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Immunohistochemistry in assessment of molecular pathogenesis of cervical carcinogenesis

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Summary

Concerning the prediction of HPV-associated cervical disease, several importance issues are related both to the management of women with diagnosed CIN and those with cervical cancer. Oncogenic HPVs are capable of contributing to the development of malignant phenotype by several different mechanisms, most of which seem to be closely interrelated. Because of the fact that these molecular interactions are mediated by proteins, the logical strategy to dissect the complex molecular pathways is to study the functions of these proteins, utilising the capabilities of immunohistochemistry (IHC).

IHC offers practically unlimited possibilities to study any target molecules, against which a monoclonal or polyclonal antibody can be raised. This review describes the IHC-based strategies used by this author to assess the molecular pathogenesis of cervical cancer and its precursors in a number of large-scale prospective cohort studies conducted during the past 25 years.

In the ongoing HPV-PathogenISS study, 13 different markers are being tested to evaluate their predictive value in distinct viral events, e.g. persistence or clearance of high-risk HPV in women treated for CIN. Apart from getting new insights into the molecular pathogenesis of HPV-associated cervical carcinogenesis, we anticipate the disclosure of individual markers, a set of markers, or an expression profile of any such marker sets that would be of clinical value as predictors of disease outcome in cervical carcinogenesis.

Key words: Human papillomavirus; Immunohistochemistry; Cervical cancer; CIN; Biomarker; Prognosis.

Introduction

The key etiological role of the high-risk human papillomavirus (HR-HPV) types in cervical cancer has been confirmed beyond reasonable doubt. HR-HPV is shown to be associated with CIN and cancer in almost 100% of cases, in contrast to the low-risk HPV (LR-HPV) types that are rarely found in these lesions [1-7]. The detailed molecular mechanisms explaining the different oncogenic potential of LR-HPV and HR-HPV have emerged only recently [2-5]. It seems that these differences are linked, at least in part, with the different functions of two important viral oncogenes, E6 and E7, which are constantly expressed in HPV-positive cancer cells, and their expression is considered mandatory to initiate and maintain the malignant phenotype [2-5, 8].

The accumulated data from prospective follow-up studies suggest that the natural history of clinical HPV infections of the uterine cervix is basically identical to that of CIN lesions, with a) progression, b) persistence, and c) regression as the main outcome measures [1, 9, 10]. However, HPV infections have special features in their life cycle that are related to the different risks of developing cervical cancer [2, 4, 11]. It seems obvious that HPV type, viral load, acquisition of new (incident) infections as well as clearance of the virus, are salient features of the life cycle of HPV infections [1, 10, 12-16], but their significance in cervical carcinogenesis is incompletely understood. Results on the accumulation of incident HPV infections are still scanty and factors predicting these events are in part controversial [17-25]. Similarly, the first studies addressing the mechanisms of viral clearance have reported conflicting findings [21-25].

Predicting disease outcome is a major challenge in modern medicine. Concerning the prediction of HPV-associated cervical disease, several issues are of importance [1, 4, 5]. These are related both to the management of women with diagnosed CIN and those with cervical cancer. In the former, two issues are closely linked with disease outcome. These are: a) curative (radical) excision of CIN, and b) clearance/persistence of HR-HPV after treatment [1, 4, 5, 31]. Apart from the well recognised risk of recurrence associated with margin involvement in the cone, the role of persistent HR-HPV as a cause of treatment failure has achieved

increasing attention in the recent literature [31-33]. Prognosis of cervical cancer, on the other hand, is determined by several predictors, and according to the recent task force on prognostic factors in cervical cancer, there is an urgent need for more specific markers capable of predicting the disease outcome in individual patients [34-36]. The same is true concerning the prognostication of the natural history of CIN lesions. Despite some promising recent progress, the disease outcome of CIN lesions (if not treated) in individual patients is still unpredictable [4, 5, 37-40].

To better respond to this challenge of predicting the viral events and the clinical disease, we need to elucidate in more detail the molecular mechanisms regulating the life cycle of HPV in the cervical epithelium. More information is needed on the pathogenesis of HPV-induced cervical disease [41] as well as the molecular biology of the events leading to cell transformation and cervical cancer [1-3, 5, 42]. Before entering the discussion about the potential strategies on how to dissect these molecular pathways, and particularly the role of immunohistochemistry (IHC) in this strategy, a brief introduction to the pathogenesis and molecular biology of HPV-associated cervical carcinogenesis is necessary.

Pathogenesis of HPV infections in the cervix

Pathogenesis is a widely misused term in the literature, utilized to describe almost any event or series of events documented by different measures in any pathological process. In classical virology, however, viral pathogenesis is defined as a concept covering the mechanisms by which viruses produce disease in their host [43]. In most cases, our current understanding on viral pathogenesis is based on experimental studies with animal models and on observations of natural human infections, HPV infections not making any exemption in that respect [1-5, 41]. While approaching the pathogenesis of viral infections, this complex entity could be sequestrated a) into chronological stages, and b) into three different levels of involvement: 1) the host; 2) the target cell; and 3) the nucleic acids and their encoded proteins [2, 3, 41, 43]. To fully understand the complex interplay between the virus and the host in viral pathogenesis, one should be able to unravel the answers to a number of specific questions at each of these three levels. In our recent HPV textbook [4], a special chapter was devoted to the molecular pathogenesis of cervical HPV infections [41]. The mechanisms involved in virus replication and cellular transformation at the level of nucleic acids and proteins are discussed in another chapter [42].

