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HGF/C-MET system pathways in benign and malignant histotypes of thyroid nodules: an immunohistochemical characterization

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Summary. Objective. Upon binding with HGF, the thyrosine-kinase receptor c-met induces cell growth, scattering and morphogenic effects via the trasducers STAT3 and phosphorylated-STAT3, PI3K/Akt, Rho. HGF, c-met and STAT3 are expressed with very high frequency in papillary thyroid carcinomas (PTC), suggesting a role in PTC. Herein we first investigate the simultaneous expression of HGF, c-met, STAT3, phosphor-STAT3, PI3K, Akt and Rho in thyroid nodules.

Design and methods. Using immunohistochemistry, we studied: 30 colloid nodules (CN), 18 hyperplastic nodules (HN), 20 follicular adenomas (FA), 15 oncocytic adenomas (OA), 20 PTC, 16 follicular carcinomas (FTC) and 6 anaplastic carcinomas (ATC).

Results. All 7 proteins were expressed in 15% of FA (with HGF, PI3K and Rho stromal reactivity) and 25% of PTC, and the combination HGF/c-met/STAT3/pSTAT3/PI3K was expressed by all PTC, each protein being expressed by tumor cells. In contrast, 13/16 FTC (81%) exhibited immunoreactivity for PI3K (both epithelial and stromal), and 100% of ATC was $PI3K^+$ (both epithelial and stromal) and Rho^+ (epithelial). Epithelial expression of PI3K correlated with the clinical behavior of histotypes and, within FTC, the proportion of $PI3K^+$ cells correlated with both the clinical and pathological stage ($r=0.95$; $p<0.001$). As for the shared epithelial expression of PI3K, this concerned approximately one-fourth of tumor cells in FTC and ATC *vs* one-thirtieth in PTC.

Conclusions. Our data may have practical implications for the targeted medical therapy of thyroid cancer arising from the follicular epithelium.

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Introduction

Hepatocyte growth factor (HGF) exerts mitogenic, motogenic, morphogenic, and antiapoptotic activities in various cell types, including follicular thyroid cells (thyrocytes), upon binding to its specific tyrosine-kinase receptor c-met (Bottaro et al., 1991; Naldini et al., 1991; Giordano et al., 1993; Weidner et al.,1993). 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, beta catenin and Rho. Such effectors are ubiquitous, in that they are expressed in all human tissues, and trigger distinct biological events, i.e. growth, scattering and morphogenesis, in epithelial cells (Graziani et al., 1991; Pelicci et al., 1995; Ponzetto et al., 1995, 1996; Ridley et al., 1995; Boccaccio et al., 1998; Potempa and Ridley, 1998; Coulonval et al., 2000; Kodama et al., 2000; Royal et al., 2000; Kitajo et al., 2003; Miao et al., 2003; Okano et al., 2003; Vandeput et al.,2003).

Among thyroid lesions, c-met and HGF are expressed at very high levels and frequently in papillary thyroid cancer (PTC) (75%-100% of cases), whereas they are never expressed in follicular (FTC) and anaplastic (ATC) thyroid cancers, and are expressed at low levels and infrequently in benign lesions (20-30%) (Di Renzo et al., 1995; Oyama et al., 1998; Trovato et al., 1998; Gentile et al., 2008). In benign lesions, HGF expression is restricted to stromal cells (Trovato et al.,

1998). The concurrent overexpression of the downstream molecule STAT3 has been shown in PTC, suggesting a major functional role of HGF/c-met/STAT3 signaling in this histotype (Trovato et al., 2003).

The PI3K pathway, and mainly downstream molecules such as Akt, p70S6K or PTEN, have been investigated in malignant thyroid tumors (Weng et al., 2001; Miyakawa et al., 2003; Hou et al., 2007; Wang et al., 2007; Shinohara et al., 2007; Abubaker et al., 2008; Hou et al., 2008; Paes and Ringel, 2008; Santarpia et al., 2008). Particularly, genetic alterations in the PI3K-related pathways were found with a very high prevalence in FTC and ATC (Miyakawa et al., 2003; Abubaker et al., 2008; Hou et al., 2008; Santarpia et al., 2008). The general conclusion of these studies is that activation of downstream components of the PI3K pathway represents a relevant event in thyroid tumor progression and dedifferentiation (Weng et al., 2001; Miyakawa et al., 2003; Hou et al., 2007; Wang et al., 2007; Abubaker et al., 2008; Hou et al., 2008; Paes and Ringel, 2008; Santarpia et al., 2008). Recently, Liu and co-workers (Liu et al., 2008) have examined the prevalence of mutations and copy number gains of a panel of genes associated with the PI3K/Atk pathway, including c-met, in a series of FTC and ATC but not PTC. They found c-met copy gain in 5/58 FTC, three of which showed immunoreactivity for phosporylated-Akt (Liu et al., 2008). No data are currently available regarding the expression of HGF/c-met signal and PI3K in PTC.

