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Neutrophil gelatinase-associated lipocalin (NGAL) immunohistochemical expression in follicular cell-derived thyroid tumors: a novel diagnostic tool?

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Summary. Discrimination of follicular cell-derived benign and malignant tumors of the thyroid is one of the major problems encountered in surgical pathology. In the present study, we evaluated the immunohistochemical expression of NGAL, an iron-binding protein involved in the infiltrative potential of cancer cells, in a cohort of tumors including 8 follicular adenomas (FA), 2 Hurthle cell adenomas (HA), 2 atypical adenomas (AA), 8 minimally invasive follicular carcinomas (MIFC), 9 widely invasive follicular carcinomas (WIFC), 3 Hurthle cell carcinomas (HC) and 8 papillary carcinomas (PC) with 5 follicular-variant PC (FVPC) and 3 not otherwise specified (PC-NOS). Our goal was to test whether evaluation of NGAL immuno-expression may be of use in the differential diagnosis of benign and malignant thyroid neoplasias. 92% of benign tumors (specificity) were negative for NGAL, whereby NGAL immuno-expression was found in 82% (sensitivity) of malignant tumors, and, specifically, in 100% of MIFC, in 87% of WIFC, in 100% of HC, in 80% of FVPC. None of the PC-NOS displayed NGAL staining. When only tumors with a follicular architecture were considered, NGAL specificity for malignant lesions was 92%; sensitivity, positive predictive value and negative predictive value were 92%, 96% and 85%. Diagnostic accuracy of NGAL expression in the differential diagnosis between benign and malignant follicular tumors was 92%. In conclusion, NGAL protein seems to represent a marker of malignant follicular cell-derived thyroid tumors, and especially of those with follicular architecture. Hence assessment of its expression might be of use with respect to differential diagnosis from follicular benign neoplasias.

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Introduction

Thyroid carcinomas account for the majority of endocrine neoplasms worldwide, and their incidence rates have steadily increased over the past few decades (De Lellis et al., 2004).

The discrimination between benign and malignant thyroid nodules represents one of the major problems encountered in diagnostic surgical pathology. Specifically, in the World Health Organization (WHO) classification, follicular carcinoma of the thyroid is defined as a malignant epithelial tumour showing follicular cell differentiation and lacking the diagnostic nuclear features of papillary thyroid carcinoma (De Lellis et al., 2004). The diagnosis is not based on architectural or cytological features, but on the presence of invasion through the capsule and/or invasion into the blood vessels. Thus, the histological criteria, though posing few problems in cases of widely invasive carcinoma, may lead to inter-observer variation among pathologists, in discriminating minimally invasive (or encapsulated) carcinoma from adenoma (Hirokawa et al., 2002). Also, the differential diagnosis of the follicular variant of papillary thyroid carcinoma from follicular adenoma or carcinoma may be challenging for the pathologist when the nuclear features of papillary carcinoma are not well developed or are only focally expressed (Elsheikh et al., 2008).

On the light of this, more objective markers able to discriminate between these tumours have been investigated, including galectin-3, HBME-1, cytokeratin-19 and matrix-metallo proteinases (MMP),

or proliferation markers such as MCM2 and Ki-67. The findings were generally encouraging and promising although some studies demonstrated inconclusive or conflicting results (Sahoo et al., 2001; Hermann et al., 2002; Erickson and Lloyd, 2004; Prasad et al., 2005; Barroeta et al., 2006; Cho et al., 2006a,b; El Demellawy et al., 2008; Buergy et al., 2009; Saleh et al., 2010). Thus additional studies are needed in the search to identify useful markers for differentiating benign from malignant thyroid nodules (Saleh et al., 2010).

