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Mechanism of progression in cervical intraepithelial neoplasia

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INTRODUCTION

CERVICAL INTRAEPITHELIAL NEOPLASIA

Classification of the cervical intraepithelial neoplasia

The cervical intraepithelial neoplasia (CIN) is a pre-cancerous lesion of uterine cervical squamous cell carcinoma (SCC) and is characterized by potentially premalignant transformation and abnormal growth, named dysplasia, of cervical keratinocytes. The histological classification system provides three CIN grades referred to as CIN1 (mild dysplasia), CIN2 (moderate dysplasia) and CIN3 (severe dysplasia) lesions. Each CIN grade remains confined to the cervical epithelium (Figure 1, A, www.lookfordiagnosis.com) and has a variable potential evolution towards the cancer. CIN1 lesions are characterized by keratinocytes with abnormal cell growth, perinuclear cytoplasmic vacuolation, named koilocytosis, and increase volume of the nucleus. This histologic change is confined to the basal third of the cervical epithelium (Figure 1, B). CIN2 lesions, compared to CIN1, are characterized by higher cytologic atypia and cellular disorganization, while koilocytosis is lower or absent. The histological abnormalities are confined to the basal two thirds of the cervical epithelium (Figure 1, C). CIN2 cervical cells show two main features: (i) atypical mitotic figures induced by aneuploidy; (ii) abnormal nucleus/cytoplasm ratio. CIN3 lesions are characterized by keratinocytes endowed with high proliferative index, immaturity and vertical orientation. Abnormal keratinocytes exceed the two thirds of the epithelium or expand throughout the thickness of the epithelium, altering the whole cyto-architecture of the tissue (Figure 1, D).

Figure 1. Histological representation of normal and CIN tissue. (A) Normal cervical epithelium; (B) CIN1, disorganization of the lower third of the epithelium; (C) CIN2,

disorganization of the lower half of the epithelium with viral infection; (D) CIN3, the disorganization of the epithelium is evident in more than 2/3 lower.

Epidemiology of the CIN lesions

The incidence of CIN lesions in a population depends on several factors such as etiopathogenetic factors, the efficiency of prevention programs, the immunologic status, and the age of the female. The World Health Organization (WHO) has estimated that the annual incidence of CIN lesions among women aged 25-65 years who undergo cervical SCC screening for the first time was 3-10% for CIN 1 and 1-5% for CIN2 and CIN3. CIN2 and CIN3 lesions are generally diagnosed in women between 25 and 35 years of age (Kumar *et al.*, 2007). On the contrary, cervical SCC is usually detected in women over 40 years of age, typically 8 to 13 years after a diagnosis of CIN3. Women can develop CIN lesions at any age?. The prevalence of CIN lesions varies from 1/10,000 in women aged 15-19 years to 1/1,000 in women aged 25-29 and then it reduces to 8/10,000 in women aged 25-29 years (WHO 2010). In developing Countries, like Nigeria, the mean age for CIN lesion is about 37 years. The prevalence is 3.6% for CIN1, 0.8% for CIN2 and 0.4% for CIN3. Such a result is due to the lack of screening methods and it mirrors the typical trend of developing Nations (Oguntayo and Samaila, 2012).

Clinical progression of CIN lesions

The temporal progression CIN1> CIN2> CIN3 represents the initial steps in tumorigenesis of cervical SCC. However, CIN lesions do not always progress towards cancer. Indeed, the histological and molecular phenotype of CIN reflects a fine balance between the factors that promote/accelerate or reduce/slow down disease progression (Melsheimer *et al.*, 2001). About 90% low-grade CIN lesions tend to regress spontaneously while 10% women progress to high grade CIN2 and CIN3 lesions. Typically, the risk of CIN progression to cancer is related to the severity of CIN lesion. Indeed, the probability that a CIN3 lesion progresses towards cervical SCC is significantly higher than CIN1. The percentage of regression and progression of different grades of CIN lesions are shown in Table 1:

Lesion	Regression	Persistence	Progression to CIN3	Progression to SCC
CIN1	57%	32%	11%	1%
CIN2	43%	35%	22%	1.5%
CIN3	32%	<56%	-	>12%

Table 1. Regression, persistence and progression to CIN3 or SCC probability of different CIN pre-cancerous lesions.

CIN lesions are believed to progress over time, evolving from low-grade neoplasia (CIN1), to high-grade neoplasia (CIN2 and CIN3), and finally to invasive carcinoma (Ostor, 1993). Accurate predictions of the progression rates of CIN lesions are limited due to a lack of sensitive markers for neoplastic progression. Untreated CIN1 lesions have about 10% progression rate to CIN3, whereas CIN2 have about 20% progression rate to CIN3 and CIN3 lesions have rates of about 12% to progress to cervical SCC (Ostor, 1993; Castle *et al.*, 2009; Moscicki *et al.*, 2010). Traditionally, CIN1 lesions are conservatively managed and followed in time, while CIN2 and CIN3 are surgically removed. Thus, it can be speculated that many women, whose CIN would not evolve to malignant lesions, are unnecessarily candidate to follow-up or even over-treated and exposed to the risks of surgical excision (Shanbhag *et al.*, 2009). These data suggest that there is a need for sensitive markers that can be used in routine pathology practice to identify CIN lesions with a high risk of progression.

HUMAN PAPILLOMA VIRUS

Human papillomavirus (HPV) is the necessary cause of both CIN lesions and cervical SCC (Park *et al.*, 1995; Schiffman and Brinton, 1995; Bosch *et al.*, 2002). HPV is a small double-stranded (ds) DNA virus belonging to Papillomaviridae family.

Papillomaviridae family is divided into 16 genres defined phylogenetically by the letters of the greek alphabet (De Villiers *et al.*, 2004). Five of them, Alpha, Beta, Gamma Mu and Nu, belong to the human papillomavirus (Figure 2). The α -Papillomavirus are predominantly mucosal, while other genres are predominantly cutaneous. Approximately 40 HPV types show ano-genital tropism and are transmitted by sexual activity. Among these, some HPV cause genital warts, while others produce persistent infections that may evolve in CIN lesions and cervical SCC (Schiffman *et al.*, 2003). For these reasons, HPVs are divided into high- and low- oncogenic risk due to their ability to induce benign genital warts or CIN lesions and cervical SCC (Table 2). High-risk HPVs are detected in 90% of cervical cancers (Clifford *et al.*, 2003; Muñoz *et al.*, 2003). High-risk HPV16 and HPV18 are detected in approximately in 50% and in 10-20% of all cervical cancers, respectively (Muñoz *et al.*, 2003). Low-risk HPVs are associated with 90% of the warts, benign squamous papillomas and CIN1 lesions, that in most cases resolve spontaneously (Muñoz *et al.*, 2003).

HPV Group	HPV type
High Risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82
Low Risk	6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108
Probably High-Risk	26, 53, 66, 68, 73, 82

Table 2. Epidemiologic classification of High-, Low- and Probably High-risk HPVs

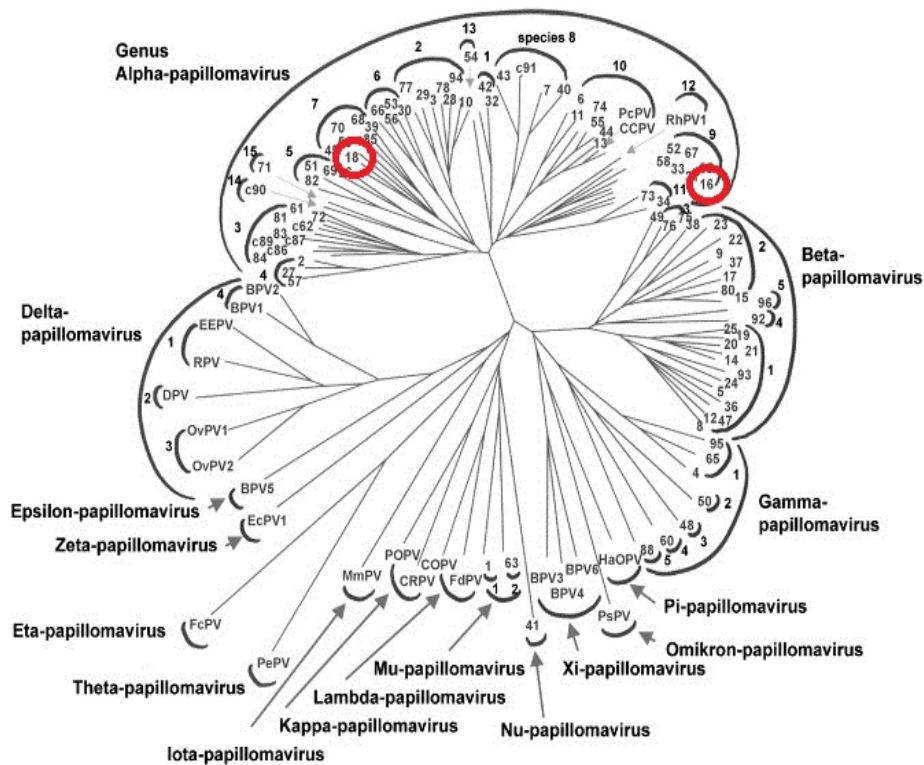


Figure 2. Phylogenetic tree of 118 types of papillomavirus. Each HPV type is identified by the number at the end of each cluster. The number above the semicircular symbol identifies the papillomavirus species. The greek symbols identify genera. HPV16 and HPV18, the main responsible of cervical cancer, are highlighted in red.

Molecular structure of HPV

HPV is a small envelope-free double-stranded (ds) DNA virus. The virion is composed by a proteic capsid (diameter=55 nm) consisting of 72 pentameric or hexameric-shaped capsomeres in icosahedral symmetry (Figure 3, <https://visualsonline.cancer.gov/details.cfm?imageid=2255>). Viral DNA is located inside the capsid. HPV genome is circular, double-stranded and it is composed of approximately 8,000 base pairs (Narisawa-Saito and Kiyono, 2007). The genetic information is contained in only one DNA filament (positive strand) whereas 8 are the coding regions are eight. Six coding regions are defined “early” and tagged E1, E2, E4, E5, E6 and E7, whereas two are called “later” and tagged L1 and L2 (Bravo and Alonso 2007).

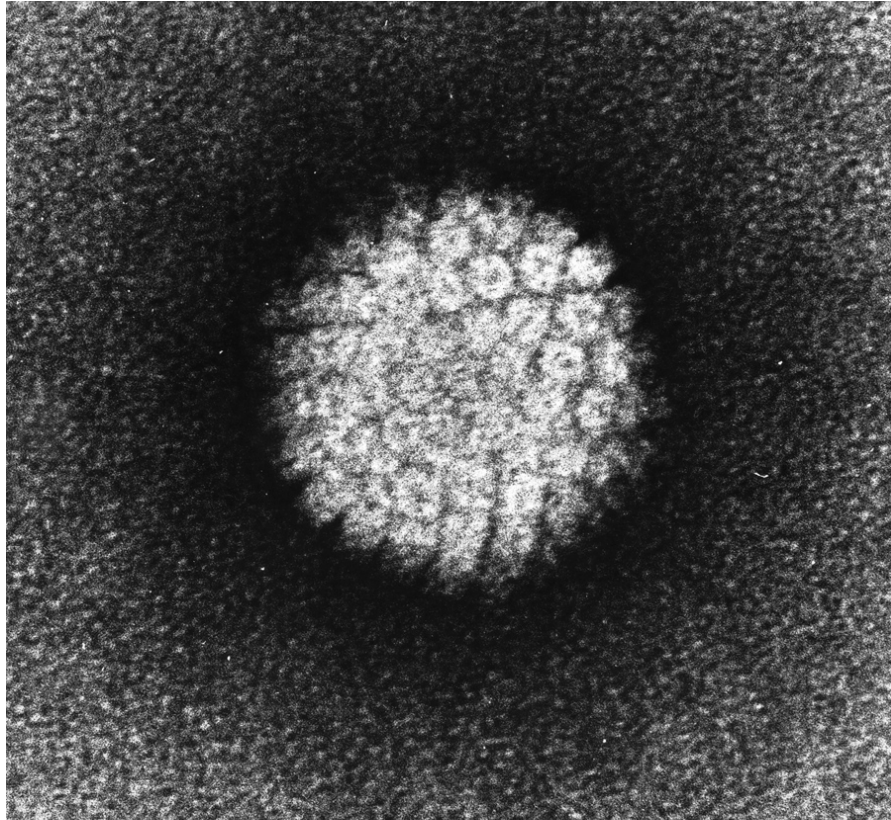


Figure 3. Electron micrograph of a HPV 16.

HPV genome can be divided into three regions: (i) Long Control Region (LCR), or upstream regulatory region (URR), which is a non-coding region required for viral replication and viral DNA encapsidation; (ii) Early region (E), that represents 45% of the viral DNA and contains the six E genes expressed early in the HPV life cycle; (iii) Late Region (L), corresponds to 40% of the viral DNA and encodes for the structural proteins of capsid, L1 and L2 (Muñoz *et al.*, 2006). The “E” and “L” regions are referred to as ORF (Open Reading Frame) and are separated by the LCR region (Muñoz *et al.*, 2006).

The viral genes are transcribed in a clockwise direction, but the transcription is not temporally sequential. The encoded proteins have the same nomenclature of genes: six nonstructural regulatory proteins, E1, E2, E4, E5, E6 and E7 that interact with the host genome and proteins to replicate viral DNA, and two structural proteins L1 and L2, expressed after replication of viral DNA. Each viral protein has a specific function in the viral replication cycle. E1 and E2 are specifically involved in DNA replication, while E4,

E5, E6, and E7 contribute to the initial destabilization of proliferation and differentiation of the host cell (Bravo and Alonso, 2007). E1 plays a major role in the initial phase of replication of the viral genome as it presents the ATP-dependent helicase activity that allows recognition and beginning of viral DNA replication. E2 gene encodes several proteins that regulate early viral gene transcription and viral DNA replication, thanks to a structural sequence that binds specific DNA regions (Wilson *et al.*, 2002). At low levels, E2 binds specific recognition sequences and activates early viral promoters, while at high concentrations it represses viral transcription by blocking the binding to transcription factors. E2 plays a key role in the direct regulation of the levels of E6 and E7 oncoproteins. Indeed, loss of E2 expression is the first stage of neoplastic transformation. Despite its name, E4 is expressed during the final phases of the viral replication cycle: it seems to interact with the keratin intermediate filaments, making them mechanically unstable and thus facilitating the release of mature virions from the keratinocytes. E5 is a highly hydrophobic protein made up of 83 amino acids that participates to viral DNA replication along with E1, E2 and E4 proteins. Its expression induces several cellular changes. Indeed, E5 enhances the signal of growth factors (Crusius *et al.*, 1998) activates MAPK pathway and down-regulates MHC class I and class II (Ashrafi *et al.*, 2005). Specifically, E5: (i) activates the EGF receptor to promote cell proliferation; (ii) enhances the activity of transcription factors, such as c-jun and c-fos thereby favoring cellular mitosis; (iii) inactivates p21; (iv) prevents apoptosis resulting in DNA damage; (v) prevents transport of the major histocompatibility complex (MHC) class I and class II. Recent works proved that E5 contributes to hyperplasia, regulates growth and invasion in cervical cancer cell lines and promotes cervical carcinogenesis in conjunction with E6 and E7 (Genther *et al.*, 2005; Maufort *et al.*, 2010). A study of Kabsch conducted, in 2002, indicates that E5 could inhibit apoptosis, forcing the cell to remain in a continuous proliferative state.

E6 and E7 are the two oncoproteins of High Risk HPVs. These two viral proteins are essential to induce and maintain cell transformation because they interfere with the normal controls of cell cycle and apoptosis. E6 is one of the first proteins to be expressed during HPV infection. This protein is formed by 150 amino acids with a molecular weight of 18 kD. Typically, E6 protein of High Risk HPVs is located in the nucleus and has no intrinsic enzymatic activity. E6 is involved in different cellular pathways, which involve a large number of different proteins: transcription factors, pro-apoptotic proteins, proteins involved in the formation and maintenance of cellular architecture, polarity and adhesion, and factors involved in DNA replication and repair. (Zur Hausen, 2002). E6 protein binds

to tumor suppressor protein p53, its principal target, thereby leading to p53 degradation (Scheffner *et al.*, 1990; Werness *et al.*, 1990). p53 plays a key role in apoptosis and cell cycle regulation, through several mechanisms: (i) it induces apoptosis in the case of irreparably damaged DNA; (ii) it activates p21 (an inhibitor of cyclin/cyclin-dependent kinase complex, cdk) consequently holding cell cycle at the G1/S regulation point on DNA damage recognition and finally blocking cell growth (Vogelstein *et al.*, 2000). Therefore, functional inactivation of p53 by E6 results in G1/S and G2/M deregulation of cell cycle control leading to abnormalities in DNA replication and genomic structure. The carboxyl (C)-terminal of E6 protein carries a short sequence that interacts with a specific set of PDZ domains included in several human proteins (Scott and Klingelutz, 2014). PDZ domains (an acronym from the initial of the proteins PSD95, DLG, and ZO1 on which they have been identified) are small domains that bind to peptide ligands through a consensus sequence XX(S/T/Y)X(V/L/M) located on target proteins. The PDZ protein family present a conserved domain that is often found in proteins located in the areas of contact between cells, such as tight junctions between epithelial cells or neural cell synaptic junctions. Furthermore, PDZ proteins are implicated in signal transduction and polarity. The members of PDZ protein family (MUPP-1, and hDlg, hSCRIB, MAGI-1, -2 and -3, GIPC, PATJ, PTPN3 and PSD95) bind the C-terminus of the protein E6 of oncogenic HPVs and are subsequently degraded in a proteasome dependent manner (Gardioli *et al.*, 1999; Glaunsinger *et al.*, 2000; Nakagawa and Huibregtse, 2000; Massimi *et al.*, 2004). As a consequence of the degradation of PDZ family proteins, cellular contact mediated by adherence junctions is lost with a consequent lack of cell polarity. These alterations were observed in High Risk HPV-associated transformed cells.

