VALUE OF IMMUNOHISTOCHEMISTRY IN CONFIRMING UNDIFFERENTIATED OVARIAN CARCINOMAS

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Summary. The present immunohistochemical study was carried out on ten cases of ovarian tumours diagnosed with usual staining as undifferentiated ovarian carcinomas. A panel of antibodies was chosen in order to confirm the epithelial origin of these tumours and to eliminate some possible ovarian metastasis. The cytokeratin AE1/AE3, EMA and BerEP4 positivity supported their epithelial origin even if thirty per cent of the cases co-expressed cytokeratin and vimentin. Even if there is not a specific marker to confirm the ovarian origin of the tumours, the CA125 and CEA positivity suggested that these carcinomas might represent a serous or a mucinous dedifferentiation. Associated with these antibodies, the calretinin, CK7 and CK20 stainings allowed the separation of these tumours from peritoneal mesotheliomas with ovarian extension and from ovarian metastasis originating in the gastrointestinal tract. The evaluation of the PCNA labelling index confirms the high degree of cell proliferation and the aggressiveness of these tumours.

Key words: undifferentiated ovarian carcinomas, immunohistochemistry.

INTRODUCTION

According to the OMS classification, undifferentiated carcinomas of the ovarian surface epithelium are defined as inclusion tumours with minimal differentiation such as mucin vacuoles, endometrial-like glands or psammoma bodies (Serov *et al.*, 1973).

Three types of undifferentiated carcinomas have been described: the classical type, small cell carcinoma of hypercalcemic type and small cell carcinoma of pulmonary type, all these types being very rare when their diagnosis is made strictly on the basis of morphological criteria (Seidman *et al.*, 2002).

Ovarian malignant epithelial tumours were shown to result from the modified mesothelial cells of the ovarian surface epithelium and over 75% of the patients with malignant ovary tumours showed, at the moment of their diagnosis, an extension of the tumour beyond the ovary (pelvis and abdominal cavity).

Moreover, 10% from the undifferentiated ovarian tumours cannot be identified only on the basis of morphological criteria, and 8% of the ovarian tumours are metastasis from the gastrointestinal tract or from the mammary gland. Their prognosis and treatment depend on their origin and it is therefore essential to

establish the origin of the carcinoma located in the abdominal cavity (Boerman *et al.*, 1991).

Taking into account all these aspects, in the present study were utilized antibodies, which helped establish the epithelial origin of the analysed undifferentiated tumours and the fact that this one was in the ovarian surface epithelium. Consequently, they helped separate these tumours from peritoneal mesotheliomas with ovarian extension and from ovarian metastasis originating in the gastrointestinal tract. To complete the study of the analysed tumors we also evaluated the degree of the tumoral cell proliferation.

MATERIAL AND METHODS

The present study has been performed on 10 cases of ovarian undifferentiated carcinomas selected from 22 cases diagnosed in a six-year period of time (1995–2000). The cases have been histopathologically diagnosed in the Craiova Laboratory of Pathology after processing specimens (tumour ovaries) issued from the Obstetrical–Gynaecological and Surgery Departments of the Craiova Emergency Hospital.

The specimens were processed using the standard method of paraffin embedding and initially stained with the usual H&E stain. Next followed the immunohistochemical treatment with the LSAB/HRP method (labelled streptavidin biotin) at the Laboratory of Histological, Histopathological and Immunohistochemical Techniques from the University of Medicine and Pharmacy, Craiova and "Victor Babeş" Institute, Bucharest. The utilized antibodies were concentrated antibodies made by *DAKOCytomation*, Denmark and ZYMED Laboratories Inc., San Francisco. The table below (Table 1) shows the performed dilutions and pre-treatment.

In order to immunohistochemically confirm the diagnosis of undifferentiated carcinoma of the ovary, all the studied specimens were initially marked with vimentin to control the primary processing of the analysed cases. First we chose a panel of antibodies to help establish the epithelial origin of the studied tumours. Thus, we opted for cytokeratin AE1/AE3, EMA and BerEP4 antibodies.

Since there is no specific marker to indicate the ovarian origin of the tumours, the differentiation of the studied tumours from possible ovarian metastasis was realized through several associated immunohistochemical reactions.

Thus, the anti-calretinin antibody was used in the present study to help, together with the BerEP4, CA125 and CEA antibodies, to eliminate a mesothelioma especially for the cases, which, at the moment of diagnosis, showed dissemination at the level of the peritoneal cavity. The, CK7 and CK20 stainings allowed the separation of these tumours from ovarian metastasis originating in the gastrointestinal tract.

