THE CYTOLOGICAL, HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF CERVICAL SQUAMOUS INTRAEPITHELIAL LESIONS

THESIS DIRECTOR:
UNIVERSITY PROFESSOR DOCTOR: SIMIONESCU CRISTIANA EUGENIA

STUDENT:
MUNTEAN MIHAELA

CRAIOVA, 2012
INTRODUCTION

For about 50 years, in developed countries, the detection of cervical cancer using the cytological Papanicolaou test had a gradual evolution; but recently this method was subject of a real revolution. In these countries, screening tests were implemented and the cervical cancer incidence was reduced with about 80%. The objective of these programs is the identification of pre-invasive and invasive lesions of the cervix. This way, this type of lesions can be found and treated in order to prevent the invasion stage acquisition. The exact quantification of this type of lesions is very important because of their different regression, persistence and progression rate which are very different for each degree of neoplasia. This could have important prognostic and therapeutic implications and could also help in clinical monitoring of patients.

ESSENTIAL BIOCHEMICAL EVENTS IN CERVICAL CARCINOGENESIS

Oncogenical high-risk types of HPV produce two proteins which have an important role in growth stimulation and transformation: the E6 and E7.

The oncoprotein E7 is mainly responsible the malignant transformation and immortality of cells infectected with oncogenical high risk HPV. The oncoprotein E6 strongly enhances the activity of E7. Analysis of E6 and E7 mutants show that the link between Rb and p53 is very important for the cellular transformation and immortality.

During the viral life cycle, apparently E2 acts as an important regulation factor of the E6-ORF and E7-ORF expression by inducing the inhibition of E6 and E7-ORF. By doing so, the cellular proliferation is controlled [276].

Breaking the link E2-ORF but keeping that between E6 and E7-ORF can cause a non-systematic expression of E6 and E7-ORF and an uncontrolled cellular proliferation.

THE MOTIVATION AND THE PURPOSE OF THE STUDY

The present study is an evaluation of the markers involved the early stage of cervical carcinogenesis in order to identify possible prognostic and therapeutic targets. Research in this domain is recent and if the desired results are obtained, this could contribute to an augmentation of patient's life quality and hope by avoiding complicated and mutilating surgeries.

SAMPLE AND METHODS

A. ANALYZED SAMPLES

The cases analyzed belong to a private medical unity of cytopathology in Slatina (Promed) and the study took place during the period of time 2007-2010. 9675 patients with a cervical cytology were examined: 917 were diagnosed with SIL or SIL-like modifications; 321 patients cytologically previously tested were examined both histopathologically and immunocytochemically; 56 were tested immunohistochemically and constituted the objective of this study.

B. EMPLOYED METHODS
For the **cytological** test, applied on 9675 patients with a cervical cytology, the Papanicolaou coloration method was employed. After the test, biological sample from 321 patients was taken by biopsies, endocervical curettage or diatermo-excision colposcopically guided. It was analyzed by the classical **histopathological** method. For each of the 321 patients, an extra-smear was collected for the **immunocytochemical** test in order to detect, using the Viroactiv-HPV HR Kit, the L1 **major capsid protein** of HPV. The **immunohistochemical** study was done on 56 patients morphologically diagnosed with cervical neoplasms and the expression of Ki-67, p16, c-erbB-2, E-cadherin, bcl-2, VEGF, CD105, CD34, D2-40 was regarded. We employed as a work method the two-stroke method, using the EnVision technique for protein amplification. The **morphometric analysis**, by the quantification method “hot spot”, was used for the quantification of the angiogenesis (CD31,CD105) and lymphangiogenesis (D2-40) potential of tumors. The **statistical analysis** used correlation coefficients (Pearson) and tests of comparison of means (test t-Student, ANOVA). To appreciate the dependency between two classification factors, the tables of incidence 2x2 have been used which were interpreted with the Chi-squared test.

**RESULTS**

**VI.1.CYTOLOGICAL STUDY RESULTS**

**VI.1.1 PERIOD OF STUDY**

The year distribution analysis of the statistical results showed that the higher incidence rate happened during the year 2008 with 2627 cases, followed in a descending order by 2007 (2546 cases), 2009 (2275 cases) and 2010 (2227 cases) respectively.

