JBUON 2018; 23(4): 1029-1040
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

RANKL, OPG, TRAIL, KRas, and c-Fos expression in relation to central lymph node metastases in papillary thyroid carcinoma

Maria V. Deligiorgi[1], Helen Mahaira[2], Christos Eftychiadis[2], Georgia Kafiri[3], Georgia Georgiou[1], George Theodoropoulos[1], Manousos M. Konstadoulakis[1], Eleni Zografos[1], George C. Zografos[1]

[1]First Department of Propaedeutic Surgery, Hippokratio General Hospital of Athens, Medical School, National and Kapodistrian University of Athens, Athens, Greece; [2]Department of Pathology, KAT General Hospital of Athens, Athens, Greece; [3]Department of Pathology, Hippokratio General Hospital of Athens, Athens, Greece

Summary

Purpose: *RANKL, OPG and TRAIL have long been pursued in cancer. Mutated KRas proteins and c-Fos overexpression – well-recognized oncogenic events – have been conceived as coordinators of RANKL, OPG and TRAIL pathways. Considering the paucity in the relevant literature, the purpose of the present study was to investigate whether the expression of these molecules configures a distinct papillary thyroid carcinoma (PTC) subgroup with adverse clinicopathological characteristics.*

Methods: *RANKL, OPG, TRAIL, KRas, and c-Fos immunohistochemical expression in relation to clinicopathological characteristics of PTC was assessed retrospectively in paraffin-embedded PTC specimens from 114 patients who underwent total thyroidectomy with simultaneous central lymph node dissection (CLND).*

Results: *Expression of RANKL, OPG, TRAIL, Kras and c-Fos was revealed in 78.6, 63.2, 61.4, 47.4, and 73.7% of PTC, respectively. As predominant KRas-expressing PTC histotype emerged the classical PTC (cPTC), comprising 66.7% of PTC. A significant correlation was demonstrated of RANKL, OPG, and TRAIL expression with central lymph node metastasis CLNM ($p=0.007$, $p<0.001$, and $p=0.002$, respectively), concerning especially cPTC as regards to RANKL ($p=0.027$) and OPG ($p=0.006$), and both cPTC ($p=0.043$) and follicular variant of PTC (FVPTC) ($p=0.049$) with regard to TRAIL. OPG expression associated significantly with multifocality ($p=0.045$). Multivariable-adjusted logistic regression models characterized TRAIL as independent predictor of CLNM (OR=10.335, 95% CI: 1.23-86.87). CLNM correlated significantly with six pairs of coexpressions: TRAIL-KRas ($p=0.011$), TRAIL-c-Fos ($p=0.006$), OPG-c-Fos ($p=0.024$), RANKL–TRAIL ($p<0.001$), RANKL–OPG ($p<0.001$), TRAIL–OPG ($p<0.001$).*

Conclusion: *The present study suggested for the first time that OPG, RANKL, TRAIL expressions, either alone or in concert involving c-Fos and KRas expression, are related to CLNM. Further research is warranted to elucidate whether the examined molecules can be endorsed as indicators of aggressive PTC behavior and guide a personalized therapeutic intervention.*

Key words: *lymph nodes, OPG, RANKL, thyroid carcinoma, TRAIL*

Introduction

Thyroid carcinoma (TC) is the most common endocrine malignancy [1], albeit a rare entity accounting for 2.1% of global cancer burden [2]. Papillary TC (PTC) is the most prevalent TC histotype, comprising 80% of TC [1] and 85% of differentiated TC (DTC) [3], with an incidence increasing world-

Correspondence to: Maria V. Deligiorgi, MD. "Hippokratio" General Hospital of Athens, Vas.Sofias 114, PC11527, Athens, Greece
Tel:+30 6947524060, E-mail: mdeligiorgi@yahoo.com
Received: 12/01/2018; Accepted: 02/02/2018

wide over the prior decades [4]. The indolent nature of DTC imposes the endorsement of treatment paradigms based on personalized risk assessment with focus on recurrence incidence hovering at 25% [5]. Recently, CLNM have gained prominence as the main culprit for not only recurrent [5] but also persistent disease, occurring in 20-90% of PTC at initial diagnosis [3].

Current guidelines for the management of DTC published by the American Thyroid Association (ATA) lay the groundwork for a continuum of risk stratification integrating the molecular landscape of PTC with the clinicopathological profile, thereof emphasizing lymph node metastases (LNM) [3], launching a realm of intense research.

The designation of nuclear factor kB (NFkB) as coordinator of TC [6,7] provides the rationale for the investigation of receptor activator of NFkB (RANK) ligand (RANKL) – a member of tumor necrosis factor (TNF) family – and its decoy receptor osteoprotegerin (OPG) – a secreted member of TNF receptor family – in PTC [8,9]. A compelling evidence supporting the critical role of RANKL in PTC is derived from an innovative pathway analysis revealing that the TNF receptor associated factor (TRAF)-6-mediated induction of NFkB, a process initiated by RANKL, is included among 87 differential pathways in PTC [10]. Originally conceived as the cornerstone of bone milieu [11], the RANKL/OPG interaction extends beyond the OPG-mediated abrogation of RANKL-induced bone resorption. The ubiquitous expression of OPG and RANKL delineates an intricate network of cross-talking pathways, governing osteoimmunology intertwined with initiation and progression of cancer [12-17].

