NILCO SIGNALING IS A NOVEL BIOMARKER IN THYROID CANCER

Bartu Badak[1], Rumeysa Özyurt[2], Nilüfer Erkasap[3], Mete Özkurt[3], Derya Güzel[4], Serdar Erkasap[1], Enver İhtiyar[1], Emine Dünda[5], Deniz Arık

[1]Department of General Surgery, Eskisehir Osmangazi University Medical Faculty, Meselik, Eskisehir, Turkey - [2]Department of Physiology, Kütahya Sağlık Bilimleri University Medical Faculty, Kütahya, Turkey - [3]Department of Physiology, Eskisehir Osmangazi University Medical Faculty, Meselik, Eskisehir, Turkey - [4]Department of Physiology, Sakarya University Medical Faculty, Sakarya, Turkey - [5]Department of Pathology, Osmangazi University Medical Faculty, Meselik, Eskisehir, Turkey

ABSTRACT

Introduction*: This study aimed to demonstrate expression changes of Notch1, Notch4, Jagged1, leptin, leptin receptor ObRb, interleukin-1a (IL-1a), IL-1β, IL1R, Vasoactive Growth Factor-A (VEGFA), VEGFR1, VEGFR2, IL-6 genes in human thyroid cancer tissues. Also, leptin, T3, and T4 concentrations in plasma of both thyroid cancer and healthy controls were compared.*

Materials and methods*: Notch1, Notch4, Jagged1, leptin, leptin receptor ObRb, IL-1α, IL-1β, IL1R, IL-6, VEGFA, VEGFR1, and VEGFR2 mRNA expressions were analyzed by qRT-PCR in human tissue of normal and thyroid tumors from 27 patients. The plasma leptin, T3, and T4 concentrations were determined was by ELISA.*

Results*: In this study, we showed that Notch4, Jagged1, leptin, IL-1α, IL1R, VEGFA, VEGFR2, and IL-6 are over-expressed in human thyroid carcinoma tissues compared with normal thyroid tissues. Also, mean plasma leptin, T3, and T4 concentration were significantly higher in thyroid cancer patients compared with healthy controls. The activation of Notch4 and Jaggedl is associated with leptin, IL-1α, and IL1R mRNA expressions, and this positive correlation can also be attributed to clinicopathological parameters. Moreover, we have shown that plasma concentration levels of leptin, T3, and T4 are related and contribute to poor survival results.*

Conclusion*: These results indicate that NILCO (Notch, IL-1 and Leptin) could be critical in the development of thyroid cancer and this is a useful clinical biomarker in thyroid carcinoma.*

Keywords: *Thyroid cancer, Biomarker, NILCO, Plasma.*

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Introduction

Thyroid cancer is one of the endocrine malignancies that has been increasing rapidly in recent years all over the world[1, 2]. In thyroid cancer, the recurrence rate is high but the mortality rate is low[2]. Although there are several histological types of thyroid cancer, papillary thyroid cancer (PTC) is the most common malignancy that accounts for 70-90% of thyroid cancers. Follicular thyroid cancer (FTC) is the second most common type of malignancy and includes about 5% of cases[3]. The pathways play a role in the potential molecular mechanisms of thyroid cancer therapy have been studied in recent years[4]. One of these pathways is Notch signaling and studies have shown that this pathway plays a role in the development of different types of cancer. At the same time, last studies have shown that the Notch pathway is associated with the development of thyroid cancer but it has not clear, yet[5-7].

Recent studies have shown that leptin affects various signaling pathways involved in cancer progression[8]. These studies have demonstrated that leptin regulates interleukin-1 (IL-1) and Notch sign-

aling in the breast, endometrial (EmCa), and pancreatic cancer cells[9,10]. In particular, the interaction between these three pathways is associated with breast cancer cell proliferation, migration, invasion, and chemoresistance[11]. A new signaling axis between NILCO was associated with breast and EmCa cancer progression[11,12]. However, there is no study related to NILCO and human thyroid cancer tissue. Based on all this data, we hypothesized that NILCO could be a critical biomarker in the development of thyroid cancer and affect the expression of proangiogenic molecules and induction of cell proliferation. To test this hypothesis, we have studied human thyroid cancer tissues.

Materials and methods

Study populations

The present study was approved by the Ethics Committee of Eskisehir Osmangazi University for Clinical Research (80558721/66) and was performed following the ethical standards of the Helsinki Declaration. Tissue samples from tumor and adjacent normal thyroid tissue (n:27) were collected within 1 year at the Department of General Surgery, Hospital of Eskisehir Osmangazi University, Eskisehir, Turkey. Plasma samples were obtained from thyroid cancer patients (n:27) and were compared with plasma samples of healthy volunteers (n:27).