Each of these three levels in viral pathogenesis poses new restraints that the virus has to surmount to proceed to the next level. Two mutually exclusive outcomes exist: 1) successful completion of all the stages results in disease, whereas 2) failure to complete these stages results in either a) abortive- or b) non-productive infection, or c) total failure to infect the cell [43]. In HPV infections, these options seem to be the following: 1) clinical infection (with potential to malignant transformation); 2) subclinical infection; 3) latent infection; or 4) no infection at all [1-5, 41]. For the viruses to survive, it is in their best interests to develop intricate mechanisms to evade the host resistance mechanisms, resulting in full-blown viral disease.

It will be a major challenge for HPV research to uncover the mechanisms leading to this untoward outcome of what basically seems to be a benign infection, usually leading to rapidly regressing harmless proliferations of the squamous epithelium, and only occasionally leading to development of CIN and cancer [1, 4, 5, 41]. At the level of the host, a series of key questions need to be unravelled to fully understand the eight different stages [43] in HPV pathogenesis [41]. Such questions include at least the following: 1) How does the virus enter the host? 2) Where does it undergo initial replication? 3) How does it spread in the host? 4) What organs and tissues are infected? 5) How is the virus transmitted? A detailed discussion of the issues related to these questions falls outside the scope of this review, but at least tentative answers are available to most of these key questions at the level of the host, unlike at the level of individual cells, where the mechanisms seem to be far more complex [41].

Before the full understanding of HPV pathogenesis at the cellular level, solutions should be available for the following questions: 1) What is the nature of the virus receptor? 2) What is the mechanism by which the virus enters the cell? 3) How does viral infection lead to alterations in host cell functions? and 4) What are the pathways and mechanisms by which the virus is released from the infected cells? It is clear that many of these issues are still far from being fully elucidated [1-5, 41, 44, 45]. A detailed discussion of the multitude of molecular mechanisms related to these events is not possible in this context; the reader is referred to the special text of the author on this subject [41].

In principle, these topics can be divided into three wider categories: 1) viral events, 2) alterations in the structure of host cells, and 3) alterations in host cell functions. Of the former, the most important are those related to: a) initiation of viral infection, b) transcription of HPV, c) translation of HPV mRNAs into viral proteins, d) HPV replication, e) assembly of HPV particles, f) maturation of HPV particles, and g) release of HPV particles from the target cell [2-5, 41, 42]. Alterations in morphology of HPV-transformed cells are linked with the changed properties of these cells, and can be categorized into a) alterations in cell surface structures, b) alterations of cytoskeleton, and c) altered cell adhesion properties.

Even more important than alterations of cell morphology, are alterations in the functions of virally transformed cells. Transformation induces a number of changes in the regulation of cell growth in vitro, including 1) decreased density regulation of cell growth, 2) decreased requirement for growth factors, and 3) decreased anchorage-dependence of cell growth. The in vivo equivalents of these altered in vitro growth properties are the events contributing to increased mobility of the transformed cells, contributing to the development of an invasive phenotype.

In brief, polypeptide growth factors bind to specific, high-affinity receptors on the cell surface, which triggers a cascade of events leading to induction of cellular DNA synthesis and cell division, through a wide variety of signal transduction pathways. Within several minutes to an hour, this leads to activation of gene expression involving up-regulation of a series of growth-related genes, many of which are cellular oncogenes *(c-onc)* [2, 3, 41]. In transformed cells, a constitutive production of growth factors renders them less dependent on external mitogenic factors, a phenomenon called autocrine growth stimulation. Among the most important polypeptide mitogens in serum are epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), and hormones such as insulin and insulin-like growth factors (IGFs). Dissecting these complex events in HPV-induced cell transformation leads us to the third level in our excursion across the HPV-associated pathogenesis, i.e., the level of molecular biology of nucleic acids and proteins [42].

Molecular biology of cell transformation by HPV

Papillomaviruses are non-enveloped, small DNA viruses with circular and double-stranded DNA genomes of approximately 7,200-8,000 base pairs. All putative protein coding sequences, called open reading frames, (ORFs) are restricted to one strand [1-5, 42]. ORF is a segment of DNA that is large enough to encode for a protein. The coding sequences have been classified as early (E) and late (L) to indicate their sequence of expression in the viral life-cycle. All PV genomes contain up to eight early (E) genes. E1, E2, E4, E5, E6 and E7 are found in most papillomavirus genomes, while E3 is found only in bovine papillomavirus 1 (BPV 1). The early genes encode for viral replication and cellular transformation and they are detectable in the proliferative areas of HPV-induced lesions. The late (L) genes, L1 and L2, code for the structural proteins and their expression is restricted to the differentiating part of the epithelium where also viral DNA replication occurs. ORFs L1 and E6 are separated by 400-1,000 base pairs that do not encode proteins, known as a long control region (LCR), but which contains promoter and enhancer DNA sequences critical in the regulation of viral replication and transcription [1-5, 8, 42].

Expression of HPV genes

HPV infection has its initiation in the basal cells of stratified squamous epithelia, where a particular combination of cellular factors interacting with the LCR starts the transcription of the viral E6 and E7 oncogenes [2, 3]. The E6 and E7 genes alter the cell cycle by their interaction and inactivation of tumour suppressor proteins: E6 binds and degrades protein p53, and E7 associates with p105Rb [8, 42]. E1 and E2 are the proteins to be synthesized next. E2 blocks the early transcription and permits E1-specific binding to the viral origin of replication (ori) located within the LCR, initiating viral genome replication. Following the course of viral infection, the E2-induced E6 and E7 down-regulation releases p53 and p105Rb proteins, and the differentiation process can continue. Then, a putative late promoter can activate the capsid genes L1 and L2, which assemble into mature virions, to be released from the infected cells [41, 42].

