant neoplasms, whereas FTC accounts for 10%–15% of cases. Another 5% is accounted for by MTC, a neuroendocrine tumor, and the remaining 1% by ATC.<sup>1</sup>

Although the majority of PTC show an indolent behavior, relapses are seen in many patients. Therefore, it is important to identify new targets for PTC treatment.<sup>2,3</sup> Several studies indicate that activating transcription factor 5 (*ATF5*, also known as *ATFx* and *ATF7*), a transcription factor of the ATF/CREB<sup>4</sup> regulates differentiation,<sup>5–7</sup> cell proliferation, and survival.<sup>8–10</sup>

Recently, we demonstrated that *ATF5* plays a potential role in osteogenic differentiation.<sup>11</sup> Previous studies showed that *ATF5* supports cell survival by regulating the expression of antiapoptotic proteins MCL-1<sup>12</sup> in glioblastoma and BCL-2<sup>13</sup> in epithelial ovarian cancer.

Moreover, Liu et al<sup>14</sup> demonstrated that Egr-1 gene was a downstream target of *ATF5* that mediates *ATF5*-dependent cell proliferation, tumorigenic transformation, and survival.

In addition, Ishihara et al<sup>15</sup> reported that *ATF5* was one of the key molecules involved in the development of oncogenic resistance to radiotherapy, enhancing both the survival ability and malignant potential of lung cancer cells.

Therefore, *ATF5* inhibition could be an attractive target for cancer therapy.<sup>15</sup> In particular, *ATF5* may be an effective method to enhance radiosensitivity in cancer cells and prevent the recurrence and progression of cancer after radiotherapy.

*ATF5* is highly expressed in several cancer types, including breast, lung,<sup>16</sup> glioma,<sup>17</sup> B-cell chronic lymphocytic leukemia,<sup>18</sup> rectal,<sup>19</sup> and ovarian cancer,<sup>13</sup> whereas it is not detectable in most normal human tissues (except the liver, where *ATF5* is highly expressed).<sup>20</sup>

Recent evidence suggests that *ATF5* is overexpressed in FTC.<sup>21</sup> In particular, Barden et al<sup>21</sup> identified the differences in expression levels of several genes, including *ATF5* in follicular thyroid adenomas and FTCs.

Despite this, however, the role of *ATF5* in PTC, MTC, and ATC has not yet been investigated. In addition, the *ATF5* gene generates two transcripts: *ATF5* isoform 1 (*ATF5*, transcript variant 1: NM\_012068.5) and *ATF5* isoform 2 (*ATF5*, transcript variant 2: NM\_001193646.1), which encodes the same single 30 kDa protein.<sup>22</sup> These isoforms are identical in their coding regions, but differ in the 5' untranslated region (UTR), designated as *ATF5*-5' UTR $\alpha$  and *ATF5*-5' UTR $\beta$ . The significance of these two transcripts is presently not known and has not yet been investigated in cancer.

Recent evidence suggests that 5' UTR $\alpha$  reduced the stability of *ATF5* mRNA. Repression of 5' UTR $\alpha$  is released

by amino acid limitation or NaAsO<sub>2</sub> exposure via eIF2 $\alpha$  phosphorylation. On the other hand, 5' UTR $\beta$  is insensitive to stress conditions.<sup>23–25</sup>

In the present study, *ATF5* mRNA and protein expression were analyzed using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) in PTC and nonneoplastic thyroid tissue.

Moreover, we included some cases of ATC in our study. We clearly demonstrated that *ATF5* was expressed more in PTC patients than in normal thyroid tissue, thus suggesting that *ATF5* could represent a suitable tool to treat PTC.

## Methods

### Samples

Fourteen cases of PTC were retrieved from the archives of the Pathology Unit at the Mediterranean Institute of Oncology. Of these, frozen and nonneoplastic thyroid tissue was available for eleven patients. Histopathological data are summarized in Table 1.

