The tyrosine kinase receptor c-met, its cognate ligand HGF and the tyrosine kinase receptor transducers STAT3, PI3K and RHO in thyroid nodules associated with Hashimoto's thyroiditis: an immunohistochemical characterization


1Unit of Endocrinology, Clinic-Experimental Department of Medicine and Pharmacology; 2Department of Human Pathology, University of Messina, Messina, Italy

Abstract

Hepatocyte growth factor (HGF) exerts proliferative activities in thyrocytes upon binding to its tyrosine kinase receptor c-met and is also expressed in benign thyroid nodules as well as in Hashimoto's thyroiditis (HT).

The simultaneous expression of HGF/c-met and three trasducers of tyrosine kinase receptors (STAT3, PI3K, RHO) in both the nodular and extranodular tissues were studied by immunohistochemistry in 50 benign thyroid nodules (NGs), 25 of which associated with HT. The ligand/tyrosine kinase receptor pair HGF/c-met and the two trasducers PI3K and RHO were expressed in NGs, regardless of association with HT, with a higher positive cases percentage in HT-associated NGs compared to not HT-associated NGs (25/25 or 100% vs 7/25 or 28%; P<0.001). HGF, PI3K and RHO expression was only stratal (fibroblasts and endothelial cells), in all 32 reactive NGs, while c-met localization was consistently epithelial (thyrocytes). Immunoreactions for HGF, c-met, PI3K and RHO were also apparent in the extra-nodular tissue of HT specimens, where HGF and PI3K were expressed not only in strimal cells but also in thyrocytes along with the c-met. Finally, a positive correlation was observed between the proportion of HGF c-met, PI3K follicular cells and the grade of lymphoid aggregates in HT. In conclusion, HGF c-met, PI3K are much more frequently and highly expressed in HT compared to NGs, and among all NGs in those present in the context of HT. A paracrine effect of HGF/c-met on nodule development, based on the prevalent stratal expression, may be suggested along with a major role of HGF/c-met and PI3K in HT. Finally, the expression of such molecules in HT may be regulated by lymphoid infiltrate.

Introduction

Hashimoto's thyroiditis (HT) is the most prevalent autoimmune thyroid disease worldwide and is characterized by variable clinical presentation with respect to proliferation of the follicular cells.1,2 Thyrocyte proliferation may be very intense in HT, thus leading to multiple nodular lesions.3,4 Increased prevalence of HT patients with associated nodular goiter remains high in moderately iodine-deficient areas such as southern Italy.5

Growth factors other than thyrotropin (TSH), and cytokines favor the development of diffuse and/or nodular goiter.1,6 However, only few studies have evaluated the role of different growth factors in the nodular variant of HT.7,11

Recently, we reported the immunohistochemical expression of the hepatocyte growth factor (HGF) in HT-associated nodular goiter specimens and demonstrated that it was more frequent and intense than what observed in non-HT goiters.8 Upon binding to its specific tyrosine-kinase receptor (HGF-R or c-met), HGF exerts mitogenic and anti-apoptotic activities in various cell types, including follicular thyroid cells.9-16 Previous studies demonstrated that HGF and c-met are expressed in hyperplastic nodules (but non in normal thyroid tissue) and are overexpressed in papillary thyroid carcinomas (PTC).15-16

