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Titlul proiectului
"Dezvoltarea școlilor doctorale prin acordarea de burse tinerilor doctoranzi cu frecvență"

Contract nr: POSDRU/88/1.5/S/52826

Beneficiar
Universitatea de Medicină și Farmacie din Craiova
DOCTORAL THESIS
SUMMARY

The role of epidermal growth factor EGF and its receptor in the prognosis of ovarian serous tumors

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CRAIOVA 2012
# CONTENTS

## INTRODUCTION

<table>
<thead>
<tr>
<th>CHAPTER I. EPIDEMIOLOGY AND RISK FACTORS IN OVARIAN CANCER</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER II. OVARIAN CARCINOGENESIS AND PROGNOSTIC FACTORS IN OVARIAN CANCER</td>
<td>12</td>
</tr>
<tr>
<td>CHAPTER III. DIAGNOSIS METHODS FOR OVARIAN CANCER</td>
<td>27</td>
</tr>
<tr>
<td>CHAPTER IV. THE HISTOLOGICAL CLASSIFICATION OF OVARIAN TUMORS</td>
<td>33</td>
</tr>
</tbody>
</table>

## THE MOTIVATION AND PURPOSE OF STUDY

<table>
<thead>
<tr>
<th>CHAPTER V. MATERIAL AND METHODS</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER VI. RESULTS</td>
<td>46</td>
</tr>
<tr>
<td>VI.A. STUDY OF THE EPIDEMIOLOGICAL AND CLINICAL DATA OF OVARIAN SEROUS TUMORS</td>
<td>46</td>
</tr>
<tr>
<td>VI.B. HISTOPATHOLOGICAL STUDY OF THE OVARIAN SEROUS TUMORS</td>
<td>51</td>
</tr>
<tr>
<td>VI.C. IMMUNOHISTOCHEMICAL STUDY OF THE OVARIAN SEROUS TUMORS</td>
<td>88</td>
</tr>
</tbody>
</table>

## CHAPTER VII. DISCUSSIONS

| VII.A. ANALYSIS OF THE EPIDEMIOLOGICAL AND CLINICAL DATA OF OVARIAN SEROUS TUMORS | 129 |
| VII.B. HISTOPATHOLOGICAL ANALYSIS OF THE OVARIAN SEROUS TUMORS | 133 |
| VII.C. IMMUNOHISTOCHEMICAL ANALYSIS OF THE OVARIAN SEROUS TUMORS | 141 |

## CHAPTER VIII. CONCLUSIONS

<table>
<thead>
<tr>
<th>REFERENCES</th>
<th>153</th>
</tr>
</thead>
</table>
INTRODUCTION

Epithelial ovarian tumors are the most common cancers in this location, the serous type representing up to 50% of this pathology. However, ovarian serous carcinomas represents 3% of cancers in females, 32% of malignant lesions in the female genital tract and 60-80% of ovarian carcinomas. Epidemiological data, the frequency of lesions and their incidence in all age groups, designate serous ovarian tumors as a major worldwide health problem.

In this direction, numerous studies have attempted to establish clinical-pathological parameters to ensure early diagnosis and differential therapy of these lesions. However, especially for carcinomas, the diagnosis is established in approximately 75% of cases in advanced stage, parameter establishing usually reserved prognosis of patients. In addition, recent studies suggest the possibility that some benign tumors (cystadenomas, cystadenofibromas) should be classified as cystic dilatation or fibromas, which would have major implications for epidemiological data. In this context, identification of biomarkers of which immunoexpression to characterize biomolecular mechanisms involved in the occurrence and progression of serous ovarian tumors, is a target of great interest in current research programs. Establishing a panel of proteins involved in multiple mechanisms of tumor development may provide a clinico-pathological model of investigation for serous ovarian tumors, as well as considering the behavior of serous carcinomas with this location.