Disruption of the E2 gene transcription (by virus integration) is usually associated with cervical cancer. In the absence of E2, E6 and E7 remain constitutively expressed, sustaining the immortality of the infected

cells and blocking the epithelial differentiation programme. In situ hybridization studies have shown that the pattern of HPV gene expression varies according to the grade of the clinical lesion. HPV early (E) proteins can be detected throughout the whole spectrum of HPV lesions. In benign lesions, E4 and E5 are usually most strongly expressed, while the expression of E6, E7 and E2 is low [8, 42]. In contrast, E6 and E7 are up-regulated in high-grade lesions [46]. Relevant for the present discussion are only the functions of the transforming HPV oncogenes, E6 and E7, and those of E5, which seem to be weakly transforming.

HPV oncoproteins and cell cycle

The molecular pathways used by these three transforming HPV proteins are different from each other [8, 42]. Accordingly, E6 can form complexes in vitro with the tumour suppressor gene product p53, sharing similarities in this respect with adenovirus E1B protein and the SV40 large T antigen. In contrast to E1B and large T which inactivate p53 through their binding, E6-p53 complex formation requires an additional cellular protein E6-AP leading to complete degradation of p53. In HR-HPV positive cells, expression of E6 proteins results in increased turnover of p53, which leads to an abrogation of p21-mediated G1/S arrest in response to DNA-damaging agents [2, 3, 8, 42, 47, 48]. The best known target of p53 is $p21^{waf1/cip1}$, an inhibitor of CDKs, which mediates the p53-induced G_1-arrest by preventing the phosphorylation of the members of the pRb protein family (see below).

On the other hand, HPV E7 protein binds to pRb and triggers the release of E2F-like transcription factors from their complex with active, hypo-phosphorylated pRb [8, 42, 47, 48]. This results in active transcription of pRb-regulated genes and subsequently promotes the progression of the G1/S-phase of the cell cycle. Thus, E7-induced inactivation of pRb imitates a process, whereby pRb is inactivated a) through gene mutation, b) deletion of the gene, or c) through enhanced phosphorylation by over-expressed cyclin-dependent kinases, CDK4 and CDK6, all three events being detected in many human cancers [2, 3, 8]. These functions are characteristic of E6 and E7 of the HR-HPV types only, in contrast of E6 and E7 of the LR-HPV types, which fail to bind p53 and pRb [2, 3, 8, 42, 47, 48].

E5 seems to be weakly oncogenic and is suggested to potentiate the transforming activity of E7 [2, 3, 8, 42]. These oncogenic functions of HR-HPV E5 are mediated by up-regulation of the EGFR [42], known to be expressed in practically all CIN lesions [44]. Both EGF-dependent and EGF-independent mechanisms have been implicated for E5 functions [49]. In either case, the key event in E5-induced cell proliferation seems to be the activation of MAP (mitogen-activated protein) kinases (MAPK) in the MAPK signalling pathway [42, 49, 50]. Once phosphorylated, MAPKs migrate into the nucleus and phosphorylate their target transcription factors including *c-fos,* myc, *Ets1, Ets2, Elk-1, c-jun* [41, 49, 50].

HPV proteins and apoptosis

Apart from the important role played by HR-HPV E6 and E7 in cell cycle regulation, these two oncoproteins are also of key importance in preventing apoptosis [2, 3, 8, 42]. Indeed, the importance of E6/p53 interaction is in preventing p53-induced apoptosis. Despite the resistance to G1 arrest, E7 expressing cells, which contain functional p53, show an enhanced sensitivity to apoptosis. E7 does not interfere with the initial steps of the p53 response however, and E7 expressing cells show enhanced expression of $p21^{waf1/cip1}$ and reduction in cyclin E- and A-associated kinase activities following DNA damage. Thus, E7 might resist the activation of programmed cell death by E2F-1, known to trigger the induction of cyclin E- and A-kinases as well. The current data also imply that p53-induced G1 arrest and p53-induced apoptosis are two separate and independent pathways [42].

Immunohistochemistry (IHC) as a research tool

As evident from above, oncogenic HPVs are capable of contributing to the development of malignant phenotype by several different mechanisms, most of which seem to be closely interrelated. Because of the fact that these molecular interactions are mediated by proteins, the logical strategy to dissect the complex molecular pathways is to study the functions of these proteins. The scope of this communication is to decipher some strategies in how to approach this subject in HPV-associated cervical carcinogenesis using IHC as the research tool.

IHC is an elegant technique that has become widely adopted both in diagnostic pathology and as a research tool since the late 1970's, following the discovery of how to produce monoclonal antibodies in commercial quantities. This technology offers practically unlimited possibilities to study any target molecules, against which a monoclonal or polyclonal antibody can be raised. Due to its technical flexibility, IHC has proven indispensable in the differential diagnosis of many human tumours in routinely processed paraffin sections equally well as it is suitable to analyse the target molecules in fresh, frozen sections, cytological smears or cultured cells. IHC has numerous technical modifications, according to the nature of the study material, i.e., whether fixed or fresh tissue or cells. The detailed presentation of these different technological modifications clearly falls outside the scope of this review, suffice it state that it is impossible to imagine cancer research of today without IHC.

