“VICTOR BABEȘ” UNIVERSITY OF MEDICINE AND PHARMACY FROM TIMIŞOARA

FACULTY OF MEDICINE

DEPARTMENT XII – OBSTETRICS AND GYNECOLOGY

BĂLAN LAVINIA

PhD THESIS

CELLULAR, MOLECULAR, AND CLINICOPATHOLOGIC ASPECTS IMPACTING CERVICAL CANCER DEVELOPMENT AND THEIR INTERRELATIONSHIP WITH THE TUMOR MICROENVIRONMENT

– A B S T R A C T –

Scientific Coordinator:

PROF. UNIV. DR. PIRTEA LAURENȚIU

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I. GENERAL PART

INTRODUCTION

Cervical cancer is the fourth most prevalent cancer among women globally, behind breast, colorectal, and lung cancer (1). In Romania, around 3,400 women are diagnosed with the disease annually, with over 1,800 dying. Romania ranks second in terms of cervical cancer incidence and mortality among EU countries (2,3).

Detecting and treating precursor lesions is crucial for preventing invasive cervical cancer, as it has an extended course of development.

The introduction of the Pap test in 1940 revolutionized the diagnosis of morphological changes in the cervical epithelium, resulting in a significant decrease in the prevalence of cervical cancer in industrialized countries (4). The International Agency for Research on Cancer (IARC) recommends that cervical cancer screening programs using the Pap test at three to five-year intervals for women aged 35-64 can reduce invasive cervical cancer by at least 80% (4). The main cause of cervical cancer is chronic infection with high-risk strains of HPV.

Cervical cancer is more prevalent among women with lower socioeconomic backgrounds due to a lack of sexual education and increased sexual partners. Smoking increases the risk by two times compared to non-smokers. Chronic immune-suppression, such as HIV or kidney transplantation, also increases the risk (3,5).

The prognosis of cervical cancer is determined by the disease's severity at diagnosis, including tumor stage, histological type, grading, tumor volume, and depth of invasion (5).

The Papanicolau and HPV tests are currently used for cervical cancer screening, but definitive diagnosis is achieved through a biopsy or cone biopsy with histological examination.

People with underlying chronic illnesses, such as cancer, are more vulnerable to severe acute respiratory syndrome (SARS-COV-19) infection. During the early phases of the COVID-19 pandemic, there was a significant drop in the identification of several forms of cancer (6, 7), with an increasing proportion of cancer diagnoses at advanced stages (8).

This negative effect was caused by shortcomings in health systems, a lack of preparedness, and resource availability, as well as an increase in COVID-19 cases, where the demand for SARS-CoV-2 testing competes with the ability to deliver HPV testing (8), compounded by a staff shortage (9).

Researchers have focused on tumor cells as the primary target for therapeutic therapies.

The tumor stroma (TSR) is a crucial part of the tumor microenvironment, comprising fibroblasts, extracellular matrix, capillaries, and inflammatory cells. It plays a significant role in cancer development, progression, metastasis, and treatment resistance (10). The peritumoral microenvironment is crucial for tumor formation and cell dispersion. Tumor invasion occurs within a tumor-host microecology, where stromal and tumor cells exchange enzymes and cytokines. The stroma supports cancer cells (11, 12).

TSR is a useful and valuable method for pathologists to obtain more predicted information from hematoxylin-eosin HE-stained slides. The tumor stroma ratio should be assessed during the initial morphopathological examination of the post-intervention diagnostic specimens.

Tumor stroma interrelation is a highly discussed issue in recent times not only in cervical cancer but also in other types of neoplasia. However, their predictive value in cervical cancer prognosis is not well known.

The study of tumor stroma offered many opportunities for future cancer therapy. Targeting components of the tumor microenvironment has huge clinical potential, especially when combined with other therapeutic agents.

Lymph node metastasis (LNM) is a common form of cervical cancer that significantly impacts patient outcomes. Patients with positive LNM have a significantly worse 5-year survival rate compared to those without metastases (91%) (13).

Sentinel lymph node biopsy (SLN) is the first site of tumor metastasis, and its pathology should reflect metastatic illness in other lymph nodes. Ultrastaging methodology and tissue processing are crucial components of SLN adoption in cervical cancer. Research has shown that SLN ultrastaging can identify 10% to 15% more patients with micrometastases (MICs), with MICs having a similar negative impact (14).

