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The Indicators of Predicting
Disease Outcome in HPV Carcinogenesis

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1. Introduction

Cervical cancer is one of the most common cancers among women worldwide, currently ranked as the third cancer causing death for females (Ferlay et al., 2010). Since the screening programs have been implemented the incidence and mortality associated with cervical cancer have declined (Gustafsson et al., 1997) but in developing countries it still remains a cause of death in women.

Although essential for the transformation of cervical epithelial cells, HPV is not sufficient, a number of other cofactors and molecular events being also incriminated (Woodman et al., 2007). Cervical HPV infection is a sexually transmitted disease which occurs in women short time after beginning the sexual life. In young women, the disease is usually transient, its prevalence decreasing around 30 years (de Sanjosé et al., 2007). A second peak of infection is found in older women and is superposed to the peak of cervical cancer incidence (Anton et al., 2011). Near 40 of the more than 140 HPV types identified so far can infect the cervix (Bernard et al., 2010). Differences in carcinogenicity of cervix specific HPV types are partially related to the expression of the E6 and E7 oncogenes which, among other functions, interfere with tumour suppressor proteins p53 and pRb, respectively (Sinal et al., 2005). By these interactions viral oncogenes abrogate the mechanisms of cell-cycle control and apoptosis.

As many studies sustain, in the absence of persistent infection the risk of cervical cancer is low (Schiffman et al., 2011). Persistent infection with high risk genotypes and the inability of the immune system to clear viral infection are the main factors contributing to tumors genesis. Thus, according to new concepts (Snijders et al, 2006), both the immune mechanisms of the host and the nature of infected cells are decisive for cervix lesions development. It takes 12-15 years before a hrHPV persistent infection may lead to cervical carcinoma, thus underlying the multistep process of cervical oncogenesis. Aberrant functions of tumour-suppressor genes as a result of their interaction with hrHPV viral genes, determine the genomic instability. These alterations cumulated with the action of various cofactors lead to progressive lesions and finally to cancer. The epithelial cells transformation is a 4 steps process, as proven by *in vitro* analysis: extended life span, immortalization, loss of anchorage and tumorigenic growth (Snijders et al, 2006). Each step is characterized by accumulation of (epi) genetic cell changes.

There is a continuing interest in the molecular mechanism of HPV oncogenic induction program, especially for clinicians who need new markers. Together with HPV DNA and PAP tests, these new markers may have additional value for risk assessment of cervical cancer and may reduce the number of biopsies. A major challenge for gynaecologists is to implement more markers in the routine practice, in order to have a better estimation of the risk a cervical lesion has to progress to cancer. Taking into account the contribution of oncogenic HPVs to malignant phenotype development by several interrelated mechanisms we evaluated the molecular markers (used in a variety of techniques) which might characterize different stages of HPV-associated epithelial lesions. The next step is to determine their suitability as "surrogate markers". In order to point host genes involvement, viral cell cycle has to be known.

2. HPV life cycle

All the HPV types infecting cervix are non-enveloped small sized viruses and have about 8000-base pairs in their double-stranded circular DNA. Their genome has overlapped open reading frames, coding for eight proteins, divided into an early (genes E1-E7) and a late region (genes L1-L2). The early genes are expressed in the infected basal cells while the late proteins are synthesized only in well differentiated cells. The early proteins have regulatory functions, being involved in HPV genome replication and transcription, cell cycle, cell signalling and apoptosis control, immune modulation and structural modification of the infected cell (Molijn et al., 2005; Sinal & Woods, 2005).

The replication cycle of HPV is tightly linked to differentiation of the epithelium it infects. For the initial infection to occur microlesions in the stratified epithelium are requested. Although some cell surface receptors have been mentioned, as heparin sulphate (Giroglou et al., 2001) and alpha-6 integrin, the controversy persists. It has been suggested that for a lesion to be maintained, the virus must infect an epithelial stem cell (Egawa, 2003). Following infection, in the basal cells of the epithelium, where low levels of viral early proteins (E1, E2, E5, E6 and E7) are observed, a low number of viral genome copies are maintained as episome. For viral DNA synthesis, beside E1 and E2 proteins that are necessary for viral replication, papilomaviruses use the host cell machinery. Viral transcription increases in differentiated cells while assembly of new virions occurs only in the squamous epithelia undergoing terminal differentiation. E4 is the first protein expressed late in infection and it may be accompanied by E5. The late proteins L1 and L2, are expressed in the granular layer of the epithelium and the assembled viruses are released from the fully differentiated cells. This pattern of gene expression is characteristic to productive infection and its pathological effect is specific for CIN 1 and some CIN 2 lesions. A deregulated E6 and E7 expression in proliferating cells and the interference of these viral oncogenes with tumour supressor genes p53 and pRB respectively, determine the cells to overcome senescence barrier and to develop a transformed phenotype (Fehrmann & Laimins, 2003). Recent studies have provided data on how different types of stimuli activate signalling pathways leading to senescence. It seems that these stimuli are funnelled through p53 and pRB (Narita et al., 2003) whose combined levels of activity determine the cells to enter senescence or to remain in a competent state for proliferation (Dirac & Bernards, 2003). Usually, the up-regulation of viral oncogenes expression is associated with viral genome integration into the host cell chromosome and with viral E2 gene disruption. Loss of the E2

leads to the uncontrolled expression of viral oncoproteins, which in turn leads to disruption of the normal cell cycle regulation and progres of HPV-associated cervical cancer (Hung et al., 2010). E6 and E7 oncoproteins are also required for the maintenance of the transformed status of infected cells (Longworth & Laimins, 2004). This pattern is characteristic for high grade CIN and carcinoma.

In terms of HPV pathogenesis, depending on the HPV genotyope involved in the infections, there are three types of clinical manifestations: (a) productive infection, characterized by virions production and a strictly regulated expression of the viral genes at well defined sites. CIN 1 represents the histological manifestation of productive infections. (b) latent/inactive infection, rarely visible, without clinical signs (asymptomatic), characterized by maintenance of the viral genome in basal layers and early viral proteins expression at levels below those necessary to produce an effective immune response. Viral persistence may be the consequence of infection in case of immune system depletion and may result from silencing expression of viral gene through methylation (Kalantari et al., 2004). (c) abortive infection, as a precursor to cancer, is associated in particular with hrHPV genotypes which induce changes in virus-infected cells causing cancer. HPV-induced cancers often arise in sites which are non-optimal for productive infection. Low risk HPV types are only occasionally associated with mucosal cancers.

3. Biomarkers – Indicators of the disease state

According to Wentzensen and von Knebel Doeberitz (2007), there are several applications for the cancer biomarkers including: (1) early detection of cancers (identification of individuals prone to develop cancer at a time point that still allows for a successful curative intervention); (2) improved reproducibility of the histopathological diagnoses (allowing for risk assessment of detected lesions and stratify intermediate lesions); (3) surveillance of persons at risk (allowing for non-invasive monitoring or reduce invasive procedures); (4) post therapy monitoring (predicting the progression and monitoring recurrences after treatment).