It is essential to realise the difference between the key research tools detecting DNA, RNA and proteins. Each of these has a distinct place in cancer research. While detection of DNA by different hybridisation techniques or PCR indicates e.g., that HPV-DNA is present, it does not necessarily imply that the virus is active. Detection of viral transcripts (mRNA) by PCR-based assays indicates that active transcription of the genome is ongoing. The end result will be proteins that arise through the translation of mRNA species into protein products. Because modifications frequently take place both in transcription and in translation, the analysis of DNA, mRNA and proteins does not necessarily measure equivalent events. IHC is used to detect the latter, i.e., functional proteins as the end products of gene activation, which are the key mediators of the molecular events leading to malignant phenotype [2-5, 8].

Strategies to study molecular pathogenesis of cervical cancer by IHC

Following the discovery of the link between HPV and cervical cancer precursors in 1976, the first application of IHC was in demonstration of HPV structural proteins in cervical biopsies, which was initiated in the early 1980's by this author [51, 52]. It was soon realised, however, that these structural proteins (L1 and L2) are only expressed in productive HPV infections, which precluded the use of this technique in detection of non-productive (transforming) infections, mostly found, e.g., in CIN lesions and cervical cancer [4, 5, 52]. As a diagnostic tool, IHC was replaced by DNA technology by the mid 1980's [4, 5, 51-53].

In the study of cervical cancer and its precursors, the applications of IHC are completely different today. Instead of detecting the virus and its structural proteins in the tissues, the main application of IHC is the intense search for the potential biomarkers that could be of predictive value for disease outcome in both cervical cancer and its precursors. Despite a substantial amount of effort made in this field, the discovery of such a perfect prognostic marker (or a set of markers) still awaits [8, 34-36]. There are several reports claiming that a certain marker or another is of prognostic significance in cervical cancer, but unfortunately, none of those have proven superior to the predictive value of the clinical FIGO stage, and few even as an independent predictor when entered in a multivariate analysis [34-36]. Despite these discouraging results, there is no doubt that the implications of any marker with independent prognostic value in cervical cancer, and even in CIN lesions, would be immense [1, 4, 5, 36]. In parallel with the increasing depth of understanding the viral life cycle and its associations with molecular events in cervical carcinogenesis, the search for novel biomarkers is more intense than ever, and a major breakthrough can be witnessed any time from now [8, 34-36].

Following is a short description of the strategies followed by this author and his research groups during the past two decades or so, in applying IHC to target intracellular pathways involved in the molecular pathogenesis of HPV-associated cervical carcinoma.

Research settings and study design

The simplest way of using IHC to study cervical carcinogenesis is to collect a retrospective series of archival (paraffin-embedded) cancer biopsies or CIN lesions, and analyse them using an antibody (or a set of antibodies) for a known biomarker. The limitations of this type of approach are evident, however. What you can achieve is to notice that the marker is differently expressed in different grades of CIN or in well- and poorly differentiated cancers, but the biological significance of the observation remains obscure, unless you can correlate the expression data with some biologically meaningful outcome measure. In cases of cancer,

such an outcome measure is usually the 5-year survival, but in cases of CIN, it is more difficult to imagine any such outcome measures if only one sample of each patient is available for analysis. In both cases reliable and complete follow-up data is needed which is not always easy to obtain even for cancer, and usually only exceptionally available for CIN lesions. In cases of CIN, it would necessitate repeated examinations of the same patient to document the disease outcome without treatment (i.e., persistence, regression, progression) or after treatment (cured, residual, recurrence) [4, 31-33]. Unfortunately, for many of the biomarkers studied in cervical carcinogenesis, this type of retrospective research setting based on archival material is the only one used so far. Needless to say, there are far more markers available today that have not yet been tested in CIN lesions or cervical cancer.

All these arguments advocate the use of a prospective cohort as the design-of-choice, when IHC is to be applied to search for clinically relevant prognostic biomarkers in this disease. As seen below, this type of setting also provides more options to correlate the IHC data with biologically relevant outcome data, not only in cancer but also in their precursors, when prospectively followed-up to evidence the disease outcome.

Study Designs

The author has been privileged in conducting several major prospective studies over the past two decades, providing a unique opportunity to apply IHC in sequential biopsies and/or baseline biopsies with disease outcome established by prospective follow-up. These major projects include the following: a) Kuopio HPV cohort study (1981-1998), b) NIS cohort study (1998-2002); c) the LAMS study (2002-ongoing), and d) the HPV-PathogenISS study (2002-ongoing). The study design of all these cohort studies has been different and the major focus has not been on IHC except in the latest of these projects (d).

The Kuopio HPV cohort study was the first and until today, prospective cohort study with the longest duration of those ever conducted on women with cervical HPV infections [4, 53]. The study has been presented in a large series of publications during the two decades of its duration, and the key results were synthesized in a recent monograph [4]. Among the over 200 reports published from this keystone study, there are several in which IHC was applied to analyse the molecular targets of HPV in cervical biopsies of these prospectively followed-up women [39, 44-46, 54-57]. The key molecules analysed include the cell cycle proteins, oncogenes, some growth factor receptors as well as several transcription factors. Despite some promising novel observations, none of those proved to be a useful predictor of disease outcome during the follow-up.

The NIS cohort study was designed to compare conventional PAP smears with HPV testing as screening tools in the low-resource settings of three new independent states (NIS) of the former Soviet Union [58, 59]. This recently concluded cross-sectional and cohort study comprises a cohort of 3,187 women (at different risk for HPV and CIN), of whom complete follow-up data are available for 887 women. In this study, a panel of ten antibodies was used to assay the expression of groups of less extensively studied proteins regulating cell cycle (cyclin-A, E2F4, p21, MIB-1, pRb/p107, pRb/p105, pRb/p130) and cell adhesion (α-, β- and E-cadherin). The reporting of the results of this study has just started, and the analysis of the IHC data is in the pipeline [58-60].

