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Immunohistochemistry of ovarian granulosa cell tumours

The value of tissue specific proteins and tumour markers

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Summary. Paraffin-embedded material of 47 ovarian tumours primarily diagnosed as granulosa cell tumours, including 2 cases of juvenile granulosa cell tumour, were studied immunohistochemically for the presence of intermediate filament proteins, epithelial membrane antigen and tumour markers. Forty-one lesions, including the 2 juvenile granulosa cell tumours, were vimentin positive, while keratin and epithelial membrane antigen expression could not be detected. Six tumours primarily diagnosed as poorly differentiated malignant granulosa cell tumours were vimentin negative, showed a mild to moderate positivity for keratin and intense positivity with the anti-epithelial membrane antigen antibody. These latter tumours were therefore classified as undifferentiated ovarian carcinomas, corresponding to their significantly poorer prognosis and shorter survival when compared with the granulosa cell tumours. Two of these six tumours were positive for carcino-embryonic antigen. Two small cell carcinomas of the ovary studied in addition expressed keratin in a proportion of tumour cells while no epithelial membrane antigen or vimentin was detectable. None of the tumours tested for alpha-fetoprotein, human chorionic gonadotrophin, human placental alkaline phosphatase and human placental lactogen, were positive.

The data indicate the value of antibodies directed against intermediate filament proteins and epithelial membrane antigen to distinguish granulosa cell tumours from poorly differentiated carcinomas, a worthwhile distinction considering the much better prognosis of granulosa cell tumours.

Key words: Granulosa cell tumour – Immunohistochemistry – Intermediate filament proteins – Tumour markers – Ovarian carcinoma

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Introduction

Ovarian neoplasms comprise a group of histomorphologically and functionally different tumour types. Most of these can be diagnosed by routine examination of adequate numbers of sections stained by hematoxylin eosin with the occasional help of special stains (Scully 1979, 1984). However, it may be difficult to differentiate between tumours lacking clear histological differentiation; i.e., the differentiation between poorly differentiated sexcord-stromal tumours from undifferentiated carcinomas or carcinoid tumours. Immunohistochemical techniques have therefore been introduced recently in the diagnosis and study of ovarian neoplasms (Kurman et al. 1984).

So far only a limited number of studies report on the diagnostic use of intermediate filament proteins in ovarian tumours (Bonazzi et al. 1983; Czernobilsky et al. 1987; Kurman et al. 1984; Miettinen et al. 1983, 1985). At present the practical contribution of tumour markers to diagnosis and serological therapy monitoring has been greatest in germ cell tumours. A few sexcord-stromal tumours (Sertoli-Leydig cell tumours) have been associated with elevated levels of alpha-fetoprotein in the serum (Benfield et al. 1982; Chadha et al. 1987; Chumas et al. 1984; Mann et al. 1986; Tetu et al. 1986; Young et al. 1984). In two cases this antigen was localized in areas with hepatocyte differentiation within a retiform Sertoli-Leydig cell tumour with heterologous elements (Chadha et al. 1987; Young et al. 1984).

The aim of the present study was to evaluate the usefulness of immunohistochemical demonstration of intermediate filament proteins and epithelial membrane antigen in a group of ovarian neoplasms primarily diagnosed as granulosa cell tumours. In addition, the presence of a number

Table 1. Commercial source of monoclonal and polyclonal antibodies used

Antibodies	Specific for	Commercial source	Dilution
rabbit anti vimentin	vimentin	Euro-diagnostics	1/50
rabbit anti keratin	keratin	Euro-diagnostics	1/50
mouse anti EMA	epithelial membrane antigen	DAKO	1/10
rabbit anti CEA	carcinoembryonic antigen	DAKO*	1/250
mouse anti AFP	alpha-1-feto-protein	Unipath	1/100
rabbit anti HCG	chorionic gonadotropin (β-chain)	DAKO	1/50
rabbit anti HPLAP	human placental alkaline phosphatase	DAKO	1/200
rabbit anti HPL	human placental lactogen	DAKO	1/400

* After absorption with liver, spleen and stomach powder

of tumour markers and oncofetal antigens was investigated.

