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Pattern of expression of intermediate cytokeratin filaments in the thyroid gland: an immunohistochemical study of simple and stratified epithelial-type cytokeratins

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Abstract The expression of simple and stratified epithelial-type cytokeratin (CK) intermediate filaments was evaluated by immunohistochemistry in a series of 41 papillary carcinomas, 10 follicular carcinomas, 2 poorly differentiated carcinomas and 34 specimens of normal thyroid parenchyma and lymphocytic thyroiditis. The aim of the study was to establish the CK profile of normal thyroid and thyroid carcinomas in order to clarify the putative application of CK immunostaining in diagnostic surgical pathology, and to evaluate whether the process of neoplastic transformation and tumour progression in the thyroid may be associated with any particular change in CK expression. Normal thyroid strongly expressed simple epithelial-type CKs 7 and 18 and, to a lesser degree, CKs 8 and 19, but did not express stratified epithelial-type CKs. The same pattern was found in lymphocytic thyroiditis, though the CK 19 immunoreactivity was stronger in these lesions than in the normal thyroid. Papillary and follicular thyroid carcinomas shared the expression of simple epithelial-type CKs 7, 8, 18 and 19. Immunoreactivity for CK 19 was frequently stronger and more widely distributed within each particular tumour in papillary than in follicular carcinomas, but it could also be detected, at least focally, in every follicular carcinoma. Strong expression of CK 19 highlighted small foci of papillary carcinoma not easily identifiable by conventional histological examination. Stratified epithelial-type CKs 5/6 and 13 were detected in a high percentage of papillary carcinomas, in contrast to their absence in follicular carcinomas and normal thyroid. The CK pattern was similar in primary and metastatic papillary carcinomas. We conclude that papillary carcinoma of the thyroid presents a distinct CK profile that may be used for diagnostic purposes.

Key words Papillary carcinoma · Follicular carcinoma · Immunohistochemistry · Cytokeratin filaments

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Introduction

Cytokeratin (CK) filaments are the intermediate filaments forming the skeleton of epithelial cells, providing a support to mantain cell integrity and the structure of epithelial tissues. Recent data suggest that CKs can act as signal transducers [10] and may play a part in cell migration by affecting cell shape and cell motility [4]. Soft human epithelia can present 20 different types of CKs, which are divided into 2 types (I and II) according to their isoelectric pH value and molecular weight [17, 18]. CKs are numbered from the highest to the lowest molecular weight within each group (type II, neutral to basic CKs, includes CKs 1–8; type I, acid CKs, includes CK 9–20). Type-I and type-II CKs are always expressed in paired form [18]. Put in a simplified way, low-molecular-weight CKs are typical of simple and glandular epithelia and high-molecular-weight CKs (epidermal CKs) are characteristic of stratified epithelia.

The expression of CK filaments has been evaluated in several normal and pathological tissues [1, 2, 7, 9, 13, 20, 22, 26, 29, 30] (see [12, 15] for reviews). The stratified epithelia of the oral [20, 26, 30] and cervical [29] mucosae, and the transitional epithelium of the urinary tract [25] are the most extensively studied examples. In these models, a change in the pattern of CK expression has been found in relation to the progression from normal to dysplastic/neoplastic squamous epithelia, and to the nuclear grade of transitional epithelial neoplasms. This change consists in the appearance, in squamous and transitional tumours, of CKs that are characteristic of

simple/glandular epithelia. The detection of a CK pattern specific for myoepithelial or basal cells has also been reported in an attempt to distinguish between benign and malignant conditions in prostate [9] and breast [7] lesions. Finally, the CK expression in different tissues and/or neoplasms has also been used to assess the organ of origin of a metastatic tumour [12, 15, 19, 22].

Several reports on the cytoskeletal components of follicular cells in the thyroid gland, either focusing on the CK pattern only [23, 24, 27] or including the detection of other cytoplasmic filaments [3, 6, 11, 16, 28, 31], have been published to date. The results of these studies are contradictory as far as the types of CKs expressed by thyroid tumours are concerned. The presence or absence of stratified epithelial-type CKs in papillary carcinomas has not been established definitively [3, 6, 11, 16]. The usefulness of a particular low-molecular-weight CK (CK 19) in the differential diagnosis between papillary and follicular carcinoma remains controversial [6, 23, 27, 31].