Neutrophil gelatinase-associated lipocalin (NGAL), also known as NRL (neu-related lipocalin), oncogene 24p3, uterocalin and lipocalin 2 (LCN2), is a 25 KDa protein which is stored in the granules of human neutrophils (Cowland and Borregaard, 1997). NGAL participates in iron trafficking (Yang et al., 2002) and increases the cytoplasmic levels of this mineral by capturing and transporting the iron particles to the inner cell after interaction with specific membrane receptors (24p3, megalin) (Goetz et al., 2000). Its role in iron delivery into cells underlies the multiple effects attributed to NGAL. Released by activated neutrophils, this protein participates in the iron depletion strategy exploited in the immune defense against bacterial pathogens (Goetz et al., 2000). In addition, NGAL seems to be involved in the growth, development and differentiation of several human tissues, as early as in the embryo, through its regulation of iron responsive-genes which are important in the differentiation of primordial cells (Gwira et al., 2005). Finally, NGAL seems to participate in cancerogenesis by favoring iron uptake from extracellular space within the malignant cells, a fundamental process for the maintenance of neoplastic cell multiplication (Devireddy et al., 2005). Consistently, NGAL synthesis is induced by factors promoting the development of neoplasias (Bratt, 2000) and its over-expression has been reported in several malignancies (Bauer et al., 2008; Iannetti et al., 2008; Barresi et al., 2010a-c, 2011). The negative prognostic value of NGAL expression in human neoplasias (Bauer et al., 2008; Barresi et al., 2010b, 2011) seems to be linked to its role in cancer progression. Indeed this protein may favor cancer progression through the (positive) modulation of matrix metallo-proteinase-9 (MMP-9), which degrades the basement membranes and extracellular matrix enabling neoplastic cell invasion (Yan et al., 2001). In addition, NGAL has been shown to enhance the invasive and metastatic potential of colon cancer cells through an iron-dependent mechanism (Hu et al., 2009).

In the light of its action in the enhancement of cancer cell invasiveness, in the present study we aimed to analyze whether NGAL immunohistochemical expression differs in follicular cell-derived thyroid tumors on the basis of infiltrative potential of neoplastic cells. Thus, our goal was to determine whether evaluation of NGAL immuno-expression may be of use in the differential diagnosis of follicular cell-derived benign and malignant thyroid neoplasias.

Materials and methods

Forty surgical specimens of primary thyroid tumors, obtained from an equal number of patients (24 female and 16 male patients; age range 20-78 years; mean age: 50.2 years), were retrieved from the files of the Department of Human Pathology, University of Messina, Italy, and tested for NGAL immunohistochemical expression. Tumors were classified according to the World Health Organization classification system criteria (De Lellis et al., 2004) into eight follicular adenomas (FA), two Hurthle cell adenomas (HA), two atypical adenomas (AA), eight minimally invasive follicular carcinomas (MIFC), nine widely invasive follicular carcinomas (WIFC), three Hurthle cell follicular carcinomas (HC) and eight papillary carcinomas (PC) with five follicular-variant PC (FVPC) and three PC not otherwise specified (PC-NOS). Among MIFC, four displayed capsular and vascular invasion (Table 1: cases 16, 17, 18, 19) while the remaining four were characterized by only capsular invasion.

NGAL immuno-expression was also analyzed in the thyroid parenchyma adjacent to the tumors, as well as in six thyroids obtained from foetuses at different gestational ages (ranging between 16 and 23 weeks) of gestation.

All cases had been formalin fixed and paraffin embedded. For each case, 4 μm thick sections were cut form the corresponding paraffin block for subsequent immunohistochemical study.

Briefly, the endogenous peroxidase activity was blocked with 0.1% H_2O_2 in methanol for 20 min.; then, normal sheep serum was applied for 30 min to prevent unspecific adherence of serum proteins. Sections were successively incubated at 4°C overnight with the primary antibody against NGAL (Santa Cruz Biotechnology, Santa Cruz, CA; w.d. 1:100). The bound primary antibody was visualized by the envision peroxidase detection system. To reveal the immunostaining, the sections were incubated in darkness for 10 min. with 3-3' diaminobenzidine tetra hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), in the amount of 100 mg in 200 ml 0.03 % hydrogen peroxide in phosphate-buffered saline (PBS). Nuclear counterstaining was performed by Mayer's haemalum. Specificity of the binding was assessed by omitting the primary antiserum or replacing it with normal rabbit serum or phosphate buffered saline solution (PBS, pH 7.4). Proximal tubules within samples of normal renal parenchyma were used as positive controls for the immunoreactions (Barresi et al., 2010a).