Materials and methods

From the files of the Dutch Ovarian Tumour Committee 33 granulosa cell tumours were retrieved. Fourteen cases primarily diagnosed as granulosa cell tumours and referred from regional hospitals to the Rotterdam Radiotherapy Institute were also included in the study. Formalin fixed and routinely paraffin embedded tissue blocks were available from the above cases. In addition, 2 tumours with a diagnosis of small cell carcinoma of the ovary were studied. The histological diagnosis was based on sections stained with haematoxylin azofloxin, using standard criteria (Fox and Langley 1976; Scully 1979). Based on an orientating pilot study and the literature we decided to study the following antigens and markers: vimentin, keratin, epithelial membrane antigen (EMA), carcino-embryonic antigen (CEA) and alpha-fetoprotein (AFP). A Grimelius stain was also performed on all tumours.

The mouse monoclonal antibodies and the rabbit antisera used in the present study are listed in Table 1.

To detect the binding of mouse monoclonal antibodies on tissue sections we applied the indirect conjugated method using a rabbit-anti mouse antiserum conjugated to horse-radish peroxidase (RAM-PO) purchased from DAKO, Denmark. The binding of rabbit antibodies was detected by a horse-radish peroxidase conjugated swine anti-rabbit antiserum (SWAR-PO) purchased from DAKO, Denmark.

Paraffin sections of 5 µ thickness were deparaffinized in xylene and absolute ethanol. Endogenous peroxidase activity was blocked by incubation in methanol containing 3% hydrogen peroxide for 30 min at room temperature. For detection of keratin and vimentin sections were subsequently subjected to proteolytic treatment using 0.1% protease (Sigma) in phosphate buffered saline (PBS) for 25 min at 37° C. Before the application of primary rabbit antisera reduction of nonspecific background staining was achieved by incubation of the sections with 20% nonimmune swine serum (NSS) diluted in PBS for 15 min. The sections were then overlaid with the appropriate dilutions of the primary antibodies for 1 h at room temperature (see Table 1), rinsed for 10 min with PBS, incubated for another 30 min with the second (conjugated) antibody and rinsed again in PBS for 10 min. The RAM-PO was diluted 1:100 in PBS containing 5% nonimmune human serum (NHS) and 5% nonimmune rabbit serum (NRS). The SWAR-PO was diluted 1:100 in PBS containing 5% NHS and 5% NSS. Antibody localization was visualized by incubation of sections with a TRIS-buffered saline solution (0.05 M, pH 7.4) containing 50 mg% 3,3 diamino benzidine-tetra-hydrochloride (Fluka, FRG) and 0.03% hydrogen peroxide. Slides were then washed with running tapwater, counterstained with haematoxylin and mounted in Malinol.

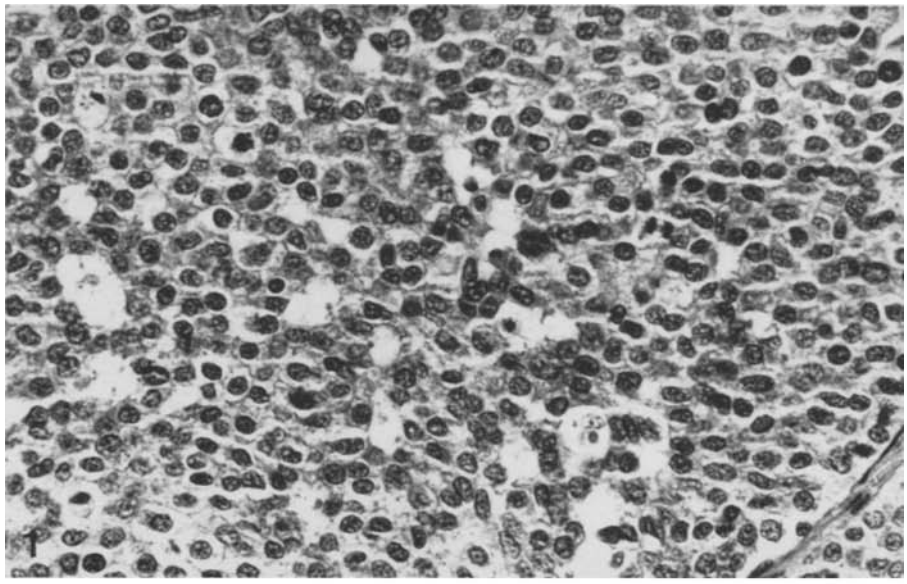
Positive controls of human chorionic gonadotrophin (HCG), human placental lactogen (HPL), human placental alkaline phosphatase (HPLAP) and EMA included sections of placental tissue. Positive controls for AFP and CEA were sections of a hepatocellular carcinoma and a colon carcinoma, respectively. Negative controls omitting the primary antibody were also employed.