We undertook the present immunohistochemical study of simple/glandular and stratified epithelial-type CKs using antibodies raised against CKs 1, 4, 5/6, 7, 8, 13, 18, 19 and 20 in a series of normal thyroid tissues, in lymphocytic thyroiditis and in carcinomas of the follicular epithelium of the thyroid gland in an attempt to further elucidate the CK pattern of these tissues and to evaluate whether the process of neoplastic transformation of the thyroid (with particular emphasis on papillary carcinoma) is associated with any specific change in the expression of CKs.

Materials and methods

Thyroid specimens were obtained from the files of The Norwegian Radium Hospital, Oslo, Norway. Thirty-four samples of macroscopically normal thyroid and 53 samples of thyroid carcinoma were included in the series. The carcinomas were classified according to the criteria of Hedinger et al. [8] and LiVolsi et al. [14] and divided into: papillary carcinoma (PC; 41 cases: 30 of the ordinary type of PC, 9 cases of the follicular variant of PC, 2 cases of Hürthle cell PC); follicular carcinoma (FC; 10 cases: 7 of the minimally invasive type of FC, 2 of them presenting features of Hürthle cell carcinoma, 3 of the widely invasive type of FC); poorly differentiated carcinoma (PDC; 2 cases, 1 with foci of anaplastic transformation). In 4 cases of PC only lymph node metastases were examined; the other 49 cases were primary PC. The primary lesion and lymph node metastases were examined in 13 cases of PC. The thyroid parenchyma in the periphery of the carcinoma was observed in 29 cases; it was normal in 25 and showed lymphocytic thyroiditis with Hürthle cell changes in 4 cases. The remaining 5 macroscopically normal thyroids consisted of material resected to achieve total thyroidectomy after a previous diagnosis of thyroid carcinoma. All these tissues were fixed in formalin and embedded in paraffin. HE-stained sections were examined in all cases.

The antibodies used in immunohistochemistry, their sources and dilutions are described in Table 1. Sections were stained using the avidin-biotin-peroxidase complex (ABC) method. Deparaffinized sections were rehydrated, washed with phosphate-buffered saline (PBS) pH 7.4, and treated with enzyme (trypsin, pronase and pepsin were used, respectively, in the reactions with CKs 1, 4, 8 and 19, CKs 5/6, 7 and 20, and CK 13). The sections were then treated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min to block endogenous peroxidase, and to unmask the epitopes of keratin 18 they were microwaved in an "antigen retrieval" solution. The sections were then incubated for 20 min with normal serum from the species in which the secondary antibody was made, in order to eliminate non-specific staining. Excess normal serum was blotted from the slides before incubation with primary antibodies for 18–22 h at 4°C. The sections were then incubated with a 1: 200 dilution of biotin-labelled secondary antibody for 30 min and ABC for 60 min (10 µg/ml of avidin and 2.4 µg/ml of biotin-labelled peroxidase; Vector, Burlingame, Calif.). Tissue was stained for 5 min with 0.05% 3′3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05 M Tris (hydroxymethyl) aminomethane (Tris) buffer at pH 7.6, containing 0.01% H_2O_2, and then counterstained with haematoxylin, dehydrated and mounted in Diatex. All series included positive controls, which were simple glandular and stratified epithelia known to be immunoreactive for the CKs used in the present work. Negative controls consisted of substituted mouse myeloma proteins of the same subclass and with the same concentration as the monoclonal antibodies.

Table 1 List of primary antibodies[a]

Antibody	Source	Dilution
CK 1	Enzo Diagnostic	1:75
CK 4	Boehringer Mannheim Biochemica	1:100
CK 5/6	Boehringer Mannheim Biochemica	1:10
CK 7	Dakopatts A/S	1:100
CK 8	Boehringer Mannheim Biochemica	1:25
CK 13	Boehringer Mannheim Biochemica	1:50
CK 18	BioGenex Laboratories	1:10
CK 19	Boehringer Mannheim Biochemica	1:50
CK 20	Progen Biotechnik GMBH	1:50

[a] All the antibodies are mouse monoclonal

A semi-quantitative method was used to score the immunoreactivity of the tissues: 0, <5% of immunoreactive cells; +, 5–25% of immunoreactive cells; ++, 25–50% of immunoreactive cells; +++, 50–75% of immunoreactive cells; ++++: >75% of immunoreactive cells. Whenever possible, the intensity of immunostaining was recorded as strong or weak.

