The Role of KISS1/KISS1R System in Tumor Growth and Invasion of Differentiated Thyroid Cancer

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Abstract. *Background/Aim: KISS1 protein and KISS1 receptor form a system that mainly promotes suppression of metastasis in various forms of cancer. We studied the relationship between KISS1/KISS1R expression and tumor progression in differentiated thyroid cancer (DTC). Materials and Methods: Thirty-three patients diagnosed with DTC were included in the study. Immunohistochemical cytoplasmic expression was evaluated for KISS1 and cytoplasmic/membranous expression for KISS1R in thyroid cancer tissues. Results: KISS1 expression was significantly higher in tumors with extrathyroidal invasion and advanced stage. KISS1R expression showed a statistically significant, moderate negative correlation with tumor size. Conclusion: Increased expression of KISS1 is possibly acquired to prevent further tumor invasiveness and formation of local or distant metastasis. It appears that malignant cells in DTC express increased levels of KISS1 as the tumor invades extrathyroidal tissues. Decreased expression of KISS1R seems to attenuate signaling of the KISS1/KISS1R system, possibly leading to tumor growth.*

Differentiated thyroid cancer (DTC), derived from thyroid follicular epithelial cells (1), is showing an increasing incidence worldwide (2). Age, gender, tumor size, multi-focality, oxyphilic variant, extrathyroidal extension and lateral node metastasis are known important factors for the prognosis of patients with DTC (3-9). However, despite earlier diagnosis and better therapeutic approaches, the mortality rate is increasing (10, 11). Extrathyroidal extension and metastatic disease are responsible for most DTC-related deaths. Immunohistochemical and molecular methods are focusing on the identification of new signatures and novel tests for more accurate staging to better determine clinical risk assessment and appropriate therapeutic strategies (12).

Tumor spreads and metastasis occurs when cancer cells disseminate from the primary site and colonize a different tissue microenvironment in a distant organ. *KISS1* was originally identified as a metastasis suppressor gene in melanoma cell lines, modulating tumor cell invasion and migration without affecting tumorigenicity (13). The human *KISS1* gene that maps to chromosome 1q32 consists of four exons and functions as a significant modulator of metastasis formation in various cancers (13-16). This gene encodes KISS1, a 145-amino-acid human precursor protein that is post-translationally processed outside of cell to Kisspeptin-54 that is further truncated to other amino acid carboxyl terminal fragments known as Kisspeptin-14, Kisspeptin-13 and Kisspeptin-10 (17). Kisspeptin-54 is a carboxy- terminal amidated peptide with 54 amino acid residues and is the ligand of a G-protein-coupled receptor known as KISS1-derived peptide receptor (KISS1R; previously designated as hOT7T175, GPR54, AXOR12). Kisspeptin-54 has agonist activity on KISS1R resulting in suppression of metastasis in human melanoma cells (18, 19). Furthermore, KISS1 is playing an emerged role in reproductive biology and benign diseases, such as hypogonadism (20), and is implicated in the pathophysiology of implantation and growth of ectopic endometrium in endometriosis (21).

Different mechanisms are implicated in the suppressive role of KISS1/KISS1R system regarding metastasis. A negative correlation exists between KISS1 and matrix metalloproteinase (MMP) activity, particularly MMP-9 and MMP-2 that are implicated in tumor invasion and metastasis (22-24). KISS1 also inhibits cellular processes, such as growth, proliferation and migration through the

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Key Words: KISS1, KISS1R, Kisspeptin, local metastasis, tumor growth, invasion, differentiated thyroid cancer.

