ORIGINAL PAPER

Expression of hepatocyte growth factor in Hashimoto's thyroiditis with nodular lesions

R.M. Ruggeri,[1] S. Sciacchitano,[2,3] F. Trimarchi,[1] G. Barresi,[4] M. Trovato[4]

[1]Dipartimento Clinico-Sperimentale di Medicina e Farmacologia, Sezione di Endocrinologia, Policlinico Universitario G. Martino, Università di Messina, Messina; [2]Ospedale San Pietro Fatebenefratelli-Associazione Fatebenefratelli per la Ricerca (AFaR), Rome; [3]Dipartimento di Medicina Sperimentale e Patologia, II Facoltà di Medicina, Università La Sapienza, Rome; [4]Dipartimento di Patologia Umana, Policlinico Universitario G. Martino, Università di Messina, Messina, Italy

Hashimoto's thyroiditis (HT) is an autoimmune thyroid disease frequently associated with hyperplastic nodules (HN)s. Hepatocyte growth factor (HGF) is expressed in benign thyroid nodules and over-expressed in malignant thyroid nodules, particularly in papillary thyroid carcinomas. To elucidate the role of HGF in the development of HNs in association with HT we evaluated, by immunohistochemistry, the expression of HGF in both nodular and extranodular tissues, obtained from 30 HTs and 15 goiter samples. Six normal thyroid glands were used as controls. All normal control tissue samples exhibited no evidence of HGF immunoreaction. HNs showed weak to moderate HGF immunoreaction, which was located exclusively in the cytoplasm of stromal cells (fibroblasts and endothelial cells). However, the percentage of positive cases was higher in HNs arisen in the context of HT, compared to HNs not associated with HT (30/30 or 100% vs 4/15 or 40%; $p<0.001$). HGF immunoreactivity was also detected in all extranodular tissues from HT specimens (30/30 or 100%), but we found some significant differences. In fact, while in HNs observed in the context of HT lesions HGF was expressed only in stromal cells, in the extranodular tissues from the same thyroid gland affected by HT it was also detected in the cytoplasm of the epithelial follicular cells. Furthermore, HTs showed a much higher HGF staining grade in the extranodular tissue compared to HNs. Finally, a clear positive correlation was observed in HT between the proportion of HGF expressing follicular cells and the grade of lymphoid aggregates of the thyroid gland. In conclusion, HGF is much more frequently and highly expressed in thyroid tissue with HT, compared to goiter. In HT glands HGF can be detected in both follicular thyroid cells and stromal cells, while in HNs, either from goiters or associated with HT, its expression is restricted only to the stromal cells. These data indicate that HGF may play a role in cell proliferation processes occurring in thyroid glands affected by HT, probably under the regulation of the lymphoid infiltrate.

Key words: hepatocyte growth factor, c-met, hashimoto's thyroiditis, hyperplastic thyroid nodules.

Correspondence: Dr. Maria Trovato,
Dipartimento di Patologia Umana, Policlinico Universitario di Messina, Padiglione D, 4° piano 98125 Messina, Italy
Tel. +39.090.2212343.
Fax +39.090.2212523.
E-mail: mariatrovato@tin.it

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Hashimoto's thyroditis (HT) is part of a large spectrum of diseases, usually classified as Autoimmune Thyroid Diseases (AITD). The various types of AITD are characterized by variable behavior with respect to thyroid cell proliferation. In the AITD associated with large goiter, the thyroid follicular proliferation may be very intense, and it can be even associated with occult foci or macroscopic areas of malignant transformation (Weetman AP *et al.*, 1994; Gasbarri A *et al.*, 2004). On the other side, in the AITD associated with atrophy, thyroid cell proliferation is virtually absent, with marked destruction of cells and follicles, but intense proliferation of stromal cells is the predominant feature, leading to fibrosis (Weetman AP *et al.*, 1994; Dayan CM *et al.*, 1996). The clinical presentation of HT associated with goiter consists in a spread bilateral rubbery enlargement of the thyroid gland, with one or more true nodular lesions arising in the context of HT, and characterized by the appearance of hyperplastic nodules (HN) at cytological examination (Hayashi N *et al.*, 1986; Jayaram G *et al.*, 1987; Li Volsi, 1990; Bidey SP *et al.*, 1999).