In addition, two cases of ATC were evaluated using IHC, and one by qRT-PCR. This study was approved by the Institutional Review Board of Mediterranean Institute of Oncology, and written informed consent was obtained from all patients.

### Total RNA extraction and reverse transcription

Total RNA was extracted from eleven normal and PTC frozen tissues and one case of ATC using the RNeasy Mini Isolation Kit (Qiagen, Valencia, CA, USA). RNA purity was assessed by measuring the ratio of the absorbance at 260 and

**Table 1** Patient characteristics

Age, years	Sex	Specimen type	T	N	M	Stage	Histological subtype	ATF5 IHC score in tumor	ATF5 IHC score in normal tissue
53	Female	Thyroid	I	0	0	I	PTC	2B	0
35	Female	Thyroid	I	0	0	I	PTC	0	1B
48	Female	Thyroid	I	0	0	I	PTC	3B	0
58	Male	Thyroid	I (m)	0	0	I	PTC	2B	0
54	Male	Thyroid	3	1a	0	III	PTC	2B	0
27	Male	Thyroid	3	I	0	I	PTC	3B	0
52	Female	Thyroid	I	0	0	I	PTC	4C	3B
80	Female	Thyroid	3	0	0	III	PTC	3B	2A
40	Female	Thyroid	2	I	0	I	PTC	3B	2C
59	Female	Thyroid	3	0	0	III	PTC	2B	2B
74	Female	Thyroid	3	0	0	III	PTC	2B	1B
31	Male	Thyroid	I	I	0	I	PTC	2A	2A
61	Female	Thyroid	I	0	0	I	PTC	4B	4A
33	Female	Thyroid	3	I	0	I	PTC	2B	2B

**Notes:** The table includes details of 14 cases of papillary thyroid carcinoma in different stages; there were 4 males and 10 females. Age range of patients was 27–80 years, with a mean age of 50 years.

**Abbreviations:** ATF5, activating transcription factor 5; IHC, immunohistochemistry; PTC, papillary thyroid carcinoma; T, tumor; N, lymph node; M, metastasis; m, multifocal.

280 nm, considering 1.8–2 as admissible range of ratios for pure RNAs. RNA quality was analyzed using Agilent 2100 Bioanalyzer RNA assays (Agilent Technologies, Santa Clara, CA, USA) and evaluated by calculating the ratio of the 28S and 18S ribosomal RNA intensity peaks. Total RNA was stored at  $-80^{\circ}\text{C}$ .

RNA samples (1  $\mu\text{g}$ ) were reverse transcribed using the High-Capacity cDNA Reverse Transcription (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Reverse transcription products were stored at  $-20^{\circ}\text{C}$ .

## Real-time RT-PCR

qRT-PCR was performed using EUR<sub>x</sub><sup>®</sup> SG qPCR Master Mix according to the manufacturer's protocol on a HT7900 instrument (Applied Biosystems). Primers were designed using Primer BLAST<sup>26</sup> and specifically recognize selected mRNAs by targeting exon–exon junctions (Table 2). *ATF5* transcriptional isoforms are differentially amplified according to dissimilarities in 5' UTRs. Specificity of the amplification products was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during PCR. Each sample was tested in triplicate, and samples were used for analysis of relative gene expression using the  $2^{-\Delta\Delta\text{CT}}$  method. Reported  $\Delta\text{CT}$  values are inversely proportional to target mRNA concentration.

## Immunohistochemistry

*ATF5* expression was analyzed using IHC on 14 PTC formalin-fixed paraffin-embedded specimens and adjacent normal thyroid tissues and two ATCs. Paraffin blocks were cut as 3- $\mu\text{m}$  sections. Tissue sections were dewaxed with xylene and rehydrated in descending concentrations of ethanol (100% and then 75%). Antigen retrieval was performed with a pH 8.0 EDTA solution at  $95^{\circ}\text{C}$  for 40 minutes

in a Decloaking Chamber (Biocare Medical, Walnut Creek, CA, USA). After rinsing briefly in phosphate-buffered saline (PBS 1 $\times$ ), sections were incubated for 10 minutes with 3%  $\text{H}_2\text{O}_2$  at room temperature in the dark and washed again.