The technique of ultrastaging for treating cervical cancer has been incorporated into standard practice and referenced in internationally recognized treatment guidelines. Various tracers, such as Technetium methylene blue (TMEB) and Indocyanine green (ICG), have been suggested for sentinel lymph node detection to increase its effectiveness. SLN ultrastaging has been shown to increase the negative predictive value by up to 100% from 91%. (15,16).

II. SPECIAL PART

1. RESEARCH MOTIVATION

Tumor stroma interrelation is a significant issue in cervical cancer and other types of neoplasia. The epithelium of the cervix develops closely with molecular signals received from the mesoderm and connective tissue during the embryonic period. This interrelation is well-controlled under normal conditions, but imbalances can cause pathological conditions.

The cervical stroma has unique features, such as hormone-dependent cellular components, mainly fibroblasts, that react to malignant transformation of overlying epithelial cells. The tumor-stroma ratio is an increasingly important criterion in evaluating cervical tumors, with a major clinical and therapeutic impact.

Fibroblasts in the tumor stroma can synthesize factors that determine and support the perpetuation of malignant transformation of epithelial cells and act on other stromal components adjacent to hyperplastic fibroblasts. Cancer-related fibroblasts (CAFs) are another aspect of fibroblasts in malignant lesions, with several types described depending on their molecular variability. These molecular types have been shown to be specific to certain molecular forms of breast cancer.

Molecular heterogeneity in stromal fibroblasts is assumed to be present in cervical cancer, but this has not been certified to date. Limited literature data exists on how certain components of the tumor stroma, such as lymphoid infiltration, have been correlated with paraclinical parameters like peripheral blood leukocyte counts at diagnosis and post-treatment. This study aims to associate diffuse lymphoid infiltrate or tertiary lymphoid structures (TLS) with peripheral blood leukocytes in both primary tumor and metastatic lymphoid lesions.

The stromal tumor ratio studied in the primary tumor will then be correlated with the presence or absence of lymph nodes to identify the stromal impact on the metastasis process. Another extremely important aspect of the tumor stroma impacting the malignant transformation of epithelial cells is the vascular compartment. Lymphovascular invasion is a parameter that is presented by the medical anatomologist

at the time of establishing the primary histopathological diagnosis but is not a criterion for choosing the therapeutic and drug methods.

Sinus histiocytosis observed in lymphoid nodes with or without metastases is also a subject not studied in the literature. It is currently unknown whether the prognostic and therapeutic impact of sinus histiocytosis in cervical cancers with lymph node metastases is known and this will be studied in the present paper by correlating the presence or absence of sinus histiocytosis in lymph nodes with metastatic tumor-stroma rapport in the primary tumor. At the same time, at both the cervical stromal level and the vascular compartment, the impact of radiation therapy on cervical cancer is also controversial. There are indications that radiotherapy will result in a marked inflammatory reaction of the cervix followed by an overactivity of the angiogenic process to form new blood vessels that may further promote the spread of tumor cells, while the impact of radiotherapy on stromal fibroblasts is unknown despite its cytotoxic effect on malignant epithelial cells.

SARS CoV 2 infection is extremely less evaluated related to its impact on cervical cancer development, progression, and metastasis. Indirect data about SARS CoV 2 impact on immune microenvironment changes of cervical cancer or the general immune milieu of the women with direct impact on a possible change of the cervical cancer development dynamics.

Predicting cervical LN metastasis (LNM) is crucial for improving survival rates and reducing recurrence. Very few small cohort studies used an ultrastaging method to assess non-SLNs; most of them only assessed SLNs. This study aimed to evaluate all lymph nodes, SLN and non-SLN, in patients with cervical cancer and high risk of LN involvement but negative intraoperative pathologic LN assessment, to increase sensitivity and diagnostic accuracy in cervical cancer with 5-year follow-up.

All of these reasons described above were the basis for the present study.