The study included 70 cases of serous ovarian tumors that were analyzed in terms of clinical and epidemiological data. Subsequently, histopathological analysis followed tumor type (benign, borderline or malignant) and for malignant tumors, degree of tumor differentiation, pattern of invasion, pTNM staging and the presence of particular aspects such as histological differentiation, inflammation and necrosis.

Immunohistochemical analysis included a number of 45 serous ovarian tumors and assessed the expression of epidermal growth factor and its receptors and their relationship with the cell cycle (p16, p53), tumor proliferation (Ki67) and angiogenesis (CD34). Also the immunoexpression of analyzed markers was evaluated and compared with main clinico-pathological parameters which were included in this study, in this sense using statistical analysis (chi square, t-student, ANOVA, Pearson index).
**Keywords:** ovarian serous tumor, benign, borderline, carcinoma, differentiation degree, immunohistochemistry, EGF, EGFR, Her2/neu, prognostic.

**Chapter I - „Epidemiology and risk factors in ovarian cancer“** - describe the incidence of ovarian cancer in the world and in Romania in relation to age, gender and related to general pathology of malignancy. They also analyze the main risk factors involved in serous ovarian tumors.

**Chapter II - „Ovarian carcinogenesis and prognostic factors in ovarian cancer“** - investigate current hypotheses on the initiation and development of ovarian serous carcinomas and the genetic support for these biomolecular events. There are also indicated the main prognostic factors for ovarian serous carcinomas that are systematized in tumor and patient related factors, respectively treatment related factors.

**Chapter III - „Diagnosis methods in ovarian cancer“** - analyze symptoms in ovarian cancer patients and paraclinical imaging or laboratory investigations, useful for the diagnosis.

**Chapter IV - „The histological classifications of ovarian tumors“** - presents the last classification from 2003 by the Working Group for ovarian tumors within the World Health Organization.

**OBJECTIVES OF THE STUDY**

The study analyzes epidermal growth factor and its receptor immunoexpression in benign, borderline and malignant serous ovarian tumors in order to identify possible relations with the biomolecular mechanisms, such as tumor proliferation, cell cycle disturbance, tumor angiogenesis. In this respect, the identification of therapeutic targets with certain value can lead to improved prognosis in patients with serous ovarian tumors.

Specific objectives of the study include:

1. Expanding knowledge of clinico-pathological immunohistochemical and morphometric factors involved in ovarian tumorigenesis, in order to deepen its mechanisms;

2. Identification of biomolecular mechanisms by which epidermal growth factor and its receptors provide tumor proliferation and interact with cell cycle proteins and angiogenesis;

3. Establishing the prognostic role of epidermal growth factor and its receptor in relation to clinico-pathological and immunohistochemical analyzed parameters.
Chapter V- „Material and Methods”- provides information about the material studied and methods used in research.

INVESTIGATED MATERIAL

This study was conducted over a period of three years (2009-2011), comprising a total of 170 cases with clinical diagnosis of ovarian tumor, the biological material being represented by surgical excision pieces obtained from patients hospitalized in the Surgery Clinic of Clinical Hospital CFR (railway) Craiova and of Obstetrics Gynecology Clinics of the Emergency County Hospital Craiova.

METHODS

In a first phase we aimed to evaluate the clinical course of patients selected by the pursuit of parameters of interest: epidemiological data (patient age, place of origin, risk factors, family history, hormone replacement therapy, nulliparous, obesity), symptoms (pelvic pain, irregular menstruation, ascites, etc.), the time elapsed until the specialty exam, clinical and laboratory data.

After excision of tumors, the biological material was investigated to identify the main macroscopic morphological features: appearance, number, size, color, consistency, indicating involvement / non-involvement of neighboring structures and loco-regional lymph nodes.

Pieces were processed by the classical technique with paraffin embedding and Hematoxylin–Eosin stain, using also special stains such as van Gieson, which allow a good classification of lesions according to histological appearance.