The third of these cohort studies is known as the LAMS (Latin American Screening) study and comprises a cohort of 12,107 women, enrolled by four clinics in Brazil and Argentina, in areas with different risks of cervical cancer [61]. The two major aims of this (EC-funded) ongoing cross-sectional and cohort study are: a) to compare eight different diagnostic tests as potential screening tools in these low-resource settings, and b) to assess whether the different risks of cancer in the different geographic areas (South/North) are due to 1) different natural history of cancer precursors, or 2) different exposure to the main risk factor, oncogenic HPV types [61]. Women with CIN lesions are prospectively followed-up, and their biopsies are subjected to IHC analysis for a selected series of biomarkers, different from those examined in the previous two cohorts. This study is ongoing, and not even these markers for IHC analyses have been selected as yet [61, 62].

The fourth of these major studies (HPV-PathogenISS) is the only one where the major focus has been on IHC testing of a series of antibodies to key molecular markers of cellular carcinogenesis, carefully selected following the strategy explained below. This study has two main components; 1) a prospective series of (HIV- and HIV+) women with cervical HPV lesions, prospectively followed-up to compare disease outcome in these two groups, and 2) a retrospective series of 300 biopsy samples (150 cervical cancer and 150 CIN), with complete follow-up for cancer patients and serial PCR data of the women after cone treatment of their CIN [31]. The study design and baseline data have recently been reported [63].

Outcome measures

Apart from the traditional disease outcome measures (survival, death), the prospective study design combined with the molecular diagnostic tools of HPV enables the assessment of a series of other outcome measures, both clinical and viral [4]. This applies particularly to CIN lesions, for which few such measures are available in archival (retrospective) series, as discussed above. These outcome measures are known as intermediate endpoint markers of cancer, i.e., conditions in the causal pathway towards cancer, and as such can be considered as surrogate markers of invasive cervical cancer [65, 66]. Such surrogates readily include CIN lesions of intermediate and high grade (CIN2 and CIN3), as well as their counterparts detected in cytological smears, i.e., HSIL. Both of these represent lesions evolved as a result of demonstrated progression of low-grade lesions (N-CIN, CIN1 or ASC-US, LSIL) to higher grade during the follow-up, as confirmed by biopsy and PAP smear, respectively. Optional outcomes of CIN are regression and persistence [1, 4, 5, 64], representing useful intermediate endpoint markers as well. Importantly, disease recurrence after radical treatment of CIN is another disease outcome with value as an intermediate endpoint marker. In invasive cervical cancer, several biomarkers seem to be of potential relevance in the assessment of the cellular mechanisms through which, e.g., the effects of neoadjuvant chemotherapy are mediated [67, 68].

In addition to these intermediate endpoint markers detected by morphological means, and representing different stages of clinical disease, a set of intermediate endpoint markers can be clearly defined for HPV infections as well. Thus, the above-listed clinical endpoint markers have their counterparts in the life cycle of HPV; viral persistence, clearance or incident infections [1, 4, 5, 10-16, 17-30, 64]. All these events are best evaluated in a prospective cohort study, using DNA detection techniques (PCR, HCII, others). In addition, the role of persistent HR-HPV as a cause of treatment failure of CIN has achieved increasing attention in the recent literature [31-33]. Thus, detection of HPV DNA in the cervix after excision of a CIN lesion is another meaningful endpoint marker in the management of this disease.

Molecular targets in cervical carcinogenesis

Like most (if not all) human malignant neoplasias, cervical carcinoma has a multi-factorial etiology. This disease is unique among human malignancies, however, in that the key etiological agent (HPV) is well established [1-7]. Despite a substantial amount of effort put into the basic research of cervical cancer during the past 25 years, many of the detailed molecular events in carcinogenesis are still incompletely understood. To better elucidate these highly complex mechanisms, it is conventional to subdivide these topics into separate entities, each representing a distinct area in molecular cancer research. In the following this subdivision into distinct areas is adhered to, while representing our strategy to target the key molecular pathways in cervical carcinogenesis using IHC-based strategies.

Cell adhesion

All cell adhesion molecules are trans-membrane glycoproteins, changing their structure upon ligand binding. They can affect cell functions through other molecules to which they are attached, some of which are part of the cytoskeleton or enzymes. There are six families of adhesion molecules: the immunoglobulin-like superfamily, cadherins, integrins, receptor protein tyrosine phophatases (RPTP), selectins and hyaluronan receptors. Most of these are cell-cell adhesion molecules, whereas integrins and CD44 mediate cell-matrix adhesion. In addition, CD44 also mediates cell-cell adhesion. These factors have not been extensively studied as prognosticators of cervical cancer so far [69, 70]. We recently demonstrated that the response to neoadjuvant chemotherapy and patient survival in cervical cancer was independently predicted by the expression of CD44v6 isoform [67], while none of the other adhesion molecules analysed had any such predictive value.

In the ongoing HPV-PathogenISS study, we selected E-cadherin as the target for our IHC analysis of the expression of cell adhesion molecules in CIN lesions and cervical cancer [63]. Cadherins are a large family of cell adhesion molecules that mediate cell-cell interactions by means of Ca^{2+}-dependent, homophilic protein-protein interactions. E (epithelial)- and N (neuronal)-cadherins are the best characterized of these [67, 70]. E-cadherin is essential for the formation and maintenance of epithelia, and loss of function of E-cadherin correlates with increased invasiveness and metastasis of different human tumours. For regular adhe-

sive function, E-cadherin has to form complexes with peripheral cytoplasmic catenins which are multifunctional proteins that are also involved in signal transduction and growth regulation; α-, β-, and γ-catenin.