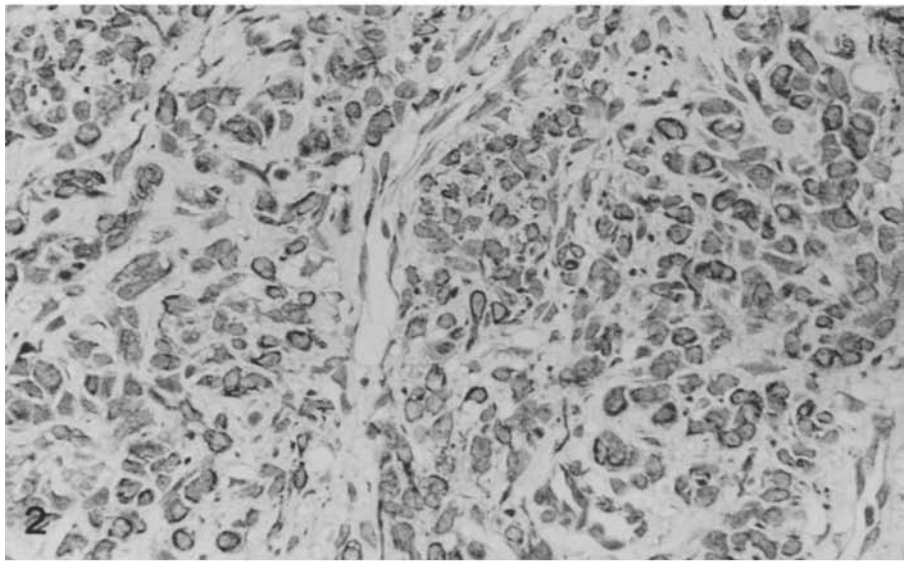
Results

Thirty-nine of the 49 neoplasms had the histological appearance characteristic of well differentiated granulosa cell tumours. These are therefore designated as typical. Twenty-five of these neoplasms were pure granulosa cell tumours with a minor theca cell component in 14 cases. The granulosa cells had scanty cytoplasm and oval or angular nuclei with a longitudinal groove, often containing a single nucleolus. The tumour cells were closely but haphazardly arranged in a dense stroma. A variety of histological patterns was observed, i.e. microfollicular, insular, trabecular and diffuse. The microfollicular pattern was characterized by Call-Exner bodies which are small cavities containing eosinophilic material. The diffuse pattern was most frequently observed in our series.

The two juvenile granulosa cell tumours typically showed a nodular pattern with follicular spaces of varying sizes containing eosinophilic material which stained positive with mucin stains. The nuclei were moderately hyperchromatic and a considerable number of mitoses were present. Nuclear grooves were rare or absent.

Six neoplasms were poorly differentiated, composed of solid areas consisting of epithelial-like cells lacking the coffee-bean nuclei and focally included spaces resembling Call-Exner bodies (Fig. 1), and they are further designated as atypical. The two small cell carcinomas were composed of closely packed cells with scanty cytoplasm and dark nuclei with prominent nucleoli. Nuclei were not grooved. Islands of tumour cells showed scat-