In accord with the Bethesda terminology, the interpretation of the smears analysis showed the following cytological categories: NLIM in 87.71% of cases, ASC-US in 5.45% of cases, ASC-H in 0.51% of cases, LSIL in 1.83% of cases, LSIL-H in 0.45% of cases, HSIL in 1.24% of cases, AGC in 1.82% of cases, carcinom scuamos in 0.17% of cases, adenocarcinom in 0.12% of cases, insatisfactory for evaluation in 0.70% of cases.

For the cytological study, we selected patients with major and minor squamose abnormalities. Not taking into consideration the carcinoms and the AGC categories, a total number of 917 patients (9.48%) were proven to be: ASC-US in 527 cases, ASC-H in 49 cases, LSIL in 177 cases, LSIL-H in 44 cases, HSIL in 120 cases.

With an important difference, the distribution on living mediums showed that women belonging to the urban medium have much higher chances 642/917 (70%) to develop such a disease compared to those from rural mediums (275/917 which is 30%).

**VI.1.2 GROUPES OF AGES**

The age distribution of patients with cellular squamoses abnormalities showed that the age groupe 31-40 is the most numerous. The most frequent result of the cytological test was ASC-US and the age group 21-30 was the predominant one. Patients having the next cytological test results: ASC-H, LSIL, LSIL-H and HSIL, the interval with a maximum prevalence was situated between 31 and 40 years.

**VI.1.3 INTERNAL CONTROL OF THE LABORATORY**
During our study we have enregistered 0.70% of smears that were not suited to be evaluated. The incidence rate of ASC-Us was of 5.45% and that of ASC-H was estimated at 0.51%. The incidence rate of ASC-H represented 8.51% of the total number of ASC identified. The fraction ASC/SIL had a value of 1.69 in our case.

**VI.1.4. THE CYTOLOGY OF SQUAMOUS ABNORMALITIES**

- The cytomorphology of ASC-US patients' smears was constituted of smears with mature squamous, intermediate (ASC-US „pure” or ASC-US „mature”), isolated or organised in small groups cells, with 2,5 to 3 times increased nuclei.
- The cytomorphology of ASC-H patients' smears was constituted of smears with atypical metaplastics cells, isolated or in small groups, non-cohesive, with a syncytial patter (about 10 cells) and atypical para-keratinized cells, also isolated or in small groups.
- The cytomorphology of ASC-H patients' smears was characterised by cells with size similar to those of superficial (S) or intermediate cells (I), with big nuclei (3 to 6 times bigger that those from normal I cells), koilocytes and dyskeratocytes.
- The cytomorphology of LSIL-H patients' smears was constituted of smears compatibles with LSIL cytology, associated with some isolated or grouped cells of ASC-H type.
- The cytomorphology of HSIL patients smears included some more severe cytological abnormalities, either concerning the true I cells or the PB cells (like CIN2). Cells with severe dyskaryosis of B (basal) and PB (parabasal) size were interpreted like CIN3.

**VI.2. THE HISTOPATHOLOGICAL STUDY OF SQUAMOUS INTRAEPITHELIAL LESIONS**

After the histopathological examination we were able to identify 177 (55.14%) squamous intraepithelial lesion cases.

Morphologically, most LSIL/CIN1 cases (70/97 cases, which is 72.17%) were plane, conventional forms. In 8 cases (8/97 cases, which is 8.25%) we described LSIL keratinized. In a small number of patients (4/97 cases, which is 4.12%) we identified the immature papillary metaplasia (PIM, immature condyloma). In 4 cases (4/97 cases, which is 4.12%) we described the inverted condyloma (endophytic form). In the case of 3 patients (3/97 cases, which is 3.13%) we identified atypical immature squamous metaplasia (AIM). In only 2 cases (2/97 cases, which is 2.06%) we diagnosed a variety of LSIL-LSIL with marked atypia (LSIL-MA).

From a morphological point of view, we classified 56 cases (56/80 cases, which is 70%) as HSIL/CIN2, the conventional CIN2 sub-type. 3 cases (3/56 cases, which is 5.36%) were diagnosed as a particular form of CIN2, the eosinophilic dysplasia.