Few reports have investigated the role of different growth factors and cytokines in the induction of thyroid follicular growth in those clinical conditions in which HT is associated with nodules (Ruggeri *et al.*, 2006). The hepatocyte growth factor (HGF), recognized as a potent mitogen for hepatocytes and exerting mitogenic, motogenic, morphogenic, antifibrotic, and antiapoptotic activities in various cell types, may be implied in inducing goitrous growth (Nakamura *et al.*, 1984; Nakamura *et al.*, 1989; Bottaro *et al.*, 1991; Birchmeier *et al.*, 1998). In fact, HGF is expressed in benign thyroid hyperplastic lesions, over-expressed in papillary thyroid carcinomas (PTC) but it is not expressed in normal thyroid tissue (Trovato M *et al.*, 1998; Scarpino S *et al.*, 2003; Mineo R *et al.*, 2004; Vesel˘ D *et al.*, 2004). Furthermore, we previously described a different localization of HGF expression in PTC compared to HN. On the basis of this evidence, we have indicated different sites of HGF production. The observed HGF localization on the stromal cells inside the HN indicates that these glandular stromal cells may constitute the natural sites of thyroid HGF production. On the contrary, in PTC the HGF expression on tumour epithelial cells is probably due to an aberrant switch of the production site occurring in thyroid malignant lesions (Trovato *et al.*, 1998).

In this study we focused our attention on the immunoexpression of HGF in goitrous HT lesions to evaluate its role in the pathogenesis of both HT and goitrous nodules associated with HT.

Materials and Methods

Tissues Collection

Thyroid tissue specimens were retrieved from the archives of the Department of Human Pathology, University of Messina, Italy. They included 6 normal thyroid glands removed at autopsy and 45 thyroid surgical samples taken from patients subjected to total or sub-total thyroidectomy for large nodular goiters. Patients were recruited at the Endocrinology Unit of the University of Messina. In 30 cases (25 females and 5 males; mean age ± SD: 52±8) a diagnosis of HNs associated with HT was made, while in 15 cases (13 females and 2 males; mean age ± SD: 55±13) only a HN with no clinical, laboratory or ultrasonographic evidence of AITD was found. All patients, were euthyroid at the time of the thyroidectomy, either spontaneously or under levo-thyroxine therapy (TSH values were 0,9 ± 0,53 μIU/mL; range 0,1 to 1,9). The 45 thyroid tissue samples were studied comparing the nodular and the extranodular tissues in the same patient. The HT lesions were classified at the histological diagnosis according to the criteria proposed by Doniach & Roitt and Li Volsi, and studied paired with the associated nodules (Doniach *et al.*, 1988; Li Volsi, 1990).

Thyroid samples were fixed in 4% formalin and routinely processed through graded alcohol and xylene to paraffin wax. Paraffin blocks of each sample were cut into 5-μm serial sections to perform Haematoxylin-Eosin (H&E) stain and immunohistochemistry.

According to morphological features of nucleus and cytoplasm, the epithelial follicular cells were classified into 3 different types, as previous described (Trovato *et al.*, 2004): 1) dark nucleus and eosinophilic cytoplasm (DN-EC); 2) clear nucleus and eosinophilic cytoplasm (CN-EC); dark nucleus and oncocytic cytoplasm (DN-OC, or Hurtle cells).

The intra-glandular inflammatory lymphoid aggregates have been evaluated as previously reported (Ruggeri *et al.*, 2006). The presence of a lymphoid aggregate was reported when at least, 150 lymphocytes and a variable number of plasma cells per high-power field were observed. The typical appearance of a lymphoid aggregate with germinal center consisted in a lymphoid aggregate arranged into well-developed follicular centers with central macrophages-like cells showing large-clear cytoplasmic appearances. The lymphoid aggregates were graded as follows: 0 = no lymphoid aggregate or at most one single, small lymphoid aggregate without germinal center in each section; I = occasional, usually small lymphoid aggregates with rare or absent germinal centers in each section; II = several, usually mixed, small and large lymphoid aggregates with some germinal center in each section; III = numerous, large lymphoid aggregates with frequent germinal centers in each section.