As is known, Rho promotes cell cycle progression from G1 to S (Noguchi et al., 1998; Coleman et al., 2004), and regulates scattering and branching morphogenesis in response to HGF/c-met activation (Kodama et al., 2000; Royal et al., 2000; Kitajo et al., 2003; Miao et al., 2003). To date, Rho has been implied in cytoskeleton reorganization and cell survival in rat thyroid cells (Barone et al., 2001), but its expression has not been studied in human thyroid tissues.

Recently, we reported the immunohistochemical expression of HGF, c-met, PI3K and Rho in benign nodular goiters associated with Hashimoto's thyroiditys (Ruggeri et al., 2010).

Herein we evaluate the immunohistochemical expression of HGF, c-met, STAT3 and its phosphorylated form (pSTAT3), PI3K and its downstream molecule Akt, and Rho in normal thyroid tissues and both benign and malignant thyroid nodules. Next, we correlate these expressions with the histology of lesions and, in the malignant nodules, with the pathological TNM and clinical stage.

Materials and mehtods

Tissue collection

One hundred and twenty-five surgical thyroid specimens were retrieved from the files of the Department of Pathology of the University of Messina, Italy. All specimens were 4% formalin-fixed and routinely processed through graded alcohol and xylene to paraffin wax. The 125 surgical specimens included the following thyroid lesions arising from the follicular epithelium, which were classified as proposed by the World Health Organization (WHO) (Hermanek and Sobin, 2002; Li Volsi et al., 2004): 30 colloid nodules (CN); 18 hyperplastic nodules (HN) of which 8 were follicular (FHN), 5 oncocytic (OHN) and 5 clear-cells (CCHN); 20 follicular adenomas (FA) (5 normofollicular, 10 microfollicular and 5 macrofollicular); 15 oncocytic adenomas (OA); 20 PTC (14 conventional and 6 follicular); 16 FTC (9 minimally invasive and 7 widely invasive); and 6 ATC.

All investigated nodular lesions were studied along with the normal non-nodular tissue from the contralateral thyroid lobe. In addition, paraffin embedded specimens of nine normal thyroids (NT) harvested at autopsy were also studied as possible control normal tissues.

Hematoxylin-eosin-stained sections of each specimen were re-evaluated by the pathologists in order to confirm the pathology diagnosis following the new WHO criteria (Li Volsi et al., 2004). The hospital Ethics Committee approved the study protocol.

Immunohistochemistry

The 134 blocks were cut into serial sections of five-micrometers each. Immunohistochemistry was performed, separately, using seven antibodies against: HGF-α (H-145, working dilution 1:100), c-met (p140 anti h-met, w.d. 1:100), STAT3 (h-190, w.d. 1:100), phosphorylated STAT3 (p-STAT3, Ser 727, w.d. 1:100), PI3K p85α (B-9, w.d. 1:200), Phospho-Akt (Ser473 D9E w.d. 1:100), Rho (Y486, w.d. 1:100). The first four antibodies are rabbit polyclonal by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), the fifth is mouse monoclonal (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), the sixth is rabbit monoclonal by Cell Signaling Technology (Cell Signaling Technology, Beverly, MA) and the seventh is rabbit monoclonal by Abcam (Abcam plc., Cambridge, UK).

Tissue sections were deparaffinized in xylene and rehydrated in alcohol. Then, the endogenous biotin was inactivated by the addition of a 0.05% (v/v) solution of streptavidin in phosphate-buffered saline (PBS), and the endogenous peroxidase activity was quenched by adding a 0.3% (v/v) solution of 3% H_2O_2/methanol for 30 min. Staining was obtained with the LSAB system (kit from Dako, Carpinteria, CA, USA). 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, Saint Louis, MO, USA) activated with 0.05% hydrogen peroxide was used to develop the end reactions. Sections were counterstained with Mayer's haematoxylin, 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 immunoabsorption 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, while specimens of lung, breast and liver carcinomas were used as positive controls for STAT3 (lung), Akt (lung), pSTAT3 (breast), PI3K (breast), and Rho (liver) immunoreaction.

