UNIVERSITY OF MEDICINE AND PHARMACY

CRAIOVA

DOCTORAL THESIS

SUMMARY

THE ROLE OF ANGIOGENESIS IN THE INITIATION AND PROGRESSION OF PREINVASIVE AND INVASIVE SQUAMOUS LESIONS OF THE UTERINE CERVIX - HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

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CRAIOVA 2013

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INTRODUCTION

Cervical cancer is the third malignant neoplasm in frequency of women around the world, representing around 13% of all cancers. In our country, cervical cancer is the first cause of death in patients aged between 25-44 years. Frequency of preinvasive and invasive lesions in the exocervix, the age groups affected and risk factors involved indicates these injuries as an important health problem in our country and around the world.

The process of carcinogenesis from the exocervix is an ideal model for research, with a spectrum of lesions which from one end presents low squamous intraepithelial lesions, followed by the high grade lesions, and at the other end, microinvasive, frank invasive and metastasing lesions. In addition to implementing preventive screening programs for early detection of carcinomas and preinvasive lesions, numerous studies have investigated the biomolecular mechanisms present in this lesional filiation. In cervical carcinogenesis, angiogenesis is an essential process that ensures the initiation and progression of neoplastic lesions, and currently provides numerous therapeutic targets, considering the complexity of the mechanisms involved.

Study of angiogenic factors and quantification of cervical angiogenesis, can provide important information about the angiogenic phenotype of preinvasive and invasive squamous lesions of the cervix. Also, the angiogenic phenotype relationship with epidemiological and pathological parameters of prognostic may provide information about evolutionary potential and can create evaluation models which constitute the basis of investigative trials for possible therapeutic targets.

The study included 665 cases that were analyzed in terms of epidemiological data. Subsequently, histopathological analysis performed on a sample of 650 preinvasive and invasive squamous lesions investigated the type and associations (dysplasia / carcinoma), the degree of lesions, particular aspects of carcinomas, the presence of vascular and perineural invasion.

Immunohistochemical study was performed on a group of 57 biopsies that were analyzed for vascular microdensity, the degree of vessels maturity and proliferation and the expression of growth factors and their receptors, comparative, for the two lesional types and in relation to the epidemiological and histopathological parameters, using statistical analysis (means, standard deviations, Student t-tests, ANOVA tests, chi square and Pearson tests).

Keywords: angiogenesis, dysplasia, carcinoma, vascular microdensity, immaturity, proliferation, growth factors, angiogenic phenotype.

CHAPTER I- „Epidemiological aspects and risk factors involved in preinvasive and invasive lesions of the uterine cervix"- describe the incidence of cervical cancer and precursor lesions in the world and in Romania in relation to age groups and related to general pathology of malignancy. It is also discussed the role sceening programs for these injuries and the impact on patients prognosis. Also, are analyzed the main risk factors involved in dysplastic and invasive cervical lesions.

CHAPTER II- „Angiogenesis - basic mechanisms of carcinogenesis"- describe historical data related to angiogenesis, tumor angiogenesis mechanisms, the role of proangiogenic and antiangiogenic factors, mechanisms of tumor angiogenic switch and the role of stromal elements in angiogenesis, experimental models used for investigation, the possibilities of quantification and perspectives of research in this direction.

CHAPTER III- „The role of angiogenesis rolul in preinvasive and invasive lesions of the uterine cervix"- emphasizes the particularities of tumor angiogenesis process in dysplastic and invasive cervical lesions in this sense being mentioned classic and recent studies.

OBJECTIVES OF THE STUDY

In this study is proposed a complex evaluation of angiogenesis in cervical preinvasive and invasive squamous lesions by using conventional methods as well as techniques represented by immunohistochemistry and morphometry.

