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Current Insight into Specific Cellular Immunity of Women Presenting with HPV16-Related Vulvar Intra-Epithelial Neoplasia and Their Partners

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1. Introduction

The premalignant lesions of vulvar intraepithelial neoplasia (VIN) involve the mucosal and/or cutaneous epithelium of the vulva. VIN may be HPV-related VIN (usual VIN) or – unrelated and represents the most frequent vulvar cancer precursors. Usual VIN occurs in adult women and commonly resembles persistent anogenital warts which are often multifocal pigmented papular lesions. It is caused by high-risk HPV (HR-HPV) types, essentially 16 in up to 91% of the cases (Srodon et al, 2006), and histologically, it is made of poorly to undifferentiated basal cells and/or highly atypical squamous epithelial cells (McClugagge et al, 2009). The involvement of the entire thickness of the epithelium defines the grade 3 of the disease (VIN3). The disease progresses towards invasion in about 3% of treated patients and 9% of the untreated ones according to a review of over 3,000 cases (van Seters et al, 2005) whereas evolution towards invasive carcinoma is observed in about 30% of untreated grade 3 cervical intraepithelial neoplasia (CIN3) patients (Ostor et al, 1993).

2. Virology

HPVs are DNA viruses with a circular double strain genome including 8 000 base pairs. The genome is divided into three regions: a Long Control Region which controls viral replication, a region coding for Early proteins (E1 to E7, including the E6 and E7 proteins that share oncogenic and transforming properties), and a region coding for Late proteins such as L1 and L2 proteins that constitute 80% and 20% of the viral capside, respectively. More than 150 HPV have been sequenced, one HPV being considered different from another when there is a difference in 10% of nucleotides coding for L1 genes.

Following a breach in the malpighian pluristratified epithelium, HPVs infect basal stem cells of keratinocytes. The virus initially remains in episomal form with synthesis of E2 protein. This protein is a major regulator of viral vegetative cycle and is required for transcriptional

regulation as well as viral DNA replication together with the E1 helicase (Desaintes et al, 1996). In contrast, E2 is generally undetectable in cancers due to a preferential integration of the viral genome in the cell genome and disruption of the E2 open reading frame (Berumen et al, 1994; Collins et al, 2009). Therefore E2 is a marker of viral infection and is specific for the early stages of the viral gene expression in infected cells. This was formally demonstrated in a recent work that showed a strong staining of the E2 protein in the intermediate differentiated layers of HPV16-infected tissues and low grade CIN (Xue et al, 2010). The high expression of HPV16 E2 in low grade lesions therefore represents a marker for HPV infection even before any clinical manifestation.

After integration of the genome of oncogenic HPVs such as HPV16 into the host genome, viral oncogenic E6 and E7 proteins are synthesized in large quantities in the inner third of the epithelium. E6 links to p53 and induces its degradation by the ubiquitin pathway and E7 links to pRB and allows the release of growth factors such as E2F.

During maturation of keratinocytes from the basal layer to the epithelial surface, viral capside proteins L1 and L2 are synthesized and expressed at the surface of mature keratinocytes in order to form a new viral particle which is able to infect adjacent healthy epithelium and to contaminate sexual partners.

3. Epidemiology of HPV16 related VIN

HPV infections occur preferentially in young women under 25 years of age (Boulanger et al, 2004). Several stages of lesions can be observed following oncogenic HPV infection. The first stage is a simple infection of keratinocytes that become koilocytes. The following stages are related to the transformation of infected keratinocytes into malignant cells. The depth at which malignant cells are found defines the disease stage. High grade squamous intraepithelial lesions as VIN3 are diagnosed on the basis of biopsy, with malignant cells in entire thickness of the epithelium

The premalignant lesions of HPV-related grade 3 intraepithelial neoplasia involve the mucosal and/or cutaneous epithelium of the vulva (usual VIN or VIN3), perineal and perianal region. Usual VIN occurs in adult women and commonly resembles persistent anogenital warts that are more often multifocal pigmented papular lesions disseminated on the vulva and/or the perianal skin than monofocal unique lesion (Figure 1).