For the evaluation of the results, we manually quantified the following indexes: (a) number of positive cases; (b) number of reactive epithelial and stromal cells per case, based on counting 1000 cells/case at 50x magnification; (c) subcellular location of the staining: membrane, cytoplasm and nucleus; (d) staining grade using a semiquantitave 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, the mean value was considered as the result.

Statistical analysis

Differences between means ± SD were analyzed by the two-tailed Student's t-test or Mann-Whitney test as appropriate, while differences between proportions were analyzed by the χ^2 test with Yates' correction for continuity. 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

Illustrative immunohistochemistry is presented in Fig. 1. In all reactive epithelial or stromal cells, the immunoreaction for HGF and c-met was detected in the membrane and cytoplasm, immunostaining of PI3K, Rho and Akt was observed in the cytoplasm, STAT3 immunoreaction was observed in the nucleus and cytoplasm while pSTAT3 immunoreaction only in the nucleus.

Expressions of HGF/c-met/STAT3/pSTAT3/PI3K/Akt/Rho pathway in normal thyroids and benign nodules

No expression of HGF, c-met, STAT3, pSTAT3, PI3K, pAkt and Rho could be detected in normal thyroid tissues from the 125 non-nodular parenchimas studied or the 9 autopsy normal thyroids.

Similarly, no immunostaining for all seven proteins was observed in the majority of CN (22/30 or 73%), HN (14/18 or 77%), and OA (10/15 or 66.6%) (Table 1). Of the 17 remaining reactive lesions, the eight CN (8/30 or 26%) and the four HN (4/18 or 22%) expressed HGF, c-met, PI3K and Rho, while the five OA (5/15or 33%) were HGF^+, $c\text{-}met^+$ and $PI3K^+$ (Table 1). All FA expressed PI3K (Table 1). Most of them were $PI3K^+$ solely (16/20 or 80%). Three FA (15%, two normofollicular and one microfollicular variants) expressed the full set of the seven proteins, while another one FA (macrofollicular variant) which reacted with six proteins failed to express Akt (Table 1).

There were little differences in localization of immunostaining among these four types of lesions. Thus, expression of HGF (which concerned 7-9% of cells), PI3K (3-15% of cells) as well as Rho (5-9% of cells) was only stromal (Table 2). Localization of c-met was consistently epithelial and restricted to 2-4% of cells (Table 2). STAT3, pSTAT3 and pAkt immunoexpressions were restricted to epithelial follicular cells of FA with an average of 3% of cells for the first two proteins, respectively, and 7% of cells for the last (Table 2).

Expressions of HGF/c-met/STAT3/pSTAT3/PI3K/Akt/Rho pathway in malignant nodules

All 7 proteins were expressed in 5/20 (25%) PTC (two conventional and three follicular variants), but the combination $HGF^+/c\text{-}met^+/STAT3^+/pSTAT3^+/PI3K^+$ was expressed by all 20 PTC (100%) (Table 1). In sharp contrast, 13/16 FTC (or 81%, 7 minimally invasive and 6 widely invasive) exhibited immunoreactivity for PI3K, and 100% of ATC was $PI3K^+$ and Rho^+ (Table 1). In addition, one $PI3K^+$ FTC (widely invasive) and one $PI3K^+$ ATC also expressed pAkt (Table 1). The reactivity against all 7 proteins observed in 5 PTC was matched by 3 FA, though the pattern of immunoexpression was different. First, HGF staining was restricted to stromal cells (8% of these cells) in the FA, while it was restricted to the thyrocytes in the PTC (an average of 23% of the cancer epithelial cells) (Table 2). Second, the same difference in localization of immunoreactivity applied to Rho, and to a much greater extent (5% of the adenoma stromal cells vs. an average of 40% of the cancer epithelial cells), as well as to PI3K, but with an inverted quantitative pattern (15% of the adenoma stromal cells vs. an average of 4% of the cancer epithelial cells) (Table 2). Third, the four HGF^+ FA and all PTC shared the epithelial expression of c-met, STAT3 and pSTAT3 but this was restricted to just 2% of the adenoma epithelial cells for each protein as opposed to 47%, 41% and 12% of the cancer epithelial cells, respectively (Table 2). Fourth, the grade of immunoreaction for each of the seven proteins was intense to very intense in the epithelial cells of PTC, while it was weak to intense in the epithelial and stromal cells of the four FA (data not shown). PTC shared with FTC and ATC the epithelial expression of either PI3K or pAkt and with ATC the epithelial expression of Rho. However, in FTC and ATC immunoreactivity for PI3K concerned approximately one-fourth (23-27%) of cells as opposed to one-thirtieth (4%) in PTC, whereas in ATC immunoreactivity for Rho concerned one-fifth of cells as opposed to approximately one-half in PTC