TNF-related apoptosis inducing ligand (TRAIL) is a member of TNF family, produced by T natural killers cells, credited with initiating the extrinsic pathway of apoptosis selectively on cancer cells, upon interaction with its signaling receptors (TRAILR1, TRAILR2) [18,19]. TRAIL has been shown to eliminate *in vitro* TC cells [20,21], while OPG, a decoy receptor for TRAIL, endows not only thyroid [22], but also breast [23] and prostate [24] cancer cells with a survival advantage. Intriguingly, a tumor-promoting non-canonical TRAIL-mediated signaling has been described recently [18].

KRas protein along with NRas and HRas proteins synthesize the p21 Ras family of small p21 GTP-binding proteins, controlling every aspect of cell biology. Oncogenic signal transduction downstream of mutated Ras proteins entails the activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/Akt pathways [25]. While Ras mutations have been considered as a hallmark of follicular thyroid cancer (FTC) [25,26], a distinct profile of PTC harboring Ras mutations has recently emerged [1]. NRas gene is the prevailing mutated Ras gene in TC [25]; however, KRas gene polymorphisms have been currently correlated with increased PTC risk [27].

c-Fos oncoprotein is a member of Fos family (c-Fos, Fos-B, Fra-1, Fra-2) heterodimerizing with members of Jun and Maf family to form transcription factor activator protein 1 (AP-1), integrating mitogenic stimuli into oncogenic transcriptional programmes [28]. Although c-Fos has been implicated in cancer, including meningioma [29], neurinoma [29], and breast cancer [30], it remains underexplored in PTC.

Herein, we hypothesized that RANKL, OPG, TRAIL, mutated KRas proteins, and c-Fos may configure a distinct group of aggressive PTC and thus refine the risk stratification schemes. Pertinent literature is exceptionally scarce, rendering further investigation imperative with the aim to facilitate the risk-adapted treatment. In order to test this hypothesis we evaluated the immunohistochemical (IHC) expression of the aforementioned molecules in correlation with adverse clinicopathological characteristics, emphasizing CLNM, as well as with coexistence of Hashimoto's thyroiditis (HT), a characteristic highly controversial and yet an issue of burgeoning research [31].

Methods

Study population

This retrospective study enrolled 114 patients with PTC who underwent thyroid surgery at Hippokratio General Hospital of Athens in Greece from 2009 to 2014. The study included patients with histologically confirmed PTC who underwent total thyroidectomy with simultaneous dissection of lymph nodes of the central cervical compartment as primary surgery. Central cervical compartment was defined according to a consensus statement [32]. Patients who underwent completion thyroidectomy and/or reoperation of recurrent disease were excluded. The tumors were classified according to American Joint Committee on Cancer (AJCC) TNM system (7th Edn) [3]. Characteristics studied included age, sex, histological PTC subtype, multifocality, capsular invasion, CLNM status, coexistence of HT, T stage and TNM stage. The study was performed in compliance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Hippokratio General Hospital of Athens (Reference number 3231.14.2.12).

Immunohistochemistry

Immunohistochemical (IHC) staining for RANKL, OPG, TRAIL, KRas, c-Fos was performed on 2-5 μm-thick sections from the original formalin-fixed paraffin-

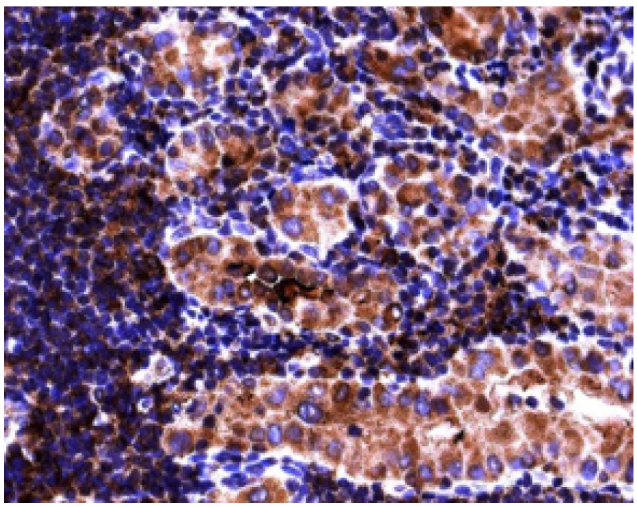

Figure 1. Cytoplasmic RANKL positive immunostaining (magnification ×40).

embedded (FFPE) tissue samples of patients' tumors according to standard protocols. Briefly, the slides were placed in oven at 60°C for 60 min and then deparaffinized, rehydrated and subjected to heat-induced antigen retrieval using a buffer solution (Target Retrieval Solution pH=9, Dako). After remaining in the buffer at room temperature for 10 min, the slides were washed first in running tap water and then in buffer solution Tris-Buffered NaCl Solution with Tween 20 pH=7.6, Dako (TBS). Immunostaining was completed using Autostainer, Dako. The slides were incubated for 60 min with the following rabbit polyclonal primary antibodies: anti-RANKL (ab9957 Abcam Cambridge, UK, dilution:1/100), anti-OPG (ab73400, Abcam Cambridge, UK, dilution:1/100), anti-TRAIL (ab9959 Abcam Cambridge,UK, dilution:1/500), anti-KRas (ab180772, Abcam Cambridge, UK, dilution: 1/100), anti-c-Fos (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution:1/100). After applying the endogenous peroxidase