Specific objectives of the study include:

- identification and definition of the morphological parameters that characterize the precursor and invasive squamous cervical lesions for use as the criteria for assessment of prognosis in immunohistochemical investigations;
- quantification of vessel neoformation in preinvasive and invasive squamous cervical lesions in order to reveal the involvement of angiogenesis in the development and progression of lesions;
- identification of the maturity degree of newly formed vessels in preinvasive and invasive squamous cervical lesions;
- establishment of the proliferative activity degree in neoformation vessels;
- identification and quantification of proteins with angiogenic role and of their receptors to the establish the proangiogenic sources, and also the angiogenic specific phenotypes;
- identification of synergistic or antagonistic molecular mechanisms modulating angiogenesis.

CHAPTER IV- „Material şi Metode”- provides information about the material studied and methods used in research.

STUDIED MATERIAL

This study was conducted over a period of 4 years (2009-2012), the biological material being represented by biopsy, conization or hysterectomy surgical pieces, obtained from patients hospitalized in the Obstetrics Gynecology Clinics of the Emergency County Hospital Craiova.

METHODS

In a first phase we followed the **epidemiological evaluation** of 665 cases in terms of distribution by year, age, environment of origin and associated risk factors.

Subsequently, **histopathological analysis** was conducted on 660 of surgical pieces and biopsy fragments which were fixed in 10% buffered neutral formalin, processed by the classical technique with paraffin embedding, sectioned at 3-5 μm and Hematoxylin–Eosin stain. It was followed the type and association of lesions (dysplasia / carcinoma), the degree of lesions, particular aspects of carcinomas, the presence of vascular and perineural invasion.

Immunohistochemical analysis was performed on a group of 57 selected biopsies from the casuistry that were analyzed for vascular microdensity (CD105), the maturity of the vessels (CD105/α-SMA), the degree of vascular and tumoral proliferation (Ki67/CD105), and also the expression of some growth factors and their receptors (VEGF, VEGFR1, VEGFR2, ANG2). The panel of antibodies used is presented below:

Antibody	Clone/ Manufacturer	Dilution	Antigenic retrieval	Positive external control
CD105 (endoglin)	SN6h/ Dako	1:1000	-	Kidney
CD105 (endoglin)	Policlonal/ Thermo Scientific	1:50	Citrate buffer, pH 6	Kidney
α-SMA (smooth muscle actin)	1A4/ Dako	1:50	Citrate buffer, pH 6	Colon
Ki67	MIB 1/ Dako	1:200	Citrate buffer, pH 6	Breast carcinoma
VEGF	C1/ Dako	1:100	Citrate buffer, pH 6	Kidney
VEGFR1 (Flt-1)	C17/ Dako	1:150	Citrate buffer, pH 6	Skin
VEGFR2 (KDR/Flk-1)	Policlonal/ Abcam	1:300	Citrate buffer, pH 6	Kidney
ANG 2 (angiopoietin)	F1/ SantaCruz Biotechnology	1:50	Citrate buffer, pH 6	Placenta

Were performed single and double reactions; for the simple reactions the working systems were represented by LSAB2 System-HRP and CSA II (Biotin-Free catalyzed Amplification System-CD105 monoclonal) for visualisation using DAB (diaminobenzidine).

In the case of double reactions were used sequentially LSAB2 systems System-HRP (for CD105, polyclonal, Ki67) and LSAB2 System-AP (for α-SMA), for visualisation using DAB (brown), respectively Vulcan Fast Red (red).

Morphometric analysis examined the microdensity of vessels marked with CD105 and α-SMA by "hot spot" method, which consisted in manual quantification qof vessels. For quantification were also calculated composite scores for VEGF, VEGFR1, VEGFR2, Ang-2, which took into account the percentage of labeled cells and the intensity of the stain. For Ki-67 was calculated the proliferation index by dividing the number of positive cells to the total cells counted at 40x microscope field.

Statistical analysis used average values, standard deviations and comparison tests (Student t, one-way ANOVA, chi square, Pearson tests), made with SPSS10 software.