Table 1
Utilized antibodies, dilution and pretreatment

Primary antibody	Antibody clone	Dilution	Antigen retrieval
Vimentin	V9	1:30	none
AE1/AE3	AE1 and AE3	1:50	HMAR
EMA	E29	1:50	HMAR
CK 7	OV-TL 12/30	1:25	HMAR
CK 20	Ks 20.8	1:25	HMAR
CA 125	OC 125	1:25	HMAR
CEA	policlonal	1:400	HMAR
BerEP4	BerEP4	1:40	Enzyme digestion
Calretinin*	policlonal	1:100	HMAR
PCNA	PC10	1:100	HMAR

*made by ZYMED; HMAR = Heat Mediated Antigen Retrieval

To complete the study of the analysed tumours we used as marker of the cell proliferation the anti-PCNA antibody and we calculated the labelling index, which reached high values in undifferentiated carcinomas.

METHODS OF RESULT INTERPRETATION

Since there is no standard method for the immunohistochemical interpretations, in order to formulate the results we used some of the models offered by previous studies.

The immunostaining **intensity** was evaluated using a four-degree-system in accordance with the model of Wauters, 1995:

– negative immunostaining (–);
– weak positive immunostaining (+);
– moderate positive immunostaining (++);
– strong positive immunostaining (+++).

We excluded from our interpretation the fields, which showed folding of the paraffin sections, necrosis or hemorrhagic infiltrate.

The immunostaining distribution was evaluated as being diffuse when more than 50% of the cells were positive and, respectively, focal when 5–50% of the cells were positive (Wauters, 1995).

For the PCNA immunostaining the cells, which showed a certain positive nuclear staining, were considered positive, while the cells with an equivoque nuclear staining were considered negative.

The **labelling index for PCNA** was determined by the number of cells having positively staining nuclei divided by the total counted cells (labelled and unlabelled) and multiplied by 100 to obtain a percentage labelling index (Guo *et al.*, 1993). A minimum number of 500 epithelial cells were counted for each case at a magnification of ×400.

RESULTS AND DISCUSSIONS

All the studied cases had (with the usual H&E staining) the aspect of the classical type of undifferentiated ovarian carcinomas (Figure 1).

IMMUNOHISTOCHEMICAL ANALYSIS FOR VIMENTIN ANTIBODY

The immunohistochemical analysis for vimentin antibody showed in all the cases a positive marking at the stromal level (presence of internal positive control and of good primary processing control) but in three of the studied cases there was a positive immunostaining at the level of the tumour cells. The intensity of the reaction to vimentin antibody in tumour epithelial cells ranged from weak (Figure 2) to strong positive (Figure 3).

This variability was in relation to the degree of the cells dedifferentiation and it was due to the different concentration of the intermediate filaments from the undifferentiated tumour epithelial cells and to the degree of epithelial cell deviation towards a mesenchymal transdifferentiation. All the specimens were focally positive and the immunostaining pattern in the stromal cells was uniformly distributed in the cytoplasm while in the tumour epithelial cells it had a paranuclear concentration. The vimentin expression in undifferentiated carcinomas is possible because, as the cells become dedifferentiated, they lose the characteristics of the origin tissue (Norton *et al.*, 1987).

The presence of vimentin in the tumour epithelial cells required the introduction of antibodies meant to help differentiate the studied tumours from tumours with mesenchymal origin, especially from mesotheliomas. Thus, anti-calretinin antibody was also used (presently considered to be the best marker for mesotheliomas) together with cytokeratin, EMA and BerEP4 antibodies (epithelial cells markers).

IMMUNOHISTOCHEMICAL ANALYSIS FOR CYTOKERATIN AE1/AE3 ANTIBODY

The immunostaining for cytokeratin AE1/AE3 was of great importance in the determination of the epithelial origin of the studied tumours since, for this marker, all the ten cases were positive at the level of tumour cells but negative at the stromal level (Figure 4).

The immunostaining was frequently diffuse and the intensity of the reaction ranged from weak, moderate (Figure 5) to strong positive (Figure 6). The pattern of staining was uniformly cytoplasmatic and often associated with a “pericellular pattern” (Figure 6).