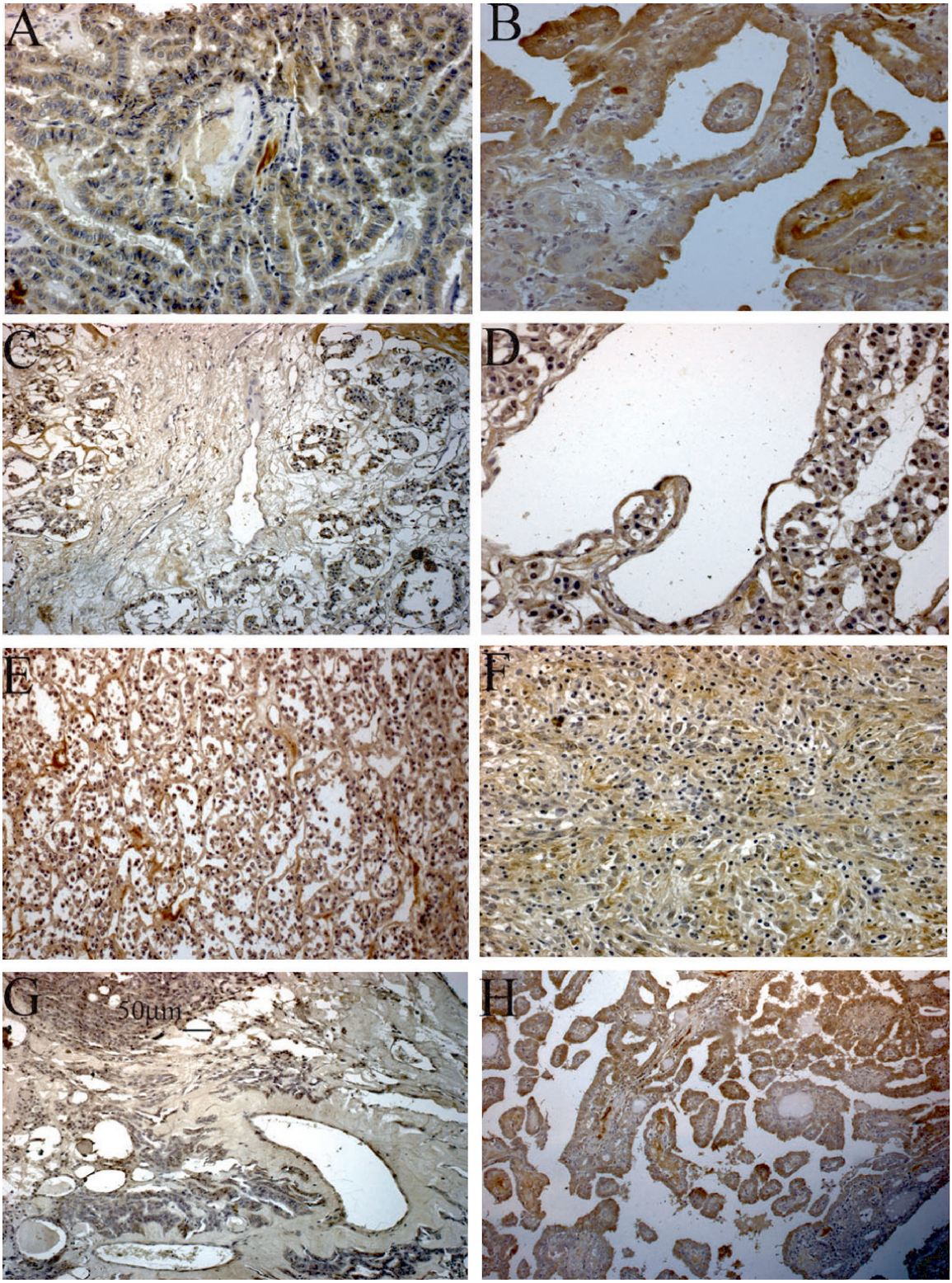

Fig. 1. HGF, c-met, STAT3, pSTAT3, PI3K, AKT and Rho immunostaining in representative thyroid nodules. **A.** Intense HGF immunoreaction in a follicular variant of papillary thyroid cancer (PTC). The HGF immunostaining is located in the membrane and cytoplasm of the cancerous epithelial cells. **B.** Very intense c-met immunostaining in a classic variant of PTC. The c-met reaction is observed in the membrane and cytoplasm of epithelial cells. **C.** Intense STAT3 immunoreaction in a follicular variant of PTC. Note the positive nuclear immunoreaction of the epithelial cells. **D.** Moderate pSTAT3 immunoreaction in a follicular variant of PTC. Note the positive nuclear immunoreaction of the epithelial cells. **E.** Intense PI3K immunoreaction in a follicular thyroid cancer (FTC). The PI3K immunoreaction is located in epithelial cells. **F.** Intense PI3K immunoreaction in an anaplastic thyroid cancer (ATC). The PI3K immunoreaction is located on the epithelial cells. **G.** Moderate Akt immunoreaction in a classic variant of PTC. The Akt immunoreaction is located in the epithelial cells. **H.** Intense Rho immunoreaction in a classic variant of PTC. The Rho immunoreaction is located in epithelial cells. A, F, x 250; B-E, G, H, x 150

Table 1. Expression of HGF, c-met, STAT3, pSTAT3, PI3K, Akt and Rho in benign and malignant thyroid nodules*.

	Thyroid nodules	No. of positive cases	Positive case no.	Immunoreactivity						
				HGF	c-met	STAT3	pSTAT3	PI3-K	Akt	Rho
BENIGN NODULES	COLLOID NODULES (CN, n=30)	8	# 1-5	+	+	-	-	+	-	+
			# 16-18	+	+	-	-	+	-	+
	HYPERPLASTIC NODULES (HN, n=18)	4								
	Follicular (FHN, # 1-8)		# 1-4	+	+	-	-	+	-	+
	Oncocytic (OHN, # 9-13)		-							
	Clear cell (CCHN, #14-18)		-							
	FOLLICULAR ADENOMAS (FA, n=20)	20								
	Normofollicular (# 1-5)		# 1, 2	+	+	+	+	+	+	+
			# 3-5	-	-	-	-	+	-	-
	Microfollicular (# 6-15)		# 6	+	+	+	+	+	+	+