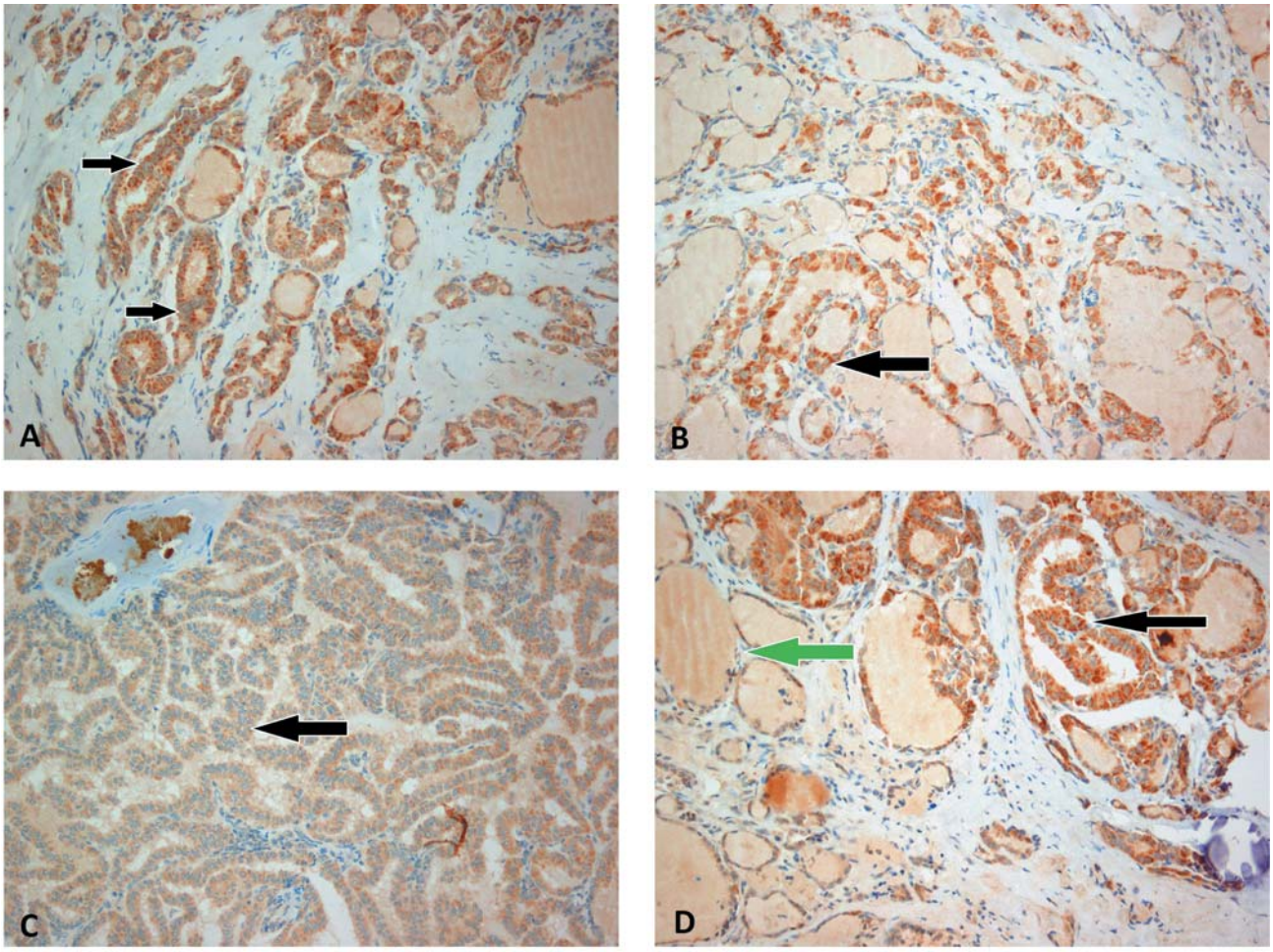

Figure 1. *A, B: Strong KISS1 immunohistochemical expression in differentiated thyroid cancer (DTC) (black arrow), (magnification ×200). C: Moderate KISS1 immunohistochemical expression in DTC (black arrow) (magnification ×200). D: Strong KISS1 immunohistochemical expression in DTC (black arrow) – no expression in normal thyroid (green arrow) (magnification ×200).*

activation of mitogen-activated protein kinase (MAPK) (25). Furthermore, Kisspeptin-10 inhibits tumor angiogenesis, a critical step of metastasis (26). KISS1R activation also may decrease the metastatic potential of cancer by increasing the expression levels of myocyte-enriched calcineurin interacting protein 1 (MCIP-1), an inhibitor of calcineurin, which promotes the vascular endothelial growth factor-regulated (EGFR) protein (27, 28). In addition, the anti-metastatic effect of KISS1 is possibly induced by increasing the expression of endothelial monocyte activating polypeptide II (EMAP-II) in tumor tissues (29). In the present study of 33 DTC cases, we show that immunohistochemical expression of KISS1 is significantly associated with extrathyroidal invasion and advanced stage and the corresponding expression of KISS1R is inversely correlated with tumor size.