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Fig. 1. Clinical presentations of usual multifocal or monofocal vulvar and preineal intraepithelial neoplasia

4. Why does usual VIN can spontaneously regress?

Although usual VIN lesions are often chronic and recurrent, they can regress spontaneously in up to 35% of young (less than 30 years) women presenting with multiple pigmented lesions within a median duration of 9.5 months (Jones et al, 2005} (Bourgault Villada, 2010). We previously studied a patient who presented with multifocal usual VIN and showed a complete clearance of viral lesions eight months after disease onset and two months after electrocoagulation of less than 50% of the usual VIN lesions (Bourgault Villada et al, 2004). Immunohistochemical study of her initial vulvar biopsy revealed a marked dermal infiltrate containing a majority of CD4+ T lymphocytes and an epidermal infiltrate made up of both CD4+ and CD8+ T cells (Figure 2). She showed also a proliferating response against one peptide from E6 protein and a high frequency anti-E6 and anti-E7 effector blood T cells by *ex vivo* IFNγ– ELISpot assay just before clinical regression (Figure 3). Such a study of blood cellular immune responses together with the analysis of vulvar biopsies obtained simultaneously and correlated to clinical outcome was not previously reported. In an anti-HPV vaccine trial conducted by Davidson and al (Davidson et al, 2003), usual VIN lesions completely regressed in a patient following vaccination. Interestingly, immunostaining of

vulvar biopsy prior to the vaccine showed a marked CD4+ and CD8+ T lymphocyte infiltrate of both epithelial and sub-epithelial sheets. One may wonder whether the regression of these patient lesions could be related to a spontaneous regression. Therefore, the observation of a CD4+ and CD8+ infiltrate within sub-epithelial and epithelial sheets in the biopsy and the visualization of very strong blood anti-HPV T cell responses in patient with usual VIN could be predictive of spontaneous clinical outcome. It may also be thought that high numbers of blood CD4+ and CD8+ lymphocytes after therapeutic vaccination could allow clearance of HPV-16 lesions in usual VIN, assuming that anti-HPV vaccine-induced T effector cells could home in the HPV cutaneous and mucosal lesions.
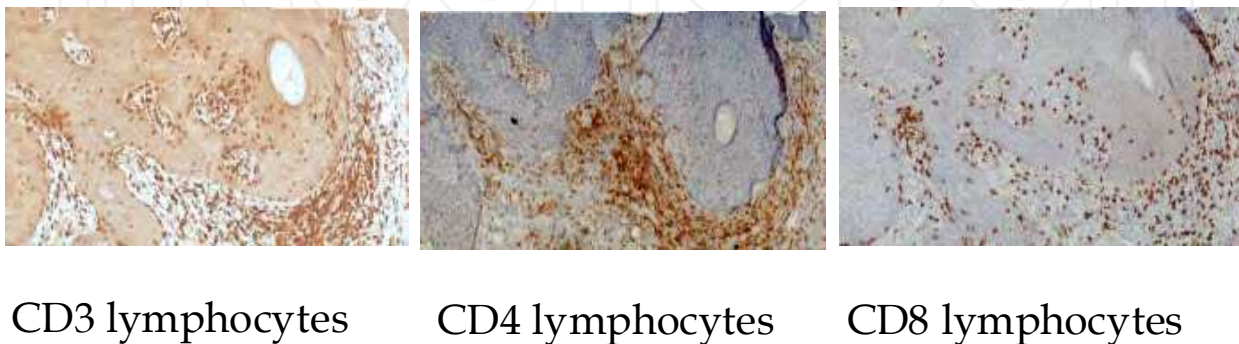
CD3 lymphocytes   CD4 lymphocytes   CD8 lymphocytes