Tissue samples

In this study, tumor and adjacent normal thyroid tissues of thyroid cancer patients were studied. All samples were obtained during routine surgery performed in patients with thyroid cancer. The study included 27 patients (8 men and 19 women) with a mean age of 51.29 years (range: 22-71 years). We reported the clinical characteristics of patients such as age, gender, pathological type, tumor stage and metastasis state (Table 1). Tumor and adjacent normal thyroid tissues, pathological type, stage of tumor and metastasis were determined by pathologists before molecular analyzes. Immediately after excision, tissue samples were fixed in RNAlater solution (Qiagen) and stored at - 80°C.

RNA extraction, cDNA synthesis, and determination of mRNA levels by quantitative reverse transcription PCR

Total RNA was isolated from tumor and adjacent normal thyroid tissues using the GeneJet RNA Purification Kit (Thermo Scientific). The concentration and purity of the RNA were measured using a NanoDrop 1000 (Thermo Scientific). 260/280 values are about 2.1. Isolated RNA samples were converted to complementary DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) at 42°C for 60 min and 70°C for 5 min according to the manufacturer's instructions. cDNA samples were stored at -80°C until analysis. Notch1, jagged1, leptin, IL-1, IL-6, VEGF-A, VEGFR2 gene expressions were measured with SYBR Green qPCR Kit (Thermo Scientific). cDNA synthesis was verified by the detection of the β-actin transcript, which was used as an internal control. Relative differences in expression were determined using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method.

Determination of Leptin, T3 and T4 plasma concentrations by enzyme-linked immunosorbent assay

Plasma leptin, T3 and T4 concentrations were determined using commercially available human-specific enzyme-linked immunosorbent assay (ELISA) kits (DIAsource ImmunoAssays S.A., Belgium) as recommended by the manufacturers. Concentrations were calculated using a plate reader system and through the recommendations using a standart curve (Awareness Technology, Inc. Martin Hwy. Palm City, USA). Results were given as ng/mL.

Statistical analysis

All statistical analyses were performed using GraphPad6. The descriptive statistisics are reported as n (sample size), mean and standard deviation for continuous variables and n (sample size), median and 25th and 75th percentiles for categorical variables. Continuous normally distributed measurements were compared across the groups by the t-test. In the comparisons between the two groups, the continuous variables that did not show normal distribution were compared by the Mann-Whitney U-test. A P value less than 0.05 (P, 0.05) was accepted as significant.

Results

Patient characteristics

We categorized 27 patients with thyroid cancer by comparing gene expression patterns of tumor samples and adjacent normal thyroid tissues after data trimming. For this purpose, we summarized the clinicopathologic profile of patients such as age, gender, pathological diagnosis, number of tumor fo-

cus, metastasis state and tumor stage in Table1. Of the tiroid cancer cases 78% were female patients and 48% are papillary carcinoma and half of the cases metastasis were observed. The majority of cases were in stage 1.

Gender (n:27)		
	Female	21 (%77,78)
	Male	6 (%22,22)
Pathological diagnosis		
	Papillary microcarcinoma	13(%48,14)
	Papillary carcinoma (classical type)	2(%7,4)
	Papillary microcarcinoma (follicular variant) Papillary carcinoma (follicular variant)	5(%18,51) 3(%11,11)
	Hurthle cell carcinoma	1(%3,7)
	Medullary carcinoma	3(%11,11)
Number of tumor focuses		
	Single	25(%92,59)
	Multiple	2(%7,41)
Metastases		
	Systemic	2(%7,40) (2 female)
	Local None	11(%40,74) (8 female/ 2 male) 14(51,85) (11 female/ 4 male)
Stage		
	I II III IV	18(%66,6) 7(%25,9) 0(%0) 2(%7,4)

Table 1: Clinical and pathological features of the patients (N=27).

Gene expressions

Notch4 mRNA abundance was significantly higher in the tumor tissue compared with the adjacent normal thyroid tissue ($P < 0.05$; Fig. 1b.). Similar to Notch4, Jagged1, Leptin, IL-1α, IL1R, IL-6, VEGFA, and VEGFR2 mRNA levels were significantly increased in the thyroid cancer tissue compared with the adjacent normal thyroid tissue ($P < 0.01$, Fig. 1c. ; $P < 0.01$, Fig. 1d; $P < 0.05$, 1f ; $P < 0.01$, Fig.1h ; $P < 0.05$, Fig. 1l; $P < 0.01$, Fig.1i; $P < 0.01$, Fig. 1k). Also, there are no differences in Notch1, ObRb, IL1-β, and VEGFR1 gene expressions between the groups (Fig. 1a, Fig.1e, Fig. 1g and Fig. 1j).