Materials and Methods

Patients and sample selection. Thirty-seven paraffin tissue blocks from patients who underwent total thyroidectomy for histologically proven thyroid cancer between January 2009 and December 2009 were retrospectively selected. The samples were used in accordance with approval of the local Ethics Committee (National and Kapodistrian University, Medical School). Finally, 33 diagnosed with DTC patients were included in the study (papillary thyroid cancer (PTC) or follicular thyroid cancer (FTC); medullary and anaplastic carcinomas were excluded).

The patients ranged in age from 16 to 80 years old (mean age for women was 48±15.1 years, mean age for men was 50.9±12.9 years). None of the patients had received any radioiodine therapy or radiation therapy prior to surgery or any neoadjuvant chemotherapy or had prior medical history of the same or any other type of cancer or family history of thyroid cancer. A copy of the official pathology report from a certified pathologist was kept on record for all cases

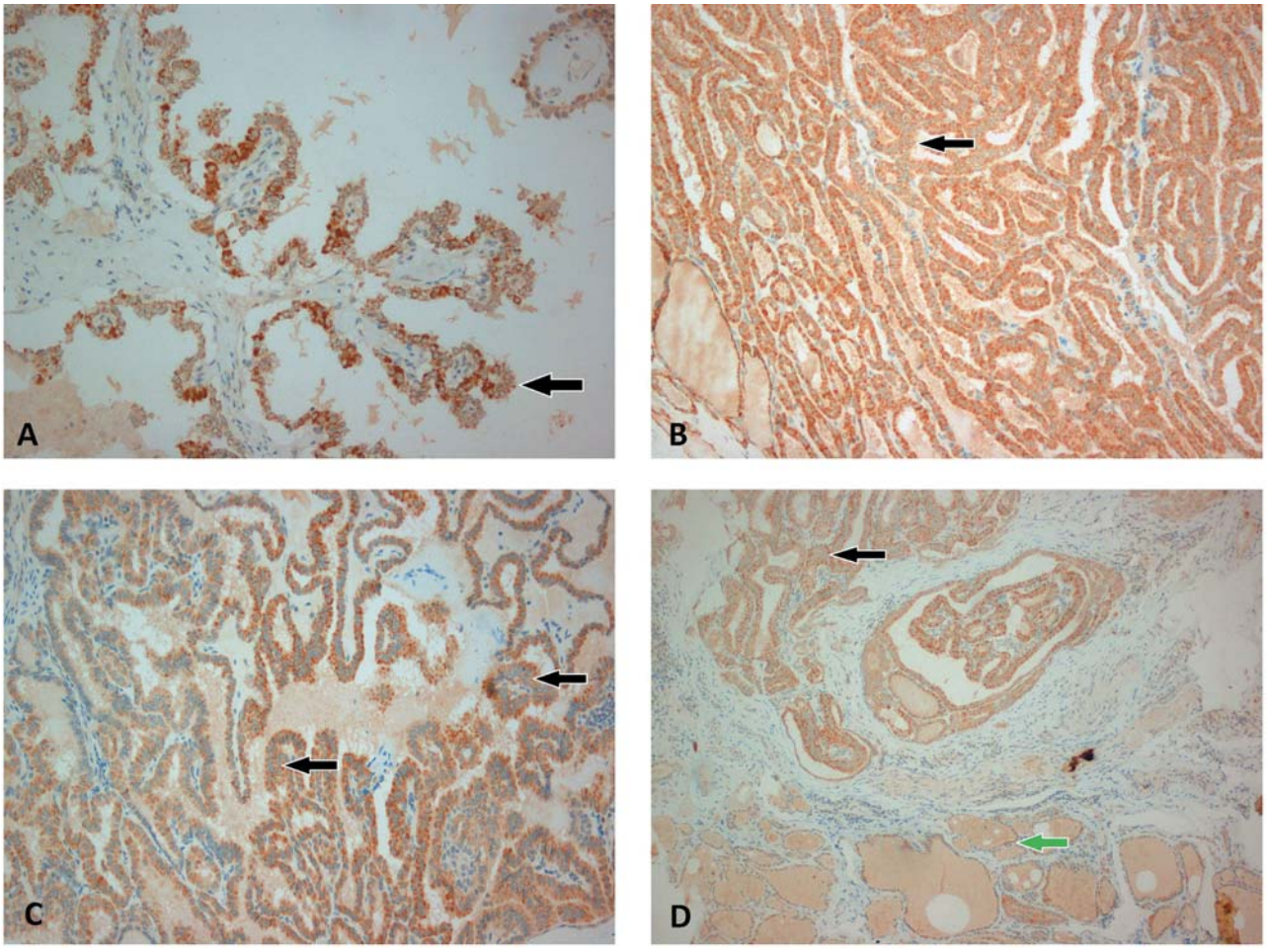

Figure 2. *A, B: Strong KISS1R immunohistochemical expression in differentiated thyroid cancer (DTC) (black arrow) (magnification ×200). C: Strong (right) and moderate (left) KISS1R immunohistochemical expression in DTC (black arrow) (magnification ×200). D: Strong KISS1R immunohistochemical expression in DTC (black arrow) – no expression in normal thyroid (green arrow) (magnification ×100).*

and all specimens were further re-evaluated and reviewed by two pathologists.

Immunohistochemical method. Sections from paraffin-embedded tissue blocks were de-paraffinized with xylene and rehydrated in a graded series of alcohols. The Bondmax automated system (Leica Microsystems, New Castle, Newcastle Upon Tyne, UK) was used for the immunohistochemical