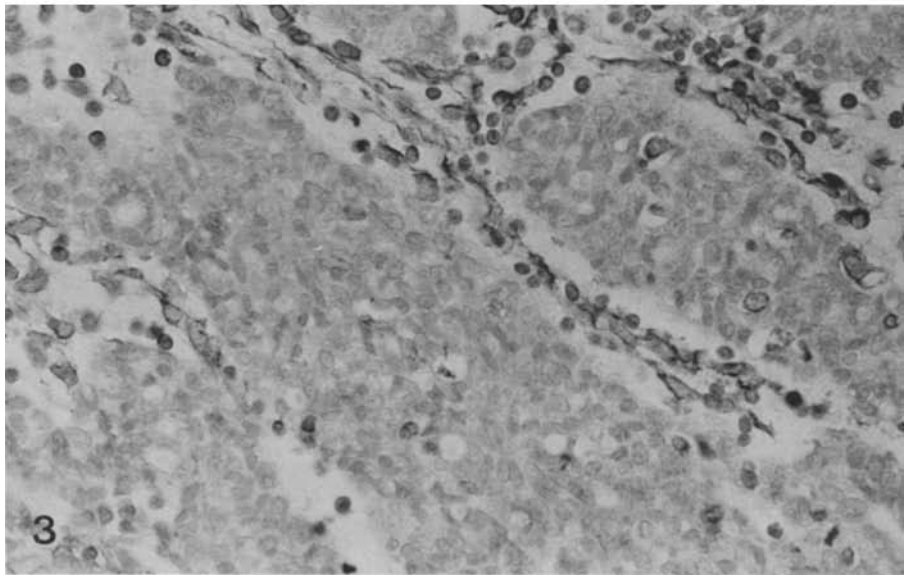


Fig. 1. An undifferentiated ovarian carcinoma initially misdiagnosed as GCT

Fig. 2. An adult type granulosa cell tumour with diffuse growth pattern showing vimentin positivity in tumour cells

Fig. 3. Same tumour as in Fig. 1 showing vimentin negative tumour cells. Vimentin is only positive in the vascular and stromal septal cells

Table 2

Pat. no.	Age (years)	Tumor diam	Hist.	Vim.	Ker.	EMA	CEA	AFP	Grim.	Patient status
1	37	?	T	+	−	−	−	−	−	A 132 mo
2	47	7.5	T	+	−	−	−	−	−	Lfu 31 mo
3	58	3.5	T	++	−	−	−	−	−	Lfu 4 mo
4	58	?	T	+	−	−	−	−	−	A 132 mo
5	50	20	T	+	−	−	−	−	−	Lfu 14 mo
6	39	5	T	+	−	−	−	−	−	A 132 mo
7	53	12	T	++	−	−	−	−	−	A 132 mo
8	47	3.5	T	+	−	−	−	−	−	A 100 mo
9	44	15	T	+	−	−	−	−	−	Lfu 24 mo
10	39	11	T	+	−	−	−	−	−	A 96 mo
11	44	28	T	+	−	−	−	−	−	A 96 mo
12	20	5	T	+	−	−	−	−	−	A 72 mo
13	51	10	T	+	−	−	−	−	−	A 60 mo
14	53	5.5	T	+	−	−	−	−	−	A 60 mo
15	75	4	T	+	−	−	−	−	−	D 39 mo ht dis
16	32	6	T	+	−	−	−	−	−	A 29 mo
17	52	8	T	+	−	−	−	−	−	A 228 mo
18	61	7	T	+	−	−	−	−	−	A 48 mo
19	61	24	T	+	−	−	−	−	−	A 32 mo
20	27	7.5	T	+	−	−	−	−	−	A 45 mo
21	66	8	T	+	−	−	−	−	−	A 135 mo
22	46	12	T	+	−	−	−	−	−	A 72 mo
23	37	16	T	++	−	−	−	−	−	A 144 mo
24	68	?	T	++	−	−	−	−	−	D 12 mo rec
25	58	10	T	++	−	−	−	−	−	A 85 mo
26	62	10	T	+	−	−	−	−	−	D 39 mo rec
27	51	9	T	+	−	−	−	−	−	D 46 mo lung ca
28	54	22	T	+	−	−	−	−	−	D 98 mo rec
29	56	?	T	+	−	−	−	−	−	A 60 mo
30	73	?	Atyp	−	+	+++	+	−	−	D 8 mo rec
31	41	?	Atyp	−	+	+++	−	−	−	D 10 mo rec
32	69	?	Atyp	−	+	+++	+	−	−	D 14 mo rec
33	74	?	Atyp	−	+	++	−	−	−	D 27 mo rec
34	53	?	Atyp	−	+	++	−	−	−	D 39 mo rec
35	41	14.5	Atyp	−	++	++	−	−	−	D 50 mo rec
36	70	8	T	+	−	−	−	−	−	A 228 mo
37	42	4	T	++	−	−	−	−	−	A 48 mo
38	48	9	T	++	−	−	−	−	−	A 24 mo
39	46	7	T	+	−	−	−	−	−	A 11 mo
40	43	30	T	++	−	−	−	−	−	A 14 mo
41	24	14	T	+	−	−	−	−	−	A 14 mo
42	54	3	T	+	−	−	−	−	−	A 84 mo
43	47	16	T	+	−	−	−	−	−	A 12 mo
44	47	4.5	T	+	−	−	−	−	−	A 15 mo
45	60	3	T	+	−	−	−	−	−	A 8 mo
46	15	33	JGCT	+	−	−	−	−	−	*
47	16	?	JGCT	+	−	−	−	−	−	*
48	26	?	SCC	−	+	−	−	−	−	*
49	28	14	SCC	−	+	−	−	−	−	*