Approximately 80% of CIN1 lesions regress spontaneously (Follen and Richards-Kortum, 2000) and usually are managed conservatively. On the other hand, CIN2 and CIN3 have a considerable risk of progression toward invasive cancer and are therefore usually treated by conization or other less invasive procedures. Anyway, despite its rather low rate of progression, CIN2 is frequently treated, fact that leads to contradictory discussions (Spitzer et al, 2006; Wright et al, 2003). In order to restrict treatment to CIN1/2 groups, there is a need for biomarkers that could discriminate between CIN1/2 with high risk of progression and those with high chance to regress spontaneously (Ozaki et al., 2011). Additional markers are also necessary to overcome diagnostic inaccuracies due to the existence of several CIN mimics, such as immature squamous metaplasia (Duggan et al, 2006; Geng et al, 1999), or to heterogeneous distribution of dysplastic lesions that can result in over- and under-diagnosis.

A hrHPV positive test without associated important cytological changes would always require monitoring. The risk of progression toward invasive cancer increases with the lesions' grade (Ostor 1993), thus, biomarkers specifically associated with disease progression, allowing for a correct triage must improve the cervical cancer screening

programs and the early detection of people at risk. Therefore, in order to be useful, the biomarkers must meet the following criteria: (1) they must be differently expressed in normal and high-risk tissues; (2) they should be synthesised in a well defined stage of carcinogenesis; (3) both the marker and its assay must be acceptable sensitive, specific and accurate; (4) they should be easily measured; (5) they should be correlated with a decrease in the cancer incidence rate (Follen and Schottenfeld, 2001). The inherent variability in interpretation between individuals has led to wide ranges in diagnostic precision between practices, but the advent of immunohistochemistry and the more recent discovery of new genes and their functions have resulted in the identification of cellular proteins that are differently expressed in tumours (Nucci et al., 2003).

3.1 Cell cycle regulation markers

The loss of cell-cycle control by HPV oncoproteins directly or indirectly interacting with the host genes (Giannoudis et al., 2000) leads to accelerated proliferation mirrored by an increased number of cells expressing specific markers. Among cyclins and cyclin dependent kinases (CDKs) that governed cell cycle, p53, Rb and CDK inhibitors (p15, p16, p18, p19, p21, p27) play a special role by arresting the cell cycle until damaged DNA is completely repaired. Therefore, the cell cycle regulators may shed light on the understanding of HPV-mediated cervical carcinogenesis (Conesa-Zamora et al., 2009).

The roles played by cyclins and CDKs in distinct phases of the cell cycle, suggest that they could be used as markers for cell proliferation in cervical malignancy (Skomedal et al., 1999).

The ability of HPV oncoproteins to disrupt growth regulatory proteins may have effects on the cyclin-dependent kinase inhibitors linked to the G1- and G2- checkpoints. As mentioned before, cell cycle deregulation in cervical carcinogenesis is by far linked to p53 and pRb. The degradation of p53 tumour-suppressor gene by E6 may result in accumulation of damaged DNA. By contrast with other cancers where p53 appears to be down-regulated by point mutations, in cervical cancer p53 expression seems to be increased; the levels of p53 in high-grade SIL (HSIL) and cervical carcinoma is higher than in low-grade SIL (LSIL) and normal cervix. There are authors who reported higher p53 expression in cervical biopsy specimens from patients without HPV infection or who where infected with low-risk HPV (Tsuda et al., 2003). 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. These results suggest that the absence of p53 immunostaining might be due to the capacity of some hrHPVs to down-regulate in different extent the p53 expression, probably via E6-induced ubiquitination. Over-expression of p53 does not seem to be related to HPV oncoprotein action, as some authors noted higher expression of p53 protein in early lesions. These data support the hypothesis of a partially protective role of the wild-type p53 in the early stages of cervical lesions (Vassallo et al., 2000) and is correlated with the observation that high levels of p53 expression are detected mostly in low-grade SILs (LSILs) and lrHPV-associated lesions (Kurvinen et al., 1996). Thus, p53 might function as a molecular marker for the risk of the progression of HPV-associated SILs. Øvestad et al, (2011) reported that CIN2–3 lesions regressed if higher epithelial pRb and p53 levels were present. Therefore, they proposed p53 and pRB as biomarkers. One paper noted that in HPV-positive or –negative low CIN

or ASCUS, in the majority of cases, p53 was overexpressed in the basal cell layer, thus presenting a positive response to viral infection or to the lesion (Cenci et al., 2005). A paper reported that the majority of CIN showed absent to focal staining while most of the invasive carcinoma showed regional to diffuse staining. A greater expression of p53 in the malignant cervical neoplasms than in the pre-malignant cervical lesions was noted, suggesting that p53 overexpression is not an early phenomenon in cervical cancer (Tan et al., 2007). p53 control the G1 transition to S phase and its abrogation affect the expression of p16, p21 and p27 cyclin inhibitors. p21, p27 and p57 CDK inhibitors inhibit the cyclin/CDK2, CDK4 complexes and causes G1 arrest. The levels of these proteins are of prognostic significance in cervical oncogenesis. p27 might be inactivated early in carcinogenesis by E7 hrHPV and may precede tumor invasion (Zur Hausen, 1994).

p16 is a tumor suppressor protein belonging to the family of INK4 cyclin-dependent-kinase inhibitors whose increased expression has been associated with HPV-infected dysplastic and neoplastic epithelium of the cervix. p16 expression is the result of negative feedback on functional inactivation of pRb by E7 HPV (Zur Hausen 1994). p16 protein normally acts like a negative regulator of cellular proliferation, but its inhibitory action is not efficient in the case of proliferating hrHPV infected cells (Dray et al., 2005). Most studies confirm p16 overexpression in all HSIL lesions but also in some normal epithelium of cervix at both protein (von Knebel Doeberitz et al., 2002) and messenger levels (Bleotu et al., 2009). On the other hand, p16 is not expressed in cervicitis nor is in squamous metaplasic epithelium (in the absence of CIN stages) (Hu et al., 2005). Still, other studies proved that p16 high level was found in cervical glandular epithelium and also in metaplasic epithelium. Therefore immunohistochemical analysis of p16 is considered a powerful tool in the identification of HPV-mediated premalignant and malignant lesions of the uterine cervix. Cytoplasm predominant localization of p16 seems to be related to the increasing histological grade of cervical lesion (Horn et al., 2006). Yıldız et al. found strong and full thickness staining for p16 in the cervix epithelium in HSIL lesions and weak and basal/rare staining in LSIL. All hrHPV-positive cases were p16-positive, but no statistically significant relationship between HPV infection and the intensity and distribution of p16 was found (Yıldız et al., 2007). Depending on the positive cells distribution, there have been recognised four patterns of p16 staining which correlate to the lesion's grade (Kostopoulou et al., 2011).