staining of samples using the KISS1 (FL-145) polyclonal antibody and the anti-GPR54 polyclonal antibody (Santa Cruz Biotechnology, Inc. Dallas, TX, USA) at 1:150 and 1:100 dilution, respectively. KISS1 (FL-145) is a rabbit polyclonal antibody raised against amino acids 1-145 representing full length KISS1 of human origin. Villous and extravillous trophoblast of the placenta were used as positive controls for both antibodies. Furthermore, negative controls were performed by omitting the primary antibody. Each slide was then evaluated independently by two blinded trained pathologists using intermediate power light microscopy (30). One representative tumor section was stained and evaluated per case.

Cytoplasmic expression was evaluated for KISS1 (Figure 1) and cytoplasmic/membranous expression for KISS1R (GPR54) (Figure 2). Immunohistochemical expression of stained proteins was classified as either grade 1 (weak intensity), grade 2 (moderate intensity) or grade 3 (strong intensity). The H scoring system, a model that incorporates both intensity and distribution of staining, was used for semiquantitative analysis of protein immunoreactivity. More specifically, the percentage of positive cells was measured in every section and multiplied by 1, 2 and 3, respectively (grade 1 score, percentage with grade 1 expression ×1; grade 2 score, percentage with grade 2 expression ×2; grade 3 score, percentage grade 3 expression ×3). A total score between 0 and 300 was obtained for each case (total score, grade 1 score + grade 2 score + grade 3 score).

Statistical analysis. All statistical analyses were performed using the SPSS 22.0 (IBM©, SPSS©, Statistics, version 22, release 22.0.0.0, 64bit; http://www-01.ibm.com/software/analytics/spss/products/statistics/). The One-Sample Kolmogorov-Smirnov Test was applied

