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Distinctive expression of STAT3 in papillary thyroid carcinomas and a subset of follicular adenomas

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Summary. Hepatocyte growth factor (HGF), HGF receptor (c-met) and the interleukin 6 (IL-6) are expressed in thyroid nodules. In extra-thyroidal tissues, the HGF/c-met and IL-6/IL-6 receptor (IL-6R) systems activate STAT3, a member of the signal transducers and activators of transcription (STATs) family. To evaluate whether either system utilizes STAT3 in thyroid nodules, we examined the immunohistochemical expression of HGF, c-met, IL-6, IL-6R, STAT3 in 6 normal thyroids and in 68 thyroid nodules.

STAT3 expression was observed in 12/12 (100%) papillary thyroid carcinomas (PTC) but in none of the follicular tumors. Among benign thyroid nodules, only 2/10 (20%) follicular adenomas (FA) were $STAT3^+$. All these 14 $STAT3^+$ cases expressed both HGF and c-met, but only 5 PTC co-expressed IL-6 and IL-6R and the 2 FA were $IL\text{-}6^+$ but $IL\text{-}6R^-$. The remaining 8 FA were all $HGF^-/c\text{-}met^-$, but $IL\text{-}6^+$; of these 8, only 2 were also $IL\text{-}6R^+$.

In conclusion, in thyroid nodules STAT3 is expressed only in PTC and a number of FA. Since these cases are consistently $HGF^+/c\text{-}met^+$ and only one-third of them co-express IL-6/IL-6R, STAT3 expression correlates with the HGF/c-met expression, not with the IL-6/IL-6R expression. The 100% rate of expression of the HGF/c-met/STAT3 signaling in PTC could be relevant for the establishment of the papillary phenotype. Because of the communeness of a HGF/c-met/STAT3 pattern between all PTC and a subset of FA, we speculate that a fraction of FA may progress to PTC.

Key words: Hepatocyte growth factor, c-met, Interleukin 6, Interleukin 6 receptor, STAT3

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Introduction

Tumors of the thyroid follicular cells represent an informative model for understanding the molecular pathogenesis of multi-stage tumorigenesis. The development and the progression of thyroid tumors is closely correlated with phenotype-specific mutations in genes involved in growth control (Fagin, 1992). Among thyroid cancers, papillary thyroid carcinoma (PTC) is associated to frequent over-expression of the c-met proto-oncogene, which, instead, is rarely expressed in follicular thyroid carcinomas (FTC) and anaplastic thyroid carcinomas (ATC) (Di Renzo et al., 1992; Oyama et al., 1998; Trovato et al., 1998).

The product of the c-met proto-oncogene is the tyrosine-kinase receptor for hepatocyte growth factor (HGF) (Giordano et al., 1989; Bottaro et al., 1991). HGF is a mesenchyme-derived pleiotropic cytokine with a heterodimeric structure consisting of a heavy α-chain and a light ß-chain. We have already reported for the first time the epithelial co-expression of HGF and c-met in PTC (Trovato et al., 1998). The binding of the specific ligand induces activation of the tyrosine-kinase domain of c-met and auto-phosphorylation of the receptor (Bottaro et al., 1991; Bardelli et al., 1994). Particularly, auto-phosphorylation of Tyr1349 and Tyr1356 provides binding sites for multiple molecules containing SH2 groups (Pozzetto et al., 1994). These molecules act as intracellular transducers by recruiting and activating several effectors, including phosphatidylinositol 3-kinase (PI3-kinase), Ras, adaptators GRB2 and SHC, the docking protein Gab1 and STAT3 (Graziani et al., 1993; Pellicci et al., 1995; Royal and Park, 1995; Ponzetto et al., 1996; Weidner et al., 1996; Schaper et al., 1997; Boccaccio et al., 1998; Maffe and Comoglio, 1998). By interacting with c-met, HGF can promote different responses (i.e. scattering, growth and morphogenesis) in epithelial cells through activation of several pathways (Bhargava et al., 1992). For instance, in Madin-Darby canine kidney cells the scattering effect of HGF/c-met is mediated by one effector (PI3-kinase), while the

proliferative action is mediated by another effector (GRB2) (Graziani et al., 1993; Royal and Park, 1995; Ponzetto et al., 1996) and the morphogenetic action is mediated by STAT3, one member of the signal transducers and activators of transcription (Boccaccio et al., 1998; Maffe and Comoglio, 1998) family.