2. SCIENTIFIC OBJECTIVES

- To identify factors with prognostic and predictive value for cervical cancer development, progression, and metastasis by using basic research methods correlated with clinic-pathologic parameters
- Assessment of tumor/stroma ratio in primary tumor and its impact on clinical pathology factors
- Impact of radiation therapy on tumor stroma ratio.
- Assessment of inflammatory infiltrate and tertiary lymphoid structures (TLS) and their interrelation with peripheral blood leukocyte levels
- Identification of common therapeutic targets expressed in both epithelial cells and stroma, PDGF, PDGFR, cd 34, Hpv.

- Characterization of cell heterogeneity in tumor stroma: Fibroblasts/Myofibroblasts
- TLS (lymphoid tertiary extractor)/ inflammatory infiltrate lymphovascular invasion on the cervical piece
- Vessels /stroma ratio assessment
- Impact of pandemic period SARS COV-2 in diagnosis and development of cervical cancer
- Accuracy of ultrastaging in the detection of micrometastases in sentinel and non-sentinel lymph nodes in cervical cancer

III. RESULTS

1. PERSONAL CONTRIBUTION: IMMUNE PECULIARITIES OF CERVICAL CANCER STROMA WITH SPECIAL EMPHASIS ON TERTIARY LYMPHOID STRUCTURE AND CORRELATION TO CLINICOPATHOLOGICAL PARAMETERS

The study investigates morphological variants of tertiary lymphoid structures (TLSs) in relation to cervical cancer development, from intraepithelial neoplastic lesions to invasive carcinomas with locoregional lymph node metastases.

Materials and methods: This retrospective analysis comprised 100 cervical cancer cases who had had total hysterectomy with lymphadenectomy in the Obstetrics and Gynecology Clinic of the Municipal Emergency Clinical Hospital of Timisoara, Romania, from 2020 to 2023. Bilateral ilio obturator lymphadenectomy and total hysterectomy were used to acquire biopsy samples. The presence of germinal centers, other stromal structures, TLS density, topography relative to the tumor lesion, and malignant cell islets are used to evaluate and classify TLS.

Results: We first globally evaluated the total number of TLSs (TLS.T). We observed topographically two places in the cervical stroma: TLS immediately peritumorally positioned and TLS away from tumor lesions. Invasive carcinomas have bigger superficial TLSs with a well-defined germinal center. As they approached the tumor, TLSs increased in size and density. We also detected a special type of TLS associated with nerve fibers, which we named tertiary lymphoid structures associated with nerves (TLS.N). The total number of TLSs did not correlate with age, but 85.71% of patients presenting TLS.N were aged between 59 and 72 years old. Our findings showed a strong correlation between age (postmenopausal, $p = 0.005$) and TLS-N presence.

Similarly, TLS parameters evolved with tumor differentiation. Only in the TLS.N group did the tumoral grading (G) 3 correlate with TLS ($p = 0.041$), while TLS.T did not correlate with G. All TLS.N. patients, except one, had lymphovascular invasion and massive histiocytosis. On the first point, TLS.N correlated with lymphovascular invasion ($p = 0.032$).

Conclusion: Tertiary lymphoid structures associated with nerves have not been previously reported in cervical cancer, and their effects on prognosis and aggression are unknown. There was a substantial association between TLSs.N presence and age over 60, suggesting it is exclusive to menopausal women. They were also substantially connected with lymphovascular invasion and G3, suggesting they may be a poor cervical cancer prognostic factor.

2. PERSONAL CONTRIBUTION: IMPACT OF SARS-COV-2 PANDEMIC ON THE DIAGNOSIS OF CERVICAL CANCER AND PRECURSOR LESIONS - A SINGLE-CENTER RETROSPECTIVE STUDY

In this study our aim was to perform a retrospective analysis of the volume of cervical screening tests, the number of patients treated with an excision method, and the incidence of invasive and non-invasive cervical during a pandemic and pre-pandemic period of 24 months.