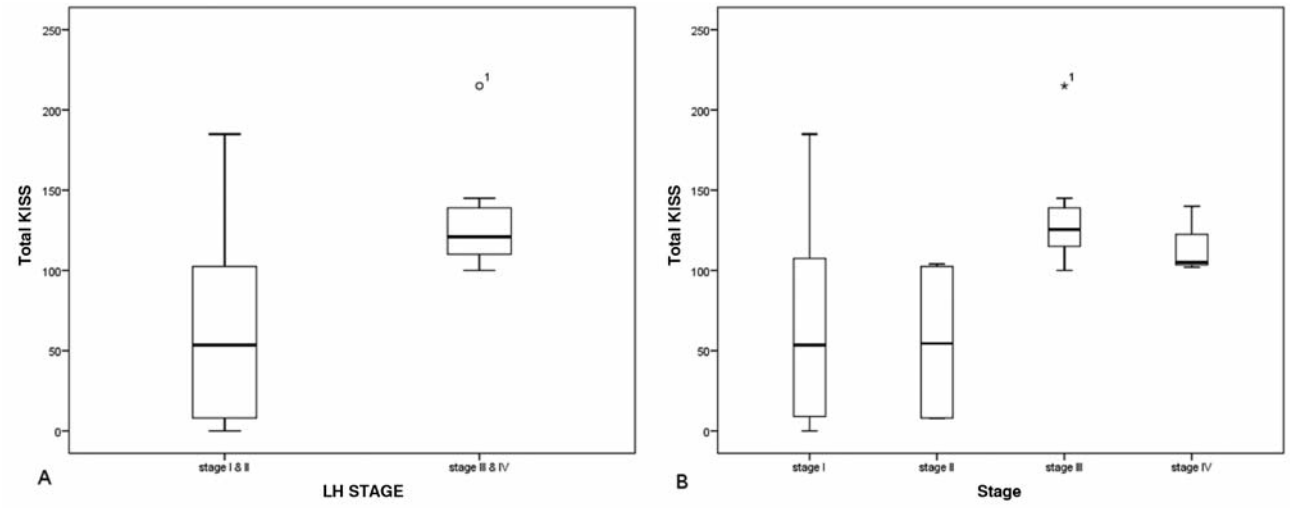

Figure 3. *A. Comparison analysis showed that median KISS1 expression was significantly higher in advanced stage (Stage III and IV) compared to early-stage thyroid tumors (Stage I and II) (p=0.000). B. Multiple comparison post-hoc analysis showed statistically significant higher expression levels of KISS1 in Stage III compared to stage I (p=0.008) and between all Stages (p=0.011). (TOTAL KISS, KISS1 expression; LH STAGE, low-high stage).*

Table I. *KISS1 and KISS1R expression status and other clinicopathological variables of the study.*

Variable	Results	Association with KISS1 expression	Association with KISS1R expression
No of patients (Age-years±SD)	33 (48±15.1)	r=0.283, *p*=0.111	r=0.692, *p*=0.083
Gender			
Females	25 (47.2±15.8)	*p*=0.674	*p*=0.403
Males	8 (50.9±12.9)		
Uni-/Multifocal			
Unifocal	17	*p*=0.428	*p*=0.751
Multifocal	16		
Tumor size (mm)- mean±SD	26±24	r=–0.115, *p*=0.526	r=–0.463, *p*=0.02*
Lymph node metastasis			
Negative	26	*p*=0.724	*p*=0.944
Positive	7		
Vessels invasion			
Negative	31	*p*=0.597	*p*=0.877
Positive	2		

*Significant at the 0.05 level.

to test the normality of distribution in all continuous variables and the choice of methods for statistical testing of continuous variables was based on whether the data permitted parametric or non-parametric analysis. The correlation between two different continuous and/or categorical ordinal variables was assessed by the Pearson correlation coefficient for continuous variables subjected to parametric analysis; the Spearman and Kendall for non-parametric analysis. Differences of KISS1 and KISS1R expression between two groups were performed using the *t*-test and Mann-Whitney-Wilcoxon test, respectively. The Kruskal–Wallis and Welch with Brown- Forsythe one-way analysis of variance was used to assess for differences between more than two groups. A p-value <0.05 was considered statistically significant; p-values of *post hoc* paired comparisons were adjusted with the Bonferroni method.

