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Wnt3a but not CDX-2 expression is associated with differentiated thyroid cancer

Gleyne Lopes Kujew Biagini[1*], Carmem Austrália Paredes Marcondes Ribas[1], Henrique Diez Higashi[1], Vanessa Yumi Hirata[1], Maria Augusta Karas Zella[1], Ivan Bartolomei[1], Giuliana Biagini[2], Luiz Martins Collaço[1]

SUMMARY

OBJECTIVE: Thyroid neoplasm incidence has increased worldwide, mostly due to the advancements in medical imaging and screening rates. The aberrant Wnt/β-catenin pathway has been identified as a key mechanism, and it has also been related to the metastatic activity of differentiated thyroid cancer. We aimed to verify the difference in the expression of Wnt3a, a canonical activator of the β-catenin signaling, and CDX-2, a transcription factor upregulated by Wnt/β-catenin pathway, in multinodular goiter and differentiated thyroid cancer and to determine their prognostic value.

METHODS: We included 194 thyroid tissue surgical specimen and their clinicopathological data: study group (differentiated thyroid cancer, n=154) and control group (multinodular goiter, n=40). Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded tissue by the primary antibodies Wnt3a and CDX-2.

RESULTS: High Wnt3a expression was significantly associated with differentiated thyroid cancer (p=0.031). CDX-2 was negative in all differentiated thyroid cancer cases (100%) and also in multinodular goiter. Wnt3a expression was significantly associated with tumors ≤20 mm (p=0.044) and with the absence of capsule invasion (p=0.031). The multivariate analyses suggested that older age (≥55), independent of capsular invasion and tumor size, was an independent prognostic factor for Wnt3a expression (p=0.058).

CONCLUSIONS: Wnt3a expression but not CDX-2 is correlated with differentiated thyroid cancer samples in comparison to multinodular goiter. Although its prognostic value was limited to tumor size and capsule invasion, a combined model in a panel of immune markers can add accuracy in the classification of challenging thyroid follicular-derived lesions.

KEYWORDS: Wnt3A protein. CDX2 protein. Thyroid cancer. Goiter. Biomarker.

INTRODUCTION

Differentiated thyroid carcinoma (DTC), the most common endocrine malignancy, detected mainly after the formation of a cervical nodule, has a low mortality rate. However, some rare histopathological variants of DTC are associated with the more aggressive disease with increased risk of tumor-related death[1]. Less is known about reliable factors that could not only predict outcomes in DTC but also help to differentiate benign proliferated cells from malignant ones[2]. Due to the increasing use of powerful imaging diagnostic tools, a great number of low-risk diseases or benign thyroid nodules are being treated with unnecessary aggressiveness, also yielding thyroid cancer over-diagnosis and ramping up comorbidities associated with its overtreatment[3].

The thyroid gland is a relatively dormant organ, and the control of regeneration of a thyroid gland is complex. It is uncommon for a benign thyroid adenoma to evolve toward a carcinoma. It seems that most carcinomas are malignant from the onset. Initiation and progression of thyroid cancer involve multiple genetic disruptions, of which mutations leading to the activation of the MAPK, PI3K/AKT, and Wnt/β-catenin signaling pathways are the most explored[4]. Aberrant positive staining for β-catenin in DTC and multinodular goiter (MNG) has been documented[5]. Wnt proteins, closely controlling Wnt/β-catenin pathways activation, contribute to the homeostasis of several tissues of epithelial origin and are implicated in vital cellular functions like stem cell regeneration, cell survival, and organogenesis[6]. Wnt1, Wnt3a, Wnt7a, or Wnt10b induces the accumulation of intracellular β-catenin by stimulating the inactivation of the formation of the β-catenin destruction complex, therefore leading to a marked cell proliferation[7].

