

## RESEARCH

# Expression of NOTCH1 in thyroid cancer is mostly restricted to papillary carcinoma

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## Abstract

The NOTCH signaling is an evolutionarily conserved signaling pathway that regulates cell–cell interactions. NOTCH family members play a fundamental role in a variety of processes during development in particular in cell fate decisions. As other crucial factors during embryogenesis, NOTCH signaling is aberrantly reactivated in cancer where it has been linked to context-dependent effects. In thyroid cancer, NOTCH1 expression has been associated to aggressive features even if its *in vivo* expression within the entire spectrum of thyroid tumors has not definitively established. A series of 106 thyroid specimens including non-neoplastic lesions, benign and malignant tumors of common and rare histotypes, were investigated by immunohistochemistry to assess NOTCH1 expression. Extent of positivity and protein localization were investigated and correlated with clinical and morphological parameters. NOTCH1 positivity was predominantly associated with papillary carcinomas and only occasionally found in follicular carcinomas. Poorly differentiated and undifferentiated thyroid carcinomas showed only a partial positivity. NOTCH1 expression pattern also seemed differently distributed according to histotype. Our data confirm a role of NOTCH1 in thyroid cancer and highlight for the first time the specific involvement of this pathway in papillary carcinomas. Our data also indicate that other thyroid malignancies do not rely on NOTCH1 signaling for development and progression.

## Key Words

- ▶ NOTCH1
- ▶ thyroid
- ▶ papillary carcinoma
- ▶ immunohistochemistry

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## Introduction

NOTCH receptors belong to a highly conserved signaling pathway. Relying on cell–cell contacts, receptors belonging to this family sense cues from the surrounding microenvironment inducing intracellular responses.

NOTCH signaling regulates many fundamental processes during development and homeostasis of a wide range of tissues. As a consequence, deregulation of this pathway has been linked to multiple human disorders, from congenital syndromes to cancer (1, 2, 3). NOTCH signaling is considered a potential target in several hematological malignancies (4) and carcinomas (5) and has a potential role in reducing tumor angiogenesis (6, 7).

Noticeably, integration of the large amount of genetic and functional data lately generated on the function of NOTCH signaling in cancer suggests a broad, often conflictual and context-dependent function (8, 9, 10, 11). Indeed, functional studies implicate NOTCH signaling as essential for many if not all hallmarks of cancer and clearly point to roles that range from oncogenic to tumor suppressive, depending on cancer type (12).

NOTCH receptors are large transmembrane proteins and NOTCH receptor activation is mediated by a sequence of proteolytic events, which cause the release of the NOTCH intracellular domain, translocation to

the nucleus and association with a DNA-bound protein (13). There are different expression patterns of the ligand and the receptors, explaining differences in signaling activity and post translational modifications, but there is a growing body of data demonstrating that NOTCH signaling is more complex than originally supposed and that its apparently contradictory role as oncogene or tumor suppressor has to be clarified yet (14).

NOTCH1 is the prototype and by far the most studied member of this family of receptors. NOTCH1 as other members of this family has been involved in the development of thyroid cancer even if with controversial role. On one side the activation of NOTCH signaling mediates growth suppression by cell cycle arrest in well-differentiated thyroid carcinomas (WDTCs) (15, 16). On the other side, NOTCH1 has been described highly expressed in WDTC with *BRAF*, *RET*/papillary thyroid carcinoma (PTC) mutation or active MAPK signaling. In this context, activated NOTCH1 signaling promotes tumor growth (17, 18). Noticeably, Kim *et al.* postulated that NOTCH signaling influences the malignant potential of thyroid cancer and observed a NOTCH1-dependent reduction of epithelial-mesenchymal transition (EMT) and cancer stem cells markers in anaplastic tumors (19). In parallel, the expression of NOTCH1 receptor seems to be related to tumor invasiveness in PTC, and it has been proposed as molecular marker of poor prognosis (20, 21). In line with this reports, we recently reported that NOTCH1 cooperates with the transcription factor DEC1 in controlling aggressiveness of PTCs (22).

Despite the involvement of NOTCH1 in thyroid carcinogenesis, still many issues remain to be addressed. In particular, the expression profile of this protein within the spectrum of thyroid tumors, benign and malignant, follicular-derived and medullary, has never been consistently investigated (23). Thus, in this work, we aimed at analyzing by immunohistochemistry the expression of NOTCH1 in a large series of thyroid neoplasms with distinct histogenesis and very different biological behavior.

