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ORIGINAL ARTICLE

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Thyroglobulin mRNA expression helps to distinguish anaplastic carcinoma from angiosarcoma of the thyroid

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Abstract The existence of angiosarcoma (AS) of the thyroid has been a matter of debate for many years, because some authors believe that most if not all ASs are in fact "angiomatoid" anaplastic carcinomas (ACs). Immunohistochemistry alone was not successful in solving the problem, since cytokeratin expression is a known occurrence in AS. Therefore, we wanted to compare nine cases of AS with ten cases of AC, assessing whether thyroglobulin (TG) mRNA was still transcribed in undifferentiated tumors and could be helpful to distinguish AC from AS. The cases were analyzed for TG mRNA expression by means of radioactive in situ hybridization. The silver grains were counted using an automated device, and their amount was compared with that of stroma, background, and peritumoral thyroid. A weak signal was present in all AC but not in AS (mean counts 35.7 and 9.6 arbitrary units, respectively: $P<0.01$). In two cases of AC, residual areas of poorly differentiated insular carcinoma had a strong signal (similar to that of peritumoral thyroid). These findings further confirm that AC and AS of the thyroid are unrelated malignant tumors.

Keywords Thyroid · anaplastic carcinoma · Angiosarcoma · Thyroglobulin · In situ hybridization

Introduction

Epithelioid angiosarcoma (AS) of the thyroid was first described in 1990 [11]. The histogenesis of these very aggressive tumors has been the matter of long standing controversies, because many authors believe that ASs are a variant of sarcomatoid carcinoma, in view of their nearly constant cytokeratin positivity in the neoplastic elements [17, 19, 25, 26]. Other authors believe that true AS of the thyroid does exist, though it is rarely observed [2, 4, 8, 9, 14, 18, 21, 31, 33, 37, 39, 40, 41]. AS is apparently unrelated to the follicular cell lineage and develops mostly in endemic goiter areas, possibly being the result of malignant transformation of goitrous nodules that underwent recurrent intranodular hemorrhagic events with subsequent thrombotic and hyperplastic endothelial proliferations [11].

So far, the dispute has not been solved due to the difficulty of recognizing an immunophenotypic profile specific of AS relative to anaplastic carcinoma (AC). In fact, vimentin is strongly expressed in AS, but AC of the thyroid also strongly expresses it [20, 24, 34, 35]. Cytokeratin immunoreactivity is generally present in AC [7, 24] but has repeatedly been shown in AS of the thyroid and of other locations [1, 11, 32]. Endothelial markers specifically identify vascular differentiation in AS. Nevertheless, factor VIII-related antigen, which is the most specific endothelial marker, is frequently passively taken up by neighboring cells. Immunoreactive thyroglobulin (TG) is generally negative in AC [5, 30, 40]. Rare cases were shown to be focally immunoreactive [27], possibly due to a residual differentiated neoplastic component or to passive absorption by tumor cells [7, 23].

Since it is known that in undifferentiated tumors of other organs, the detection of the specific mRNA (rather than the protein) can better demonstrate hormonal expression (e.g., chromogranin A in small cell lung carcinoma) [13], we wanted to assess whether TG mRNA expression was present in cases of AS and AC of the thyroid, despite the absence of TG immunoreactivity. Accordingly, ten cases of AC and nine cases of AS of the thyroid gland were investigated by means of radioactive in situ hybridization (ISH) to reveal TG mRNA.

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Materials and methods

Cases

Ten cases of ACs and nine ASs of the thyroid were collected from the pathology files of the University of Turin (16 cases) and Bologna (three cases) during the period 1984–1997. The three cases from Bologna had been previously reported [11]. All cases were reviewed and reclassified according to the World Health Organization (WHO) classification [15]. Surgical specimens were fixed in formalin and embedded in paraffin. Serial sections were cut for conventional stainings, for immunohistochemistry, and for ISH. Clinical and follow-up data were obtained from all patients.

Immunohistochemistry

Immunohistochemistry was used to define the immunophenotypic profile of the tumors, using the avidin-biotin peroxidase complex according to Hsu et al. [16]. The following primary antibodies were used: cytokeratin (clone KL1; Immunotech; Marseille, France; diluted 1:200), vimentin (clone V9; Dako; Glostrup, Denmark; diluted 1:30), factor VIII-related antigen (polyclonal; Dako; diluted 1:1000), CD34 (clone QBend10; Novocastra Laboratory; Newcastle, UK; diluted 1:50), CD31 (clone JC/70A; Dako; diluted 1:50), calcitonin (polyclonal; Ortho Diagn.; Raritan, N.J.; diluted 1:1), and thyroglobulin (polyclonal; Dako; diluted 1:3000).