This “pericellular pattern” suggested the epithelial origin of these tumors. The “pericellular pattern”, described as being of higher intensity at the periphery of the tumoural cells, is not observed in mesenchymal tumours so its presence is

considered to be in favour of the epithelial origin of the tumour (Hammond *et al.*, 1991). Three of the studied cases co-expressed cytokeratin AE1/AE3 and vimentin. The co-expression of the vimentin and of the cytokeratin is frequently found in several types of carcinomas while sex cord stromal tumours (such as the granulosa cell tumour with which is made the differential diagnosis of some undifferentiated ovarian carcinoma) are vimentin positive but usually cytokeratin negative (Dabbs *et al.*, 1986).

For a correct differential diagnosis between the undifferentiated ovarian carcinomas and the diffuse type of granulosa cell tumour it is necessary to use inhibin antibody which is negative in undifferentiated ovarian carcinomas but positive in granulosa cell tumour (Pelkey *et al.*, 1998).

It was not possible to introduce the inhibin antibody in the present study, but the antibodies used were sufficient to exclude the granulosa tumour because all of the studied cases were AE1/AE3 and EMA positive (see below). The granulosa tumours are vimentin positive and EMA negative (Soslow and Isaacson, 2002).

IMMUNOHISTOCHEMICAL ANALYSIS FOR EMA ANTIBODY

All the studied carcinomas were EMA positive. The stromal cells didn't present epitopes for this marker. EMA was diffusely positive in all the cases with predominant membranar staining (Figure 7) sometimes associated with cytoplasmatic staining.

Eight cases showed strong intensity immunostaining and the other two-presented moderate intensity immunostaining. "True" EMA reactivity (*i.e.*, that which generally equates with epithelial differentiation) must be cell membrane-based (Cerilli and Wick, 2002).

EMA showed the acinar differentiations, which had not been observed on the usual staining (Figure 8). In these acinar areas the positivity was strong and of luminal type, similar to the typical luminal pattern of EMA in endometroid carcinomas.

The results are in accordance with the studies that reported that all the analysed undifferentiated ovarian carcinomas were strong positive for the EMA antibody (Seidman *et al.*, 2002) and this antibody emphasized acinar differentiation, often where it was not easily observed in haematoxylin and eosin preparations (Hammond, 1991).

Vimentin, cytokeratin AE1/AE3 and EMA confirmed the epithelial origin of the analysed tumours and excluded the sex cord stromal tumours.

IMMUNOHISTOCHEMICAL ANALYSIS FOR CA125 ANTIBODY

Seven of the studied cases showed epitopes for this antibody at the level of the tumour cells. In five of these cases the distribution of CA125 staining was focal

with heterogeneous type intensity and the pattern of staining was membranar (Figure 9).

It is important to mention that the heterogeneous type intensity is characterised by the presence in the same tumour of areas with strong, moderate and weak intensity together with completely negative areas. Even if CA125 is not a specific marker of the ovarian origin of the tumours, this type of heterogeneous staining was in favour of the ovarian origin.

Some studies have shown that the specimens of ovarian undifferentiated cell carcinomas stained with CA125 frequently showed heterogeneous focal positivity or only isolated cells were specifically stained with CA125 (Neunteufel and Breitenecker, 1989).

In the other two cases the distribution of CA125 staining was diffuse with strong intensity immunostaining and with a membranar and cytoplasmatic pattern too (Figure 10).

The ovarian undifferentiated carcinomas reactivity to CA125 is similar to that of the serous carcinomas. Thus, 70% of the undifferentiated carcinomas react to CA125 (Neunteufel and Breitenecker, 1989).

However, the results reached by the specialists vary within wider limits, some of them indicating that only 46% from these carcinomas are CA125 positive (Silva *et al.*, 1991).

IMMUNOHISTOCHEMICAL ANALYSIS FOR CEA ANTIBODY

The immunohistochemical analysis for CEA antibody showed that only the CA125 negative cases were CEA positive.

This distribution of positivity for the two antibodies suggested the possibility of a mucinous dedifferentiation in the CEA positive cases and of a serous dedifferentiation in the CA125 positive cases.

It seems that CEA is an indication of the mucinous type dedifferentiations since it was detected in 83% of the mucinous carcinomas, which were, at the same time, negative for CA125 (Neunteufel and Breitenecker, 1989).

The distribution of CEA staining was focal, the intensity of the reaction being weak and the pattern of the stain being of the cytoplasmatic type for all the positive specimens (Figure 11).