CHAPTER V- „Results" and CHAPTER VI- „Discussions" indicate the results obtained in the study, which are reported to classic and recent data from literature.

Epidemiological study indicated a maximum incidence of lesions in the fourth decade of life, with an average age in diagnosis of 35,4 years for CIN lesions respectively 53,2 years for squamous cell carcinoma. In this study, were found differences in the distribution of cases by environment of origin, and associated risk factors were identified in 65,4% of cases being represented by smoking, oral contraceptives and HPV infection in both CIN lesions and in carcinomas

Literature data indicate an average age of diagnosis for squamous cell carcinoma of approximately 51 years, while for dysplastic lesions, this age is between 25-35 years, and this is the reason why cervical cancer screening is indicated for patients with age 25-30 years **[7]**. Some studies have noted that low-grade dysplastic lesions have greater tendency to regress, whereas high-grade lesions most likely persists or progresses **[168]**. Literature data indicate a synergic action for risk factors, there are so-called multifactorial hypothesis of cervical cancer, which supports the interaction of inflammatory lesions with hormonal disturbances and exposure to chemicals, including those contained in cigarette smoke and lead to the appearance of lesions **[24]**.

Histopathological study revealed the presence of CIN lesions in 79,2% of cases, carcinomas in 6,3% of cases and associations in 14,5% cases, especially for high-grade lesions; microcarcinoma was present associated in invasive frank lesions in a proportion of 11,1%. High-grade precursor lesions (58,9%) and poorly differentiated carcinomas (48.1%) were the most common, the classic appearance of keratinized or nonkeratinized occurring in 85.2% of analyzed cases. It ss described the presence of basaloid and papillary carcinomas in 14.8% of cases, and and also of perineural and vascular invasion in 5,9%, respectively 10,3% of the cases.

Accurate histological grading CIN lesions is important for therapeutic attitude as it is different for the CIN I, CIN II and CIN III **[115]**. In case of cervical squamous carcinomas, was proposed a complex grading system for evaluation, which included assessment of the keratinization degree, cell nuclear pleomorphism, pattern of invasion and host tissue response (inflammatory infiltrate) **[95]**. Regarding the keratinized and non-keratinized forms, often can be

seen combinations of these, which indicates that there is a broad spectrum of biological injuries **[168]**.

The literature data indicate that the majority of cervical squamous carcinomas developed from precursor lesions, about 2/3 of the untreated CIN lesions progressing to an invasive carcinoma in a variable period of time which can range from 3 to 20 years, being rarely reported cervical squamous cell carcinomas developed quickly without a history of precursor lesions **[99, 168]**. Also this evolutionary aspects of dysplastic lesions underlines the importance of careful search for high grade lesions and microinvasive foci on serial histological sections **[32]**. The literature indicates that the probability of vascular invasion increases with the depth of tumor invasion, presence of neoplastic emboli representing an independent risk factor **[168]**. Furthermore, perineural invasion is an independent prognostic indicator for cervical carcinoma **[123]**.

Immunohistochemical analysis followed to characterize and comparative quantification of angiogenesis in dysplastic and invasive cervical lesions in relation with epidemiological and histopathological parameters of interest.

CD105 immunostaining was used to quantify vascular microdensity being identified in all cases in the cytoplasm of endothelial cells. CD105 MVD increased with grade lesions, but with no differences between carcinomas and dysplasia which indicates the presence of vascular neoformation process in both lesion types.

Vascular microdensity as method for the quantification of tumor angiogenesis has been associated with patient prognosis, an increased number of neoformation vessels was associated with an increased risk of tumor progression and metastasis, as well as lower survival period **[38]**. The link between CD105 and tumor angiogenesis was suggested by numerous studies that found an overexpression of the protein in endothelial cells in tumors localized in the colon, breast, lung, brain tissue, prostate as well in uterin ecervix, in comparison with normal tissues **[179]**.