E6 protein is also able to induce cell immortalization via telomerase activation and specifically activating the expression of the catalytic subunit of telomerase, i.e. telomerase reverse transcriptase TERT. Telomerases are RNA-dependent polymerases that add telomere repeats to the ends of chromosomes (Klingelutz *et al.*, 1996; Kiyono *et al.*, 1998; McMurray and McCance, 2003; Howie *et al.*, 2009). They are ribonucleoproteins made up by RNA and a protein component, which is the reverse transcriptase subunit TERT. This subunit catalyzes the addition of deoxynucleotides in a TTAGGG sequence to the ends of telomeres (Shampay and Blackburn, 1988). Thereby preventing the degradation of the chromosomal ends during DNA replication. Both proliferative stem cells and cancer cells express human TERT (hTERT). The expression of hTERT allows telomerase activity and suppression of cell senescence in germ cells. The E6 of some HPV

types is able to activate the TERT promoter and this mechanism is strongly associated with cancer (Van Doorslaer and Burk, 2012). Even though the exact molecular mechanism of promoter activation is still unclear, it probably involves E6AP binding (Gewin and Galloway, 2001). Furthermore, E6 activates the transcription of hTERT through the transcription factor c-myc (Veldman *et al.*, 2001) that displaces the USF transcriptional repressor from the E box in the TERT promoter (McMurray and McCance, 2003). According to other models, E6 and E6AP bind to a repressor of TERT transcription called NFX1-91, inducing its degradation. The interaction between E6-E6AP and NFX1-91 allows myc to bind to the hTERT promoter and activates it (Gewin *et al.*, 2004). As a consequence of the interaction with E6-E6AP, NFX1-91 is ubiquitinated and degraded, thereby removing transcriptional repression at the TERT promoter. Conversely, a splice variant of NFX1 referred to as NFX1-123 is unable to interact with E6 and hence transcriptional repression is kept despite HPV infection (Katzenellenbogen *et al.*, 2007). Furthermore, NFX1-123 stabilizes TERT transcripts in HPV-16 E6 expressing cells by binding to poly-(A) binding proteins (Katzenellenbogen *et al.*, 2007; Katzenellenbogen *et al.*, 2009). Interestingly, E6 is able to bind directly TERT proteins, (Liu *et al.*, 2009) even though the biological consequences of this interaction are still unknown (Liu *et al.*, 2009).

HPV E7 is a phosphoprotein made up of 100 amino acid residues that is not encoded by all papillomaviruses. E7 is composed by three domains: conserved region (CR) 1, CR2 and CR3. The E7 amino terminus region contains two regions corresponding to the small portion of CR1 and nearly the entire CR2, whose sequence is similar to the adenovirus (Ad) E1A (Phelps *et al.*, 1988). The CR1 and CR2 domains are separated by a non-conserved sequence of variable size and amino-acidic composition. The CR3 is located at the carboxyl terminal that contains a zinc-binding site important for dimerization. Such a site is made up by two CXXC domains separated by 29-30 amino acid residues (Barbosa *et al.*, 1989; McIntyre *et al.*, 1993). When one or both the cysteine residues of one zinc-binding site is mutated, the virus is no longer able to immortalize human keratinocytes in the dermis (HFK) and transform rodent cells (Dyson *et al.*, 1989). E7 could presents post-translational modifications. Indeed, E7 contains a consensus phosphorylation site for: (i) protein kinase C (PKC) on threonine 7 in the CR1 homology domain (Liang *et al.*, 2008); (ii) casein kinase II (CKII) in the CR2 domain (Firzlaff *et al.*, 1989; Barbosa *et al.*, 1990); (iii) unknown kinase in CR3 domain (Massimi and Banks, 2000). E7 can be either carboxyl terminally polyaminated or amino (N)-terminally ubiquitinated and subsequently degraded via proteasome (Reinstein *et al.*, 2000; Jeon *et*

al., 2003). This oncoprotein is usually located in the nucleus, but can be potentially shuttled between the two cellular compartments, thanks to nuclear export sequences (Knapp *et al.*, 2009). E7 interacts with several cellular proteins: transcription factors and proteins that remodel chromatin, negative regulators of the cell cycle, and components of the innate immune response. As previously reported, E7 has oncogenic activity as it efficiently immortalizes human keratinocytes via the combined action with E6 (Felsani *et al.*, 2006). In addition to that, E7 binds pRb and other members of Rb family: p107 and p130 (Dyson *et al.*, 1989; Davies *et al.*, 1993). E7 associates to pRB through the conserved LXCXE sequence motif within CR2 domain (Dyson *et al.*, 1989). pRb is a nuclear tumor suppressor phosphoprotein belonging to the pocket protein family, whose members have a interacting region with various proteins (Korenjak and Brehm, 2005). pRb prevents excessive cell growth by inhibiting cell cycle progression and can be considered a “molecular brake” of the transition from G1 to S phase. pRb regulates cell cycle progression through the interaction with E2F/DP, a dimer of E2F protein and a dimerization partner (DP) protein (Wu *et al.*, 1995) that pushes the cell cycle into S phase (Funk *et al.*, 1997). The active form of pRB normally binds and inactivates E2F/DP, blocking cell cycle in G1 phase. Furthermore, the complex pRb-E2F/DP interacts with several chromatin remodeling enzymes such as acetylases and methylases, thereby silencing numerous promoter of genes involved in the progression from G1 to S phase. On the contrary, when the cell is ready to divide, Rb is inactivated through phosphorylation, allowing the progression from G1 to S phase. The other two members of retinoblastoma family that interact with E7 (p107 and p130) are involved in the control of the different phases of the cell cycle. Specifically, p130 has a regulatory function during the G0/G1 phase, while p107 is active in the G1/S transition. E7 is able to bind p107 and p130, allowing cell progression from G1 to S phase and enhancing cellular proliferative characteristics (Dyson *et al.*, 1989; Howie *et al.*, 2009). E7 is able to interact with the cyclin-dependent kinase, CDK2 dependent cyclin A and E that normally regulate cell cycle (Arroyo *et al.*, 1993). Furthermore, it associates to inhibitors of cyclin/cdk complex as p21 and p27, thereby blocking their inhibitory action and fostering the activity of the complex cyclin/cdk (Funk *et al.*, 1997). p16INK4a is an inhibitor of cyclin/cdk complex, whose overexpression normally induces cycle arrest. E7 counteracts the inhibitory role of p16INK4a through mechanisms that have not been completely clarified yet. For this reason, p16INK4a was considered as a molecular marker for the CIN pre-cancerous lesions (Razmpoosh *et al.*, 2014). Furthermore, E7 is able to bind histone deacetylases (HDACs), a

class of enzymes that remove acetyl groups ($O=C-CH_3$) from the ϵ -N-acetyl lysine amino acid on the histones and increase their ability to bind to DNA, thereby enhancing genetic transcription. The interaction between E7 and HDACs causes chromatin packing and consequently blocks transcription. Since pRb normally binds and recruits HDAC at level of E2F inducible promoters, E7 can prevent histone deacetylation also in an indirect way, i.e. through the inhibition of pRb (Brehm *et al.*, 1999). Furthermore, in the case of persistent HPV infection, HDACs recruitment allows E7 to silence specific genes, such as interferon regulatory factor 1 (IRF1), whose expression is important in immune response (Park *et al.*, 2000). Since the viral genome is unable to remain permanently in episomal form when a mutation in the E7-HDAC binding site of E7 is induced (Longworth and Laimins, 2004), it has been deduced that the association with HDAC allows E7 to maintain the viral DNA in episomal form. The molecular mechanism of such an interaction is still unclear, but it has been proposed that HDAC directly deacetylates of E2F, causing loss of function. Another important action of E7 is the transactivation of the enzyme phosphatase Cdc25, which allows the dephosphorylation and activation of complex cyclin/cdk and is required for cell cycle progression (Jinno *et al.*, 1994). Another capacity of E7 is the ability to induce genomic instability. In fact, several HPV-positive cancer cells contain different aneuploidies, indicating that changes in the number of chromosomes are important events in tumor progression. The presence of E7 is sufficient to induce an abnormal increase in the number of chromosomes in primary human keratinocytes (Duensing *et al.*, 2000). Mutated E7 proteins that do not bind or degrade pRb, but are associated with p107, induce centrosome abnormalities during cell division (Duensing and Munger, 2003). These anomalies are also observed in cells lacking p53 and pRb and in embryonic fibroblasts of knock-out mice for pRb, p130 and p107. It has been speculated that a combination of family members of pRb or other factors can induce centrosome abnormalities during HPV infection (Duensing and Munger, 2003).

The late proteins L1 and L2 have a structural function. L1 is the major viral capsid protein common to all the HPV protein. L1 self-assemble into 72 pentamers and is the most exposed region of the capsid and the target of the majority of neutralizing antibodies. L2 protein is highly variable among different types of HPV. This viral protein plays mainly regulatory and structural. In fact, it presents a nuclear localization signal and it is involved into the selective viral DNA encapsidation.

The life cycle of HPVs in uterine cervix

HPV infections are mainly common in keratinocytes of the skin or mucous membranes presenting pluristratified epithelium, the only tissue in which they can replicate. In particular, HPV infection starts with the initial contact of the virus with micro-abrasions of the cutaneous or mucous membranes that expose segments of the basement region of the pluri-stratified epithelia, composed by stem elements (figure 1, A; Figure 4). Following the same process, genital HPV attack the uterine cervix, whose covering surface is thin and hence particularly susceptible to suffer from micro-abrasions, making it even more vulnerable to infection. As far as we know, the virus enters in the basal cells through endocytosis, even though the exact mechanism (classical endocytosis or receptor-mediated endocytosis) is still unclear. Presumably the heparan sulfate proteoglycans, located at the cell surface and in the extracellular matrix, mediate the initial phase of cell infection (Joyce *et al.*, 1999; Patterson *et al.*, 2005). As for other viruses, (Chung *et al.*, 1998; Summerford *et al.*, 1999) it seems that secondary receptors as efficient as the integrin receptor $\alpha 4\beta 6$ are needed for HPV infection (Evander *et al.*, 1997; Bossis *et al.*, 2005). After receptor recognition, the process of endocytosis occurs through clathrin-coated vesicles. After the entrance of the virus into the cytoplasmic compartment (Culp and Christensen, 2004), the capsid protein is disassembled in the lysosomes and the viral DNA is transferred into the nucleus through the minor capsid protein L2 (Day *et al.*, 2003). The viral DNA remains in episomal form in the nucleus in the number of 10-100 copies/cell equivalent and this process does not usually cause cytological abnormalities (Schiller *et al.*, 2010). HPV early genes E1/E2 are expressed at low levels in the nucleus and trigger the replication of the viral genome, which is almost completely mediated by the replicative machinery of the host keratinocyte (Figure 4). The life cycle of HPV is intimately associated with the proliferative activity and differentiating status of the host keratinocyte. In fact, quick cellular replication is necessary for an increased viral progeny. E2 protein activates and controls the expression of E6 and E7 that stimulate the host cell to proliferate, thereby stimulating cell growth.

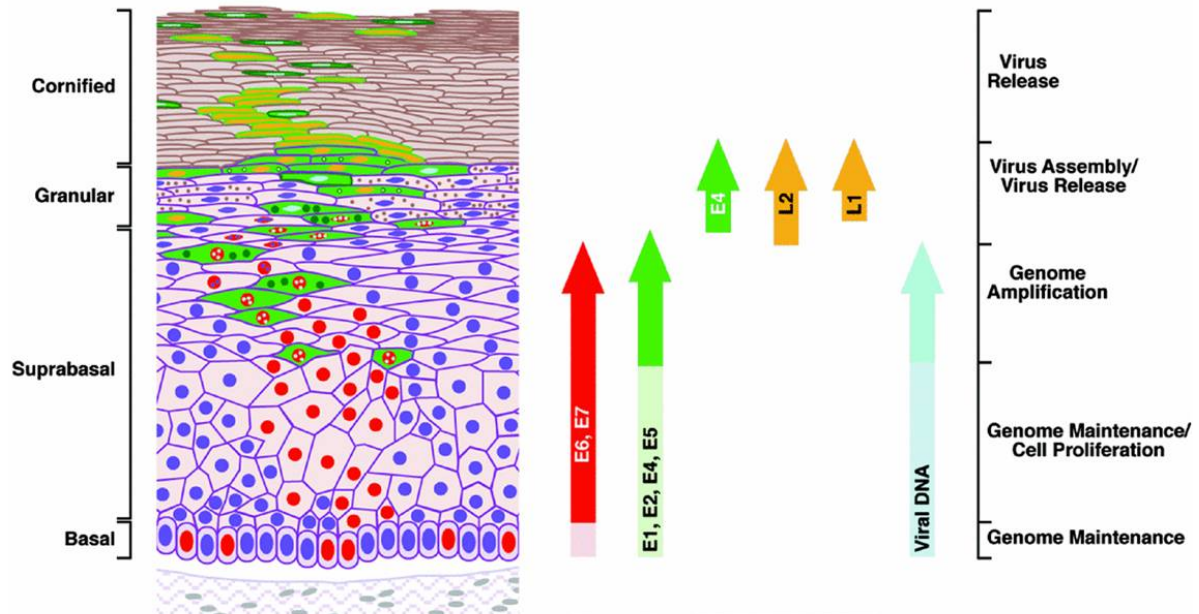


Figure 4. HPV life cycle. Graphical representation of the key events in HPV life cycle: from the first interaction with the basal layer by the virions to the DNA replication, viral gene expression and viral release.

Furthermore, E6 and E7 proteins induce a block of cell cycle progression keeping the cell in an undifferentiated state. The final effect is an increased proliferation of undifferentiated and infected keratinocytes (Doorbar *et al.*, 2012). The viral cycle follows the natural maturation of the epithelium, where basal keratinocytes progressively differentiate while moving towards the most superficial layers. In the intermediate cell layers, viral DNA replication stops and the expression of E4 and E5 early genes starts (Figure 4, <http://www.clinsci.org/cs/>). The proteins transcript of E4 activate the expression of the late genes L1 and L2, while and proteins transcript of E5 encapsidates the viral DNA within the virion (Doorbar *et al.*, 2012). The virus is completely assembled in the squamous cells of the upper layer of the pluri-stratified epithelium, where the keratin is destroyed and virions are released by dead cells (Figure 4). The infectious process is slow as it takes about 12–24 hours for the initiation of transcription and the final passage of life cycle is the exfoliation of superficial keratinocytes and subsequent release of HPV virions into the surrounding cervix environment (Doorbar *et al.*, 2012). The complete life cycle of HPV from the first interaction with the basal layer to the viral release, requires about 6-12 weeks.

HPV and cell proliferation

During the full replicative cycle of low-risk HPV the genome remains in episomal form and the production of mature viral progeny occurs. On the contrary, during high-risk HPV infections viral DNA is integrated into the DNA of the host cell. This molecular mechanism has a double effect: it decreases viral progeny on the one hand and it increases proliferative capacity in the host cell on the other hand. Indeed, the integration occurs at the level of the E2 ORF, blocking the repressive action of E2 on viral oncoproteins E6 and E7 and leading to uncontrolled expression. As recently reported, E6 and E7 stimulate cell cycle progression by inhibiting the activity of important regulatory proteins of the cycle, p53 and pRb, with well known molecular mechanisms (Munger *et al.*, 1989; Sheffner *et al.*, 1990). E5 seems to be involved in the stimulation of cell proliferation. In fact, it can inhibit apoptosis and lead the cell to a high proliferative status.

HPV and tumorigenesis

A crucial aspect of the replicative cycle of cervical high-risk HPVs is the possibility to induce persistent infections. As previously reported, the integration of viral DNA into the DNA of the host cell causes the disruption of E2 gene sequence and leads to the uncontrolled and continuous production of viral oncoproteins E6 and E7. Consequently, the epithelial cells that express E6 and E7 have a growth advantage compared to those that contain the viral DNA in episomal form. This is the first mechanism in the multistep process of cervical neoplastic transformation. E6, E7 and E5 are critical for the tumorigenic process, as they stimulate cell proliferation, induce cell survival and modulate keratinocytes differentiation, as confirmed by experimental models. In fact, when the expression of E6 and E7 is inhibited in cultured transformed cells the malignant phenotype is reverted, while the activation of E2 in *in vitro* experiments blocks cell proliferation in cervical SCC cell lines (Goodwin *et al.*, 1998; Jiang and Milner, 2005). During high-risk HPVs infection, the tumorigenic process changes the histological characteristics of the cervical squamous pluri-stratified epithelium. However, the viral DNA is present only in the episomal form and the expression levels of oncoproteins E6 and E7 are relatively low (Schiller *et al.*, 2010). This phase is generally referred to as CIN1 lesion. Conversely, when the viral DNA begins to integrate in shuffle mode and becomes equally available in the

episomal and integrated form, the cervical pre-neoplastic lesion acquires tumorigenic potential (CIN2). Finally, cervical keratinocytes of CIN3 lesions are no longer able to regulate their own proliferative and differentiation capacity, due to the uncontrolled expression of E6 and E7. Epidemiological and experimental data indicate that the presence of High-risk HPVs in cervical keratinocytes is necessary yet not sufficient to induce cervical SCC. Indeed, other genetic and epigenetic events would be implicated in the multifactorial process of neoplastic transformation (Zur Hausen, 2002), justifying the presence of several functional and structural abnormalities of both oncogenes and tumor suppressor genes as well as epigenetic modifications in cervical SCCs. As to the proto-oncogenes, cell mutations and/or gene amplification have been identified in the genes encoding for the subunits of PIK3CA, RAS, MYC, ErbB2 and cIAP1 (Zhang *et al.*, 2002; Imoto *et al.*, 2002; Bertelsen *et al.*, 2006). As to the tumor suppressor genes, loss of PTEN, CADM1 and NOTCH seems to be associated with tumor progression (Cheung *et al.*, 2004; Overmeer *et al.*, 2008; Vande and Klingelutz, 2013). The epigenetic deregulation and its role in cervical pre-neoplastic CIN progression or in SCC will be dealt in detail in the following chapter.