CDX-2, a transcription factor of the caudal homeobox family, upregulated by Wnt3a, plays an important role in the differentiation and polarization of epithelial cells. Given that the thyroid gland develops from foregut endoderm, the inhibition of endoderm posterior pattern that leads to promoting

[1]Faculdade de Medicina da Escola Evangélica Mackenzie, Medical Research Institute – Curitiba (PR), Brazil.
[2]Instituto Carlos Chagas, Fundação Oswaldo Cruz – Curitiba (PR), Brazil.
*Corresponding author: gleyne.biagini@fempar.edu.br
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anterior foregut polarization is necessary to driving thyroid progenitor cells to differentiated follicular epithelial cells[8]. This is accomplished, in part, by CDX-2 expression[9].

The ability of the adult thyroid gland to regenerate in response to injury represents an important homeostatic process. The representative Wnt3a and CDX-2 interplay is illustrated in Figure 1.

In DTC, CDX-2 nuclear expression has been described in cases of columnar cell variant, with still no definition about its relevance in thyroid tumor aggressiveness[10,11]. To date, it has not been studied in MNG, hence, its absence correlates with less histological differentiation and advanced staging in colorectal malignant tumors shedding light on a tumor suppression function[12].

The expression of CDX-2 in association with Wnt3a in both MNG and DTC has not been studied previously.

This research aimed to compare the immunostaining of MNG and well-differentiated follicular thyroid neoplasms for Wnt3a and CDX-2 and to determine their prognostic value in DTC.

METHODS

This is a cross-sectional retrospective study, carried out at University Evangelical Mackenzie Hospital, in Curitiba, PR, Brazil. Total thyroidectomy (TT) was performed between the years 2002 and 2017. DTC's demographic information, clinical data, treatment, and follow-up were collected. Two pathologists reviewed the blocks.