The assessment of immuno-stained section was performed by two independent pathologists, blinded to the clinico-pathological data. NGAL expression was based on the presence of cytoplasmic and membranous staining. The intensity of staining (IS) was graded as (0) negative, (1) weak, (2) moderate, (3) strong; the area of staining positivity (ASP), recorded as percentage of neoplastic positive cells, was assessed by following

values: 0 (< 10%), 1 (11-25%), 2 (26-50%), 3 (51-75%), 4 (>75%), according to the procedure previously described (Barresi et al., 2010a,c). Then, an intensity-distribution (ID) score was generated for each case by multiplying the values of IS and ASP. Cases displaying an ID score 0 were considered as negative for NGAL.

Fisher exact test was performed to assess the statistical correlations between NGAL expression and the various clinico-pathological parameters, such as age and sex of the patients and the T stage or type (benign vs malignant) of the tumors. A probability (P) value less than 0.05 was considered statistically significant. Data were analysed using the SPSS package version 6.1.3 (SPSS Inc., Chicago, IL, USA).

The percent of malignant lesions with positive staining for NGAL of the total malignant lesions (sensitivity), the percent of unstained benign lesions of the total benign lesions (specificity), the percent of malignant lesions with positive staining of the total lesions with positive staining (positive predictive value), and the percent of benign lesions with negative staining to NGAL of the total lesions with negative staining (negative predictive value) were calculated. The diagnostic accuracy (true positive+true negative/ all positive+ all negative) of NGAL staining was also assessed.

Sensitivity, specificity, negative and positive predictive values, as well as diagnostic accuracy of NGAL staining in the differential diagnosis between benign and malignant tumors with follicular architecture, were also assessed by excluding papillary carcinomas from the analysis .

Results

No NGAL staining was observed in the follicular cells of the thyroid parenchyma adjacent to the analyzed tumors (Fig. 1). On the contrary, cytoplasmic NGAL immuno-expression with moderate intense staining (2+) was encountered in all the fetal thyroids (Fig. 2).

Clinico-pathological characteristics as well as NGAL immuno-expression data relative to the analyzed thyroid neoplasias are summarized in Table 1.

A variable NGAL staining was evidenced in 24 out of the 40 thyroid tumors included in the cohort, and specifically in 1/8 (12%) of FA, in 8/8 (100%) of MIFC (Fig. 3a), in 7/8 (87%) of WIFC (Fig. 3b), in 4/5 (80%) of FVPC (Fig. 3c), in 3/3 (100%) of HC (Fig. 3d). None of the PC-NOS displayed NGAL staining (Fig. 3e). 7/8 (87%) of FA were negative for NGAL (Fig. 3f).

In all the positive tumors NGAL immuno-reactivity was present in the cytoplasm of the neoplastic cells. In the only NGAL positive FA, staining was weak (1+) and involved 20% of neoplastic cells (Table 1). In the HC, in all but one positive MIFC (case n. 20) and in all but one WIFC (case 23), NGAL labelling was widespread and involved 100% of neoplastic cells (Table 1). In MIFC n. 20 (Table 1), NGAL immunostaining was limited to the neoplastic cells at the capsular infiltrative front.

With reference to positive FVPC, half were stained by anti-NGAL antibody in 100% of cells, while in the other half NGAL immuno-expression was confined to 25% of neoplastic cells.

At statistical analysis through Fisher exact test, no statistically significant associations emerged between NGAL expression and age and gender of the patients or T of the tumors (Table 2). NGAL expression appeared to be significantly more frequent in the malignant tumors in comparison to benign ones (P= 0.000006) (Table 2), even when only lesions with follicular architecture were considered (P= 0.000001) (Table 2).

NGAL expression was specific (specificity: 92%) for carcinoma. The probability that a malignant thyroid tumor expressed NGAL (sensitivity) was 82%; the