24 lesions from the total number of HSIL (24/80 cases, which is 30%) were considered as HSIL/CIN3 (including severe dysplasia and in situ carcinoma), most of them being a morphological CIN3 sub-type with big and non-keratinized cells.

Very rarely (2/24 cases, which is 8.33%) we have encountered the morphological variety -CIN3 with small anaplastic cells or the morphological forme -CIN3 with big keratinized cells (1/24 cases, which is 4.17%)

The cases with multiple associated lesions we reported the diagnostic of the most
severe lesion. These cases were identified in 14.29% of CIN2 (8/56) and 62.5% of CIN3.

VI.3. CORELATIONS BETWEEN THE DIFFERENT LESIONALS CYTO-HISTOLOGICAL CATEGORIES. PERFORMANCE OF PAPANICOLAOU TEST

In the studied group, 10.26% of NLIM smears were false-negatives.

In the case of patients with an ASC-US cytology, the dysplasia rate for the histological threshold CIN2+ was of 15.58% (12.99% CIN2 and 2.59% CIN3). In the ASC-H category, we identified a dysplasia rate of 56% for CIN2+ (24% CIN2 and 32% CIN3). Patients with a cytological LSIL presented a dysplasia rate for CIN2+ of 27.63% (26.31% CIN2 and 1.32% CIN3). In the case of patients with a LSIL-H cytology, we cytological identified 62.5% of CIN2+ (50% CIN2 and 12.5% CIN3). HSIL patients have provided a CIN2+ dysplasia rate of 73.68% (21.05% CIN2 and 52.63% CIN3).

For the evaluation of Papanicolaou test performance and in the cytological detection of CIN2+, we have calculated the sensitivity, specificity, the positive predictive value and the accuracy of Pap-test. For the cytological threshold of ASC-US+, LSIL+ and HSIL we obtained a highly negative corelation between the sensitivity and the specificity of the cervical cytologic. In our study, the positive predictive value increased with the increase of the cytological interpretations threshold. The biggest value of accuracy was detected fot the LSIL+ threshold (68.84%).

VI.4. THE IMMUNOCYTOCHEMICAL STUDY OF SQUAMOUS INTRAEPITHELIAL LESIONS

The immunocytochemical colorations of smears obtained from 321 patientes, showed 56 positives immunocolorations belonging to the next cytological categories: 4 from NLIM, 20 from ASC-US, 3 from ASC-H, 23 from LSIL, 3 from LSIL-H and 3 from HSIL.

The 144 histologically negatives cases (non-CIN) were L1- on a cytological correspondance.

From the total number of 97 cases histologically confirmed as CIN1, 46.39% (45/97) were provided by immunocytocolorations L1+ and the rest of 53.61% (52/97) were immunocytocolorations L1-.

19.64% (11/56) of CIN2 histologies were associated with the immunocytocoloration L1+, the most part of CIN2 histologies, 80.36% (45/56) having a L1-immunocytochemical correspondent. All cytological categories (24/24, 100%) which were histological CIN3 were immunocytochemically L1-.

VI.5. THE IMMUNOHISTOCHEMICAL STUDY OF SQUAMOUS INTRAEPITHELIAL LESIONS

From a total number of 177 pre-invasives studied lesions, only 56 were investigated by immunohistochemical tests. They included CIN-type lesions with different severity degrees, 24 CIN1 cases, 18 CIN2 cases and 14 CIN3 cases.
VI.5.1 THE KI-67 IMMUNOEXPRESION STUDY IN SQUAMOUS INTRAEPITHELIAL LESIONS

All the CIN studied cases (56/56 cases, which 100%) were Ki-67 positive, so that there was no lesion reported with a score of 1. A score of 2 was strictly accorded in low degree neoplasia cases. The next cases reported a score \( \geq 3 \): high degree neoplasia cases, CIN2,3. The percent of positive cells and the reaction intensity increased in the same time as the histopathological degree of CIN and extended from the basel layer to superficial epithelial layers. The ANOVA test indicated significant differences in Ki-67 immunoexpression, depending on the differentiation degree of lesions \([F(2,53)= 104,96, p=0,000]\).

