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SUMMARY

***THE ROLE OF GROWTH AND
PROLIFERATION FACTORS IN
PROGRESS OF SEROUS AND
MUCINOUS OVARIAN CARCINOMAS***

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KEY WORDS: low grade serous ovarian carcinoma, high grade serous ovarian carcinoma, mucinous ovarian carcinoma, serous borderline tumors, borderline mucinous tumors, growth and proliferation markers.

INTRODUCTION

Ovarian epithelial tumors are the most common ovarian cancer, accounting for approximately 60% of all ovarian tumors, of which carcinomas account for about 90% of ovarian malignancies [73,183]. A relatively recent study that only followed the incidence of carcinoma-derived ovarian surface epithelium indicates high-grade serous carcinoma to be by far the most common type of carcinoma [216].

On the other hand, based on the prevalence and mortality, serous carcinoma is the most important type of ovarian cancer, representing the most primitive ovarian carcinomas with bad prognosis [405].

Subsequent division of various subtypes of epithelial tumors in benign, borderline and malignant forms is based on the fact that borderline tumors have intermediate architecture and cytology between the benign and malignant ones belonging to the same type of epithelial cells but with better prognosis at the same stage compared to their malignant counterparts.

The study performed on 54 cases of malignant ovarian tumors, serous and mucinous followed a full and detailed evaluation of the ovarian carcinogenesis process using classic methods of investigation as well as modern techniques such as immunohistochemistry and morphometry.

A. THE STATE OF KNOWLEDGE

The chapters I to III cover the most important notions about: ovarian carcinoma epidemiology, risk factors (reproductive history, age, obesity, metabolic syndrome, smoking, ethnicity, pelvic inflammatory disease, endometriosis, hormone replacement therapy, family history and genetic factors, nutrition, environmental factors), pathogenesis of low and high grade serous ovarian carcinomas, pathogenesis of mucinous ovarian carcinoma.

B. PERSONAL RESEARCH

CHAPTER IV. PURPOSE AND SPECIFIC OBJECTIVES.

MATERIAL AND METHODS

This paper aims to evaluate ovarian carcinogenesis in order to identify some of the factors involved in the unfavorable progression of some of the borderline tumors or ovarian carcinoma, serous and mucinous in order to identify possible prognostic and / or therapeutic targets.

This study was performed retrospectively and prospectively for a period of 3 years between 2013-2015 and included 54 cases of ovarian malignancies, both borderline tumors and serous and mucinous carcinomas and followed the analysis of some histopathological and immunohistochemical parameters for these tumor types.

For this purpose, we have watched the following objectives:

- Expanding knowledge of histopathological and immunohistochemical features of borderline tumors and of serous and mucinous carcinomas.
- identification and definition of histopathological parameters.
- identifying the most reliable growth and proliferation markers in ovarian, serous and mucinous malignant tumors.

The **material** studied was represented by the anatomo-pathological records from which we extracted information on patients age, the macro and microscopic parameters of the selected ovarian tumors.

In addition, for the retrospective cases I used the paraffin blocks and the histological samples found in the archives of the Pathology Laboratory of the Emergency County Hospital Craiova. For present cases, the surgical excision pieces fixed in 10% buffered formalin, after registration the macroscopic parameters, we put them to the usual histological processing by the paraffin inclusion technique.

Subsequently, the cases investigated histopathologically have been subjected to the immunohistochemical examination within the Morphopathology Laboratory of the UMF Craiova.

The **study methods** used were: histopathological analysis, immunohistochemical analysis and statistical analysis.

The **histopathological analysis** was performed on a number of 54 malignant serous and mucinous tumors and aimed to identify their main histopathological parameters in relation to prognosis. The histopathological parameters included the following criteria for assessment: for the borderline tumors: histological variety, the presence / absence of stromal microinvasion, the presence / absence of lympho-vascular invasion, the presence / absence of epithelial implants; for the carcinomas: histological variety, lesion degree, presence / absence of peritoneal metastases, pTNM staging.