Fig. 2. Immunohistochemical study of the vulvar biopsy just before spontaneous regression
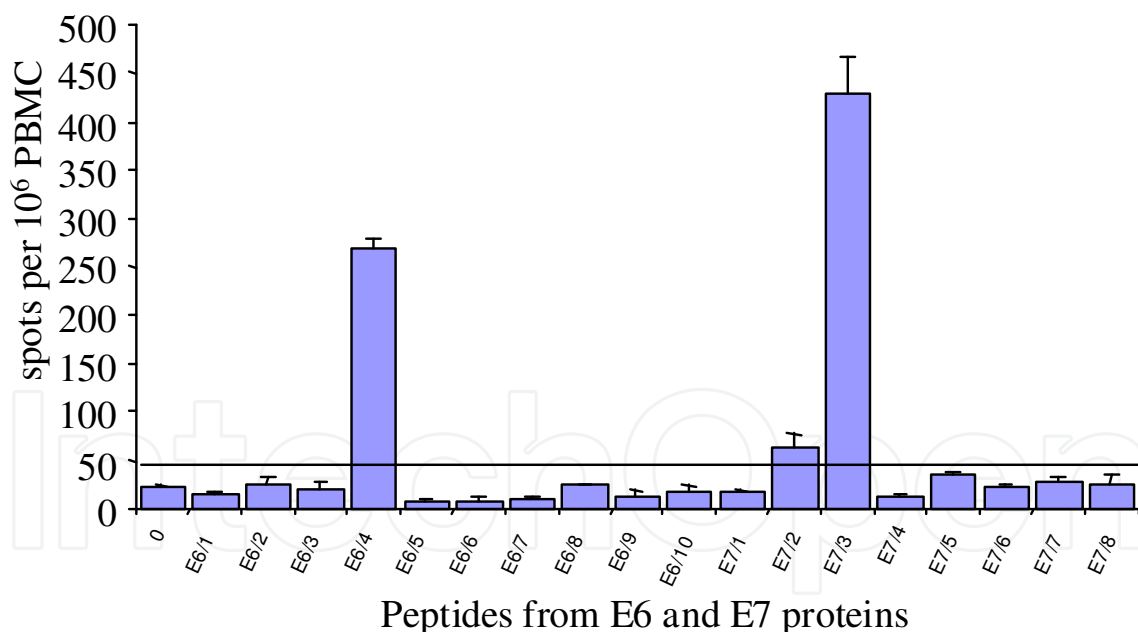
Fig. 3. IFNγ–ELISpot assay performed just before clinical regression

5. What is the exact role of cellular HPV16-specific T-cell responses?

Cellular immunity (CD4+ and CD8+ T-cells) plays a key role in the defense against all HPV-induced infections or lesions by destroying HPV-infected or -transformed keratinocytes. Indeed, the incidence of HPV infections and diseases significantly increases with CD4+ T cell impairment in immunosuppressed such as transplanted (Arends et al, 1997) or HIV-infected

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patients (Sun et al, 1997). In asymptomatic HPV16 infections, most women resolve spontaneously their infection without clinical disease concomitantly with blood anti-HPV16 Th1 CD4+ T cell responses (Welters et al, 2003). Similarly, regression of condyloma is associated with a dense epithelial cellular infiltrate made up of both CD4+ and CD8+ T lymphocytes with a Th1 cytokine profile as measured by cytokine mRNAs in interferon (IFN)-treated condylomas (Coleman et al, 1994). Proliferative CD4+ T cell responses are also associated with spontaneously regressive CIN3 (Kadish et al, 1997). The evolution of CIN3 towards invasive cancers is featured by a decrease of CD4+ cellular infiltrate, an increase of CD8+ T lymphocytes (Ghosh et al, 1992) with impairment of HPV16-specific CTL responses which could be related to a down- regulation of MHC class I molecules on HPV-16-infected cells and to the appearance of suppressive T lymphocytes (Treg) and a loss of blood anti-HPV-16 CD4+ activity. In high grade cervical intraepithelial neoplasia (CIN), positive intra-dermal reaction after intra-dermal injection of 5 HPV-16 E7 large peptides correlated with the spontaneous clearance of the lesions, which further indicates the presence and the very important role of HPV specific CD4+ T lymphocytes (Hopfl et al, 2000).

6. Anti-E2 T-cell responses are a marker of clinical viral control

We recently tested in a longitudinal study of 18 months, by proliferative assays, intracellular cytokines synthesis and IFNγ–ELISpot, the cellular immune responses against the HPV16 E2 protein that is early synthesized after HPV infection when the virus is episomal in eight women presenting with HPV16-related usual VIN and their healthy male partners (Jacobelli et al, 2011, unpublished data). In six women, we showed that anti-E2 polyfunctional CD4 T-cell responses (proliferative responses and synthesis of IFNγ and/or IL2) appear when the clinical lesions heal after treatment or when the HPV infection remains silent. In the women presenting with persistent lesions, no proliferation was observed.