Cyclins such as cyclin D1 are subjected to molecular alterations that characterize cervical carcinoma. Cyclin D1 forms a complex with CDK4 or CDK6 to carry out the phosphorylation of pRb. The phosphorilation of pRb leads to the release of the transcriptional factor E2F which, in turn, induces the expression of proteins required for S phase. Since D type cyclins and E7 HPV possess similar binding regions for pRb and pRb related pocket proteins, inactivation of pRb either by the cyclin/CDK complexes in G1 or by interaction with the E7 hrHPV may result in a decreased expression of cyclin D1. There are controversial results on the role of cyclin D1 in cervical carcinogenesis and clinical outcome (Goia et al., 2010). Although some studies reported no correlation between the expression of D1 cyclin and dysplasia (Goia et al., 2010) some authors associated overexpression of cyclin D1 with a poor prognosis in cervical carcinoma (Bae et al., 2001). An increase in cyclin D1 expression was observed from CIN 3 to invasive carcinoma as a consequence of the inability of overexpressed p16 to inactivate CDK4/6, the partner of cyclin D1. The authors suggested that the immunohistochemical expression of cyclin D1 might be a marker in cervical cancer progression only if restricted beyond to the lower third layer (Conesa-Zamora et al, 2009).

Moreover, cytoplasmic staining is more noticeable in the basal and parabasal layers, suggesting a recently acquired alteration related to a high-grade dysplasia and the progression of the disease (Baldin et al., 1993). A significant increase in the cytoplasmic staining associated with an increasing degree of dysplasia was reported by Carreras et al. (2007) due to the fact that the number of cells in the S phase increases in severe lesions. This finding gives support to the cytoplasmic expression of p16 in lesions of higher grade since p16 is the inhibitor partner of cyclin D1 and they often appear together (Zhao M., et al., 2006). The relationship between cyclin D1 expression and an increasing degree of dysplasia was not as remarkable as that seen for p16 but was statistically significant.

Cyclin B1 complexes solely CDK1 (cdc2) to form the mitosis-promoting factor, which regulates the G2–M transition and is the primary regulator of mitosis. p53 was shown to prevent the G2–M transition by decreasing cyclin B levels. Some authors reported significantly cyclin B1 expression in invasive cervical cancer than in normal cervical tissue (Zhao P., et al., 2006).

Cyclin E is expressed and associated with CDK2 near the G1-S boundary. Cyclin E hyperexpression is related to pRB inactivation by E7HPV but can not be correlated with hrHPV types. Recent studies have shown that cyclin E antigen correlates with the presence of HPV in atypical squamous epithelial cells. The association is stronger in LSIL, thus recommending this potential biomarker as a useful tool in distinguishing between benign/reactive changes from preneoplastic squamous atypia (Weaver et al., 2000). Much more common in cervical lesions *versus* non-neoplastic epithelium, cyclin E sensitivity may hamper its use as a marker (Crum, 2000). Other authors consider cyclin E together with p16 as the most sensitive tools for LSIL and HSIL detection respectively (Keating et al., 2001). In ThinPrep samples, cyclin E and p21 expression seems to correlate better with HSIL (Moore et al., 2005). The Cyclin E/CDK2 complex phosphorylates p27, tagging it for degradation, thus promoting expression of **cyclin A** with progression toward the S phase. A close association between hrHPV and cyclin A1 was noted (Goia et al, 2010). This could be explained by its complex up-regulation by both E7 and E6 oncoproteins, making cyclin A active in both S phase and late G2 phase of the cell cycle. Cyclin A1 might be a useful marker of cell proliferation most notably orchestrated by the capability of E7 to abrogate the inhibitory activity of p21CIP1/WAF1/SDI1 on CDK and proliferating cell nuclear antigen–dependent DNA replication necessary for the G1-S transition (Erlandsson, F. et al, 2006). Studies on cyclin A as prognostic marker are limited.

Immunohistochemistry investigation of several cell markers (cyclin D1, cyclin E, CDK4, p53, mdm-2, p21, p27, p16, Rb and Ki-67) revealed that aberrations involving p27, cyclin E, CDK4 and p16 are early events in HPV 16 and 18-associated cervical carcinoma, whereas cyclin D1 and p53 pathway abnormalities are considered late events. Therefore, the authors considered that immunohistochemical tests for p16 and cyclin E could be useful in early diagnosis of cervical carcinoma (Bahnassy et al., 2007).

E7 HPV binds to and blocks the function of p21 and p27 in a p53-independent manner (Zehbe et al., 1999). Both p21 and p27 are involved in cell cycle arrest through binding to G1 phase cyclin–CDK complexes. E7 hrHPV modulates the cytoplasmic localization of p27 and inhibits p21 function. Some studies showed that p21 immunoreactivity seems to be significantly correlated with high grade stage of cervical disease. The protein was reported to be significantly increased in CIN3 and *in situ* carcinoma. On the other hand, p27 was

reported to decrease from normal cases to carcinoma (Cheung et al., 2001). On the other hand, Huang et al. (2010) reported that p21 expression decreased in HPV 18 positive carcinomas. The fact that aberrant expression of p27, cyclin E and p16 are early events in carcinogenesis (while p21 occur late in cervical carcinogenesis) might be of interest for early diagnostic and for monitoring patients with cervical dysplasia (Bahnassy et al, 2007). No established relationship was found between p27 expression and cell proliferation in cervical cancer (Kumar & Verma, 2006).

3.2 Proliferation markers

Those cells with high proliferative activity that allow the accumulation of transforming mutations are more likely to be associated with premalignant and malignant tissues. The proliferative aspects distribution in specific layers (basal vs. parabasal vs. superficial) might indicate growth-regulatory mechanisms; thus the relation between proliferation and growth deregulation is of interest (Heatley, 1998). Proliferation in the normal cervix, in cervical intraepithelial neoplasia and in invasive cervical carcinomas can be assessed by a range of techniques requiring technologies of varying sophistication and accessibility, considered in four broad groups (Heatley, 1998): (l) mitotic counts on routinely processed histological sections and cytological smears; (2) *in vivo* or *in vitro* incorporation of tritiated thymidine in actively proliferating cells, by injection into the cervix and by incubation with fresh tissue, respectively; (3) immunohistochemical techniques for Ki-67 labelling index and AgNOR counts; (4) flow cytometry.

In the normal cervix, mitotic activity (quantified in routinely processed histological sections and cytological smears or by tritiated thymidine incorporation) is usually confined to the basal and parabasal layers, but in CIN the numbers and height of mitotic figures within the epithelium increases. The presence of mitotic figures is part of routine diagnosis and biomarker value is discussed as a whole.

Flow cytometry DNA content analysis on minced biopsy specimens (Melsheimer et al. 2004) or smear (Tong et al., 2009) allow the association between aneuploidy and dysplasia degree. lrHPV types tend to be associated with polyclonal lesions whereas hrHPV types are associated with monoclonal lesions (Park et al, 1996). Ploidy appears to be a measurable biomarker and a good predictor of the biological behaviour with a better predictive value than the histopathologic characteristics (Follen and Schottenfeld, 2001). It is considered that in advanced dysplastic lesions, aneuploidy precedes HPV integration, further supporting the notion that integration of viral genomes is the consequence and not the cause of chromosomal instability and transformation (Melsheimer et al. 2004). Therefore, an increased aneuploid DNA value together with the increase in grades of cervical dysplasia are specific prognostic markers of malignancy (Kashyap & Das, 1998); flow cytometric analysis of DNA ploidy may be a potential means providing a strategic diagnostic tool for early detection of cervical cancer (Melsheimer et al. 2004). The combination of DNA ploidy (determined by cytometric test) with HPV screening and cytology is an optimal method to detect progressive lesions since it has the highest sensitivity and specificity (Singh et al. 2008).