This type of staining supports the ovarian origin of the studied tumours. Yet, since CEA can't confirm the ovarian origin, the differentiation of gastrointestinal metastasis was made with CK7 and CK20 (see below).

A weak or a focal staining for CEA supports the ovarian origin of the tumours while this type of staining is very rare in tumours with gastrointestinal origin, which are intense and diffusely positive for CEA (Hammond *et al.*, 1991).

In adenocarcinomas, the reaction to CEA is located at the luminal pole as well as in the cytoplasm but tends to concentrate at the cytoplasmatic level, as the

tumours tend to dedifferentiate, *i.e*. to have a high histological degree (Goldstein and Silverman, 2002).

IMMUNOHISTOCHEMICAL ANALYSIS FOR BEREP4 ANTIBODY

Seven of the studied tumours expressed epitopes for this antibody. The staining was moderate and diffuse, with a membranar pattern associated with a granular cytoplasmatic one (Figure 12).

The BerEP4 positivity pleaded for the epithelial nature of the tumours taking into account the fact that this marker makes the distinction between the epithelium and the mesothelium (Latza *et al.*, 1990) though 5–20% from the mesotheliomas show focal staining for this antibody (Gaffey *et al.*, 1992).

Since three of the cases were vimentin positive suggesting a possible mesenchimal or even mesothelial origin, it was important to introduce the BerEP4 antibody in this study to help clarify, together with the other antibodies, the epithelial differentiation of these tumours.

The fact that not all the studied cases expressed epitopes for BerEp4 was due to the weak degree of differentiation of these tumours. In malignant tumours of the ovarian surface epithelium, the expression of BerEp4 is correlated with the degree of differentiation of the tumours but not with the clinical stage (Cherchi *et al.*, 2001).

IMMUNOHISTOCHEMICAL ANALYSIS FOR CALRETININ ANTIBODY

All of the studied tumours were negative for this marker. The positivity of the ovarian surface epithelium (for the cases where it was present on the diagnosis specimens) was very helpful since it represented the positive internal control.

The calretinin negativity of all the cases was extremely useful for the elimination of the mesotheliomas with ovarian extension. Though rare cases of ovarian surface epithelial tumours were calretinin positive, positivity was not reported in the undifferentiated carcinomas of the ovary. In the calretinin positive ovarian surface epithelial tumours, the positivity was clearly different from the diffuse, intense, nuclear pattern positivity found in the mesotheliomas (Hammar, 2002).

However, in two cases calretinin positive cells were observed in the tumoural stroma. These cells, grouped on small areas, showed either moderate or intense staining. The pattern of the staining was either cytoplasmatic or cytoplasmatic and nuclear (Figure 13).

These cells were interpreted as fibroblastic because of their morphological aspect and by the presence of such calretinin positive cells at the ovarian level have already been reported (Dabbs, 2002).

IMMUNOHISTOCHEMICAL ANALYSIS FOR CK7 ANTIBODY

All the cases of undifferentiated ovarian carcinomas were positive for CK7 antibody. The intensity of the reaction was maximum in seven of the cases and moderate in the other cases. The tumoural stroma was negative. The distribution of the staining was diffuse and the pattern was cytoplasmatic, frequently associated with a "pericellular" pattern, which characterizes the epithelial tumours (Figure 14).

The results of the present study correspond to those already registered by the specialists who have reported that all the undifferentiated ovarian carcinomas were CK7 positive (majority showing diffuse staining) and CK20 negative (Wauters, 1995).

The CK7 positivity in all studied cases confirmed once more their epithelial nature and was also useful for the determination of the ovarian origin.

IMMUNOHISTOCHEMICAL ANALYSIS FOR CK20 ANTIBODY

All the studied cases were CK20 negative in comparison with the positivity of the external control (colon). The correlation of the results obtained after the immunostaining with CK20 and CK7 allowed the exclusion of ovarian metastasis originating in the gastrointestinal tract.

CK20 used together with CK7 allowed the differentiation of the primary ovarian tumours from ovarian metastasis (Soslow and Isaacson, 2002).

However, the cases of ovarian carcinomas CK7 positive and CK20 negative must be differentiated from the mesotheliomas, which have the same staining profile for these two cytokeratines (Wauters, 1995).

The calretinin negativity in all the studied cases allowed the elimination of a mesothelioma.

IMMUNOHISTOCHEMICAL ANALYSIS FOR PCNA ANTIBODY

All the studied cases were PCNA positive. The values of the PCNA labelling index ranged from 40% to 95%. Eight cases had a PCNA labelling index higher than 50% (Figures 15 and 16).