## Materials and methods

### Case selection

The study was conducted by the Pathology Unit of the Arcispedale Santa Maria Nuova-IRCCS in Reggio Emilia, Italy, and was approved by the Local Ethics Committee (namely, Comitato Etico di Area Vasta Emilia Nord, protocol Rf-2016-02364167, approved 29th June 2017)

that authorized the use of anonymized archive tissue. One hundred six surgically excised thyroid samples were selected from the archive of the Pathology Unit and re-evaluated according to the current 2017 WHO classification (24). Clinicopathological information are summarized in Table 1 and Supplementary Table 1 (see section on supplementary data given at the end of this article).

### Immunostaining

Immunohistochemistry was performed on a representative 4µm formalin-fixed, paraffin-embedded section from each case, as previously described (25), including both neoplastic and normal thyroid tissues.

Rabbit polyclonal NOTCH1 antibody (Cell Signaling, clone D1E11, code #3608) and monoclonal Ki67

**Table 1** Clinical and pathologic features of the patients (N = 106).

	N (%)
Age at diagnosis, years (mean, 95% CI)	54 (19–85)
Gender	
Females	67 (63.2)
Males	39 (36.8)
Histological diagnosis	
NH	2 (1.9)
FA	5 (4.7)
HA	5 (4.7)
HTT	6 (5.7)
PTC	48 (45.3)
FTC	12 (11.3)
HCC	5 (4.7)
MTC	9 (8.5)
PDTC	6 (5.7)
ATC	8 (7.5)
Stage (AJCC)	
NA	19 (17.9)
I	61 (57.5)
II	12 (11.3)
III	0 (0)
IV	14 (13.3)
Metastases (lymph node + distant)	
Yes	28 (26.4)
No	60 (56.6)
NA	18 (17)
Number of metastases	
Single	13 (12.3)
Multiple	13 (12.3)
NA	80 (75.4)

ATC, anaplastic thyroid carcinoma; FA, follicular adenoma; FTC, follicular thyroid carcinoma; HA, Hürthle cell adenoma; HCC, Hürthle cell carcinoma; HTT, hyalinizing trabecular tumor; MTC, medullary thyroid carcinoma; NA, not available; NH, nodular hyperplasia; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma.

antibody (clone MIB1, Agilent) were used at 0.33 µg/mL and 46 mg/mL respectively, according to manufacturer's instructions. In a previous work we confirmed the NOTCH1 antibody specificity by performing Western blot analysis on thyroid cancer cell lines transfected with siRNA against NOTCH1 (22).

As positive controls, we stained samples from human colic mucosa and other epithelia, in accordance with the indications of the antibody distributors. As negative control, we used the normal thyroid tissue adjacent to the lesions examined, which was consistently negative in all cases. To further verify the specificity of the antibody, according to the Tumor Protein Atlas (<https://www.proteinatlas.org/ENSG00000148400-NOTCH1/tissue/ovary>), we additionally performed NOTCH1 immunostaining on three cases of normal ovarian tissue, three cases of peripheral nerves in traumatic neuroma, three cases of hyperplastic lymphoid tissue of the tonsil and three cases of normal pancreatic tissue, including both exocrine pancreas and islets of Langerhans. As expected, we did not observe any staining in the ovarian stroma (Supplementary Fig. 1A), peripheral nerve (Supplementary Fig. 1B), lymphoid germinal centers (while squamous epithelium was reactive, Supplementary Fig. 1C) and islets of Langerhans (on a background of positive exocrine pancreas, Supplementary Fig. 1D), thus confirming the specificity of the staining.

The tissue sections were pre-treated with heat antigen retrieval in EDTA buffer for 72'. The staining was developed on a Ventana Bench-Mark ULTRA platform using the OptiView DAB detection Kit (Ventana-Roche, Tucson, USA). Slides were counterstained with hematoxylin.

The entire section from each case was scored for signals quantification by two pathologists (SP and MR). The signal was defined as absent (neg, 0%), focal (+, 1–29%), moderate (++, 30–50%) and diffuse (+++, >50%).

Pattern of positivity was recorded as luminal (i.e. on the apical pole of the neoplastic cells), membranous and cytoplasmatic.