Some proliferating molecules were investigated by immunohistochemistry like biomarkers in cervical precursor lesions: PCNA, Ki67, ICBP90, etc.

Ki-67 is a nonhistone protein expressed in the nucleus during the whole cell cycle, except for the early phases of G0 and G1. It constitutes a marker for proliferating cells and was associated with severe dysplasia and cervical cancer (Sarian et al., 2006). Ki-67 stains positive in the parabasal, basal and intermediate layers of condylomas, in the basal and parabasal layers of CIN; in addition, cells positive for Ki-67 staining are identified in intermediate and superficial layers of the squamous epithelium. Ki-67 expression correlates positively with histologic grade and distinguishes non-diagnostic atypia from SIL. The grade and pattern of Ki-67 expression in precursor lesions are still topics of debate (Vijayalakshmi et al., 2007). Even Ki-67 is not as specific as p16 for precancerous lesions, its immunohistochemical diffuse pattern is associated with a severe lesion. A lot of studies were conducted in order to establish the objective, reproducible, and reliable use of Ki-67 in the classification of dysplastic changes in cervical epithelium. Expression of Ki-67 (MIB-1) in the upper layers/superficial layers of the epithelium corroborated with more than 15% of basal cells positive for MIB-1 staining can be used to distinguish condyloma from inflammation or squamous metaplasia (Mittal and Palazzo, 1998). Ki-67 evaluation can be also a valuable adjunct in the distinction of CIN from normal or benign cervical squamoepithelial lesions (Kruse et al., 2002; Keating et al., 2001). MIB-1 expression in the basal and the upper-third layer proved useful in grading SIL with equivocal mitotic index (Popiolek et al., 2004). Immature squamous metaplasia can be MIB-1 cluster positive, but this false-positive case showed a special staining pattern, different from CIN: 1) MIB-1 staining in the nuclei is not diffuse (as in CIN) but clumped; 2) positive nuclei are somewhat less densely packed than in CIN (Kruse et al., 2002). Noteworthy is that the presence of a cluster of at least two MIB-1-positive nuclei (MIB-C) in the upper two thirds of the epithelial thickness is a highly sensitive and specific marker to discriminate between normal epithelium and low-grade squamous intraepithelial lesion (Pirog et al., 2002). Cauterized dysplastic/condylomatous epithelium showed significantly greater expression of MIB-1 than cauterized normal epithelium, being a good marker (Mittal 1999).

Recent studies showed that two cell cycle–related proteins, minichromosome maintenance protein-2 (MCM2) and topoisomerase II-α (TOP2A) are overexpressed in cervical cancer. **Minichromosome maintenance protein 2** drives the formation of pre-replicative complexes in G1 phase; overexpression of MCM2 provides the link between oncogenic HPV infection and the molecular event of cervical dysplasia (Rihet et al, 1996). **DNA TOP IIA** is a nucleic enzyme that plays an important role in DNA replication, transcription, recombination, condensation, and segregation through interaction with the double-helix DNA (Ofner et al, 1994). **ProEx C** is a cocktail of two monoclonal antibodies that targets the expression of these two proteins (MCM2 and TOP II A) and its increased expression is associated with HSIL lesions. The ProExC signal is intense, diffuse and comprises the entire thickness of the epithelium in HSIL, with a variable pattern of the staining in LSIL, and is usually negative in reparative or reactive immature squamous metaplasias (Pinto et al., 2010). ProEx C positive expression seems to be more specifically associated with SIL than Ki-67 positive expression (Conesa-Zamora et al, 2009). ProExC immunostaining, when compared with p16 immunostaining, have similar specificity for CIN 2+ and higher specificity for CIN 3+ but lower sensitivity for CIN 2+ and CIN 3+ (Guo et al., 2011). Some reports suggested that ProExC can be more selective and informative for the progression of low-grade (CIN1) and moderate-grade (CIN2) lesions than Ki67 (Beccati et al., 2008).

ICBP90 protein is a member of a nuclear proteins family with DNA-binding properties involved in DNA replication. ICBP90 detection, used as a proliferation marker (Hopfner R, et al, 2000), gives information only concerning the number of cells that entered the cycle without any indication on the duration of the cycle. ICBP90 like Ki67 was linked to the development of an HSIL. 97.6% of HSILs positively stained for ICBP90; thus confirms it as a useful proliferation marker (Lorenzato et al., 2005). If an hrHPV type was present, the association of a suspect DNA profile with a positive proliferation marker could predict the presence of HSIL in a very accurate way. On the other hand the association of a suspect DNA profile with the presence of ICBP90-positive cells and MIB-1 in the upper two thirds of the epithelium could help to discriminate between an LSIL and an HSIL with high PPV, (Lorenzato et al., 2005).

3.3 Markers of epithelial organization and differentiation

The life cycle of human papillomaviruses (HPVs) is tightly linked to the differentiation program of the host's stratified epithelia that it infects. Viral infection induces changes in squamous differentiation, which is reflected by the pattern of cytokeratin polypeptide expression. By studying this pattern in relation with the presence of the virus, it was obtained an indication on the influence the virus has in individual cells, on the cellular differentiation. For example, E1-E4 contributes to different processes in both the early and late stages of the virus life cycle (Nakahara et al., 2005). The viral E1-E4 protein contributes to the replication of the viral genome as a nuclear plasmid in basal cells. Expression of the HPV-16 E1–E4 protein in human keratinocytes results in the total collapse of the cytokeratin matrix, but nuclear lamins and tubulin and actin networks are unaffected (Doorbar et al., 1991). On the other hand, the presence of the skin-type cytokeratins CK1 and CK10 in condylomata accuminata derived from anogenital skin (HPV 6 and HPV 11 positive) was decreased, whereas CK13 and to a lesser degree CK4 appeared increased (Mullink et al., 1991).

HPV infection may alter the differentiation status of the epidermis leading to a major expression of K14 in the basal and suprabasal layers (like in HPV infected normal epithelium), up to the more superficial layers (like in epidermodysplasia verruciformis, EV) (Barcelos & Sotto, 2009). Comparing the morphologic distribution of cytokeratins 13 and 14 and involucrin in naturally occurring low-grade SILs and high-grade SILs infected with a variety of HPV types, Southern et al., (2001) observed that: (1) the absence of cytokeratin 14 expression is associated with high-risk HPV infection and occurs more frequently in high-grade SILs; and (2) dedifferentiation, with loss of cytokeratin 13 or involucrin expression occurs only in high-grade lesions. Also, it was noted that in most LSILs infected with lrHPVs, cytokeratin 14 expression was present in all epithelial layers, with fewer lesions; the expression confined to the basal/parabasal layers with focal loss of expression. By contrast, only in few cases (11%) of LSILs infected with hrHPVs, cytokeratin 14 expressions was present in all epithelial layers; most of these lesions showed cytokeratin 14 expression confined to the basal/parabasal layers (56%), with a number of lesions showing focal loss of expression (33%) (Southern et al 2001). Loss/reduction of cytokeratin 14 protein expression level is associated with transformation but not immortalization because in HPV16 immortalized keratinocytes downregulation of cytokeratin 14 occurred only at a transcriptional level (the protein level remaining normal) (Bowden et al, 1992). In

epidermodysplasia verruciformis CK1/10 showed retarded or negative expression and e-cadherin is diminished in superficial koilocytotic cells' foci, more superficially in EV. Positive staining for K16 and K4 was observed in normal HPV infected epithelium as in EV (Barcelos & Sotto, 2009).