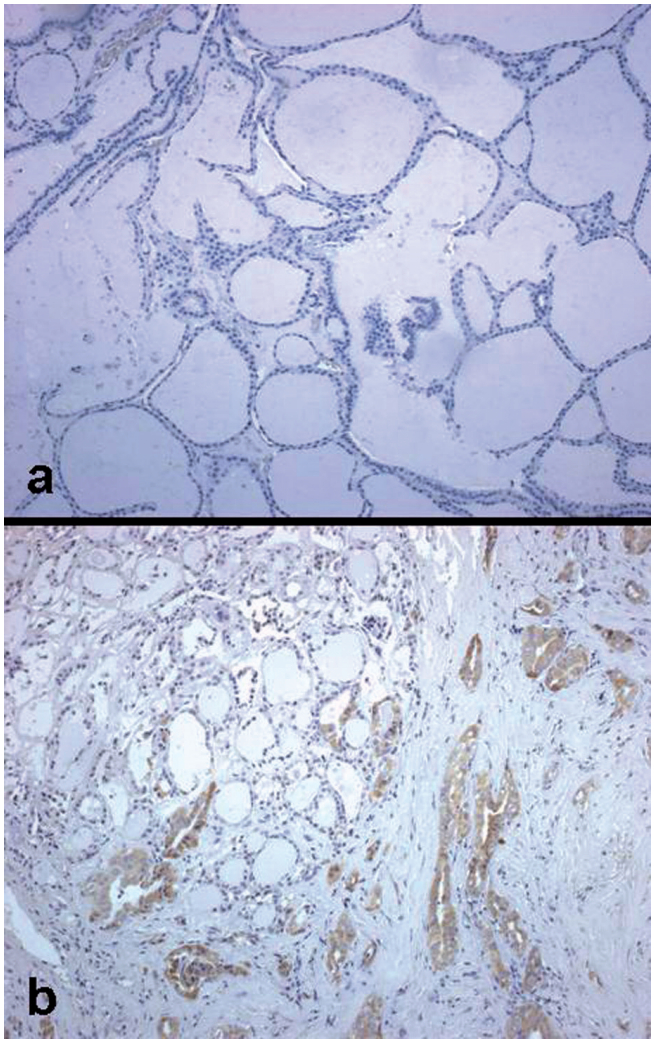

Fig. 1. a. Absence of staining for NGAL in thyroid parenchyma adjacent to one of the analyzed tumors. NGAL stain. **b.** Absence of staining for NGAL in thyroid parenchyma adjacent to one of the analyzed tumors (left) and NGAL immuno-expression in the neoplastic glands. NGAL stain. x 100

probability that a positive NGAL thyroid neoplasia was a carcinoma (positive predictive value) was 96%; the probability that a negative tumor was a benign one (negative predictive value) was 69%. Diagnostic accuracy of NGAL expression in the differential diagnosis between benign and malignant thyroid tumors was 85%.

When only tumors with a follicular architecture were taken into consideration, NGAL specificity for malignant lesions was 92%; sensitivity, positive predictive value and negative predictive value were 92%, 96% and 85%. Diagnostic accuracy of NGAL

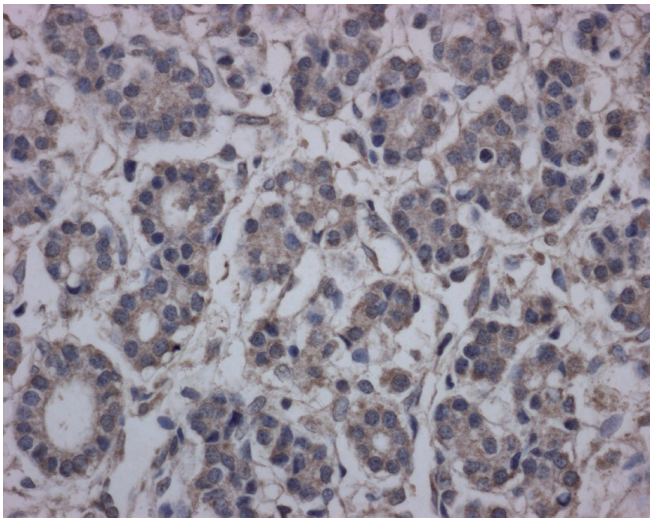

Fig. 2. NGAL immuno-expression in the cytoplasm of fetal thyrocytes. NGAL stain. x 400

Table 1. Clinico-pathological characteristics and NGAL immuno-expression relative to the forty analized tumors.