In situ hybridization

Sections (5-µm thick) were deparaffinized twice for 5 min in xylene, hydrated through a standard series of ethanols to phosphate-buffered saline (PBS), and digested with 0.2 mg proteinase K (Sigma)/ml in 0.1 M Tris-HCl and 50 mM ethylene diamine tetraacetic acid (EDTA) pH 8.0 for 15 min at 37°C. After blocking twice for 5 min in ice-cold 0.1 M glycine/PBS, sections were treated with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine pH 8.0 for 10 min, rinsed for 3 min in 2× sodium saline citrate (SSC), and dehydrated. An RNA probe corresponding to bases 836–1245 of the TG complementary DNA (cDNA) [22] was labeled with ^{33}P-UTP (Amersham International, Zurich, Switzerland) by transcription of a polymerase chain reaction (PCR)-generated template containing at each end a suitable phage RNA polymerase promoter [38]. Briefly, two primers (sequences are shown in Table 1) were used to amplify the portion of the TG cDNA encompassing positions 838–1245. As starting material, we used a small amount of plasmid no. 2, which is described elsewhere [3] and was kindly provided by Dr. J.L. Berge-Lefranc (Marseille, France). This facilitated the procedure.

The 457-base pair PCR product contained the amplified TG sequence flanked by two unique restriction sites (*Pst*I and *Sst*I) and two promoters specific for the T7 and the SP6 RNA polymerases, respectively. Cutting the PCR product with *Pst*I and transcribing with SP6 RNA polymerase will yield a 413-base RNA probe complementary to the TG mRNA. Cutting the same PCR product with *Sst*I and transcribing with T7 RNA polymerase will yield an RNA probe of the same length as the first probe but of complementary sequence, which was used as negative control. For the hybridization in parallel sections, both (anti-sense and sense) labeled probes (final concentration 105 cpm/µl) were boiled for 3 min in 10 mM Tris-HC1 pH 8.0, 1 mM EDTA, and 500 µg/ml tRNA from *Escherichia coli* (Sigma), rapidly chilled on ice were added to a mixture containing 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 8.0, 5 mM EDTA, 1× Denhardt's solution, and 10% dextran sulphate (Sigma). The hybridization mix was applied to sections and covered with silicon-treated coverslips. Slides were then incubated overnight at 42°C under prewarmed mineral oil. After hybridization, the oil was removed by chloroform washes, and dried slides were washed 1 h in 2×SSC at room temperature (RT), then digested with 20 µg RNase A/ml and 10 U RNase T1/ml (Boehringer Mannheim, Rotkreuz, Switzerland) in 0.5 M NaCl, 10 mM Tris-HCl for 30 min at 37°C. After washing in 2×SSC for another 30 min, slides were washed first in 0.1×SSC for 10 min at 42°C and then in 0.1°SSC for 30 min at RT. The sections were then dehydrated. Slides were exposed for 2–3 weeks to Ilford K2 nuclear emulsion (Ilford, Fribourg, Switzerland), developed in D19 (Kodak), fixed in Unifix (Kodak), and mounted.

Quantitative analysis of labeling density

Silver grains of autoradiographic emulsion were counted in tumor cells, normal thyroid follicles, and stromal areas using the image analysis system Quantimet 500 (Leica, Cambridge, UK). The analyzer was calibrated to count silver grains measuring 0.15–0.35 µm^2 in an area of 4200 µm^2 (objective: planar 100×; eye-pieces: planar 10×). In each case, five different fields of the tumor, of normal thyroid (when available), and of stroma (usually capsular fibrous tissue or vessel wall) were counted. In selected cases, the corresponding areas in the negative control slides (hybridized with the sense probe) were counted. The mean value among the five counts was recorded. It represented an arbitrary unit corresponding to the number of grains per given area. In two AC cases, areas of residual poorly differentiated (insular) carcinoma were also counted.

Results

Clinico-pathological data. Six female and four male patients were included in the AC group, aged 38–79 years (median 56.5 years). ACs were ill-defined masses, measuring 3–11 cm in their greatest axis (mean 5.5 cm). Extension to perithyroidal tissues was present in four of them. All cases were constituted by large irregular cells, some having multinucleation. These were cytokeratin and vimentin positive and TG and calcitonin negative when analyzed using immunohistochemistry. In two cases (case 9 and case 10) residual foci of poorly differentiated (insular) carcinoma (strongly immunoreactive for TG) were found (Fig. 1). In the AS group, two male and seven female patients, aged 52–84 years (median 70 years), were included. The tumors were large, ill-defined masses measuring 4–10 cm (mean 6.2 cm). ASs were characterized by anastomosing vascular channels

Table 1 Sequence of the primers used for the generation of the thyroglobulin template using the polymerase chain reaction. The sequences of the promoters are in italics, the restriction sites are underlined, and the thyroglobulin proper sequence is in bold