Regauer & Reich (2007) found the following profile for the CK17: when antibodies against CK17 were used, the columnar endocervical epithelium showed no staining; basal keratinocytes in ectocervical glycogenated squamous epithelium have inconsistently, focally and weakly CK17 expression in cytoplasm; the proliferating cells of immature squamous metaplasia stain positive for CK17 in the subcolumnar reserve cells and in the proliferating basal and suprabasal cells, and negative for columnar cells; the mature metaplastic epithelium presents decreased or even lack of CK17 expression; high-grade dysplasia show concomitant staining of both CK17 and p16. They sustain a mutually exclusive immunohistochemical profile of CK17 and p16 that allows the separation of immature metaplasia with or without reactive atypia (characterized by strong, uniform CK17 staining of the proliferating cells with concomitant p16 negativity) from CIN III (characterised by strong diffuse staining of p16 in all dysplastic proliferating cells). The dual expression of CK17 and p16 in atypical squamous lesions with metaplastic features can sustain the hypothesis that CIN III alternatively may develop via HPV infection of metaplastic cells.

Considering all these aspects, we can say that in order to establish the correct diagnostic, all potential markers involved in epithelial reorganization and differentiation must be synchronic evaluated with other proliferating markers.

Plasma membrane expression of caveolin-1 (Cav-1), a constituent of lipid rafts and regulator of cell signalling, increases by the E5 HPV16 oncoprotein through the C-terminal 10 amino acids of E5. Moreover, E5 induces a 23- to 40-fold increase in the lipid raft component, ganglioside GM1, on the cell surface and mediates a dramatic increase in caveolin-1/GM1 association, a potential mechanism for immune evasion by the papillomaviruses (Suprynowicz et al., 2008). This phenomenon is very important in productive infection, in production of viral progen. But, an inverse relationship between Cav-1 expression and transformation has been clearly established. Cav-1 levels are reduced in transformed cells and forced reexpression of Cav-1 could abrogate anchorage-independent growth in transformed cells (Williams & Lisanti, 2005). In HPV transformed cells, caveolin-1 is downregulated and its expression is reduce by E6 HPV (Razani et al, 2000). In a small percentage of cervical cancer tumors caveolin-1 silencing occurs via promoter methylation (Chan et al., 2003; Dueñas-González et al, 2005).

E-cadherin is a transmembrane protein with a cytoplasmic domain connected to the actin cytoskeleton through association with cytoplasmic proteins, α-, β-, and γ-catenins (Piepenhagen & Nelson, 1993; Hinck et al, 1994). E-cadherin is essential for cell-to-cell junction and for cellular adhesion, being responsible for cellular interconnection, segregation of cell types, differentiation, epithelial polarization, cell stratification, signaling, cell motility and proliferation. Loss of E-cadherin function or expression has been implicated in cancer progression and metastasis. Down-regulation of E-cadherin was closely associated with progressive CIN and cell proliferation (Branca et al., 2006a) in HPV-positive tissue but not in the HPV-negative tissue (Samir et al., 2011). E-cadherin downregulation result in an increased cellular motility and metastasis.

Tissue transglutaminase 2 (TG2), member of calcium dependent enzymes family (Peng et al., 1999), is a cytosolic protein that catalyzes the formation of a covalent bond between the gamma carboxamide group of peptide bound to glutamine residue and the primary amino group of a wide variety of proteins leading to their post-translation modification (Boehm et al., 2002). TG2 induce polyamination of retinoblastoma protein, protecting it from caspase-mediated cleavage (Boehm et al., 2002), but also of HPV E7 disruption (the interaction of HPV E7 and pRb is a major step for cervical carcinogenesis) (Jeon et al. 2003).

TG2 levels in CIN1 are high, reflecting cellular response to inflammation and HPV infection and facilitating cell survival by its interaction with proteins involved in cell proliferation and cell death. In CIN2 and CIN3, the immunoreactivity for TG2 remains at moderately high levels, but typical for these cases, as compared with CIN1, it appears to be specifically expressed into nucleus. These nuclear translocations of TG2 in the high-grade CIN were associated to cell survival or anti-apoptotic phenotype, and therefore aid the progression or persistence of the CIN lesion. TG2 is a potentially useful marker for low-grade dysplasia or CIN1 lesions because there are clear differences between normal and CIN1 epithelium. On the other hand, TG2 represents one potential biomarker due to the nuclear/nucleo-cytoplasmic staining of TG2 in high-grade dysplasia (CIN2/3); that type of staining is not observed in normal cervical epithelium or generally in CIN1 lesions (Gupta et al., 2010).

3.4 Transcription factors and cell signalling pathway

Transcription factors are the principal modulators of gene expression and are involved in various processes controlling normal and transformed cell behaviour. Viral transcription is known to be positively regulated by glucocorticoid hormones via the up-stream regulatory region, which may partly explain its contributing effect to the transformation (Gloss et al, 1987; Mittal et al, 1993). However, in the complex picture of cervical cancer, hormone actions represent only one cofactor of HPV transformation. Thus, whatever the mechanism leading to carcinogenicity, there is a complex activation of transcription factors.

Previous studies demonstrated a functional involvement of the AP1 transcription factor in HPV-induced cervical carcinogenesis. Transcriptional activation of HPV in a keratinocyte-specific manner, depend on specific interaction between several nuclear factors and specific sites from LCR of certain HPV genotype. AP1 appears to be a common regulator of various HPV types expression, which act directly or through additional HPV type-specific that cooperate with AP1 to achieve full activation of virus gene expression (Butz & Hoppe-Seyler, 1993; Mack & Laimins, 1991; Kyo et al., 1995; Chan et al., 1990; Chong et al., 1991). Transcriptional regulation of HPV16 is activated also by NF1, TEF- 1, TEF-2 and Oct1 factors (Chan et al., 1990; Chong et al., 1991; Ishiji et al., 1992).

In HPV productive infection, E6/E7 transcripts were found to be expressed in most cellular layers with a reduced level of expression in the differentiated cells. E6/E7 expression was shown to correlate with AP1 factors distribution, suggesting that AP1 plays a significant role in the expression of viral oncogenes in uterine cervix differentiating epithelia. Co-expression of two proliferation inductors, AP1 and E6/E7, in undifferentiated cell layers might create a positive regulatory loop, contributing to the maintenance of initial HPV infection and subsequent activation in basal and suprabasal cellular layers (Kyo et al., 1997).

Aiming to obtain information about alterations in the expression of AP1 family members, de Wilde et al. (2008) found that, starting from immortal stages, c-Fos, Fra-2 and JunB expression became up regulated towards tumorigenicity while Fra-1, c-Jun, Notch1, Net and CADM1 became down regulated. They established that if the onset of deregulated expression of various AP1 family members became already manifest during the immortal state, a shift in AP1 complex composition appeared as a late event associated with tumorigenicity (de Wilde et al., 2008).