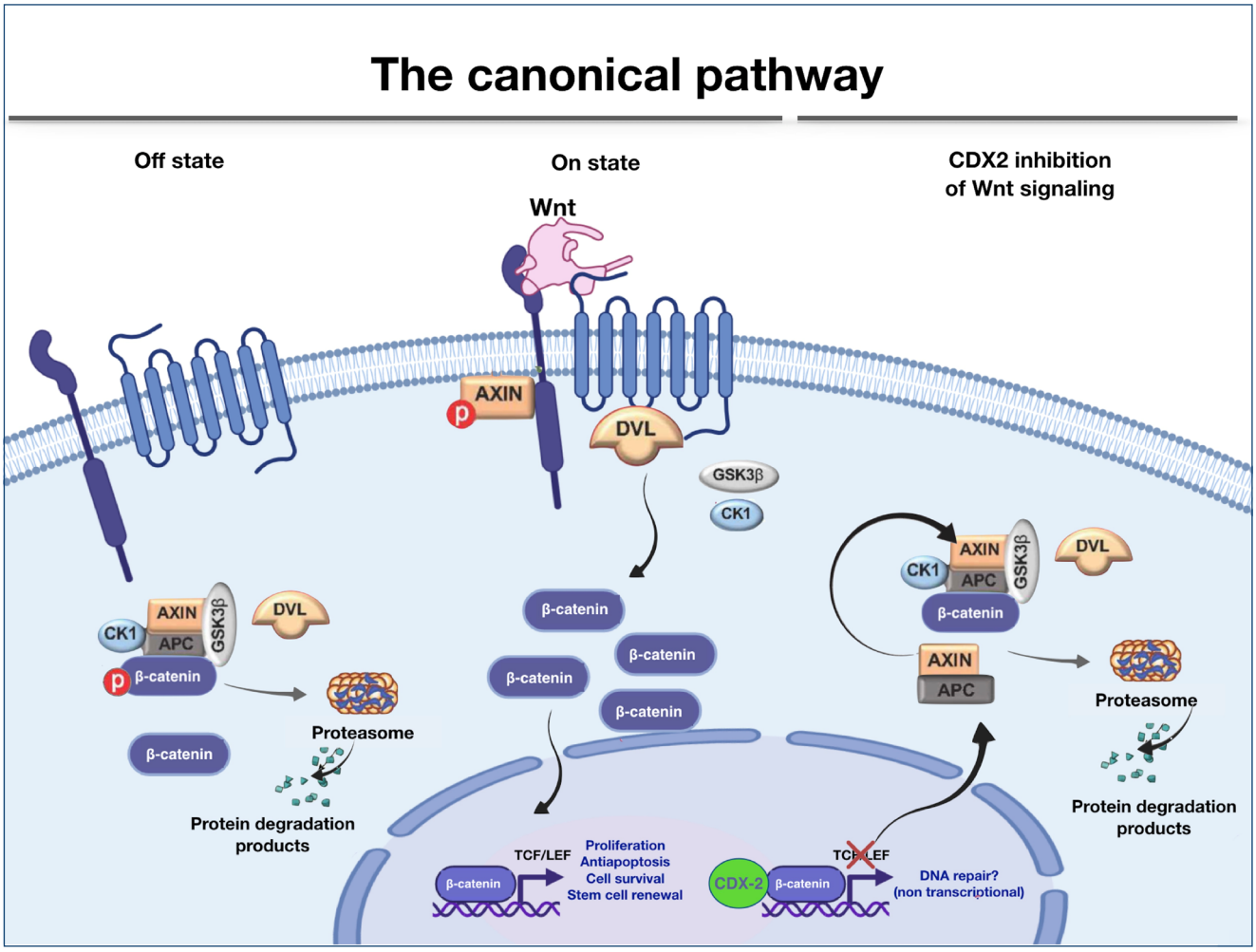

Figure 1. Schematic representation of the interaction between Wnt3a and CDX-2 during the genesis of the thyroid and cellular regeneration. (1) Off state: In a dormant state, when the cells are not dividing, the β-catenin is degraded. (2) On state: Wnt3a stimulates the inactivation of the formation of the β-catenin destruction complex (formed by casein kinase 1 – CK1, glycogen synthase kinase 3 – GSK3, axin protein, and APC protein), which causes intracellular accumulation of β-catenin. This accumulation leads to the activation of target genes of the Wnt/β-catenin pathway, responsible for controlling cell survival, proliferation, and stem cell renewal. (3) CDX inhibition of Wnt signaling: the CDX2 transcription factor, important for the morphogenesis of the thyroid follicular epithelial cells, is believed to act as a blocking factor of the Wnt/β-catenin signaling pathway synergistically. Created with BioRender.com.

The sample consisted of 154 patients with the diagnosis of DTC and 40 patients with MNG, selected from the Anatomic Pathologic database. The demographic information, clinical data, treatment, and follow-up of patients with DTC were collected between January 2018 and December 2019. TT was performed between the years 2002 and 2017.

Formalin-fixed, paraffin-embedded thyroid surgical specimens were sent for immunostaining. For immunohistochemical (IHC) evaluation, multi-sample blocks were built using the Tissue Tek Quick-Array™ handpiece. Coupled clamps allowed to extract sections of 1–3 mm each from the most representative area of the tumor. Each multi-sample block yielded 5 µm thickness slides that were subsequentially submitted to the immunoperoxidase technique performed using the Benchmark Ultra™ instrument. Incubation with the primary antibodies lasted 20 min at room temperature. The primary antibodies used were membrane Wnt3a (GeneTex, CA, USA; diluted 1:100) and nuclear CDX-2 (CellMarque, CA, USA; diluted 1:200). Amplification was conducted by Ultraview Universal DAB Detection Kit® following the manufacturer's instructions. The immunostained TMA sections were evaluated and scored under a light microscope by two pathologists at different time points. Immunostaining for nuclear CDX-2 was scored in a three graded scale: score 0, weak staining in <10% of the tumor cells; score 1, moderate staining ≥10–75% of the tumor cells, and score 2, strong staining in ≥75% of the tumor cells. Positive membrane Wnt3a was considered when marked in ≥10% of the tumor cells.

Informed consents were collected under Institutional Review Board-approved protocols of the Hospital Universitário Mackenzie do Paraná (nº 1.999.671) in Curitiba, Paraná, Brazil.

Statistical analysis

Continuous and categorical variables were compared using the Student's t-test and chi-square test. For the multivariate analysis, the logistic regression model was adjusted including the ones with value <0.25. Wald test was used to determine the significance of the variables, and the estimated association measure used was the odds ratio. The $p<0.05$ indicated a statistical significance. Data were analyzed using Stat/SE version 14.1 (StataCorpLP, USA).