Similarly to activation of the other ligand/tyrosine kinase receptors, activation of the HGF/c-met signaling system recruits several intracellular effectors, including phosphatidylinositol 3-kinase (PI3K), Ras, adaptators GRB2 and SHC, the docking protein Gab1, the member of the signal transducers and activators of transcription family STAT3, β catenin and RHO.17-23 Such effectors are ubiquitous, as they are expressed in all human tissues, and trigger distinct biological events, i.e. growth, scattering and morphogenesis, in epithelial cells.22-23 Concerning thyroid oncology, expression of these ubiquitous effectors (for instance, STAT3, PI3K) has been investigated mainly in malignant thyroid tumors arising from the follicular epithelium,23,24 but rarely together with expression of HGF/c-met.25-26 The few data available on the expression of such molecules in thyroid nodules of HT patients are mainly focused on the possible association between HT and thyroid cancer.27 Larson and co-workers reported that the PI3K pathway components p-Akt, Akt1, and Akt2 were highly expressed in HT and HT-associated PTC, as well as in non-PTC, but not in the normal follicular epithelium. On this basis, they suggest that the PI3K/Akt pathway activation might represent a common molecular mechanism between the chronic autoimmune inflammation of thyroid and PTC.27-28

Moreover, two recent studies related PI3K expression/activation with the mechanisms of immunity response.29-30 It is well known that autoimmune thyroid diseases is related with the balance between T helper types 1 and 2 (Th1 and Th2) responses, by an involvement of Toll-like receptors (TLR).31-32 The subunit p85 of PI3K participates, in this framework, in the ligation with TLR controlling the Th1/Th2 balance for an advantage of Th1 cytokines.33 Moreover, PI3K regulatory subunits p85 regulates the motility of T and B lymphocytes.34 Here we evaluate the immunohistochemical expression of ligand/tyrosine kinase receptor pair HGF/c-met and three trasducers of activated tyrosine kinase receptors (STAT3, PI3K, RHO) in benign thyroid nodules, half of which developed in the context of background HT. To the best of our knowledge, the expression of these molecules has not been previously examined in HT.

Materials and Methods

Tissues collection

Fifty surgical thyroid specimens were retrieved from the files of our Pathology Department, while 5 normal thyroids (NT) were harvested at autopsy. All specimens were 4% formalin-fixed and paraffin-embedded. The 50 surgical specimens were from patients diagnosed and followed up at our Endocrine Unit and included benign nodular goiters (NG), of which 25 were associated with HT. In such patients, the diagnosis of HT was based on the currently accepted clinical, laboratory (circulating thyroid antibodies) and ultrasonographic criteria. All nodular lesions were studied along with the non...
nодular tissue from the contralateral thyroid lobe (Table 1).

Hematoxylin-eosin-stained (H&E) sections of each specimen were re-evaluated by the pathologists in order to confirm the HT diagnosis, evaluate the intra-glandular inflammatory lymphoid aggregates and catalogue the lesions by cytopathological morphology. HT was diagnosed using histopathologic criteria of Li Volsi.20

The lymphoid aggregates were categorized as previously reported.11 Briefly, aggregates made up of at least 150 lymphocytes and a variable number of plasma cells per high-power field, were considered as lymphoid aggregates and scored from 0 to 3 (0 = no lymphoid aggregate or at most one single, small lymphoid aggregate without germinal center in each section; 1 = occasional, usually small lymphoid aggregates with rare or absent germinal centers in each section; 2 = several, usually mixed, small and large lymphoid aggregates with some germinal center in each section; 3 = numerous, large lymphoid aggregates with frequent germinal centers in each section).

Cellular morphology was classified according to the nuclear and cytoplasm features at light microscopy, as previously reported.11 Based on these morphological features, the thyrocytes of each lesion were classified into three different cellular types: i) dark nucleus and eosinophilic cytoplasm (DN-EC); ii) dark nucleus and oncocyctic cytoplasm (DN-OC or Hurtle cells); iii) clear nucleus and eosinophilic cytoplasm (CN-EC) (Table 1).