Nuclear factor-kappa B (NF-kB) is a ubiquitously expressed transcription factor, which has an important role in intracellular regulation of immune response, inflammation, and cell cycle regulation (Nair et al., 2003; Niederberger & Geisslinger, 2010; Hayden & Ghosh, 2011). NF-κB is one of the targets through which HPVs could interfere with the transcriptional control in cervical carcinogenesis (Spitkovsky et al., 2002; Fontaine et al., 2000). The oncogenic HPVs action on NF-kB through several ways: 1) there is a functional NF-kB binding site within the HPV16 LCR (long control region), at position 7554–7563, acting as an effective repressor of HPV transcription (Fontaine et al., 2000); 2) involvement of viral oncogene: hrHPV E7 inhibits NF-kB activation and nuclear translocation and prevents its binding to the responsive DNA elements, e.g. the LCR of hrHPV. On the other hand, hrHPV E6 inhibits NF-kB (p65)-dependent transcriptional activity within the nucleus, thus further contributing to the escape of hrHPV from the transcriptional control of NF-kB (Spitkovsky et al., 2002; Nees et al., 2001). Thus, NF-kB is one of the targets through which these HPVs could interfere with the transcriptional control in cervical carcinogenesis.

Using immunohistochemistry it was demonstrated that NF-kB is constitutively activated in high-grade CIN (Nair et al., 2003). In term of stain pattern, the intensity of cytoplasmic NF-kB expression increased along with the increasing grade of CIN, being most frequent in invasive carcinomas. There was no detectable nuclear NF-kB expression in the normal cervix or CIN1 and CIN2 lesions; an intense nuclear expression appears very rare, even in CIN3 and cervical cancer and is related to hrHPV (Branca et al., 2006b). Like biomarker, increased/normal cytoplasmic NF-kB expression can distinguish CIN with high specificity, but low sensitivity. On the other hand, the nuclear expression suffers from lower sensitivity. Studies accomplished by Branca et al, (2006b) clearly demonstrated that neither cytoplasmic nor nuclear NF-kB staining is a significant predictor of the clearance/persistence of hrHPV types after treatment of CIN.

The ERK/MAPK cascade has been reported to be activated in cervical cancer cell lines both by hrHPV and by some low-risk HPV types. In normal squamous epithelium of the cervix as well as in metaplastic squamous cells, Branca et al (2004) found weak cytoplasmic ERK1 expression confined mostly to the parabasal layers. Intranuclear staining is detected in CIN lesions that increase in both intensity and extent towards higher grade CIN lesions.

ERK1 expression showed poor specificity for predicting hrHPV, and do not have a practical value as a predictor of hrHPV in cervical cancer and its precursors. Although E5 HPV seems to mediate overexpression and activation of the ERK/MAPK signalling cascade, multiple other mechanisms that mediate the activation of ERK/MAPK pathways might be involved. Despite the fact that ERK1 expression seems to be an early marker of cervical carcinogenesis, it is not a specific marker of hrHPV in CIN and cervical cancer, and does not predict disease outcome in the latter.

3.5 Apoptotic markers

Apoptosis or programmed cell death is initiated by two types of biological signals: (1) extrinsic - the specific ligands activate their receptors (Fas-Fas ligand interaction) and (2) intrinsic – mitochondrial pathway used in response to non-specific stimuli (such as alteration of DNA, radiation and osmotic stress), leading to release of cytochrome c. Both paths lead to the activation of caspase 3.

In HPV infected cells, inhibition of apoptosis may be a mechanism to promote viral persistence (Kanodia, 2007). HPVs exhibit several mechanisms for overcome the apoptotic program. E6 binding to p53 stimulates p53 degradation thus preventing p53-dependent apoptosis of infected cells. On the other hand, it suppresses FasL E5 mediated apoptosis and is associated with reduction to half of the Fas expression.

Fas (APO-1/CD95) system regulates diverse physiological and pathological mechanisms for apoptosis. Interaction between Fas ligand and Fas receptor induces cell death, mechanism that could help to destroy HPV-infected keratinocytes. Facilitated cellular proliferation (Das et al., 2000), through reduced Fas mediated apoptosis has been described in cervical carcinogenesis by immunohistochemistry (Reesink-Peters et al., 2005) and polymerase chain reaction (Das et al., 2000). On the other hand, the paracrine overproduction of Fas-L could facilitate tumour progression by inducing apoptosis of the immune cells usually expressing Fas in their membrane, such as CD8 and natural-killer cells. During HPV induced cervical carcinogenesis two Fas-related mechanisms may be taken into consideration: (a) suppression of apoptosis in infected keratinocytes by downregulation of Fas-R expression; and (b) active immunosuppression by Fas-L overproduction by tumor cells (Das et al., 2000; Griffith et al., 1995). Granular cytoplasmic and membranous Fas-R stains are identified by immunohistochemistry in the normal cervix, and their loss has been reported in approximately 50% of squamous intraepithelial lesion (SIL) and SCC (Jones & Munger, 1996; Lerma et al., 2008). Fas-R expression by tumor cells seems to be unrelated to the stage or quantity of the lymphoid infiltrate and it is a constitutive event independent of tumor progression (Lerma et al., 2008). Fas-L immunostaining in tumor cells is directly correlated with the tumor stages: 36.4% in stage I, 50% in stage II, and 75% in stage III, and inversely correlated with the presence of a florid lymphoid infiltrate. This suggest that Fas-L production by tumor cells results in decreased lymphoid cell reaction and might be a defence mechanism of the tumor against host's immunity (Lerma et al., 2008).

Bcl2 protein is localized in the mitochondrial membrane, the endoplasmic reticulum and in the nucleus. Bcl2 is an oncoprotein blocking cell apoptosis that can be induced by the absence of growth factors, alterations in DNA, viral infection, lymphokines action, cytostatic drug or radiation therapy. Its overexpression permits the malignant transformation of the cells and extends the survival potential of malignant cells.

The prognostic value in predicting lesion progression is disputed. Guimarães et al., (2005) found by immunohistochemical techniques that expression of Bcl2 in HPV-infected cervical biopsies is not useful for predicting the progression of HPV-related SIL. On the other hand, Singh et al., (2009) noted cytoplasmic expression of Bcl2 protein in cervical dysplasia, a various intensity of immunoreactivity between different cytological grades of cervical smears and an association with the presence of HPV16/HPV18.

Follow-up data revealed that cases with high-risk HPV and co-induced expression of apoptosis-regulatory proteins presented a trend to progressive disease (Sing et al., 2009). These data confirm the observations of Fonseca-Moutinho et al., (2004), that Bcl2 is an independent factor in defining low risk of progression for CIN 3 and co-expression of estrogen receptor, progesterone receptor, and Bcl2 may be a useful tool in identifying the CIN 3 lesions with low risk of progression toward cervical cancer. Bcl2 has a more important value in cancer, the ratio of Bcl2 to Bax expression determining the survival or the death following an apoptotic stimulus. In order to establish a new predictor of the outcome of radiotherapy for human cervical carcinoma, Harima et al., (1998) established that the increased Bcl2 expression after radiotherapy is correlated with poor survival, while increased Bax expression after radiotherapy is correlated with good survival; these findings suggest that the levels of Bax and Bcl2 expression after radiotherapy are useful prognostic markers in patients with human cervical carcinoma (Harima et al., 1998). Some studies confirm that evaluation of Bax and Bcl2 expressions provide independent prognostic information for the clinical course of the disease and therefore could be developed as prognostic indicators for cervical cancer: Bax expression was associated with good survival while Bcl2expression was associated with poor survival, and combination of Bcl2+/Bax+ was significantly associated with poorer disease free survival (Wootipoom et al., 2004).