			# 7-15	-	-	-	-	+	-	-
	Macrofollicular (# 16-20)		# 16	+	+	+	+	+	-	+
			# 17-20	-	-	-	-	+	-	-
	ONCOCYTIC ADENOMAS (OA, n=15)	5	# 1-5	+	+	-	-	+	-	-
MALIGNANT NODULES	PAPILLARY CARCINOMAS (PTC, n=20)	20								
	conventional variant (# 1-14)		# 1, 2	+	+	+	+	+	+	+
			# 3-14	+	+	+	+	+	-	+
	follicular variant (# 15-20)		# 15-17	+	+	+	+	+	+	+
			# 18-20	+	+	+	+	+	-	+
	FOLLICULAR CARCINOMAS (FTC, n=16)	13								
	minimally invasive (# 1-9)		# 1-7	-	-	-	-	+	-	-
	widely invasive (# 10-16)		# 10	-	-	-	-	+	+	-
			# 11-16	-	-	-	-	+	-	-
	ANAPLASTIC CARCINOMAS (ATC, n=6)	6	# 1	-	-	-	-	+	+	+
			# 2-6	-	-	-	-	+	-	+

* Thyroid benign and malignant lesions have been classified on histological bases as proposed by the World Health Organization (WHO) in WHO Classification of Tumours: Pathology and Genetics of Tumours of Endocrine Organs (Li Volsi et al., 2004)

Table 2. Expression of HGF, c-met, STAT3, pSTAT3, PI3K, Akt and Rho in epithelial and stromal cells of the different histotypes of thyroid lesions.

	Thyroid nodules (no of positive cases)	Tumor cells*	Mean percentage ± SD of +ve cells*						
			HGF	c-met	STAT3	pSTAT3	PI3K	Akt	Rho
BENIGN NODULES	COLLOID NODULES (CN, n= 8)	epithelial	-	3 ± 0.5	-	-	-	-	-
		stromal	7 ± 3	-	-	-	4 ± 1	-	6 ± 2
	FOLLICULAR HYPERPLASTIC NODULES (FHN, n= 4)	epithelial	-	5 ± 1	-	-	-	-	-
		stromal	9 ± 3	-	-	-	6 ± 3	-	9 ± 3
	FOLLICULAR ADENOMAS (FA, n= 20)	epithelial	-	2 ± 0	2 ± 0	2 ± 1	-	7± 1	-
		stromal	8 ± 3	-	-	-	15 ± 3	-	5 ± 0
	ONCOCYTIC ADENOMAS (OA, n = 5)	epithelial	-	2 ± 1	-	-	-	-	-
		stromal	8 ± 3	-	-	-	8 ± 3	-	-
MALIGNANT NODULES	PAPILLARY CARCINOMAS (PTC, n= 20)	epithelial	23 ± 11	47 ± 16	41± 12	12 ± 5	4 ± 1	4 ± 1	40 ± 14
		stromal	-	-	-	-	-	-	-
	FOLLICULAR CARCINOMAS (FTC, n = 13)	epithelial	-	-	-	-	23 ± 9	5 ± 0	-
		stromal	-	-	-	-	7 ± 2	-	-
	ANAPLASTIC CARCINOMAS (ATC, n = 6)	epithelial	-	-	-	-	27 ± 8	3 ± 0	20 ± 5
		stromal	-	-	-	-	6 ± 1	-	-