VI.5.2 THE P16 IMMUNOEXPRESION STUDY IN SQUAMOUS INTRAEPITHELIAL LESIONS

The p16 immunoexpression was proven to be positive in 82,14% of the total number of examined samples. The negative results were exclusively low degree neoplasia cases, CIN1. The p16 expression pattern and the reaction intensity gradually increased from CIN1 (score \( \geq 2 \)), to CIN2 (score \( \geq 3 \)) and than to CIN3 (score of 4). It had a specific location and was proportional to the lesions degree of severity. The ANOVA test indicated significant differences in p16 immunoexpression, depending on the differentiation degree of lesions \([F(2,53)= 98,32, p=0,000]\). The Chi-squared test showed significant differences in p16 immunoexpression depending on the proliferation index Ki-67, \( \chi^2(6, N = 56) = 50,5, p = 0.000 \). Pearson test indicated a significant positive linear correlation between the values of Ki-67 and p16, \( r(54)=0,830, p=0,000 \).

VI.5.3 THE IMMUNOHISTOCHEMICAL EVALUATION OF C-ERBB-2 IMMUNOEXPRESION IN SQUAMOUS INTRAEPITHELIAL LESIONS

The c-erbB-2 immunoexpression showed positives results in 14,3% of the total number of examined cases, exclusively identified for the CIN2+ threshold, distributed as follows: CIN2 in 11,1% of cases and CIN3 in 42,9% of cases. The ANOVA test indicated significant differences of c-erbB-2 immunoexpression in CIN1 lesions compared to that in CIN2 and CIN3 lesions, \([F(2,53)= 18,27, p=0,000]\). The Chi-squared test showed significant differences in c-erbB-2 immunoexpression depending on the proliferation index Ki-67, \( \chi^2(4, N = 56) = 28,1, p = 0.000 \) and on p16 score, \( \chi^2(4, N = 56) = 28,1, p = 0.000 \).

VI.5.4 THE IMMUNOHISTOCHEMICAL EVALUATION OF E-CADHERIN IN SQUAMOUS INTRAEPITHELIAL LESIONS

The E-cadherin expression resulted positive in 89,3% of the total number of CIN cases, being distributed as follows: 100% of CIN1 number of cases cases, 100% of CIN2 number of cases and 57,1% of CIN3 number of cases. The aberrant expression of E-cadherin was proportionally correlated with the degree of neoplasia, having the highest
values in cases with aggressive lesions (50% in CIN3). The ANOVA test indicated significant differences of E-cadherin immunoexpression in CIN1 lesions compared to that in CIN2 and CIN3 lesions, \(F(2,53)= 91.09, p=0.000\). The Chi-squared test showed significant differences in E-cadherin immunoexpression depending on the proliferation index Ki-67, \(\chi^2(6, N = 56) = 25.17, p = 0.000\).

VI.5.5. THE IMMUNOHISTOCHEMICAL EVALUATION OF BCL-2 IN SQUAMOUS INTRAEPITHELIAL LESIONS

Positives results for the bcl-2 were reported in 35.7% of the total number of examined cases, in the limit of a score of 3. The immunoexpression increased with the lesion's degree of severity, from 8.3% in CIN1 cases, ranging to 44.4% in CIN2, and even to 71.4% in CIN3 cases. The bcl-2 immunocoloration pattern identified in our study, was exclusively a basa one. The ANOVA test indicated significant differences of bcl-2 immunoexpression depending on the differentiation degree of lesions, \(F(2,53)= 12.65, p=0.000\). The Chi-squared test showed significant differences in bcl-2 immunoexpression depending on the proliferation index Ki-67, \(\chi^2(6, N = 56) = 25.17, p = 0.000\). Pearson test indicated a significant positive linear correlation between the values of Ki-67 and Bcl-2, \(r(54)=0.646, p=0.000\).

VI.5.6. THE VEGF, CD105 (ENDOGLIN), CD34 AND D2-40 IMMUNOEXPRESION IN SQUAMOUS INTRAEPITHELIAL LESIONS

The VEGF immunoreaction was identified at a cytoplasmic level in 37 cases (66%). In CIN1-type of lesions, the average index of positives results was of 25.5%, in CIN2 of 50.3% and in CIN3 was of 78.5%, respectively. The ANOVA test indicated significant statistical differences between the average index values for obtaining positives results for VEGF immunoexpression, depending on the differentiation degree of lesions, \(F(2,53)= 83.47, p=0.000\) and on Ki-67 proliferation index \(F(2,53)= 31.93, p=0.000\).