Vim = vimentin; Ker = keratin; EMA = epithelial membrane antigen; CEA = carcinoembryonic antigen; AFP = alpha-fetoprotein; Grim = grimelius staining; T = typical; Atyp = atypical; A = alive; D = died; mo = months; Lfu = lost to follow-up; ht dis = heart disease; rec = recurrence; JGCT = juvenile granulosa cell tumour; SCC = small cell carinoma; * see text page 10

tered follicle-like structures containing eosinophilic material. Numerous mitotic figures were seen.

Data on immunohistochemical findings on paraffin-embedded tumour material and clinical follow-up are summarized in Table 2. All the typical granulosa cell tumours were distinctly positive for vimentin (Fig. 2). However, the 6 tumours with atypical histological features were essentially negative for vimentin, although in 3 of the 6 tumours widely scattered vimentin positive tumour cells were found. Vimentin staining was strong in the vascular endothelial cells and stromal elements

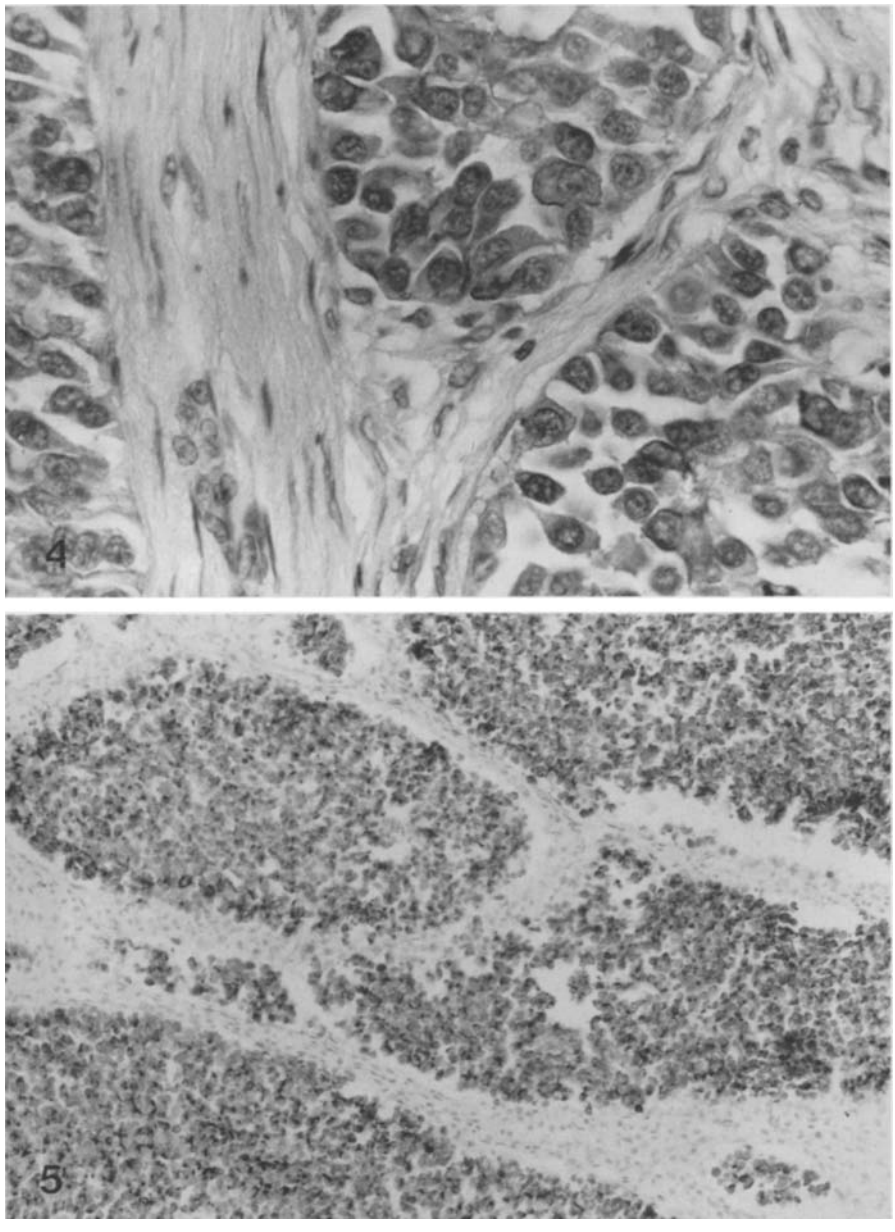


Fig. 4. An ovarian carcinoma with solid epithelial areas originally diagnosed as granulosa cell tumour showing moderate positivity of tumour cells for keratin

Fig. 5. An ovarian carcinoma originally diagnosed as granulosa cell tumour showing intense positivity of tumour cells for epithelial membrane antigen

(Fig. 3). The 2 small cell carcinomas did not express vimentin.