SP6-SstlAS
5'-*GAT-TTA-GGT-GAC-ACT-A TA-GAA-TAC*-**GAG-CTG-GTT-GAT-CGT-GGG-TG**-3'
T7-PstlS
5'-*TCT-AAT-ACG-ACT-CAC-TAT-AGG-GAG*-**ACT-GCA-GAG-ACG-GTT-CCT-CGC**-3'

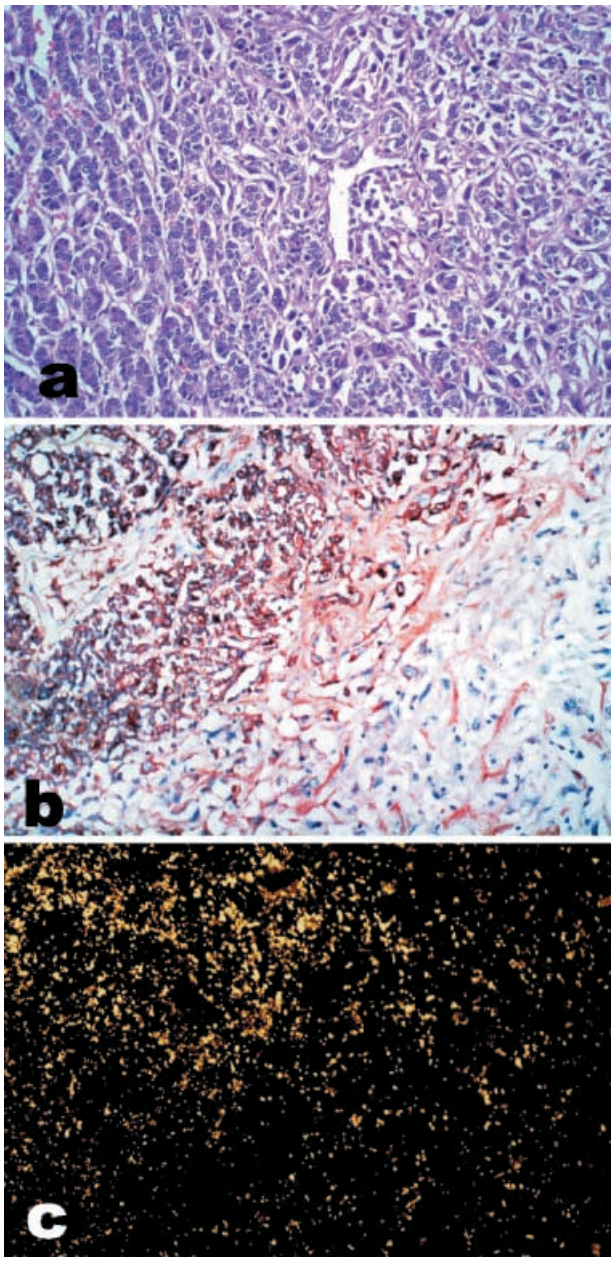

Fig. 1 Case 9. **a** Anaplastic carcinoma with a residual component of poorly differentiated carcinoma (**a**; *left*). The immunoreactivity for thyroglobulin is present in the poorly differentiated area only (**b**; *upper left*), while the in situ hybridization (ISH) reaction showed a strong signal for thyroglobulin mRNA in the poorly differentiated areas (**c**; *upper left*) and a weak signal in anaplastic carcinoma areas (c; *bottom right*). **a** Hematoxylin and eosin; **b** immunoperoxidase; **c** radioactive ISH, dark field. 250×