Blood proliferative T-cell responses against HPV16 E2 peptides have been also observed in 50% of healthy women, who presumably previously cleared HPV16 infection (de Jong et al, 2004) and in 9 out of 22 regressive CIN3 cases (Dillon et al, 2007). In another studies, the lack of anti-E2 proliferative responses was reported in 16 of 18 patients (89%) affected with usual VIN lesions (Davidson et al, 2003) and in 7 of 8 and 9 of 12 women affected with CIN3 (Dillon et al, 2007; de Jong et al, 2004).These observations reinforce the strong role of T-cells in the control of HPV replication.

7. Why the male partners do not have any HPV16-related lesions?

Men are vectors of oncogenic HPV infection (Buckley et al, 1981; Giuliano et al, 2011). However, while HPV infection was found in 71 to 90% of the partners of HPV-infected women (Hippelainen et al, 1994; Nicolau et al, 2005), only 52% harbored the same HPV subtypes (Reiter et al, 2010). Moreover, penile intra-epithelial neoplasia is rare and detected in less than 2% of the men in contact with oncogenic HPV (Giraldo et al, 2008). We thus analyzed HPV infection and anti-HPV16 E2 blood T-cell responses in asymptomatic male partners chronically exposed to HPV16 during sexual intercourses with their wives affected with usual VIN (Jacobelli et al, 2011, unpublished data). We had hypothesized that male partners exposed to replicative HPV16 could develop immunologic responses against the

early E2 viral protein and thus clear infection. In the absence of condom usage for at least 6 months, the male partners of women presenting with usual VIN could be contaminated by HPV16. HPV16 and HPV27 (a cutaneous HPV) were identified in genital sampling gathered by cytobrush in only two of the eight healthy partners. Such a prevalence of contamination by HPV16 is similar to the one usually observed in male partner of oncogenic HPV-infected women (Reiter et al, 2010). In this male population, we have chosen to study anti-HPV16 E2 T-cell responses because E2 protein is an early highly expressed protein. E2 is bigger than E6 and E7 proteins and induces more T-cell responses than E6 and E7. Therefore looking for an E2-specific response is then more sensitive. In addition, E2 is required for replication and the detection of E2-specific T cell responses is the signature of viral replication. The study of anti-E2 T-cell responses is then more appropriate for the early phases of HPV16 infection as supposed in male partners of women having usual VIN. We have observed HPV16-E2-specific proliferative responses in seven and intracellular cytokine synthesis of single IFNγ, dual IFNγ/IL2 and single IL2 in six out of the seven partners. Since there is no E2 protein in the viral particle, the high frequency of E2-specific T cells responses in partners of women with usual VIN demonstrates that the virus replicate in males.

These E2 specific T-cell responses indicate a striking correlation in all male partners but two between the absence of the HPV-related lesion. The presence of spontaneous E2-specific proliferative T-cell responses and single IFNγ, dual IFNγ/IL2, single IL2 T-cell producers was previously described in other viral systems (Harari et al, 2006; Pantaleo et al, 2006). These polyfunctional anti-E2 T-cell responses could be due to an efficient presentation of viral antigens by dendritic cells present in mucosal tissue and it is tempting to speculate that E2-specific responses are responsible for the clearing of the lesion. Therefore, spontaneously HPV control is related to the presence of memory polyfunctional CD4+ T-cells in male partners.

8. Why the prophylactic vaccine could be useful in men?

The analysis of E2 specific T cell responses is a sensitive and reliable tool to analyze disease progression and the natural history of HPV infection. In six out of eight male partners, the presence of T-cell proliferative responses and single IL2, dual IFNγ/IL2, single IFNγ memory T-cells against HPV16 E2 peptides was concomitant to the control of genital HPV lesions despite HPV16 exposure. These results are reminiscent of those described in Gambian prostitutes exposed to HIV with presence of anti-HIV cytotoxic T lymphocytes without any detectable HIV (Rowland-Jones et al, 1995). Such anti-viral immune T cells responses thus reflect an undetectable viral infection. Our experimental results demonstrate for the first time that, although not clinically detectable, HPV16 can replicate in men and can induce a strong memory T cell response against one of an early viral protein. The presence of polyfunctional (IL2, IFNγ/IL2 and IFNγ secretions and proliferation) anti E2 CD4+ T-cell responses in asymptomatic men unambiguously establishes that E2 is a marker of HPV infection even when undetectable lesions. Responses represent correlates of protective antiviral immunity in HPV infection. Monofunctional (production of IFNγ by IFNγ-ELISpot) "anti-E2 T-cell" responses does not allow HPV16 control. These results suggest that male are an important reservoir of HPV and provide a strong argument in favor of prophylactic HPV vaccination of young men with VLPs to decrease HPV16 infection in men, viral transmission from men to women and thus fight against the spread of mucosal HPV diseases in the population.