EPIGENETIC AND CANCER

It is well known that epigenetic alterations, i.e. improper DNA methylation and histone modification, are strongly involved, as other molecular phenomena, like genetic mutations or gene expression changes, in a cell's transformation to cancer (Novak, 2004). Furthermore, the DNA methylation and histone modifications defects could be operate alone or in concert with several other molecular alterations involved in cancer development.

Epigenetics can be defined as the study of all heritable chemical modifications that affect the phenotype without altering the genotype. Indeed, it is defined as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” (Russo *et al.*, 1996). Furthermore, epigenetics modification may have short- or long-term and even trans-generational effect. Epigenetics modification are well-established and common phenomena in all cells and are involved in the regulation

of gene expression. At the basis of the epigenetics process there are the regulation of chromatin remodeling and regulation of gene expression that are accomplished through two related mechanisms: DNA methylation and posttranslational histone modifications. From a biological standpoint, epigenetics modifications are phenomenon that plays a key role in a diversity of processes, such as embryonic development, immune system response, infertility and cancer development. However, aberrant epigenetic modifications may have the same negative effect of a gene mutation, because the expression of the DNA is altered. Consequently, aberrant epigenetic modifications may be associated, or induces, disease. While the genetic code is considered static, i.e. it is the same in each cell for the entire life of the organism, the epigenetics phenomena are dynamic, tissue-specific and provides to change the phenotype because of various factors such as environmental factors (Li *et al.*, 2006). This dynamic state may suggest the possibility of reversibility of epigenetic changes, and then a possible therapeutic target for a certain number of diseases. (Egger *et al.*, 2009)

Aberrant CpGs methylations, hypo- and hypermethylation, have long been associated with diseases and cancer. DNA methylation is a biochemical process based to the addition of a methyl group to the S-adenosyl-methionine to the 5' position of cytosine belonging to CpG DNA dinucleotide (Biermann and Steger 2007). This activity is mediated by DNA methyltransferases (DNMTs) family enzymes. Specifically, the family of enzymes DNMT1 is involved in the maintenance of methylation patterns (Bestor *et al.*, 1988); whereas, enzymes DNMTs 3a and 3b (Okano *et al.*, 1999) are required for de novo methylation activity (Lei *et al.*, 1996; Okano *et al.*, 1999). Another protein, DNMT3L, is homologous to DNMT3s, but has no catalytic activity and assists the DNMTs by increasing their ability to bind DNA and stimulating their activity (Chedin *et al.*, 2002; Suetake *et al.*, 2004). Takai and Jones formulated the accepted definition of a CpG island as a 500, or higher, base pair segment of DNA with a G+C equal to, or greater than, 55% and with a CpG frequency of at least 0.65 of the statistically expected value (Takai and Jones, 2002). In mammals 70% to 80% of CpG cytosines are methylated (Jabbari and Bernardi 2004). CpG islands are located preferentially in high concentrations within promoters genes (Bird *et al.*, 1995) with a frequency about 40% (Fatemi *et al.*, 2005), indicating that they are involved in the regulation of gene expression (Deaton and Bird, 2011). Indeed, DNA hypomethylation in CpG islands correlates with gene expression (Biermann and Steger, 2007), whereas DNA methylation in CpG islands is associated with transcriptional repression (Deaton and Bird, 2011). Hypermethylation in CpG islands

located at gene promoter region cause gene suppression because: (i) DNA methylation itself prevents binding of transcriptional factors (Choy *et al.*, 2010); (ii) methylated DNA is bound by methyl-CpG-binding domain proteins (MBDs) (Nan *et al.*, 1993), which, subsequently recruit other factors involved in gene expression inhibition (Illingworth and Bird, 2009; Thomson *et al.*, 2010);

In general, these epigenetic modifications, causes defective gene expression, improper condensation and chromosomal instability (Attila and Lorincz, 2014). In cancer cells, both of these alterations could coexist in concert or alone. As previously reported, CpG islands are typically located within, or close to, gene promoter and are involved in gene expression regulation. Whereas CpG islands preceding tumor suppressor gene promoters are generally unmethylated, in cancer cells it is possible that an abnormal increase of methylation level at this region induce gene silencing. Indeed, hypermethylation of tumor suppressor gene promoters is often associated with the silencing of those genes. The tumorigenic process is induced when genes that regulate the cell cycle are silenced, allowing cells to increase their proliferative capacity and reproduce uncontrollably (Esteller, 2007). Two important genes that acquire hypermethylation were two cell-cycle inhibitor referred to as p16 and p15 in different types of cancer (Viet *et al.*, 2007). Various genes involved in DNA repair, such as O-6-methylguanine-DNA methyltransferase (MGMT), undergo defective methylation in many types of carcinomas (Esteller *et al.*, 1999; Weller *et al.*, 2010). Furthermore, another DNA-repair gene found to be hypermethylated is MLH1. This gene was detected whit this defective epigenetics aberration in gastric cancer (Li *et al.*, 2014). Others suppressor genes known to be hypermethylated during cancer progression were: RB, Cyclin-dependent kinase inhibitor; the Von Hippel–Lindau tumor suppressor gene VHL; and E-cadherin, a calcium-dependent cell-cell adhesion glycoprotein (Greger *et al.*, 1989; Santini *et al.*, 2001). Typically, during cancer development, while hypermetilation generally occurs in a single molecular target, DNA hypomethylation is a general phenomenon. Genomic instability is the principal consequence, but sometime transcriptional activation of oncogenes may occur. Indeed, aberrant hypomethylation of CpG islands located at proto-oncogene promoters could lead to an increase of expression of these genes. In normal cell, repetitive genomic sequence, such as LINE, SINE, IAP and Alu elements are highly methylated. These genomic sequences ensure genomic integrity/stability and improper hypometylation phenomena could induce undesired mitotic recombination. Indeed, loss of DNA methylation in SAT2 (juxtacentromeric satellite 2) and SAT α (centromeric satellite α) was detected in breast

cancer and may contribute to progression of ovarian cancer (Widshwendter *et al.*, 2004). DNA loss of methylation of individual gene is rather in common. Indeed, frequently, most of promoter regulatory region are under this epigenetic control, belong to tissue specific genes. For instance, a cancer/testis (CT) antigens, expressed in normal condition in adult male testis, are epigenetically and aberrantly activated in various types of human cancers (Caballero and Chen, 2009). Furthermore, in colorectal cancer and melanoma, the CT antigens MAGE are reactivated by promoter hypomethylation (Weber *et al.*, 1994).

High-risk HPVs and epigenetics deregulation in cancer

The multifactorial process of cervical SCCs high-risk HPVs induced, includes, among the various aspects, epigenetic changes in the host genome. The apoptotic pathway presents numerous genes, which epigenetic aberrations are involved with the onset of cervical SCCs. The gene coding two members of tumor necrosis factor receptor superfamily, referred to as decoy receptors (DcR1/DcR2) can be the target for abnormal methylation that leads to their silencing in cervical SCCs, suggesting that cervical cancer cells may obtain a growth advantage, probably due to the down-regulation of decoy receptor DcR1/DcR2 (Van Noesel *et al.*, 2002; Shivapurkar *et al.*, 2004). Interestingly, despite cervical SCCs show down-regulation of hTERT mRNA, a study has found a correlation between reduced expression, and catalytic subunit activity, with hTERT gene promoter demethylation (Guilleret and Benhattar, 2003). p73, a member of the p53 family, involved in cellular response to DNA damage induced by radiation and chemotherapeutic agents, presents two independent promoters that have opposite activities. One of this two promoters presents within the exon 1, is rich in CpG dinucleotides and its transcriptional silencing through hypermethylation represents a mechanism for inactivation of this gene in cervical SCCs (Liu *et al.*, 2004). As previously reported, a lot of number of cell cycle-related genes is deregulated during cancer development. Aberrant methylation of the p16 gene promoter occurs in situ as well as in invasive tumors with a frequency of ranged between 10-100% (Nakashima *et al.*, 1999). Furthermore, p16 hypermethylation is progressively most frequent during CIN pre-cancerous lesions progression. Indeed is present in 17.6% of CIN I, 42.1% of CIN II, 55.0% of CIN III, and 65.0% of invasive

cancers (Huang *et al.*, 2011). Two other genes cycle-related genes epigenetically involved in cervical SCCs onset and progression are CCNA1 and the fragile histidine triad (FHIT). The CCNA1 gene encodes Cyclin A1, which regulates the cell cycle. CCNA1 downregulation due to promoter hypermethylation was detected in 60% and 93.3% of microinvasive cancers, and invasive cancers, respectively (Yang *et al.*, 2010). FHIT is another protein involved in cell cycle regulation and apoptosis. Epigenetic silencing of this gene by promoter hypermethylation is common in cervical cancer (Ki *et al.*, 2008). Furthermore, the signal transduction pathway Wnt, named for its most upstream ligands, the Wnts, is epigenetically deregulated during cervical SCC development. In this pathway were detected promoter aberrant methylations in PTEN (Cheung *et al.*, 2004), E-cadherin (Widschwendter *et al.*, 2004) and APC (Virmani *et al.*, 2001) in various cervical SCCs cases. Two genes belonging to the Fanconi anemia (FA)-BRAC pathway referred to as BRCA and FANCF present aberrant methylation in their promoter in cervical SCCs (Marsit *et al.*, 2004; Narayan *et al.*, 2003). Furthermore, in a study conducted in patients with SCC, BRCA1 promoter hypermethylated cases present a frequency of 6.1%, in invasive SCCs, whereas FANCF hypermethylation rate was 30%. Thus, hypermethylation of these genes was mutually exclusive in the analyzed cases, suggesting the important role of epigenetics aberrations in this pathway for cancer. (Narayan *et al.*, 2004). Other molecular pathways that have genes, which promoter carries epigenetics deregulations include: mismatch repair, metastasis/cell death, cell differentiation and DNA repair (Narayan *et al.*, 2003; Virmani *et al.*, 2001).

MOLECULAR STUDIES OF CIN PROGRESSION

The molecular bases involved in the pre-cancerous CIN lesions progression to cervical SCC were previously investigated in different studies. These studies were carried out at both tissue and cellular level using microarray analysis or HPV DNA transfection assays, respectively.

In order to compare gene expression signature on biopsies from cervical SCC with normal tissues, different studies were performed using microarray technique. (Cheng *et al.*, 2002; Chen *et al.*, 2003; Wong *et al.*, 2003; Rosty *et al.*, 2005; Wong *et al.*, 2006; Chao *et al.*, 2006). However, in all these studies have not been investigated the molecular mechanisms involved in the progression of CIN lesions to the cervical SCC. These mechanism were subsequently investigated in other works when were used, in addition to cervical SCC and normal tissues, CIN biopsies as well (Gius *et al.*, 2007; Arvantis and Spandidos, 2008; Song *et al.*, 2008; Rajcumar *et al.*, 2011). A work conducted by Gius and colleagues shows that proliferative/immunosuppressive genes, such as p16INK4a, KIF23 and CENPF are up regulated in CIN1 lesions, probably due to the epithelial response to human papillomavirus infection, while proangiogenic stromal/epithelial interaction genes, such as HINT1, TAGLN and TBX19 and proinvasive genes, such as DSG3, MMP3 are mainly up-regulated in CIN2 and CIN3 lesions, respectively. These results suggest a cooperative signaling interaction between stroma and tumor cells. Finally, the signature pattern detected in CIN3 and SCC probably represents epithelial tumor cell overcrowding (Gius *et al.*, 2007).

Microarray studies were performed on biopsies of tumor and normal tissue. This study model may affect the actual gene expression profile of the type of cell under study, as keratinocytes, in this case. Such a problem has to be ascribed to tissue samples - in particular cervical CIN or normal tissue samples - that contain a significant number of stromal cells and contaminants derived from the host immune cells, such as monocytes, dendritic cells and lymphocytes.

In vitro HPV-transfected human skin keratinocytes represent good models to mimic the molecular and morphological characteristics of cancerous cells (Pirisi *et al.*, 1987;; Zyzak *et al.*, 1994; Creek *et al.*, 1995; Borger *et al.*, 2000; Akerman *et al.*, 2001; Chang and Laimins, 2000; Nees *et al.*, 2001; Oh *et al.*, 2001). In vitro models of HPV-transfected cells showed loss of differentiation, overexpression of EGF receptor and resistance to

TGF-beta-induced growth inhibition (Pirisi *et al.*, 1988). Several studies have applied microarray technology to this model with the aim of identifying the molecular processes involved in HPV-induced transformation and tumor development (Ruutu *et al.*, 2002; Duffy *et al.*, 2003; Kravchenko-Balasha *et al.*, 2009). However, HPV-transfected keratinocytes may not mirror the actual *in vivo* situation because they do not derive from the uterine cervix, but from other parts of the body. Moreover, keratinocytes were not naturally infected by high-risk HPVs. Consequently, the molecular pathways detected in this model could be different from those involved in HPV induced progression to cervical SCC.

Only few gene-expression studies have been performed on cervical keratinocytes that were naturally infected with HPV, i.e. *in vitro* neoplastic HPV-keratinocytes derived from CIN lesions (Gray *et al.*, 2010). In those studies, the gene expression profile was investigated in HPV16-infected keratinocytes derived from low-grade CIN1 lesions (Gray *et al.*, 2010). A work of Nees and colleagues used, as a study model, primary cultures of ectocervical keratinocytes obtained from cervical tissue from hysterectomies. The cells used were infected with retroviruses expressing E6 and E7 genes of HPV16 (Nees *et al.*, 2001). In 1989 Stanley and colleagues set up a human cervical keratinocyte cell line, referred to as W12, from a low-grade cervical lesion histologically diagnosed as CIN1. This keratinocyte cell line represented a good model to study the natural history of cervical neoplasia. In fact, the same model was used to identify groups of genes that carried expression changes due to HPV-16 integration (Alazawi *et al.*, 2002). Santin and colleagues analyzed gene expression on *in vitro* cultures of cervical keratinocytes using microarray assay. Even though cultured keratinocytes did not derive from CIN pre-cancerous lesions, the study was conducted on 15 primary cervical cell lines: 11 HPV-16 or HPV-18 positive cervical SCC primary cultures and 4 cell lines of normal cervical keratinocytes (Santin *et al.*, 2005).

OBJECTIVE AND AIMS OF THE STUDY

Overall, the main objective of my study was to investigate the molecular mechanisms occurring in neoplastic progression of CIN2 and CIN3 cervical pre-cancerous lesions. To this purpose, HPV16-CIN2 and HPV16-CIN3 keratinocytes derived from high-grade CIN2 and CIN3 pre-cancerous lesions were investigated by microarray analysis and DNA methylation of gene promoters. Aims of this study were:

I

To set up a cell culture protocol able to derive pre-neoplastic and normal cervical keratinocytes from small tissue fragments of naturally high-risk HPV-infected CIN2 and CIN3 lesions and normal uterine cervix, respectively. Cultures of CIN and normal cervical keratinocytes were stained with immunofluorescence technique in order to investigate expression of epithelial and cervical markers.

II

To investigate the gene expression profile of HPV16-CIN2 and HPV16-CIN3 keratinocytes. To this purpose, HPV16-CIN2 and HPV16-CIN3 keratinocytes and the corresponding normal cervical keratinocytes were subjected to microarray analysis, Real-Time Quantitative RT-PCR, and Immunohistochemistry analysis.

III

To analyze the mRNA expression levels and DNA methylation status of target genes in HPV16-CIN2 and HPV16-CIN3 keratinocytes. To this purpose, HPV16-CIN2 and HPV16-CIN3 keratinocytes and the corresponding normal cervical keratinocytes were subjected to Real-Time Quantitative RT-PCR, and bisulfite sequencing analysis.

MATERIALS AND METHODS

CERVICAL UTERINE SPECIMENS

Small tissue fragments (2-3 mm³) were taken from CIN biopsies, CIN2 or CIN3 pre-cancerous lesions, after surgery excision. The corresponding surrounding normal tissues were also provided. The patients had undergone electrosurgical conisation under colposcopic examination using 5% acetic acid and Lugol's iodine solution, which stains the pathologic tissue with a white color, whereas stains normal tissue brown (Stafl and Wilbanks, 1991). CIN and normal specimens were selected and divided by the gynecologist during surgery and CIN specimens were classified by pathologists according to international criteria (Horvat *et al.*, 2008). Informed written consent was obtained from all patients in accordance with our institutional guidelines.

CIN2, CIN3 AND NORMAL TISSUE PREPARATION

Each tissue fragment was transferred into a 50 ml centrifuge tube and submerged in an ice-bath containing 20 ml of DMEM:F12 transporting medium (with L-glutamine, 15 mM HEPES and 3.151 g/L glucose; Lonza, Milan, Italy) with 200 U ml⁻¹ Penicillin/Streptomycin (10,000 U ml⁻¹ penicillin, 10,000 U ml⁻¹ streptomycin; Lonza, Milan, Italy), 0.25 µg ml⁻¹ Amphotericin B (250 µg ml⁻¹; Lonza, Milan, Italy). Under a

sterile hood, the transporting medium was removed and the tissue fragments were rinsed with 10 ml of sterile PBS 1X (without calcium or magnesium; Lonza, Milan, Italy,) and mixed manually 3-4 times. The samples were centrifuged at 400 g at RT for 10 minutes and the PBS 1X was discarded. The samples were washed again twice and afterwards the PBS 1X was discarded. The tissue fragments were lifted with tweezers and placed in a 10 cm Petri-dish. Afterwards the tissue fragments were finely cut with a disposable blade. During cutting, it was followed the direction of the blade edge, and the tissue was not dragged laterally; to cut the tissue in all directions, the Petri-dish was turned around periodically. Then, DMEM:F12 medium (with L-glutamine, 15 mM HEPES and 3.151 g/L glucose; Lonza, Milan, Italy), 4.5 ml, was deposited on the Petri-dish and the minced specimens were aspirated and transferred into a T25 flask (Corning, Pero, Italy); 500 μ l of 2000 u/ml Type II collagenase enzyme solution (final concentration 200 u/ml, Sigma-Aldrich, Milan, Italy) were added and tissue digestion was performed at 37 °C, with 5% CO₂, for 24 hrs.