Immunoreaction *CD105 / α-SMA* was used to assess the degree of maturity of neoformation vessels, the α-SMA stain being identified in all cases in pericytes and stromal myofibroblasts. α-SMA MVD was higher in low-grade CIN lesions (not statistically significant) and well-differentiated carcinomas (statistically significant) compared with high-grade lesions, in which the degree of vascular immaturity was superior. The vascular immaturity degree was proportional with the microdensity of newly formed vessels.

The pericytes were associated with microvascular stability, although biomolecular mechanisms involved are not fully known **[165]**. Some studies have noted that loss of pericytes and even altering their connections are responsible for vascular instability, the presence of bleeding and increased risk of metastasis **[14]**.

Ki67 immunostaining was used to determine the degree of tumor (Ki67) and vascular (Ki67/CD105) proliferation, being present in the nuclear level in all cases. Ki67 proliferation index was significantly higher in dysplastic and invasive high-grade lesions and in carcinomas compared with dysplastic lesions. In the case of CIN lesions, the mean value of the number of CD105 positive vessels that had proliferative activity was 8,5%, and respectively 15,6% in the case of carcinomas.

Although endothelial cells are activated from the initial stages of angiogenesis, a biomolecular process proven to be present in preinvasive cervical lesions, most of these are negative for Ki67, indicating a low proliferative activity **[145]**. Also, in the case of cervical carcinomas, neoformation vessels with proliferative activity are relatively rare **[145]**.

VEGF immunoexpression was identified in 89,4% of cases analyzed in the cytoplasm of dysplastic and neoplastic cells, with significantly higher differences in CIN III lesions and high-grade carcinomas and also in carcinomas when compared with dysplastic lesions. There are significant differences of VEGF score deppending on Ki67 proliferation index in carcinoams, and also with MVD CD105 both in CIN lesions and in carcinoma.

Most studies in the literature indicate an increase of VEGF expression in invasive, aggressive tumors, aspect observed in gastric, ovarian, colorectal, breast, lung carcinomas **[44]**. It is demonstrated involvement of HPV in cervical tumor angiogenesis in a VRGF dependent manner, because due tu oncogenic proteins, on the one hand lead to the inactivation of tumor suppressor genes (p53, pRb) and the second, factor HIF-1, trigger the vascular neoformation process is activated **[147]**.

VEGFR1 immunoreaction was identified in 82,4% of analyzed cases in the cytoplasm of dysplastic and neoplastic cells, with significant differences of VEGFR1 score deppending on the degree of carcinomas differentiation, lesions in which the VEGFR1 score values were significantly higher than CIN lesions.

VEGFR2 immunostaining was identified in 78,9% of analyzed cases without statistical differences of score deppending on parameters of interest, including VEGF and VEGFR1 stain.

In principle, VEGFR1 activation induces endothelial cell migration without significant cellular proliferative effects, and there are soluble forms of VEGF inhibition **[159]**. The VEGF-VEGFR2 is the most important stimulator of tumor angiogenesis and also ideal target for angiogenic therapy **[46]**. Some clinical trials performed on samples from patients with colorectal cancer, breast, kidney and lung and receiving anti-VEGF-A/VEGFR therapy, resulted in a statistically significant increase of survival free interval with minimal side effects **[163]**.

ANG-2 immunostain was identified in 64,9% of investigated cases at the perinuclear cytoplasm of dysplastic and neoplastic cells, the scores being significantly lower in CIN lesions when compared with carcinomas, respectively in poorly differentiated carcinomas compared to the well / moderately differentiated ones.

Angiopoetin 2 (ANG2) is an endothelial specific growth factor that antagonizes the ANG1 activity, promotes pro-inflammatory vascular activity and has a role in the destabilization of endothelium and vascular remodeling. Overexpression of Ang-2 in tumors is associated with reducing number of pericytes which are in direct contact with the endothelial cells **[68, 211]**. In the presence of VEGF, angiopoietin 2 promotes vascular growth and branching, while in the absence of VEGF leads to endothelial cell destruction and regression of newly formed vessels **[72, 110]**.