Results

Clinicopathological characteristics. Staging was performed in accordance with the American Joint Committee on Cancer (AJCC) (31). Sixteen of the 33 DTC (48.5%) were classified as stage I, 4 (12.2%) as Stage II, 10 (30.3%) as Stage III and 3 (9%) as Stage IVA. Patients' clinicopathological data are listed in Table I. Thirty-three patients with DTC were included in the study, 25 were female and 8 were male. Seventeen of 33 were solitary thyroid cancers and 16 were multifocal. The mean tumor size was 26mm (±24 mm). Lymph node metastasis was present in 7 patients, vessel

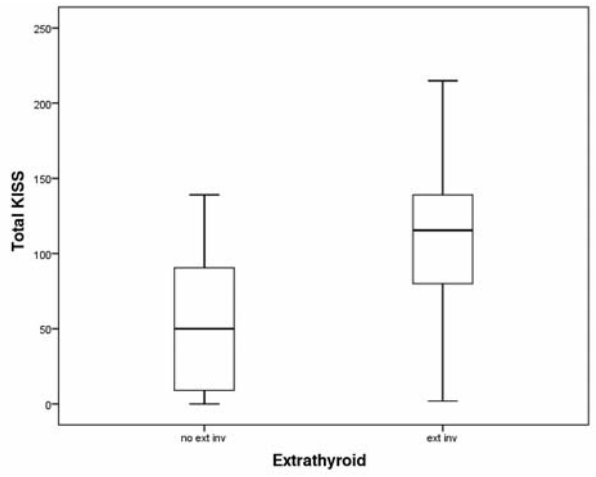

Figure 4. *KISS1 expression was significantly higher in tumors that were extended beyond the thyroid gland capsule when compared with tumors that were not extended beyond the thyroid gland capsule (p=0.012). (TOTAL KISS, KISS1 expression; ext inv, extrathyroidal invasion).*

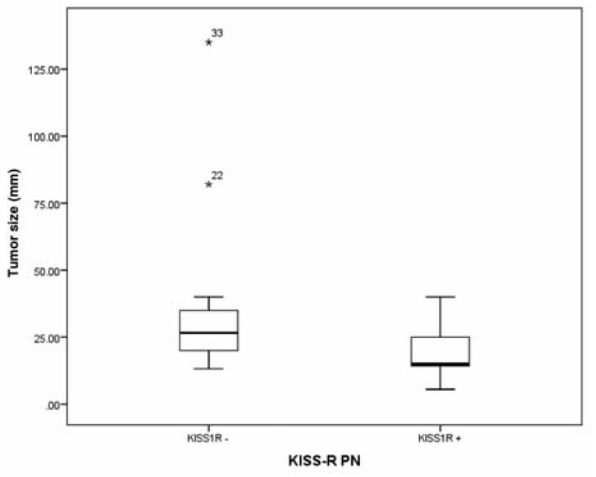

Figure 5. *KISS1R positive IR was associated with smaller DTC tumors (p=0.046). (IR, Immunoreactivity; DTC, Differentiated Thyroid Cancer; asterisks represent outliers (cases that fall well outside the range of the other values).*

invasion in 2 patients and extrathyroidal invasion was found in 22 patients.

KISS1 expression is correlated with extrathyroidal invasion and advanced stage of disease. For the 33 cases of thyroid tissue immunohistochemistry, KISS1 immunoreactivity (IR) was scored between 0 and 215 (mean=89.85±57.81). Staining was scored as 0 for negative cases (IR=0), +1 for mild staining (IR=1-39) and +2 for intense staining (IR≥40). Of the 33 cases, 26 had +2 staining intensity, 6 stained at +1 and 1 case was negative for kisspeptin-IR. The negative (0) and mildly reactive (+1) cases were grouped for additional statistical analysis and assigned the designation 0 and considered KISS1-negative, while the +2 cases were considered KISS1-positive and designated as 1.

KISS1 expression did not significantly differ between different genders (p=0.674). There was no significant association between KISS1 expression and patient's age (r=0.283, p=0.111) and no significant association between KISS1 expression and tumor size (r=-0.115, p=0.526). Presence or absence of lymph node metastases or infiltration of adjacent vessels did not significantly affect KISS1 expression levels (p=0.724 and p=0.597 respectively).

Comparison analysis showed that median KISS1 expression was significantly higher in advanced-stage (Stage III and IV) compared to early-stage thyroid tumors (Stage I and II) (p=0.000) (Figure 3A). In addition KISS1 positive IR was associated with advanced stage of DTC (Stages III and IV) (p=0.027). In Stage I and II of DTC there were 7 KISS1 negative and 13 KISS1 positive cases.

