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PRECANCEROUS CERVICAL LESIONS AND IMMUNOMARKERS FOR THEIR PROGNOSIS

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ABSTRACT. Cervical cancer is a disease which still affects too many women, even if we live in the XXIst century. Pap smear represented for few decades an important tool in the diagnosis of this pathology and is consider the most important screening method with a good acceptance from the patients. We know the importance of human papilloma virus (HPV) infection in cervical malignant pathology and its evolution. The diagnosis of presence of HPV-high risk type can give a prognosis for precancerous lesions. These lesions appear with months or years before malignant affections of the cervix and if we put the diagnosis of an increased cell proliferation, or we demonstrate the infection with HPV-HR, we can use this time to monitories or give the appropriate treatment for the precancerous lesions. There are immunomarkers for these kind of investigations and from them, we used Ki-67 and p53, trying to demonstrate the usefulness of immunochemical markers in the diagnosis and prognosis of cervical precancerous lesions.

Keywords: cervical precancer, HPV, p53, Ki-67, immunomarkers

INTRODUCTION

Cervical cancer is unfortunately very frequent, being the second most common cancer at women in the worldwide, and the most important cause of women's death in Romania. For that reason, there are investigations which are made to diagnose the precancerous cervical lesions, but the Pap smear was the one who represented a revolution in laboratories tests for cervical cancer. For many years, this simple but very efficient test represented the best tool for the first steps in diagnosis of cervical dysplasia.

The HPVs are a family of DNA viruses with more than 150 genotypes. More than 40 of these genotypes infect the anogenital tract and they are the cause of local diseases from genital wart to invasive cancers. The types which are considered more carcinogenic in humans are HPV-16 and HPV-18. Integration of HPV DNA into a host genome has been hypothesized to play an important role in the carcinogenesis of HPV-related carcinomas. It is considered that high grade lesions might have origin in mild dysplasia or can arise directly from infection by high risk HPV types (HR-HPV). High-grade squamous intraepithelial lesions are usually monoclonal and present HR-HPV genotypes in 90% of cases. In these lesions is a high expression of viral oncoproteins E6 and E7, which are responsible for the induction and maintenance of transformed types cervical cancer cells. (Masciullo et al., 2007)

Knowing that HPV is the promoter in cervical cancer, we have to use this information in the monitoring of the patients with precancerous lesions highlighted by Pap smear. SIL is the morphological manifestation of HPV replication and protein expression. Moreover, the presence of active squamous metaplasia appears to play an important role in the support of viral replication. The guidelines proposed that once screening was initiated, Pap smear should be made at every 3 years if three consecutive annual Pap smears were normal, except for "high risk" women who should be screened annually.

Because the adolescent cervix is structurally and biochemical different of adult women cervix, is more vulnerable to HPV infection, favored by multiple sexual intercourse, sexually transmitted disease, smoking. Once sexual activity is initiated, active immature squamous metaplasia occurs. This process is characterized by replacement of the columnar epithelium by rapidly proliferating immature squamous epithelium. This rapidly proliferating cellular population is presumably vulnerable to HPV infection resulting not only in replication of the virus, but also accompanying viral induced genetic alterations in the host metaplastic squamous epithelium, which if infection persists can lead to HSIL. (Moscicki, 2007)

Approximately, 50% of adolescents and young women acquire a cervical HPV infection within 5–7 years after initiating sexual intercourse, with the highest risk factor being a recent new sexual partner (Moscicki et al., 2001). Low grade squamous intraepithelial lesions (LSIL) are the morphological manifestation of cervical HPV infections and high rates of LSIL would therefore be expected in this group. The frequency of LSIL is much higher in adolescents than in older women (>30 years). However, it is important to emphasize that HPV detection in adolescents is most commonly associated with normal cytology. More than three-quarters of infected adolescents have normal cytology (Moscicki et al., 2001)

The cervical lesions associated with high risk HPV type (HR-HPV), have an increased risk for an evolution more or less fast to cervical cancer. The HPV infection is diagnosed by PCR test, but there are a lot

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of immunomarkers which can be used to put in evidence the potential of proliferation of the lesion. This is a sign for bad prognosis and is possible to appreciate the evolution of a precancerous lesion to cervical cancer.