* The count of the reactive epithelial and stromal cells was based on evaluation of 1000 cells/case, using a 50x magnification.

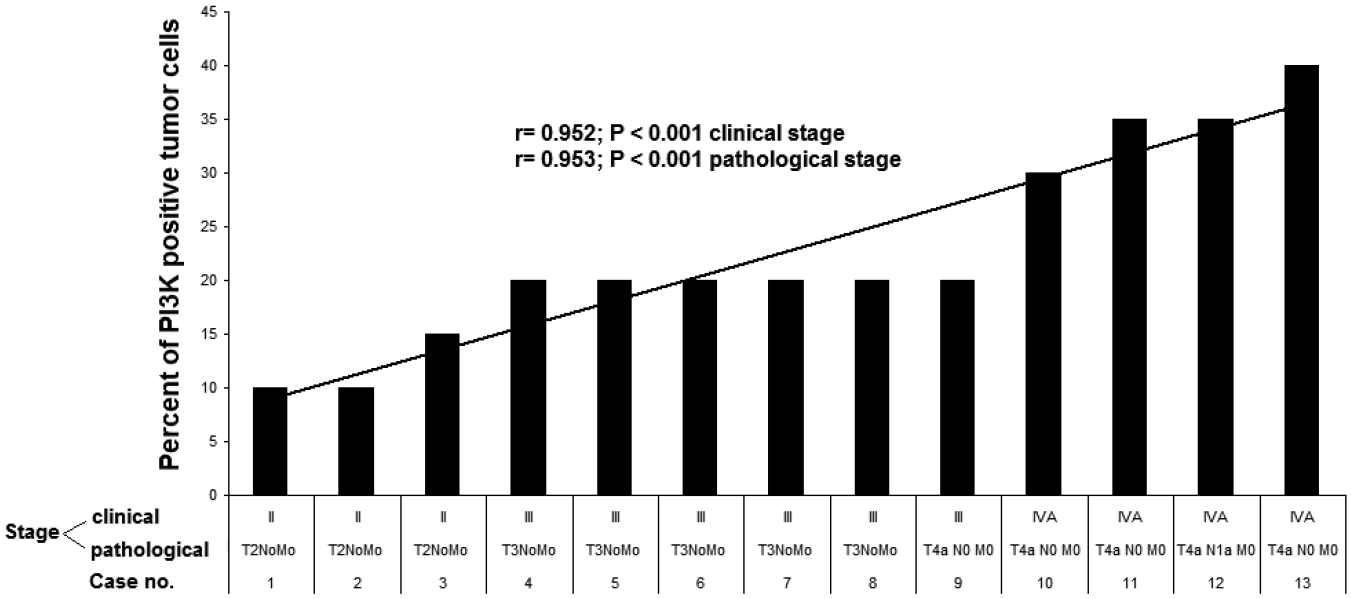

Fig. 2. Correlation of PI3K expression with the clinical or pathologic stage in follicular carcinomas. Because the Arabic digit in the pathological stage numbering corresponds to the Roman digit in the clinical stage numbering (2 → II; 3 → III; 4 → IV), the Pearson's coefficient of correlation applies to both the clinical stage and the pathological stage. Pathologic (pTNM) and clinical stages have been determined, according to the revised criteria of the American Joint Committee of Cancer (Hermanek et al., 2002). Follicular carcinomas were confined inside the thyroid in cases 1 through 6. In cases 7 through 13 the tumor extended outside the thyroid capsule, invading the surrounding soft tissues. Only case 10 had laterocervical lymph node metastases.

(Table 2). pAkt distribution was quite similar in the three lesions (3% to 5% of tumour cells).

Correlations

In the 20 PTC, a comparison was possible based on stage (that is, between the seven T2 and the thirteen T3 lesions) or subtype (that is, between the 14 conventional and the six follicular variants). The follicular variant cancers had values of c-met (56.5±18.2% vs. 42.5±14.2%, p=0.05) and Rho (53.3±14.6% vs. 34.3±10.5%, p=0.006) expression significantly greater than the conventional variant ones.