Table II. *Descriptive statistics and comparisons of KISS1 expression.*

TOTAL KISS1	N	Mean	Std. Deviation	*p*-Value
Stage I and II	20	64.60	57.922	p=0.000**
Stage III and IV	13	128.69	30.062	
Stage I	16	66.94	60.207	I *vs*. III, p=0.008**
Stage II	4	55.25	54.573	
Stage III	10	132.60	32.129	
Stage IV	3	115.67	21.127	
Total	33	89.85	57.813	p=0.011*
no ext inv	11	54.64	46.680	p=0.011*
ext inv	22	107.45	55.509	

(TOTAL KISS, KISS1 expression; ext inv, extrathyroidal invasion). *Significant at the 0.05 level. **Significant at the 0.01 level.

In Stage III and IV of DTC there were 13 KISS1 positive cases and no negative. Further post hoc multiple comparison analysis between every stage of DTC showed statistically significant higher expression levels of KISS1 in Stage III compared to Stage I (p=0.008) and between all stages (p=0.011) (Figure 3B).

Of the 33 DTC cases, 11 were found with tumors that were confined in the thyroid gland and 22 were identified with tumors that were extended beyond the thyroid gland capsule. However, KISS1 expression was significantly higher in tumors with extrathyroidal invasion when compared to tumors that were not extended beyond the thyroid gland capsule (p=0.012) (Figure 4) (Table II).

KISS1R expression is inversely correlated with tumor size. For the 33 cases of thyroid tissue immunohistochemistry, KISS1R immunoreactivity (IR) was scored between 0 and 52 (mean, 7.43±11.15). Staining was scored as 0 for negative cases (IR=0), +1 for mild staining (IR=1-5) and +2 for intense staining (IR>6). Of the 33 cases, 9 had +2 staining intensity, 7 stained at +1, 7 cases were negative for KISS1R-IR and 10 cases were uninterpretable. The negative (0) and mildly reactive (+1) cases were grouped for additional statistical analysis and assigned the designation 0 and considered KISS1R negative, while the +2 cases were considered KISS1R positive and designated as 1.

KISS1R expression did not differ significantly between different genders (p=0.403). There was no significant association between KISS1R expression and patient's age (r=0.692, p=0.083). KISS1R expression showed a statistically significant, moderate negative correlation with tumor size (r=–0.463, p=0.02). In addition KISS1R positive IR was associated with smaller DTC tumors (p=0.046, Figure 5).

Presence or absence of lymph node metastases, infiltration of adjacent vessels and extrathyroidal invasion did not significantly affect KISS1R expression levels (p=0.944, p=0.877 and p=0.398, respectively). In addition there were no statistically significant differences in KISS1R expression between advanced stage (Stage ≥III) as compared to early stage thyroid tumors (Stage ≤II) (p=0.91) (Table II).

Discussion

It is well-recognized that metastasis, the major cause of cancer-related deaths, is due to a subset of cells that have left the primary tumor and are often behaviorally distinct from cells remaining at the site of tumor origin (32). KISS1 expression may suppress the metastatic potential of cancer cells, demonstrating its suppressive potential on metastasis in the majority of cancers, without affecting tumorigenicity. In addition reduced expression of KISS1 is correlated with poor clinical outcome (33).

The objective of the present study was to evaluate the utility of KISS1 and KISS1R immunohistochemistry in predicting tumor progression and local metastasis. We found that KISS1 expression levels are significantly higher in advanced staged tumors (Stage ≥III) when compared to first stages (Stage ≤II) of thyroid cancer (p=0.000). Furthermore, increased KISS1 expression was found in tumors with extrathyroidal invasion (p=0.012). We also found that KISS1R expression negatively regulates tumor size since there is a statistically significant, moderate negative correlation with tumor size (r=–0.463, p=0.02). This result indicates that reduced expression of the receptor possibly facilitates tumor growth. Several limitations regarding the sample size of the present study should be acknowledged. In addition, because the number of patients diagnosed with distant metastasis of DTC is comparatively rare and rarely biopsied, no metastatic lesions were included in order to examine the expression of KISS1/KISS1R in metastatic sites compared to primary tumors.