All the typical granulosa cell tumours, including the 2 juvenile granulosa cell tumours examined were keratin negative. The 6 atypical neoplasms were keratin positive, the intensity of staining being mild to moderate in 10–50% of the tumor cells (Fig. 4). In both small cell carcinomas a proportion of tumor cells showed positive staining reaction.

All granulosa cell tumours including the juvenile granulosa cell tumours were negative for EMA. The 6 atypical tumours revealed a distinct and intense positivity for EMA (Fig. 5) in the majority of tumour cells. The 2 small cell carcinomas examined were EMA negative.

Only 2 of the 6 tumours classified as atypical were CEA positive. None of the typical granulosa cell tumours were CEA positive. The juvenile granulosa cell tumours and small cell carcinomas were also negative.

None of the typical or atypical tumours tested for AFP were positive. Juvenile granulosa cell tumours and small cell carcinomas were also negative.

The 6 granulosa cell tumours tested for HCG, HPLAP and HPL as a part of the orientating pilot

study showed negative results. Grimelius: Staining performed on all the typical and atypical tumours as well as the small cell carcinomas was negative. Survival data are listed in Table 2.

Discussion

In ovarian tumour histopathology granulosa cell tumours constitute a major problem of differential diagnosis especially with respect to undifferentiated carcinomas (Young and Scully 1982). We therefore studied a large series of tumours putatively diagnosed as granulosa cell tumour immunohistochemically, with a clinical follow-up period up to 19 years.