RESULTS

Of the 154 patients with DTC, 125 (81.2) were woman, with a mean age of 45.7±13.53 years. Papillary thyroid cancer (PTC) was the most common histological variant (n=112, 72.7%). These data are presented in detail in Table 1. Most cases were classified as TNM class I (n=132, 85.7%). Low-risk MRSS ATA 2009 (Modified Risk Stratification System from American Thyroid Association Guidelines 2009) was diagnosed in the majority (n=88, 57%). Central lymphadenectomy was performed in 104 (67.5%) cases, and bilateral cervical exploration was performed in 21 (13%) cases. A total of 22% (n=35) of patients did not receive radioiodine remnant ablation (RRA), and 7.1% (n=11) and 68% (n=105) did undergo either low-dose (30 mCi) or high-dose RRA (100–200 mCi), respectively. Group MNG, mean age 59.5 years, was mostly composed of women (92.5%). Although gender distribution was not different, age was statistically distinct between groups: 59.5 years in MNG and 45.2 years in DCT ($p<0.01$).

Table 1. Baseline characteristics of 154 patients with MRSS ATA 2009: modified stratification system.

Age (years)	
Mean±SEM	45.7±13.53
Median (range)	43.4 (19.2–85.2)
Histology, n (%)	
PTC classic variant	112 (72.7)
PTC follicular variant	32 (20.8)
PTC oncocytic variant	3 (1.9)
PTC tall cell variant	2 (1.3)
Follicular	5 (3.2)
Cervical lymphadenectomy, n (%)	
No	29 (18.8)
Central	104 (67.5)
Central and lateral	21 (13.0)
Bilaterality, multifocality, and capsular invasion, n (%)	
Bilateral	38 (24.7)
Multifocal	57 (37)
Capsular invasion	47 (30.5)
TNM stage, n (%)	
I	132 (85.7)
II	11 (7.1)
III	10 (6.5)
IVc	1 (0.6)
MRSS ATA 2009, n (%)	
Low	88 (57.1)
Intermediate	45 (29.2)
High	21 (13.6)
^{131}I ablative dose (mCi)	
Mean±SEM	86.7±57.8
Median (range)	100 (0–200)

CDX-2 expression was negative in 145 (94%) patients, and the results were inconclusive in 9 (5.8%) patients. Group MNG also showed the absence of CDX-2 expression in all cases.

The expression of Wnt3a in the group MNG was positive in only 7 (17.5%) cases and negative in 33 (82.5%) cases. The demographics and clinical features of the patients in the DTC study group and MNG are presented in Table 2.

Wnt3a expression was inconclusive in 33 (21.4%) cases. Among the other 121 cancerous tissues, 45 (37.2%) cases showed high Wnt3a expression. A significant difference was detected between DTC and MNG (p=0.031). Wnt3a expression was significantly associated with smaller tumors (p=0.044) and the absence of capsule invasion (p=0.031).

In a multivariate analysis, tumor size was significantly associated (p=0.024) with Wnt3a expression, while capsule invasion presented only a trend association (p=0.052). Age ≥55 years old, independent of capsular invasion and tumor size <2 cm, also showed a statistically significant trend (p=0.058).

DISCUSSION

In this research, we found increased expression of Wnt3a in DTC compared to the significant lower expression in MNG, indicating that IHC analysis of Wnt3a staining is a valuable tool for differentiating DTC from proliferative benign thyroid tumors. To the best of our knowledge, this is the first time that these markers were studied comparing thyroid carcinomas and benign thyroid hyperplasia blocks. Similarly, a study investigated Wnt3a expression in PTC samples and compared it to their paracancerous tissues[13]. In this case, the results of IHC showed that the positive expression rates of Wnt3a in PTC tissues were significantly higher. Interestingly, in their report, the expression of Wnt3a is correlated with TNM stage, differentiation, extramembranous invasion, and lymph node metastasis, but not with tumor size. In our study, however, Wnt3a overexpression was correlated with less aggressive characteristics, according to TNM-AJCC, in a significant manner. It was detected more in small tumors, less than 2 cm, and in those without capsule invasion.