Immunohistochemistry

Immunocytochemistry was performed, separately, by the rabbit polyclonal antibody against HGFα (H-145, 1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Tissue sections were deparaffinized in xylene and rehydrated by serial passages in graded alcohol. Endogenous biotin (EB) was inactivated by addition of 0.05% (v/v) solution of streptavidin in phosphate–buffered saline (PBS), and endogenous peroxidase activity was blocked by incubation of the slides in a 0.3% v/v solution of 3% H_2O_2/methanol for 30 min. Staining was obtained with a standard labelled biotin-streptavidin-peroxidase method (LSAB kit from Dako, Carpinteria, CA, USA). The colour reaction was developed using 3,3′ diaminobenzidine (DAB) as chromogen. The slides were counterstained with Mayer's haematoxylin, dehydrated and mounted. Specificity was assessed by omitting the primary antiserum or by replacing the primary antiserum with normal goat or rabbit serum. In each of these conditions, no staining was evident.

Table 1. Lymphoid aggregates grade and expression of HGF in hyperplastic nodules and Hashimoto's thyroditis.

		Lymphoid aggregate grade	*Positive cases*	*Staining cellular localization*				*Staining score*				
				DN-EC	*DN-OC*	*CN-EC*	*Stromal cells*	*Absent*	*Weak but distinct*	*Moderate*	*Intense*	*Very intense*
Normal thyroid (n=6)		0	0/5	0	0	0	0	5	0	0	0	0
Nodular Goiter (n=15)	Hyperplastic nodules (n=15)	0	4/15	0	0	0	13±6	11	2	2	0	0
	Extranodular tissue (n=15)	0	0/15	0	0	0	0	15	0	0	0	0
Hashimoto's	Hyperplastic nodules (n=30)	0	30/30	0	0	0	12±8	0	10	20	0	0
Thyroiditis			30/30	57±19	24±24	21±14	11±8	0	0	12	8	10
(Nodular Variant) (n=30)	Extranodular Tissue (n=30)	I (n=12)	12/12	38±9	5±9	13±11	14±10	0	0	8	3	1
		II (n=15)	15/15	68±12	40±24	27±14	10±6	0	0	4	5	6
		III (n=3)	3/3	80±0	20±0	20±0	5±0	0	0	0	0	3

**The proportion of positive cells was calculated based on evaluation of 1000 epithelial cells using 50X magnification. Semiquantitative grading of immunostained cells distribution was scored as described under Materials and Methods.*

An immunoabsorption test was performed to confirm the specific immunoreactivity of the antibody. Liver specimens were used as positive controls of the HGF immunoreaction. Moreover, some frozen sections of the normal thyroid tissues, HN and HT included in this study were used as control for the HGF immunohistochemical reaction.

Histological and immunohistochemical evaluations were performed twice and blindly by two different pathologists (MT, GB), with an inter-observer concordance of nearly 100%. Where minimal inter-observer discrepancies were present, the mean value was considered as the result. For the evaluation and comparison of the results, the following criteria were used: a) number of positive cases; b) site of immunostaining: epithelial or stromal; c) number of positive epithelial cells per case, based on counting 1000 cells using a 50X magnification; and d) semiquantitative grading of staining using a scored system from 0 to 4+ (0 = absent; 1+ = weak but distinct; 2+ = moderate; 3+ = intense; 4+ = very intense).

Statistical analysis

Once tested for normal distribution and variance, data (mean ± standard deviation) were analyzed by the two-tailed Student's *t*-test, χ^2 test with Yates' correction for continuity and linear regression analysis. The level of statistical significance was always set at $p<0.05$.

Results

Histopathology

All 45 HNs showed histological features of HN and the cellular type more frequently observed showed DN-EC features. The majority of HNs were characterized by either large or small follicles, filled with colloid and composed of small flat or cubic cells, with dark nuclei and eosinophylic cytoplasm (DN-EC). Others showed cystically dilated follicles, resulted in a papilliferous structure called *Polster di Sanderson*. No intranodular lymphoid aggregates were observed (grade 0) in any HN, either HT or non-HT associated (Table 1).

The HT showed small follicles with scarce, dense, pink colloid delimitated by cuboidal follicular cells. DN-EC cells were the major cell type present in all HT, but in most cases DN-OC cells, and occasionally CN-EC cells, were also evident. Lymphoid aggregates were graded as I (12/30 or 40%), II (15/30 or 50%) or III (3/30 or 10%) (Table 1).

Immunohistochemistry

HGF immunohistochemical reaction was similar in both frozen and paraffin sections. HGF immunostaining was not detected in normal thyroid tissues such as the control normal thyroids and contralateral lobe to the 15 HNs CN (Table 1).