Case	sex	age	diagnosis	NGAL IS	NGAL ASP	NGAL ID score	T
1	F	48	follicular adenoma	0	0	0	
2	M	60	follicular adenoma	0	0	0	
3	F	63	follicular adenoma	0	0	0	
4	M	61	follicular adenoma	0	0	0	
5	M	64	follicular adenoma	1	1	1	
6	M	46	follicular adenoma	0	0	0	
7	F	40	follicular adenoma	0	0	0	
8	M	52	follicular adenoma	0	0	0	
9	F	66	Hurthle cell adenoma	0	0	0	
10	F	20	Hurthle cell adenoma	0	0	0	
11	F	48	atypical follicular adenoma	0	0	0	
12	F	35	atypical follicular adenoma	0	0	0	
13	F	40	minimally invasive follicular carcinoma	2	4	8	T1
14	F	49	minimally invasive follicular carcinoma	3	4	12	T1
15	F	56	minimally invasive follicular carcinoma	2	4	8	T1
16	F	58	minimally invasive follicular carcinoma	2	4	8	T1
17	F	49	minimally invasive follicular carcinoma	1	4	4	T1
18	F	45	minimally invasive follicular carcinoma	1	4	4	T1
19	M	50	minimally invasive follicular carcinoma	1	4	8	T1
20	F	47	minimally invasive follicular carcinoma	1	2	2	T1
21	M	55	follicular carcinoma	0	0	0	T2
22	F	65	follicular carcinoma	2	4	8	T1
23	F	78	follicular carcinoma	1	1	1	T2
24	F	54	follicular carcinoma	3	4	12	T2
25	F	52	follicular carcinoma	2	4	8	T2
26	F	46	follicular carcinoma	2	4	8	T2
27	F	62	follicular carcinoma	3	4	12	T1
28	M	55	follicular carcinoma	2	4	8	T2
29	F	67	follicular carcinoma	3	4	12	T2
30	F	38	Hurthle cell carcinoma	2	4	8	T1
31	M	42	Hurthle cell carcinoma	2	4	8	T1
32	F	32	Hurthle cell carcinoma	2	4	8	T1
33	M	35	papillary carcinoma	0	0	0	T1
34	M	29	papillary carcinoma	0	0	0	T2
35	M	44	papillary carcinoma	0	0	0	T2
36	F	43	papillary carcinoma follicular variant	2	4	8	T1
37	M	40	papillary carcinoma follicular variant	0	0	0	T2
38	M	71	papillary carcinoma follicular variant	2	1	2	T1
39	F	66	papillary carcinoma follicular variant	3	1	3	T1
40	F	38	papillary carcinoma follicular variant	3	4	12	T1

IS: Intensity of Staining; ASP: Area of Staining Positivity; ID: Intensity Distribution; F: female; M: male.