Materials and Methods: The study compared 404 patients who underwent cervical cone biopsy for

cervical cancer. The study examined patients' specimens based on histopathological characteristics and categorized cervical lesions based on pap smears. Results: There was a statistically significant age difference between the two study periods. The mean difference was 32 years before the pandemic and 35 years during the pandemic (p-value > 0.05). The biggest patient loss ratio identified by age group was in the 50–59-year group, with a 14.53% loss in the pre-pandemic period and a 9.1% loss in the pandemic period. In the pandemic period, patients from rural areas presented in the clinical trial with a lower rate of 39.52% (83 patients) vs. 60.47% (127 patients) in urban areas. A higher percentage of patients experiencing cervical bleeding as a clinical manifestation in the pandemic period vs. the pre-pandemic period, with an increase in more severe lesions in the pandemic period, had a statistical significance of 8% more newly diagnosed compared to the pre-pandemic period. Conclusions: The addressability of the patients during the COVID period was not affected in a drastic way in our study. We encountered a decrease in appointments in the age group of 50–59 years and a decrease in patients with rural residences. In our study, we found an increase in cervical bleeding as a reason for consultation in the pandemic period with a higher lesion degree, both on a pap smear and on a cervical biopsy.

3. PERSONAL CONTRIBUTION : MUTUAL INFLUENCE OF CERVICAL CANCER TUMOR-STROMA INTERACTION EVALUATED BY PDGF-BB AND PDGF R-BETA EXPRESSION

We aim to evaluate in this study the relationship between Platelet-derived growth factor PDGF expression in tumor cells and tumor stroma receptor expression, as well as the effects of PDGF overexpression on angiogenesis and stromal lymphangiogenesis.

Materials and Methods: The study was conducted on tissue material collected from the cervix by targeted biopsies from discernible macroscopic lesions, cone pieces, and post-operative material of patients with premalignant intraepithelial lesions and invasive lesions. This was a retrospective study that included 46 archival paraffin-embedded specimens of cervical carcinoma selected for our purpose.

Results: In the quasi-normal cervical epithelium adjacent to cervical tumors, platelet-derived growth factor (PDGF-BB) expression was heterogeneous with a decrease in expression from the basal to the superficial squamous layer. The highest expression intensity was recorded in the basal layer where PDGF-BB had an intense expression.

The expression of PDGF in stromal cells was greater than the expression of markers by the transformed neoplastic cells. PDGF BB, PDGFR-alpha, and beta were expressed in tumor cells in 41 (89.1%), 25 (54.35%), and 46 (100%) respectively.

In CIN 3-type lesions as well as microinvasive PDGFR-alpha lesions, it has been extensively expressed across the epithelial malignancy area with an enhancement in cells exposed to the tumor stroma.

The beta-receptor was also highly expressed in CIN 3 lesions. Tumor embolisms from invasive lesions exhibited a divergent expression of PDGFR-alfa and beta-expression in the sense that tumor embolisms were highly positive for PDGFR-beta and negative for PDGFR-alfa.

PDGF assessment for microinvasive cancer. At this point, the expression of tumor mass markers was not detected in 6.25% of cases. Low expression was accomplished in 31.25% of cases, moderate in 43.75% of cases, and strong in 18.75% of cases, respectively. In pre-invasive carcinoma lesions, the expression of PDGF was

more pronounced in the basal epithelial mass area, specifically in the microinvasion area at the front.

Another stage of our study was the evaluation of the vascular and lymphatic microdensity of the tumor stroma adjacent to the cases included in the study. Blood vascular microdensity (MVD) ranged from 20-84 blood vessels/microscopic field x 200 with a mean of 41.78 blood vessels. No correlation was found between PDGF and PDGFR beta expression and MVD. PDGFR alpha expression correlated with both MVD and LMVD (lymphatic vascular microdensity) ($p<0.036$ and 0.05, respectively). We found a significant correlation between PDGFR beta expression and both intratumoral and peritumoral LMVD ($p<0.045$, and 0.032, respectively).

Conclusions : Our findings suggest that PDGF mediates the interaction of cancer cells with cancer-associated fibroblasts, promoting cancer cell proliferation in a paracrine way. This has implications for innovative combinatorial cancer therapy.

4. PERSONAL CONTRIBUTION : FEASIBILITY AND DIAGNOSTIC ACCURACY OF ULTRASTAGING IN THE DETECTION OF MICROMETASTASES IN SENTINEL AND NON-SENTINEL LYMPH NODES IN CERVICAL CANCER: A SINGLE-CENTER RETROSPECTIVE STUDY WITH A FIVE-YEAR FOLLOW-UP PERIOD

This study aimed to evaluate patients with cervical cancer by ultrastaging all the lymph nodes (LN), sentinel LN (SLN), and non-SLN, to increase the sensitivity of the detection of LN metastases and the diagnostic accuracy in cervical cancer with a five-year follow-up.