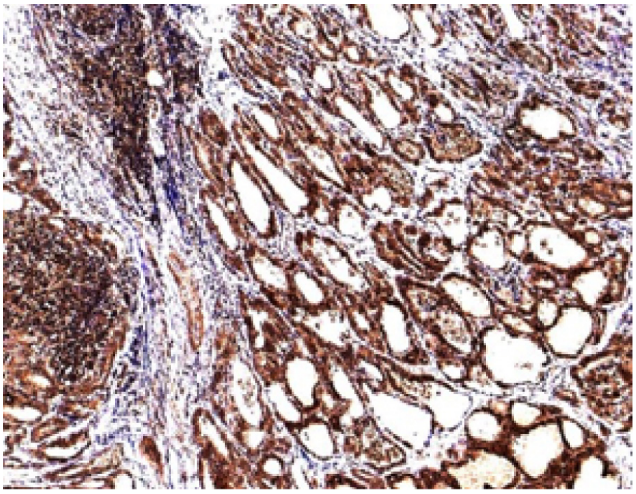

Figure 2. Cytoplasmic OPG positive immunostaining (magnification ×10).

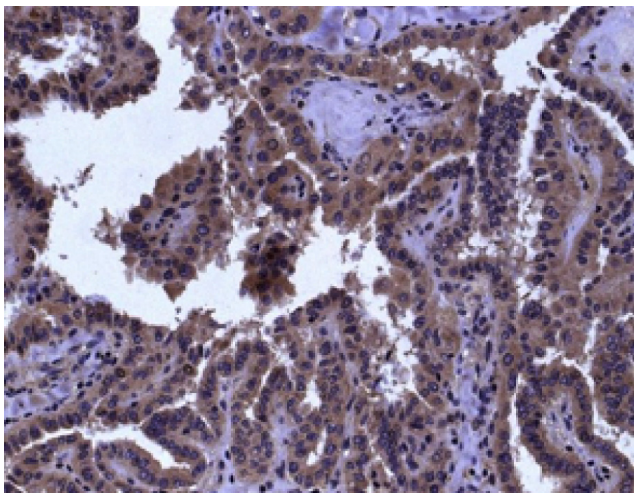

Figure 4. Cytoplasmic KRas positive immunostaining (magnification ×20).

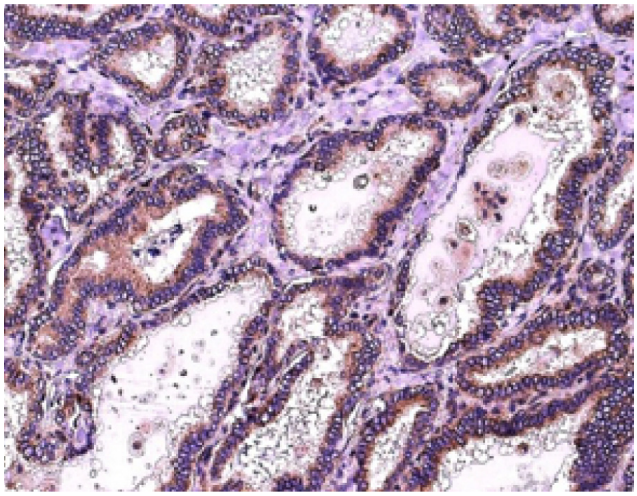

Figure 3. Cytoplasmic TRAIL positive immunostaining (magnification ×20).

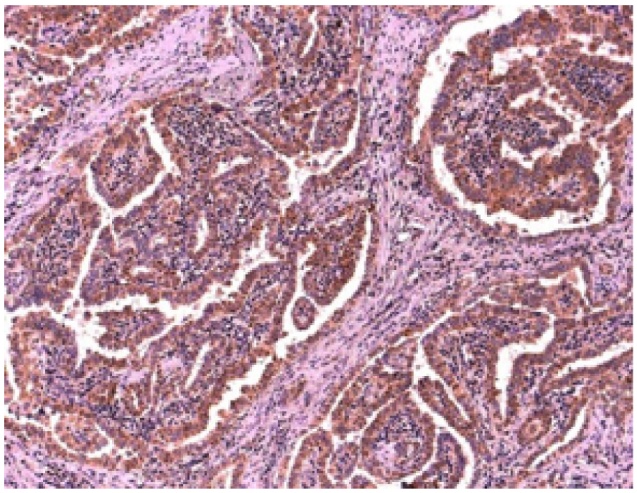

Figure 5. Cytoplasmic c-Fos positive immunostaining (magnification ×10).

blocking reagent Dako, the slides were incubated with a peroxidase-conjugated polymer (EnVision™ detection system peroxidase/DAB, Dako). Immunoreactions were visualized using diaminobenzidine (DAB, Dako) as chromogen. Finally, the slides were washed in dextrose-in-water solution (DW), counterstained with hematoxyline, washed in running tap water, dehydrated, cleaned and mounted. The appropriate positive and negative controls were also prepared.