All HN showed a weak or moderate HGF immunoreaction, which was located exclusively in the cytoplasm of stromal cells, such as fibroblasts

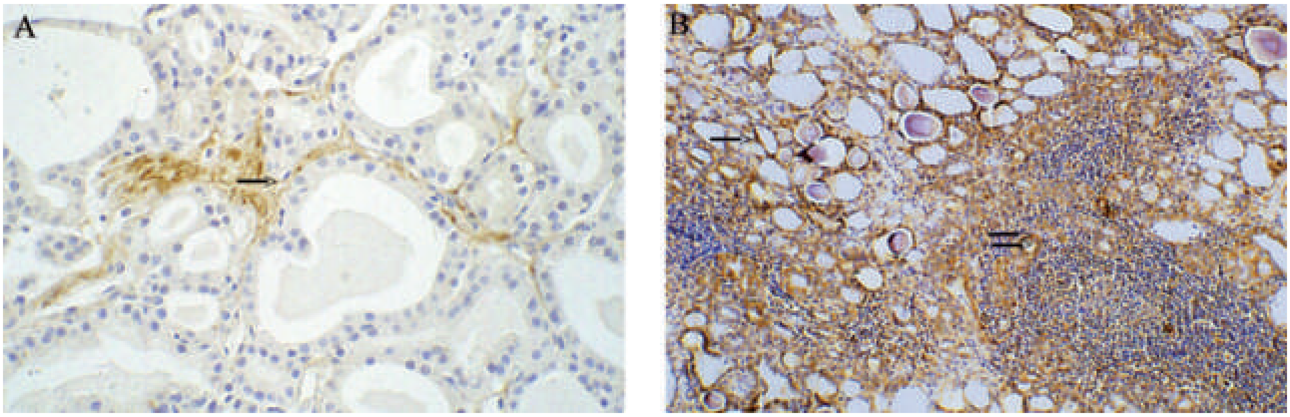

Figure 1. HGF immunostaining in a sample of hyperplastic nodule and Hashimoto's thyroiditis. Panel A: moderate HGF immunoreaction on stromal cells (fibroblasts and endothelial cells) of a hyperplastic nodule (positive cells appear brown). (Original magnification: 250 X). Panel B: intense HGF immunostaining in a sample of Hashimoto's thyroiditis. The immunoreaction is located in the cytoplasm of epithelial cells (brown deposits in cytoplasm) showing cytological features of follicular cells with DN-EC (arrow) or CN-OC (double arrow). Note the absence of HGF reactivity in the cytoplasm of lymphocytes (Original magnification: 150 X).

and endothelial cells (Table 1) (Panel A of Figure 1). However, the expression of HGF was recognized more frequently in HNs associated with HT compared to HNs not associated with HT (4/15 or 40% vs 30/30 or 100%; p<0.001) (Table 1).

Similarly to HN, all HT showed HGF immunostaining, too. Nevertheless, significant differences were observed when we compared the HGF immunoreaction in HN and HT lesions. In fact, in HT, apart from the stromal cells, HGF was expressed in the cytoplasm of epithelial follicular cells with DN-EC, DN-OC and CN-EC features (Table 1) (Panel B of Figure 1). Further, the HGF staining grade observed in HT was higher than in HN, because it was ranging from moderate to very intense in HT while it was always moderate in HN.

In HT lesions, HGF immunoexpression was detected mostly in the DN-EC cells (Table 1). In fact, the proportion of HGF+ DN-EC cells overcame those of HGF+ DN-OC, CN-EC and stromal cells, respectively, (p<0.001, in all cases). Further, considering the grade of lymphoid aggregates of HT, the proportion of DN-EC+ cells was positively correlated with the increase of lymphoid aggregates grade.

Discussion

Nodular lesions frequently arise in the context of HT, and are due to the compensatory growth of new follicles in response to the follicular destruction caused by the autoimmune reaction (Li Volsi, 1990). It is well known that the main cause of the simple goiter is an abnormal follicular stimulation by the increase in TSH serum levels, but there is a long list of growth factors and cytokines possibly involved in the development of goiter. Nevertheless, few reports have investigated the role of such different growth factors and signaling systems in the induction of thyroid follicular growth, and specifically in those clinical conditions in which nodular goiter is associated with HT (Ruggeri *et al.*, 2006). In the present study, we analyzed HGF expression as a possible new marker of the nodular hyperplasia associated with HT.