Immunohistochemistry

The 55 blocks were cut into serial sections of five micrometres each. Immunohistochemistry was performed, separately, using rabbit polyclonal antibodies against HGF-α (H-145, working dilution (w.d.) 1:100), c-met (B-9, w.d. 1:100) and STAT3 (B-9, w.d.1:100), all three purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); a mouse monoclonal antibody against PI3K p85α (B-9, w.d. 1:200), purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and a rabbit monoclonal antibody against RHO (Y486, w.d.1:100), purchased from Abcam plc., Cambridge, UK. Tissue sections were deparaffinized in xylene and rehydrated in alcohol. Next, the endogenous biotin activity was quenched by adding a 0.3% (v/v) saline (PBS), and the endogenous peroxidase activity was quenched by adding a 0.3% (v/v) solution of 3% H2O2/methanol for 30 min. Staining was obtained with the LSAB system (kit from Dako, Carpinteria, CA, USA). 3',3'-diaminobenzidine (DAB, Sigma) activated with 0.05% hydrogen peroxide was used to develop the end reactions. Sections were counterstained with Mayer’s hematoxylin, dehydrated and mounted.

Specificity of the antibody binding was assessed by omitting the primary antiserum or by replacing the primary antiserum with normal rabbit or mouse serum. In each of these conditions, no staining was evident. In addition, an immunoblotting test was performed to confirm the specific immunoreactivity of each antibody. Specimens of normal tissues of bladder and liver were used as positive controls for HGF and c-met immunoreaction, respectively. Positive controls for STAT3, PI3K and RHO immunoreaction were performed by using specimens of lung, breast and liver carcinomas, respectively. The evaluation of the results was based on: i) number of positive cases; ii) number of reactive epithelial and stromal cells per case, based on counting 1000 cells/case at 50X magnification; iii) subcellular location of the staining: membrane, cytoplasm and nucleus; iv) staining grade using a semiquantitative score system from 0 to 4+ (0, absent; 1+, weak but distinct; 2+, moderate; 3+, intense; 4+, very intense). Histological and immunohistochemical evaluations were done twice and blindly by two pathologists (M.T., G.B.) with an inter-observer concordance of nearly 100%. Where minimal inter-observer discrepancies were present, data were averaged.

Statistical analysis

Data were tested for normal distribution and variance (mean ± SD) and analyzed by the twotailed Student’s t-test, ANOVA and χ2 test with Yates’ correction for continuity, when appropriate. The association between two variables was analyzed by the linear regression analysis. The level of statistical significance was always set at P < 0.05.

Results

Histopathology and cellular morphology

All the 50 benign NGs showed the histological features of colloid nodules characterized by either large or small colloid-filled follicles built by cells with small flat or cubic shape. Instead, cellular hallmarks of these lesions were the epithelial follicular cells with DN-EC features, even if in most cases of HT-associated NGs, DN-OC cells and seldom CN-EC cells too were observed (Table 1). The predominant cellular type in HT showed features of DN-EC cells. Intra-nodular lymphoid aggregates were absent (grade 0) in all 50 NGs. Extra-nodular lymphoid aggregates were the main histological evidence of HT. These aggregates were graded as 1 (10/25 or 40%) or 3 (3/25 or 12%). As previously specified in the Materials and Methods paragraph, the 25 HT specimens were from patients diagnosed at our Endocrine Unit, and all of them have circulating anti-thyroid antibodies.

Immunohistochemistry

Illustrative immunohistochemistry for HGF, c-met, PI3K and RHO is presented in Figure 1. No expression of HGF, c-met, STAT3, PI3K and RHO was detected in normal thyroid tissue, namely the control normal thyroids and the contralateral lobe to the 25 NGs not associated with HT. Similarly, no immunostaining for STAT3 was observed in all nodular lesions.

Table 1. Expression of HGF, c-met, PI3K and RHO on epithelial and stromal cells in a series of 25 colloid nodular goiters (NGs) and of 25 nodular Hashimoto’s thyroiditis (HT) examined along with the corresponding extra-nodular thyroid tissue.