For the **immunohistochemical processing** there were selected 54 cases, where we watched the expression of growth factors such as EGFR, HER2 and HER3 as well as proliferation factors such as Ki67, p53 and p16. The immunohistochemical study was an enzyme detection type using LSAB-HRP (Labelled Streptavidin-Biotin 2 System Horseradish Peroxidase, Dako, code K0675) as a working method. The result of the immunohistochemical reactions consisted in microscopic visualization by their brown staining of the investigated antigens using chromogen DAB (code 3467, Dako).

The sections obtained from the immunohistochemical processing were examined at the Nikon Eclipse 90i microscope (Nikon, Apidrag, Bucharest) equipped with a 5 megapixel color camera, apocromatic planes (x10, x20, x40) and narrow band fluorescence filters. The captured images were purchased at different zoom ranges using Nikon NIS-Elements dedicated software. For each antibody, we performed in tandem both positive external control and negative external control for both using the same immunohistochemical technique. Positive external control I performed on normal tissues containing the investigated antigen, which were processed under the same conditions as lesion specimens.

The antibodies used in the present study are presented in the table together with the clone and source of their origin, the dilution used, as well as the unmasking and the tissue used for external control.

Panel with the antibodies used in the immunohistochemical study

Antibody	Clone/Source	Dilution	Unmasking	External Control
EGFR	E30	1:1000	without unmasking	oral mucosa
HER2	polyclonal	1:250	Citrat, pH 6	breast carcinoma
HER3	DAK-H3-IC	1:100	Tris-EDTA, pH 9	ileum
Ki67	MIB 1/Dako	1:100	Tris-EDTA, pH 9	amygdala
p53	DO-7	1:50	Tris-EDTA, pH 9	amygdala
p16	DC-468/Dako	1:100	Citrate, pH 6	cervical HSIL

For the **statistical analysis** there were used mean values, standard deviations and comparative tests (t-Student, Anova unifactorial, Chi square, Pearson) which were performed using the SPSS10 software. *The t-Student test* was used to compare the averages of the analyzed categories. *The ANOVA test* was used to analyze variance of a numeric variable under the influence of a grouping variable using the Analyze, Compare means, One-Way Anova commands. *The Chi square test* was used to interpret incidence tables. The data were appreciated from the point of view of the dependence between the two classification factors. The test indicated whether there was a link (mutual influence) between two factors, the commands used in the software being Analyze, Descriptive Statistics, CrossTabs. *The Pearson test* was used to assess the distribution of the actual immunohistochemical marker values.

**CHAPTER V AND VI.
RESULTS AND DISCUSSIONS**

The discussions show the results of the study that are reported in the literature.

The **histopathological analysis** of the 54 serous and mucinous malignant tumors revealed: 41 cases of serous malignant tumors and 13 cases of malignant tumors. Both lesion types included both borderline tumors and carcinomas. Thus, the 41 serous malignant tumors included 14 cases of borderline serous tumors and 27 serous carcinomas, and the 13 cases of mucinous malignant tumors analyzed included 6 cases of

borderline mucinous tumors and 7 mucinous carcinomas. Of the 41 malignant serous tumors, 27 cases were serous carcinomas and 14 borderline tumors. With respect to the age of patients diagnosed with malignant serous tumors, we found that while serous borderline tumors were predominantly diagnosed in patients aged 31-78 years, serous carcinomas were predominantly diagnosed in patients between the age of 41 -81 years.

And the literature have reported similar results of our study, as the borderline serous ovarian tumors are diagnosed in patients older than than those with ovarian cystadenoma, but are usually much younger than the patients with invasive ovarian carcinoma [14]. Most patients are approximately 40 years old at the time of diagnosis [122]. By comparison, the mean age of patients diagnosed with invasive ovarian carcinoma is 60 years [2].

In our study of the 14 cases of borderline serous tumors, 12 cases belonged to the typical form and only 2 cases of micropapillary form. These results coincide with the data in the literature, where the serous subtype was divided by about 90% - in the typical form, the rest being represented by the micropapillary form (10%) [454].