STAT3 is a cytoplasmic protein which is inactive when monomeric (Deker and Kovarik, 1999). Following interaction with c-met either directly through the SH2 domain, or indirectly through the Gab1 bridges, STAT3 becomes phosphorylated and able to homo- and hetero-dimerize. In such activated form, STAT3 translocates into the nucleus and induces the expression of target genes the promoters of which are located in regions containing the functional regulatory sequences SIE (sis-inducible element) (Boccaccio et al., 1998).

STAT3 protein is also involved in the transduction of interleukin 6 (IL-6)/gp-130 signal, but with mechanisms that are different from those described above for the HGF/c-met complex. IL-6R is a type-I membrane protein with one α-chain and two gp130 signal-transducing subunits. By interacting with the α-chain, IL-6 induces homodimerization and autophosphorylation of gp-130, and activation of the associated Janus protein tyrosine kinases (JAKs) (Hirano, 1998). In turn, JAKs and the phosphorylated gp130 induce homo/hetero-dimerization of the phosphorylated STAT3 in the cytosol and its subsequent nuclear translocation (Hirano, 1998). Once in the nucleus, heterodimers of STAT3 may bind nuclear SIE DNA sequences (Ogata et al., 1997). IL-6 expression has been reported in normal thyroid epithelial cells, in colloid nodules (CN), in follicular adenomas (FA), in PTC and also in autoimmune thyroid disorders (Zheng et al., 1991; Watson et al., 1994; Bartalena et al., 1995; Kayser et al., 1995; Basolo et al., 1998). No expression of IL-6R was found in cell cultures of normal and neoplastic thyrocytes (Basolo et al., 1998).

To evaluate the possible involvement of STAT3 in thyroid oncogenesis arising from the follicular epithelial cell, we have examined the expression of HGF, c-met, IL-6, IL-6R and STAT3 in normal and pathological thyroid tissues.

Materials and methods

Thyroid specimens

Six normal thyroids (NT) harvested during autopsy and 68 surgical thyroid specimens were retrieved from the files of the Departments of Pathology of the University of Messina, Italy. These were the same cases we have studied previously for another purpose (Trovato et al., 1999).

All specimens were 4% formalin-fixed and paraffin-embedded. The 68 surgical specimens included the following thyroid lesions, classified as proposed by the Armed Forces Institute of Pathology (AFIP) (Rosai et al., 1990): 10 colloid nodules (CN); 10 follicular hyperplasias (FH); 10 follicular adenomas (FA) (2 normofollicular, 2 microfollicular, 2 microfollicular toxic, 3 macrofollicular, and 1 hyalinizing trabecular); 10 oncocytic adenomas (OA); 12 papillary carcinomas (PTC) (6 conventional, 2 follicular, 1 oncocytic, and 1 tall cell); 10 follicular carcinomas (FTC) (5 minimally invasive type, and 5 widely invasive type); and 6 undifferentiated (anaplastic) carcinomas (ATC). All lesions were studied paired with the corresponding perinodular normal tissue. Hematoxylin-eosin-stained sections of each specimen were re-evaluated by the pathologists to confirm the presence of the diagnosed disorder.