Histopathological study included a total of 70 cases of serous ovarian tumors for which we followed tumor type (benign, borderline or malignant) and for the malignant lesions the degree of tumor differentiation, pattern of invasion, pTNM staging and the presence of particular aspects such as histological differentiations, inflammation and necrosis.

Immunohistochemical analysis included a number of 45 serous ovarian tumors and assessed the expression of EGF and its receptors 1 and 2 and their relation to the cell cycle (p16, p53), tumor proliferation (Ki67) and angiogenesis (CD34). Also immunoexpression of analyzed biomarkers was evaluated and compared with the main histopathological prognostic parameters, represented by the degree of tumor differentiation and tumor stage.

Working systems used were the CSA II biotin-free catalyzed Amplification System (code K1497) for EGFR and LSAB™ + Kits, Universal (code K0679) for the other antibodies, for visualisation using DAB (diaminobenzidine).
For quantification were calculated index of positivity (IP) and proliferation index values, by dividing the number of positive cells per 100 cells counted in each case being evaluated 1,000 cells; this values were useful for assessing the types of lesions. Her2/neu immunostain was quantified according to the criteria of the American Society of Clinical Oncology / College of American Pathologists (ASCO / CAP) for breast carcinoma. The immunostain for blood vessels was done using an panendotelial marker (CD34), quantified using the microvessel density (MVD). In this sense "hot spot" morphometric method was used, which consisted in manual quantification of vessels.

**Statistical analysis** used average values, standard deviations and confidence intervals, and comparison tests (Student t, unifactorial ANOVA, chi square, Pearson) made with SPSS10 software.

The panel of antibodies used is presented below:

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>CLONE</th>
<th>DILUTION</th>
<th>RETRIEVAL</th>
<th>POSITIVE CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>polyclonal/SDX</td>
<td>1:300</td>
<td>Citrate buffer, pH 6, pH 6</td>
<td>Placenta</td>
</tr>
<tr>
<td>EGFR</td>
<td>polyclonal /Sigma</td>
<td>1:1000</td>
<td>Without exposure</td>
<td>Placenta</td>
</tr>
<tr>
<td>HER2/NEU</td>
<td>polyclonal /Dako</td>
<td>1:300</td>
<td>Citrate buffer, pH 6</td>
<td>Mammary carcinoma</td>
</tr>
<tr>
<td>KI67</td>
<td>MIB 1/Dako</td>
<td>1:100</td>
<td>Citrate buffer, pH 6</td>
<td>Mammary carcinoma</td>
</tr>
<tr>
<td>P53</td>
<td>DO-7/Dako</td>
<td>1:50</td>
<td>Citrate buffer, pH 6</td>
<td>Tonsil</td>
</tr>
<tr>
<td>P16</td>
<td>E6H4</td>
<td>ready to use</td>
<td>Epitope Retrieval Solution/ Kit</td>
<td>HSIL cervix</td>
</tr>
<tr>
<td>CD34</td>
<td>QBEnd10,/Dako</td>
<td>1:30</td>
<td>Citrate buffer, pH 6</td>
<td>Kidney</td>
</tr>
</tbody>
</table>
Chapter VI- „Results” and Chapter VII- „Discussions” indicate the results obtained in the study, which are reported to recent data from literature.

In the clinico-epidemiological study the benign serous tumors had a mean age of diagnosis of 42.3 years, the lesions being more commonly unilateral (85%) with an average size of 15.06 cm. Serous borderline tumors had a mean age of diagnosis of 47.5 years and were more frequently unilateral (66.6%) with an average size of 12.16 cm. Malignant serous tumors had a mean age of diagnosis of 55.7 years, and the lesions were more commonly unilateral (55%) with an average size of 13.22 cm. The literature indicates a younger age of diagnosis for benign tumors, which are encountered including in pediatric pathology (Morowitz M, 2003). In a retrospective study that analyzed borderline serous tumors over a period of 19 years, the average age of diagnosis was 46.9 ± 16.7 years (Fauvet R, 2012). This age is slightly higher than the serous cystadenomas (Lazar E, 2009). Plaxe SC et al. in 2008 indicates that the average age of diagnosis for low grade ovarian carcinomas was 55.5 years compared with those for the high degree where average age was 62.6 years (Plaxe SC, 2008).