Interpretation of IHC staining

IHC staining was evaluated by microscopy by two expert pathologists. The intensity of staining was scored as no staining (0), weak (1+), moderate (2+) and strong (3+) and the percentage of positive cells with each individual staining intensity was assessed visually. A semiquantitative scoring system was applied to assess IHC expression, evaluating simultaneously the staining intensity and the percentage of positive cells, as previously described [33]. In particular, for each slide, an H score, ranging from 0 to 300, was calculated according to the following formula: 1x [percentage of cells stained weakly(1+)]+2x [percentage of cells stained moderately(2+)] +3x [percentage of cells stained strongly(3+) [33].

The RANKL, OPG, TRAIL and c-Fos positive expressions were split into high or low expression in reference to their respective median levels of H scores as follows: low expression (0<H score<median H) and high expression (H score≥median). KRas IHC expression was dichotomized in negative expression (H score=0) and positive expression (H score>0). Representative immunostainings of RANKL, OPG, TRAIL, KRas and c-Fos are shown in Figures 1-5.

Statistics

Statistical analyses were conducted using SPSS (IBM Statistical Package for Social Sciences v. 21.0, Chicago, IL). Parameter distributions were not normal as indicated by Kolmogorov-Smirnov test. Pearson's chi-square test, or Fisher's exact test were used to analyze categorical data when the number of cases in a category was <10. Multivariable-adjusted logistic regression models controlling for sex, age, and T stage were applied to assess the association between the presence of CLNM and the expressions of the examined molecules. A p value of less than 0.05 was considered statistically significant.

Results

The clinicopathological characteristics of the study population are depicted in Table 1. The expression of the studied molecules according to histological PTC subtype is presented in Table 2. RANKL, OPG, TRAIL, KRas and c-Fos expression was observed in 78.6, 63.2, 61.4, 47.4 and 73.7% of PTC, respectively.

The expression of RANKL, OPG and TRAIL demonstrated a significant association with CLNM (p=0.007, p<0.001, and p=0.002, respectively; Tables 3 and 4). To identify the histological PTC subtype for which the aforementioned associations are significant we analyzed the expression of OPG, RANKL and TRAIL according to CLNM status separately in classical PTC (cPTC) and follicular variant of PTC (FVPTC). Tall cell variant of PTC (TCV PTC) was excluded from analysis due to small number

Table 1. Clinicopathological characteristics of the study population

Characteristics	*n (%)*
Age	
Mean (SD), years	39.27 (13.68)
<45	72 (63.2)
≥45	32 (28.1)
Sex	
Male	20 (17.5)
Female	92 (80.7)
Histological PTC subtype	
cPTC	76 (66.7)
FVPTC	32 (28.0)
TCV PTC	6 (5.3)
Multifocality	
Yes	58 (50.9)
No	54 (47.4)
Capsular invasion	
Yes	66 (57.9)
No	48 (42.1)
CLNM status	
Positive	54 (47.4)
Negative	60 (52.6)
Hashimoto's thyroiditis	
Yes	70 (61.4)
No	44 (38.6)
T stage	
1a	42 (36.8)
1b	26 (22.8)
2	4 (3.6)
3	42 (36.8)
TNM stage	
I	98 (85.9)
II	2 (1.8)
III	14 (12.3)

n: number of patients, PTC: papillary thyroid carcinoma, cPTC: classical papillary thyroid carcinoma, FVPTC: follicular variant of PTC, TCV PTC: tall cell variant of PTC, CLNM: central lymph node metastases

Table 2. IHC expression of molecules according to histological PTC subtype in the study population

IHC expression	*Histological PTC subtype*			
	CPTC *n (%)*	*FVPTC* *n (%)*	*TCV PTC* *n (%)*	*Total PTC* *n (%)*
RANKL				
Negative	18 (24.3)	6 (18.8)	0 (0.0)	24 (21.4)
Low	36 (48.6)	8 (25)	2 (33.3)	46 (41.1)
High	20 (27)	18 (56.3)	4 (66.7)	42 (37.5)
Total	74 (97.4)	32 (100)	6 (100)	112 (100)
OPG				
Negative	32 (42.1)	10 (31.3)	0 (0.0)	24 (36.8)
Low	14 (18.4)	2 (6.3)	2 (33.3)	18 (15.8)
High	30 (39.5)	20 (62.5)	4 (66.7)	54 (47.4)
Total	76 (100)	32 (100)	6 (100)	114 (100)
TRAIL				
Negative	32 (42.1)	12 (37.5)	0 (0.0)	44 (38.6)
Low	22 (28.9)	8 (25)	6 (100)	36 (31.6)
High	22 (28.9)	12 (37.5)	0 (0.0)	34 (29.8)
Total	76 (100)	32 (100)	6 (100)	114 (100)
KRas				
Negative	40 (52.6)	16 (50)	4 (66.7)	60 (52.6)
Positive	36 (47.4)	16 (50)	2 (33.3)	54 (47.4)
Total	76 (100)	32 (100)	6 (100)	114 (100)
c-Fos				
Negative	22 (28.9)	6 (18.8)	2 (33.3)	30 (26.3)
Low	22 (28.9)	6 (18.8)	0 (0.0)	28 (24.6)
High	32 (42.1)	20 (62.5)	4 (66.7)	56 (49.1)
Total	76 (100)	32 (100)	6 (100)	114 (100)

IHC: immunohistochemical, PTC: papillary thyroid carcinoma, cPTC: classical papillary thyroid carcinoma, FVPTC: follicular variant of PTC, TCV PTC: tall cell variant of PTC

of tumors. The association of RANKL and OPG expression with CLNM proved to be significant concerning cPTC (p=0.027 and p=0.006, respectively), while TRAIL expression associated significantly with CLNM in both cPTC (p=0.043) and FVPTC (p=0.049) (Table 5). OPG expression correlated significantly with multifocality (p=0.045) (Table 4). No significant correlation of RANKL, OPG and TRAIL with any of the remaining clinicopathological characteristics was observed.