Materials and methods: This is a retrospective study of 14 cervical cancer cases from 2017 to 2019 at the Municipal Emergency Clinical Hospital of Timisoara, Romania. The cases were selected based on their high risk of LN involvement but negative intraoperative pathologic LN. After re-evaluating all paraffin block biopsy samples from 29 cases, 14 cases were included in the study, which met all criteria for ultrastaging on surgical biopsy samples.

Results: Patients' ages included in the study ranged from 43 to 70 years (median: 57.14 years). According to the International Federation of Gynecology and Obstetrics (FIGO) staging, the majority of the patients were

in stage IB: seven cases (50%). The study revealed a positive correlation between patient age and FIGO staging, with Pearson's correlation coefficient of 0.707 and a p-value of less than 0.05, indicating that older patients were more likely to be diagnosed with a higher FIGO stage. The mean follow-up was 34.5 months, and the median follow-up was 36 months (range: 6-60 months). We obtained 167 nodes, with a mean of 11.92 nodes/case. Twenty-one LN were found to be positive with the ultrastaging method. We detected 11 LN with macrometastases (MAC) (52.38%), seven with micrometastasis (MIC) (33.3%), and three with tumor cell islets (14.4%). That would be 13% of newly diagnosed ultrastaging cases as positive nodes. This ultrastaging method detected nodal MIC in 8 (57.1%) out of the 14 patients, who initially tested negative for LN involvement using the routine Hematoxylin and Eosin (HE) method. The detection of micrometastases in these patients underscored the superior sensitivity of ultrastaging, which was further highlighted by the subsequent relapse of four (28.57%) out of these eight patients. The study also found no correlation between the FIGO standardization and the number of MIC found in these patients.

Conclusions: Predicting LN metastasis (LNM) in cervical cancer is crucial for improving survival rates and reducing recurrence. Very few small cohort studies used

an ultrastaging method to assess non-SLNs; most of them only assessed SLNs. We showed in our study that the ultrastaging method, both in the case of SLN and non-SLN, is superior compared with H&E analysis, with a 13% rate of new positive nodule diagnosis. Metastatic involvement of non-SLN was found in over 50% of all cases (8/14) according to the ultrastaging method. Additionally, our study confirms that the sensitivity of SLN ultrastaging is high for the presence of both MIC and MAC in SLN. As a result, we feel that ultrastaging is the most effective method for SLN analysis in patients with early-stage cervical cancer, and bilateral detection is preferable, significantly reducing false-negative results. The routine use of SLN along with ultrastaging would lead to more accurate surgical staging and better oncological follow-up of cases.

IV. GENERAL CONCLUSIONS AND ORIGINAL CONTRIBUTIONS

This doctoral thesis aims to conduct a multidisciplinary study of cervical cancer that will combine microscopic aspects with clinical-pathological data in order to define new diagnostic and therapeutic targets. The study included both clinical and experimental research in the context of cases selected during the COVID-19 pandemic. In this aspect, we can consider that the objectives of scientific research have been achieved and even exceeded by the unscheduled insertion in scientific research of the impact of SARS-CoV2 on the evolution and prognosis of cervical cancer. In this original aspect of the thesis, this research has exceeded the clinical and experimental limits, taking into account the social factor not studied in most malignant lesions. Such a social event, through its components, can cause an unfavorable evolution of cervical cancer only by its presence, increasing the number of cases diagnosed at the first presentation of the patient and being part of high pathological categories. This aspect, at least in our geographical area, can be considered one of the original contributions of our PhD thesis.

Another objective of our study was to focus on the stroma of cervical cancer. Usually, the study of cervical cancer is focused on the malignant epithelial component, from diagnosis to the specific tests needed for therapy and the prediction of prognosis. This PhD thesis had as its main objective the cellular components of stroma not studied in the literature but which recently have had an impact on the response to therapy very important, and to a lesser extent the malignant epithelial component only inserted and studied in close interrelation with tumor stroma (histopathological types) of cervical cancer.