Afterward, the sections were permeabilized with Triton 0.4% for 30 minutes. Subsequently, the nonspecific binding of the antibodies was blocked with 4% blocking serum (bovine serum albumin; Sigma Aldrich Co., St Louis, MO, USA) for 30 minutes. The sections were next stained with a rabbit anti-*ATF5* antiserum (Novus Biologicals, Littleton, CO, USA) at a ratio of 1:500 in a humidified chamber overnight at  $4^{\circ}\text{C}$ .

The following day, sections were washed three times (5 minutes each) in PBS 1 $\times$ .

Visualization was achieved by exposing the sections to Envision + Dual Link System-HRP (Dako Denmark A/S, Glostrup, Denmark) for 30 minutes at room temperature. The sections were then incubated with diaminobenzidine solution and rinsed with PBS 1 $\times$ . The slides were counterstained with Harris's hematoxylin for 1 minute at room temperature and, after dehydration, sealed with a drop of mounting medium.

Interpretation of staining was done using a semiquantitative system. The percentage of stained cells was assessed as follows: 1=1%–25%; 2=26%–50%; 3=>51%–75%; 4=76%–100%, whereas staining intensity was scored as follows: A = low; B = mid; C = strong (Table 1).

## Statistical analyses

Differences in *ATF5* transcripts expression between normal and tumor samples were assessed using paired Student's *t*-test. *ATF5* staining has been evaluated comparing both the percentage of *ATF5*-positive cells and the intensity of the signal in tumor tissues and adjacent area using Fisher's exact test. Comparisons resulting in *P*-values  $<0.05$  were reported as statistically significant. Statistical calculation was performed using the software R (version 3.2.1, Lucent Technologies, Murray Hill, NJ, USA).<sup>27</sup>

**Table 2** List of primers used in real-time PCR analysis

Target transcript	GenBank ID	Primer sequence	Temp Fwd/Rev ( $^{\circ}\text{C}$ )	Prod size (bp)
<i>ATF5</i> transcript variant 1	NM_012068.5	Fwd: CAGGAAATTCTGCAAGCAAGGAA Rev: CGGCGACACTCTTCCCTCTG	60/62	220
<i>ATF5</i> transcript variant 2	NM_001193646.1	Fwd: TGTCCTCGGATCACAGTCTCT Rev: AAGTGGGAAGACTCCATGGCTG	60/60	201
GAPDH	NM_002046.5	Fwd: GCTCTCCAGAACATCATCCCTGCC Rev: GCGTTGTCATACCAGGAAATGAGCTT	65/63	347
TUBB	NM_178014.3	Fwd: GCGCATTCCAACCTTCCAG Rev: CCCAGAACTTGGCACCGAT	59/60	166

**Abbreviations:** *ATF5*, activating transcription factor 5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction; TUBB, tubulin beta chain; Prod, product; bp, base pair; Fwd, forward; Rev, reverse.

## Results

### ATF5 expression in PTC

mRNA levels of *ATF5* isoforms 1 and 2 were examined by real-time PCR analysis on eleven PTC and adjacent normal samples using the primers listed in Table 2.

Our results indicate that *ATF5* isoform 1 mRNA level was significantly higher ( $P=0.0477$ ) in PTC when compared to adjacent normal tissue ( $\Delta$ CT medians: 3.3 for tumor and 3.9 for normal); *ATF5* isoform 2 was not statistically significant ( $\Delta$ CT medians: 3.3 for tumor and 3.5 for normal). Also, there was a statistically significant difference ( $P=0.046$ ) when both isoforms were considered together ( $\Delta$ CT medians: 6.6 for tumor and 7.3 for normal). These data are shown in Figure 1.

Such findings suggest that *ATF5* is expressed more in PTC patients than in normal samples, and hence this could be an attractive therapeutic target for PTC cancer treatment.