Neither KRas nor c-Fos expression showed a significant association with any of the examined characteristics (analytical data not shown).

Multivariable-adjusted logistic regression models controlling for age, sex and T stage revealed a significant correlation of TRAIL expression with CLNM (OR:10.335, 95% CI: 1.23-86.87) (Table 6).

Analyzing the pairs of coexpressions of the examined molecules according to CLNM status, 6 pairs of coexpressions demonstrated a significant correlation with CLNM: TRAIL–KRas (p=0.011), TRAIL–c-Fos (p=0.006), OPG–c-Fos (p=0.024), RANKL–TRAIL (p<0.001), RANKL–0PG (p<0.001), TRAIL–OPG (p<0.001) (Table 7).

Discussion

To our knowledge, the present study demonstrated for the first time a significant correlation of OPG, RANKL, TRAIL, either alone or in coexpression implicating KRas and c-Fos expression, with CLNM, designating TRAIL as an independent

Table 3. Correlation of IHC expression of RANKL and TRAIL with clinicopathological characteristics

Characteristics	*IHC expression*							
	RANKL				*TRAIL*			
	Negative n (%)	*Low n (%)*	*High n (%)*	*p value*	*Negative n (%)*	*Low n (%)*	*High n (%)*	*p value*
Age (years)				0.241				0.118
<45	20 (19.6)	28 (27.5)	24 (23.6)		24 (23.1)	30 (28.8)	18 (17.3)	
≥45	2 (2)	14 (13.7)	14 (13.7)		16 (15.4)	4 (3.8%)	12 (11.5)	
Sex				0.577				0.775
Male	2 (1.8)	10 (9.1)	8 (7.3)		6 (5.4)	8 (7.1)	6 (5.4)	
Female	22 (20)	34 (30.9)	34 (30.9)		38 (33.9)	28 (25)	26 (23.2)	
CLNM status				**0.007**				**0.002**
Negative	22 (19.6)	20 (17.9)	16 (14.3)		36 (31.6)	14 (12.3)	10 (8.8)	
Positive	2 (1.8)	26 (23.2)	26 (23.2)		8 (7)	22 (19.3)	24 (21.1)	
Histological PTC subtype				0.256				0.107
cPTC	18 (16.1)	36 (32.1)	20 (17.9)		32 (28.1)	22 (19.3)	22 (19.3)	
FVPTC	6 (5.4)	8 (7.1)	18 (16.1)		12 (10.6)	8 (7)	12 (10.6)	
TCPTC	0 (0)	2 (1.8)	4 (3.6)		0 (0)	6 (5.3)	0 (0)	
Multifocality				0.078				0.265
No	18 (16.4)	16 (14.5)	20 (18.2)		26 (23.2)	12 (10.7)	16 (14.3)	
Yes	6 (5.5)	30 (27.3)	20 (18.2)		18 (16.1)	24 (21.4)	16 (14.3)	
Capsular invasion				0.725				0.497
No	8 (7.1)	20 (17.9)	20 (17.9)		18 (15.8)	12 (10.5)	18 (15.8)	
Yes	16 (14.3)	26 (23.2)	22 (19.6)		26 (22.8)	24 (21.1)	24 (21.1)	
Hashimoto's thyroiditis				0.943				0.633
No	8 (7.1)	18 (16.1)	16 (14.3)		18 (15.8)	16 (14)	10 (8.8)	
Yes	16 (14.3)	28 (25)	26 (23.2)		26 (22.8)	20 (17.5)	24 (21.1)	
T stage				0.379				0.476
1a	6 (5.4)	18 (16.1)	16 (14.3)		20 (17.5)	8 (7)	14 (12.3)	
1b	10 (8.9)	10 (8.9)	6 (5.4)		6 (5.3)	10 (8.8)	10 (8.8)	
2	0 (0)	0 (0)	4 (3.6)		2 (1.8)	0 (0)	2 (1.8)	
3	8 (7.1)	18 (16.1)	16 (14.3)		16 (14)	18 (15.8)	8 (7.0)	
TNM stage				0.681				0.183
I	20 (17.9)	42 (37.5)	34 (30.4)		36 (31.6)	36 (31.6)	26 (22.8)	
II	0 (0)	0 (0)	2 (1.8)		0 (0)	0 (0)	2 (1.8)	
III	4 (3.6)	4 (3.6)	6 (5.4)		8 (7)	0 (0)	6 (5.3)	

p values depicted in bold denote statistical significance. IHC: immunohistochemical,PTC: papillary thyroid carcinoma, cPTC: classical papillary thyroid carcinoma, FVPTC: follicular variant of PTC, TC: tall cell variant of PTC, CLNM: central lymph node metastases

predictor for CLNM in PTC. Moreover, this study yielded novel insights into the histological profile of KRas-expressing PTC as well as the significant association of OPG with PTC multifocality.