3.6 Markers of chromosomal stability

Cells that escape from senescence by gene inactivation continue to divide and suffer telomeres loss reaching the second proliferative block, stage 2 of mortality (M2); this is characterized by massive cell death caused by critical shortness of telomeres and telomeres dysfunction. The telomerase is a RNA-dependent DNA-polymerase that synthesizes telomeres DNA and provides molecular bases for unlimited proliferative potential. Telomerase activity is absent in most normal somatic cells but present in more than 90% of tumor cells and immortalized cells *in vitro* (Kim et al., 1994). Regarding cervical neoplasia, it is not clear whether telomerase is activated during the progression of this disease or whether HPV16 infection activates directly telomerase *in vivo*. Most studies on cervical tissue or cervical swabs indicate telomerase activation only after progression to intraepithelial lesion (Nowak, 2000). Taken together, *in vitro* and *in vivo* studies suggest that infection with HPV16 and the concomitant expression of E6 is associated with telomerase activation. Cervical lesions containing hrHPV in early stages does not present telomerase activity or present it at low levels (Nowak, 2000). Some results show that telomerase activity appears only when E6 is expressed at elevated levels. The hTERT expression (telomerase catalytic subunit) is in agreement with E6 hrHPV role in telomerase activation, but this association lost its significance due to strong association between hTERT and stage of intraepithelial lesion. One feasible explanation may be done by the recent experiments concerning the E6/ E7 dynamics and telomerase expression along with the progressive grades (Peitsaro et al., 2004). The initially high levels of E6 decrease dramatically, while hTERT mRNA expression and telomerase activity increase by 10 and respectively 4 fold. This means that the telomerase activation by E6 HPV is an early event and selection of clones with increased telomerase activity will lead to tumor progression and this intimate association with gradual lesions hides hTERT association with E6 hrHPV. Taking into account that HPV infections have been associated with cervical cancer, telomerase activity may be a central mechanism by which HPV infections can lead to malignant transformation

of cervical mucosa. Immunohystochemical studies on biopsy specimens have shown that normal epithelium is completely negative for hTERT or presents a profile scoring positive cells in parabasal layer. More positive cells were observed in the squamous metaplasia epithelium and sometimes in suprabasal metaplasia proliferating cells. Positive immunostaining was nuclear and limited to a few cells and stromal immunoreactivity was strictly associated to lymphocytes having constitutive hTERT expression. CIN lesions and cancer present a different pattern: hTERT-positive nuclei are present in all the layers of epithelium, but occasional cytoplasm expression can appear (Branca et al., 2006c). Paradoxically, there was a decrease in the nuclear staining intensity of hTERT in squamous cell carcinomas (Frost et al., 2000; Yan et al., 2004) with an increase in cytoplasm staining (Jarboe et al., 2002). Disturbance of the normal translocation mechanisms of hTERT to the nucleus, associated with cervical mucosa malignant transformation may be responsible for these differences in the expression pattern. Although it has been demonstrated that the loss of hTERT immunostaining may be associated with the deregulation of normal translocation mechanisms of hTERT to the nucleus, some authors suggest another mechanism involved in a reduced hTERT expression in human cancers: inactivation at the transcription level (Bleotu et al, 2010). The heterogeneity of both sensitivity and specificity in telomerase detection seen between different studies is due to: sample size (smear/lavage versus cervical biopsy), contamination with blood or necrotic cells (including telomerase inhibitors, which can lead to false-negative results) (Wang et al., 2004) or haemoglobin (a powerful inhibitor of PCR reaction). False positive results in strong inflammatory reactions may occur due to inflammatory infiltration.

More recently, some **epigenetic modifications** were associated with cervix HPV infection. Modifications encompass three types of changes: chromatin modifications, DNA methylation and genomic imprinting, each of which is altered in cancer cell.

Generally, HPV infections are followed by epigenetic changes such as methylation of viral genes or host genome. The pattern of HPV genes methylation varies depeding with the viral life cycle, the presence of disease and possibly the viral type. The *de novo* methylation of HPV DNA could be a host defence mechanism or a strategy that the virus uses to maintain a long-term infection, or both. Aberrant methylation of CpG islands in the promoter regions of tumour suppressor genes (TSG) is one of several epigenetic changes which contribute to carcinogenesis (Kumar & Verma, 2006). Viral oncogenes can induce tumor suppressor gene methylation following activation of DNA methyltransferases. For some genes, the prevalence of methylated forms increases with disease severity; for others, methylated forms are only detected in women with invasive disease. In a recent paper, aberrant DNA methylation was found as an early event in carcinogenesis and as an additional molecular marker for the early diagnosis. Among all studied genes, three were found as potential biomarkers of cervical cancer risk (hypermethylation of CDH13, DAPK1 and TWIST1 promoters) (Missaoui et al., 2011).

Aberrant methylation of the p16 gene occurs early within tumor cell populations. p16 is more frequently methylated in advanced tumors (Wong et al., 1999) thus suggesting that its reactivation could have therapeutic value. In a study performed on 62 cases of squamous cell carcinomas, Cheung et al showed that promoter methylation of PTEN was found in 58% of patients with persistent disease while those who died of the disease had a significantly higher percentage of PTEN methylation. Thus, PTEN was considered an important

significant predictor for both total and disease-free survival after controlling age, pathologic grade and clinical stage (Cheung et al., 2004). Another studied gene was E-cadherin which methylation frequency in cervical cancer varies between 28 and 80.5% (Widschwendter et al., 2004a). It appears that E cadherin methylation have prognostic significance, cases with no promoter methylation having a better outcome in univariate and multivariate analyses. New studies are focused on identification of the methylation status of several genes present in the serum or plasma of patients with cervical cancer with regard to their prognostic significance.

In a study on 93 serum samples using the methyLight technique MYOD1 promoter methylation was strongly associated with shorter, disease-free and overall survival (Widschwendter et al., 2004b). Data suggesting that methylation of gene promoters in patients with cervical cancer is a common phenomenon have been reported. Strong correspondence between DAPK, p16, and MGMT genes methylation in serum and in primary tumors was noted thus allowing the discovery of a potential biomarker in sera. It has been found that parallel testing of HPV and PAX1 methylation in cervical swabs confers an improved sensitivity than HPV testing alone (80% vs. 66%) without compromising specificity (63% vs. 64%) for HSIL/SCC (Yang et al., 2003; Lai et al., 2008). When PAX1 methylation marker is tested alone, the specificity for HSIL/SCC is 99%. These data encourage further studies to identify a set of methylated genes that would have prognostic significance as surrogate markers.