The CD105 immunomarking was identified in all the analyzed cases. MVD CD105 in the case of CIN lesions increased in the same time with the decrease of the pre-invasive lesions differentiation degree, values ranging from 3 to 28 elements/microscopic field (MF). ANOVA test indicated significant statistical differences between the average values of MVD CD105, depending on the differentiation degree of lesions \(F(2,53)= 137.08, p=0.000\). Also, it indicated significant differences between the average values of MVD CD105 depending on Ki-67 proliferation index, \(F(2,53)= 16.02, p=0.000\).

The CD34 immunoexpression was identified in all the analyzed cases. The density of MVD-CD34 had higher values in the case of low differentiation CIN lesions and the number of vessels identified per microscopic field was between 7 to 30 elements/MF. ANOVA test indicated significant statistical differences between the average values of MVD CD34, depending on the differentiation degree of lesions \(F(2,53)= 112.85, p=0.000\) and on Ki-67 proliferation index, \(F(2,53)= 14.46, p=0.000\).

The D2-40 immunoreaction was identified in 100% of the number of analyzed lesions. The cytoplasmic marking was present in the case of cells that have bounded
lymphatic spaces and in the case of basal epithelial cells. The number of marked lymphatic vessels varied, ranging from 1 to 12 elements/MF. ANOVA test indicated significant statistical differences between the average values of LVD D2-40, depending on the differentiation degree of lesions [F(2,53)= 33,84, p=0,000] but also insignificant differences between the LVD values in CIN2 and CIN3 respectively [F(1,30)= 1,94, p=0,173] which suggests that there are statistical differences between high degree and low degree lesions.

VII. DISCUSSIONS

The results of the cytological study respect the Bethesda System which recommends that the ASC-US frequency should be maximum 5% of the total cytological cases, ASC-US should represent 5-10% of the ASC diagnostics and the SIL incidence should not exceed 2-3 times [235].

Establishing a sure diagnostic – for squamous intraepitelial lesions, especially for the CIN1 and CIN2 diagnostics – is known to be difficult [43], as professional histopathologists have proven that there is a significant variability in quantifying the cervical histological specimens [204]. The coexistence of multiple lesions in the same histological specimen is often confirmed [27], but it is commonly accepted that generally a complexity of lesions is not reported because, in most cases, it is not clinically relevant [254].

A comparison between cytological and histopathological results is essential in cytopathological laboratories and it is recommended by the European guides in order to insure their quality, to develop the performances of the laboratory and especially to avoid false-negative results [291].

Several authors choose the viral capsid HPV-L1 as a prognostic marker to identify the potential of a malignant squamous neoplasia [120], others suggest that HPV-L1 should be used as a screening instrument, to classify cases like L1+, L1- as lesions with respectively low and high malignant potential [107].

It has been proven that the Ki-67 immunomarking, at an index of over 30% is highly predictive for malignant or premalignant lesions [45], and Mayerhofer K and his collaborators consider in their study that the ki-67 overexpression is an indicator of biological aggression [175].

In this study, the positivity of p16 test in all the 56 CIN cases, was found in 82.14% of the samples, the smallest values corresponding to CIN1. These results confirm the reports made by Tringler B et al or Simionescu C et al [272, 245].

Pinion SB et al, found that c-erbB-2 overexpression is more frequently reported in high-grade lesions (CIN3), suggesting that this oncoprotein is implicate in the early stages of carcinogenesis [211].

Although a direct relation between the expression of the E-cadherin and the degree of CIN wasn’t found in this study, an abnormal coloration of the membrane and cytoplasm was observed, especially in aggresive types of lesions.

Recent studies show the bcl-2 expression in the basal, parabasal, intermediate and superficial layers predicts a progressive evolution towards a highly severe lesion, therefore, the significance of the bcl-2 expression for the diagnostic and prognostic of inner epithelial cervical lesions remains to be debated.
It has been proven over the last years that the VEGF expression, in CIN as well as in invasive carcinoma, is associated with microvascular density (MVD) [154].

In this study, like in other reports, the evaluation of the lymphatic vessel density could suggest an early lymphangiogenesis in cervical carcinogenesis [262].