Most patients with serous ovarian tumors were from urban areas (55.7%) and were associated with risk factors like family history, hormone replacement therapy, nulliparity and obesity, factors that are discussed in the literature (Riman T, 2001 , Jordan SJ, 2007). Ovarian stimulation therapy, diet, smoking, oral contraceptives, occupational exposure represents other risk factors that are described in the literature the data being controversial in the sense of different epidemiological results for different analyzed cohorts (Salehi F, 2008).

Histopathological analysis of serous ovarian tumors revealed benign lesions in 62.9% of cases, the borderline in 8.5% and 28.6% of malignant lesions, similar to those in the literature (Lazar E, 2009; Tavassoli FA, 2003).

Cystadenoma and cystadenofibroma were the most common benign lesions. Most of borderline tumors were noninvasive. The most common histological subtype of ovarian serous carcinoma was the papillary cystadenocarcinoma (85%), high-grade type being more common (80%). The literature indicates that serous ovarian borderline tumors can be found with benign tumors as papillary cystadenoma (Russell P, 1997), and ovarian serous carcinomas 75% of low grade (Vang R, 2009). Recent studies indicates a grading system that divides serous ovarian carcinoma of low grade and respectively high-grade lesions, which is easy to apply, reproducible and based on their molecular classification (Ayhan A, 2009).
Low grade carcinomas indicated a glandular, micropapillary or cribriform pattern of invasion, while for high-grade carcinomas the insular and solid patterns were most frequently observed. In general, in cases of carcinoma the insular or solid pattern of invasion means aggressive tumors with high metastatic potential and numerous recurrences (Samaratunga H, 2005, Peng CW, 2010). Most carcinomas analyzed were classified as stage IB (T1bN0M0, 45%), followed by stage IA (T1aN0M0, 40%), stage IIA (T2aN0M0, 10%) and stage IIIA (T3aN0M0, 5%). Ovarian serous carcinoma represents an attractive target for ovarian carcinogenesis research since most are not in an advanced stage at the time of diagnosis (Brown PO, 2009). However from the stage II tumor development is rapid and reserved prognosis (Brown PO, 2009). In 31.2% of high-grade serous ovarian carcinomas were identified areas with endometrioid, transitional, microcystic or clear cell differentiation.

**Immunohistochemical analysis** investigated the expression of EGF, EGFR and Her2/neu in relation to markers involved in tumor progression and with some histopathological prognostic parameters for serous ovarian tumors.

**EGF (epidermal growth factor)** immunoexpression was identified for 88.8% of serous tumors analyzed in the membrane and peripheral cytoplasm. The immunostain did not differ in relation to tumor type (benign, borderline, malignant) or depending on degree and stage of malignant tumors. Xu Z et al, in a 2010 study conducted on ovarian malignant cell cultures have indicated that synergistic action of TGFβ (transforming growth factor) and EGF induce an invasive phenotype of these cells in vitro (Xu Z, 2010).

**EGFR (epidermal growth factor receptor)** immunoreaction was identified in 46.6% of the analyzed serous tumors at the membrane level. We found significant differences between benign tumors and borderline tumors of EGFR immunoexpression membrane on the one hand and serous ovarian carcinomas on the other hand, and also depending on their degree of differentiation, high-grade carcinomas indicated the highest scores. Most recent studies indicate the association of EGFR overexpression with high aggressiveness and reserved prognosis of ovarian carcinoma (Brustmann H, 2008, Suo Z, 2004).