Immunohistochemistry

Five-μm serial sections of the selected blocks were used for immunocytochemical studies. Immunocyto-chemistry was performed, separately, using the rabbit polyclonal anti-phospho-specific-STAT3 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), the rabbit polyclonal antibodies against HGFα or c-met (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and with the monoclonal antibodies against IL-6 or IL-6Rα (Sigma, St. Louis, MO, 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. Subsequently, the slides were subjected to the antigen retrieval technique as described by Gown (Gown et al., 1993). Slides were first placed in 10 mM citrate buffer adjusted to pH 6.0 with 2 M sodium hydroxide, and then microwaved for 15 min (power set at 500 watts). Microwave exposure was broken into three equal time periods and, at the end of the first cycle, 50 ml of distilled water was added to the slide in order to prevent loss of fluid from boiling. Each primary antibody, diluted 1:100 in 10mM PBS, was added and slides were incubated overnight at +4 °C. Staining was obtained with a standard labeled biotin-streptavidin-peroxidase method (LSAB kit from Dako, Carpinteria, CA, USA). The color reaction was developed using 3,3'-diaminobenzidine (DAB) as chromogen. The slides were counterstained with Mayer's hematoxylin, 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.

Immunocytochemical staining was evaluated twice and independently by three pathologists (M.T., M.G., G.B.), who were blind to clinical data, with an inter-observer agreement of 100%. For the evaluation and comparison of the results, the following criteria were used: a) number of positive cases; b) number of positive epithelial cells per case, based on counting 1000 cells using a x50 magnification; c) site of positivity: epithelial

or stromal; d) sub-cellular location of the epithelial staining: plasmamembrane, cytoplasm and nucleus; and e) semiquantitative grading of staining using a scored system from 0 to 4+ (0, absent; +, weak but distinct; ++, moderate; +++, intense; ++++, very intense).

Statistics

Once tested for normal distribution and variance, data (mean ± standard deviation) were analyzed by the two-tailed Student test or Mann-Whitney rank sum test, as appropriate. Differences between proportions were analyzed by Fisher's exact test. The level of statistical significance was always set at $P<0.05$.

Results

Illustrative immunohistochemical features are shown in Fig. 1, while all data are summarized in Tables 1 and 2.

Normal tissue, colloid nodules and follicular hyperplasias

No positive immunostaining for STAT3, HGF, c-met and IL-6R was detected in the two lobes of the six normal thyroids (Table 1) and in the unaffected perinodular tissue of all 68 nodules. Moderate IL-6 reactivity was observed in 2/6 (33%) normal thyroids (Table 1) and concerned 20% of the follicular cells .

In all cases of CN and FH, STAT3 was always undetectable, while HGF and c-met were detected in approximately one-third of either histotype (CN=3/10; FH=4/10) (Table 1). HGF was localized in stromal cells while c-met was localized in follicular cells. In either histotype, the proportion of cells expressing the ligand (HGF) outnumbered the proportion of cells expressing the cognate receptor, but expression of the two proteins was similarly weak to moderate (Table 1). IL-6 was undetected in all HGF^+/c-met^+ CN or FH, but was weakly expressed in one-fifth of the cases, and limited to the same proportion (7%) of follicular cells. None of the 10 CN or FH showed IL-6R reactivity (Table 1).

Oncocytic adenomas

All ten cases were STAT3 negative, but 4 (40%) were HGF^+, 4 (40%) c-met^+, 8 (80%) IL-6^+ and 5/10 (50%) IL-$6R^+$ (Table 1). The frequency of co-expression of the ligand and the receptor was similar in the HGF/c-met system vs the IL-6/IL-6R system (4/4 or 100% vs 5/8 or 63%, $P=0.49$). As in CN and FH, HGF expression was restricted to one-tenth of stromal cells, one-fiftieth of follicular cells expressing c-met. Reactivity for both IL-6 and IL-6R was epithelial (tumor follicular cells), and again the cells expressing the ligand outnumbered the cells expressing the receptor. Immunostaining of the 4 proteins was always weak to moderate.

Follicular adenomas

Two cases (20%) were $STAT3^+$, HGF^+, c-met^+, IL-6^+, but IL-6R negative (Table 1). Of the remaining 8 cases, all were IL-6^+, two were IL-$6R^+$ (Table 1). Thus,

Table 1. Expression of the two ligand/receptor systems HGF/c-met and IL-6/IL-6R, and the signal transducer protein STAT3 in normal thyroid tissues and benign nodules[a,b,c].