Although RANKL, OPG and TRAIL have long been pursued in cancer, only two previous studies have addressed their expression in malignant thyroid tissue [8,9].

Herein, the RANKL expression in PTC revealed concordance with the observation of Sood et al. [8]. These data build upon the existing literature, substantiating RANKL expression in various cancer types, including breast, prostate, colorectal, lung, bladder and gastric cancer [14]. On the contrary, Heymann et al. showed no RANKL expression in PTC [9].

In contrast with the study of Sood et al. [8], we demonstrated a significant association of RANKL expression with CLNM, concerning particularly cPTC, implying that RANKL promotes the metastatic process. This finding appears rational, considering the *in vitro* and *in vivo* RANKL-induced migration of RANK-expressing cancer cells [34] as well as the implication of RANKL in epithelial mesen-

Table 4. Correlation of IHC expression of OPG with clinicopathological characteristics

Characteristics	*OPG IHC expression*			*p value*
	Negative n (%)	*Low n (%)*	*High n (%)*	
Age (years)				0.401
<45	22 (21.2)	14 (13.5)	36 (34.6)	
≥45	16 (15.4)	4 (3.8)	12 (11.5)	
Sex				0.133
Male	2 (1.8)	4 (3.6)	14 (12.5)	
Female	40 (35.7)	14 (12.5)	38 (33.9)	
CLNM Status				**<0.001**
Negative	36 (31.6)	2 (1.8)	22 (19.3)	
Positive	6 (5.3)	16 (14)	32 (28.1)	
Histological PTC subtype				0.338
cPTC	32 (28.1)	14 (12.3)	30 (26.3)	
FVPTC	10 (8.8)	2 (1.8)	20 (17.5)	
TCV PTC	0 (0)	2 (1.8)	4 (3.5)	
Multifocality				**0.045**
No	26 (23.2)	12 (10.7)	16 (14.3)	
Yes	16 (14.3)	6 (5.4)	36 (32.1)	
Capsular invasion				0.122
No	20 (17.5)	2 (1.8)	26 (22.8)	
Yes	22 (19.3)	16 (14)	28 (24.6)	
Hashimoto's thyroiditis				0.505
No	14 (12.3)	10 (8.8)	20 (17.5)	
Yes	28 (24.6)	8 (7)	34 (29.8)	
T stage				0.723
1a	16 (14)	4 (3.5)	22 (19.3)	
1b	8 (7)	4 (3.5)	14 (12.3)	
2	2 (1.8)	2 (1.8)	0 (0)	
3	16 (14)	8 (7)	18 (15.8)	
TNM stage				0.640
I	34 (29.8)	16 (14)	48 (42.1)	
II	0 (0)	0 (0)	2 (1.8)	
III	8 (7)	2 (1.8)	4 (3.5)	

p values depicted in bold denote statistical significance. IHC: immunohistochemical, PTC: papillary thyroid carcinoma, cPTC: classical papillary thyroid carcinoma, FVPTC: follicular variant of PTC, TCV PTC: tall cell variant of PTC, CLNM: central lymph node metastases

chymal transition (EMT), angiogenesis [35], cancer cell migration and invasion [36]. Our data endorse the current literature sustaining the aggressive phenotype of various RANKL-expressing cancer types, involving prostate cancer [37], hepatocellular cancer [38] and osteosarcoma [39]. Consistent is the notion that RANKL, produced either by cancer cells or tumor microenvironment, consolidates the "seed and soil" concept of metastasis [14]. As the mechanistic underpinning of the prometastatic potential of RANKL could be conceived the activation of crucial kinases, including PI3K/AKT/mammalian target of rapamycin (mTOR) and MAPK [p38, c-Jun N-terminal kinases (JNK), ERK1/2], downstream of RANKL/RANK interaction, leading to activation of pivotal transcription factors including NFkB and Fos/jun [14,17]. If the RANKL-induced decrease of E-cadherin demonstrated in hepatocellular carcinoma [40] is confirmed in PTC, the association of loss of E-cadherin with TC aggressive phenotype [41] might reflect the RANKL prometastatic activity. However, our finding contradicts the antitumor aspect of RANKL revealed in breast cancer [42], hinting at the dichotomous RANKL signaling [42].

Table 5. Correlation of RANKL, OPG, TRAIL IHC expression with CLNM status according to histological PTC subtype

IHC expression	*Histological PTC subtype*					
	cPTC			*FVPTC*		
	Negative CLNM n (%)	*Positive CLNM n (%)*	*p value*	*Negative CLNM n (%)*	*Positive CLNM n (%)*	*p value*
RANKL			**0.027**			0.324
Negative	16 (21.6)	2 (2.7)		6 (18.8)	0 (0)	
Low	16 (21.6)	20 (27)		4 (12.5)	4 (12.5)	
High	6 (8.1)	14 (18.9)		10 (31.3)	8 (25)	
Total	38 (51.4)	36 (48.6)		20 (62.5)	12 (37.5)	
OPG			**0.006**			0.069
Negative	26 (34.2)	6 (7.9)		10 (31.3)	0 (0.0)	
Low	2 (2.6)	12 (15.8)		0 (0.0)	2 (6.3)	
High	12 (15.8)	18 (23.7)		10 (31.3)	10 (31.3)	
Total	40 (52.6)	36 (47.4)		20 (62.5)	12 (37.5)	
TRAIL			**0.043**			**0.049**
Negative	24 (31.6)	8 (10.5)		12 (37.5)	0 (0.0)	
Low	10 (13.2)	12 (15.8)		4 (12.5)	4 (12.5)	
High	6 (7.9)	16 (21.1)		4 (12.5)	8 (25)	
Total	40 (52.6)	36 (47.4)		20 (62.5)	12 (37.5)	

p values depicted in bold denote statistical significance. IHC: immunohistochemical, PTC: papillary thyroid carcinoma, cPTC: classical papillary thyroid carcinoma, FVPTC: follicular variant of PTC, CLNM: central lymph node metastases