VIII. CONCLUSIONS

-This study presents a certain uniformity of solicitation in cytological testing, between 2007 and 2010.
-The cytological study highlights a total percentage of 11.58% of patients with cytological anomalies, and the incidence of 1.24% of cytological category HSIL suggests a high risk of HSIL for these patients.
-There has been a significant participation of women from urban zones (70%), in comparison with those from rural zones (30%).
-Maximum age interval was of 31-40 years, except for the ASC-US category, where it was of 21-30 years.
-Quality control of the laboratory highlighted values which were compatible with those recommended by the Bethesda System.
-The LSIL cytomorphology represented the most reproducible cytological category, whereas the ASC-H one was the most heterogeneous.
-Morphologically, 72.17% of LSIL/CIN1 were represented by plane, conventional lesions, and in 27.83% of the cases we were able to identify particular morphological aspects of these lesions.
-Most of the HSIL/CIN2 cases (94.64%) were classical conventional morphological ones, and a small percentage of these (5.36) were diagnosed as eozinophilic dysplasia.
-In this study, 87.5% of HSIL/CIN3 lesions were represented by the subtype with big nonkeratinized cells, 8.33% by the small anaplastic cells type and only 4.17% were represented by the big keratinized cell type.
-When associated lesions were found, the lesion of highest degree was reported.
-Cyto-histological correlations showed for the CIN2+ histological threshold a rate of dysplasia equal to or higher than that reported in international studies.
-LSIL-H may represent a distinctive cytological category in Bethesda terminology.
-Evaluation of the Papanicolau test in the histological detection CIN2+ has given results which are similar to those of international studies.
-The HPV-L1 immunocoloration contributed to the arbitration of the therapeutic attitude or the subsequent monitor of the patient.
-The Ki-67 imuno-expression proved to be positive in all studied lesions, with significant statistical differences of the ki-67 expression depending on the CIN degree (p=0.000).
-The p16 imuno-reaction was positive in 82.14% of the examined samples. Negative results (17.86%) were given exclusively by CIN1 neoplasias.
-The c-erbB-2 expression was positive in 14.3% of the cases and it was exclusively identified at the CIN2+ threshold, significantly different in CIN1 from CIN2, CIN3 (p=0.000) and from ki-67 (p=0.000) and p16 score (p=0.000).
-E-cadherine expression was positive in 89.3% of the CIN cases. The abnormal expression was highest in the aggressive lesions (50% of CIN3). Statistical analysis proved significant differences of the expression in CIN1 from CIN2,3 (p=0.000) as well as from the proliferate index ki-67 (p=0.000).
Bcl-2 was positive in 35.7% of cases, with an exclusively basal pattern, below score of 3. The expression was very high in high degree lesions. Statistical analysis indicated the existence of an antiapoptotic mechanism of cell proliferation.

- Statistical analysis between Ki-67 and p16, C-erbB2 and E-cadherine indicate the existence of alternative mechanisms that insure lesion progression.

- VEGF expression was positive in 66% of CIN cases. The medium index of positive cases was highest in CIN3 (78.5%), thus statistically proving that there is a linear relationship between the CIN degree and the VEGF expression as well as between VEGF and proliferation index ki-67 (p=0.000).

- CD105 marking was identified in all 56 CIN cases, with statistically significant differences between medium values of MVD CD105 and the degree of neoplasia (p=0.000) and the proliferation index ki-67 (p=0.000).

- CD34 expression was found in all CIN cases, its highest medium value being reported in CIN3 (23.1 ± 2.93/MF). Statistically significant differences were found between the medium value of MVD CD34 and the degree of differentiation of lesions (p=0.000), as well as the index ki-67 (p=0.000).

- Statistic analysis suggests that angiogenesis represents an essential mechanism for proliferation of lesions and sustains the viability of CD105 and CD34 for the study of cervical angiogenesis.

- D2-40 expression, identified in all the cases, grows in an important manner and progressively along with the degree of cervical neoplasia and with the existence of statistic differences between high and low degree lesions.
SELECTIV BIBLIOGRAPHY


254. **Stoler MH.** It is more complicated than you think: doctor, which HPV-type caused this lesion?. HPV Today. Newsletter of Human Papillomavirus. 2010 Feb; 20: 4-6.