### ATF5 expression in ATC

mRNA levels of *ATF5* isoforms 1 and 2 were also examined by real-time PCR analysis on one ATC and adjacent normal samples. The results showed an increased expression of

both isoforms in ATC patients than in normal sample (data not shown).

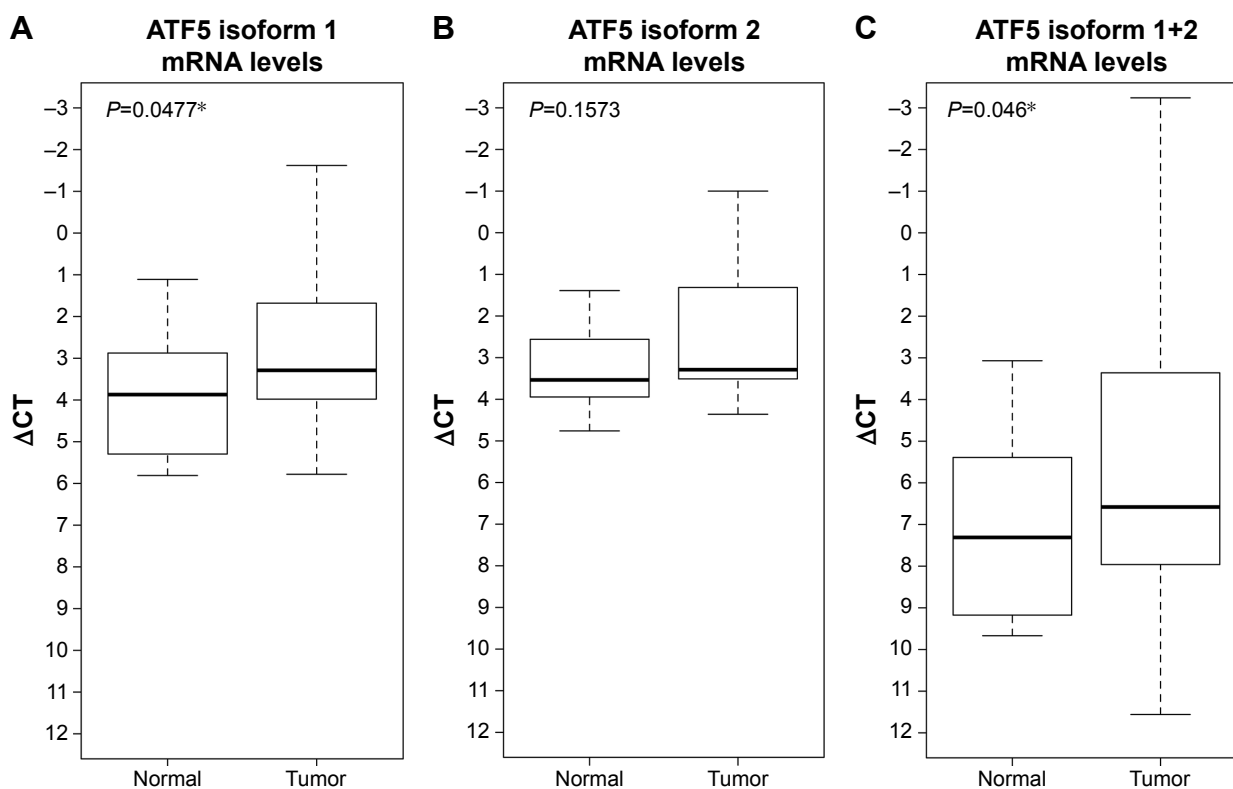
### IHC ATF5 expression

To confirm these data, we performed an anti-ATF5 immunohistochemical analysis on 14 PTC specimens. We found higher ATF5 expression in PTC when compared to adjacent normal thyroid tissue (Figure 2), confirming the data obtained from real-time PCR analysis.

Representative staining of ATF5 protein expression in normal and PTC tissues is shown in Figure 3. Afterward, we performed an anti-ATF5 immunohistochemical analysis on two ATC specimens. Interestingly, we observed a strong expression of the protein (data not shown).