CIN2, CIN3 AND NORMAL CULTURE SETUP

After digestion, cell suspension was gently mixed to optimally disaggregate all tissue fragment residues, and then transferred the into a 10 ml conical tube and centrifuged at 400g at RT, for 10 minutes. The supernatant was discarded and the cell pellet was washed twice with 2 ml of PBS 1X. The cell pellet was suspended in 5 ml of DMEM:F12 complete medium (with L-glutamine, 15 mM HEPES and 3.151 g/L glucose; Lonza, Milan, Italy) with 200 U ml⁻¹ Penicillin/Streptomycin (10,000 U ml⁻¹ penicillin, 10,000 U ml⁻¹ streptomycin; Lonza, Milan, Italy), 0.25 μ g ml⁻¹ Amphotericin B (250 μ g ml⁻¹; Lonza, Milan, Italy) and 10% v/v of Fetal Bovine Serum (FBS, Lonza, Milan, Italy) and seeded in a T25 flask. Cell cultures were incubated at 37 °C, with 5% CO₂. After two days, the cell suspension was transferred into a 10 ml conical tube whereas the attached cells were washed twice with 5 ml of PBS 1X; then, 5 ml of fresh DMEM:F12 complete medium was added. Cell suspension was centrifuged at 400g at RT for 10 minutes. The cell pellet was

washed twice with 2 ml of PBS 1X, then suspended in 5 ml of DMEM:F12 complete medium and transferred into a new T25 flask (T2 flask). The cell suspension in the T2 flask was incubated at 37 °C, with 5% CO₂, and leaved to attach for 2 days. After two days, the cell suspension of the T2 flask was discarded, the attached cells were washed twice with PBS1X and 5 ml of fresh DMEM:F12 complete medium was added.

CIN2, CIN3 AND NORMAL PRIMARY COLONY EXPANSION

Colonies grown in T1 and T2 flask were analysed with Inverted Nikon TE2000E microscope; the larger colonies, and made up of small cells, were selected for expansion. DMEM:F12 complete medium was removed and cells were washed twice with 5 ml of PBS 1X. The upper surface of the T1 and T2 flasks was opened at the top with a red-hot sterile disposable blade. The flasks were carefully opened in order to avoid dropping plastic fragments onto the layer where the cells were located. After discarding of the PBS1X, glass cylinders (height 10 mm, external diameter 9 mm, internal diameter 7 mm; Elettrofor s.a.s., Borsea, Italy) were used to isolate the single colonies. Glass cylinders were sealed with a silicone rubber and placed around the colonies; after pressing lightly down, so that the cylinders adhered well to the bottom of the T25 flasks, cells were detached with 50 µl of 0.05% w/v Trypsin (from bovine pancreas; Sigma-Aldrich, Milan, Italy)/0.01% w/v Ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Milan, Italy) in a PBS 1X solution, at 37°C for 5 minutes. After incubation, most of the cells from different colonies were completely detached. However, cells from some colonies were difficult to detach and further 3-5 minutes of incubation at 37°C were needed. Then, cell suspension from each colony was recovered and seeded in a well of 6 well culture plate (Corning, Pero, Italy) with 2 ml of DMEM:F12 complete medium. Cells were left to attach overnight at 37 °C, with 5% CO₂. After incubation, the DMEM:F12 complete medium was changed with 2 ml of DMEM:F12 complete medium/defined Keratinocyte Serum Free Medium (dKSFM, Invitrogen, Monza, Italy) (ratio 1:1) (DMEM:F12/dKSFM) medium. Cells were incubated at 37 °C, with 5% CO₂ and the DMEM:F12/dKSFM medium was changed every

3 days. Cell cultures became 80% confluent typically in 4 days for CIN2 and CIN3 keratinocyte colonies and in 7 days for normal keratinocyte colonies. In order to obtain huge amounts of normal and neoplastic keratinocytes, the subconfluent cells were detached from each well with 500 μ l Trypsin/EDTA solution at 37 °C for 5 minutes, and then transferred into a new T25 flasks with 5 ml DMEM:F12/dKSFM medium. Cell cultures were incubated at 37 °C, with 5% CO₂ and the medium was changed every 3 days. CIN2 and CIN3 keratinocyte colonies grew more quickly than normal keratinocyte colonies. Therefore, 10 days were sufficient to reach sub-confluence for CIN2 and CIN3 keratinocytes and 15 days or more were needed in order for sub-confluence to be reached by normal keratinocytes.

IMMUNOFLUORESCENCE ASSAYS

Single CIN3 and normal colonies were isolated with cloning rings, keratinocytes were subdivided onto different coverslips and cells were grown on microscope slides. Keratinocytes were fixed by immersing the slides in jars filled with paraformaldehyde solution (4% formaldehyde and 0.5% Triton X-100 in PBS 1X) and incubated at 37°C for 20 min. Keratinocytes were then blocked with 10% goat serum in PBS 1X at 37°C for 1h. Subsequently keratinocytes were incubated with different mouse anti-human monoclonal antibodies (mabs). To determine the epithelial and cervical markers, immunofluorescence staining with K5, K14, K17, and K19 keratins and with p63 (Dako SpA, Milan, Italy) was performed, as previously described (Quade *et al.*, 2001; Radu *et al.*, 2002; Martens *et al.*, 2004; Harper *et al.*, 2007; Tudor *et al.*, 2007). The substitution of primary antibodies with PBS 1X served as a negative control. Digital images from a Nikon TE2000E microscope were captured using the ACT- 1 software for the DXM1200F digital camera (Nikon, Florence, Italy). The percentage of cells expressing different keratin markers in the colonies was quantified by counting 1,000 cells in four randomly selected fields/colony.

DNA ISOLATION

DNA was isolated from a small fraction of CIN2, CIN3, and normal specimens and from corresponding CIN2, CIN3, and normal keratinocytes according to standard procedures, as described (Barrandon and Green, 1987; Bononi *et al.*, 2012; Rotondo *et al.* 2012). All DNA was stored at -80°C until the time of analysis. Furthermore, before molecular analysis, all DNA was first quantified using the Nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE USA). To test the suitability of the extracted DNA for PCR analysis, isolated DNA was PCR amplified with β -globin primers (Pancaldi *et al.*, 2009).

HPV DETECTION AND GENOTYPING

Purified DNA from CIN2, CIN3, and normal specimens and from corresponding cultured CIN2, CIN3 and normal keratinocytes was amplified for HPV sequences with the general primers GP5-GP6, which enable detection of HPV -6b, -11, -16, -18, -31, -33 genotypes. PCR analysis was carried out with 500 ng human genomic DNA (Barrandon and Green, 1987; Bononi *et al.*, 2012; Martini *et al.*, 2004). HPV PCR product sizes were 139 bp for HPV -6b, -11 and -33 genotypes, 142 bp for HPV -16 and -31 genotypes, and 145 bp for HPV -18 genotype. PCR products were electrophoretically separated on 2.5% agarose gel. To further assess PCR product specificity, a restriction endonuclease analysis of HPV sequences was performed with *RsaI* digestion (Barrandon and Green, 1987; Bononi *et al.*, 2012). DNA digestion was performed at 37°C for 2 h. The digested DNA products were electrophoretically separated on 20% acrylamide gel, and DNA fragment size GP5-GP6 amplified DNA of CIN and HPV PCR (positive control) products were compared.

RNA ISOLATION

Total RNA was extracted from CIN2, CIN3, and normal keratinocytes by use of a RNazol kit (Life Technologies, Milan, Italy), according to the manufacturer's instructions. All RNA was stored at -80°C until the time of analysis. Furthermore, before molecular analysis, all RNA was first quantified using the Nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE USA).

WHOLE HUMAN GENOME EXPRESSION DETECTION BY OLIGO MICROARRAY

RNA from CIN2, CIN3 and normal keratinocytes was hybridized on Agilent whole human genome oligo microarray (Agilent Technologies, Palo Alto, CA). This microarray consists of 60-mer DNA probes which have been synthesized in situ and represent 41,000 unique human transcripts. One-colour gene expression was performed according to the manufacturer's procedure. Briefly, RNA quality was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies). Low quality RNA (RNA integrity number below 7) was excluded from microarray analyses. Labelled cRNA was synthesized from 500 ng of total RNA using the Low RNA Input Linear Amplification Kit (Agilent Technologies) in the presence of cyanine 3-CTP (Perkin-Elmer Life Sciences, Boston, MA). Hybridizations were performed at 65°C for 17 hours in a rotating oven. Images at 5 µm resolution were generated by the Agilent scanner, and Feature Extraction 10.5 software (Agilent Technologies) was used to obtain the microarray raw data.

MICROARRAY DATA ANALYSIS

Microarray raw data were analyzed by use of GeneSpring GX 10 software (Agilent Technologies). Data transformation was applied to set all the negative raw values at 1.0, followed by normalization at the 75th percentile. A filter on low gene expression was used to keep only the probes expressed in at least one sample (flagged as Marginal or Present). Then, samples were grouped according to their differentiation status and compared. Differentially expressed genes were selected as having a 1.5-fold expression difference between their geometrical mean in two or more groups of interest, and a statistically significant p-value (<0.05) according to ANOVA (analysis of variance) and Benjamini and Hochberg correction for reduction of false-positive values. Differentially expressed genes were employed for sample cluster analysis by use of the Pearson correlation as a measure of similarity. The microarray raw data have been deposited at ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>) under accession number E-MTAB-2019.

REVERSE TRANSCRIPTION QUANTITATIVE REAL-TIME PCR (RT-QPCR)

The differential expression of selected genes in CIN2, CIN3 and normal keratinocytes was validated by Reverse Transcription quantitative real-time PCR (RT-qPCR). Briefly, 300 ng total RNA was reverse transcribed with a random hexamer primer using High Capacity RNA-to-cDNA Kit (Roche Applied Science, Milan, Italy), according to the manufacturer's instructions. qPCR monitoring was performed with the ABI 7500 Fast Real Time PCR system (Roche Applied Science) and Power SYBR Green PCR Master Mix (Roche Applied Science). The following eight genes were investigated: RARB (Bohlken *et al.*, 2009), IRF6 (Restivo *et al.*, 2011), TIMP3 (Bernot *et al.*, 2010), APOC1 (Oue *et al.*, 2004), MSX1 (Chetcuti *et al.*, 2011), PHGDH (Liu *et al.*, 2013), C-JUN (De-Castro *et al.*, 2004) and p63 (Yalcin-Ozuysal *et al.*, 2010). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as an internal control (Chetcuti *et al.*,

2011). Each assay was performed in triplicate. Data analysis was performed with the 2^{- Ct}

method.

IMMUNOHISTOCHEMICAL (IHC) ANALYSIS

Immunohistochemical (IHC) analysis was performed on paraffin-embedded specimens from 30 patients with CIN lesions (10 CIN1, 10 CIN2 and 10 CIN3), from 4 with invasive squamous cell cervical cancer, and from 10 with normal cervical tissue. The IHC staining was performed by use of the Multimeric Detection Kit (Universal DAB Detection Kit Ultraview, Roche Tissue Diagnostics (CH), on a BenchMark XT immunostainer (Roche T.D.). Paraffin-embedded tissue sections (4 µm thick) were stained with mouse monoclonal 3PGDH antibody sc-100317 (Santa Cruz Biotechnology, Santa Cruz, CA) (dilution, 1:50). HeLa cells, processed as tissues, ie. pelleted, fixed and paraffin-embedded, were used as positive controls, as recommended by the manufacturer. Staining intensity and the distribution of staining were assessed by two pathologists. Staining was graded as negative (no staining) and as weak, moderate, or strong intensity.

SODIUM BISULFITE TREATMENT OF DNA

DNA from CIN2, CIN3 and normal keratinocytes was treated with sodium bisulfite using the Epiect Bisulfite kit (Qiagen, Milan, Italy) as previously described ([Rotondo et al., 2013](#)). Sodium bisulfite treatment induces the conversion of unmethylated cytosines of DNA to uracil, while leaving the 5-methylcytosines unchanged. Samples were then purified using DNA purification columns (Epiect Bisulfite kit, Qiagen).

RARB METHYLATION PCR PRIMERS DESIGN

RARB methylation PCR primers were designed using the MethPrimer informatics software (Li and Dahiya, 2002). Briefly, MethPrimer is a software for methylation designing PCR primers for methylation investigation. In a first step, this software is able to search for all CpGs in a limit input sequence of 5 Mbp. In a second step the software can design primers within the input sequence through general parameters changes, like product Size, primer T_m, etc. (Li and Dahiya, 2002).

BISULFITE TREATED DNA PCR OF RARB AND IRF6 PROMOTER REGION

The methylation assay was performed at the promoter region of RARB and IRF6 genes. 150 ng of sodium bisulfite-treated CIN2, CIN3 and normal keratinocytes DNA was amplified at the RARB and IRF6 (Botti *et al.*, 2011) loci by Bisulfite treated DNA PCR. The RARB promoter region studied contained 14 CpG islands whereas the IRF6 promoter region studied contained 25 CpGs.

DNA CLONING AND SEQUENCING

Amplified products were purified with the QIAquick PCR Purification Kit (Qiagen) and then cloned with the TOPO TA cloning kit (Invitrogen), using the Turbo Competent *E. coli* bacteria strain (EuroClone) and the pCR 2.1-TOPO vector (Invitrogen), according to manufacturer's instructions. Selection of bacterial clones containing the fragment of interest was performed using selective LB growth medium with ampicillin (100 µg/ml,

Sigma-Aldrich). For each DNA sample, 10 positive clones were selected for sequencing analysis. Single clones were sequenced using automated ABI Prism Genetic (Analyzer Applied Biosystems).

STATISTICAL ANALYSIS

The observed RARB and IRF6 epigenotype frequencies (i.e. methylated CpGs) were compared between groups using the chi-square trend test with Yates' correction. All statistical analyses were carried out using Graph Pad Prism version 5.0 for Windows (Graph Pad, La Jolla, CA, USA). *P*-values < 0.05 were considered statistically significant.

RESULTS

HPV-DNA ANALYSIS OF CIN2, CIN3 AND NORMAL SPECIMENS AND KERATINOCYTES

In a first step of our experiments, high-risk HPV16 presence in CIN2 and CIN3 specimen was investigated. To this purpose, CIN and normal specimens, were screened by PCR for HPV DNA sequences. All normal samples were negative for HPV sequences (Figure 5, A). CIN2 and CIN3 keratinocyte specimens tested positive for HPV16 sequences were selected for the present study (Figure 5, A, B). The DNA fragment sizes for HPV types are shown in the table 3. Subsequently, in a second step, in order to confirm HPV detection and genotyping, the same DNA analysis was performed in CIN2 and CIN3 keratinocytes. Previous CIN specimens data were confirmed.

HPV type	Total length (bp)	Length of RsaI restriction fragments (bp)
HPV-6	139	30
		42
		67
HPV-11	139	30
		109
HPV-16	142	30
		42
		70
HPV-18	145	30
		38
		77
HPV-31	142	30
		112
HPV-33	139	30
		39
		70

Table 3. DNA fragment sizes for HPV types spanned by GP5/GP6 primer set and fragment lengths generated by RsaI restriction enzyme digestion.

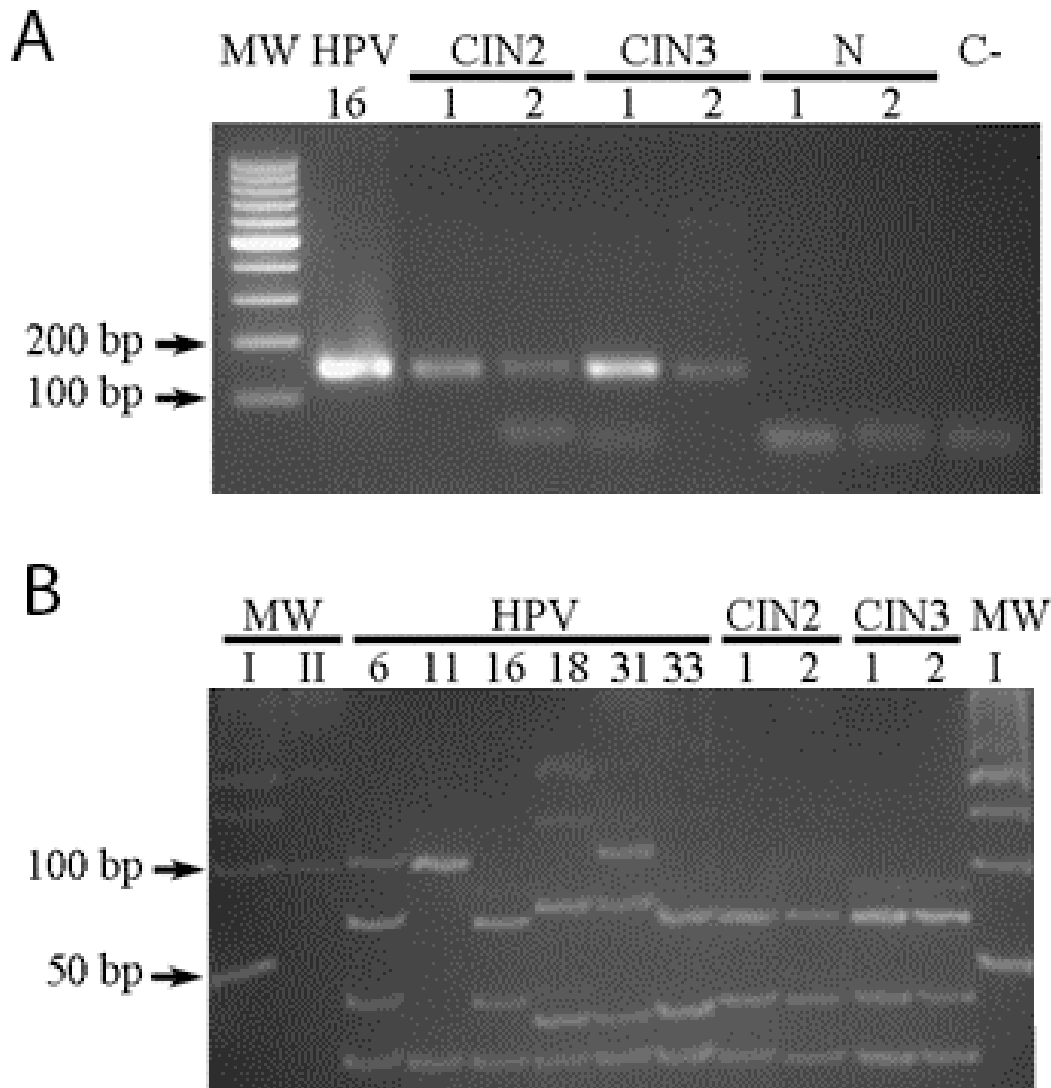


Figure 5. HPV PCR and HPV genotyping. A: the agarose gel shows HPV PCR results obtained from the CIN2, CIN3, and normal keratinocyte DNA specimens. HPV PCR products are only visible in the CIN2 and CIN3 samples (lanes 1 and 2). MW: molecular weight markers are 100 bp (left); HPV-16: PCR positive control; C-: negative control of the PCR reaction without DNA template. B: polyacrylamide gel shows HPV genotyping of HPV PCR products from the CIN2 and CIN3 specimens. The CIN2 (lanes 1 and 2) specimens and CIN3 (lanes 1 and 2) are positive for HPV-16. Fragment lengths are reported in table 3. M.W.: molecular weight markers are 100 bp (M.W. I) and 50 bp (M.W. II) ladder. HPV-6b, -11, -16, -18, -31 and -33: HPV controls.