SPECIMENS	HGF/c-met SIGNAL		IL-6/IL-6R SIGNAL		STAT3
	Ligand	Receptor	Ligand	Receptor	
Normal thyroids (n=6)	0	0	n=2 (++, c), 20%	0	0
Colloid nodules (n=10)	n=3 (++, stroma), *8±3%*	n=3 (++, m), 3%, p=0.0033	n=2 (+, c), 7%	0	0
Follicular hyperplasia (n=10)	n=4 (++, stroma) *9±3%*	n=4 (++, m), 5±1%, p=0.057	n=2 (+, c), 7%	0	0
Oncocytic adenomas (n=10)	n=4 (+/++, stroma), *9±3%*	n=4 (+, m), 2±1%, p=0.002	n=8 (+/++, c), 19±11%	n=5 (+/++, c), 8±3%, p=0.027	0
Follicular adenomas (n=10)					
normofollicular (n=2)	0	0	n=2 (++, c)	n=1 (++, c)	0
microfollicular (n=3)	n=1 (+, m, c; +, stroma	n=1 (+, m)	n=3 (++, c)	n=1 (++, c)	n=1 (+++; c, n)
microfollic, toxic (n=2)	0	0	n=2 (++, c)	0	0
macrofollicular (n=2)	n=1 (+, m, c; +, stroma)	n=1 (+. m)	n=2 (+, c)	0	n=1 (+++; c, n)
hyalinizing trabecular (n=1)	0	0	n=1 (+, c)	0	0
all variants (n=10)	10% (n=2)	2% (n=2)	5±2% (n=10)	1% (n=2)	2% (n=2)

[a]: abbreviations refer to the subcellular location of the immunostaining: c, cytoplas; m, plasma membrane; n, nucleus. The intensity of the epithelial staining was scored on a five-point scale from 0 (absent) to 4+ (very intense) as specified in Materials and Methods. [b]: The proportion of positive cells was calculated based on evaluation of 1000 cells using x50 magnification. Cell are epithelial (i.e. follicular) or stromal (i.e. fibroblasts). Data for stroma cells are reported in italics. Data are expressed as mean±standard deviation. The p value refers to the comparison with the cognate ligand. [c]: The two IL-6 positive colloid nodules and follicular hyperplasia were HGF/c-met negative. Of the 8 IL-6 positive oncocytic adenomas, 5 of which being IL-6R positive, only 4 were HGF/c-met positive; the other 4 were HGF/c-met negative. Of the 10 IL-6 positive follicular adenomas, 2 of which being IL6R positive, only 2 were HGF/c-met positive; the other 8 were HGF/c-met negative. Only the 2 follicular adenomas HGF/c-met postive were also STAT3 positive.

the rate of co-expression of the ligand and the receptor was identical in both the HGF/c-met system and the IL-6/IL-6R system (2/10 and 2/10, respectively).

Except for HGF, which had both a stromal and epithelial location, STAT3, c-met, IL-6 and IL-6R had all an epithelial-restricted location in the neoplastic cells. As seen above for other histotypes, ligand-expressor cells prevailed over receptor-expressor cells. Except for the intense expression of STAT3, which had both cytoplasmic and nuclear location, the other proteins were expressed at weak to moderate levels.

Papillary thyroid carcinoma (PTC)

All 12 PTC (100%) were STAT3$^+$, HGF$^+$, and c-met$^+$ but only 5 (42%) co-expressed IL-6 and IL-6R, while another 5 expressed IL-6 but not the cognate receptor (Table 2). Thus, the rate of ligand/receptor co-