Although, epidemiological studies show that more than 80% of HPV infections are benign and cleared within 12–18 months (Hildesheim et al, 1994), a fraction of infections persists and can initiate cellular transformation. It is the persistent infection with high-risk type HPV that is necessary (but not sufficient) for the development of squamous carcinomas of the cervix and their precursor intraepithelial lesions (Ho et al., 2004, Ferenczy et al., 2002). Premalignant lesions of the cervix are characterized by abnormal cellular or epithelial architecture in the areas surrounding the junction between the squamous and columnar epithelium (the transformation zone) of the uterine cervix. Nuclear enlargement, hyperchromasia, binucleation, presence of abnormal mitoses, high nuclear to cytoplasm ratios, koilocytosis, abnormal epithelial differentiation, increased mitotic activity, and irregular cellular orientation are all typical features of dysplasia (Hannah H. Alphs et al., 2007).

Even if LSIL and HSIL (high grade squamous intraepithelial lesion) are associated with HPV, they can also regress and especially at higher rates in adolescents compared with older women (Syrjanen et al., 1992; Nassiel et al., 1983; Nash et al., 1987). Studies in adult women show that 60–80% of LSIL will spontaneously regress and 20–30% will progress to HSIL. The slower rate of regression of LSIL observed in older women compared with adolescents is most likely because of an infection that is already persistent. Persistence of viral infection has been shown in many studies to be necessary for the development of significant precancers - HSIL (Moscicki et al., 1998; Koutsky et al., 1992; Brown et al., 1999). Another reason may be that the chance of the LSIL being misclassified is higher (the lesion is actually HSIL) in the older women because the prevalence of histological HSIL is higher in this older age group. In more recent studies (Lee at al., 1998; Schlecht et al., 2003) about 80–90% of LSIL was shown to regress in adult women.

The progression rates of squamous intraepithelial lesions remain unknown because all studies are time-limited. Cox et al. in 2003 showed that 12.8% of cervical lesions diagnosed at patients, would progress from LSIL or ASCUS/HPV-positive to HSIL within 2 years (Lee at al., 1998; Cox et al., 2003) In a longitudinal study of adolescents and young women (Moscicki et al., 2004), only 3% of LSIL in adolescents and young women progressed to HSIL within 3 years. A retrospective chart review of adolescents less than 19 years of age with cytological LSIL found that 31% progressed to HSIL by 36 months (Wright et al., 2005)

The most commonly HPV involved in the etiology of cervical dysplasia and cervical cancer are HPV 16 and HPV 18. Some specialized studies shows that more frequent and with increased aggressiveness in cervical adenocarcinoma is HPV 18 (Constantinescu et al., 2007) and in squamous cervical epithelium an aggressive trend and frequency is for HPV16. (Sahebali et al., 2003)

The most part of low grade squamous intraepithelial lesion (LSIL) will regress spontaneously and from high grade squamous intraepithelial lesion (HSIL), only 10-20% will have an evolution to cancer. (Masciullo et al., 2007) The molecular changes take place in time and if they are detected and treated early, the cervical cancer is prevented. For the diagnosis of potential progressively of precancerous lesions to invasive cancer, we can use some immunohistochemical markers which are specific for proliferation.