Statistical analysis was performed using the Chi-square test after the Yates' correction. Two values were considered significantly different when the p value was less than 0.05.

Results

The results are summarized in Table 2. The cellular localization of immunoreactivity, which was similar with all the antibodies tested and in all the tissues examined, was cytoplasmic: usually diffuse or, rarely, dot-like. A membrane reinforcement was also frequently present. Immunostaining of control reactions gave satisfactory results.

Expression of CK1 and CK4 was not observed in normal thyroid or lymphocytic thyroiditis. Immunoreactivity for CK 4 was found in 1 case of PC (+).

CK 5/6 were not detected in normal thyroid parenchyma or lymphocytic thyroiditis. The same is true for FC and PDC. In PC, expression of CK 5/6 was detected in 27 cases (Fig. 1). The number of immunoreactive cells was quantified from + to ++++. The expression of CK 5/6 was more frequently detected in neoplastic follicles/papillae within a desmoplastic stroma (Fig. 1A) and

Table 2 Expression of CKs in the different types of thyroid tissues

	Normal thyroid (n=30)	Hashimoto's thyroiditis (n=4)	Papillary carcinoma (n=41)	Follicular carcinoma (n=10)	Poorly differentiated carcinoma (n=2)
CK 1	0	0	0	0	0
CK 4	0	0	1	0	0
CK 5/6	0	0	27	0	0
CK 7	30	4	41	10	2[a]
CK 8	30[b]	4	41	10	2
CK 13	0	0	14	0	0
CK 18	30	4	41	10	2
CK 19	30[b]	4	41	10	2
CK 20	0	4[c]	1[c]	1[c]	0

[a] In one case of poorly differentiated carcinoma, no immunoreactivity for CK 7 was observed in the foci of anaplastic transformation
[b] The immunostaining of normal thyroid for CKs 8 and 19 was frequently heterogeneous. Areas of unreactive follicles were present in most of the cases, mainly for CK 19
[c] A very weak and focal immunostaining was observed in the Hürthle cells of Hashimoto's thyroiditis and in Hürthle cell carcinomas.

in the periphery of the tumour adjacent to fibrotic areas; in 4 cases the immunoreactivity was also seen in the neoplastic cells lining papillary structures in the bulk of the tumours (Fig. 1B). Immunoreactivity in a few scattered cells was detected in areas of squamous metaplasia resembling epidermal horn pearls (Fig. 1C).

CK 7, CK 8 and CK 18 were expressed in all normal thyroids and in all cases of lymphocytic thyroiditis and malignant tumours, with a +++/++++ score. The immunoreactivities obtained for CK 7 and CK 18 were always very strong and homogeneous in normal thyroid, thyroiditis, FC (Fig. 2A) and PC (Fig. 3A). Squamous metaplastic foci in PC were negative (CK 18; Fig. 3A) or positive only in very rare cells (CKs 7 and 8). Expression of CK 8 was observed in the same tissues as CK 7 and CK 18, but the intensity of staining was weaker and more heterogeneous. In the normal thyroid, unreactive follicles for CK 8 were frequently detected. No difference in immunostaining was found between FC and PC. CK 7 was not expressed in the areas of anaplastic transformation in 1 case of PDC.

Expression of CK 13 was not detected in the normal thyroid, lymphocytic thyroiditis, FC or PDC. CK 13 immunoreactivity was only detected in PC (14 cases). The immunoreactivity score was + in all cases, and the localization of the positive cells was similar to that observed with CK 5/6.

CK 19 was detected in the normal thyroid (score + to ++++), the intensity of the reaction being very weak and heterogeneous (Fig. 4). CK 19 was also detected in lymphocytic thyroiditis, with a stronger, but also heterogeneous, immunoreaction in the follicular cells. PC showed a strong immunoreactivity for CK 19 (Fig. 4), with a ++++ score in all but 1 case (Table 3). This case, scored as ++, was a follicular adenomatous tumour presenting few foci of nuclei with the characteristics of PC ; the expression of CK 19 in these foci contrasted with the negativity/weak expression in the adjacent lesion (Fig. 5). CK 19 was also detected in the areas of squamous metaplasia of PC, where its expression was strong and universal