Although the causal relationship between hrHPV infection and cervical cancer is demonstrated, HPV infection alone is not sufficient to induce the malignant transformation. Other genetic alterations, such as miRNAs, are required. MicroRNAs (miRNAs) are ~22 nt single-stranded, non-coding RNAs that generally negatively regulate their target mRNAs at a posttranscriptional level. The different expression of miRNAs in cervical cancer cells or tissues as compared with normal controls has been reported, and candidate miRNAs functioning as oncogenes (including miR-21, miR-127, miR-146a, miR-199a) and tumor suppressors (including miR-34a, miR-143, miR-145, miR-200a, miR-218) in cervical cancer carcinogenesis have been suggested (Lee et al., 2011). Using TaqMan MicroRNA Arrays, McBee and colab (McBee, et al, 2011) found that 18 miRNAs were overexpressed and 2 underexpressed (miRs-218 and 433). Only five miRNAs (miR-21, miR-135b, miR- 223, and miR-301b) may have the potential to be used as markers for progression from dysplasia toward invasive cervical disease. Some miRNAs might be down regulated in cervical HPV infection through methylation (Botezatu et al., 2011).

3.7 Markers of immune recognition

HPV presents some features that allow a specific immune behaviour: bypass the immune response, persist in the lower genital tract, induce and promote the progression of cervical cancer. These characteristics are related to (i) preferential localization of intraepithelial HPV infection, (ii) the absence of viral infection on the impact keratinocytes, (iii) the ability of HPV to interfere with innate immunity, and (iv) the expression of late proteins antigens responsible for generating antibody response. The virus uses some mechanisms to evade immune system: (1) it maintain slow infection levels so that only a low amount of virus is exposed to the immune system; (2) it exploits the redundancy of genetic code; (3) it mimics host proteins; (4) it modulates the antigen presentation; (5) interfere with IFN; (6) inhibition

of cytokines and chemokine profile; (7) distortion of adhesion molecules; (8) modulation of adhesion molecules; (9) prevention of apoptosis; (10) inhibition of APC migration.

Several studies have analyzed immunohistochemically different sub-types of immune cells in tumor tissue biopsies (TCD4, TCD8, TCD3, BCD20, CD45, CD57, CD68, etc.). The immune response to cervical neoplasia varies with the extension of the disease. In SIL, typically associated with persistent HPV infection and high viral load, the lymphocyte subpopulations percentages estimated by image analysis were 41% for CD4, 45% for CD8, 7% for CD20, and 7% for CD56 (Kobayashi et al. 2004). Most researchers revealed the essential role intra-and peri-tumor cells play in the favourable development of cervical cancer, suggesting that certain survival predictors in cervical cancer relapse may be involved (Nedergaard et al., 2007, 2008). Such predictors are: inflammatory infiltrate (role in promoting long-term survival), low CD3 (role in predicting relapse), high CD8 cell density (involved in cervical cancer favourable prognosis) and low CD4 cell (involved in advanced stages of disease) (Nedergaard et al., 2007, 2008; Bell et al., 1995; Bethwaite et al., 1996; Chao et al., 1999).

Cytokine profile distortion leads to inappropriate immune response, which may have immunosuppressive effects (failure to eliminate infection in host). HPV infections are focal and detection of systemic cytokine serum levels are not associated with the clearance or persistence of HPV infection (Hong et al., 2010).

Evaluation of cytokines may predict high-risk HPV clearance or persistence in untreated patients with mild dysplasia or less. Among HPV-infected women, IFN-gamma is significantly associated with E6, E7 HPV16 and high-risk HPV viral load in the uterine cervix. Thus, increased intralesional IFN-gamma may be considered a prognostic marker for oncogenic potential of high-risk HPV (Song et al., 2007). The multivariate logistic regression analysis showed that IFN-gamma-positive results were significantly associated with clearance of high-risk HPV after 12 months of follow-up, suggesting that intralesional IFN-gamma may be a prognostic marker for clearance of high-risk HPV (Song et al., 2008).

Most studies regarding cytokine profiles in HPV associated cancers indicate that Th2 cytokines correlate with progression toward invasive tumors (Bais et al., 2005). So, cervical tumors infiltrating lymphocytes have predominantly Th2/Tc2 polarity and regional lymph nodes seem to have a high proportion of T cells. However, this is due to the tumor rather than to the mechanisms of circumvention used by HPV. Comparing the cytokine profiles in cervical secretions of normal cervicitis, presenting or not HPV DNA, it were described high levels of IL-10 in HPV + samples (Azar et al., 2004). The early increase of IL-10 in cervical lesions can induce the inhibition of immune response against HPV infection. These data suggest that the cytokine profile distortion to an immunosuppressive profile can be induced by HPV and can cause the development of cancerous lesions (Kanodia et al., 2007). Several cytokines have been shown to reduce HPV transcription; this repression involves TGF-β, interleukin 1 and TNF-α (H. zur Hausen, 2002). TNF-α repression is lost during malignant conversion.

4. Conclusions

HPV associated cancer still remains a cause of death in women. Epidemiological studies and laboratory data confirmed that persistent infections with high risk human papillomaviruses

(hrHPV) cause virtually all cases of invasive cervical cancer. Cofactors and additional molecular events are essential for the transformation of cervical epithelial cells. These events imply cell changes that can be quantified in order to evaluate the correct status of the disease. An ideal marker should be easy to assay in a not invasively collected sample and should have a good sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). To investigate the cancer risks associated with HPV infection, sensible tools to asses the risk hierarchysation are requested to be developed, since the cytological assessment alone is not sufficient to classify cervical dysplasia. Sometimes, in order to discriminate between productive and transforming infection, a combination of biomarkers is required. Such combination will allow a correct risks hierarchysation. Although there are many studies focused on new potential biomarker for cervical cancer, until now few are validated by the scientific community and health care units.

5. Acknowledgements

6. References

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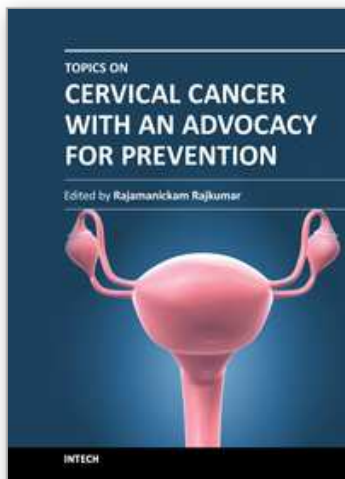
Topics on Cervical Cancer With an Advocacy for Prevention

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Cervical Cancer is one of the leading cancers among women, especially in developing countries. Prevention and control are the most important public health strategies. Empowerment of women, education, "earlier" screening by affordable technologies like visual inspection, and treatment of precancers by cryotherapy/ LEEP are the most promising interventions to reduce the burden of cervical cancer.Dr Rajamanickam Rajkumar had the privilege of establishing a rural population based cancer registry in South India in 1996, as well as planning and implementing a large scale screening program for cervical cancer in 2000. The program was able to show a reduction in the incidence rate of cervical cancer by 25%, and reduction in mortality rate by 35%. This was the greatest inspiration for him to work on cerrvical cancer prevention, and he edited this book to inspire others to initiate such programs in developing countries. InTech - Open Access Publisher plays a major role in this crusade against cancer, and the authors have contributed to it very well.

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