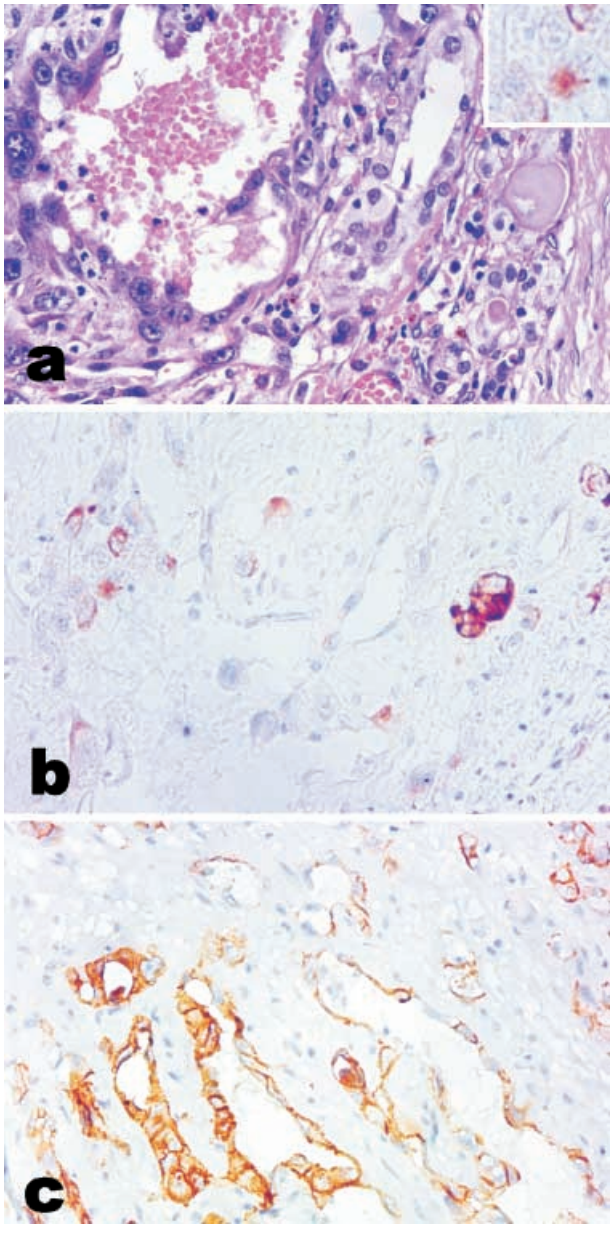

Fig. 2 Case 11. Angiosarcoma of the thyroid. Irregular vascular spaces lined by pleomorphic cells having prominent nucleoli are present in this tumor (**a**). The tumor cells focally express cytokeratin (**b**) and are strongly immunoreactive for CD31 (**c**). **a** Hematoxylin and eosin; **b** and **c** immunoperoxidase, 400×

(Fig. 2), intermingled with solid areas made of large pleomorphic cells. These had ovoid nuclei and prominent nucleoli. Extensive areas of necrosis and hemorrhage were also recognized. All AS diffusely expressed vimentin and endothelial markers (factor VIII-related antigen, CD31, and CD34). Cytokeratin was expressed in six of nine cases (focally in three of these), but no TG immunoreactivity was ever detected.

At follow-up (available for 17 cases), all patients but one (operated on because of AC 10 years ago and currently free of disease) died of their disease 1–54 months (mean 10 months) after diagnosis. The clinico-pathological data of the currently studied cases are summarized in Table 2.

Table 2 Clinico-pathological data and thyroglobulin gene expression in anaplastic carcinoma (AC) and angiosarcoma (AS) of the thyroid. *IHC* immunohistochemistry; *CK* cytokeratin; *TG* thyroglobulin; *ISH* in situ hybridization; *DOD* died of disease; *NED* no evidence of disease; *n.a.* not available (no peritumoral normal follicles available for grain count)

Number	Diagnosis	Gender/age (years)	Size (cm)	Stage	Follow-up (months)	IHC		ISH for TG (no. of grains × area)[a]		
						CK	TG	Tumor	Thyroid	Tumor/thyroid
1	AC	Female/54	4	pT3	DOD 3	+	–	15.4	n.a.	n.a.
2	AC	Male/60	6	pT3	DOD 12	+	–	18.0	56.8	0.31
3	AC	Female/45	10	pT3	DOD 15	+	–	15.8	97.2	0.16
4	AC	Female/79	4	pT3	DOD 3	+	–	42.5	n.a.	n.a.
5	AC	Female/59	4	pT3	DOD 1	+	–	57.8	n.a.	n.a.
6	AC	Male/49	4	pT4	DOD 26	+	–	63.6	223.6	0.28
7	AC	Female/48	5	pT3	NED 84	+	–	15.2	n.a.	n.a.
8	AC	Male/66	7	pT4	DOD 1	+	–	36.0	284.8	0.13
9	AC[b]	Female/63	11	pT4	DOD 2	+	–[b]	49.0	297.6	0.16
10	AC[b]	Male/38	12	pT4	DOD 15	+	–[b]	44.1	272.4	0.16
11	AS	Female/82	10	pT3	DOD 1	+ focal	–	4.0	86.1	0.05
12	AS	Female/70	7	pT3	DOD 5	+ focal	–	4.6	52.0	0.09
13	AS	Female/53	4	pT3	DOD 7	+ focal	–	6.1	123.6	0.05
14	AS	Female/68	7	pT3	DOD 13	–	–	30.0	184.0	0.16
15	AS	Male/64	4	pT3	DOD 7	–	–	6.2	256.0	0.02
16	AS	Female/79	7	pT3	DOD 54	–	–	7.0	51.2	0.13
17	AS	Female/42	8	pT4	lost	+	–	5.1	73.2	0.07
18	AS	Female/84	4	pT3	lost	+	–	5.6	63.0	0.09
19	AS	Male/77	5	pT4	DOD 2	+	–	7.8	n.a.	n.a.