CHAPTER VII- „Conclusions” indicates the study conclusions.

Epidemiological analysis

- the incidence variance between the analyzed years was 11%;
- the maximum incidence of lesions was observed in the fourth decade of life, with an average age in diagnosis of 35,4 years for CIN lesions respectively 53,2 years for squamous cell carcinoma;
- the risk factors identified in 65,4% of cases were represented by smoking, oral contraceptives and HPV infection;

Histopathological analysis

- CIN lesions represented 79,2%, carcinomas 6.3% and their association 14.5%; microcarcinoma was identified in 11,1% of invasive analyzed lesions;
- high-grade precursor lesions predominated being identified in 58,9% of cases;
- in relation to tumor grade, the most common were poorly differentiated squamous cell carcinoma, followed by moderately differentiated, the classic appearance of keratinized or nonkeratinized carcinomas being observed in 85,2% of cases;
- were identified basaloid carcinomas (8.8%) and papillary squamous cell carcinoma (6%) cases, and also the presence of vascular (5.9%) and perineural (10.3%) invasion;

Immunohistochemical analysis

- CD105 MVD values were significantly higher in high-grade lesions, with no statistical difference between dysplasia and carcinomas, which indicates a vascular neoformation constant process during tumor progression;
- the degree of vascular immaturity quantified with CD105/α-SMA reaction was higher in high-grade lesions and proportionally with the density of neoformation vessels;
- in the case of CIN lesions, the mean value of the number of CD105 positive vessels that had proliferative activity was 8,5%, and 15.6 % in the case of carcinomas, with no difference in the degree of lesions, which may suggest the existence of a constant vascular proliferative compartment;
- the vessels with proliferative activity showed some morphological maturity aspects;
- VEGF immunostaining was associated with grade lesions, Ki67 index and CD105 MVD, being higher for carcinomas;
- there were significant differences of VEGFR1 score deppending on the degree of carcinomas differentiation, lesions with significantly higher values of VEGFR1 score than CIN lesions ;
- higher scores of VEGFR1 stain to the tumor cells compared with VEGFR2 suggests autocrine regulation mechanisms of angiogenesis cervical achieved mainly through VEGFR1;
- ANG -2 immunoreactivity was significantly lower in CIN lesions compared with carcinomas, respectively in poorly differentiated carcinomas compared to the well / moderately differentiated ones, which can be attributed to already destabilized vascular network;

- immunohistochemical profile characterized by CD105 positivity, tumoral and vascular Ki67, VEGF, VEGFR1 and negativity of α -SMA and ANG -2 corresponded to moderately / poorly differentiated carcinomas, designating aggressive lesions;
- in case of CIN III lesions, angiogenic profile was more similar to that of cervical squamous carcinomas than precursor lesions, characterized by high levels of CD105 MVD, high degree of immaturity and vascular and tumor proliferation and VEGF and Ang -2 scores specific to an aggressive immunophenotype;
- for squamous lesions of the uterine cervix, assessment of vessels microdensity (CD105), maturity (α -SMA), proliferation (Ki67) and stabilization (ANG -2) of newly formed vessels and the assessment of VEGF and VEGFR1 are useful to characterize aggressive angiogenic phenotypes caught in preinvasive or invasive stages.

Selective references

[7] Arbyn M, Antoine J, Valerianova Z, Mägi M, Stengrevics A, Smailyte G, Suteu O, Micheli A. Trends in cervical cancer incidence and mortality in Bulgaria, Estonia, Latvia, Lithuania and Romania. Tumori. 2010;96(4):517-23.

[14] Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev. 2005;15(1):102-11.

[24] Castellsagué X, Muñoz N. Chapter 3: Cofactors in human papillomavirus carcinogenesis--role of parity, oral contraceptives, and tobacco smoking. J Natl Cancer Inst Monogr. 2003;(31):20-8.