## Statistical analysis

Statistical analysis was performed using R Software v 3.5.1. Analysis of the frequency of NOTCH1 positivity was performed by contingency tables and significance was calculated by Fisher's exact test. Differences were considered statistically significant with a *P* value <0.05.

## Results

### Patients' features

One hundred six thyroid tumors, spanning the entire spectrum of thyroid lesions were analyzed (Table 1 and Supplementary Table 1). Sixty-three percent of patients were female (67 out of 106) and the average age was 54±15 years. Two samples were nodular hyperplasia (NH), five samples were follicular adenomas (FA), five were Hürthle cell adenoma (HA) and six were hyalinizing trabecular tumors (HTT). The remaining were malignant lesions. 68.1% were classical well-differentiated thyroid cancers, papillary thyroid carcinoma (PTC, *n*=48, 54.5%) and follicular thyroid carcinoma (FTC, *n*=12, 13.6%). 15.9% were scarcely differentiated tumors, including poorly differentiated thyroid carcinoma (PDTC, *n*=6, 6.8%) and anaplastic thyroid carcinoma (ATC, *n*=8, 9.1%). The remaining 31.9% of samples encompassed rare histological variants of thyroid cancer including Hürthle cell carcinomas (HCC, *n*=5, 5.7%) and medullary thyroid carcinoma (MTC, *n*=9, 10.2%). Twenty-eight malignant tumors had developed metastasis (26.4%) and 13 of these had multiple metastatic lesions (46.4%).

**Table 2** Results of NOTCH1 expression.

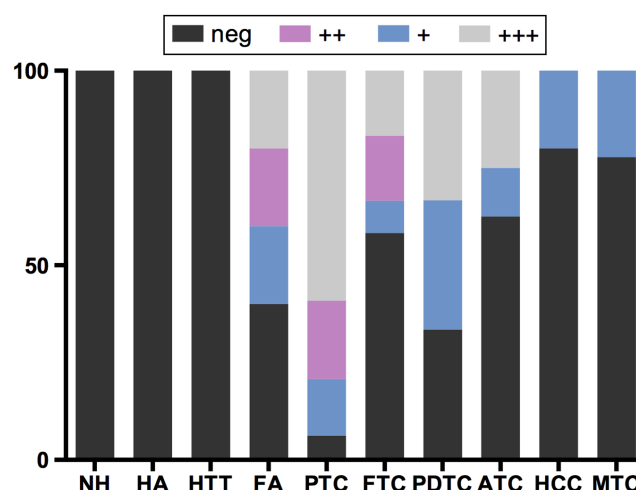
NOTCH1 immunostaining	Non-neoplastic/benign tumors (%)	Malignant tumors (%)	N (%)
Negative	15 (83.2)	28 (31.8)	43 (40.6)
+	1 (5.6)	14 (15.9)	15 (14.2)
++	1 (5.6)	3 (3.4)	4 (3.7)
+++	1 (5.6)	43 (48.9)	44 (41.5)
TOT	18 (100)	88 (100)	106 (100)
NOTCH1 pattern	Non-neoplastic/benign tumors (%)	Malignant tumors (%)	N (%)
Cytoplasmic	3 (100)	11 (18.3)	14 (22.2)
Luminal	0	48 (80)	48 (76.2)
Cytoplasmic + luminal	0	1 (1.7)	1 (1.6)
TOT-positive cases	3 (100)	60 (100)	63 (100)

Metastases were associated primarily with ATC ( $n=7$ ), PTC ( $n=9$ ) and MTC ( $n=3$ ).

### Immunohistochemical expression of NOTCH1

Tables 2 and 3 summarize the results of this analysis. Both positivity and pattern of expression were evaluated. NOTCH1 expression was significantly associated with malignant phenotype ( $P<0.0011$ ). Sixty-eight percent of malignant lesions (60 out of 88; 45 PTC, 5 FTC, 4 PDTC, 3 ATC, 2 MTC and 1 HCC) were positive for NOTCH1, while only 16.6% of positive non-malignant samples (3 out of 18) scored positive for this marker. NOTCH1 positivity in the non-malignant lesions were restricted to FA. 69.8% of positive lesions showed NOTCH1 expression in more than 50% of cancer cells (Supplementary Table 2).