To the best of our knowledge, few studies have thus far evaluated the role of KISS1 system in thyroid cancer (25, 27). Ringel *et al*. demonstrated higher levels of expression of KISS1R in papillary cancers compared to follicular cancers, with the latter known to have a greater metastatic potential (25). Furthermore, Stathatos *et al*. showed that exposure to Kisspeptin-54 inhibited the growth and migration capabilities of thyroid cancer cells (27). In the present study, although KISS1 expression was not clearly associated with any clinicopathological or demografic factors (age, gender, tumor size, lymph node metastases or infiltration of adjacent vessels), increased KISS1 expression was shown to correlate significantly with extrathyroidal invasion and advanced stage (Stage ≥III). However, it seems that cancer cells in DTC express more KISS1 as the tumor increases in diameter and/or invades in extrathyroidal tissue leading to regional metastasis. These results indicate a correlation between KISS1 expression and thyroid cancer progression.

Another result of the present study was that KISS1 expression was significantly higher in tumors that have spread to extrathyroidal tissue when compared to earlier stages of metastatic process like cell detachment and migration from the primary tumor but still confined in the thyroid gland. The present findings suggest that expression of KISS1 is positively correlated with extrathyroidal tumor invasion and indicate that gain of expression of the *KISS1* gene may play an important role in thyroid cancer progression. Given the known tumor suppressive role of KISS1 expression, it is possible that a gain of expression of KISS1 gene is acquired to prevent further tumor expansion and formation of distant metastases. Since there is an up-regulation of KISS1 expression in extrathyroidal tumors, a suppressive role of KISS1/KISS1R at first stages of extrathyroidal cancer progression is possible in order to prevent distant metastasis. The expression of KISS1 may be increased to down-regulate the metastatic potential of DTC possibly through the decreased growth and migration capabilities of thyroid cancer cells.

Another finding is that the expression of KISS1R is decreasing as the tumor is growing. Consequently the binding and activation of KISS1R by kisspeptins cannot lead to an effective KISS1/KISS1R signaling. Attenuation of this signaling possibly promotes tumor growth of DTC. Thyroid cancer cells may acquire an effective system to inhibit the potency to metastasize and loss of this regulatory mechanism has been associated with cancer progression. The expression of these transcripts may represent a paracrine and/or autocrine response to the cancer.

There is increasing interest to use KISS1 expression as a prognostic marker of cancer (34). Kisspeptin could be used as a useful biomarker in the identification of cancers with increased metastatic ability and for the early detection of micrometastatic extrathyroidal invasion. Our results also suggest that KISS1 expression may be a useful tool for classification of thyroid cancer and that gain of KISS1 expression could be a powerful prognostic indicator in thyroid cancer patients. As such, KISS1 and KISS1R seem to modulate the metastatic potential of cancers cells, rendering these agents as promising targets for novel and effective therapeutic agents in the field of cancer treatment, especially in KISS1R-positive cancers. They could also be used as inhibitors of micrometastatic growth leading to prevention of malignant relapse. Further investigational studies are needed to comprehend changes in the expression profile of KISS1 and KISS1R at the gene and protein level for the potential tumor suppressor role of KISS1 in DTC that could serve as a mechanism against migration when cancer cells attempt to metastasize. Also, more studies are required in order to investigate whether patients with increased KISS1 levels should be administered different treatment regimens. The gain of KISS1 appears to play an important role in the progression of extrathyroidal invasion metastasis and loss of KISS1R seems to promote tumor growth.

In conclusion, our study adds further evidence regarding the role of KISS1 and KISS1R in the progression of thyroid cancer. This study revealed a significant relationship between KISS1 expression and extrathyroidal invasion and advanced stage and an inverse correlation between KISS1R and tumor size. Further investigational studies are required to better elucidate the role of the Kisspeptin system as a regulator of tumor growth and extrathyroidal invasion in DTC.

Conflicts of Interest

There are no conflicts of interest and the Authors received no specific funding for this article.

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