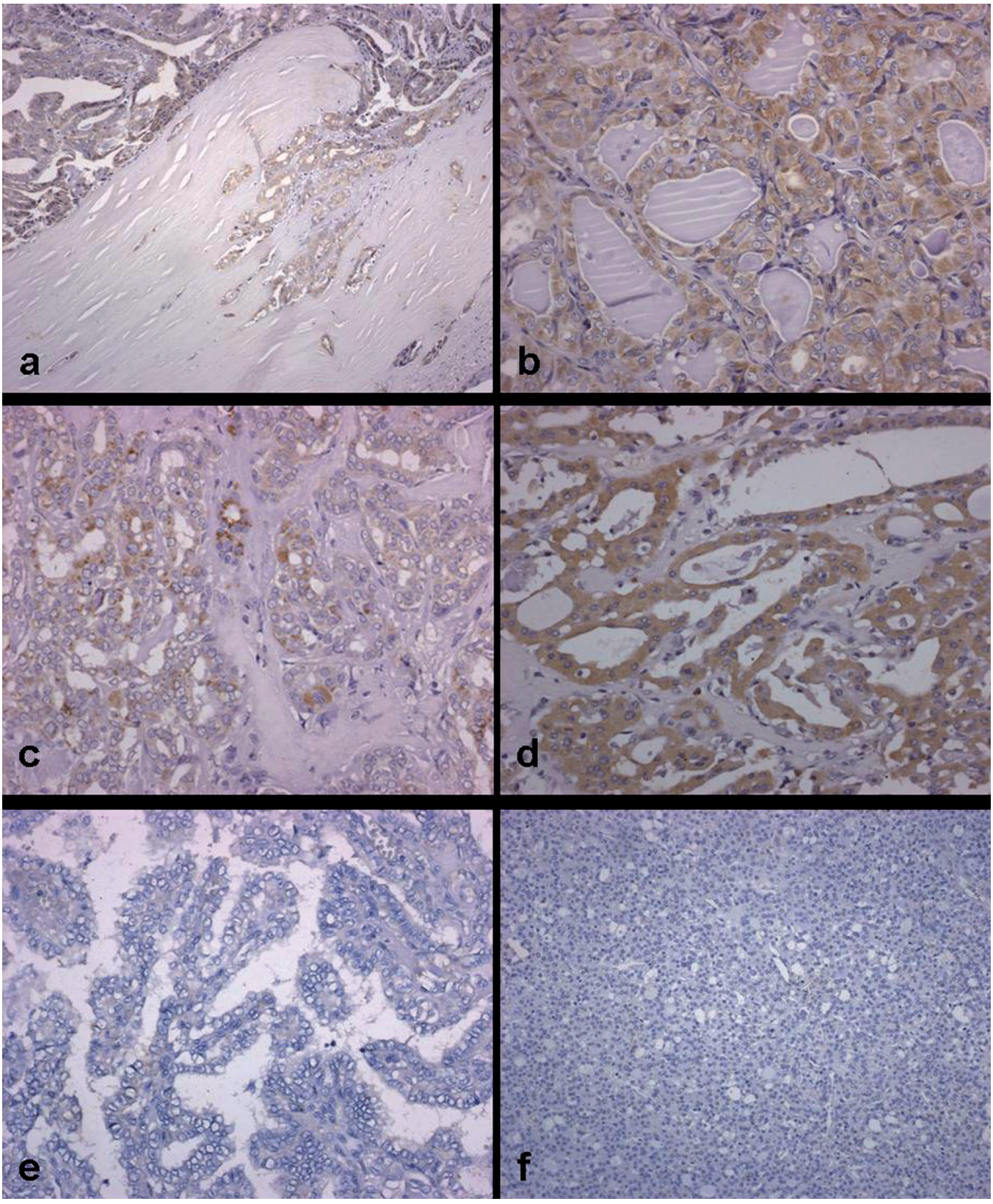

Fig. 3. NGAL positive staining in the cytoplasm of neoplastic cells in MIFC **(a)** (NGAL stain), in WIFC **(b)** (NGAL stain), in FVPC **(c)** (NGAL stain) and in HC **(d)** (NGAL stain). **e.** No NGAL immuno-expression was observed in PC-NOS (NGAL stain). **f.** Absence of NGAL immuno-expression in FA (NGAL stain). a, f, x 100; b-e, x 200

Table 2. Statistical correlations between NGAL immuno-expression and clinico-pathological variables investigated through Fisher exact test.

Parameter	NGAL immuno-expression		P
	Absent	present	
Sex			
male	9	7	
female	7	17	0.109
Age			
≤50 years	10	12	
>50 years	6	12	0.525
Tumor			
Benign	11	1	
malignant	4	24	0.000006
Follicular tumor			
benign	11	1	
malignant	2	23	0.000001
T			
1	6	16	
2	4	6	0.682

expression in the differential diagnosis between benign and malignant follicular tumors was 92%.

Discussion

In the present study we analyzed NGAL immunohistochemical expression in different thyroid epithelial tumors derived from follicular cells. Our aim was to test whether the evaluation of the immuno-expression of this protein might be of use in the differential diagnosis of these neoplasias.

NGAL expression had been previously evaluated by immunohistochemistry and real time PCR in anaplastic thyroid carcinoma and in a few cases of papillary and follicular carcinomas (Iannetti et al., 2008); nonetheless, to the best of our knowledge, it had been never examined in thyroid adenomas or in MIFC up to now. In line with the findings reported by Iannetti and coll. (2008), we did not evidence NGAL immuno-staining in thyroid parenchyma adjacent to the tumor in all cases. By contrast, NGAL staining of moderate intensity was extensively found in all the fetal thyroids submitted to the immunohistochemical procedure. It had been previously demonstrated that NGAL is involved in the growth, development and differentiation of the kidney, as early as in the embryo, through its regulation of iron responsive-genes (Yang et al., 2002; Gwira et al., 2005). Expression of this protein in the fetal thyrocytes and its absence in the normal adult gland suggest that NGAL may also exert a role in thyroid embryogenesis and development, similarly to that described in the kidney (Yang et al., 2002; Gwira et al., 2005).