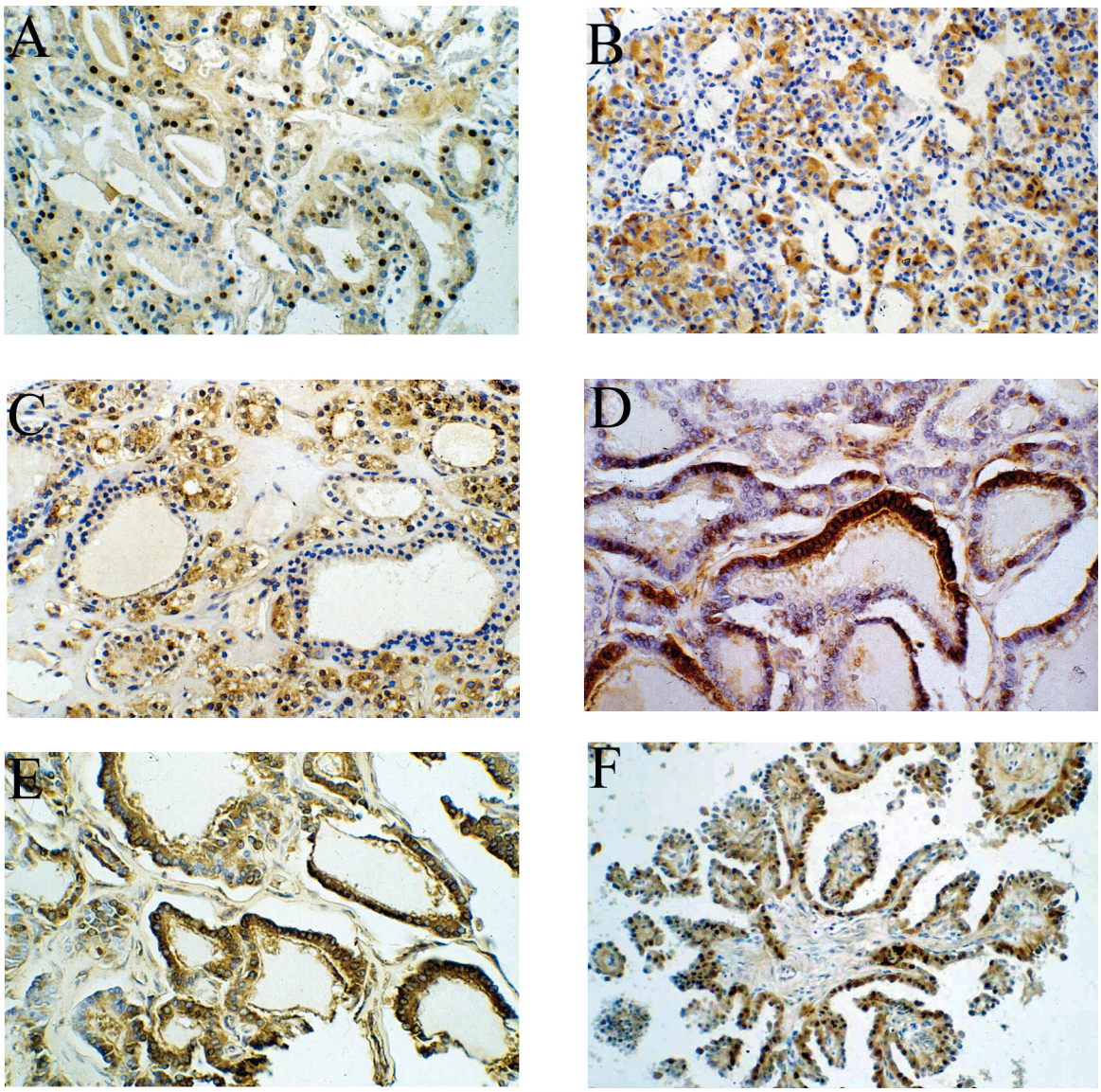

Fig 1. Representative immunohistochemistry. **A:.** STAT3 stain in follicular adenoma. The intense immunostaining is detected in the cytoplasm and nucleus of the neoplastic follicular cells. **B.** IL-6 positive immunoreaction in oncocytic adenoma. The intense stain is observed in the cytoplasm of the tumor oncocytic cells. **C:** IL-6R immunostaining in a follicular adenoma. The stain is moderate and seen in the cytoplasm of the neoplastic follicular cells. **D.** HGF-α-positive immunoreaction in a follicular variant of papillary thyroid carcinoma. The intense staining is located in the plasma membrane and cytoplasm of the tumor papillary cells. **E.** c-met-positive immunoreaction in a follicular variant of papillary thyroid carcinoma. The intense stain is located in the plasma membrane and cytoplasm of the tumor papillary cells. **F.** STAT3 immunostaining in the conventional variant of papillary thyroid carcinoma. The intense stain is observed in the cytoplasm and nucleus of the tumor papillary cells. x 40

expression was greater for the HGF/c-met system than for the IL-6/IL-6R system (100% vs. 42%, P = 0.004).