Table 2. Characteristics of subjects and Wnt3a expression in multinodular goiter and differentiated thyroid carcinoma.

Variable		Groups		p-value
		MNG (n=40)	DTC (n=121)	
Age	Years	59.5±14.1	45.2±13.1	<0.001
Gender	Female	37 (92.5)	100 (82.6)	0.199
Wnt3a	Negative	33 (82.5)	76 (62.8)	0.031
	Positive	7 (17.5)	45 (37.2)	

These results highlight a possible role of the canonical activator Wnt3a acting as a tumor suppressor as reported in some other tissues. In non-solid neoplasms, Nygren et al. showed B-cell precursor acute lymphoblastic leukemia (B-ALL) cell death induction by Wnt3a[14]. Also, by activating canonical Wnt signaling in ALL cells, the Wnt3a is known to inhibit the proliferation of B-ALL cell lines[14]. In melanoma, Wnt3a has been related to enhanced apoptosis[5], and in hepatocellular carcinoma, it has been shown to improve hypoxia-induced epithelial–mesenchymal transition (EMT)[15].

Perhaps, the uncommon aggressiveness and low mortality rate seen in DTC have deployed researchers toward studying more aggressive tumors, in an attempt to unveil the Wnt/β-catenin pathway role in cancer. This could explain why the literature is scarce relating Wnt family members to DTC.

In one of these few reports, using cell culture, Cassinelli et al. showed that in human primary thyrocytes, RET/PTC1 promotes β-catenin transcriptional activity to drive thyrocyte neoplastic transformation, indicating that RET–PTC fusions also trigger the Wnt pathway in an independent manner of Wnt members activation[16]. Recently, in a study with co-cultured cells using thyroid cancer cells and alternatively activated cells-like tumor-associated macrophages, the knockdown of Wnt3a reduced the proliferation and migration of thyroid cancer cells[17]. Even though these findings might oppose to those of ours, it is important to note that this study was performed using cell culture, which is done in an artificial environment that does not always reproduce the in vivo scenario.

Taking all these data together, it could be possible that Wnt3a expression, correlated to malignant tissues with better outcomes, was lost as an epiphenomenon of tumor dedifferentiation and, therefore, was not detected in some cases, rather than being a true pathogenic factor.

In our groups, CDX-2 expression was negative in all benign and in DTC samples. To date, CDX-2 expression has been seen only in tumors with columnar cell[18] and cribriform morular variant histology, the former associated with more aggressive behavior[19]. Since our cohort was composed only of one representative case of each above-mentioned variant and both were classified as low-risk cases, the absence of CDX-2 expression was predictable.

There are some limitations in our study. The non-neoplastic thyroid samples were not representative of other thyroid hyperplasia or even normal thyroid tissue, to better understand the above-mentioned markers. We also could not determine whether the Wnt3a expression is prognostic or a predictive factor due to the indolent behavior of most of our cases.

CONCLUSION

This study shows that Wnt3a expression but not CDX-2 is correlated with DTC samples in comparison to MNG. Although its prognostic value was limited to tumor size and capsule invasion, a combined model in a panel of immune markers can add accuracy in the classification of challenging thyroid follicular-derived lesions.

AUTHORS' CONTRIBUTIONS

GLKB: Conceptualization (Equal), Formal Analysis (Lead), Investigation (Lead), Methodology (Equal), Project administration (Lead), Resources (Lead), Supervision (Lead), Writing – original draft (Lead), Writing – review & editing (Lead). **CAPMR:** Funding acquisition (Lead), Project administration (Equal), Supervision (Equal). **HDH:** Data curation (Equal), Investigation (Equal). **VYH:** Data curation (Equal), Investigation (Equal). **MAKZ:** Data curation (Equal), Investigation (Equal). **IP:** Data curation (Equal), Investigation (Equal). **GB**: Project administration (Equal), Writing – original draft (Equal), Writing – review & editing (Equal). **LMC:** Conceptualization (Equal), Funding acquisition (Equal), Project administration (Equal), Resources (Supporting).

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