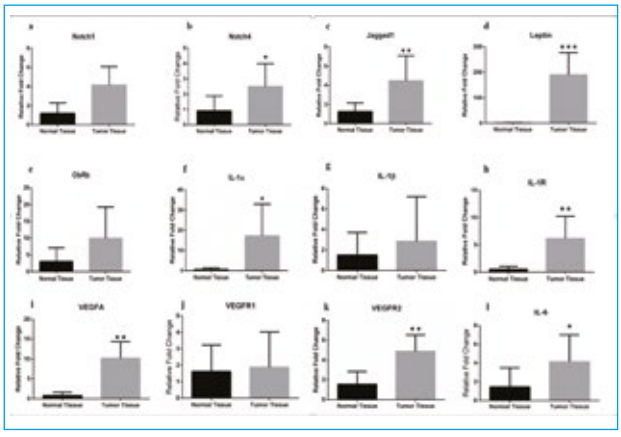

Fig. 1: qRT-PCR expression of Notch1 (a), Notch4 (b), Jagged1(c), Leptin (d), Leptin receptor (ObRb) (e), IL1-alpha (f), IL1-beta (g), IL1R (h), VEGF-A (i), VEGFR1 (j), VEGFR2 (k), IL6 (l) in normal tissue and tumor tissues. β actin was used as internal control. Data are representative results derived from a minimum of three independent experiments. *P < 0.05 and **P < 0.01.

Leptin, T3 and T4 plasma concentrations

Mean plasma leptin, T3 and T4 concentration were significantly higher in thyroid cancer patients compared with healthy people. Data for plasma leptin T3 and T4 concentration are reported in Fig. 2a, Fig. 2b and Fig. 2c.

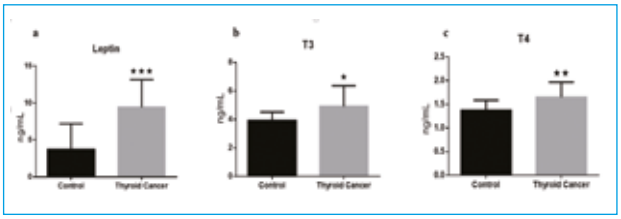

Fig. 2: Plasma leptin (a), T3 (b) and T4 (c) concentrations. Thyroid cancer patients compared to the normal healthy subjects; *P < 0.05, **P < 0.01 and ***P < 0.001.

Discussion

In the present study, our aim was to evaluate NILCO as a biomarker in the development of thyroid cancer which can affect the expression of proangiogenic molecules and induction of cell proliferation.

Our results show that the induction of thyroid cancer along with VEGF/VEGFR-2 was highly dependent on the interaction between NILCO in thyroid cancer tissues. Our results show that NILCO can be a clinical target of drugs and agents targeting signaling can be used as potential cancer therapeutics. Moreover, the inclusion of validated NILCO biomarkers in clinical trials in the future can help identify patient populations that are likely to respond to this class of drugs.

Previous studies have shown that NILCO molecules are expressed in breast and EmCa tissues[8, 13]. Also, a functional leptin-Notch interaction has been reported to be required for pancreatic cancer progression and growth[9]. However, the activation of the NILCO signal may be different according to tumor type and this crosstalk has not yet been studied in thyroid cancer. In this study, we found that NILCO and related molecules are important in the development of thyroid cancer and may be associated with clinical-pathological features.

Notch signaling regulates many basic processes during the development and homeostasis of different types of tissues. As a result, the failure of this pathway leads to many important human diseases, such as cancer[14, 15]. Therefore, Notch signals are considered a potential target in various hematological malignancies[16] and cancers[17] and play an important role in reducing tumor angiogenesis[18]. Notch1 is a prototype and is the most studied member of this re-

ceptor family by now. Although the role of Notch1 is controversial, it has a potential impact on the development of thyroid cancer. Ferretti et al. reported that the activation of the Notch signal mediates growth suppression by cell cycle arrest in well-differentiated thyroid carcinomas (WDTC)[19]. In another study, Notch1 was highly expressed in WDTC with PTC[20]. That is, the role of Notch in thyroid cancer is still poorly understood. Correlated with Yamashita et all. we also showed that Notch1, Notch4, and Jagged1 expressions increased in thyroid tumor tissues compared to adjacent normal thyroid tissues (Fig. 1a, Fig. 1b, and Fig. 1c) and significantly associated with papillary microcarcinoma since 13 of the 27 lesions showed expression of these genes. On the other hand, like the work of Geers et al.,[21] we have shown that tumor thyroid tissues expressed a higher level of Notch4 than normal thyroid tissues. This increase in Notch4 expression may be associated with leptin. As previous studies have shown that leptin can regulate Jagged1 and Notch4 in human cord blood CD34+ cells and early differentiated human endothelial cells (HUVEC)[22].