**Her2/neu (EGFR2)** immunoexpression was identified in 46.6% of the serous analyzed tumors at the membrane level. Her2/neu expression indicated significant differences in benign, borderline and malignant low grade serous ovarian tumors compared to high grade where were found the highest scores. EGFR and Her2/neu expression analysis revealed a linear correlation...
and no statistical relationship with EGF expression of the two receptors. We have not found significant variations EGFR and Her2/neu immunostain according to tumor stage, pattern of invasion and other clinical-pathological analyzed parameters. Ginath S indicated that HER-2/neu is overexpressed in ovarian serous carcinomas compared with other histological types of malignant ovarian surface tumors while in normal ovary and benign ovarian tumors the protein expression is reduced (Ginath S, 2001).

*Ki67* immunoreaction was identified at the nuclear level in 34 of serous analyzed tumors, which represented 75.5%. Ki67 proliferation index indicated variations depending on grade and tumor stage, the high values being present in high-grade carcinomas in advanced stage of disease. Also, the proliferation of malignant and benign lesions was higher than borderline. In 2005, in a study conducted by O'Neill CJ Ki67 proliferation index was significantly different in low grade carcinomas (23%) compared with high-grade ones (55%) (CJ O'Neill, 2005). Ki67 relationship with EGF receptors can be attributed to direct involvement in stimulating cell proliferation, aspects suggested by some studies (O'Neill CJ, 2005, Vang R, 2009).

p53 immunoexpression was identified at nuclear level in 21 cases, which represented 46.7% of the cases analyzed. p53 immunoexpression indicated differences in relation to tumor type (benign, borderline, malignant), and the degree of tumor differentiation, high scores belonging to high-grade carcinomas. In 2004 Skírnisdóttir I ae al. analyzed the expression of EGFR and p53 on a group of 226 surface ovarian carcinomas and proposed their stratification into three groups – low risk (well-differentiated, and negative for p53 and EGFR), intermediate risk (well-differentiated, p53/EGFR positive or poorly differentiated and p53/EGFR negative) and high-risk (poorly differentiated and p53/EGFR positive) (Skírnisdóttir I, 2004).

*p16* immunoreaction was identified in the nuclear and cytoplasmic level in 14 cases of ovarian serous tumors which represented 31.1% of the analyzed group. p16 immunoexpression identified differences in the degree carcinomas, diffuse and intense stain being specific to high grade lesions. We have not identified differences in relation to histological type, tumor stage or the invasion pattern. P53, P16, EGFR and Her2/neu diffuse immunostain was characteristic to high grade carcinomas. In a study by O'Neill CJ et al. in 2007, they indicated significant differences in p16 immunostain in high-grade serous carcinomas compared with low grade and borderline tumors (O'Neill CJ, 2007).
CD34 immunoreactivity was found in the membrane of endothelial cells in all analyzed serous ovarian tumors. We found differences in CD34 MVD values between benign, borderline and malignant high-grade lesions, with no significant differences between low grade carcinomas and borderline tumors. We found a positive linear correlation between CD34 MVD values and EGFR, Her2/neu and Ki67 values. Chen AP analyzed in 2009 the relationship of EGFR with angiogenesis in ovarian carcinomas, indicating a higher rate of EGFR positivity and increased MVD in ovarian carcinomas and borderline tumors than normal and benign specimens, existing a correlation between the two parameters (Chen AP, 2009).

Chapter VII- „Conclusions” indicates the study conclusions.

- Clinico-epidemiological study indicated that benign tumors were larger and the borderline tumors and carcinomas were diagnosed at older ages.

- Most patients with serous ovarian tumors were from urban areas (55.7%) and associated risk factors like family history, hormone replacement therapy, nulliparity and obesity.

- Histopathological analysis of serous ovarian tumors revealed benign lesions in 62.9% of cases, the borderline in 8.5% and malignant lesions in 28.6%.