With reference to the neoplasias included in the present study, NGAL immuno-expression was detected in 82% of malignant tumors and in 8% of benign ones. Immunostaining was localized at the cytoplasm of the neoplastic cells, similarly to that observed in colorectal carcinoma (Bauer et al., 2008; Barresi et al., 2010b, 2011). This may indicate that NGAL has been internalized to the inner cell following its binding to membrane receptors. Specifically, all MIFC, all but one WIFC and half of the analyzed PC were stained by anti-NGAL antibody, whereas only one of the analyzed adenomas displayed NGAL immuno-expression. Thus, NGAL expression in thyroid tumors appears to be similar to that of other iron-binding proteins such as lactoferrin, ferritin and transferring (Tuccari and Barresi, 1985; Barresi and Tuccari, 1987). We may hypothesize that NGAL expression may reflect augmented iron requirement in the cancerogenesis of thyroid carcinomas. Coherently, in a recent review article, the role of iron overload in carcinogenesis, through the induction of oxidative DNA damage, has been emphasized (Toyokuni, 2009); furthermore, it is known that iron depletion leads to cell cycle arrest and apoptosis (Le and Richardson, 2002).

NGAL expression by the neoplastic cells of infiltrative thyroid tumors may also mirror the acquirement of invasive properties, which differentiate thyroid follicular adenomas from carcinomas. Interestingly, in one of the MIFC, staining was observed in the capsular infiltrative area of the tumor, which is in line with the NGAL role as an enhancer of the invasive potential of cancer cells. NGAL may enhance neoplastic cell invasiveness by positively regulating the activity of MMP-9, which degrades the basement membranes and extracellular matrix, thus liberating vascular endothelial growth factor (VEGF), and enabling angiogenesis, invasion and metastasis (Yan et al., 2001; Fernandez et al., 2005; Lee et al., 2005). Indeed, by forming the NGAL/MMP-9 complex, NGAL can protect MMP-9 from proteolytic degradation; this would trigger an enhancement of the enzymatic activity of MMP-9 and explain the enhanced tumoral invasiveness and diffusion associated with NGAL over-expression (Yan et al., 2001). Besides, a statistically significant higher MMP-9 expression has been documented in MIFC in comparison to FA or adenomatous goiters (Erickson and Lloyd, 2004). In addition, NGAL has been demonstrated to increase invasiveness of cancer cells through an MMP-9-independent process (Hu et al., 2009). Indeed, it has been shown to enhance the invasive and metastatic potential of colon cancer cells by decreasing e-cadherin-mediated cell-cell adhesion through an iron-dependent mechanism (Hu et al., 2009).

The significantly higher frequency (P= 0.000006) of NGAL immuno-expression which we found in malignant thyroid tumors in comparison to benign ones may be of use in the differential diagnosis of these lesions. Indeed, NGAL expression appeared to be specific (specificity: 92%) for carcinoma and represented a sensitive method (sensitivity: 82%) for the identification of thyroid malignant tumors. Sensitivity, negative and positive predictive values, as well as diagnostic accuracy of NGAL expression in the

differential diagnosis between benign and malignant thyroid tumors increased when only the tumors displaying a follicular architecture were considered (sensitivity: 92%; positive predictive value: 96%; negative predictive value: 85%; diagnostic accuracy: 92%). Indeed, though malignant, all three PC-NOS included in our cohort were negative for NGAL, while NGAL staining was evidenced in 80% of FVPC. Besides, FVPC genetic profile seems to share some similarities with that of FC, rather than with PC-NOS (Zhu et al., 2003; Ghossein, 2009). Thus, NGAL staining might be particularly useful in the discrimination between thyroid tumors with a follicular pattern.

In conclusion, according to our preliminary findings, NGAL protein, which is involved in the acquirement of cancer cell invasive potential, seems to represent a marker of malignant follicular cell-derived thyroid tumors, and especially of those with follicular architecture. Hence, assessment of its expression might be of use with respect to differential diagnosis of the latter from benign neoplasias. The presence of this protein in fetal thyrocytes, as well as in malignant tumors derived from follicular cells, together with its lack in the adult normal thyroid, may indicate NGAL as an oncofetal marker in thyroid tumors.

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