As also supported by the linear regression analysis mentioned above and shown in Fig. 2, the aggressive behavior of carcinomas correlated with the proportion of cancer epithelial cells that expressed PI3K. Indeed, this proportion was 16±4.7% in the microinvasive FTC but it was twice as much in the widely invasive FTC (30±8.3%; p<0.04) and in the ATC (27±4%, p<0.01). By contrast, the PI3K-positive epithelial cells of PTC were 4±1%, five-fold less than in microinvasive FTC (p<0.0001), and none of the benign nodules had epithelial expression of PI3K.

Since FTC encompassed three stages (II, III and IVA), we were able to stratify the individual percentage of PI3K positive cells based on stage. We show that such percentage increased progressively from group II though group IV (11.6±2.8 though 35±4, p<0.0001). The same highly significant relationship, as evaluated by linear regression analysis, is illustrated in Fig. 2. No such correlation with malignancy was apparent upon analyzing the stromal expression of PI3K (data not shown).

Of the several possible relationships between any two variables that we have evaluated, there were no other statistically significant correlations.

Discussion

In this study we have shown a correlation between benign and malignant nodules, as well as among different hystotypes of thyroid lesions, and the immunohistochemical expressions of HGF/c-met/STAT3/pSTAT3/PI3K/pAkt/Rho. These findings are in line with our previous reports on HGF/cmet and STAT3 expression in thyroid nodules (Trovato et al., 2003).

In the series of thyroid lesions previously analyzed, we have shown the immunohistochemical co-expression of HGF and c-met in PTC thyrocytes, raising the possibility of c-met activation through an autocrine loop (Trovato et al., 1998). Next, we have shown concurrent overexpression of the downstream molecule STAT3 in PTC, so that the pathway HGF/c-met/STAT3 was overexpressed in 100% of PTC and 0% of either FTC or ATC, suggesting that such an autocrine signaling pathway might be one of the relevant phenotypic

features of PTC (Trovato et al., 2003). Normal thyroid tissue and benign nodules were STAT3 negative, except for a few follicular adenomas (FA). Indeed, this subset of STAT3+ve FA co-expressed HGF, c-met and STAT3, a finding that led us to hypothesize that these adenomas may progress to PTC (Trovato et al., 2003). Our present study on another series of 125 thyroid lesions offers some new knowledge.

Moving rightwards, from benign lesions encompassing five histotypes (CN, FHN, FA and OA) through malignant histotypes (PTC, FTC and ATC), our immunohistoistochemistry study detected a pattern for the trio HGF/PI3K/Rho stromal expression in the benign lesions and epithelial expression in the malignant lesions.

In the said 26% of CN, 22% of FHN and 20% of FA, approximately 8% of stromal cells expressed HGF, thus providing the ligand for a paracrine interaction with the c-met expressed on a very limited number of epithelial cells. However, this possible interaction was not accompanied by the epithelial expression of any of the three downstream signals investigated (STAT3, PI3K and Rho) in the full group of benign lesions, whereas it was accompanied by expression of STAT3 and pSTAT3 in a number of FA epithelial cells equal to that which expressed c-met. On the other hand, because in the same 26% of CN, 22% of FHN and 20% of FA there was stromal expression of PI3K and Rho, in the absence of stromal expression of c-met, PI3K and Rho expression is independent of HGF/c-met signaling.

Only 5/15 OA had epithelial cells immunoreactive. Immunoreaction concerned c-met only and just 2% of the adenoma epithelial cells. Because these 5 OA also had a stromal-restricted expression of both HGF and PI3K, which concerned approximately 8% of the OA stromal cells, but no stromal expression of c-met, the stromal expression of PI3K should be consequential to signalings other than the HGF/c-met.

The rate with which PI3K expression occurred was small in benign histotypes (20-33%) except for FA (80%). Crossing the benignity line, the PI3K expression rate was either the highest (FTC, 81%; PTC, 100%; ATC, 100%) and with both stromal and epithelial location. In FTC, we found that 10% to 40% of the cancer epithelial cells expressed PI3K, depending on the stage of the disease. Such direct correlation of PI3K with malignancy can be generalized to thyroid carcinomas arising from the follicular epithelium, because PI3K epithelial expression was displayed only by the carcinomas, as opposed to adenomas or pre-adenoma lesions, and with an increasing gradient that matches their gradient in clinically malignant behaviour (PTC << FTC < ATC). In FA and PTC, the expression of pAkt appeared only in the reactive cases showing the full signalling network of HGF pathway. These findings associated with the absence of pAkt expression in FA and PTC showing PI3K alone let us hypothesize that pAkt expression in these lesions is linked to the activation of HGF signal. In contrast, in FTC and ATC, pAkt expression was associated to PI3K but not linked to the HGF pathway.