9. How to cure usual VIN? Therapeutic vaccines

Preventive vaccines do not address the current need for better treatment for women previously infected by HPV 16 or 18. Other types of vaccines must be used to increase or induce new specific anti-HPV cellular immunity (CD4+ and CD8+ T lymphocytes) in order to kill transformed epithelial cells. Several approaches can be used in this aim. To stimulate cytotoxic or antiviral CD8+ T lymphocytes, the vaccines must target the cytoplasm of dendritic cells. The degradation of vaccine antigens by proteasomes results in short peptides that can bind to HLA class I molecules and migrate at the surface of dendritic cells. To stimulate CD4+ T lymphocytes, endocytosis of vaccinal antigens is essential, followed by degradation of antigens by lysosome/endosome in large peptides that associate with HLA class II molecules before migrating at the surface of dendritic cells. All these therapeutic vaccines must target E6 and E7 viral proteins and contain recombinant viruses (vaccinia viruses for example), DNA or peptides.

Recently, an open clinical trial was performed by the Melief's group (Kenter et al, 2009) in twenty women presenting with usual VIN using 13 large peptides spanning the whole E6 and E7 proteins. Forty five percent of complete (9/20 women) and 25 % (5/20) of partial remission were observed 12 months after immunization. These important results would be even more interesting if the investigators had included a placebo group (Bourgault Villada, 2010a). A new trial with a placebo group is currently under way.

Vaccinia virus was also used in a recombinant vaccine containing E6 and E7 genes from HPV16 and HPV18 (TA-HPV) to vaccinate usual VIN patients. A clinical complete or partial response was observed in 8/18 treated women (Davidson et al, 2003). More recently, vaccination against usual VIN was also performed with another recombinant vaccinia virus, TA-L2E6E7 from HPV16 (Daayana et al, 2010). Two months before vaccination, 19 women were treated by topical imiquimod and then vaccinated by intramuscular route with 3 doses of recombinant vaccinia virus. Imiquimod is an immunomodulator that increases the synthesis of type I IFN by dendritic cells after its fixation to the TLR7 in human dendritic cells. Complete remission was obtained in 58% of vaccinated women.

10. How to determine the epitopic regions for a therapeutic vaccine?

In a study including 16 women presenting with usual VIN, we have determined the strongly immunogenic regions from HPV16 E6 and E7 proteins for CD4+ and/or CD8+ T lymphocytes (Bourgault Villada et al, 2010b). Among 18 large peptides of the proteins E6 and E7, two were recognized in proliferative assays as immunodominant by T cells from 10 out of 16 women (62%) at the entry in the study, namely E6/2 (aa 14-34) and E6/4 (aa 45-68) peptides. Four other peptides, E6/7 (aa 91-110), E7/2 (aa 7-27), E7/3 (aa 21-40) and E7/7 (aa 65-87) were recognized by only 12% of the women in proliferative or IFNγ–ELISpot tests. The regions of E6 and E7 proteins implicated in T cell recognition during HPV infection were not yet well defined because of the usually low frequency of anti-HPV blood T cell responses and of the difficulties of their study.

In protein E6, some peptides included in, including or overlapping our peptides E6/2 (aa 14-34) and E6/4 (aa 45-68) have already been described as preferentially recognized by CD4+ T cells. Among them, peptide E6 42-57 that is restricted by HLA-DR7 has already

been identified (Strang et al, 1990). Regions E6 1-31, 22-51 and 24-45 can be also immunogenic for CD4+ T cells as shown in CIN or sexually active healthy women (Kadish et al, 1997). The region E6 42-71, which includes peptide E6/4 (aa 45-68), has also been described as a target of proliferative responses in CIN patients (Kadish et al, 1997). Another E6 111-158 region was previously described as inducing proliferative responses in infected asymptomatic subjects or in patients with CIN3 (Kadish et al, 1997; Strang et al, 1990) as well as E6 127-141 peptide in healthy young women (Gallagher et al, 2007). Similarly, peptides E7 43-77, E7 50-62 and E7 58-68 which are restricted by DR3, DR15 and DR17, respectively, were defined as epitopic peptides for CD4 + T cells (Strang et al, 1990; van der Burg et al, 2001; Wang et al, 2009). E7 region 51-98, including our E7/7 (aa 65-87) peptide, is also very immunogenic for proliferating T lymphocytes (de Gruijl et al, 1998; Luxton et al, 1996; Nakagawa et al, 1996).