[32] Dalla Palma P, Giorgi Rossi P, Collina G, Buccoliero AM, Ghiringhello B, Gilioli E, Onnis GL, Aldovini D, Galanti G, Casadei G, Aldi M, Gomes VV, Giubilato P, Ronco G; NTCC Pathology Group. The reproducibility of CIN diagnoses among different pathologists: data from histology reviews from a multicenter randomized study. Am J Clin Pathol. 2009;132(1):125-32.

[38] Duff SE, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. FASEB J. 2003;17(9):984-92.

[44] Ferrara N. The role of vascular endothelial growth factor in pathological angiogenesis. Breast Cancer Res Treat. 1995;36(2):127-37.

[46] Folkman J, History of Angiogenesis in: Figg DF, Folkman J (eds.), Angiogenesis- An Interogative Approach From Science To Medicine, Springer, New York, 2008.

[68] Hammes HP, Lin J, Wagner P, Feng Y, Vom Hagen F, Krzizok T, Renner O, Breier G, Brownlee M, Deutsch U. Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. Diabetes. 2004;53(4):1104-10.

[72] Hammes HP, Lin J, Wagner P, Feng Y, Vom Hagen F, Krzizok T, Renner O, Breier G, Brownlee M, Deutsch U. Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. Diabetes. 2004;53(4):1104-10.

[95] Krinstensen G, Trope C. Modern prognostic factors in cervical cancer with special attention to early disease. CME J Gynecol Oncol. 2001;6:196-203.

[99] Kruse AJ, Baak JP, Janssen EA, Kjellevold KH, Fiane B, Lovslett K, Bergh J, Robboy S. Ki67 predicts progression in early CIN: validation of a multivariate progression-risk model. Cell Oncol. 2004;26(1-2):13-20.

[110] Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos

GD. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science. 1997;277(5322):55-60.
[115] Martin CM, O'Leary JJ. Histology of cervical intraepithelial neoplasia and the role of biomarkers. Best Pract Res Clin Obstet Gynaecol. 2011;25(5):605-15.
[123] Memarzadeh S, Natarajan S, Dandade DP, Ostrzega N, Saber PA, Busuttil A, Lentz SE, Berek JS. Lymphovascular and perineural invasion in the parametria: a prognostic factor for early-stage cervical cancer. Obstet Gynecol. 2003;102(3):612-9.
[145] Raica M, Cimpean AM, Ribatti D. Angiogenesis in pre-malignant conditions. Eur J Cancer. 2009;45(11):1924-34.
[147] Recommendations on cancer screening in the European union. Advisory Committee on Cancer Prevention. Eur J Cancer. 2000;36(12):1473-8.
[163] Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, Lilenbaum R, Johnson DH. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med. 2006; 355:2542–50.
[165] Sennino B, Falcón BL, McCauley D, Le T, McCauley T, Kurz JC, Haskell A, Epstein DM, McDonald DM. Sequential loss of tumor vessel pericytes and endothelial cells after inhibition of platelet-derived growth factor B by selective aptamer AX102. Cancer Res. 2007;67(15):7358-67.
[168] Simionescu C, Cernea N, Maragaritescu Cl, Georgescu C, Iliescu D. Patologia colului uterin. Editura Medicală Universitară Craiova, 2009.
[179] Takahashi N, Haba A, Matsuno F, Seon BK. Antiangiogenic therapy of established tumors in human skin/severe combined immunodeficiency mouse chimeras by anti-endoglin (CD105) monoclonal antibodies, and synergy between anti-endoglin antibody and cyclophosphamide. Cancer Res. 2001;61(21):7846-54.
[211] Zhang L, Yang N, Park JW, Katsaros D, Fracchioli S, Cao G, O'Brien-Jenkins A, Randall TC, Rubin SC, Coukos G. Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer. Cancer Res. 2003;63(12):3403-12.