Recently, in studies of various cancer types, one of the Notch regulators has been reported to be leptin[12, 23]. In vitro papillary thyroid cancer studies of leptin released from adipose tissue and known to be a pro-angiogenic cytokine have been reported to increase expression of leptin and Ob-Rb[24]. Also, leptin increased IL-1 and VEGF / VEGFR2 system as well as Notch1-4 / JAG1 / Dll-4 gene expressions that are important in the Notch signaling pathway. Thus, in this study, the increase of VEGF / VEGFR2 in breast cancer and leptin induction of proliferation/ migration were reported to be associated with NILCO interaction (11). According to our results, the level of leptin gene expression in tumor tissue was increased compared to normal tissue (Fig. 1d). Also, leptin plasma concentration was found to be significantly higher in tumor patients than healthy subjects (Fig. 2a.), and mean plasma T3 and T4 concentration was significantly higher in thyroid cancer patients compared with healthy people (Fig. 2b and Fig. 2c). These results show that the plasma concentration of leptin is highly associated with T3 and T4 and contributes to poor survival outcomes.

Leptin regulates inflammatory cytokines such as IL-1 in various tissues and pathological conditions[25]. Since both leptin and IL-1 are inflammatory and proangiogenic factors that regulate VEGF, the relationship between IL-1 and leptin may be critical for tumor angiogenesis[26]. Gonzales et al. showed that by inhibiting the IL-1 receptor, a leptin-mediated increase of both VEGF and VEGFR2 was inhibited, suggesting that the proangiogenic role of leptin in breast cancer is mediated by IL-1 (26). In our study, increased mRNA levels of IL1 and VEGF, as well as leptin, have shown that these cytokines could actively crosstalk in thyroid cancer eliciting proinflammatory and proangiogenic effects that contribute to cancer growth. (Fig. 1f, Fig. 1h, Fig. 1i, and Fig. 1k).

For patients with well-differentiated thyroid cancer, the most powerful predictors of disease-specific mortality include tumor size, lymph node, and distant metastases, extrathyroidal spread, multifocality, and gender(27). These factors are embedded in staging systems designed to predict clinical outcomes. We have found that clinical features associated with poor outcomes are strongly associated with high NILCO gene expression levels.

As a result, NILCO crosstalk outcome mRNA expression levels in thyroid cancer are parallel to the levels of proangiogenic and pro-inflammatory cytokines IL1 and VEGF.

Overall, NILCO is also important in thyroid cancer as interactions where signals from pro-angiogenic, pro-inflammatory, and developmental factors combine to stimulate cell growth and tumor angiogenesis.

Conclusions

In conclusion, NILCO components and VEGF are differentially expressed in thyroid cancer and clinical features associated with poor outcomes are strongly associated with high NILCO gene expression levels. For the first time, we are reporting it can be a direct relationship between NILCO and thyroid cancer. Therefore, treatments to eliminate NILCO may lead to the development of new therapeutic interventions. Further research is needed to determine the biomarker and potential therapeutic values of NILCO and to target expression in thyroid cancer.