STAT3, HGF, c-met, IL-6 and IL-6R were detected in the papillary neoplastic elements of follicular origin, where each protein had the same subcellular location as in FA. HGF was also detected in stromal cells. PTC was the histotype with the greatest proportion of cells expressing each of the five proteins, as well as the histotype where the number of cells expressing the receptor was similar or greater than cells expressing the ligand. Most of the times, the extent of expression of the 5 proteins was intense to very intense (Table 2).

Follicular thyroid cancer (FTC) and anaplastic thyroid cancer (ATC)

All FTC and ATC were STAT3, HGF, c-met, IL-6, and IL-6R negative (Table 2).

In brief, STAT3 expression occurred only in $HGF^+/c\text{-}met^+$ tumors, although there were tumors $HGF^+/c\text{-}met^+$ but $STAT3^-$. The $HGF^+/c\text{-}met^+/STAT3^+$ tumors included all 12 PTC and 2/10 FA; the HGF+/$c\text{-}met^+/STAT3^-$ tumors included 3/10 CN, 4/10 FH and 4/10 OA. In the 14 $STAT3^+$ tumors, activation of STAT3 is indicated by the positive reactivity with a specific antibody against its phosphorylated form and the nuclear location, since only activated STAT3 translocates into the nucleus (see Introduction).

Discussion

Upon binding to specific cell receptors, growth factors and cytokines activate intracellular signaling pathways which are important to maintain cell homeostasis (Ihle, 1996). Following binding to the corresponding receptor, HGF and IL-6 can activate STAT3, but via different mechanisms (see Introduction). In response to HGF, STAT3 binds SIE DNA sequences of the c-fos gene promoter and, as a result, it enhances branching morphology but neither scattering nor proliferation (Boccaccio et al., 1998; Maffe and Comoglio, 1998). Even though it has been reported that in breast carcinoma cells STAT3 and c-Src synergistically induce HGF expression (Hung and Elliott, 2001), most of the literature describes the opposite; namely, that in several cell types STAT3 is a downstream molecule activated by HGF/c-met (Shaper et al., 1997; Boccaccio et al., 1998; Maffe and Comoglio, 1998). It is via STAT3 that HGF/c-met enhances branching morphology (Boccaccio et al., 1998; Maffe and Comoglio 1998) and tumorigenic growth (Shaper et al., 1997; Zhang et al., 2002). In response to IL-6, STAT3 can bind three types of nuclear DNA sequences [IL-6 like cytokine response elements, GAS (INF-γ- activated sequence) and SIE (Ogata et al., 1997)], and by doing so it regulates cellular differentiation (Minami et al., 1996; Ogata et al., 1997).

In the present study on thyroid nodules, we found that STAT3 expression was restricted to two histotypes: PTC (all) and FA (a minority). All 12 PTC and the 2 $STAT3^+$ FA co-expressed HGF and c-met, but only 5 PTC co-expressed IL-6 and IL-6R. Thus, STAT3 expression correlates with (and thus, is induced by) HGF/c-met, but not with IL-6/IL6-R. However, approximately one-third of totally benign colloid nodules, hyperplastic nodules and oncocytic adenomas were $HGF^+/c\text{-}met^+$ without being $STAT3^+$, suggesting that HGF/c-met does not act via STAT3 in these three histotypes. Moreover, in a proportion of colloid nodules, follicular hyperplasias and oncocytic adenomas, HGF and c-met may promote growth, while in PTC this signaling promotes morphogenesis, because morphogenesis is a STAT3-mediated event (Boccaccio et al., 1998; Maffe and Comoglio, 1998).