<table>
<thead>
<tr>
<th>Thyroid lesions</th>
<th>Positive cases</th>
<th>Reactive* cells</th>
<th>Percentage of positive cells (mean ±SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colloid nodular goiters (NGs, n = 25)</td>
<td>7 (28%)</td>
<td>Epithelial (DN-EC)</td>
<td>0 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stromal</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>NGs associated with HT (n = 25)</td>
<td>25 (100%)</td>
<td>Epithelial (DN-EC)</td>
<td>0 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stromal</td>
<td>12 ± 8</td>
</tr>
<tr>
<td>Extra-nodular thyroid tissue of HT (n = 25)</td>
<td>25 (100%)</td>
<td>Epithelial (DN-EC)</td>
<td>58 ± 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stromal</td>
<td>24 ± 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN-EC</td>
<td>21 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stromal</td>
<td>11 ± 8</td>
</tr>
</tbody>
</table>

*Thyroid lesions were catalogued by cytological morphology of thyrocytes based on nuclear and cytoplasm features at light microscopy.** as detailed in Material and Methods. Based on these morphological features, the cells of each lesion were categorized as: i) dark nucleus and eosinophilic cytoplasm (DN-EC); ii) dark nucleus and oncocyctic cytoplasm (DN-OC); iii) clear nucleus and eosinophilic cytoplasm (DN-OC); iv) clear nucleus and eosinophilic cytoplasm (DN-EC); v) clear nucleus and Oncocyctic cytoplasm (CN-EC). The percent of the reactive epithelial and stromal cells was based on evaluation of 1000 cells/case, using a ×100 magnification. Values pertaining to stromal cells are typed in italics.
with HT. However, significant differences in the distribution of immunoreactions could be detected by comparing the NGs arising in the context of HT with the HT-unassociated NGs. The expression of HGF, c-met, PI3K and RHO was detected more frequently in the former than in the latter nodules (25/25 or 100% vs 7/25 or 28%; P<0.001) (Table 1). However, there were no differences in the localization of immunostaining between the two types of lesions. Thus, in all 32 reactive NGs, HGF, PI3K and RHO expressions were only stromal (fibroblasts and endothelial cells) and cytoplasmic, while the localization of c-met was consistently epithelial (thyrocytes), both membranous and cytoplasmic. The percentage of reactive cells and the intensity of their immunoreactivity were similar in both types of NGs. Thus, immunoreactivity for HGF, PI3K and RHO concerned 5-10% of stromal cells, and intensity of immunostaining was weak or moderate. In the same 32 nodules, a weak c-met immunoreaction was restricted to 3-8% of thyrocytes (Table 1).

The c-met reactive epithelial cells of all 32 nodules showed the cytological features of DN-EC cells.

Expressions of HGF/ c-met/ STAT3/PI3K/RHO in the extra-nodular tissue of HT

Immunoreactions for HGF, c-met, PI3K and RHO were also observed in the extra-nodular tissue of HT specimens, but their expression differed from what observed in NGs (Table 1).

Indeed, while stromal expression of HGF, PI3K and RHO matched the corresponding stromal expression in NGs, the cytoplasm of thyrocytes also expressed HGF and PI3K, an epithelial immunoreactivity that was absent in all NGs. HGF and c-met were detected in thyrocytes with DN-EC, DN-OC and CN-EC features, while PI3K was expressed by epithelial follicular cells with the sole DN-EC features (10-35%) (Table 1 and Figure 1). Hence, thyrocytes with DN-EC features expressed the three molecules (HGF, c-met and PI3K), while both DN-OC and CN-EC thyrocytes expressed only HGF and its receptor c-met. Among thyrocytes immunoreacting for both HGF and c-met, the major proportion of HGF and c-met +ve cells showed DN-EC features (HGF +ve DN-EC cells: 58±20%, range 30 to 80%; c-met +ve DN-EC cells: 19±5%, range 10 to 25%; P<0.001 vs the corresponding percentages of DN-OC and CN-EC cells) (Table 1). Moreover, all HT lesions showed an intense stromal immunostaining for HGF (which concerned 5-40% of cells) as well as PI3K and RHO (5-15% of cells) (Table 1). Finally, the staining grade observed for the four proteins in HT was higher than that observed in NGs, because it was moderate to very intense as compared with weak to moderate (data not shown).