Analysing the 47 tumours with antibodies to keratin, EMA, CEA and vimentin, 6 tumours could be isolated by their differential immunohistochemical staining pattern when compared with the remaining tumours (Table 2). These 6 tumours were mildly to moderately positive for keratin, expressed EMA strongly, while 2 of the 6 tumours were CEA-positive. In contrast, the tumour cells of the remaining 41 tumours only expressed vimentin. Histologically, the group of 6 tumours with deviant immunostaining pattern consisted of poorly differentiated tumours which lacked, most conspicuously, the coffee-bean nuclei. Furthermore, the follow-up study demonstrated that all patients with this particular tumour type died between 8 and 50 months. The remaining 41 patients had a favourable prognosis with only 10% mortality. On the basis of histological features, immunohistochemical findings and follow-up data we suggest that those tumours with expression of the epithelial markers keratin, EMA and keratin or CEA should be classified as undifferentiated carcinomas rather than atypical granulosa cell tumours. Three of these tumours contained sporadic tumour cells with vimentin expression. This finding does not contradict the presumed epithelial differentiation of these tumours as expression of vimentin in undifferentiated ovarian carcinomas has been observed previously (Mann et al. 1986).

Recently, Czernobilsky et al. (1987), showed by biochemical analysis and by immunostaining of frozen sections that keratin intermediate filaments may occur in granulosa cell tumours. Expression of keratin in other tumours of non-epithelial differentiation such as smooth muscle has also been demonstrated recently (Brown et al. 1987; Norton et al. 1987). Apparently, expression of keratins in a particular tumour is not entirely indicative of epithelial differentiation. The discrepant results of our study and that of Miettienen et al. (1985) on the one hand and those of Czernobilsky on the other may be explained by a lesser sensitivity of the immunoperoxidase staining for keratins on formalin-fixed paraffin-embedded material as compared to acetone fixed frozen sections. Furthermore, the use of monoclonal antibodies specific for a particular keratin protein may lead to better immunostaining results than can be obtained by application of the polyclonal multispecific antikeratin antiserum used in our study.

The 2 juvenile granulosa cell tumours in our material showed an identical staining pattern as the typical granulosa cell tumours, that is to say they were intensely vimentin-positive but lacked detectable expression of keratin and EMA or CEA. Thus, adult type and juvenile granulosa cell tumours could not be separated on basis of different immunohistochemical staining patterns with the antibodies used in this study.

The 2 small cell carcinomas of the ovary examined did not show a detectable expression of vimentin or EMA, while in both cases a proportion of cells stained with anti-keratin.

In our series EMA appears to be next to vimentin the marker of choice to discriminate between undifferentiated ovarian carcinoma and poorly differentiated sex cord-stromal tumours. Similarly, other authors have suggested that in comparison to CEA and keratin, EMA was the most sensitive marker of epithelial differentiation in formalin-fixed tissue (Sloane et al. 1983). With regard to specificity, the reactivity of anti-EMA antibodies with lymphoid cells and lymphomas has been noted (Delsol 1984). The expression of CEA and/or keratin by the EMA-positive tumours virtually excludes a possible diagnosis of malignant lymphoma of the ovary in the material presented here.

Because a carcinoid tumour should be considered in the differential diagnosis of a granulosa cell tumour our negative results with Grimelius stain made the diagnosis of carcinoid less likely.

All tumours tested for AFP were negative in contrast to the 8 cases of Sertoli-Leydig cell tumours (androblastomas) with evidence of AFP production described in the literature (Benfield et al. 1982; Chadha et al. 1987; Chumas et al. 1984; Mann et al. 1986; Tetu et al. 1986; Young et al. 1984) indicating that out of the group of sex cord-stromal tumours andro-blastomas are more likely to produce AFP.

Nouwen et al. (1985) demonstrated the presence of HPLAP immunohistochemically in benign, borderline and malignant ovarian tumours including a well differentiated and a poorly differentiated granulosa cell "carcinoma" (sic). This discrepancy

could be the result of difference in histological criteria used for diagnosis of granulosa cell tumours. In contrast to their results the 6 granulosa cell tumours in our series tested for HPLAP were negative. None of the remaining tumour markers were positive in any of the granulosa cell tumours tested. Therefore serological estimation of these markers seems to us of no value in the follow-up of these patients.

This study indicates the value of the use of antibodies directed against EMA and intermediate filament proteins to distinguish granulosa cell tumours from undifferentiated ovarian carcinomas. Therefore their use should complement the routinely used histological techniques in making such a distinction when necessary. This is worthwile, considering the much better prognosis of granulosa cell tumours.

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