[a] TG mRNA expressed as arbitrary units corresponding to the number of silver grains of autoradiographic emulsion counted in tumor fields and peritumoral thyroid parenchyma (pre-defined area of 4200 μm²; see also Materials and methods

[b] Case associated with a residual poorly differentiated (insular) carcinoma component, having thyroglobulin immunoreactivity

In Situ Hybridization

The radioactive ISH reaction for TG mRNA presented a considerable heterogeneity among individual cases, probably related to metabolic conditions and/or to the formalin fixation time and nucleic acid preservation after paraffin embedding. The intensity of signal in normal thyroid follicles served as an "internal control" to evaluate the staining in those cases having peritumoral thyroid parenchyma. The results are summarized in Table 2. TG mRNA was found in all ACs, although the intensity of the staining was rather low and restricted to individual groups of cells (Fig. 3). The number of grains counted in tumor fields containing positively stained cells averaged 35.5 arbitrary units (range 13.2–63.6 arbitrary units; see Materials and methods), compared with an average of 11.6 grains in stromal/fibrous areas (less than one-third of tumor areas). Peritumoral thyroid follicles had a strong expression of TG mRNA, corresponding to a mean of 205.4 grains. Comparing these figures, it appears that the TG mRNA tumor/normal follicle ratio is 0.20 (range 0.16–0.31). As a negative control, ISH for TG with a sense probe was performed. No signal was found, and the count of the scattered grains yielded scores corresponding to 1–5% of those counted in the same areas of parallel slides processed with the specific probe (Fig. 4). The two cases of AC, having a residual poorly differentiated (insular) carcinoma component (also strongly immunoreactive for TG), showed a strong ISH signal for TG mRNA in that component, similar to that observed in peritumoral thyroid follicles and six times more intense than that of neighboring AC areas (Fig. 1).

AS did not show any TG mRNA expression (grain count of 4–7 arbitrary units; Fig. 5) except for case 14, in which a moderate density of grains was observed in focal tumoral areas. The corresponding normal thyroid counts ranged from 51.2 to 256 arbitrary units (mean 112.2 arbitrary units), which yielded a tumor/normal thyroid ratio of 0.08 (range 0.02–0.16). Peritumoral stromal fibrous areas and tumor areas in control slides processed with the sense probe (not shown) had no TG mRNA signal.

Comparing the silver grain counts in AC and AS cases, a statistically significant difference was found between the TG mRNA expression in AC and AS. The former had a tumor/normal thyroid ratio of 0.20 versus 0.08 in the latter ($P<0.05$). Even comparing absolute silver grain counts of ACs versus ASs (a mean of 35.5 grains in the former versus 8.5 in the latter), the difference was statistically significant ($P<0.01$).

Correlations

The expression of TG mRNA was apparently not related to clinico-pathological parameters. The single case of AC, having a long survival, did not contain a higher amount of TG mRNA. Cytokeratin-positive areas in AS did not display any TG mRNA signal.