Distribution of NOTCH1 positivity per histotype is reported in Fig. 1 and Table 3. NOTCH1 positivity was significantly associated with PTCs. 93.75% of PTCs tested were positive for NOTCH1 and 82.2% of these (37 out of 48) showed NOTCH1 positivity in over 50% of cancer cells (Fig. 1). NOTCH1 expression was also observed in 41.6% of FTCs (5 out of 12), 66.7% of PDTC (4 out of 6) and 37.5% of ATC (3 out of 8). By contrast NOTCH1 was limitedly expressed in MTC (two out of nine) and



**Figure 1** Distribution of NOTCH1 positivity in thyroid lesions. Histograms represent the percentage of negative and positive cells according to histotypes (+ = 1–29%, ++ = 30–50%, +++ > 50%). ATC, anaplastic thyroid carcinoma; FA, follicular adenoma; FTC, follicular thyroid carcinoma; HA, Hürthle cell adenoma; HCC, Hürthle cell carcinoma; HTT, hyalinizing trabecular tumor; MTC, medullary thyroid carcinoma; NH, nodular hyperplasia; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma.

HCC (one out of five) and in less than 10% of cells. As all the analyzed samples were surgical specimens, normal thyroid tissue was present in all cases and was consistently negative for NOTCH1 (Fig. 2).

Next, we focus our attention on localization of NOTCH1 positivity (Table 3). Strikingly, all positive PTCs showed luminal localization of NOTCH1 regardless of the sub-histotype (classic, tall cell, follicular) as the staining was stronger in the cell surface toward the lumen of the follicles or the papillae (Fig. 3). Luminal localization of NOTCH1 was largely associated to PTCs, while in the other histotype NOTCH1 was detected primarily in the cytoplasm.

Among the NOTCH1-positive samples, three PDTCs (75%) showed a granular cytoplasmic positivity (Fig. 4). In one PDTC, cytoplasmic granularity was thick and dot-like (Fig. 4D). Both NOTCH1-positive MTCs showed cytoplasmic positivity even if the staining was focal and limited to few neoplastic elements (Fig. A and B). Finally, positive FA, FTC and AC showed a delicate granular positivity in the cytoplasm (Fig. 5).

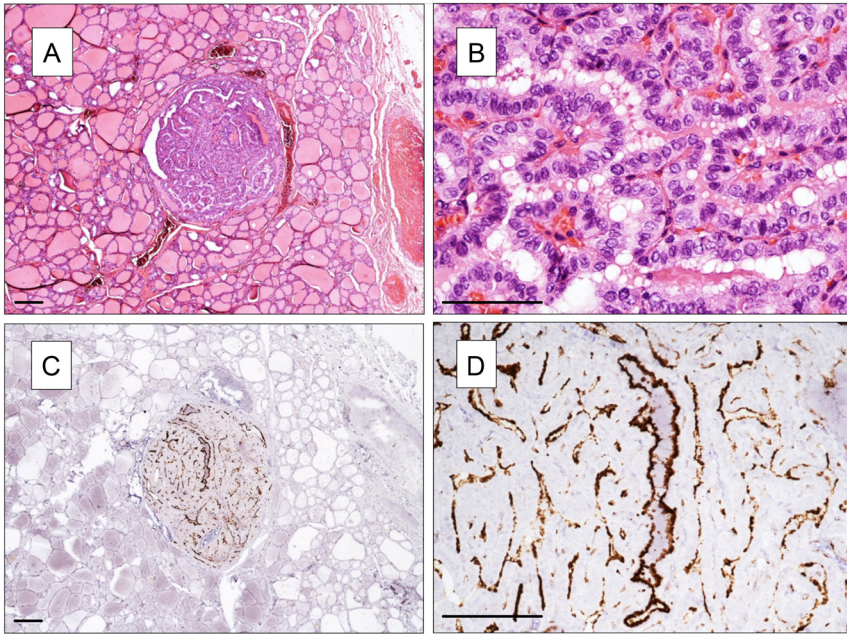
To explore whether high NOTCH1 expression could reflect higher proliferation rate of these tumors, we explored Ki67 expression in the 48 PTCs included in this analysis. In all samples, Ki67 positivity was limited (below 10% of cancer cells) independently to NOTCH1 positivity. This is not surprising, since it was already reported that Ki67 levels in WDTCs are low (26).