The putative role of HGF in inducing thyroid cellular growth is based on our previous observations (Trovato *et al.*, 1998; Trovato *et al.*, 1999, Trovato *et al.*, 2003). In these previous studies we demonstrated the presence of HGF expression in goitrous samples and its absence in normal thyroid tissue. In our present study, we were able to confirm the absence of HGF expression in normal thyroid tissue by using true normal thyroid glands removed at autopsy. In addition, we found that HGF is more frequently expressed in HNs when they are found in the context of HT compared to those detected in goiter showing no evidence of HT. This result suggests a role of HT background in the development of HNs. However, the localization of HGF inside the nodular lesions is independent from the presence of HT and is always detected in stromal cells (Trovato *et al.*, 1998; Trovato *et al.*, 1999, Trovato *et al.*, 2003).

Conversely, a different localization of HGF expression was found when we compared, in the same thyroid gland affected by HT, intranodular versus extranodular tissue samples. In fact, inside the HN, the expression of HGF was only found in stromal cells, whereas, in HT areas outside the nodule, the HGF immunoreaction involved both stromal and epithelial cells.

The distinction we made of the follicular epithelial cells of HT, according to the nuclear and cytoplasmic features, (see Materials and Methods section), allowed us to identify the subgroup of follicular cells showing DN-EC features as the cell type more frequently involved in the epithelial pathway of HGF. In these DN-EC cells we found a positive correlation between the HGF expression and the degree of lymphocytic infiltration. In fact, the number of HGF reactive DN-EC cells increases in HT cases showing a lymphoid aggregates grade II or III with respect to grade I. These data suggest that in HT the lymphocytes infiltrate could play a role in inducing HGF expression on DN-EC follicular cells.

The switch of HGF expression from stromal to epithelial cells has been previously described in two malignant thyroid lesions, namely, PTC and medullary thyroid carcinomas (MTC) (Trovato *et al.*, 1998; Trovato *et al.*, 1999, Trovato *et al.*, 2003). Considering all these studies we detected HGF expression in DN-EC cells of HT, CN-EC cells of PTC and malignant C cells for MTC. The cell-type specific restriction of HGF immunoreaction led us to speculate on the ability of HGF to activate proliferative pathways, leading to benign or malignant proliferation only in such type of cells. Regarding the HGF reactivity observed in the DN-OC cells of HT, we found this observation in contrast with our previous results concerning the absence of HGF epithelial expression in oncocytic adenomas (Trovato *et al.*, 1998; Trovato *et al.*, 1999, Trovato *et al.*, 2003). This discrepancy may be due to the fact that, in the context of HT, the cells with DN-OC features, as well as those with CN-EC appearances, arise from a process of cellular metaplasia; whereas, in the context of oncocytic adenomas, the DN-OC cells are the product of a nodular benign proliferation. Further specific studies are necessary to identify the molecular mechanisms leading to the metaplasia or tumor growth.

Our results reinforce the hypothesis that common and overlapping molecular mechanisms occur in the development of the hyperplastic and neoplastic nodular lesion in the context of thyroid chronic autoimmune inflammation. In this regard, it is worthy to note that many molecular alterations, typically associated with thyroid tumors, such as RET/PTC rearrangements (Rhoden *et al.*, 2006), overexpression of c-met protein (Ruco *et al.*, 2001), and over-expression of the antiapoptotic molecule galectin-3 (Gasbarri *et al.*, 2004) have been also detected in HT. The occurrence of HGF expression inside the nodular lesions and in the extranodular tissues of thyroid glands with HT as well as its overexpression in many different type of thyroid cancers, suggest that this growth factor may represent a possible early marker and a target for the future development of specific antitumoral treatment.

In conclusion, all data reported in this study induce to consider HGF as a goitrous factor relevant for thyroid non-neoplastic and neoplastic lesions. Its epithelial expression in Hashimoto's thyroiditis and its stromal expression in hyperplastic nodule may play a relevant role in the proliferative processes associated with chronic autoimmune thyroiditis.

References

Bidey SP, Hill DJ, Eggo MC. Growth factors and goitrogenesis. J Endocrinol 1999;160:321-32.

Birchmeier C, Gherardi E. Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol 1998; 8: 404-10.

Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, Aaronson SA. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science 1991;251:802-4.

Dayan CM, Daniels GH. Chronic autoimmune thyroiditis. N Engl J Med. 1996; 335:99-107.

Doniach D, Roitt MI. Human organ specific autoimmunity: personal memories. Autoimmunity 1988;1:11-3.