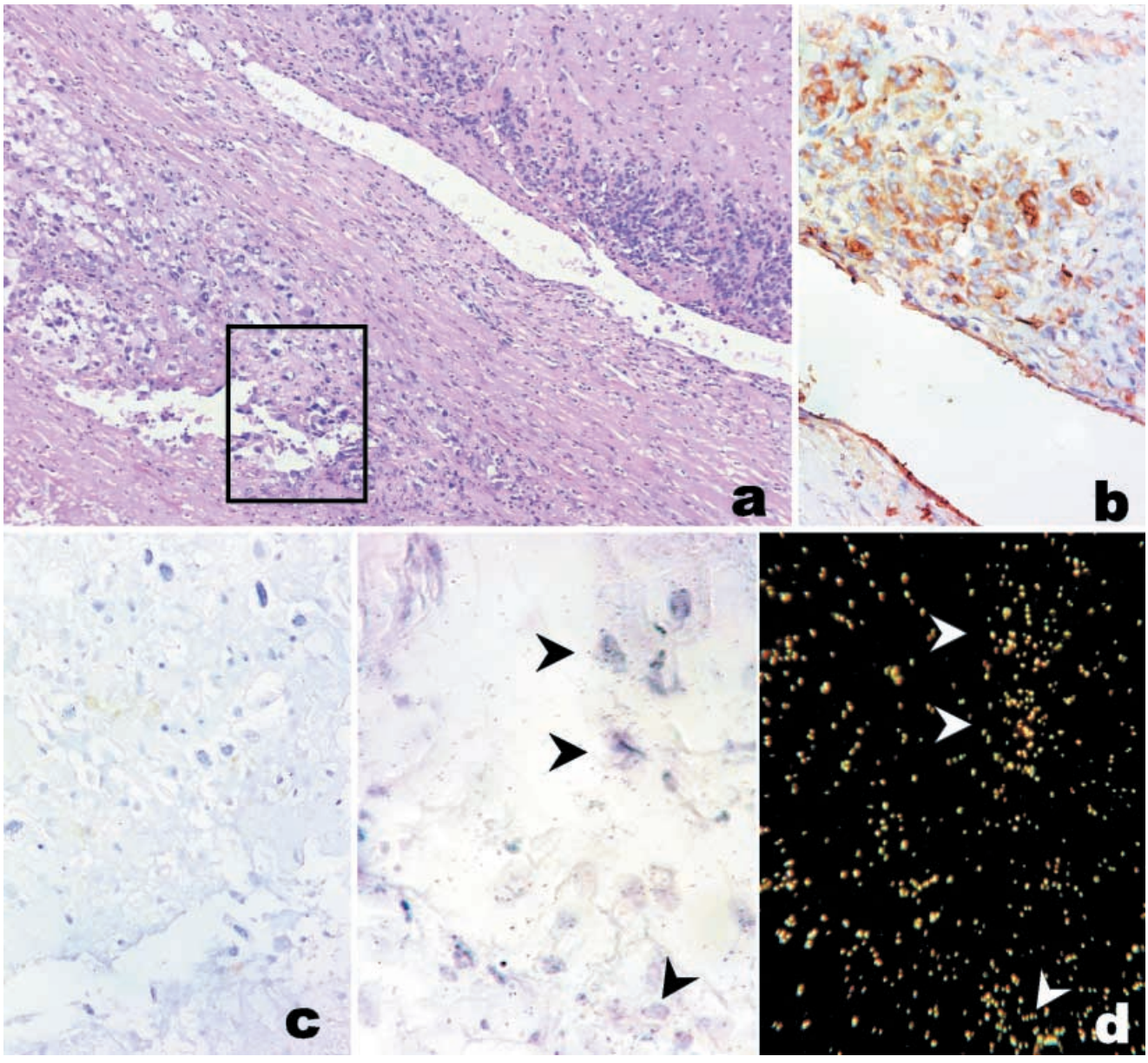

Fig. 3 Case 8. Anaplastic carcinoma. The tumor is partially capsulated and is made of markedly atypical cells arranged in irregular groups (**a**). Thyroglobulin immunoreactivity is present in peritumoral follicles (**b**) but not in the tumor cells. **c** Corresponds to the *squared area* in (**a**). The same area shows individual tumor cells (*arrowheads*) weakly expressing thyroglobulin mRNA, as detected using radioactive in situ hybridization (ISH). **d** Bright and dark fields of the same cells. **a** hematoxylin and eosin, 100×; **b** and **c** immunoperoxidase, 250×; **d** radioactive ISH, 400×

Discussion

By means of ISH, we have shown that TG mRNA is transcribed at low levels in AC of the thyroid but not in AS. The absence of TG mRNA expression in AS further supports the idea that they are true sarcomas, not related to the follicular lineage from which ACs derive.

Since TG immunoreactivity is not a feature of undifferentiated thyroid tumors, we chose a different approach and analyzed the expression of the TG gene. TG mRNA was demonstrated in AC by means of ISH, a finding which is apparently in contrast with previous reports [10, 12], in which five cases of AC were found to be negative for TG mRNA. The technique employed by these authors was Northern-blot analysis, and it is possible that the tumor extract did not contain enough mRNA to be detected using this procedure. A similar discrepancy was observed when comparing the results of different techniques to demonstrate chromogranin A mRNA in colorectal tumors [28]. When the level of transcription is low, it may well be that Northern-blot analysis turns out to be negative. Indeed, a non-radioactive ISH for TG mRNA, using a digoxigenin-labeled probe, gave a negative or very weak staining in most cases of the present series (data not shown). Unfortunately, the highly sensi-