## Discussion

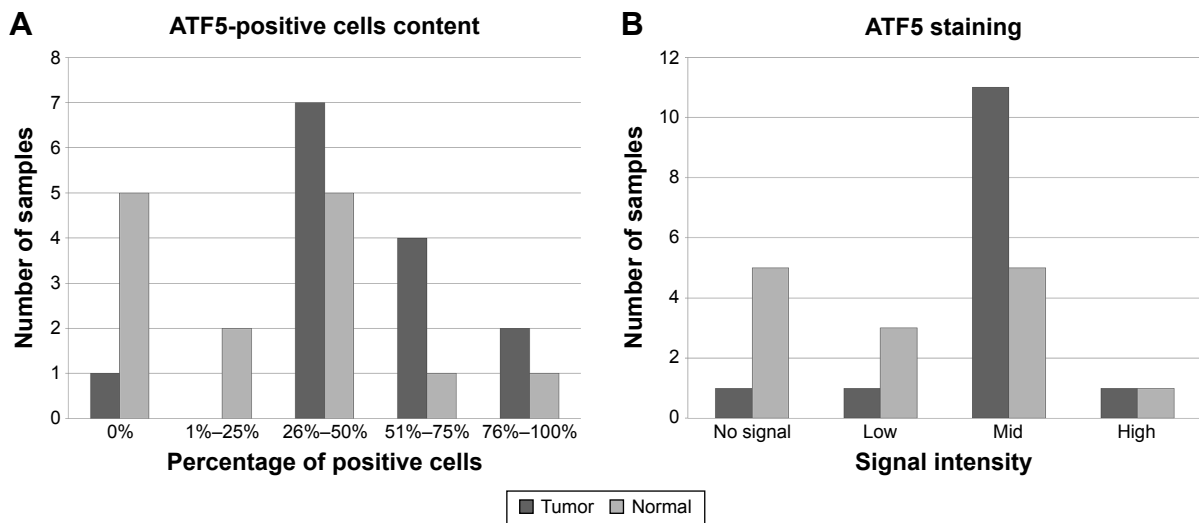
*ATF5* is a stress response transcription factor whose expression is regulated by posttranscriptional, translational, and posttranslational regulation in response to cellular stresses. Although studies have reported that *ATF5* is involved in the progression of several tumor types, its expression in PTC has never been studied. Our results are in line with previous data,



**Figure 1** ATF5 isoforms mRNA levels in PTC and adjacent normal sample by qRT-PCR.

**Notes:**  $\Delta$ CT measurements for (A) ATF5 isoform 1 (medians: 3.3 for tumor and 3.9 for normal), (B) ATF5 isoform 2 (medians: 3.3 for tumor and 3.5 for normal), and (C) both isoforms (medians: 6.6 for tumor and 7.3 for normal) in eleven PTC sample and adjacent normal sample. \*indicates  $P<0.01$ .

**Abbreviations:** ATF5, Activating transcription factor 5; PTC, papillary thyroid carcinoma; qRT-PCR, quantitative reverse transcription polymerase chain reaction.



**Figure 2** Increased ATF5 expression in immunohistochemical analyses performed on PTC patients.

**Notes:** Immunohistochemical analysis of ATF5 expression in 14 formalin-fixed paraffin-embedded specimens from PTC patients and adjacent normal tissue. Differential immunohistochemical expression of ATF5 in normal and tumor tissue. (A) indicates the percentage of positive cells, while in (B), the staining intensity.

**Abbreviations:** ATF5, activating transcription factor 5; PTC, papillary thyroid carcinoma.

which reports that in general, the expression level of *ATF5* is significantly higher in malignant tissues than their normal counterparts. The only exception appears to be hepatocellular carcinoma cells, which express lower levels of *ATF5* than normal liver cells.<sup>20</sup>

Furthermore, several studies have reported that high *ATF5* expression levels may correlate with poor prognosis in cancer patients.<sup>12</sup> In our study, however, there was no significant association between *ATF5* expression and size of the tumor, local extension, lymph node involvement, or distant metastasis.