Table 3 Comparison of the CK 19 immunoreactivity in papillary and follicular carcinomas

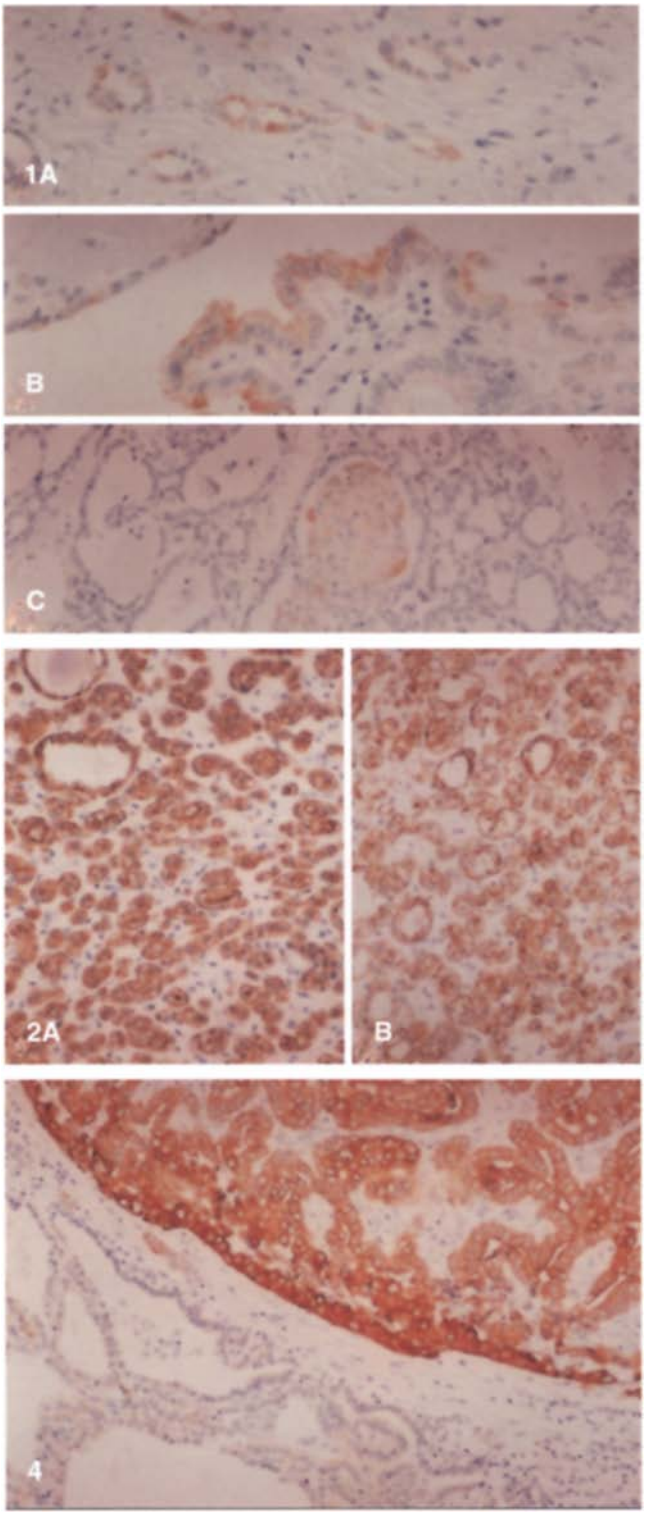
	++	+++	++++
Papillary carcinoma (n=41)	1	0	40
Follicular carcinoma (n=10)	0	3	7

$P<0.001$

(Fig. 3B). CK 19 expression was present in all cases of FC (Fig. 2B), with a score of ++++ in 7 cases and a score of +++ in the remaining 3 cases (Table 3); the intensity of immunostaining varied from weak to strong, frequently in the same tumour. PDC also expressed CK 19. In 3 cases of non-neoplastic thyroid gland resected in order to achieve total thyroidectomy after a diagnosis of thyroid cancer (PC in all cases) and in 4 cases of apparently normal thyroid parenchyma in the vicinity of PC, small foci of cells expressing CK 19 strongly were detected (Fig. 6). Although this was not apparent in the primary examination of the corresponding HE slides, a careful search established the presence of clarification or irregularity of the nuclei in the strongly CK 19-positive foci.