The characterization of E6 and E7 HPV-16 epitopes and the HLA restriction of their recognition by CD8+ T lymphocytes are more precise: E6 29-38, E7 11-20, E7 82-90 and E7 86-93 epitopes are presented by HLA-A2 (Evans et al, 2001; Ressing et al, 1995, 1996), E6 80-88 and E7 44-52 by HLA-B18 (Bourgault Villada et al, 2000) and E6 49-57 by HLA-A24 (Morishima et al, 2007). In women who cleared HPV 16 infection, cytotoxic T lymphocytes (CTL) responses are directed against epitopes preferentially located in the N-terminal half of the E6 protein (region 16-40) (Nakagawa et al, 2005). In this fragment, the dominant epitope E6 29-37 is restricted by HLA-B48, E6 31-38 by HLA-B4002 and the subdominant epitope E6 52-61 by HLA-B35 (Nakagawa et al, 2007). The same group had also shown that the peptide E6 33-42 61 is recognized by CD8+ T lymphocytes in association with HLA-A68, peptide E6 52-61 in association with HLA-B57 and –B35, peptide E6 75-83 in association with HLA-B62, peptide E7 7-15 in association with HLA-B48 and peptide E7 79-87 in association with HLA-B60 (Nakagawa et al, 2004, 2007; Wang et al, 2008). In addition, E7 7-15 is also able to bind HLA-A2 and -B8 to be recognized by CTL (Oerke et al, 2005; Ressing et al, 1995). From the latter results, two hot spots of CD8+ T-cell epitopes in protein E6 may be located in the regions E6 29-38 and 52-61 and another one in protein E7 (E7 7-15) (Nakagawa et al, 2007). Nevertheless, a poor immunogenicity of E7 protein was observed in many studies during both HPV 16 infection and after peptidic vaccination using long peptides spanning both E6 and E7 (Kenter et al, 2008; Welters et al, 2008) such as those used in our study.

The epitopes E6/2 (aa 14-34) and E6/4(aa 45-68) hence could be strongly recognized by CD4+ and / or CD8+ T lymphocytes and could be particularly relevant in the design of a peptide vaccination. We may hypothesize that the T cell responses that we observed were able to contain the tumor cells into the epithelium. Therefore, E6/2 (aa 14-34) and E6/4 (aa 45-68) peptides could play a major role in the protection against invasive cancer by stimulating T lymphocytes. Specific CD4+ T-cells play an essential role in the defense against HPV in particular in women presenting with usual VIN and their male partners.

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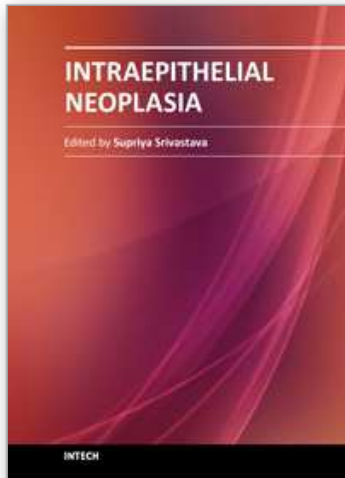
The book "Intraepithelial neoplasia" is till date the most comprehensive book dedicated entirely to preinvasive lesions of the human body. Created and published with an aim of helping clinicians to not only diagnose but also understand the etiopathogenesis of the precursor lesions, the book also attempts to identify its molecular and genetic mechanisms. All of the chapters contain a considerable amount of new information, with an updated bibliographical list as well as the latest WHO classification of intraepithelial lesions that has been included wherever needed. The text has been updated according to the latest technical advances.This book can be described as concise, informative, logical and useful at all levels discussing thoroughly the invaluable role of molecular diagnostics and genetic mechanisms of the intraepithelial lesions. To make the materials easily digestive, the book is illustrated with colorful images.

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