The present data are in line with data from other groups providing strong evidence that aberrant activation of PI3K/Akt pathway plays an extensive role in thyroid tumorigenesis, particularly in FTC and ATC, and promotes thyroid cancer progression as the genetic alterations of this pathway accumulate (Weng et al., 2001; Miyakawa et al., 2003; Hou et al., 2007, 2008; Wang et al., 2007; Abubaker et al., 2008; Paes and Ringel, 2008; Santarpia et al., 2008). Activating mutations and PI3K gene amplification, known to be associated with increased PI3K signaling, have been identified in thyroid cancer. In FTC, the frequency of mutations in the "hot spot" domains of PI3K has ranged from 6 to 13% depending on the population (Hou et al., 2007; Wang et al., 2007; Paes and Ringel, 2008). Moreover, 24-58% of FTC have reported to harbor PI3K gene amplification (defined as 4 or more copies) (Hou et al., 2007; Wang et al., 2007; Paes and Ringel, 2008). In PTC, the frequency of mutations in PI3K appears to be lower than FTC, with occurrence rates ranging from 0-3%. Similarly to FTC, gene amplification of PI3K is more common than mutations in PTC, but its frequency is lower than in FTC with occurrence rates ranging from 5-53% (Hou et al., 2007; Wang et al., 2007; Abubaker et al, 2008; Paes and Ringel, 2008). Moreover, an increased immunohisto-chemical expression of the downstream kinase Akt, has been reported (Miyakawa et al., 2003). Of significant interest have been the recent findings that ATC frequently harbor mutations and gene amplification in PI3K. In a study on fifty ATC patients by Hou, et al, 58% of ATC harbored almost one of the three studied genetic abnormalities (PI3K mutations, PI3K gene amplifications and/or PTEN mutations) associated with increased PI3K signaling. In comparison, 31% of benign follicular adenomas, 55% of FTC and 24% of PTC in this study had one of these abnormalities. The authors thus concluded that the data supported a role for PI3K signaling in thyroid tumorigenesis, particularly for FTC, and in thyroid cancer progression toward ATC. In a second study that included samples from 36 ATC patients from Santarpia and coworkers, PI3K mutations were found in five (14%) ATC, one of which had mutations in both differentiated and anaplastic areas, gene amplifications in 14 (39%), and PTEN mutations in 2 (6%) ATC. By immunohistochemistry, pAkt expression was demonstrated in 22 (61%) ATC, either in the cytoplasm (all positive cases) or in the nucleus (17 cases) of tumor cells. Cytoplasmatic staining for pAkt was noted also in PTC. The authors concluded that PI3K alterations were the most relevant events during thyroid cancer progression. A variety of other mechanisms has been thought to be responsible for activation of PI3K signaling in thyroid cancer, including signaling through oncogenes, such as RET/PTC or RAS, and signaling through other receptor tyrosine kinases known to be commonly overexpressed in thyroid cancers, such as c-

met, IGF-1, and VEGF receptors (Kondo et al, 2006). Liou and coworkers found a high prevalence of genetic alterations (mutations and/or gene amplification) of several tyrosine kinase receptors that could activate the PI3K/Akt pathway, including c-met, in a series of FTC and ATC (but not PTC). Particularly, they found c-met copy gain in 5/58 FTC, three of which showed immunoreactivity for pAkt (Liu et al., 2008). No other data are available in the literature on a possible link between HGF/c-met and PI3K/Akt expression/activation. Our data confirm that PI3K pathway activation is a crucial event in FTC and ATC progression, but it is likely driven by signals other than HGF/c-met in these hystotypes, whereas an PI3K-independent activation of pAkt via HGF/c-met may occur in FA and PTC.

There are at least two practical implications of these findings: prognostic and therapeutic. At prognostic level, the greater the proportion of FTC tumour cells that express PI3K, the more advanced the malignancy is. At a therapeutic level, it would make sense to test viable compounds that inhibit the expression of PI3K in the epithelial component of thyroid cancer as a novel modality of targeted medical treatment. With a similar reasoning, in the much needed effective drugs to treat ATC one would test a compound that inhibits both PI3K and Rho expression or that blocks their action downstream.

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