**Table 3** NOTCH1 positivity according to histotype.

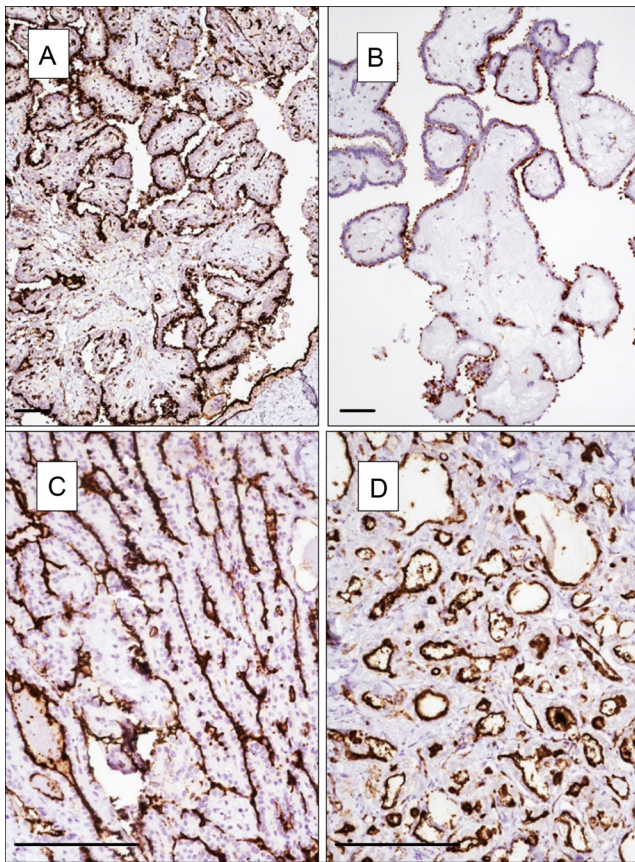
Positivity (TOT)	Pos (%)	Neg (%)
NH (2)	0 (0)	2 (100)
FA (5)	3 (60)	2 (40)
HA (5)	0 (0)	5 (100)
HTT (6)	0 (0)	6 (100)
PTC (48)	45 (93.75)	3 (6.25)
FTC (12)	5 (41.6)	7 (58.3)
HCC (5)	1 (20)	4 (80)
MTC (9)	2 (22.2)	7 (77.8)
PDTC (6)	4 (66.7)	2 (33.3)
ATC (8)	3 (37.5)	5 (62.5)
Pattern (TOT-NOTCH1+)	Cytoplasmic (%)	Luminal (%)
FA (3)	3 (100)	0 (0)
PTC (45)	0 (0)	45 (100)
FTC (5)	3 (60)	2 (40)
HCC (1)	1 (100)	0 (0)
MTC (2)	2 (100)	0 (0)
PDTC (4)	3 (75)	1 (25)
ATC (3)	3* (100)	0 (0)

\*One case of ATC shows both cytoplasmic and luminal positivity. ATC, anaplastic thyroid carcinoma; FA, follicular adenoma; FTC, follicular thyroid carcinoma; HA, Hürthle cell adenoma; HCC, Hürthle cell carcinoma; HTT, hyalinizing trabecular tumor; MTC, medullary thyroid carcinoma; NH, nodular hyperplasia; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma.