CK 20 was not detected in normal thyroid parenchyma. In lymphocytic thyroiditis, a faint immunoreactivity was found focally in cells with oxyphilic features. A similar immunoreactivity has also been detected in 2 Hürthle cell tumours (1 papillary and 1 follicular carcinoma).

The immunostaining pattern was similar in the follicular and ordinary variants of PC. In this latter group, no differences were found between papillary and follicular areas, and the same is true of the immunoreactive pattern of primary PCs and their respective lymph node metastases. The immunoreactivity was similar in minimally invasive and widely invasive types of FC.



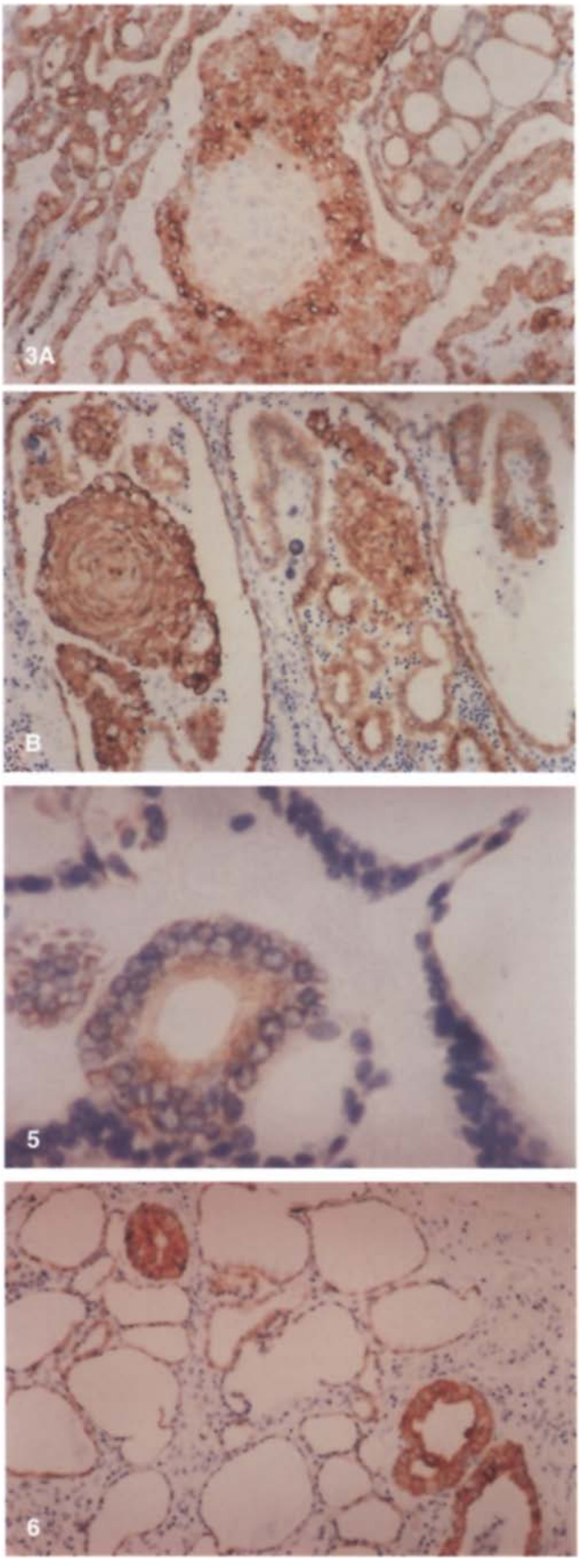


Fig. 1A–C Different patterns of immunoreactivity for CK 5/6 in papillary carcinoma. **A** Staining in neoplastic follicles within a desmoplastic stroma. **B** Staining in the cells lining papillae. **C** Staining in a focus of squamous metaplasia. CK 5/6 immunostaining; original magnification: **A**, **B** ×200, **C** ×100

Fig. 2A, B Two sections of a follicular carcinoma displaying intense immunoreactivity. **A** For CK 18. CK 18 immunostaining, original magnification ×100. **B** For CK 19. CK 19 immunostaining, original magnification ×100