Comparing the proportion of DN-EC cells expressing the HGF, c-met and PI3K with the grade of lymphoid aggregates, a positive correlation was observed for the three protein [HGF:

<table>
<thead>
<tr>
<th>Group</th>
<th>HT (n=25)</th>
<th>Positive cases</th>
<th>Reactive cells*</th>
<th>Percentage of positive cells (mean ±SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>epithelial</td>
<td>HGF</td>
<td>c-met</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DN-EC</td>
<td>37±8</td>
<td>14±4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FN-OC</td>
<td>4±8</td>
<td>2±4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN-EC</td>
<td>14±12</td>
<td>5±4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stromal</td>
<td>15±11</td>
<td>0</td>
</tr>
<tr>
<td>Group B</td>
<td>11-25 epithelial</td>
<td>DN-EC</td>
<td>71±11</td>
<td>22±2</td>
</tr>
<tr>
<td></td>
<td>(Lymphoid</td>
<td>FN-OC</td>
<td>38±22</td>
<td>13±7</td>
</tr>
<tr>
<td></td>
<td>aggregate grade</td>
<td>CN-EC</td>
<td>26±13</td>
<td>10±4</td>
</tr>
<tr>
<td></td>
<td>2 and 3) (n=15)</td>
<td>stromal</td>
<td>7±5</td>
<td>0</td>
</tr>
</tbody>
</table>

*lymphoid aggregates were scored from 0 to 3 (0=no lymphoid aggregate; 1=occasional lymphoid aggregates with rare or absent germinal centers in each section; 2=several lymphoid aggregates with some germinal center; 3=numerous lymphoid aggregates with frequent germinal centers), as detailed in Material and Methods. “Thyroid follicular cells were categorized as: i) dark nucleus and eosinophilic cytoplasm (DN-EC); ii) dark nucleus and oncocytic cytoplasm (DN-OC); iii) clear nucleus and eosinophilic cytoplasm (CN-EC), based on nuclear and cytoplasm features at light microscopy,” as detailed in Material and Methods. #The count of the reactive epithelial and stromal cells was based on evaluation of 1000 cells/case, using a 50X magnification. Values pertaining to stromal cells are typed in italics.

### Figure 1

**A:** Very intense HGF immunoreaction in a sample of HT with lymphoid aggregates defined as grade 2. The HGF immunostaining is located on the cytoplasm of thyrocytes (brown deposits) showing morphological characteristics of DN-EC cells. **B:** Intense c-met immunostaining in a sample of HT with grade 2 lymphoid aggregates. The c-met reaction is observed on the membrane and cytoplasm (brown deposits) of follicular DN-EC cells. **C:** Moderate PI3K immunoreaction in a sample of HT with grade 2 lymphoid aggregates. The immunoreaction is located in the cytoplasm of epithelial DN-EC cells (brown deposits). **D:** Moderate RHO immunoreaction in a sample of NG not associated with HT. The RHO immunostaining is located on the cytoplasm of stromal cells (original magnification: X 130, reference bar = 153 µm).
infiltrate.
molecules in HT may be regulated by lymphoid
centers (P<0.001). This observation rein-
grates were occasional with rare or absent germi-
inal centers (lymphocytic infiltrate grade 2 and 3),
as compared to the 10 cases with lymphocytic
infiltrate grade 1, in which the lymphoid aggreg-
es were more pronounced in the 15 cases with presence of germi-
nal centers (lymphocytic infiltrate grade 2 and 3),
as compared to the 10 cases with lymphocytic
infiltrate grade 1, in which the lymphoid aggreg-
es were occasional with rare or absent germi-
nal centers (P<0.001). This observation rein-
forges the hypothesis that the expression of such
molecules in HT may be regulated by lymphoid
infiltrate.