All tumors in our study were diagnosed in stage I of the disease. The authors of a study reported that most serous borderline tumors are diagnosed in early stages, with 70-80% being diagnosed in stage I compared to 25% of ovarian invasive carcinomas [416].

Regarding the borderline mucinous tumors in our study, after analyzing the 6 cases, we found that they were diagnosed in reproductive patients, with the incidence increasing in the fourth decade with an average age of 47.5 years. All mucosal tumors analyzed were in the first stage of the disease.

Our results are consisting with the literature. Mucinous borderline tumors occur in a very broad age range (9-70 years), with an average age of 35 years [14].

In our study carcinomas were diagnosed in 34 cases, of which serous carcinomas represented 27 cases (79.41%), the mucinous ones being identified in only 7 cases (20.58%). Data from literature reported serous ovarian carcinoma as the most common malignant ovarian tumor (60% of cases) [356].

Of the 27 malignant serous ovarian tumors, 3 tumors were low-grade serous carcinomas, and 24 tumors were high grade serous carcinoma. Data from the literature highlights the appearance that from the two major subtypes of serous ovarian carcinoma,

high-grade serous ovarian carcinoma represent the most aggressive form diagnosed in patients of all ages (especially in the advanced stage), being also the most common subtype of carcinoma ovarian seros [80]. In contrast, low-grade serous carcinoma is a rare subtype, representing only a small proportion (5-10%) of all serous ovarian cancers [62,480].

We found that patients with serous carcinoma were diagnosed at age of 41-81 years, with an average age of 62 years for both serous carcinoma groups. For patients diagnosed with low-grade serous carcinoma the mean age was 56.5 years, and for patients diagnosed with high-grade serous carcinoma, the mean age was 67.5 years.

Similar results have also been found in the literature. Plaxe SC. et al. performed a study comparing the patients characteristics diagnosed with low grade serum carcinoma or high grade serous carcinoma. In the first group (patients diagnosed with serum low grade ovarian carcinoma), it was observed that the mean age at diagnosis was 55 years, and for the second group (patients diagnosed with high-grade serous carcinoma) the mean age was 63 years old [356].

No patient in our study was diagnosed in stage I or IV, most patients (24 cases), were in stage III disease.

Data from the literature confirms that the diagnosis of patients in stage I disease is unusual and rare [73], and the presentation at this stage is in the form of an asymptomatic ovarian / pelvic mass [140]. Up to 95% of patients in stage III-IV disease were of serous subtype [73]. In one study, most cases of low-grade serous carcinoma were diagnosed in stage II-IV disease, only one patient was in stage I. For patients with high-grade serous carcinoma, most of them were in advanced stage disease [8].

In our study, from the 24 cases of high-grade serous ovarian carcinoma, half of them were associated with peritoneal metastases.

Data from the literature reported that peritoneal metastases are the most common cause of death in ovarian cancer patients. There are a large number of patients who are diagnosed with peritoneal metastases due to delayed early diagnosis of ovarian cancer [90]. Another study found that the most patients diagnosed with high-grade serous carcinoma and found in advanced stage had metastatic disease [370]. Approximately 85% of patients with serous ovarian carcinoma present with peritoneal metastases [133,511].

In our study, 7 patients were diagnosed with primitive ovarian mucinous carcinoma. The mean age at diagnosis was 62 years (the age range between 50-78 years). All patients were diagnosed in stage I disease.

Ovarian mucinous carcinoma accounts for less than 5% of all malignant ovarian tumors [394], and is diagnosed in very wide-range patients, including little girls and adolescents [427].

The **immunohistochemical analysis** followed expression of growth factors such as EGFR, HER2 and HER3, as well as proliferation factors such as Ki67, p53 and p16.

EGFR expression analysis revealed the positivity of the reaction in half of the cases, distributed in all tumor groups analyzed. The EGFR positivity was higher in the carcinoma group than in the borderline tumors (51.2% vs. 7.3%). Negative cases corresponded to both tumor types. In mucinous tumors, the number of positive EGFR was lower, also lower for borderline tumors compared to carcinomas (7.7% vs.15.4%). The data from the literature confirms the results of our study, and EGFR is overexpressed in serous carcinomas and varies in different studies between 4-100% of cases [372,168].