Discussion

Confirming previous reports on the CK pattern of the normal thyroid gland [3, 6, 11, 16, 23, 24, 27, 28, 31], we observed the constant and high expression of simple epithelial-type CKs 7 and 18 and, to a lesser degree, of CKs 8 and 19. There was absence of expression of keratins characteristic of stratified epithelia, such as CKs 1, 4, 5/6 and 13, and also CK 20, in normal thyroid tissues. CKs 8 and 18 are the first keratins to appear during embryonic development and are usually expressed in a paired form in almost all simple epithelia [15, 17]. The tissue distribution of CK 7 is narrower than that of CKs 8 and 18, although it has a similar specificity [15]. The strong and constant expression of CKs 7 and 18, and the weak expression of CK 8 observed in normal thyroid in our study seem to indicate that, in this organ, CKs 8 and 18 are not expressed as a preferential pair, the primary pair apparently being formed of CKs 7 and 18. As expected, no CK 1 (specific for keratinizing epidermis), CK 4 and its usual partner CK 13 (present in the upper layers of non-keratinizing stratified epithelia), or CK 5/6 (present in the basal layers of non-keratinizing stratified epithelia) were found in the normal thyroid gland. The same is true for CK 20, which is present in transitional and simple epithelia, but with a very limited tissue distribution, which makes it useful as a marker for determining the origin of metastatic carcinomas [15, 19]. The low-molecular-weight CK 19 is a major component of simple epithelia and a minor component of stratified epithelia, where it is expressed only in the basal cell layer. We have confirmed previous observations [6, 23, 27] showing that CK 19 is weakly and focally expressed in the normal follicular epithelium of the thyroid gland.

The weak immunoreactivity observed for CK 20 in Hürthle cells of lymphocytic thyroiditis and of follicular and papillary carcinomas is probably non-specific and related to the high content of mitochondria.

Follicular carcinomas presented a pattern of CK expression similar to that of normal follicular cells. Expression of CK 19 was detected in a high percentage of neoplastic cells in all cases, which is in keeping with the results obtained by Dockhorn-Dworniczak et al. [6] and contrasts in part with the data of other groups [23, 24, 27]. These described the absence of expression of CK 19 (or the expression in very few cells) in their cases of FC, at variance with the strong and universal expression of CK 19 in PCs, suggesting a role in the differential diagnosis between these carcinomas [23, 27]. Our finding of CK 19 expression in all cases of FC we have studied argues against such a statement, although we have confirmed the preferential and usually stronger expression of CK 19 in PCs than in FCs.

The PCs in our series expressed simple epithelial-type CKs 7, 8, 18 and 19, with a particularly impressive presence of CK 19, confirming previous observations [6, 23, 27, 31]. Moreover, we were able to demonstrate the presence of high molecular weight CKs 5/6 and 13 in most of the cases of PC. The cells immunoreactive for CKs 5/6 and 13 tended to concentrate in the areas of greater interaction between tumour cells and the extracellular matrix, that is in desmoplastic areas and in the periphery of the tumours, although they could also be observed delineating papillary and well-defined follicular structures. The areas exhibiting strong immunoreactivity for CKs 5/6 and 13 were not only foci of squamous metaplasia, but also corresponded to disrupted follicles or papillae.

The squamous metaplastic foci of PC presented an interesting pattern of CK expression. Simple epithelial-type CKs were not demonstrated in these areas. CK 19, a simple epithelial-type CK that was found in the basal layers of stratified epithelia, was strongly and universally expressed in the foci of squamous metaplasia. In contrast to this, the typical epidermal CKs 5/6 and 13 were observed only in a few scattered cells, and CKs 1 and 4 were not observed at all. From our results the CK pattern in squamous metaplasia of papillary carcinoma can be described as an intermediate one between those of simple and of stratified epithelia.