These high values are due to the high degree of dedifferentiation and of proliferation of the epithelial cells in the analysed undifferentiated carcinomas.

The PCNA labelling index ranges (according to the degree of differentiation of the tumour) from 30% to more than 90% at the malignant serous ovarian tumours, and from 45% to more than 95% at the malignant mucinous ovarian tumours.

The values of the PCNA labelling index are unlikely to have a prognostic value in ovarian carcinomas (Guo *et al.*, 1993).

CONCLUSIONS

The present immunohistochemical study, which completed the histological study carried out on usual staining, was very useful in confirming the epithelial origin of the studied tumours and in eliminating possible ovarian metastasis.

The vimentin used as primary processing control was stromal positive in all the cases. This confirmed the good quality of the primary processing which made possible the submission of the specimens to immunohistochemical staining.

Thirty per cent of the undifferentiated ovarian carcinomas expressed both epithelial and mesenchymal markers as a result of the dedifferentiation of the tumoural cells. The cytoplasmatic pattern associated with the pericellular pattern of the AE1/AE3 staining was in favour of their epithelial nature since this type of positivity is not found in the mesenchymal tumours.

The EMA positive staining, which has frequently shown acinar structures, distinguishes the undifferentiated ovarian carcinomas from the granulosa cell tumours and points towards an epithelial origin.

Seventy per cent of the cases were CA125 positive and CEA negative and thirty per cent were CEA positive and CA125 negative. This result suggests that these carcinomas may represent a serous or mucinous dedifferentiation.

Calretinin, BerEP4, CK7 and CK20 immunostainings completed the present immunohistochemical study of undifferentiated ovarian carcinomas allowing their separation from peritoneal mesotheliomas with ovarian extension and from ovarian metastasis originating in the gastrointestinal tract.

The evaluation of the PCNA labelling index confirmed the high degree of cell proliferation and the aggressiveness of these tumours.

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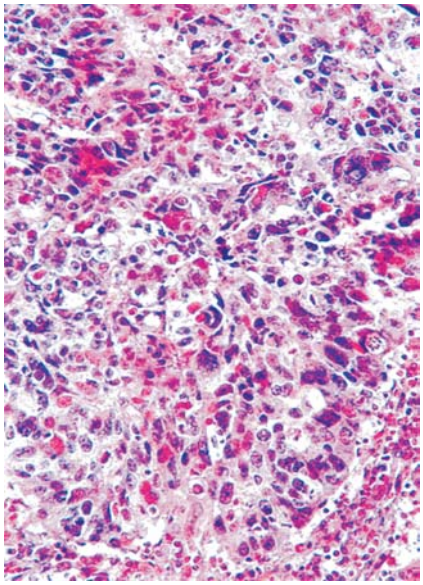

Figure 1 – Undifferentiated ovarian carcinoma, classical type (H&E staining, ×100)

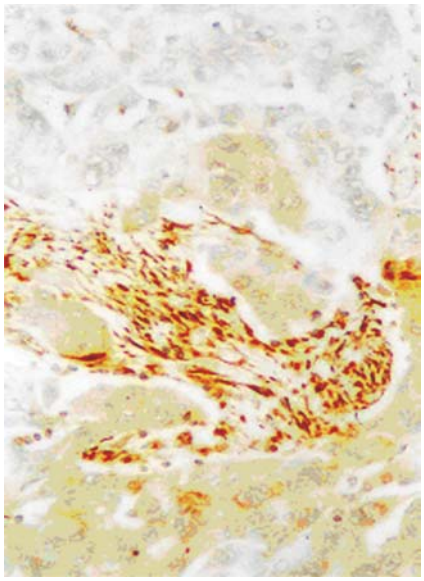

Figure 2 – Undifferentiated ovarian carcinoma, weak focal positivity (+) of the tumoural cells (vimentin, ×200)

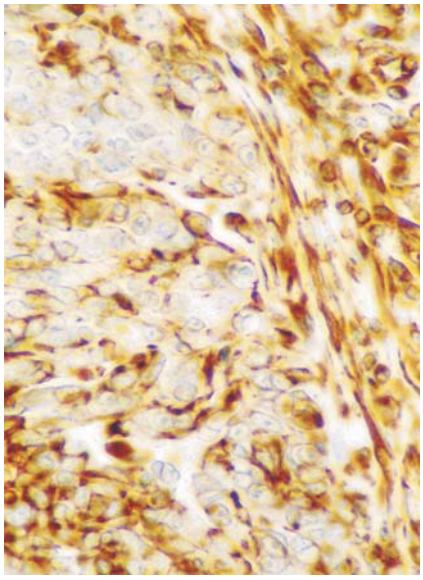

Figure 3 – Undifferentiated ovarian carcinoma, strong positivity (+++) of the tumoural cells (vimentin, ×400)