There are relatively few studies that have analyzed the EGFR expression in borderline serous tumors. One study reported protein expression in a significantly higher proportion in carcinoma compared with borderline tumors, respectively 69% versus 18% ($p < 0.004$) [479].

HER2 expression analysis revealed the positive reaction in 37% of cases. For serous malignant tumors, the HER2 positivity was observed in a small number of borderline tumors compared to carcinomas (21.4% vs. 51.8%). In the case of malignant mucinous tumors, we found the negativity expression in borderline tumors and a small number of HER2 positive cases for mucinous carcinomas (42.8%).

According to literature data, a large study involving 783 patients with ovarian cancer or borderline tumors reported overexpression of Her2 in 35% of the tumors [330]. The percentage of patients with Her2 / neu positive ovarian cancer varies considerably in the various individual studies, ranging from 8% to 66%[431,115,41,50,245,293,371,411].

HER3 expression analysis revealed the positivity reaction in 59.2% of the cases, distributed in all tumor groups. For serous malignant tumors, the HER3 positivity was observed in a small number of borderline tumors compared to carcinomas (57.1% vs.

62.9%). In the case of mucinous malignant tumors, we found HER3 positivity in 3 cases of borderline tumors (50%) and in 4 cases for mucinous carcinomas (57.1%).

Overexpression of HER3 was observed in 53.4% of ovarian cancer patients [450].

Ki67 expression analysis revealed the positivity reaction in 92.6% of the cases, distributed in all tumor groups. Ki67 negative tumors corresponded to the borderline group, serous and mucinous. For serous tumors, we noticed the Ki67 positivity in a smaller number of serous borderline tumors compared to carcinomas that were totally positive. Thus, we found positivity in 12 borderline tumors (85.7%) and in 27 serous carcinomas (100%). In the case of mucinous tumors, we found Ki67 positivity in 66.6% of cases and in all cases of mucinous carcinomas. Similar to our results, several studies reported higher expression of Ki-67/MIB-1 in ovarian carcinomas compared to borderline and benign tumors [177,139].

P53 expression analysis revealed the positivity reaction in 37 cases (68.5%), distributed in all tumor groups. For serous tumors, p53 positivity was observed in a small number of serous borderline tumors (28.6%) compared to carcinomas (96.3%). In the case of mucinous tumors, the p53 positive cases number was lower, and also lower for borderline tumors compared to carcinomas, respectively 3 cases (50%) for borderline tumors compared to 4 cases for carcinomas (57.1%). In several studies, overexpression of p53 was observed in at least 50% of advanced stage ovarian cancers [157,106]. P53 mutation or overexpression was also studied in ovarian borderline tumors, while p53 mutations were not observed in two studies [496,221], overexpression of p53 was reported with values between 0-50% [164,210,282,217,106]. In one study, immunoreaction for p53 was present in 37.7% of all tumors, predominantly in mucinous carcinoma if the values was high (mean score 52.3%), in contrast to borderline tumors (mean 15,5%) [195].

P16 expression analysis revealed the positivity reaction in 33 cases (61.1%), distributed in all tumor groups. For serous tumors, the p16 positivity was observed in a small number of serous borderline tumors (21.4%) compared to carcinomas (100%). In the case of mucinous tumors, the p16 positive cases number was lower, also lower for borderline tumors (16.6%) compared to carcinomas (28.5%). According to the literature, similar to serous carcinomas, the borderline serous tumors are frequently positive for p16

[7,327], immunoblotting for p16 being intense and non-uniform with nuclear and cytoplasmic pattern [7]. In mucinous carcinomas, immunoblotting for p16 is focal or absent [430].