References

1) Han, J., et al., miR-215 suppresses papillary thyroid cancer proliferation, migration, and invasion through the AKT/GSK-3beta/Snail signaling by targeting ARF-GEF1. Cell Death Dis, 2019. 10(3): p. 195.
2) Matrone, A., et al., Differentiated Thyroid Cancer, From Active Surveillance to Advanced Therapy: Toward a Personalized Medicine. Front Endocrinol (Lausanne), 2019. 10: p. 884.
3) Pishdad, R., et al., Coexistence of Two Different Thyroid Malignancies: A Collision Phenomenon. Cureus, 2020. 12(4): p. e7539.
4) Piana, S., et al., Expression of NOTCH1 in thyroid cancer is mostly restricted to papillary carcinoma. Endocr Connect, 2019. 8(8): p. 1089-1096.
5) Gallo, C., et al., The bHLH transcription factor DEC1 promotes thyroid cancer aggressiveness by the interplay with NOTCH1. Cell Death Dis, 2018. 9(9): p. 871.
6) Kim, H.J., et al., The Role of Notch1 Signaling in Anaplastic Thyroid Carcinoma. Cancer Res Treat, 2017. 49(2): p. 509-517.
7) Yuan, L., et al., Relationship between the upregulation of Notch1 signaling and the clinical characteristics of patients with papillary thyroid carcinoma in East Asia: a systematic review and meta-analysis. Cancer Cell Int, 2019. 19: p. 5.
8) Lipsey, C.C., et al., Oncogenic role of leptin and Notch interleukin-1 leptin crosstalk outcome in cancer. World J Methodol, 2016. 6(1): p. 43-55.
9) Harbuzariu, A., et al., Leptin-Notch signaling axis is involved in pancreatic cancer progression. Oncotarget, 2017. 8(5): p. 7740-7752.
10) Battle, M., et al., Obesity induced a leptin-Notch signaling axis in breast cancer. Int J Cancer, 2014. 134(7): p. 1605-16.
11) Guo, S. and R.R. Gonzalez-Perez, Notch, IL-1 and leptin crosstalk outcome (NILCO) is critical for leptin-induced proliferation, migration and VEGF/VEGFR-2 expression in breast cancer. PLoS One, 2011. 6(6): p. e21467.
12) Daley-Brown, D., et al., Leptin-induced Notch and IL-1 signaling crosstalk in endometrial adenocarcinoma is associated with invasiveness and chemoresistance. World J Clin Oncol, 2019. 10(6): p. 222-233.
13) Daley-Brown, D., et al., Type II Endometrial Cancer Overexpresses NILCO: A Preliminary Evaluation. Dis Markers, 2017. 2017: p. 8248175.
14) Lobry, C., P. Oh, and I. Aifantis, Oncogenic and tumor suppressor functions of Notch in cancer: it's NOTCH what you think. J Exp Med, 2011. 208(10): p. 1931-5.
15) Nowell, C.S. and F. Radtke, Notch as a tumour suppressor. Nat Rev Cancer, 2017. 17(3): p. 145-159.
16) Gu, Y., M. Masiero, and A.H. Banham, Notch signaling: its roles and therapeutic potential in hematological malignancies. Oncotarget, 2016. 7(20): p. 29804-23.
17) Venkatesh, V., et al., Targeting Notch signalling pathway of cancer stem cells. Stem Cell Investig, 2018. 5: p. 5.
18) Kofler, N.M., et al., Notch signaling in developmental and tumor angiogenesis. Genes Cancer, 2011. 2(12): p. 1106-16.
19) Ferretti, E., et al., Notch signaling is involved in expression of thyrocyte differentiation markers and is down-regulated in thyroid tumors. J Clin Endocrinol Metab, 2008. 93(10): p. 4080-7.
20) Yamashita, A.S., et al., Notch pathway is activated by MAPK signaling and influences papillary thyroid cancer proliferation. Transl Oncol, 2013. 6(2): p. 197-205.
21) Geers, C., I.M. Colin, and A.C. Gerard, Delta-like 4/Notch pathway is differentially regulated in benign and malignant thyroid tissues. Thyroid, 2011. 21(12): p. 1323-30.
22) Polus A., P.E., Dembinska-Kiec A Differences in leptin, VEGF, and bFGF-induced angiogenic differentiation of HUVEC and human umbilical blood CD34+ progenitor cells. 2003.
23) Colbert, L.S., et al., NILCO biomarkers in breast cancer from Chinese patients. BMC Cancer, 2014. 14: p. 249.
24) Uddin, S., et al., Leptin-R and its association with PI3K/AKT signaling pathway in papillary thyroid carcinoma. Endocr Relat Cancer, 2010. 17(1): p. 191-202.
25) Catalano, S., et al., Evidence that leptin through STAT and CREB signaling enhances cyclin D1 expression and promotes human endometrial cancer proliferation. J Cell Physiol, 2009. 218(3): p. 490-500.
26) Gonzalez, R.R., et al., Leptin signaling promotes the growth of mammary tumors and increases the expression of vascular endothelial growth factor (VEGF) and its receptor type two (VEGF-R2). J Biol Chem, 2006. 281(36): p. 26320-8.
27) Eustatia-Rutten, C.F., et al., Survival and death causes in differentiated thyroid carcinoma. J Clin Endocrinol Metab, 2006. 91(1): p. 313-9.

Corresponding Author:
Bartu Badak
Department of General Surgery, Eskisehir Osmangazi University Medical Faculty, Meselik, Eskisehir, Turkey
Email: drbartu@gmail.com
(Turkey)