The tumorsuppressor gene p53 is implicated in the development of cervical cancer, because the p53 protein is inactivated by the E6 oncoprotein of human papillomavirus. This protein- p53, is an housekeeping gene able to recognize when DNA damage has occurred in a cell, arresting that cell in G1 phase of the cell cycle and allow for DNA repair or, if repair is not possible, to lead that cell into cell death. Even if mutation or deletion of the p53 gene is one of the most common genetic abnormalities in malignancies, in cervical cancer these are rare (Thomas et al., 1999) Instead, E6 binds with and inactivates p53, causing its degradation through the ubiquitin system (Scheffner et al., 1993). The inactivation of p53 by HPV E6 oncoprotein also leads to the upregulation of cyclin B (Kaufmann et.al., 1997), which regulates transition from G2 to M phase.

E6 oncoproteine has a transforming action relatively weak compared to E7, but has the ability to link up with a wide variety of regulatory proteins such as p53. This is an oncogene with role of guardian of the cell cycle and encodes p53 protein that is in the nucleus, being there in 2 forms: fosforilated and unfosforilated. Protein p53 has 2 major functions - transcription and inhibition of DNA replication by interfering with proteins needed for DNA biosynthesis. Protein p53 is activated when transcriptional genome occur in the DNA damage and has the characteristics of a suppressor gene tumor, his loss being associated with the occurrence of malignity. P53 is one of the E6 and E7 targets. In normal cells, p53 level is low, and p53 disappears in tumor cells, not specific changes induced by HPV infection. In uninfected HPV cells, the p53 response is increased to DNA or cell damaged and to aberrant proliferation of cells. Increased level of p53 induces cell blocking phase G1 cell cycle, this being an opportunity to repair DNA damage caused by stress, and to remove dead cells. In the HPV-infected cells, the p53 protein is low because of the binding E6 – p53 and degrades quickly, loosing the repair mechanism for p53. (Ardeleanu et al., 2007)

For high cervical lesions, it was monitorised p53 in time. A low frequency p53 positive cells in the basal epithelial layer, was associated with a high risk of

Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 20, issue 2, 2010, pp. 87-93

progression of lesion. (Wentzensen et al., 2006) Protein p53 has an important role in inducing apoptosis in response to DNA damage and is lost or mutated in more than 50% of human cancers. However, in cervical cancers, p53 mutations are rarely found. Although, one would expect low amounts of p53 protein in HPV positive tumors, several studies have reported over-expression of p53 in cervical neoplasm, without a correlation between p53 expression and positive HPV, tumor type, lymph node status, and disease-free or overall survival. (Kumar et al., 2006)

The link between p53 and cervical carcinoma induced by HPV infection has been studied more, trying to define p53 as a marker for progression of cervical lesions. It was used a batch of patients with cervical lesions and another one with normal cervical epithelium. These were investigated immunocytochemically for aberrant expression of p53 and bcl-2, and for the presence of HPV 6-11 and HPV 16-18 it was used PCR method. The immunoreactivity for p53 has been depending on the grade cervical lesion, the intensity increased with reactivity level of dysplasia. A significant positive correlation has occurred with the presence and types of HPV. It argues in this study (Grace et al., 2003), the possibility of using the p53 as marker for squamos cervical carcinoma associated with the HR-HPV infection.

Hiller et al. in 2006 try to demonstrate the link between p53 and HR-HPV strains, examining E6 protein from 19 strains of HPV. HR-HPV - 16, 18, 33, 35, 39, 49, 51, 52, 56, 58, 66, were capable of in vitro degradation of p53. LR-HPV - 6, 11, 44, 54, 61, have not degraded p53, and strains 53, 70 and 82 that are not clearly classified as risk in literature, have induced degradation of p53. This suggests the existence of significant functional differences between the behavior of the virus in vitro and in vivo.

The Ki-67 protein plays an important role in cell proliferation. Its antigen is expressed during the cell cycle with the exception of the G0 phase, and has been used as a marker for proliferation in various tumors, including cervical carcinoma. Previous studies have shown that application of Ki-67 immunoquantitative analyses of CIN1 and CIN2 in histological biopsies has strong independent predictive value for grade, presence of oncogenic HPV, and progression of the disease (Kruse et al., 2001; 2003). The best Ki-67-feature combination to predict whether a subsequent higher CIN grade or cancer will be detected in the follow-up, is the 90th percentile of the stratification index (Si90) and the percent of Ki-67 positive cells in the middle third layer of the epithelium (Kruse et al.,2003). Ki-67 prognostic value exceeds that of CIN grade (as CIN1 or CIN2) and the presence of HR-HPV types assessed by PCR, highlighting its clinical relevance in cervical cancer outcome.