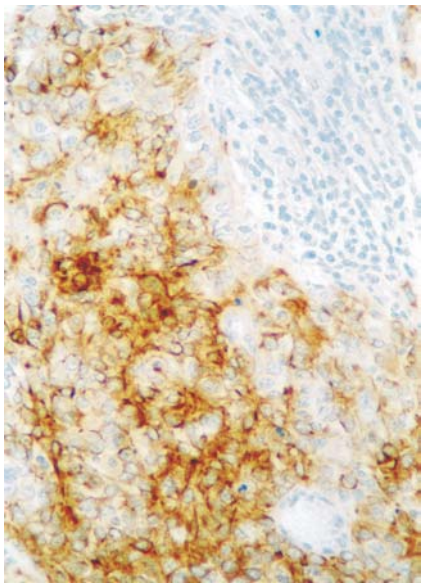

Figure 4 – Undifferentiated ovarian carcinoma, strong positivity (+++) of the tumoural cells, negative stromal cells (AE1/AE3, ×200)

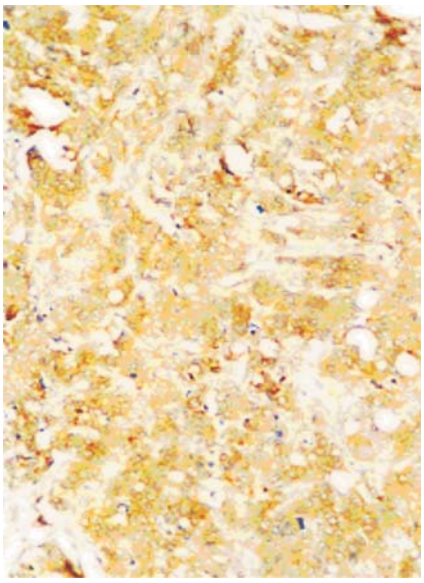

Figure 5 – Undifferentiated ovarian carcinoma, moderate diffuse positivity (++) (AE1/AE3, ×100)

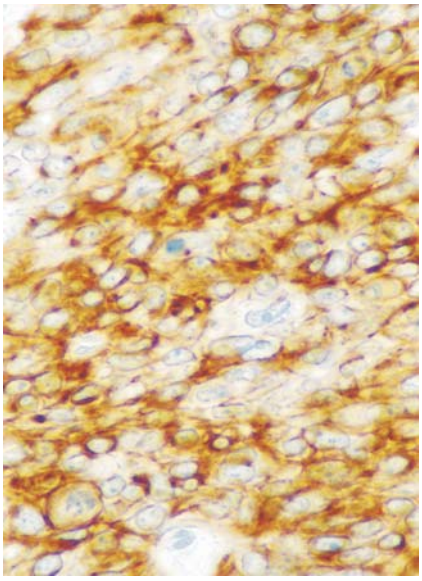

Figure 6 – Undifferentiated ovarian carcinoma, strong diffuse positivity (+++) (AE1/AE3, ×400)

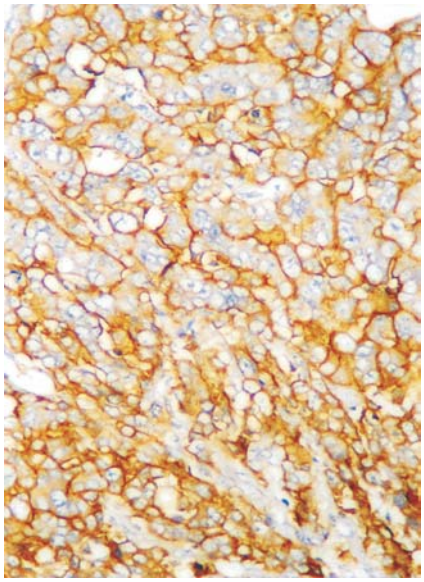

Figure 7 – Undifferentiated ovarian carcinoma, strong diffuse positivity (+++) (EMA, ×200)