One of the prerequisites for the release of carcinoma cells from the primary site might be a defect in intercellular adhesion mediated by the absence of E-cadherin expression. Thus, expression of E-cadherin might be an important parameter for the determination of the invasive potential of epithelial neoplasms, and for the transition of a benign to a malignant neoplasm. In our study, the expression of E-cadherin is being analysed in different grades of CIN and in cervical cancer, to assess its role as a potential progression marker of the disease, as well as in prognostication of two outcomes; survival in cancer and HPV clearance/persistence after treatment of CIN. The results are currently pending, and the future direction of these analyses depends on the observations made with E-cadherin IHC.

Invasion, angiogenesis and metastases

By definition, invasiveness and capability of sending distant metastases are the two essential determinants of malignant growth. The mechanisms regulating these two processes are highly complex and several different pathways are involved, acting independently or interrelated to each other. Accordingly, numerous molecular markers are available to analyse these events using IHC. In our study, we selected a strategy to use three different markers of the invasive potential of both cervical cancer and its precursor lesions. These include: a) MMP-2 (type IV collagenase or gelatinase A); b) TIMP-2 (tissue inhibitor of matrix metalloproteinase-2); and c) VEGF-C (vascular endothelial growth factor-C).

Loss of negative growth regulation and high invasive potential are often associated with abnormal expression of matrix matalloproteinases. MMP-2 is one of the signalling mechanisms for cells to begin migration, triggered by the splitting of laminin-5 and exposure of an integrin-binding site on this component of the basement membrane (BM). Usually, an altered form of laminin is found in tumours and in tissues being otherwise altered. The activated form of MMP2, however, is not found in benign tumours. Thus, detection of this enzyme is a possible early indicator of tumour activity. Until now, few studies are available on MMP-2 in cervical cancer and its precursors [71, 72], usually analysed together with its tissue inhibitor TIMP-2. We selected the same strategy, having TIMP-2 as the second antibody in assessing the mechanisms related to invasive potential and its inhibition in our lesions. TIMP-2 specifically inhibits the proteolytic (MMP-2-mediated) invasiveness of tumour cells and normal placental trophoblast cells as well. Thus, MMP-2 and TIMP-2 antibodies can be used together to evaluate the balance between the invasive potential and its tissue inhibition in CIN and cancer lesions [71, 72]. Linked with the type-specific HPV data, new insights in understanding the eventual differences in the aggressiveness of these lesions are to be anticipated.

In addition to these proteolytic mechanisms (MMPs), angiogenesis (neovascularisation) seems to play a key role in tumour invasiveness. There are several well characterised angiogenic factors, of which we selected VEGF-C as our target molecule because it is incompletely analysed in cervical cancer as yet [73, 74]. VEGF-C forms primarily non-covalent linked dimers that can phosphorylate VEGFR-3 (vascular endothelial growth factor receptor-3). During early embryogenesis, all endothelial cells express VEGFR-3, while in adult tissues, VEGFR-3 expression disappears from the vascular endothelial cells and is observed only in the lymphatic endothelium. In adult tissues, VEGFR-3 expression is induced upon tumour implementation, suggesting an important role for this receptor in tumour angiogenesis. Both VEGF-C and VEGF-D bind to and activate VEGFR-3 [73, 74].

The end result of angiogenesis and invasion is the development of metastasis which is the hallmark of a malignant tumour. This process can be predicted using IHC for the known markers of an anti-metastatic gene, e.g., nm23-H1 (NDP Kinase ß) [75, 76]. The antibody selected for our study recognizes nucleotide diphosphate (NDP) kinase-ß. The NDP kinase/nm23 gene was first identified in mouse-derived melanoma cell lines with high and low metastatic potential, and it was proposed as an anti-metastatic gene. It is now well established that nm23-H1 and nm23-H2 are metastasis-suppressor genes, implicated in the control of metastatic processes of malignant cells. Levels are reduced in tumours with high metastatic potential, and high levels correlate with favourable prognosis, e.g. in breast cancer, which has been most intensely studied for this antibody. On the other hand, only a few studies are available on nm23-H1 expression in cervical cancer [75, 76].

Cellular receptors

A wide diversity of cellular receptors are involved in malignant transformation and maintenance of the transformed phenotype [1-5]. Accordingly, a plethora of antibodies are available against well characterised cellular receptors with great functional diversity. Being in line with the above-listed markers, we selected an antibody which recognizes a 67kDa protein, identified as the high affinity laminin receptor. Laminin is one of the major glycoproteins of the BM, and displays multiple biological activities which are mediated through its interactions with specific cell membrane receptors [77, 78]. Up-regulation of laminin receptor is reportedly an independent prognostic factor in breast cancer and correlates with the dissemination of the tumour cells in bone marrow. Thus, laminin receptor appears to play an important role in tumour invasion and metastasis. This was one of the reasons to select this receptor as one of our targets, while complementing the tools used to analyse invasion, angiogenesis and metastases, listed above. The other major reason was that laminin receptor has rarely been studied in cervical cancer and its precursor lesions [77, 78].

Cell proliferation

Cell proliferation is one of the first signs of cell transformation, detected early in the causal pathway to cancer. Apart from conventional morphological measures (mitotic activity), several specific markers are available for IHC analysis of cell proliferation. The choice between these is mostly based on personal preferences rather than on differences in their technical performance [55]. For our current analysis, we selected a monoclonal antibody that recognizes the 36kD DNA polymerase delta accessory protein, known as proliferating cell nuclear antigen (PCNA). This is one of the traditional markers of cell proliferation that accurately identifies the proliferation status of tumour tissue, and has been shown to be of prognostic value in several human malignancies. In cervical cancer, however, this proliferation marker has been less extensively studied [68, 79, 80]. In addition, PCNA is a marker for cells in early G1 and S phases of the cell cycle, and as such well complements the other markers of different phases of the cell cycle selected for our study, as will be explained later.