Ki-67 is a nuclear protein expressed in cells that grows in active phases of the cell cycle, being considered a marker of proliferation and is one of those markers which may diagnose the cervical malignant proliferation, the immunoquantitative determination representing an adjunct diagnosis in grading CIN. Using the index of stratification (IS) mentioned before, is showed how deeply Ki-67 nucleus are located in epithelium, as it is higher, there is more advanced CIN, and the presence of Ki-67 in the cervix biopsy indicates incipient lesions progression to CIN 3. (Constantinescu et al., 2007)

MATERIALS AND METHODS

We used these immunomarkers to check their efficiency for the diagnosis and prognosis, on the casuistry from Clinical Hospital of Obstetrics Gynecology from Oradea. We took in study the patients admitted in our hospital between 2004- 2006, with the diagnosis of cervical dysplasia or suspicion cervical cancer.

In 2004 were admitted 115 patients with the diagnosis mentioned before. 50 of them (43.48%) were from rural areas and 65 of patients (56.52%) were from urban.

Distribution by age showed a predominance of fourth decade of age -34 patience (29.57%), followed at very low difference by the sixth decade – 28 patients (24.35%), and the fifth decade -26 patience (22.61%). Than came the seventh decade – 15 patients (13.01%), the third one – 8 patients (6.96%) and the eighth decade with 3 patients (2.61%). We had one patient of 81 years old with suspicion of cervical cancer which was confirmed after the biopsy. Unfortunately, from all these patients there were diagnosed 36 cases of squamous carcinoma and 3 adenocarcinoma of endocervix. Totally, there were 33.91% patients with cervical cancer from all the casuistry of cervical pathology in the year 2004. Carcinoma in situ, CIS, appeared at 7 patients (6.09%), CIN3 was diagnosed at 5 patients with ages predominant in the 5^{th} and 4^{th} decade and CIN2 associated with koilocitosis were 6 cases, patients belonging to the 4^{th} and 3^{rd} decade of age.

In 2005, in the hospital were admitted 228 patients with the same pathology above mentioned, 88 patients from rural areas (38.0%) and 140 patients from urban (61.0%).

The distribution of this casuistry on age group, demonstrated the predominance of the 5th and 4th decade of age – 74 patients (32.46%), respectively 69 patients (30.26%), followed by the 3^{rd} decade – 53 patients (23.25%), 6^{th} decade -26 patients (11.40%) and the seventh and eighth decades with 3 patients each (1.32%). From all this casuistry, we diagnosed 8 cases (3.51%) with squamous cervical cancer, 3 patients (1.32%) with adenocarcinoma of endocervix, 3 patients (1.32%) with CIS, 8 patients (3.51%) presented CIN3 associated with koilocitosis and 10 patients (4.39%) with CIN2. Totally we found 14 cases of cancer (6.14%) and 18 cases (7.89%) precancerous lesions.

Finally, in 2006 there were 220 patients admitted in hospital with cervical pathology- precancerous or

cancerous lesions. From these, 88 patients were from rural areas (40%) and 132 patients from urban (60%).

In this year, we had a predominance of casuistry in the forth decade of age – 86 patients (39.09%), followed by the fifth decade – 57 patients (25%), sixth decade – 37 patients (16.82%) and the third decade – 31 patients (13.60%). In the seventh decade there were 5 patients (2.27%) and in the eighth – 4 patients (1.82%). The suspicion of cervical squamous cancer was confirmed at 5 patients (2.27%), 7 carcinoma in situ (3.18%), 2 adenocarcinoma in situ (0.91%), 1 case of carcinoma adenosquamous (0.45%) and 16 patients with CIN3 (7.27%), 15 patients with CIN2 (6.82%), from each of these last 2 groups, in 9 situations respectively 10 situations, was put in evidence the koilocitosis.