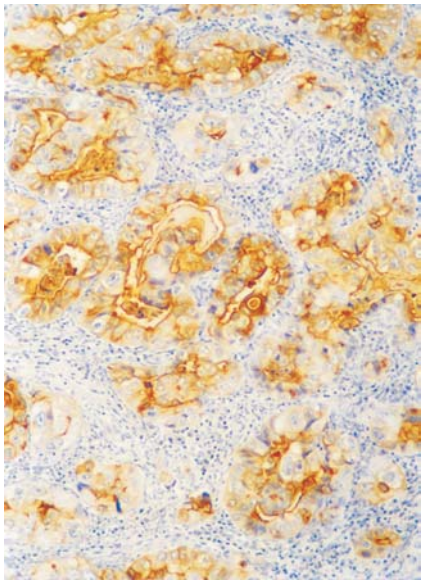

Figure 8 – Undifferentiated ovarian carcinoma, acinar differentiations (EMA, ×200)

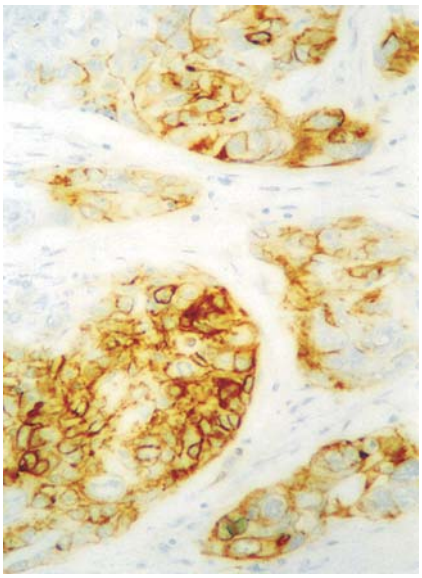

Figure 9 – Undifferentiated ovarian carcinoma heterogeneous focal positivity, membranar pattern (CA125, ×200)

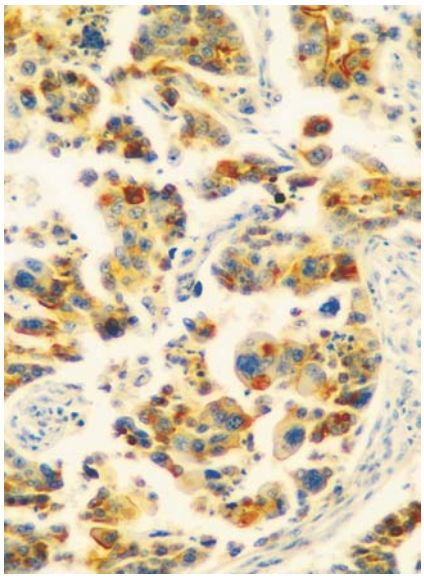

Figure 10 – Undifferentiated ovarian carcinoma, strong diffuse positivity (+++), with membranar and cytoplasmatic pattern (CA125, ×200)

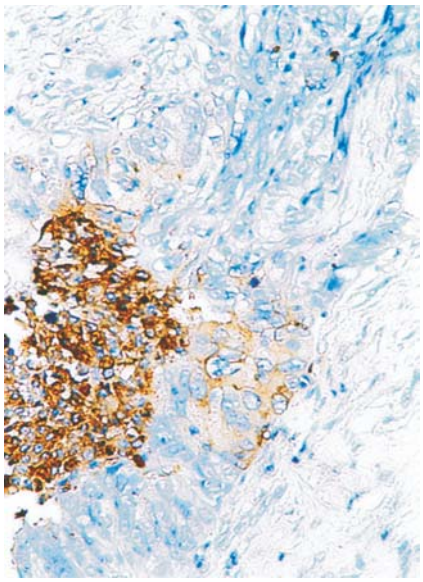

Figure 11 – Undifferentiated ovarian carcinoma, weak focal positivity (+) (CEA, ×200)

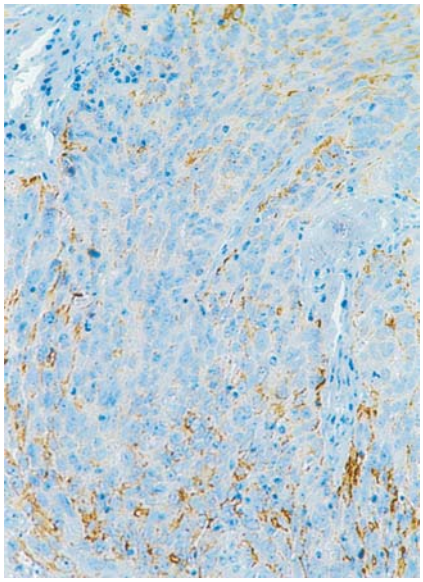

Figure 12 – Undifferentiated ovarian carcinoma moderate difuse positivity (++), with membranar and granular cytoplasmatic pattern (BerEP4, ×100)