CIN2, CIN3 AND NORMAL KERATINOCYTES CULTURES SETUP

The protocol is developed in two main steps: (i) CIN and normal keratinocyte primary culture preparation, (ii) CIN and normal keratinocyte primary colony expansion. CIN tissue fragments are obtained from CIN patients during the excision of a cone-shaped portion of preneoplastic tissue under colposcopic examination. Since excised CIN tissue specimens include surrounding normal tissue, a small fragment of normal cervical tissue can be taken from each corresponding CIN tissue specimen. The isolation of all the total cells from the small CIN and normal cervical tissue relies on a collagenase digestion step. Then, separated cells are washed, counted and seeded in T25 flasks (T1) with DMEM:F12 medium and 10% foetal bovine serum (FBS). Approximately, 1×10^4 - 2×10^5 cells are isolated from each CIN and normal tissue fragment. After seeding, cells are left to attach for 48 h; then, any unattached cell suspension is recovered, washed and reseeded for a further 48 h in new T25 flasks (T2). This re-seeding procedure allows keratinocytes endowed with slow attachment capability to be rescued later and therefore enables total primary colony numbers per specimen to be increased. Representative primary keratinocyte colonies from a CIN3 specimen are shown in figure 6. CIN2 and CIN3 tissues give the best primary cultures, producing approximately 200-400 colonies/tissue. Normal cervical tissues give rise to a lower number of colonies, ranging from 50 to 80 colonies/tissue. Frequently, T2 flasks develop many more colonies than T1 flasks. The duration of the procedure is 3-4 weeks; however, CIN2 and CIN3 keratinocyte colonies can be well visible in 2-3 weeks. Keratinocytes and fibroblasts are well distinguishable in primary cell cultures. Indeed, keratinocytes grow forming colonies whereas fibroblasts proliferate sparsely (Figure 6, A), or in disordered cell clusters or in parallel bundle groups (Figure 6, C). Keratinocyte colonies grow surrounded by fibroblasts (Figure 6, B, C) or isolated (Figure 6, A). CIN2, CIN3 and normal primary cultures develop three different types of colonies, which are classified on their cell content and morphology: type I colonies contain cells which are irregularly sized, flattened or spindle-shaped and are loosely spaced (endowed with a low proliferation rate) (Figure 6, A); type II colonies consist of small, compact and uniform sized cells (endowed with a high proliferation rate) (Figure 6, B); type III colonies contain smaller, more compact and more uniform size cells than those of type II (endowed with a very high proliferative rate) (Figure 6, C). CIN2 and CIN3 primary cultures develop approximately 70% type II/type III colonies and 30% type I colonies, whereas normal primary cultures 50% type II and 50% type I colonies. Type II

and III colonies can be expanded efficiently since they are endowed with a high proliferative capability.

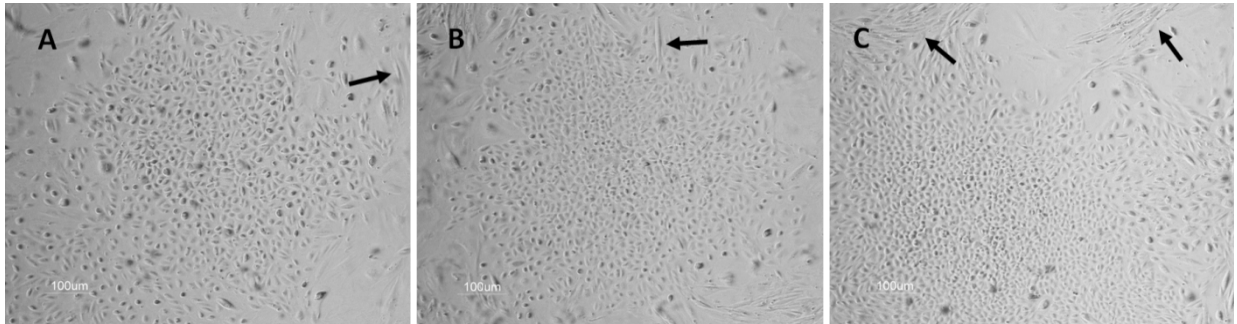


Figure 6. Keratinocyte primary colonies of a CIN3 culture. (A) A type I colony. Arrow indicates a fibroblast; (B) a type II colony. Arrow indicates a fibroblast; (C) a type III colony. Arrows indicate parallel bundle groups of fibroblasts.

IMMUNOFLUORESCENCE CHARACTERIZATION

Immunofluorescence characterization on round coverslip was assessed in CIN3 (Figure 7) and normal primary and expanded colonies. All CIN3 and normal colonies were analyzed for K14, K17, and K19 keratins and p63 expression. The CIN3 primary colonies react strongly for K14 (Figure 7, A) , K17 (Figure 7, B) , K19 (Figure 7, C) as well as for p63 in all cells (Figure 7, D). In some normal primary colonies K14 and K19 stained strongly in all cells whereas K19 reacted with a moderate signal in only 50% of cells. Furthermore, staining for p63 was negative. The CIN3 and normal colonies expanded with the new mixture DMEM-F12/dKSFM (1:1 ratio) cell culture medium stained strongly for K14, K17, K19, and p63 (Figure 7, A-D, insert), and for K14 and K19. Expanded colonies maintain the same staminal and epithelial markers as the primary colonies from which they originated as shown in figure 7.

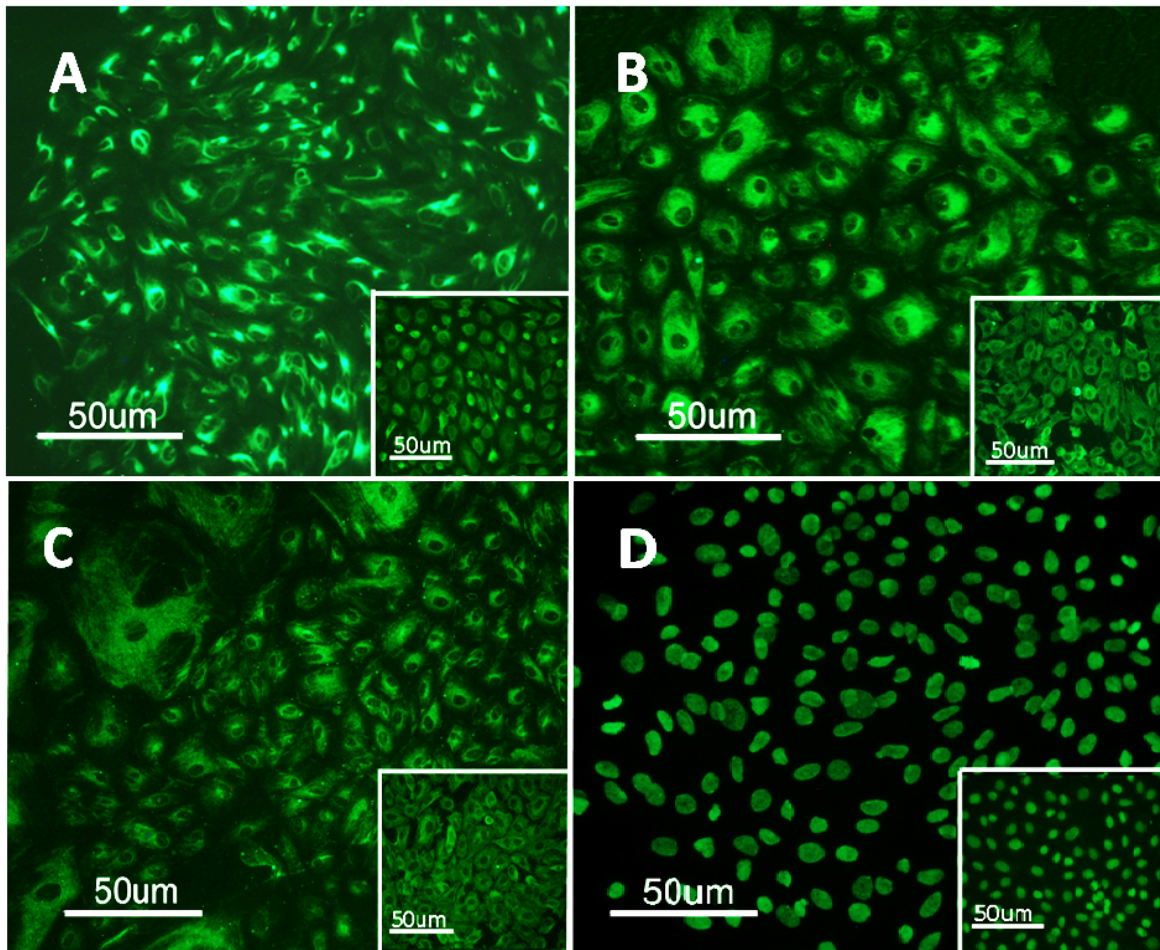


Figure 7. Immunofluorescence staining. Panels A-D: immunofluorescence staining of type III colonies from a CIN3 culture. Cells react strongly to K14 (A), K17 (B) and K19 (C) keratins (markers of cervical keratinocytes; cytoplasmic signal) and p63 (D) (marker of cervical staminal keratinocytes; nuclear signal). Keratinocytes from type III colonies expanded in the new medium mixture maintain high expression of K14 (panel A, inset), K17 (panel B, inset), K19 (panel C, inset) and p63 (panel D, inset).

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN CIN KERATINOCYTES

To identify genes associated with progression of CIN lesions, it was examined the gene expression profiles in two CIN2, two CIN3, and two normal keratinocytes obtained

by tissue cultures from four CIN patients. The genes modulated during the progression from normal to CIN2 keratinocytes and from CIN2 to CIN3 keratinocytes were investigated. One hundred and thirteen genes were significantly down-regulated in CIN2 keratinocytes compared with normal keratinocytes, and 211 genes were down-regulated in CIN3 compared with CIN2 keratinocytes ($P < 0.05$ and fold-change > 2). A consistent down-regulation from normal to CIN2 keratinocytes, and from CIN2 to CIN3 keratinocytes, was observed for the following 23 genes: *INHBB*, *SLC38A11*, *IRX2*, *RARB*, *TIMP3*, *ALDH1A3*, *ABCB4*, *EFNB2*, *C6orf168*, *ATP2A3*, *FMO3*, *FMO4*, *NCAM2*, *TLR2*, *IRF6*, *SYNM*, *HIST1H2AC*, *FRMPD4*, *LIMS3*, *C1orf96*, *FBXO32*, *HIST2H2BE* and *IFIT2* (Table 4 and Figure 8, A). One hundred seventy-five genes were significantly up-regulated in CIN2 keratinocytes compared with normal keratinocytes, and 94 were up-regulated in CIN3 keratinocytes compared with CIN2 keratinocytes ($P < 0.05$ and fold-change > 2). A consistent up-regulation from normal to CIN2 keratinocytes and from CIN2 to CIN3 keratinocytes was detected for 14 genes: *FOXD2*, *SCARA5*, *OLFM1*, *LPAR1*, *SFRP2*, *MSX1*, *APOC1*, *KLF2*, *TAGLN*, *SFXN2*, *TMEM54*, *PHGDH*, *SPOCD1*, and *ARNTL2* (Table 4 and Figure 8, B). Genes consistently up- or down-regulated during transition from CIN2 to CIN3 were used to perform a hierarchical clustering analysis (Figure 9). Normal, CIN2 and CIN3 keratinocytes grouped in three different clusters and showed a distinct gene expression pattern (Figure 9).

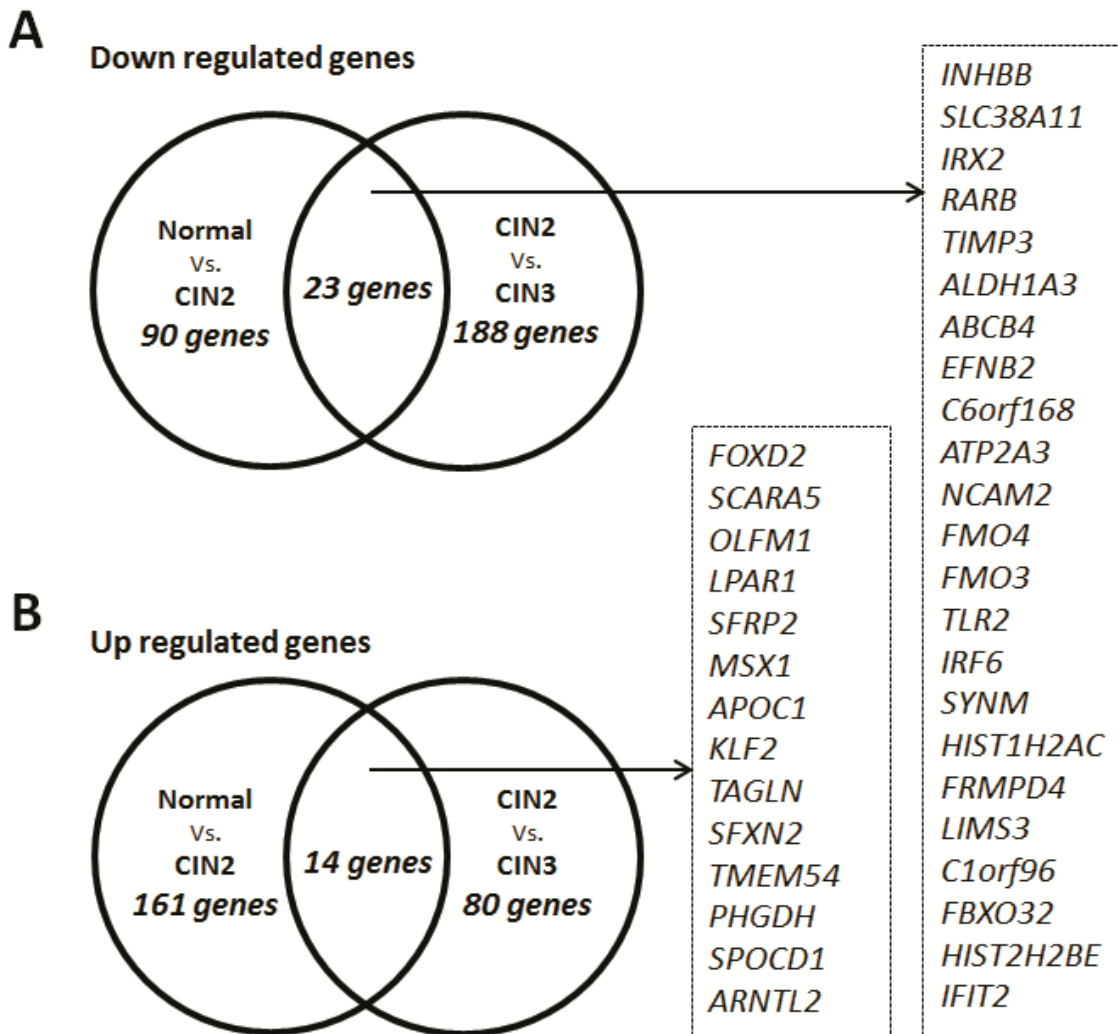


Figure 8. Genes differentially expressed revealed by microarray analysis. *A:* Intersection between the 113 genes down-regulated ($p < 0.05$ and $FC < 0.5$) in CIN2 keratinocytes (CIN2) compared with normal keratinocytes and the 221 genes down-regulated ($p < 0.05$ and $FC < 0.5$) in CIN3 keratinocytes (CIN3) compared with CIN2 keratinocytes (CIN2). Twenty-three genes are in common. *B:* Intersection between the 175 genes up-regulated ($p < 0.05$ and $FC > 2$) in CIN2 keratinocytes (CIN2) compared with normal keratinocytes, and the 94 genes up-regulated ($p < 0.05$ and $FC > 2$) in CIN3 keratinocytes (CIN3) compared with CIN2 keratinocytes (CIN2). Fourteen genes are in common.