Due to immunotherapy as an innovative therapeutic measure in different types of malignancies, including cervical cancer, we considered it useful to focus scientific research on the inflammatory component of cervical cancer and more specifically on a structure that appeared before or during immunotherapy, respectively, tertiary lymphoid structures (TLS). On this issue, as an original part, we can mention the identification for the first time of a particular type of TLS, namely TLS associated with nerves—the microscopic structure that was not described previously. We have demonstrated in this study a statistically significant correlation between the presence of this particular TLS type and the biological clinical parameters of patients with cervical cancer. Of these biological clinical parameters, we found the most important were lymphovascular invasion, peripheral lymphocyte counts, monocytes, and age. Relative to age, we have demonstrated significant differences in the presence of this inflammatory structure organized from the cervical stroma to the premenopausal and menopausal stages. This can also be considered a matter of the originality of this thesis.

Many innovative therapies are targeted primarily by the family of PDGF factors and their corresponding receptors, which are little studied in cervical cancer. For this reason, we considered it useful to include in our study the two respective PDGF

components: the PDGF-BB ligand and the PDGFR-beta receptor. Their expression on the epithelial component prior to this study was well known. Through this study, we demonstrated the heterogeneity of the expression of the two markers in the stroma of cervical cancer. This chapter of the research was limited to a descriptive evaluation of the stromal cell types, in which they were positive and the vascular layer was positive as well. If the TLS assessment concerned the immunological component of the stroma, the PDGF and PDGFR studies covered the vascular component through their two representatives, respectively, the blood and lymphatic vascularization. The results of the PDGF study were limited to the identification of statistically significant correlations between blood and lymphatic microdensities in the cervical tumor stroma. These correlations indirectly suggested the involvement of PDGF in stimulating the lymphatic and blood vasculature and implicitly the promotion of local and remote lymphovascular dissemination, common in advanced cervical cancer at the time of diagnosis.

Another original contribution to note in this thesis was the correlation of the presence of tertiary lymphoid structures with peripheral levels of inflammatory cells of the leukocyte group, in particular lymphocytes and monocytes. Linked to this, one of the technical drawbacks of this study was the impossibility of taking blood samples and storing them in a tissue bank for further use in order to dose the adjacent serum inflammatory factors that may have provided further information on the correlation between periphery and stromal tissue adjacent to cervical cancer.

Predicting lymph node metastasis (LNM) in cervical cancer is crucial for improving survival rates and reducing recurrence.

An original contribution to note in this thesis is the fact that with the ultrastaging procedure, we demonstrated improved sensitivity in detecting LN involvement. Although international societies promote the treatment, it has yet to be standardized, and there are currently no guidelines available for the ultrastaging method. Our study used an ultrastaging method to assess non-SLNs; most studies only assessed SLNs.

We feel that ultrastaging is the most effective method for SLN analysis in patients with early-stage cervical cancer, and bilateral detection is preferable, significantly reducing false negative results.

Another technical and economic disadvantage of the present study is the interposition during the study of the COVID-19 pandemic, which delayed the execution of the research but in particular restricted the carrying out of the fundamental research of the present study, as well as the restriction of the patient's presentation to periodic checks and the cessation of surgical interventions for a limited period. Based on this technical-economic disadvantage and not being a programmed objective, we thought it useful to introduce the chapter on the impact of the SARS-COV-2 pandemic on cervical cancer in the area where we are working. SARS-COV-2 gave us a new insight into the fact that restricting the access of patients to screening and diagnosis leads to the diagnosis of cervical cancers in more advanced stages, followed by a worsening of the patient's prognosis in the long term.

The present study, through its results, has opened up new avenues of research in the field. It can be considered a preliminary study in terms of the immune and vascular assessment of the cervix stroma. The remaining outstanding issues in this study are the lack of immunophenotyping of nerve-associated TLS and classic TLS to observe differences in lymphocyte cellularity that may have an impact on immunotherapy in cervical cancer. Furthermore, failure to detect serum PDGF levels in cervical cancer patients makes it impossible to obtain a complete picture of the impact of this growth factor on vascular and lymphatic microdensity, implicitly on the objective evaluation of the profile and rapidity of lymphovascular invasion with an immediate and remote

prognostic role. All the outstanding issues mentioned above are future research directions for this study.

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