Because of the 100% sensitivity and - if we momentarily disregard the 2 follicular adenomas (see below)- the 100% specificity of the $HGF^+/c\text{-}met^+/STAT3^+$ signaling in PTC, we believe that involvement of this signaling is more relevant for the establishment of such morphological variety of

Table 2. Expression of the two ligand/receptor systems HGF/c-met and IL-6/IL6R, and the signal transducer protein STAT3 in malignant thyroid nodules[a,b].

THYROID CARCINOMAS	HGF/c-met SIGNAL		IL-6/IL-6R		STAT3
	Ligand	Receptor	Ligand	Receptor	
Papillary carcinomas (n=12)					
conventional (n=8)	n=8 (+++/++++; m, c)	n=8 (+++/++++; m, c)	n=8 (++/+++; c)	n=5 (+/++; c)	n=8 (+/++++; c, n)
follicular variant (n=2)	n=2 (++++; m, c)	n=2 (++++; m, c)	n=2 (++; c)	0	n=2 (+++/++++; c, n)
oncocytic variant (n=1)	n=1 (++++; m, c)	n=1 (+++; m, c)	0	0	n=1 (++; c, n)
tall cell variant (n=1)	n=1 (+++; m, c)	n=1 (+++; m, c)	0	0	n=1 (+; c, n)
all variants (n=12)	27±12% (n=12)	68±19% (n=12), p<0.001	26±12% (n=10)	24±14% (n=5)	46±17% (n=12)
Folliclar carcinomas (n=10)	0	0	0	0	0
Anaplastic carcinomas (n=6)	0	0	0	0	0

[a]: abbreviations refer to the subcellular location of the immunostaining: c, cytoplasm, m, plasma membrane; n, nucleus. The intensity of epithelial staining was scored on a five-point scale from 0 (absent) to ++++ (very intense) as specified under Materials and Methods. [b]: The proportion of positive cells was calculated based on evaluatioon of 1000 cells using x50 magnification. Data are expressed as mean±standard deviation. The p value refers to the comparison with the cognate ligand.

differentiated thyroid cancer (namely, the papillary phenotype) than the ret and trk gene rearrangements. Indeed, rearrangements of ret or trk have been reported in ≤40% and ≤10% of non-radiation-associated PTC, respectively (Sugg et al., 1996; Bongarzone et al., 1998). In addition, ret rearrangements were detected in tumors other than PTC, particularly in follicular adenomas (Ishizaka et al., 1991; Bounacer et al., 1997; Cinti et al., 2000; Sheils et al., 2000a,b; Elisei et al., 2001). Moreover, the oncogenic drive of RET/PTC oncogenes is relatively early and weak, based on their detection in micropapillary thyroid cancer (Sugg et al., 1998). The expression of the HGF/c-met/STAT3 in 100% of PTC and in no FTC and ATC supports our previous results that PTC and FTC/ATC have a different genetic background (Trovato et al., 1999).

In view of the said sharing of ret/PTC rearrangement between a number of PTC and a number of follicular adenomas, and our previous data on loss of heterozygosity (LOH) (Trovato et al., 1999), our present data on the STAT3/HGF/c-met expression in 20% of follicular adenomas provide a novel insight in the context of the progression of thyroid adenomas to thyroid carcinomas.

In fact, while a subset of follicular adenomas started losing genetic material on the long arm of chromosome 7, including the locus for HGF/c-met, another subset retained heterozygosity just as did all PTC. Because all follicular carcinomas exhibited LOH on 7q, the first subset of follicular adenomas represents adenomas which could progress to the follicular carcinoma stage, which is in keeping with the largely held schema of thyroid oncogenesis (Fagin, 1992). In contrast, the second subset of follicular adenomas could progress to papillary carcinomas. Papillary foci of hyperplasia in FA and follicular patterns in PTC (up to the establishment of the follicular variant of PTC) (Akslen and LiVolsi, 2000) would attest to the possibility that the FA to PTC transition might indeed occur.

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