From all this casuistry, we took in work specimens from patients with nonmalignant cervical pathology (cervicitis) which were admitted in hospital for investigation of a possible precancerous lesion and specimens from patients diagnosed with precancerous lesions. We made immunohistochemical tests using from immunomarkers the two above mentioned: Ki-67 and p53. There were selected 10 specimens from benign pathology of cervix, and 10 specimens from cervical precancerous lesions (totally in 3 years being 29 CIN 2 and 31 CIN 3).

For immunohistochemy, the specimens were simultaneous processed with primary mouse antibodies in less than an hour. We used mouse monoclonal anti-human antibody Ki-67, clone MIB-1 and mouse anti-human protein p53, clone DO-7. The pattern of immunostaining is nuclear in both cases. The examined tissues were obtained by cervical biopsy, fixed in formalin and then paraffin-embedded. These are serial sectioned with the microtome Shandon ME (15 sections/ block, 3µ thickness) for the primary diagnosis and intraexperimental evaluation.

Ki-67: Monoclonal Mouse Anti-Human Ki-67 antigen (DAKO code No. M7240) at a dilution of 1:100 was applied on three-micron thick, formalin-fixed, paraffin embedded tissue sections. This was performed by using 15-minute heat-induced epitope retrieval in 10 mmol/L citrate buffer at pH 6.0, followed by 30 minutes incubation at room temperature with the primary antibody. Finally, after dewaxing and rehydration, the slides were counterstained with haemotoxylin and eosin. The same technical personnel performed the immunohistochemical staining for all the cases.

p53: Monoclonal mouse anti-human p53 protein (DAKO code No. M7001), at a dilution of 1:50 was applied on three-micron thick, formalin-fixed, paraffin embedded tissue section. This was performed by using 20-minute heat-induced epitope retrieval in Dakocytomation target retrieval solution, followed by 30 minutes incubation at room temperature with the primary antibody. This was followed by staining using the DAKO LSAB+/HRP and EnVision+/HRP kits. Streptavidin and diaminobenzidine (DAB) were then added. Finally, after dewaxing and rehydration, the slides were counterstained with haemotoxylin and eosin. The same technical personnel performed the immunohistochemical staining for all cases.

RESULTS AND DISCUSSIONS

The most affected group age of patients with cervical pathology - cervical intraepithelial neoplasia and microinvasive cervical carcinoma, was the fourth decade of age followed very close by the fifth decade of age group, corresponding to results from medical literature (Lazăr et.al, 2007).

For the specimens from benign cervical lesions, the Ki-67 positive immunostaining was 25% (5 cases from 20), or 50% if we consider just the cervicitis. For the precancerous lesions appeared a positive staining for Ki-67 of 45% (9 cases from all 20 got in the study), or 90% from precancerous lesions.

On the other side, the immunostaining for p53 in benign cervical lesions was positive in 15% (3 cases from the 20 cases in study) or 33,33% from benign lesion group. In cervical dysplasia, the positive p53 staining was in 45% (7 cases from all 20), or 70% considering only the dysplasia group.

In this study, Ki-67 expression showed a tendency to correlate positively with histological grade. The same correlation is often reported in the literature.(Sarian et al., 2006; Qiao et al., 2005; Bahnassy et al., 2007) However, owing to its high expression even in normal tissues, Ki-67 is not as specific as p16 in the identification of precancerous lesions. In this context, a diffuse pattern, which is associated with the severity of the lesion, may help to distinguish normal or benign cases from precursor lesions of cervical carcinoma.