CONCLUSIONS

The study comprised 54 ovarian malignant tumors, of which 41 cases were serous and 13 cases were mucinous, and allowed to observe the following:

The histopathological study:

- Serious malignant tumors included 14 cases of borderline serous tumors and 27 cases of serous carcinomas, and the 13 cases of mucinous malignant tumors analyzed included 6 cases of borderline mucinous tumors and 7 cases of mucinous carcinomas.

- The 14 cases of borderline serous tumors investigated corresponded mostly to typical forms of neoplasia, respectively 12 cases and only in 2 cases of serous micropapillary carcinomas, for which we noticed:

- Stromal microinvasion in 4 cases, which accounted for 28.57% in the analyzed case.

- The lymph-vascular invasion was identified in only 2 cases (14.28%).

- The epithelial implants in the peritoneum were present in 3 cases (21.42%), in 2 cases non-invasive and invasive in one case.

- Serous carcinomas were present in 27 cases, constituting 65.85% of malignant serous tumors and 50% of the case study analyzed.

- Histopathologically corresponded in 2 cases of low grade carcinomas (7.40%) and in 25 cases of high grade serous carcinomas (92.59%).

- Peritoneal metastases were present in 12 cases of the 27 serous carcinomas, all associated with high-grade tumors;

- The analysis of the 13 cases of malignant mucinous showed as in the case of serous malignant tumors the predominance of mucinous carcinomas with 7 cases (53.84%) compared to borderline mucinous tumors which were present in 6 cases (46.15%) .

- The borderline tumors have associated:

- Stromal microinvasion present in one case (16.66%).
- Pseudomixoma ovary was identified in 5 tumors (83.33%).
- Mucinous carcinomas presented:
 - In 5 cases we observed aspects associated with mucinous tumors filiation, ranging from benign neoplasias (cystadenoma or cystadenofibroma) to borderline and up to malignant tumors;
 - The tumor invasion covered 3 aspects: expansive (28.57%), infiltrative (42.85%) or mixed (28.57%).
 - The pTNM framing for all 14 cases of borderline serous tumors corresponded to stage I disease, as well as for all mucinous malignant tumors. Serous carcinomas corresponded in 3 cases of stage II and 24 cases of stage III disease.

The immunohistochemical study:

- EGFR expression analysis revealed the positivity reaction in half of the investigated tumors (50%), more frequently in carcinomas compared to borderline tumors, especially in the serous type.
 - Frequent EGFR expression in ovarian tumors requires continuous follow-up of EGFR inhibitors for ovarian cancer therapy, but perhaps following more rigorous selection and stratification criteria for patients.
 - The HER2 expression analysis revealed the positive reaction in 17 cases (31.5%), all of which were analyzed in all tumor groups except borderline mucinous tumors; the highest IP values of HER2 were present in high serous carcinomas.
 - The HER3 expression analysis revealed the positive response in 25 cases (46.2%), all of which were distributed in all tumor groups; the highest IP values of HER3 were present in poorly differentiated serous carcinomas;
 - The ErbB receptors analysis in serous and mucinous ovarian malignancies indicated their coexpression in both lesion subtypes, borderline tumors and carcinomas; the lowest incidence, regardless of the lesion subtype, was Her2.
 - The HER3 overexpression in a larger proportion of cases compared to those overexpressing HER2 or EGFR, suggests that HER3 may be a possible therapeutic target in ovarian cancer.

- The Her2 positivity for a small number of ovarian mucinous carcinomas questions the potential of anti-Her2 therapy for this group of neoplasms.
- Analysis of ki67 expression revealed the positive reaction in 50 cases (92.6%), all of which were distributed in all tumor groups; negative cases for ki67 corresponded to borderline, serous and mucinous tumors; the highest IP values of KI67 were present in poorly differentiated serous carcinomas.
- p53 and Ki-67 can be used as markers to assess the aggressive behavior of mucinous carcinomas and to distinguish them from borderline mucinous tumors.
- Serous carcinomas typically exhibited a high p16 expression; there was a statistically higher p16 expression in serous carcinomas compared to other morphological types.
- The p16 expression in both benign and borderline and malignant serous tumors suggests that it may be an early event of serous tumorigenesis.

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