Table 6. Predictors of positive CLNM in multivariable-adjusted logistic regression models

Variables	*Reference*	*OR*	*95% CI*	*p value*
Age (years)	<45	0.109	0.008-1.411	0.09
Sex	Male	0.105	0.005-2.157	0.144
T stage	T1a	3.939	1.055-14.711	**0.041**
KRas	Negative expression	0.219	0.017-2.808	0.243
c-Fos	Negative expression	0.398	0.11-1.44	0.16
RANKL	Negative expression	3.58	0.778-16.48	0.102
TRAIL	Negative expression	10.335	1.23-86.87	**0.032**
OPG	Negative expression	1.028	0.218-4.836	0.972

The final model is controlling for age, sex and T stage. p values depicted in bold denote statistical significance. CLNM: central lymph node metastases, CI: confidence interval, OR: odds ratio.

While contradicting Heymann et al. [9], we concurred with Sood et al. [8], unraveling the expression of OPG in PTC, enriching the repertoire of OPG-expressing malignancies, which encompasses breast, prostate, gastric, bladder [43], colorectal and pancreatic cancer [16], multiple myeloma and giant cell tumors [43]. Contrasting with Sood et al. [8], we demonstrated a significant association of OPG expression with CLNM, especially regarding cPTC. This finding suggests the prometastatic potential of OPG, harmonized with the previously reported aggressiveness of OPG-expressing cancer, involving breast, prostate, gastric, colorectal and pancreatic cancer [16]. Our data are anticipated, considering the recently revolutionized oncogenic and prometastatic dynamics of OPG, synthesized by proangiogenic, pro-proliferative and prosurvival signaling [12,13,15,16]. This notion is strengthened by mass spectrometry analysis in inflammatory and aggressive breast cancer cells, unveiling several proteins-partners of OPG controlling initiation and progression of cancer via regulating cell metabolism, transcription, translation, growth, proliferation, differentiation, organization of cytoskeleton, cell cycle and DNA repair [16]. Considering that integrins are well-recognized partners of OPG [15],

Table 7. Correlation of pairs of coexpressions of molecules with CLNM status

Coexpressions	*Positive CLNM n (%)*	*Negative CLNM n (%)*	*p value*
KRAS- c-Fos	22 (19.3)	22 (19.3)	0.482
RANKL–KRas	26 (22.8)	18 (15,8)	0.129
TRAIL–KRas	26 (22.8)	10 (8.8)	**0.011**
OPG–KRas	26 (22.8)	10 (8.8)	0.11
RANKL–c-Fos	36 (32.1)	30 (26.8)	0.194
TRAIL–c-Fos	34 (29.8)	16 (14)	0.006
OPG–c-Fos	34 (29.8)	20 (17.5)	**0.024**
RANKL–TRAIL	46 (40.4)	20 (17.5)	**<0.001**
RANKL–OPG	46 (40.4)	22 (19.3)	**<0.001**
TRAIL–OPG	44 (38.6)	20 (17.5)	**<0.001**

p values depicted in bold denote statistical significance. CLNM: central lymph node metastases

it would be interesting to investigate whether the pro-proliferative effect of avβ6 integrin recently demonstrated in TC cells [44] is ascribed to OPG signaling.

On the contrary, Vik et al. reported an inverse relation between OPG and risk of cancer [45], raising the question whether OPG *per se* counterbalances rather than favors tumor progression.

The TRAIL expression in PTC supports the study of Sood et al. [8]. However, in contrast with Sood et al. [8] we found a significant correlation of TRAIL expression with CLNM, concerning both cPTC and FVPTC. Our findings suggest that TRAIL reinforces the metastatic process, a notion strengthened by the significant association of TRAIL with CLNM in multivariable-adjusted logistic regression models. Given that previous studies in cervical squamous cell [46] and renal cell carcinoma [47] supported the inherent antitumor potential of TRAIL, our data appear rather unprecedented. A tenable explanation of this discrepancy could be that the cancer cells evade the TRAIL-induced tumor immune surveillance through complex pathways, involving the OPG-mediated inhibition of TRAIL [22,23], and progress, co-opting the non-canonical TRAIL signaling. In that respect, the intriguing prometastatic potential of TRAIL may corroborate the non-canonical TRAIL signaling, wherein cancer cells resistant to TRAIL-induced apoptosis hijack, through mechanisms as yet unidentified, pivotal kinases downstream of TRAIL/TRAILR interaction, involving JNK, p38, ERK, protein kinase C (PKC), and PI3K/AKT, and divert them towards prosurvival, pro-proliferative, and promigratory pathways [18]. The scarcity of relevant literature concerning PTC necessitates the continuation of research.