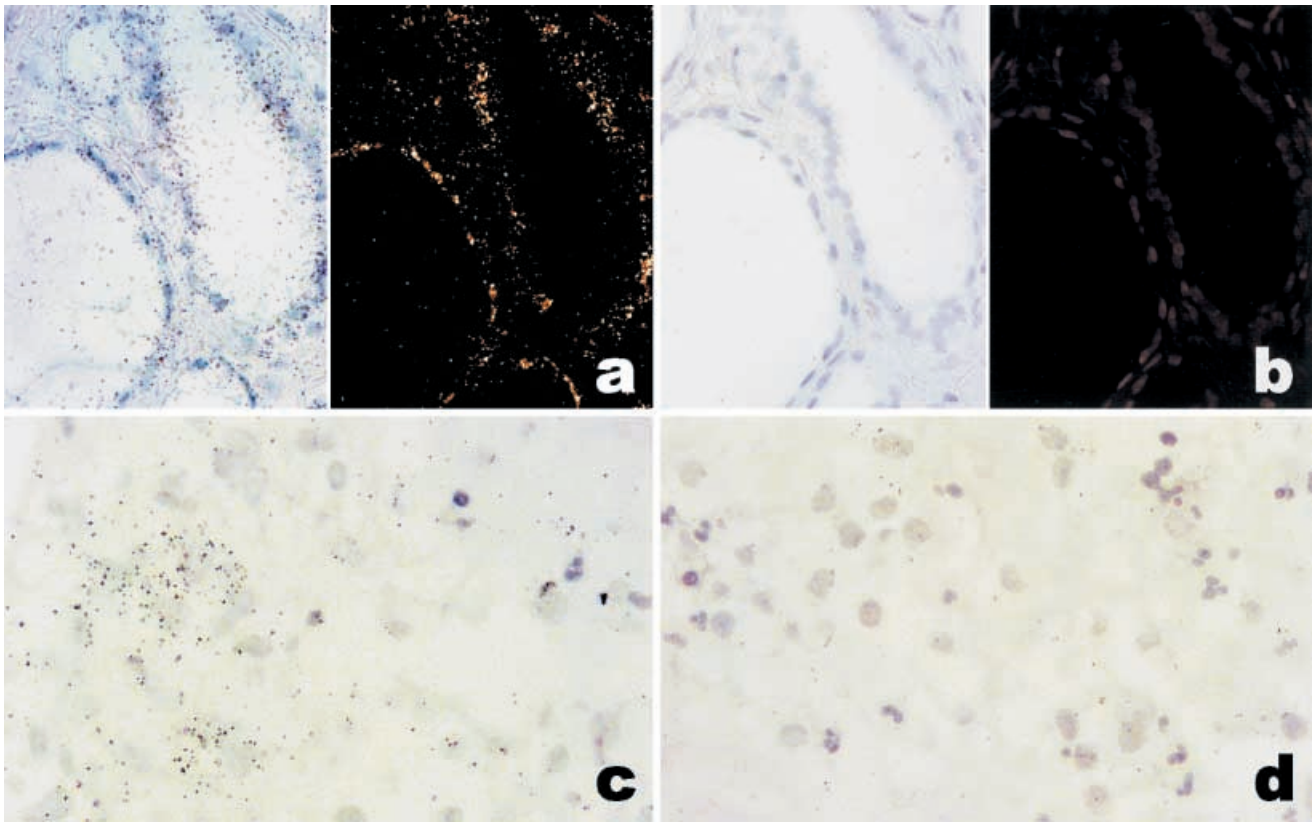

Fig. 4 Control experiments in the in situ hybridization procedure. Case 6. Peritumoral thyroid follicles are strongly positive for the specific antisense thyroglobulin probe (**a**), whereas no signal was detected using a sense probe (**b**). Anaplastic carcinoma cells are focally positive when the specific probe was applied (**c**) but not in a parallel section stained with the sense probe (**d**). **a** and **b** bright and dark fields, 400×; **c** and **d**, bright fields, 400×