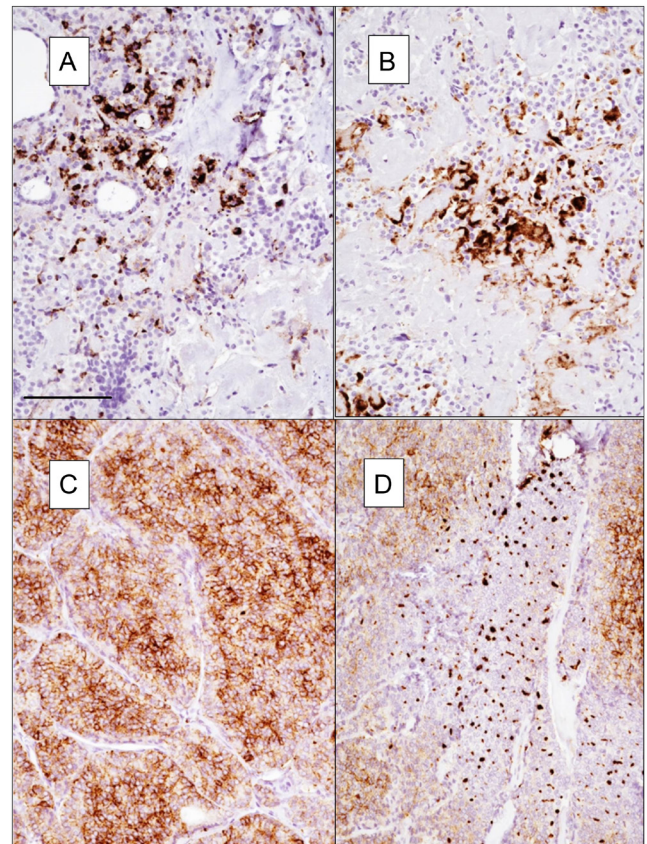




**Figure 2**  
NOTCH1 expression in a papillary microcarcinoma and in the normal surrounding thyroid tissue. (A and B) H&E staining (in B microcarcinoma at higher power view). (C and D) NOTCH1 staining. NOTCH1 positivity is restricted to microcarcinoma (in the center; in D at higher power view), while normal thyroid tissue is negative. (A, B and C) Magnification 40×, (B, C and D) 100×. Scale bar 100 μm.

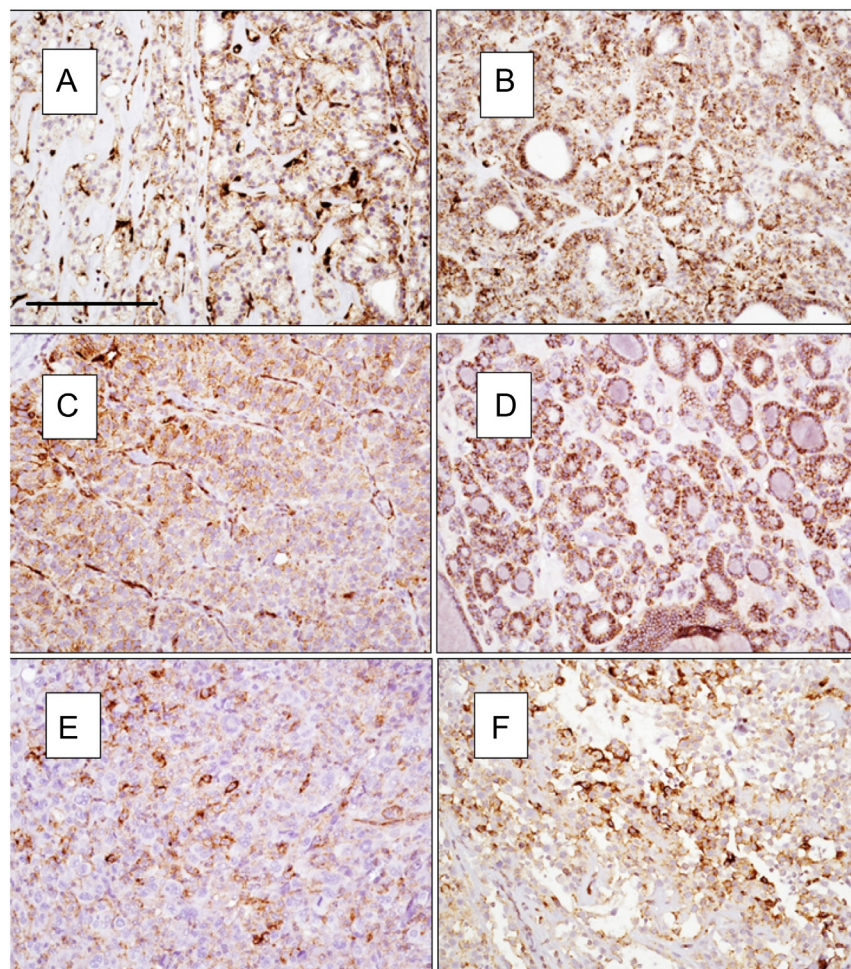


**Figure 3**  
Pattern of NOTCH1 positivity in PTC classic type (A and B), tall cell variant (C) and follicular variant (D). (A and B) Magnification 40×, (C and D) 100×. Scale bar 100 μm.