Due to limited knowledge on each individual Ras protein in PTC, KRas expression is interpreted in the context of the whole spectrum of Ras proteins [48]. Considering the recently proven reliability of IHC NRas expression to identify NRas mutated thyroid neoplasia [49], IHC KRas expression is presumed to reflect KRas mutations. Our observation of KRas expression in 50% of FVPTC consolidates the previously reported molecular profile of FVPTC [25,50,51]. A novel finding is the KRas expression in almost half of PTC (47.4%), encountered in noteworthy proportions of cPTC and TCV PTC (47.4% and 33.3%, respectively). Importantly, among KRas-expressing PTC, the most prevalent subtype was the cPTC (66.7%), followed by FVPTC (29.6%) and TCV PTC (3.7%), challenging the tenet that the Ras-positive PTC is virtually FVPTC [52]. Collectively, our data suggest that KRas-expression in PTC expands beyond FVPTC.

Our observation of c-Fos overexpression in PTC paves the way for further research to address the paucity in the relevant literature. Two previous studies [53,54] showed high levels of c-Fos mRNA in DTC, whereas Liu et al. observed decreased c-Fos mRNA expression in PTC [55]. However, these investigators did not evaluate the levels of c-Fos protein, hindering the comparison with our observation. c-Fos oncoprotein merits further exploration in PTC in view of its implication in malignant transformation, proliferation, cell apoptosis and inflammatory milieu via forming AP-1 complex, activating the expression of target-genes harboring AP-1 sites (consensus sequence 5-TGAG/CTCA-3) [56]. More light should be shed on the equivocal role of c-Fos in cancer, synthesized by a tumor-promoting aspect – proangiogenic, proinvasive, and antiapoptotic – versus an antitumor perspective, inhibitory of cell cycle and pro-apoptotic.

Considering that multifocality is a common PTC feature encountered in 18-87% of PTC [57],

especially in 40% of papillary thyroid microcarcinoma [58], the significant correlation of OPG expression with multifocality is of paramount importance. Assuming that multifocality reflects the tumor burden, conceived either as multicentricity or intrathyroidal spread of a single tumor, harboring increased risk of recurrence and mortality [57], its association with OPG expression may consolidate the tumor-promoting potential of OPG. However, multifocality is not unanimously recognized as indicator of tumor aggressiveness [57], hampering the interpretation of our result.

The significant correlation of CLNM with the coexpressions TRAIL–KRas, TRAIL–c-Fos, OPG–c-Fos, RANKL–TRAIL, RANKL–OPG and TRAIL–OPG may indicate the synergistic effect of the aforementioned molecules ascribed to their cross-talk that orchestrates the enhancement of the metastatic potential of cancer cells. Downstream of RANKL, OPG, TRAIL, and mutated KRas proteins, the activation of PI3K/AKT and MAPK cascades in concert with the overexpression of c-Fos [14,16,18,19] could be conceived as a point of convergence of oncogenic and tumor-promoting pathways driven by these molecules, rationalizing their synergistic effect. In this setting, cancer cell survival pathways triggered by the interactions OPG/aVβ5 integrin and OPG/Type II RANKL entail the activation of Ras proteins [16]. Most importantly, supportive of the prometastatic cross-talk between TRAIL and KRas is the *in vitro* Ras-stimulated conversion of TRAIL death receptors into invasion-inducing receptors, suppressing the Rho-dependent kinase (ROCK)/LIM pathway [59]. Interestingly, a vicious cycle connecting OPG with c-Fos described in mouse [60] corroborates the OPG–c-Fos coexpression. Intriguingly, the significant association of CLNM with RANKL–OPG coexpression alludes to the predominance of the *bona fide* prometastatic role of OPG over the OPG-mediated abrogation of RANKL.

Certain limitations of the present study should be acknowledged, especially the relatively small sample size, the focus on only three histological PTC subtypes, technical issues inherent in immunohistochemistry, and the lack of mRNA expression evaluation or genomic data. However, given the scarcity of the relevant literature, our findings create interesting hypotheses enriching our perception of the molecular status of metastatic PTC, provided that they are further investigated.

From a translational viewpoint, our findings, if validated, could yield the rationale for the embracement of studied molecules as predictors of CLNM, enabling the personalization of treatment. Considering the elusive preoperative and intraoperative detection of CLNM [61], the incorporation of the examined molecules in the molecular profile of PTC with a propensity to metastasize to lymph nodes may enhance the precise risk stratification of PTC with no clinical evidence of CLNM (cN0) and tailor the surgical strategy concerning the controversial prophylactic central lymph node dissection. Moreover, exploiting this molecular constellation as therapeutic target in the context of metastatic PTC is a promising perspective that remains to be illuminated.

Conclusion

In conclusion, this study yields significant preliminary evidence that OPG, RANKL and TRAIL not only *per se* but also in concert with mutated KRas proteins – extending beyond the territory of FVPTC – and c-Fos expression could be envisaged as novel indicators of CLNM in PTC, while OPG is also implicated in multifocality. Further research to decipher and harness the examined molecules in PTC is awaited with anticipation.

Acknowledgements

The authors would like to thank Mr Elias Bitarchas and Mrs Maria Antoniadou for expert technical support.

Conflict of interests

The authors declare no conflict of interests.

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