Discussion

True nodular lesions frequently arise in the
context of HT, as consequence of a hyperplastic
follicular growth, which leads to an increase in
number and size of follicles. Several growth fac-
tors and cytokines are known to cooperate with
TSH in thyroid nodular growth, but few data are
available on their expression in nodular lesions
associated with HT.10,11

In the present study, we report that the expres-
sion of the HGF/c-met system is associated with
that of the tyrosine kinase receptors transducers
PI3K and RHO in both NGs and HT lesions, while
the normal thyroids do not express any. These
findings are in line with the known ability of HGF
to induce cellular growth, as well as with our pre-
vious reports in which we observed that HGF and
its receptor c-met are expressed in goitrous sam-
plies as well as in thyroid tissues with HT, but
undetectable in normal thyroid tissue.12,13 Our
present study offers some new knowledge.

First, we demonstrated the expression of the
tyrosine kinase receptors transducers PI3K and
RHO in both HNs and HT lesions expressing the
HGF/cmet system, but we detected a more fre-
quent expression of HGF, c-met, PI3K and RHO in
the NGs associated with HT, either with scarce
lymphoid aggregates or with germinal centers, as
compared to non HT-associated NGs.

Another major result of our study is the differ-
et localization of HGF and PI3K expressions in
NGs and HT, even when occurring in the same
thyroid gland. In the NGs, approximately 10%
of stromal cells expressed HGF, thus providing the
ligand for a paracrine interaction with the c-met
expressed on a limited number of epithelial cells.
However, this possible interaction was not
accompanied by the epithelial expression of any
of the three transducers investigated (STAT3, PI3K
and RHO). In fact, both PI3K and RHO expres-
sion was restricted to approximately 8% of the
stromal cells of NGs regardless of coexisting HT.
Therefore, the cellular localization of such mole-
cules seems to be independent from the HT
lesion associated with the nodular goiter. Further,
the expression of PI3K and RHO in strom-
al cells appears to be independent from activa-
tion of the HGF/c-met signaling in this cellular
type, since there was no stromal expression of c-
meth. In HT, HGF and PI3K immunoreactions
involved both stromal and epithelial cells, raising
the possibility of c-met activation through an
autocrine loop with subsequent involvement of
PI3K.

The characterization of the follicular epithelial
cells of HT based on the nuclear and cytoplasmic
features allowed us to identify the follicular cells
with DN-EC features as the cellular type specifi-
cally involved in the epithelial pathway of HGF, c-
met and PI3K. Indeed, the epithelial co-expres-
sion of HGF, c-met and PI3K was observed only
in such cells, while the other reactive epithelial cel-
types expressed only the ligand and the
receptor.

These evidences suggest that the HGF/c-met
system may play a role in the development of
nodular goiter, and more specifically in the
goitrous growth of HT. Moreover, these data sug-
gest that HGF acts as a goitrous growth factor in
a paracrine fashion in HT-associated or non HT-
associated NGs, but both in a paracrine and
autocrine fashion in HT. The expression of the
three molecules (c-met, PI3K and especially
HGF) is much more pronounced in nodules asso-
ciated with diffuse HT, proved by the presence of
germinal centers, rather than in HT with scarce
lymphoid aggregates. In those latter, however,
the expression of the investigated molecules is
significantly higher with respect to non HT-NGs.

Finally, the positive correlation we found
between the immunoistochemical expression of
HGF, c-met and PI3K in DN-EC cells and the
lymphoid aggregates lead us to postulate that HGF, c-
met and PI3K signals may have a role also in the
mechanism of thyroid autoimmunity, as support-
ed by two recent studies relating PI3K expres-
sion/activation with the mechanisms of the
immunity response.35,36 In this way, the expres-
sion of PI3K in DN-EC thyrocytes could be
involved in the cascade of immune events, such
as in presenting antigens and recalling up lymph-
cocytes.

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