**Figure 4**  
Pattern of NOTCH1 expression in MTC (A and B) and PDTC (C and D). In PDTC cytoplasmic granularity is thick and dot-like. Magnification 100×. Scale bar 100 μm.



**Figure 5**

Pattern of NOTCH1 positivity in FA (A and B), FTC (C and D) and ATC (E and F). Magnification 100 $\times$ . Scale bar 100  $\mu$ m.

## Discussion

NOTCH signaling is an evolutionarily conserved intercellular pathway regulating interactions between adjacent cells. NOTCH1 is one of the four known members of this family. As the others, NOTCH1 protein is characterized by N-terminal EGF-like repeats followed by a Lin-12/NOTCH repeat (LNR) domains, specific of this family which prevents signaling activation. Once engaged by its ligand, NOTCH1 exposes a site of cleavage that allows ADAM17, and then  $\gamma$ -secretase cleavage that releases in the cytoplasm NICD, the activated form of NOTCH1. NICD is then translocated to the nucleus where, by the cooperation with transcriptional co-factors, controls gene expression (13). NOTCH1 signaling plays a crucial role during embryonic development being involved in cell fate commitment and differentiation (27). In cancer, NOTCH1 has been shown to play multiple and often controversial functions (28). This is evident also in the setting of thyroid cancer where, in spite of multiple

reports, a definitive consensus on NOTCH1 involvement is still missing.

Yu and colleagues have shown that NOTCH1 mRNA levels decrease in thyroid tumor cells as compared to normal thyroid and that patients with lower NOTCH1 expression had a significantly higher recurrence rate ( $P=0.038$ ) (21). By contrast, Kim *et al.* reported that the expression of activated NOTCH1 was higher in ATCs than in PTCs indicating a role of this pathway in aggressive evolution of thyroid cancer (19). According to these authors, inhibition of NOTCH1 significantly reduced proliferation and migration of ATC cells, but not PTC cells. In contrast with this observation several reports indicate a loss of NOTCH1 expression in ATCs (15, 29, 30), and we recently showed that NOTCH1 participates to the acquisition of aggressive features in PTC cells (22). Beside these controversies, no information on NOTCH1 expression was available in rare histological subtypes. Here, we showed that (1) NOTCH1 expression is restricted to tumor cells and significantly associated with malignant

phenotype in follicular-derived tumors since only 3 of the 18 benign lesions showed expression of this protein; (2) NOTCH1 expression is largely associated with PTC, while its expression in poorly differentiated tumors is less consistent; (3) rare variants including the C-cells-derived medullary carcinomas do not express NOTCH1 or have very low NOTCH1 levels and likely do not rely on this signaling for their progression. Due to the high rate of positivity observed in the PTC group, we did not notice significant association with clinical-pathologic features of these tumors. In this regard our data are in partial contradiction with the meta-analysis reported by Yuan *et al.* that reported a positive association of NOTCH1 expression in PTCs with lymph node metastasis, tumor size, capsular invasion and clinical stage (31).

Being NOTCH1 proteolytically processed and translocated into the nucleus upon activation, analysis of its localization in human tumors has been used to predict NOTCH1 pathway activation (32). In this regard our data indicate that in PTCs in which NOTCH1 is consistently and widely expressed, this protein is retained in the membrane. Noticeably NOTCH1 expression does not mark the entire membrane surface of thyrocytes, but it is restricted to the luminal surface of the papillae, even when their structure is partially compromised by the irregular neoplastic growth. Luminal expression of NOTCH1 is instead lost in poorly differentiated and undifferentiated lesions, likely as reflex of the cellular morphological changes imposed by the 'undifferentiation' process. In our analysis, we did not observe nuclear localization of NOTCH1, and in most of the non-PTC tumors, this protein seems to be localized in the cytoplasm with a granular pattern. We do not have an explanation regarding the biological meaning of this localization, but it is possible that it reflects retention of this protein in the reticulum, where it may be processed for degradation. If, as it is plausible, NOTCH1 cytoplasmic localization underscore a non-functional protein, thus our data indicate that NOTCH1 signaling, in the setting of thyroid carcinomas, operates exclusively in the PTC variants.

Even if further analyses are needed, our data add information on the potential function of NOTCH1 signaling on thyroid cancers and point to a specific role of this pathway on well-differentiated and most common variants of this tumors.

#### Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EC-19-0303>.

#### Declaration of interest

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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