Our results show a correlation between p53 expression and histological grade. Conflicting results on this topic have been published: some studies showed a significant correlation of p53 expression with CIN 3 or carcinoma compared with normal cervix or LSIL, (Bahnassy et al., 2007) whereas others showed no significant association. (Hiller et al., 2006) Our results agree with the first studies. In many human cancers, p53 is generally overexpressed as a consequence of point mutations that disrupt its transcriptional activity and cause down-regulation of proteins whose expression is induced by p53; point mutations in the p53 gene cause overexpression of an inactive form of the protein. However, the mechanism for p53 inactivation in cervical carcinogenesis is different: the E6 HPV oncoprotein causes p53 ubiquitination, and, subsequently, degradation of p53 occurs in the proteosome. Different types of p53 alterations, such as point mutations that cause over-expression or E6-induced degradation, which causes down-regulation, may hamper the correct evaluation of p53 immunohistochemical analysis in HPV-induced cervical lesions. Another reason for the lack of consistent association between p53 and grade of dysplasia may be the different role of HPV genotypes

Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 20, issue 2, 2010, pp. 87-93

in altering p53, an increased p53 expression appearing in infection with low-risk HPV genotypes (Tsuda et al., 2003). A significantly increased expression of p53 in advanced-stage cervical carcinoma implies that inactivation of p53 is associated with tumor progression.

CONCLUSIONS

In our study, we could see the importance of gynecological screening with one of the most simple, but very important test - Pap smear, or how we name it: the Babes-Papanicolaou test (FBP). The casuistry in our country shows the increase frequency of cervical precancer or worst - cervical cancer. Unfortunately, the most of the patients are diagnosed in advanced stages- II, III or even stage IV. After in our outpatient hospital it was introduced the Pap smear as free investigation and the population was sensitized for the importance of this, the number of patients which came for this investigation at gynecologist has increased. In the situation above mentioned, there were diagnosed more precancerous lesions after the cervical screening started to work and the frequency of patients with cervical cancer admitted in hospital decreased slowly.

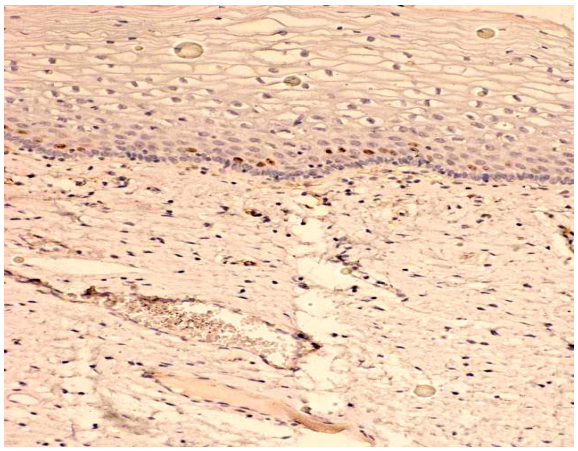

Fig. 1 Positive Ki-67 immunostaining of cervical benign lesions (x200)

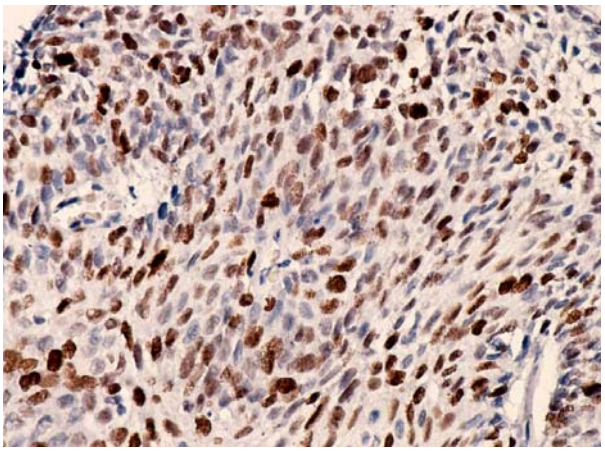

Fig. 2 Positive Ki-67 immunostaining of precancerous cervical lesions – CIN 2-CIN 3 (x400)