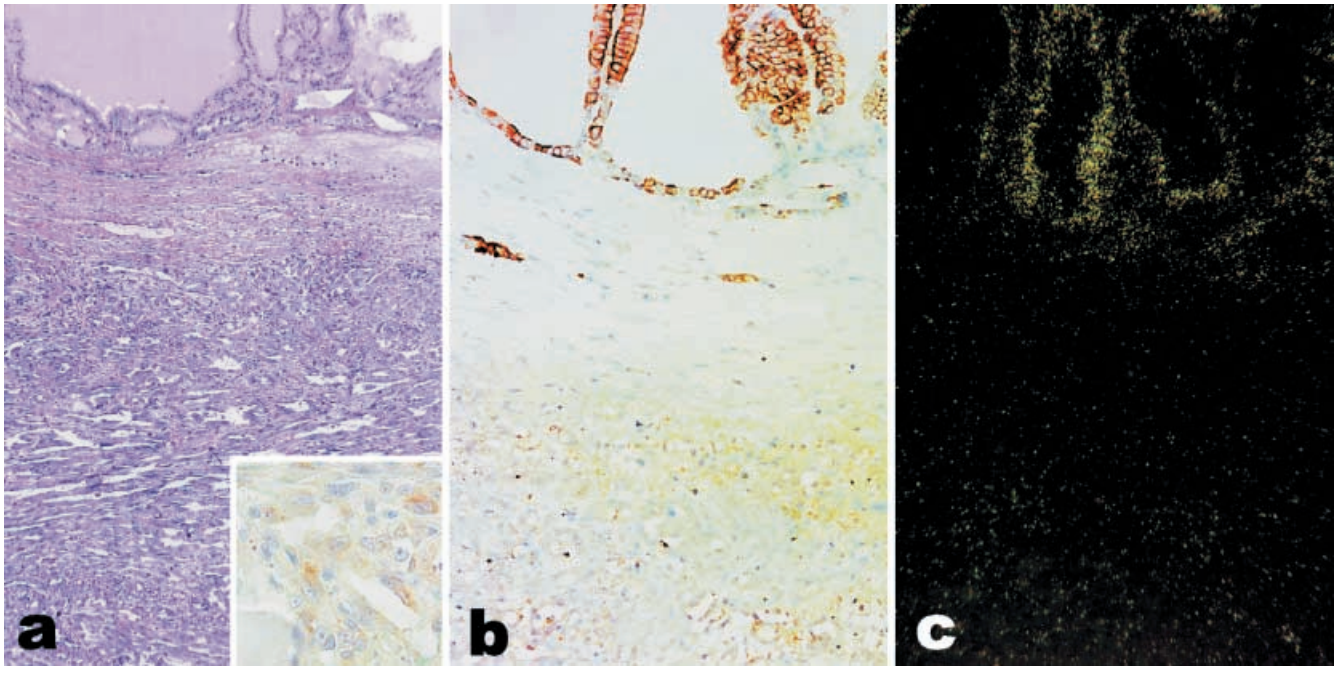

Fig. 5 Case 15. Angiosarcoma of the thyroid. This tumor has solid areas intermingled with the classical anastomosing vascular channel growth pattern (**a**). Tumor cells diffusely express factor VIII-related antigen (*inset*). No thyroglobulin was expressed by this tumor, as shown using immunohistochemistry (**b**) and in situ hybridization (ISH; **c**). Peritumoral follicles (**b** and **c**, *top*) served as a positive internal control. **a** Hematoxylin and eosin, 100×; **b** immunoperoxidase; **c** radioactive ISH, 250×

tive reverse transcriptase (RT)-PCR technique could not be employed due to lack of frozen AC and AS specimens in our series.

We relied more on radioactive ISH, which provided a stronger signal and, in addition, allowed us to quantify the amount of TG mRNA in each tumor by counting silver grains of the autoradiographic emulsion. Far from being an exact way of counting the number of mRNA copies, this procedure may provide information on the level of TG gene transcription in AC. The arbitrary units indirectly indicated the amount of mRNA of tumor cells, compared with that of stromal areas, peritumoral thyroid parenchyma (when available), and the corresponding negative control slide (stained with the sense probe) of each case.

The signal for TG mRNA was expressed as the ratio between tumor and thyroid parenchyma counts. A statistically significant difference was found when comparing the scores obtained in these different areas. The ratio between the number of grains in tumor fields versus normal thyroid (0.20) indicated that AC had an approximately fivefold reduction of TG mRNA signal. Conversely, AS had virtually no signal for TG mRNA, and the ratio was very low (0.08).

Our data showed that AC and AS have different degrees of TG gene expression. This is not a definite proof of the proposed existence of AS as a separate entity from AC [35], because it cannot be excluded that negative tumors are indeed sarcomatoid AC that have completely lost the ability to transcribe the TG gene. Nevertheless, this information is to be added to other pieces of evidence showing morphological, ultrastructural, and (in part) immunocytochemical differences between the two groups of tumors [8, 9, 11, 21, 39,40].

The vast majority of ASs have been reported in alpine areas [8, 14, 40], although small series from non-alpine areas have been recently described [11, 21]. The present series contained cases from alpine and non-alpine regions, and no differences were observed in terms of TG expression. Six cases of the present series originated from the Piedmont region in North-Western Italy, a mountain area where goiter used to be endemic due to iodine deficiency. All cases were associated with long-standing nodular goiter and regressive changes, including intranodular hemorrhage, may have provided the anatomic substrate for the development of the vascular tumors [11]. Prominent endothelial hyperplasia was reported in goiter nodules that underwent intranodular hemorrhage and re-organization phenomena of the Masson's hemangioma type [36]. This event may represent a precursor condition for subsequent neoplastic transformation. A genetic analysis of different (hyperplastic and neoplastic) vascular lesions of the thyroid might better clarify this point.

Two cases of AC had a residual poorly differentiated (insular) carcinoma component. Such association was found in the primary tumor of one case and in the locally recurrent tumor of the other. The insular component was identical to that described in the literature [6, 29]. TG production was maintained throughout the insular areas, as shown using both immunohistochemistry and ISH. When moving from insular to undifferentiated areas, no TG immunoreactivity was found, as opposed to the TG mRNA signal, which dramatically decreased – relative to insular areas – but did not disappear.

From a clinical standpoint, both AC and AS are usually associated with a dismal prognosis. Though being of follicular cell origin, serum TG is generally not as elevated as it is in well- or poorly differentiated carcinomas, and radioiodine uptake is usually rather poor in such tumors. Therefore, apart from its role in the differential diagnosis of AC from AS, the identification of residual TG gene activation in cases of AC may be useful for screening those cases possibly or partially responsive to radioiodometabolic therapy.

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