Gene symbol	Gene Bank acc. no.	Function
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23 down regulated genes		
INHBB	NM_002193	Immune response
SLC38A11	NM_173512	Amino acid transport
IRX2	NM_033267	DNA binding
RARB	NM_000965	DNA binding
TIMP3	NM_000362	Proteolysis
ALDH1A3	NM_000693	Apoptotic process
ABCB4	NM_018850	Metabolic process
EFNB2	NM_004093	Cell-cell signaling
C6orf168	NM_032511	Unknown
ATP2A3	NM_174953	Nucleotide binding
FMO4	NM_002022	Metabolic process
NCAM2	NM_004540.3	Cell-cell adhesion
FMO3	NM_001002294	Metabolic process
TLR2	NM_003264	Immune response
IRF6	NM_006147	Transcription factor
SYNM	NM_145728	Cytoskeleton constituent
HIST1H2AC	NM_003512.3	DNA binding
FRMPD4	NM_014728	Protein binding
LIMS3	NM_033514	Zinc ion binding
C1orf96	NM_145257	Embryonic development
FBXO32	NM_058229	Protein ubiquitination
HIST2H2BE	NM_003528	DNA binding
IFIT2	NM_001547	Immune response
14 up regulated genes		
FOXD2	NM_004474	DNA binding
SCARA5	NM_173833	Transmembrane transport
OLFM1	NM_006334	Cell junction
LPAR1	NM_012152	Signal transduction
SFRP2	NM_003013	Cell-cell signaling
MSX1	NM_002448	DNA binding
APOC1	NM_001645	Metabolic process
KLF2	NM_016270	DNA binding
TAGLN	NM_001001522	Organ development
SFXN2	NM_178858	Transmembrane transport
TMEM54	NM_033504	Unknown
PHGDH	NM_006623.3	Metabolic process
SPOCD1	NM_144569.5	Transcription
ARNTL2	NM_001248002.1	DNA binding

Table 4. Consistently down-modulated and up-modulated genes identified in CIN2 and CIN3 keratinocytes.

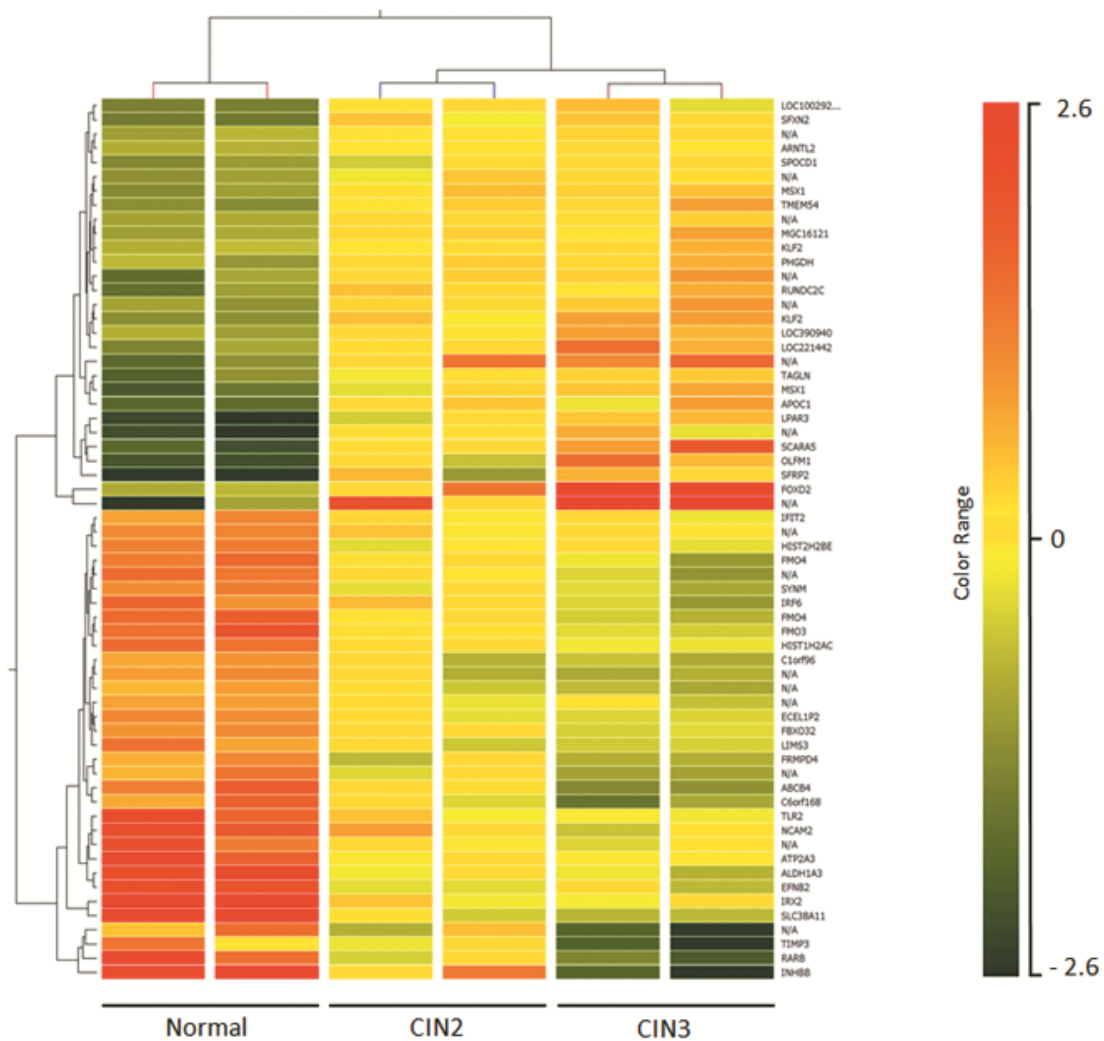


Figure 9. Cluster analysis of normal keratinocytes, CIN2 and CIN3 keratinocytes performed in accordance to the expression of commonly modulated genes, both annotated (with gene symbol) and not annotated (N/A). Genes are in rows, samples in columns. The colors of the genes represented on the heat map correspond to the values normalized on miRNA average expression across all samples (see color bar); up-regulated miRNAs are in red, down-regulated miRNAs in green.

VALIDATION OF MICRARRAY DATA BY RT-QPCR

To confirm the gene expression data identified by microarray analysis, total RNA isolated from the two CIN2, two CIN3 and two normal keratinocyte specimens was subjected to QRT-PCR analysis. Three consistently down-regulated genes from the 23 candidate genes (*RARB*, *IRF6*, and *TIMP3*) and three consistently up-regulated genes from the 14 candidate genes (*APOC1*, *MSX1*, and *PHGDH*) were chosen. Representative amplification plot is showed in figure. In accordance with microarray analysis, the mRNA expression level of *RARB*, *IRF6*, and *TIMP3* was significantly decreased from that of normal and CIN2 keratinocytes and from that of CIN2 and CIN3 keratinocytes (Figure 11). Specifically, the *RARB* gene was significantly down-regulated by 8.9-fold and 29.2-fold in CIN2 and CIN3 keratinocytes, respectively, compared to normal keratinocytes (Figure 11). As for *RARB*, *IRF6* also resulted down-expressed: 3.6-fold and 7.2-fold in CIN2 and CIN3 keratinocytes, respectively, compared to normal keratinocytes (Figure 11). Similarly, *TIMP3* gene resulted significantly down-regulated by 5.6-fold and 13.7-fold in CIN2 and CIN3 keratinocytes, respectively, compared to normal keratinocytes (Figure 11). Differences in *RARB*, *IRF6* and *TIMP3* mRNA expression levels between CIN2 and CIN3 keratinocytes were statistically significant ($p < 0.0001$).

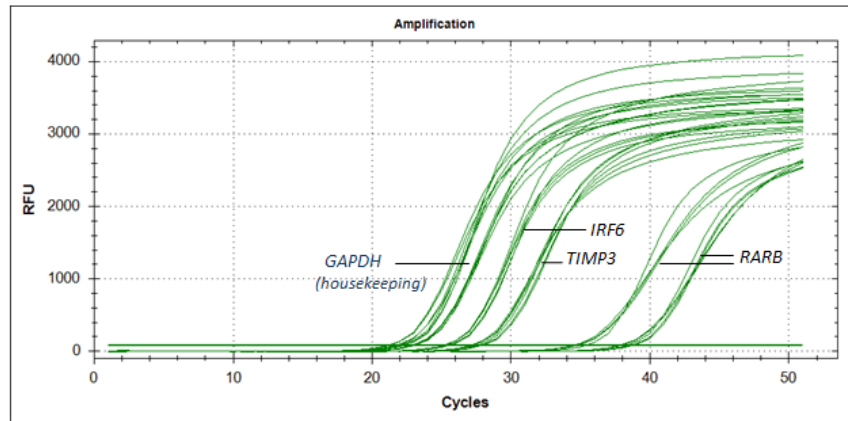
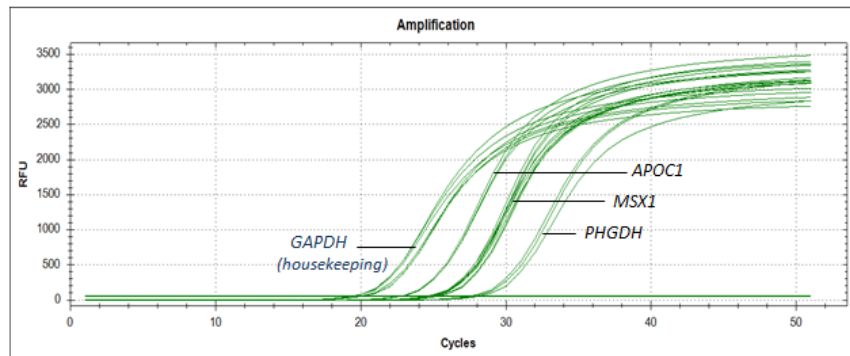
A**B**

Figure 10. Representative real-time quantitative RT-PCR amplification plot of: (A) RARB, IRF6, TIMP3, and the housekeeping gene GAPDH and (B) APOC1, MSX1, PHGDH and GAPDH.

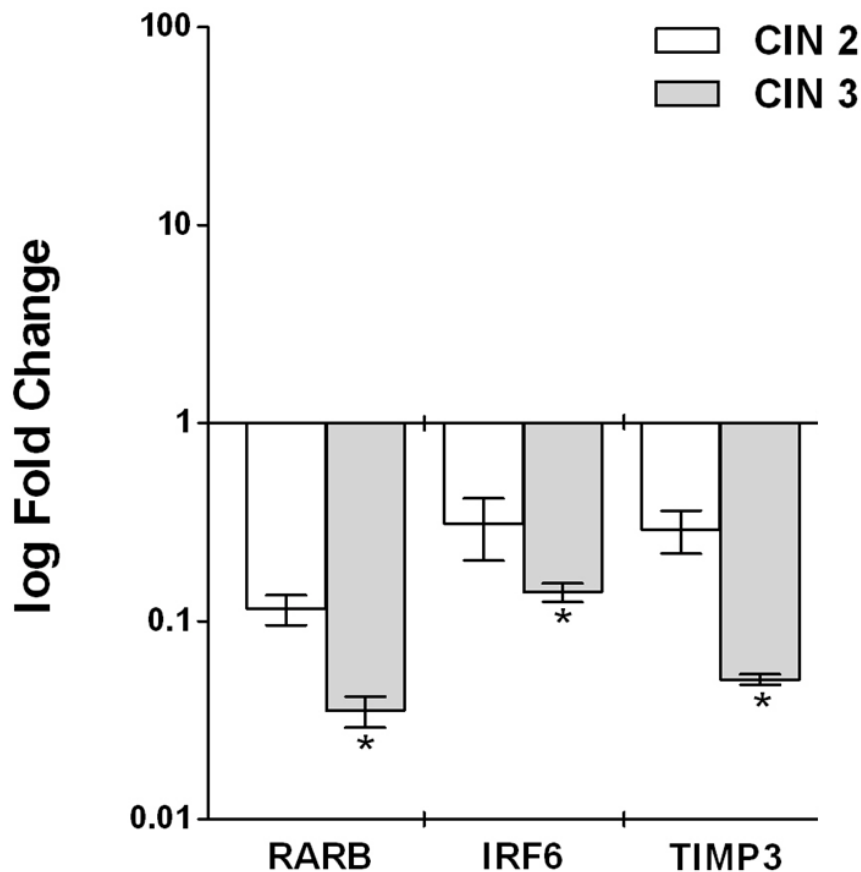


Figure 11. Differential expression of *RARB*, *IRF6*, *TIMP3* genes measured with real-time quantitative RT-PCR in specimens from CIN2 and CIN3 keratinocytes. Relative expression was calculated by use of the $\Delta\Delta C_t$ method. Data are expressed as a relative fold change ($2^{-\Delta\Delta C_t}$) over the value of normal specimens. *GAPDH* gene was used as internal control. * $P < 0.0001$ versus CIN2.

In contrast, the expression levels of *APOC1*, *MSX1* and *PHGDH* mRNA continuously increased both from normal to CIN2 keratinocytes and from CIN2 to CIN3 keratinocytes (Figure 12). Specifically, the *APOC1* gene was significantly up-regulated by 7.4-fold and 10-fold in CIN2 and CIN3 keratinocytes, respectively, compared to normal keratinocytes (Figure 12). Similarly, up-expression of *PHGDH* was also detected. This gene resulted up-regulated by 5.6-fold and 10.6-fold in CIN2 and CIN3 keratinocytes, respectively, compared to normal keratinocytes (Figure 12). *MSX1* gene resulted significantly up-regulated by 3.5-fold and 14-fold in CIN2 and CIN3 keratinocytes, respectively, compared to normal keratinocytes (Figure 12). Differences in *APOC1*, *PHGDH* and *MSX1* mRNA expression levels between CIN2 and CIN3 keratinocytes were

statistically significant ($p < 0.0001$). These findings indicate that the data obtained from the microarray analysis are reliable and that these 37 genes may be significant contributors in the progression of high-grade HPV16 CIN lesions.

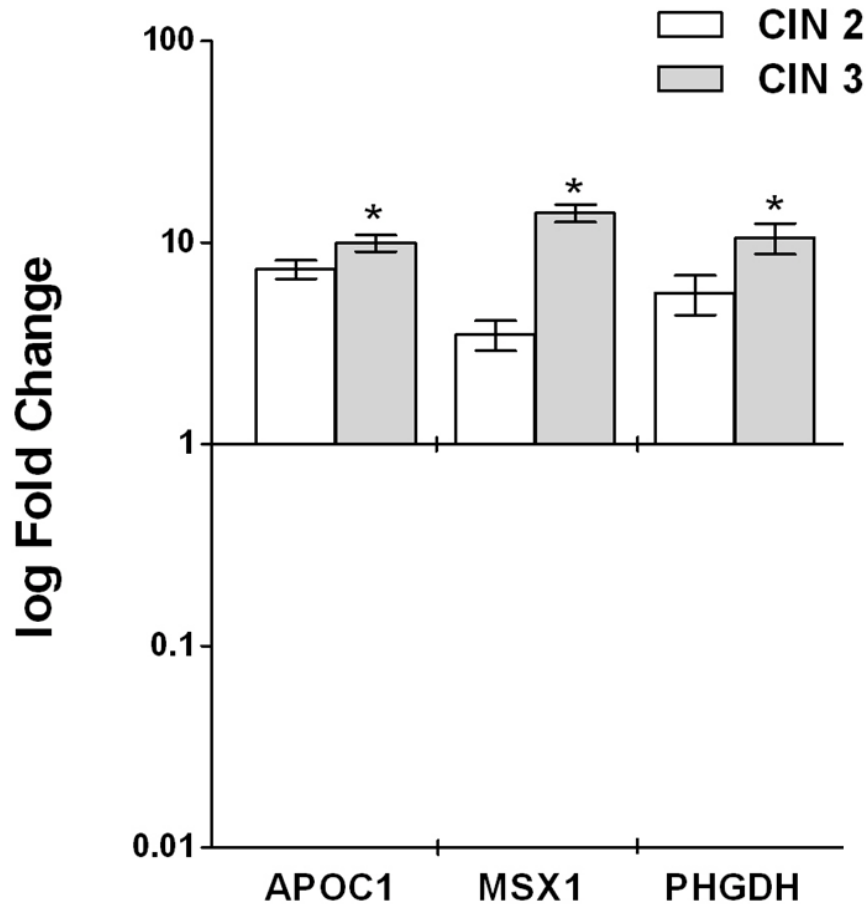


Figure 12. Differential expression of *APOC1*, *MSX1*, *PHGDH* genes measured with real-time quantitative RT-PCR in specimens from CIN2 and CIN3 keratinocytes. Relative expression was calculated by use of the $\Delta\Delta C_t$ method. Data are expressed as a relative fold change ($2^{-\Delta\Delta C_t}$) over the value of normal specimens. *GAPDH* gene was used as internal control. * $P < 0.0001$ versus CIN2.

IHC ANALYSES

The IHC staining of CIN and normal cervical tissue samples validated PHGDH protein expression. PHGDH staining was cytoplasmic. As shown in figure 13, in normal

tissues, PHGDH was weakly expressed and only in the proliferative compartment of the epithelium. In cervical cancer tissue, strong staining was present in all tissue sections. Strong staining of dysplastic cells was present in 9/10 CIN3, 7/10 CIN2 and 10/10 CIN1 lesions; one out of ten CIN3 and 3/10 CIN2 lesions showed moderate staining of dysplastic cells (data not shown). PHGDH staining extended to the superficial layers in CIN3 cases, to the mid layer in CIN2 lesions, and to the lower third of the epithelium in CIN1 lesions, corresponding to the extension of the dysplastic proliferative compartment in the different types of lesions. Nondysplastic cells in CIN cases were stained only weakly or not at all. The specificity of IHC staining for PHGDH protein in CINs and cervical cancers indicates that PHGDH is likely associated with tumorigenesis.