Transcription

Transcription is the process whereby the genetic information of DNA is transcribed to messenger RNAs, or RNA transcripts. Transcription of HPV oncogenes E6 and E7 is one of the most intensely studied subjects in cervical carcinogenesis, and seems to be under the control of a highly complex array of cellular proteins known as transcription factors [1-5]. We have previously analysed some of the conventional transcription factors in CIN lesions using IHC [45]. Discouraged by the failure to establish a valuable prognostic marker among these conventional transcription factors (Skn-1, Oct-1, AP-2), we decided to select one of those described more recently, i.e., NF-kappaB (NFkB) [81, 82].

In resting cells, NFkB is retained in the cytoplasm bound to inhibitory proteins of the IkB family. Degradation of IkB proteins occurs with cell activation by a variety of signals, including inflammatory cytokines, bacterial lipopolysaccharides as well as oxidative and fluid mechanical stress. NFkB plays a role in the development of numerous pathological states. Activation of NFkB induces gene programs leading to transcription of factors that promote inflammation, such as leukocyte adhesion molecules, cytokines, and chemokines. Members of the rel/NFkB family of transcription factors are involved in regulation of cellular responses, including growth, development, and inflammatory response. Complexes of p50 (NF-kB1) or p52 (NF-kB2) are generated through the processing of p105 and p100 precursors, respectively. The homo- and heterodimer formed through combinations of NFkB/Rel proteins bind distinct kB sites to regulate the transcription of different genes. The HPV16 E6 protein also seems to stimulate expression of multiple genes known to be inducible by NFkB and AP-1. E6 seems to enhance the expression of functional components of the NFkB signal pathway and increases the binding of NFkB and AP-1 DNA consensus binding sites [82]. Until now, however, no data are available on the possible prognostic value of NFkB in cervical cancer.

Cell cycle regulation

Some discussion has already been made concerning the important role played by HPV oncoproteins in cell cycle regulation [2, 3, 8, 42, 47-50]. Because of the complexity of cell cycle regulation, a wide selec-

tion of markers are available to target the different regulatory proteins. To avoid repeating the work done with the conventional cell cycle markers (p53, pRb), we selected one of those newcomers, topoisomerase-IIα (Topo II) for the present analysis. In mammalian cells, Topo II consists of two isozymes, Topo IIα (170KD) and Topo IIβ (180KD). The latter is expressed constantly throughout the cell cycle, whereas the expression of Topo IIα is cell cycle-regulated, peaking in the G2 to M phase and declining to a minimum at the end of the M phase [83, 84].

Topo IIα plays an important role in the synthesis and transcription of DNA as well as in chromosomal segregation during mitosis. It is reported to be a sensitive and specific marker of the late S-, G2- & M phases in transformed and developmentally regulated normal cells. Topo IIα has also been implicated in drug resistance of tumour cells. Topo IIα amplification has been associated with an increased Topo IIα protein level in breast cancer cell lines and primary tumours, whereas Topo IIα gene deletion decreased the Topo IIα protein level markedly. In cervical cancer and its precursors, IHC has rarely been used to analyse the expression of Topo IIα, and practically no data are available on its associations with HPV oncoproteins in cell cycle regulation [83, 84].

Reference was made before to HPV E7-induced inactivation of pRb which imitates a process, whereby pRb is inactivated a) through gene mutation, b) deletion of the gene, or c) through enhanced phosphorylation by over-expressed cyclin-dependent kinases, CDK4 and CDK6, all three events being detected in many human cancers [2, 3, 8]. Under normal conditions, the activity of CDK4 and CDK6 is strictly regulated by several CDK inhibitors, one of which is $p16^{INK4a}$ [8, 42, 47, 48]. This CDK inhibitor seems to be inactivated in many human cancers either by mutation, deletion or hyper-methylation of the gene, resulting in reduced expression of the $p16^{INK4a}$. Being uninhibited, CDK4 and CDK6 increase their activity leading to premature phosphorylation and inactivation of pRB. Because expression of $p16^{INK4a}$ is regulated by negative feedback from pRb, reduced or lost pRb function results in up-regulation of $p16^{INK4a}$ expression in such cells. However, when pRb is inactivated at the nucleic acid or protein level, such cells are released from the growth-inhibitory stimuli of the CDK inhibitor $p16^{INK4a}$, and continue to proliferate even in the presence of high levels of $p16^{INK4a}$ [8]. Accordingly, inactivation of pRb through binding with E7 of the HR-HPV types should result in up-regulated expression of p16INK4a, and the latter could represent a specific biomarker of cells expressing HPV E7 [8], with widespread implications, e.g., in screening. In our study, we selected $p16^{INK4a}$ antibody to analyse the dynamics of cell cycle regulation in different grades of CIN and cervical cancer, to establish whether this marker is of potential use as a predictor of HPV clearance/persistence or cancer prognosis.

Apoptosis

One of the key characteristics of normal cells is their elimination by programmed cell death or apoptosis [85]. On the other hand, failure to do so is a typical feature of malignant cells, and this inhibition of apoptosis has been extensively studied over the past years. There are several well established regulators of apoptosis (either inhibitors or mediators), which can be analysed using IHC. For the present study, we decided to analyse two of those, telomerase and survivin.