Gasbarri A, Sciacchitano S, Marasco A, Papotti M, Di Napoli A, Marzullo A, et al. Detection and molecular characterization of thyroid cancer precursor lesions in a specific subset of Hashimoto's thyroiditis. British J Cancer 2004; 91:1096-104.

Hayashi N, Tamaki N, Yamamoto K, Senda M, Yonekura Y, Misaki T, et al. Real-time ultrasonography of thyroid nodules. Acta Radiol Diagn (Stockh) 1986;27:403-8.

Jayaram G, Marwaha RK, Gupta RK, Sharma SK. Cytomorphologic aspects of thyroiditis. A study of 51 cases with functional, immunologic, and ultrasonographic data. Acta Cytol 1987;31: 687-93.

Li Volsi V. Lymphocytes in the thyroid. In: Li Volsi V, eds. Surgical Pathology of the Thyroid. W.B. Saunders Company, Philadelphia, 1990:pp. 68-97.

Li Volsi VA. The pathology of autoimmune thyroid disease: a review. Thyroid 1994; 4:333-39.

Mineo R, Costantino A, Frasca F, Sciacca L, Russo S, Vigneri R, et al. Activation of the hepatocyte growth factor (HGF)-Met system in papillary thyroid cancer: biological effects of HGF in thyroid cancer cells depend on Met expression levels. Endocrinology. 2004;145: 4355-65.

Mizukami Y, Michigishi T, Nonomura A, Nakamura S, Ishizaki T. Pathology of chronic thyroiditis: a new clinically relevant classification. Pathol Ann 1994; 29:135-58.

Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun 1984;122:1450-59.

Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature 1989; 342: 440-43.

Rhoden KJ, Unger K, Salvatore G, Yilmaz Y, Vovk V, Chiappetta G, et al. RET/papillary thyroid cancer rearrangement in nonneoplastic thyrocytes: follicular cells of Hashimoto's thyroiditis share low-level recombination events with a subset of papillary carcinoma. J Clin Endocrinol Metab 2006; 91:2414-23.

Ruco LP, Stoppacciano A, Ballarini F, Prat M, Scarpino S. Met protein and hepatocyte growth factor (HGF) in papillary carcinoma of the thyroid: evidenze for a pathogenetic role in tumorigenesis. J Pathol 2001;194: 4-8.

Ruggeri RM, Barresi G, Sciacchitano S, Trimarchi F, Benvenga S, Trovato M. Immunoexpression of the CD30 Ligand/CD30 and IL-6/IL-6R signals in thyroid autoimmune diseases. Histol Histopathol 2006; 21:249-56.

Scarpino S, D'Alena FC, Di Napoli A, Ballarini F, Prat M, Ruco LP. Papillary carcinoma of the thyroid: evidence for a role for hepatocyte growth factor (HGF) in promoting tumour angiogenesis. J Pathol 2003;199:243-50.

Trovato M, Villari D, Bartolone L, Spinella S, Simone A, Violi MA, et al. Expression of the hepatocyte growth factor and c-met in normal thyroid, non-neoplastic, and neoplastic nodules. Thyroid 1998;8:125-31.

Trovato M, Fragetta F, Villari D, Batolo D, MacKey K, Trimarchi F, et al. Loss of heterozygosity of the long arm of chromosome 7 in follicular and anaplastic thyroid cancer, but not in papillary thyroid cancer.J Clin Endocrinol Metab 1999;84:3235-40.

Trovato M, Grosso M, Vitarelli E, Ruggeri RM, Alesci S, Trimarchi F, et al. Distinctive expression of STAT3 in papillary thyroid carcinomas and a subset of follicular adenomas. Histol Histopathol 2003;18: 393-99.

Trovato M, Ulivieri A, Dominici R, Ruggeri RM, Vitarelli E, Benvenga S, et al. Clinico-pathological significance of cell-type-specific loss of heterozygosity on chromosome 7q21: analysis of 318 microdissected thyroid lesions. Endocr Relat Cancer 2004;11:365-76.

Vesely D, Astl J, Lastuvka P, Matucha P, Sterzl I, Betka J. Serum levels of IGF-I, HGF, TGFbeta1, bFGF and VEGF in thyroid gland tumors. Physiol Res 2004; 53:83-9.

Weetman AP, Mc Gregor AM. Autoimmune thyroid disease: further developments in our understanding. Endocr Rev 1994; 15:788-830.