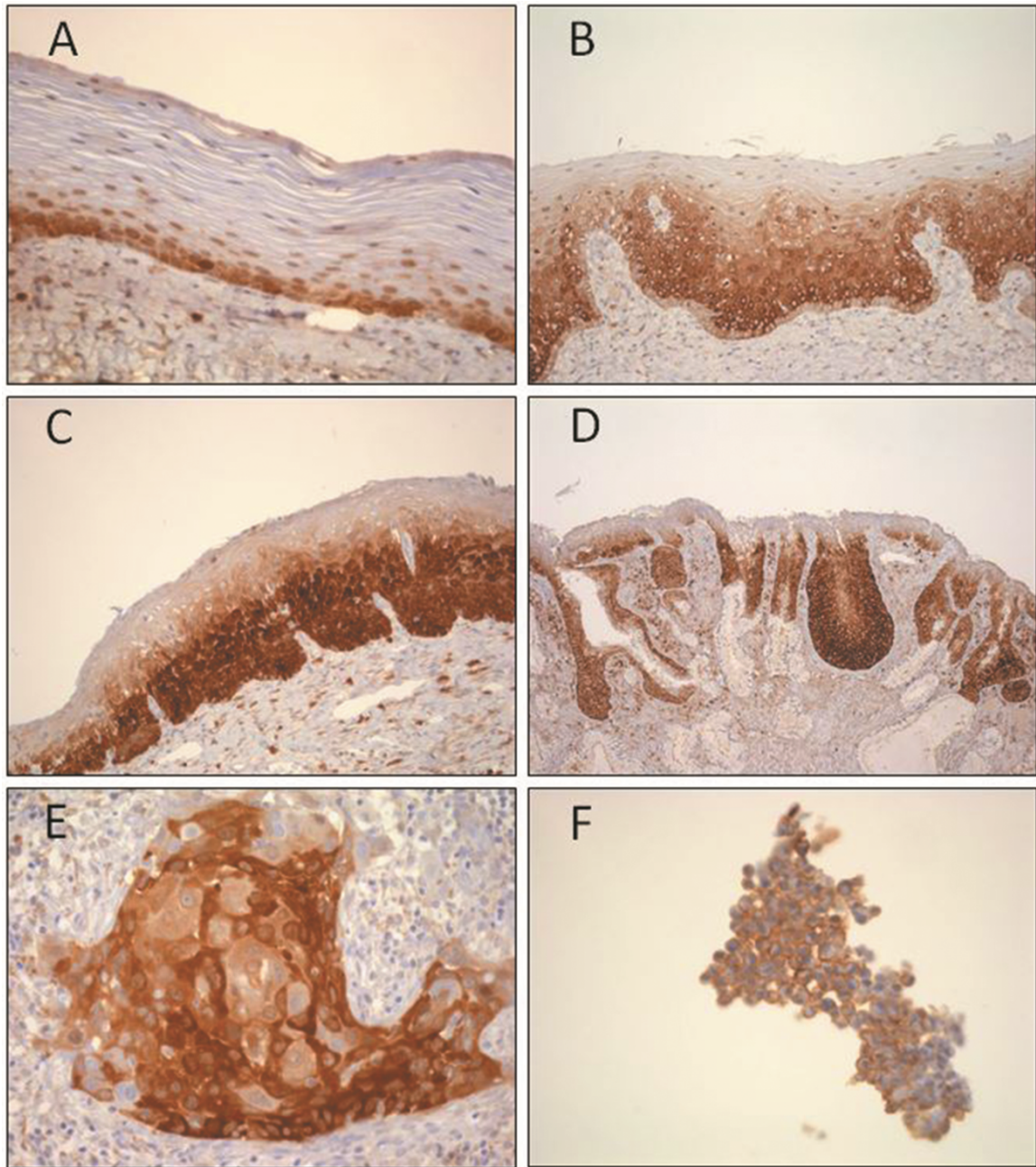


Figure 13. Immunohistochemical analysis of PHGDH in CIN, cervical cancer, and normal tissues. Immunohistochemical analysis with anti-PHGDH polyclonal antibody confirmed the elevated expression of this protein in dysplastic cells from CIN1 (B), CIN2 (C), CIN3 (D) lesions, in tumor cells from invasive carcinoma (E), and in HeLa cells (F) compared with the expression in proliferative cells from normal cervical tissue (A).

RARB, C-JUN, IRF6 AND P63 GENE EXPRESSION ANALYSIS

In order to explore the expression trend of RARB, c-Jun, IRF6 and p63 mRNAs, 10 CIN2, 10 CIN3, positive for HPV16, and 10 normal keratinocyte specimens was subjected to QRT-PCR analysis. As reported previously in this work (page 47), the mRNA expression level of RARB and IRF6, resulted significantly decreased from that of normal and CIN2 keratinocytes and from that of CIN2 and CIN3 keratinocytes (Figure 14, B) ($P < 0.0001$). Specifically, RARB was significantly down-regulated by 9.3-fold and by 35.8-fold in CIN2 and CIN3 keratinocytes, respectively, compared to normal keratinocytes (figure 3, B). Differences in RARB expression levels between CIN2 and CIN3 keratinocytes were statistically significant ($p > 0.0001$). In contrast, the amount of c-Jun mRNA was found to correlate with the degree of pre-neoplastic CIN lesion: the lowest in CIN2 (4.3-fold up-regulated compared to normal) and the highest in CIN3 (22 up-regulated compared to normal) (Figure 14, B) ($P < 0.0001$). Indeed, differences in c-Jun expression levels between CIN2 and CIN3 keratinocytes were statistically significant ($p > 0.0001$). As for RARB, IRF6 mRNA was Similarly down-regulated by 2.9-fold and 6.7-fold in CIN2 and CIN3 group, respectively, compared to normal keratinocytes (figure 15, B). Contrariwise, p63 was significantly high expressed by 3.6-fold and by 15-fold in CIN2 and CIN3 keratinocytes, respectively, compared to normal keratinocytes (figure 15, B). Differences in IRF6 and p63 expression levels between CIN2 and CIN3 keratinocytes were statistically significant ($p > 0.0001$).

RARB AND IRF6 PROMOTER METHYLATION ANALYSIS

The methylation status of the RARB and IRF6 loci was investigated by sequencing analysis of the cloned PCR products. A total of 120 clones were investigated for the DNA methylation status of RARB and IRF6 loci in bisulfite-treated DNA samples from CIN keratinocytes. RARB and IRF6 clones derived from two HPV16-CIN2, two HPV16-CIN3 and two normal RARB and IRF6 PCR products (10 from each sample) were randomly selected for DNA sequencing. The overall distribution of the different clones within CIN keratinocytes, within CIN2 and normal as well as within CIN3 and normal keratinocytes was evaluated. Only RARB or IRF6 hypermethylated clones i.e., clones showing 50% (or more than 50%) methylated CpG islands, were taken into account in this analysis. RARB

hypermethylation was detected in 9/11 (45%) of the clones from the CIN2 keratinocytes and in 16/20 (80%) of the clones from the CIN3 keratinocytes. Whereas, in normal keratinocytes, RARB hypermethylation was never detected. The difference in RARB hypermethylation between CIN2 and CIN3 keratinocytes was statistically significant ($P < 0.05$, Figure 14, A, Figure 15) as well as difference between CIN2 and normal keratinocytes ($p < 0.01$, Figure 14, A, Figure 15) and CIN3 and normal keratinocytes ($p < 0.0001$, Figure 14, A, Figure 15). IRF6 hypermethylation was detected in 6/20 (40%) of the clones from the CIN2 keratinocytes and in 11/20 (55%) of the clones from the CIN3 keratinocytes. Whereas, as for RARB, in normal keratinocytes, it was never detected (Figure 14, A, Figure 15). No significant differences in IRF6 hypermethylation frequencies were evaluated between CIN2 and CIN3 keratinocytes ($p > 0.05$, Figure 16, A, Figure 17). However, the difference between CIN2 and normal keratinocytes ($p < 0.05$, Figure 16, A, Figure 17) as well as CIN3 and normal keratinocytes ($p < 0.0001$, fig. 3. A) resulted statistically significant.

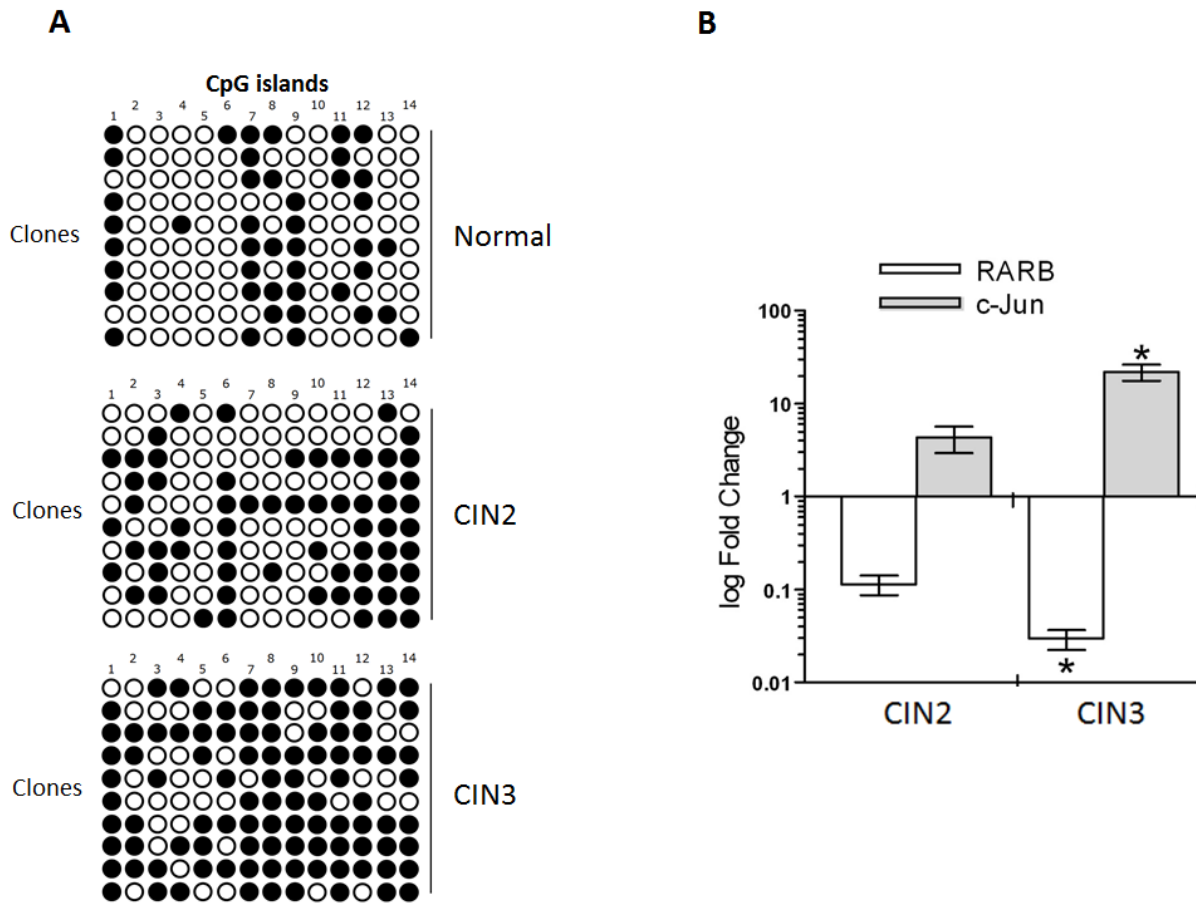


Figure 14. *RARB* promoter methylation pattern and *RARB* and *c-Jun* differential expression. (A) Bisulfite-PCR sequencing for (A) *RARB* gene promoter region in one representative Normal, CIN2 and CIN3 cultured keratinocytes. Filled-in and clear circles represent methylated and unmethylated CpG islands, respectively. The CpG islands within the *RARB* locus is numbered on the upper side of the circles. Each line represents one clone. For the analysis, were considered ten clones per sample. (B) *RARB* and *c-Jun* differential expression measured by real-time quantitative RT-PCR in specimens from CIN2 and CIN3 keratinocytes. Relative expression was calculated using the $\Delta\Delta C_t$ method. Data are expressed as a relative fold change ($2^{-\Delta\Delta C_t}$) over the value of normal specimens. * $P < 0.0001$ versus CIN2.

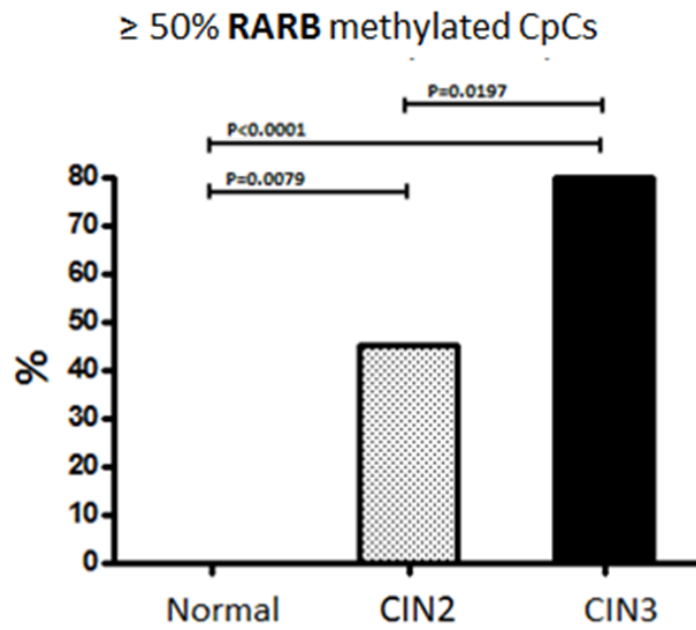


Figure 15. Frequency of *RARB* hypermethylation clones in normal, CIN2 and CIN3 keratinocytes. Only clones showing 50% or more than 50% methylated CpG islands were taken into account in this analysis

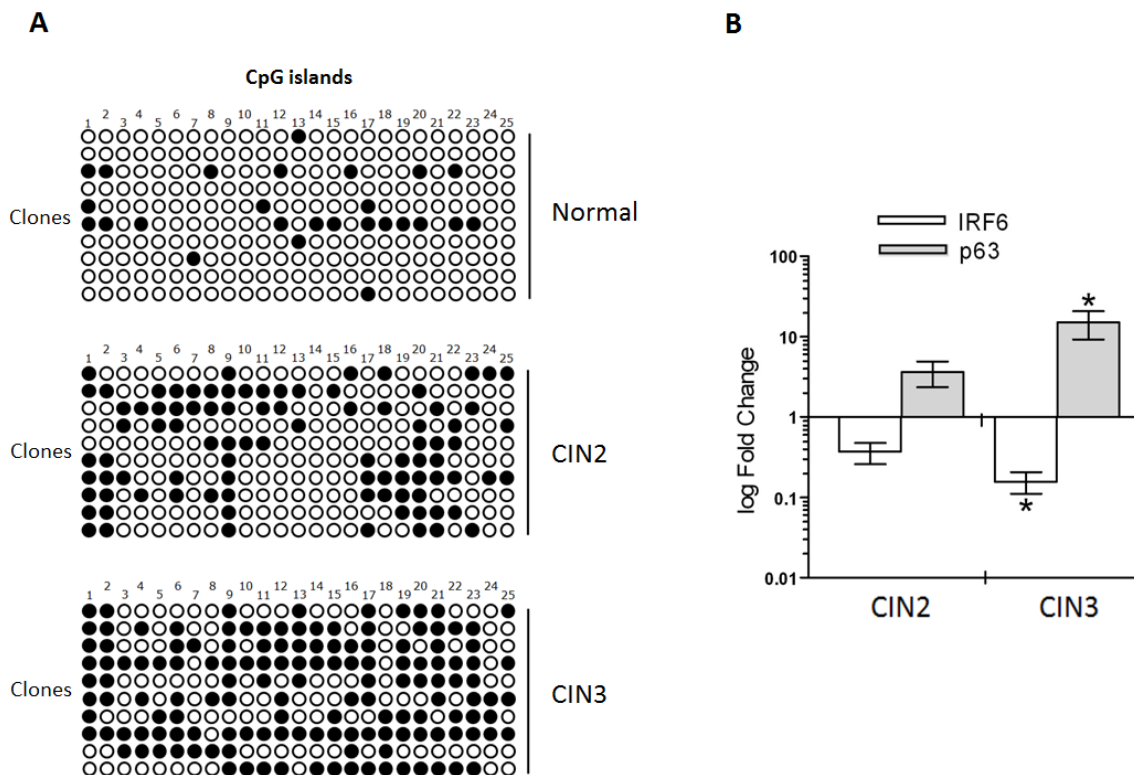


Figure 16. IRF6 promoter methylation pattern and IRF6 and p63 differential expression. (A) Bisulfite-PCR sequencing for (A) IRF6 gene promoter region in one representative Normal, CIN2 and CIN3 cultured keratinocytes. Filled-in and clear circles represent methylated and unmethylated CpG islands, respectively. The CpG islands within the IRF6 locus is numbered on the upper side of the circles. Each line represents one clone. For the analysis, we considered ten clones per sample. (B) IRF6 and p63 differential expression measured by real-time quantitative RT-PCR in specimens from CIN2 and CIN3 keratinocytes. Relative expression was calculated using the $\Delta\Delta C_t$ method. Data are expressed as a relative fold change ($2^{-\Delta\Delta C_t}$) over the value of normal specimens. * $P < 0.0001$ versus CIN2.