The presence or absence of stratified epithelial-type CKs in PCs (excluding the foci of squamous metaplasia) has been a matter of discussion [3, 6, 11, 15, 16, 23, 24]. Miettinen et al. [16] demonstrated the presence of such keratins in PC, as well as in squamous metaplastic foci of chronic thyroiditis. Their findings were confirmed by Buley et al. [3] and by Henzen-Logmans et al. [11], although these authors concluded that the positive areas they observed always corresponded to squamous metaplasia. Raphael et al. [23, 24] also reported the presence of high-molecular-weight CKs in PC when such filaments were sought in frozen material or by means of microwave antigen retrieval in paraffin sections. All these immunohistochemical studies were performed using anti-epidermal-type keratin antibodies with a broad range of immunoreactivity and thus do not allow the establishment of a precise pattern of expression of high-molecular-weight CKs in PC. Dockhorn-Dworniczak et al. [6] did not confirm the findings of Buley et al. [3] and Henzen-Logmans et al. [11] in a study using both immunofluorescence and gel electrophoresis techniques. The negative results of Dockhorn-Dworniczak et al. [6] may

◀ **Fig. 3A** Absence of expression of CK 18 in a focus of squamous metaplasia of a papillary carcinoma, in contrast to the intense staining in the papillary and follicular areas of the carcinoma. CK 18 immunostaining, original magnification ×100. **B** Strong positivity for CK 19 in a similar focus of the same papillary carcinoma. CK 19 immunostaining, original magnification ×100

Fig. 4 The strong expression of CK 19 in this papillary carcinoma contrasts with the weak positivity for this CK in the normal thyroid. CK 19 immunostaining, original magnification ×100

Fig. 5 Expression of CK 19 can be observed only in the cells exhibiting the characteristic nuclear features of papillary carcinoma. These cells were found amidst an otherwise typical follicular adenoma. CK 19 immunostaining, original magnification ×400

Fig. 6 Strong immunoreactivity for CK 19 is evident in these foci of intra-thyroidal dissemination of a papillary carcinoma. CK 19 immunostaining, original magnification ×100

reflect the relatively small amount of epidermal-type CKs in PC. The low number of cases studied by these authors [6] may have also influenced the results. In a recent review, Miettinen [15] suggested that the finding of epidermal type CKs in PC is probably due to their high content of CK 19, which could cross-react with the antibodies used in the detection of high-molecular-weight CKs. Our findings do not confirm this assumption, since such a cross-reaction could not explain the different pattern of expression of CK 19 and CKs 5/6 and 13 in squamous metaplastic foci. Moreover, if such a cross-reaction were present, one would expect to observe immunoreactivity for epidermal-type CKs in follicular carcinomas presenting a strong expression of CK 19, which has not been confirmed by our observations.

Despite the different pattern of expression of epidermal keratins in papillary and follicular carcinoma, we think that the relatively small number of cells within each particular case of PC depicting such immunoreaction argues against the routine application of CKs 5/6 or 13 in the differential diagnosis between papillary and follicular carcinoma. In this we differ from Miettinen et al. [16] and Raphael et al. [23, 24]. We concur, however, with these authors [16, 23, 24] that, whenever present, such an immunoreaction strongly indicates the diagnosis of PC.

Our study shows a change in the pattern of CK expression in PC of the thyroid, consisting on the appearance of epidermal-type CKs in an organ normally presenting only simple epithelial-type CKs. This change was detected in areas of great interaction with the extracellular matrix. We found a tendency to the expression of CK 5/6 in clinically more aggressive cases of PC (data not shown); however, a definitive conclusion could not be drawn from the present study because there were too many clinically aggressive cases. Moreover, the identical pattern of expression of CKs in primary PCs and their lymph node metastases in our series seems to indicate that the metastatic process is not drastically influenced by CK filaments. A similarity in CK pattern between primary and metastatic tumours has also been reported in transitional cell carcinomas [25]. Whether the change in the keratin profile of PC is induced by the interaction with the extracellular matrix [26] and/or mediated by oncogene(s) (as for CKs 8 and 18 and *ras* oncogene in experimental models [5, 21]), remains to be seen.

An interesting finding was the detection of minute foci of PC by means of CK 19. CK 19 immunostaining may be useful in the detection of small foci of PC – so-called in situ PC – arising in benign lesions or in normal thyroid parenchyma and in the diagnosis of discrete intrathyroidal neoplastic dissemination. The clarification of the putative meaning of such "early" expression of CK 19 is beyond the scope of the present study.

We have shown that the study of CK intermediate filaments may be useful in diagnostic surgical pathology of the thyroid. The finding of high-molecular-weight CKs in a thyroid tumour is a strong argument in favour of the diagnosis of PC. Immunostaining for CK19 does not allow the differential diagnosis between PC and FC, although it is preferentially expressed in the former. It can also be used to detect small foci of PC.

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