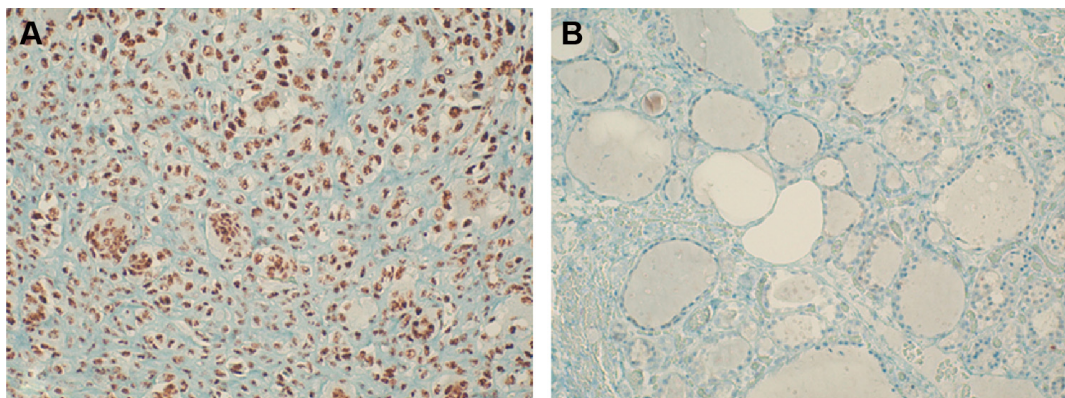
Interestingly, we observed a strong expression of the protein in some ATC included in our study. ATC is an undifferentiated carcinoma characterized by an aggressive behavior

and a poor prognosis. Further analysis with real-time PCR confirmed an increased expression of both isoforms.

Moreover, more data on ATC would be necessary to confirm our data in a larger cohort of patients.

The main biological function of *ATF5* is related to apoptosis, and several studies have been conducted so far to investigate the role of *ATF5* as a possible therapeutic target. Interference with *ATF5* function or expression in glioma cells and breast cancer cell lines caused marked apoptotic cell death.<sup>16,28</sup> Moreover, loss of function of *ATF5* in conjunction with paclitaxel treatment elicited apoptosis of pancreatic carcinoma cells.<sup>29</sup>

In addition, Karpel-Massler et al,<sup>30</sup> demonstrated that a dominant-negative *ATF5* peptide induces apoptosis in glioblastoma, triple-negative breast cancer (MDA-MB-436),



**Figure 3** Immunohistochemical expression of ATF5 in PTC.

**Notes:** Immunohistochemical expression of ATF5 in papillary thyroid carcinoma (A), 20 $\times$ , and adjacent normal thyroid tissue (B), 20 $\times$ .

**Abbreviations:** ATF5, activating transcription factor 5; PTC, papillary thyroid carcinoma.



hormone-refractory prostate cancer (PC3 and DU145), resistant non-small-cell lung cancer (H1975), BRAF (V600E)-mutated melanoma (A375), and pancreatic carcinoma (PANC-1). This dominant-negative *ATF5* also showed in vivo efficacy in reducing the growth of a range of tumor types in xenograft models.

To our knowledge, this study provides the first evaluation of *ATF5* expression and identification of its two isoforms in PTC and normal tissue. Targeting of *ATF5* could potentially become a viable therapeutic approach for patients diagnosed with PTC.

## Conclusion

In conclusion, our data revealed that *ATF5* mRNA expression and protein was higher in PTC compared to normal thyroid tissue. Our results unambiguously demonstrate the existence of both *ATF5* isoforms in PTC.

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## Author contributions

LV designed the study, analyzed the results, and wrote the manuscript. CLR performed IHC and contributed to data analysis. SF and GC performed real-time polymerase chain reaction experiments and contributed to data analysis. SS provided the surgical samples and clinical data. EA performed IHC. LM and CC analyzed IHC and critically revised the manuscript. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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