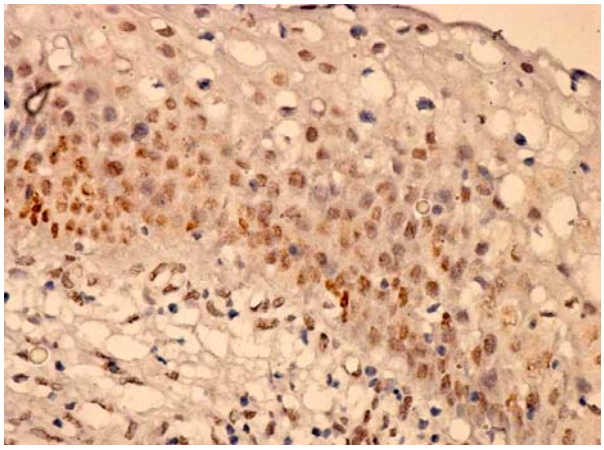

Fig. 3 Positive immunostaining for p53 of cervical benign lesions – CIN 2 (x200)

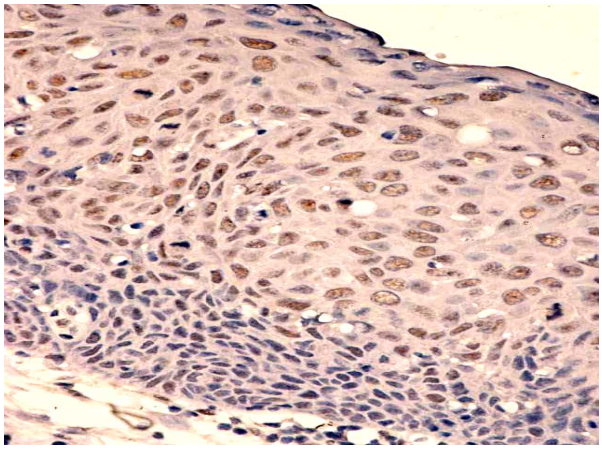

Fig. 4 Positive immunostaining for p53 of precancerous cervical lesions – CIN2-CIN 3 (x400)

The most frequent age group of patients diagnosed with cervical precancerous and cancerous pathology was in the 4th and 5th decade, but in the 3rddecade even if there were few cases of cervical cancer, they were in advanced stages –III and IV, which was really tragic. This situation demonstrates once more the importance of cervical screening and the prevention methods for HPV infection to avoid the malignant

cervical pathology at so young patients and not only that.

The patients diagnosed with precancerous lesions must be investigated more to put in evidence the presence or not of HPV infection. If HPV is present in cervical lesions the probability of these to have a progression to neoplasia is increased and the prognosis of the patients with cervical dysplasia and HPV infection is reserved when they don't follow the medical indications and management. The prognosis is worse if we talk about HPV high risk type (HPV-HR).

In management of patients with cervical precancerous lesions, after the diagnosis of L-SIL or H-SIL at Pap test, the next step could be the highlighting of HPV-HR infection in the cervix and the proliferation index. If the proliferation rate is increased, the possibility of aggressive evolution in cervical dysplasia is increased too. The presence of HPV-HR in cervical precancerous lesion gives too a risk for evolution to cancer.

Ki-67 used as proliferation marker is very useful, because we can appreciate the proliferation rate in every lesion and from this information we can have a prognosis of that lesion and the best management which is appropriate for the patient. In our study, this immunomarker demonstrated its efficiency; the results showing the staining for Ki-67 which increase in the same time with the lesion grade.

For p53, on our results we had a correspondence between the increasing immunostaining for oncoprotein p53 and the histological grade of cervical lesion. In literature, the results about the utility of p53 are not clarified yet, there are different opinions, but for our study, they are correlated with Ki-67 and good to appreciate the prognosis of cervical precancerous lesions.

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