- Cystadenoma (47.7%) and cystadenofibroma (38.7%) were the most common benign lesions.

- In 83.3% of cases were noninvasive borderline tumors, sometimes associated with benign lesions (33.3%) or being present focally in carcinomas (33.3%).

- The most common histological subtype of ovarian serous carcinoma was the papillary cystadenocarcinoma (85%), high-grade lesions being more common (80%).

- Low grade carcinomas presented a glandular, micropapilar or cribriform pattern of invasion, while for high-grade lesions insular (37.5%) and solid (31.2%) patterns were the most frequently observed.

- Most examined carcinomas were classified as stage IB (T1bN0M0, 45%), followed by stage IA (T1aN0M0, 40%), stage IIA (T2aN0M0, 10%) and stage IIIA (T3aN0M0, 5%).

- Immunohistochemical analysis indicated an EGF immunostain without difference compared to tumor type (benign, borderline, malignant) or the degree and stage of malignant tumors.
- We found significant differences between EGFR immunoexpression in benign and borderline tumors on the one hand and serous ovarian carcinomas on the other hand, and also depending on their degree of differentiation, high-grade carcinomas indicated the highest scores.
- Her2/neu expression indicated significant differences in benign, borderline and malignant low grade serous ovarian tumors compared with high-grade, where we observed the highest scores.
- EGFR and Her2/neu expression analysis revealed a linear correlation and no statistical relationship with EGF expression of the two receptors.
- Ki67 proliferation index indicated variations depending on grade and tumor stage, the highest values being present in high-grade carcinomas and in advanced disease. Also, the proliferation of malignant lesions was higher than benign and borderline ones. We found positive linear correlation between Ki67 and EGFR immunoexpression and also between Ki67 and HER2/neu.
- p53 immunoexpression indicated differences depending on tumor type (benign, borderline, malignant), and degree of tumor differentiation, high scores belonging to high-grade carcinomas.
- p16 immunoexpression identified differences depending on the degree of carcinomas, diffuse and intense immunoreactions being specific to high grade lesions.
- Analysis of the relationship between immunostains of epidermal growth factor receptor EGFR and Her2/neu with proteins involved in cell cycle such as p53 and p16 indicated:
  - P53 positive linear correlation with EGFR and Her2/neu in serous ovarian carcinoma, the diffuse p53 receptor stain and high scores being characteristic to high-grade lesions.
  - Significant differences in p16 expression depending on receptors scores, high-grade carcinomas presenting diffuse and intense p16 immunostain and high scores of EGFR and Her2/neu.
- We found differences in CD34 MVD values between benign, borderline and malignant high grade tumors, with no significant differences between low grade carcinomas and borderline tumors.
- Adenofibromas were negative for most analyzed markers which may indicate a particular phenotype and different progression routes from the rest of benign tumors.
- Positivity of benign tumors (cystadenomas, cystadenofibromas) for EGFR and Her2/neu as well as for Ki67, p53, p16, CD34, demonstrates the evolution potential of these lesions.
• We found the variability and heterogeneity of borderline tumors immunopositivity, which supports a particular biomolecular behavior and the current clinico-pathological classification.

• Clinical parameters, pattern of invasion, inflammation were not correlated with analyzed biomarkers, which limits their usefulness in assessing the malignant behavior of ovarian serous tumors.

• Association of tumor stage (FIGO) with only some of the parameters analyzed (Ki67), may indicate the existence of some molecular mechanisms of tumor growth and progression in different stages of ovarian carcinogenesis.

• Association of tumor grade with EGFR, HER2/neu, Ki67, p53, p16 and CD34 MVD recommends this histopathological parameter as the most important in assessing tumor aggressiveness.

• Statistical relations of the two growth factors with tumor proliferation, cell cycle and tumor angiogenesis recommended them as ovarian serous carcinomas therapeutic targets.

BIBLIOGRAPHY