Ever since the discovery that telomeres are longer in cancer cells and telomerase is activated in immortal cells, telomerase has been associated with oncogenesis. To explain the importance of telomerase [86, 87], some background information is necessary. In normal cells, the 3'-ends of chromosomes are capped with telomere sequences (TTAGGG; 6-26 nucleotides in length) by ribonucleoprotein telomerase during DNA replication. Telomerase is an unusual RNA-dependent DNA polymerase that uses an RNA component to specify the addition of telomere. The telomeric RNA contains a sequence complementary to TTAGGG. In ciliated protozoa and yeast, telomere length is constantly maintained by regulating the activity of telomerase. Many normal mammalian cells do not express telomerase, however, resulting in shortening of telomeres with each cell division, and ultimately causing chromosomal instability, aging and cell death (apoptosis). Introduction of telomerase into normal human cells has been shown to extend normal cell life by about 20 cell doublings. Recent evidence suggests that *c-myc* up-regulates the catalytic subunit of telomerase, TERT. The latter also cooperates with HPV E7 in cell immortalization [2-5]. These data prompted us to select telomerase as one of our targets in assessing the regulation of apoptosis, related to oncogenic HPV in the present series.

Another regulator of apoptosis that has attracted interest only recently is a protein known as survivin [88, 89]. Survivin encodes a structurally unique inhibitor of apoptosis (IAP). Survivin expression is turned off during foetal development and is not found in non-neoplastic adult human tissues. It becomes abundantly re-expressed in transformed cells and in all of the most common cancers of lung, colon, pancreas, breast and prostate in vivo. Survivin appears to be at the crossroads of cell death and cell division, involved in cytokinesis while also suppressing apoptosis. The expression of survivin in cervical cancer and its precursors has been incompletely studied [88, 89], and its associations and potential interactions with high-risk HPV types is practically unexplored as yet.

Cell signalling pathways

Practically all extra-cellular signals (both stimulatory and inhibitory) are transmitted into the nucleus via different signal transduction pathways (cascades), of which there are several for different purposes. Studying all these would not be practical and in fact impossible, because of the wide variety of markers available. To maintain the link with HPV oncogenes, we selected one of the key markers of the mitogen activated protein kinase (MAPK) signalling cascade, ERK1, the rational being as follows.

MAPKs consist of several subgroups, including the ERK, JNK, and p38 kinases. The members are regulated by many different extracellular signals ranging from cytokines, growth factors (like EGF), and neuropeptides. The pathways regulated by the MAPKs control a broad array of cellular responses ranging from survival, cell proliferation, and apoptosis. Interestingly, the oncogenic functions of HR-HPV E5 are mediated by up-regulation of the cell surface receptors for EGR (EGFR) [42], known to be expressed in practically all CIN lesions [44]. Both EGF-dependent and EGF-independent mechanisms have recently been implicated for E5 functions [49]. In either case, the key event in E5-induced cell proliferation seems to be an activation of MAPK in the MAPK signalling pathway [42, 49, 50]. This links the HPV E5 protein to the intracellular phosphorylation cascade, initiated by growth factor receptors, like EGFR, which upon binding with their ligands activate oncogene *ras* on plasma membranes. This evokes recruitment of another oncogene, *raf*, to the cell membrane, leading to activation of MEK (MAP-ERK) kinases. MEK kinases in turn phosphorylate MAPKs, which migrate into the nucleus and phosphorylate their target transcription factors including *c-fos*, myc, *Ets1, Ets2, Elk-1, c-jun* [41, 49, 50]. In addition to several others, the MAPK subfamilies include a pair of 42-44kd protein kinases, known as extracellular signal-regulated kinases or ERK1 and ERK2. Of these two, we selected the former as a marker of the MAPK signaling pathway, sharing this intriguing link with the HPV E5 oncoprotein [42, 49, 50].

Expected outcome

Using the panel of 13 antibodies selected following the strategy discussed above, the primary aim was to explore the potential usefulness of these markers as predictors of the outcome measures at two different levels; clinical disease and viral events. The former include a) progression of CIN from low-grade to high-grade, evaluated by the eventual different marker expressions in CIN1 through CIN3, and b) disease outcome in cervical cancer patients. The current study design (HPV-PathogenISS) also enables us to evaluate the predictive value of these markers in distinct viral events, e.g., persistence or clearance of HR-HPV in women treated for CIN and followed-up by serial PCR analysis, as detailed before [31]. The research strategy proceeds by analysing each of the 13 markers individually using uni- and multivariate analysis and the outcome measures at these two levels. As the final step, an ambitious attempt will be made to analyse all 13 markers combined, to assess whether a set of markers or some particular expression profile of several distinct markers would be of value as an independent predictor, exceeding the power of conventional predictors (e.g., FIGO stage). At this writing, the analysis of individual markers is ongoing, whereas some of the others still await the completion of IHC staining.

Conclusions

The adopted research strategy represents the first systematic approach to evaluate the key events in the molecular pathogenesis of HPV-associated cervical carcinogenesis by using IHC. With our panel of 13 carefully selected antibodies, coverage has been obtained for a wide range of key intracellular pathways known

to be involved in cell transformation. Utilising well characterised clinical material including both precancerous and cancerous lesions, different outcome measures can be used as dependent variables in univariate and multivariate analysis, to disclose the potential predictive factors of these outcomes of interest (disease prognosis, viral persistence/clearance). Apart from getting new insights into the molecular pathogenesis of HPV-associated cervical carcinogenesis, we anticipate the disclosure of individual markers, a set of markers, or an expression profile of any such marker sets that would be of clinical value as predictors of disease outcome in cervical carcinogenesis.

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Appendix

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