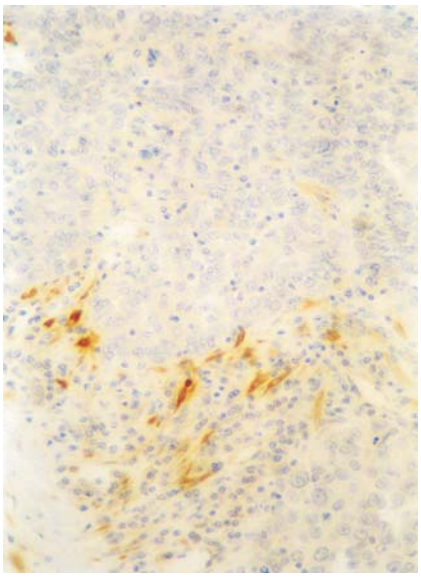

Figure 13 – Undifferentiated ovarian carcinoma, negative staining (-) of the tumoural cells and positive stromal cells (calretinin, ×200)

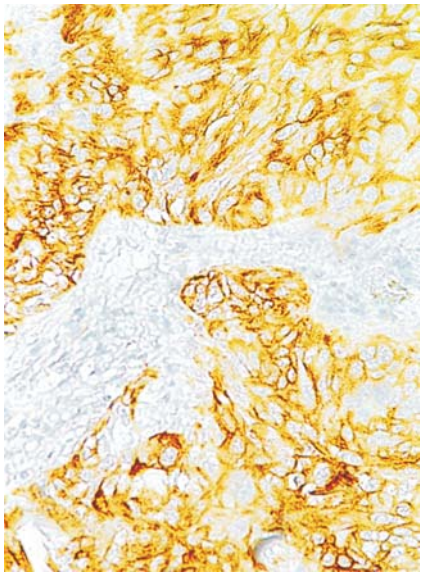

Figure 14 – Undifferentiated ovarian carcinoma, strong diffuse positivity (+++), with cytoplasmatic pattern (CK7, ×200)

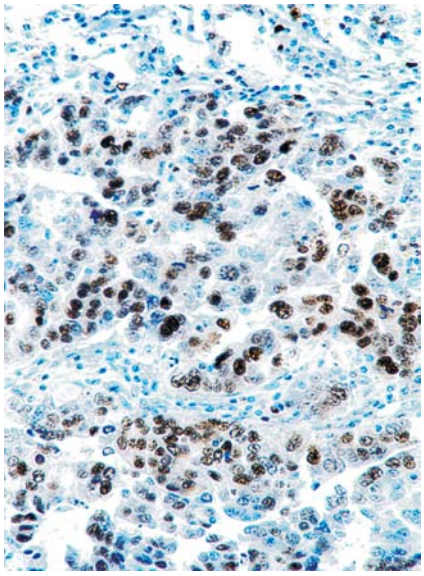

Figure 15 – Undifferentiated ovarian carcinoma, PCNA labelling index higher than 50%, ×200

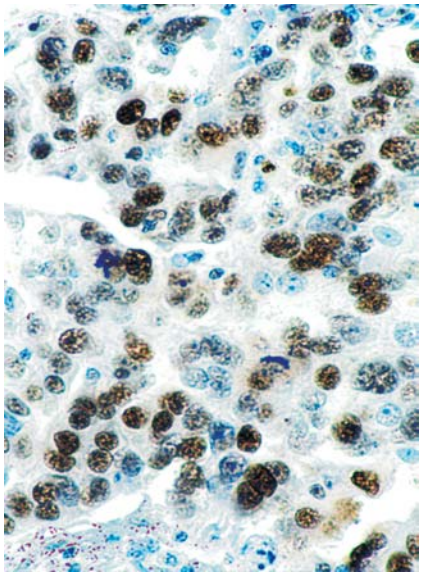

Figure 16 – Undifferentiated ovarian carcinoma, PCNA labelling index higher than 50%, ×400