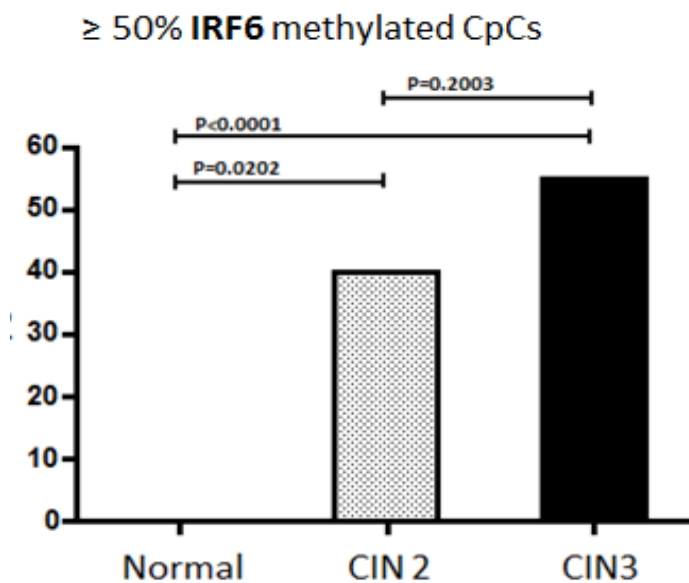


Figure 17. Frequency of IRF6 hypermethylation clones in normal, CIN2 and CIN3 keratinocytes. Only clones showing 50% or more than 50% methylated CpG islands were taken into account in this analysis

DISCUSSION

CIN AND NORMAL KERATINOCYTES CULTURES

Recognition of the role of Human Papilloma Virus (HPV) as the etiologic agent in cervical cancer has focused attention on the mechanisms inducing transformation in cervical keratinocytes (Zur Hausen, 1996; Togtema *et al.*, 2012; Lindel *et al.*, 2012; Herfs *et al.*, 2012; Kaczkowski *et al.*, 2012; Tan *et al.*, 2012; Malinowski, 2005). Due to difficulties in propagating HPV in culture (Angeletti, 2005; Pyeon *et al.*, 2005. McLaughli and Meyers ,2005). Molecular characterization of naturally infected-HPV keratinocytes derived from cervical preneoplastic lesions is a promising cell study model for elucidating HPV-induced cancerogenesis in the human cervix. The HPV transformation process occurs

in multiphase steps which are clinically diagnosed as cervical intraepithelial neoplasia (CIN) -1, 2 and 3 (Mitchell *et al.*, 1996; Schiffman *et al.*, 2007). Two published methods have been previously described for culturing keratinocytes derived from CIN1-3 lesions as well as from normal cervical tissues (Freshney and Freshney, 2002; Green, *et al.*, 1977). With one method, keratinocytes are grown in co-culture with mouse fibroblasts which are inactivated with mitomycin C or gamma rays (feeder cells) and submerged in culture medium (Freshney and Freshney, 2002). The alternative approach regards the employment of defined keratinocyte-specific medium which allows keratinocyte expansion without the use of fibroblast feeder layers (Green, *et al.*, 1977). However, there are some limitations to culturing CIN and normal cervical keratinocytes by these methods. Firstly, a large number of pure CIN or normal keratinocytes, i.e. free from cervical fibroblasts, is needed to start primary cultures. Whilst an adequate number of normal cervical keratinocytes can be isolated from cervical epithelium obtained from hysterectomy specimens (Narisawa-Saito and Kiyono, 2007) CIN tissue specimens are usually very small and do not allow a sufficient number of pure keratinocytes to be isolated for culturing purposes. Secondly, the procedure for isolating pure keratinocytes requires several steps, which increase the risk of cell culture contamination.

Therefore, the development of a rapid and easy method of enabling cultures of CIN and normal cervical keratinocytes to be grown using small amounts of starting material was considered useful. The search for a new culturing method began with the observation that seeding the total number of cells isolated from a complete enzymatic digestion of CIN or normal cervical tissues, a typical range of morphologically differing keratinocyte colonies, based on their cell content and general morphology, could be obtained. Additional studies from our laboratories demonstrated that by sufficiently diluting cells during primary propagation, CIN and normal keratinocyte colonies develop clonally. Furthermore, different CIN and normal keratinocyte primary clonal colonies consisted of small, compact and uniform size cells, indicating that cultured keratinocytes were endowed with high proliferative potential (Barrandon and Green, 1985, 1987). On the basis of these observations, a cell culturing method which employs small CIN and normal cervical tissue fragments to derive primary CIN and normal keratinocyte clonal colonies capable of further expansion was set up.

Although two different methods for CIN and normal cervical keratinocyte cultures have been reported, a very few studies regarding cellular and molecular characterization of naturally infected HPV CIN1-3 or normal cervical keratinocytes, have been published to

date. These studies have been carried out in W12 and CIN 612 cells, two naturally HPV16-infected keratinocyte lines derived from CIN1 lesions (Rader *et al.*, 1990; Bedell *et al.*, 1991; Gray *et al.*, 2010; Dall *et al.*, 2008). The reason for lacking of researchers on CIN keratinocytes is that previous culturing methods work well when large pieces of CIN or normal cervical tissues are employed, but these are rarely obtained from gynaecological surgery. By using murine fibroblasts as feeder layers, the number of CIN or normal keratinocytes seeded must maintain a 1:2 density ratio with the murine fibroblasts co-cultured in vitro (for an example, $1 \times 10^4/\text{cm}^2$: $2 \times 10^4/\text{cm}^2$) (Rader *et al.*, 1990; Freshney and Freshney, 2002). Alternatively, the number of CIN or normal keratinocytes seeded must be at a density of $2 \times 10^5/\text{cm}^2$ (Barrandon and Green, 1987), using the specific keratinocyte defined medium without murine fibroblasts. The main procedure for isolating pure keratinocytes includes dermal sheet elimination, by dispase digestion or tissue scraping, and two consecutive digestions of cervical epithelial tissues by trypsin-EDTA. Due to the small size of CIN tissues, CIN keratinocytes are also isolated following migration from micro-dissected CIN explants. However, the major problem in this latter procedure is that CIN keratinocytes are rescued at a low number and are frequently highly contaminated by cervical fibroblasts, which do not allow primary cultures to be started successfully.

The protocol described in this work is very simple compared to previous CIN and normal tissue culture methods. CIN and normal cervical keratinocyte primary colonies can be obtained in a single technical passage, i.e. after tissue digestion. Consequently, the time required is highly reduced. Moreover, our culture method enables CIN and normal keratinocytes to be grown at a clonal rate by seeding the cells at a high dilution during primary propagation. This is an important aspect since CIN lesions are clonally heterogeneous, due to the multiphase steps of HPV-induced carcinogenesis; therefore, clonal CIN keratinocyte cultures allow different CIN keratinocyte clones of the preneoplastic specimen to be faithfully reproduced in vitro. Finally, primary CIN and normal keratinocyte clones can be expanded at highly proliferative rates over different passages with no sign of differentiation. When taken altogether, these results may provide important findings on the mechanisms induced by HPV during onset/progression of cervical neoplasia. CIN2, CIN3 and normal cervical primary culture preparation provides the onset of a series of problems due mainly to the presence of endogenous microbiological contaminants, bacteria and fungi, which are naturally present in the cervical region. The contamination of primary cultures is relevant due to this aspect. Notably, the higher the cervical preneoplastic grade, the higher the endogen microbial contamination and therefore

the risk of culture contamination. CIN2 and CIN3 tissues give the best primary culture results as the higher the preneoplastic grade of keratinocytes, the higher the ability to attach and proliferate clonally. On the contrary, keratinocytes from normal tissues attach slowly and differentiate rapidly and much fewer primary colonies can be obtained than with high grade CIN2-CIN3 tissues. Nevertheless, the successful rate of normal primary keratinocyte cultures is approximately 5-6 out of 10 and nearly 10 out of 10 of CIN2-CIN3 primary keratinocyte cultures. This protocol is based on a cellular culture method, which was used in a recent publication of my lab (Bononi *et al.*, 2012) and employs the total number of cells, i.e. fibroblasts and keratinocytes, isolated from small tissue fragments of CIN or normal cervical tissue samples. The isolation of all the cells overcomes the issues arising from isolating pure populations of keratinocytes including low cell numbers and exogenous microbial contamination, and avoids keratinocyte loss from starting tissues. Although the presence of live fibroblasts is considered detrimental to keratinocyte cultures, due to overgrowth being caused (Freshney and Freshney 2002), this problem is avoided by seeding the all cells at a very high dilution, i.e. in large culture surfaces. In this way, primary keratinocyte colonies can be obtained before fibroblasts colonize the cultures. On the other hand, high cell dilution allows keratinocytes to reach optimal density for clonal growth (Barrandon and Green, 1985, 1987). Furthermore, were formulated a new medium mixture which permits CIN and normal keratinocyte colonies to be expanded singly at a highly proliferative rate for different passages without signs of differentiation. Immunofluorescence staining showed that CIN and normal keratinocyte primary colonies, as well as expanded colonies, expressed K14, K17, and K19 indicating their common origin from basal and parabasal layers of the cervical epithelia (Smedts *et al.*, 1990, Martens *et al.*, 2004; Akgül *et al.*, 2007). P63 stained strongly, with nuclear localization, in CIN3 keratinocytes suggesting that those keratinocytes present stemness characteristics. Indeed, p63 has previously been detected in stem cells from CIN and normal ectocervical epithelia (Quade *et al.*, 2001; Martens *et al.*, 2004). Therefore, naturally HPV infected keratinocyte clones have the potential to provide valuable insights into the mechanisms induced by HPV during cervical cancerogenesis. Since cultured cells represent a useful model for investigating the onset/progression of the carcinogenesis processes (Goldstein *et al.*, 2011; Karst and Drapkin, 2012) and cellular and molecular studies on naturally HPV-infected keratinocytes have been poorly reported, our protocol may provide opportunities for exploring mechanisms which occur during CIN transition, as well as gene expression

changes in progression toward cervical cancer in vitro, which to date is far from having been elucidated (Woodman *et al.*, 2007; Galloway, 2009)

GENE EXPRESSION CHANGES AND CIN PROGRESSION

In this study, gene expression changes were investigated, for the first time, in naturally HPV16-infected CIN2 and CIN3 keratinocytes by microarray analysis. Since the majority of high-grade HPV16-CIN lesions are the result of 2-dimensional intra-mucosal extension of a single monoclonal cell population infected by the oncogenic HPV (Ueda *et al.*, 2003), two CIN keratinocyte colonies, representative of all neoplastic keratinocytes comprising the CIN lesion, were chosen from each CIN tissue culture for the analysis of microarray. Moreover, in order to avoid the risk of a selection bias inherent in any long term in vitro growth, were collected CIN and normal keratinocyte colonies from primary cultures. Were used DNA microarray technology in order to compare changes of gene expression, which characterize two key stages of progression of HPV16-cervical lesions in vivo. It was encouraging that the hierarchical clustering analysis of the data neatly grouped CIN keratinocytes together according to their status: CIN2 or CIN3. Moreover, the finding that our dataset contained many expected gene expression changes (such as those involving cell proliferation and differentiation) affirmed validity of the dataset. Therefore, the results obtained in CIN2 and CIN3 keratinocytes by microarray analysis likely parallel the molecular changes that underlie progression to high-grade HPV16 CIN. Notably, the number of genes down-regulated in CIN3 keratinocytes compared with the number in CIN2 keratinocytes (n=211) was almost double that in normal keratinocytes compared with CIN2 keratinocytes (n=113). In contrast, 175 genes were increased significantly in CIN2 compared with normal keratinocytes, and 94 genes were increased in CIN3 compared with CIN2 keratinocytes. These findings suggest that the majority of genes down-regulated and up-regulated during the progression of cervical neoplasia affect the transition from CIN2 to CIN3 lesions and from normal to CIN2 lesions, respectively.

Twenty-three under-expressed and 14 over-expressed genes, which may play an important role in CIN progression, were selected. These genes are associated with HPV-mediated transformation, including the down-regulation of antiviral immune response-inducible genes and up-regulation of proliferation-related genes. Indeed, INHBB, TLR2, TIMP3, RARB, IFIT2 and EFNB2 were consistently under-expressed, whereas APOC1 and PHGDH were consistently overexpressed in CIN2 and CIN3 keratinocytes. Notably, most of the down-regulated genes, such as INHBB, TLR2, TIMP3 and RARB, are the target of promoter methylation in various tumours (De Oliveira *et al.*, 2011; Gu *et al.*, 2008; Karpinski *et al.*, 2011; Wang *et al.*, 2005), including HPV-related cancers (Stephen *et al.*, 2010), high-grade CIN lesions (Terra *et al.*, 2007), and HPV cervical cancers (Sun *et al.*, 2011), suggesting an important role of these genes in tumour development. Therefore, it is possible that the consistent decrease in mRNA expression in CIN2 and CIN3 keratinocytes accounts for increasing CpG-island methylations at the gene promoter level. In line with this, HPV16 can induce cellular gene promoter hypermethylation (Leonard *et al.*, 2012). These findings led me to explore, in the next step of my investigation, the putative link between gene expression, at mRNA levels, and gene promoter methylation status in high-grade CIN keratinocytes. A major feature of CIN2 and CIN3 keratinocytes is the consistent overexpression of genes that play a role in tumour invasiveness. Indeed, consistent up-regulation of LPAR1, a pro-angiogenesis gene (Hayashi *et al.*, 2012), and ARNTL2 (Mazzoccoli *et al.*, 2012), MSX1 (Chetcuti *et al.*, 2011), and TAGLN (Rho *et al.*, 2009), which are pro-invasive genes, was present in progression from CIN2 to CIN3 keratinocytes. These findings are in agreement with results of a recent microarray study in which transition to CIN2 stage coincided with the activation of pro-angiogenesis pathways, whereas the transition to CIN3 and then to invasive cancer was characterized by a pro-invasive gene expression (Gius *et al.*, 2007). Therefore, these up-regulated genes detected in CIN2 and CIN3 keratinocytes likely are associated with cervical cancer and may be prognostic markers for CIN progression. An interesting characteristic of CIN2 and CIN3 keratinocytes is the disruption of cell-differentiation programs. Indeed, a series of differentiation-induced genes, such as ALDH1A3 (Muzio *et al.*, 2012), IRX2 (Lewis *et al.*, 1999), IRF6 (Botti *et al.*, 2011), SYN1 (De Souza Martins *et al.*, 2011), and ATP2A3 (Korosec *et al.*, 2009), were down-regulated in late-stage CIN keratinocytes. On the other hand, stemness-related genes, such as KLF2 (Gillich *et al.*, 2012), were increased in the transition of CIN keratinocytes from CIN2 to CIN3. These data are in accordance with the oncogenic activities of HPV16 E6-

E7 viral oncoproteins that dysregulate the cell cycle through differentiation program disruption (Rosty *et al.*, 2005). Finally, two up-regulated genes, SCARA5 and SFRP2 change in opposite directions in HPV-related tumors or other cancers (Huang *et al.*, 2010). Further investigations are needed to clarify the role of these genes in CIN progression. I focused on one of the up-regulated genes, PHGDH, which encodes an enzyme that controls the flux from glycolysis into the serine biosynthesis pathway, although a novel non-metabolic role of PHGDH also has been reported. Various studies have found increasing PHGDH expression in human astrocytomas according to increasing tumour grade (Liu *et al.*, 2013), and PHGDH recently was found overexpressed in cervical cancer (Locasale *et al.*, 2011). These observations prompted me to investigate PHGDH expression in CINs and invasive cancer in order to explore its contribution to cervical carcinogenesis. Expression of PHGDH mRNA was continuously increased during conversion from normal to CIN2 keratinocytes and from CIN2 to CIN3 keratinocytes. Consistently, a strong staining of PHGDH was observed in dysplastic cells from almost all CIN lesions and in tumour cells from all cervical cancer tissues, whereas expression was less and only in the proliferative compartment of the epithelium from normal tissues, a finding which suggests that the protein expression of this gene is enhanced according to the degree of malignant transformation. In conclusion, this microarray study revealed 37 down-expressed or over-expressed genes which may contribute to progression of CIN. mRNA expression of one of the up-regulated genes, PHGDH, was significantly greater in CIN2 keratinocytes than in normal keratinocytes and in CIN3 keratinocytes than in CIN2 keratinocytes. In addition, protein expression of PHGDH increased from CIN1 to cancer according to the degree of malignant transformation. Thus, PHGDH likely plays an important role in the initiation and progression of cervical tumorigenesis and may be a prognostic marker for progression of CIN to invasive cancer.

GENES PROMOTER METHYLATION AND CIN PROGRESSION

Together with genetics changes, epigenetic alteration, such as improper DNA promoter methylation, have an important role in cancer onset and progression. Indeed, the CpGs methylation status of some gene promoter regions is a significant prognostic variable

for various tumor types, including cervical SCC (Van Noesel *et al.*, 2002; Shivapurkar *et al.*, 2004). Particularly, aberrant promoter methylation play a important role in downregulation of tumor suppressor genes in SCC. This phenomenon could lead to cell transformation or cervical SCC formation. As previously reported, a certain number of tumor suppressor genes such as p16 (Nakashima *et al.*, 1999), CCNA1 (Yang *et al.*, 2010) PTEN (Cheung *et al.*, 2004), E-cadherin (Widschwendter *et al.*, 2004) and APC (Virmani *et al.*, 2001) present improper hypermethylation in cervical carcinogenesis. Furthermore, p16 hypermethylation was detected progressively higher in CIN pre-cancerous lesions progression (Huang *et al.*, 2011). Using DNA cloning and bisulfite sequencing, it was investigated the epigenetic status of RARB and IRF6 promoter region in neoplastic progression of CIN pre-cancerous lesions. These genes have been suggested to be implicated as actual or potential tumour suppressor genes, and are aberrantly methylations of their promoters were detected -in various different cancers (Piperi *et al.*, 2010; Botti *et al.*, 2011). The RARB gene encodes for retinoic acid receptor beta, a member of the thyroid-steroid hormone receptor superfamily of nuclear transcriptional regulators, which binds the retinoic acid. Interaction of retinoic acid with RARB receptor induces cell growth and differentiation as well as embryonic morphogenesis (Soprano *et al.*, 2004). RARB improper methylation was detected in several tumors, like breast (Park *et al.*, 2012), prostate (Serenaite *et al.*, 2015) and ovarian cancer (Flanagan *et al.*, 2013) and oropharyngeal SCC (Van Kempen *et al.*, 2014). Moreover, methylation defects in RARB promoter region were detected in CIN3 biopsies (Vasiljević *et al.*, 2014) and in primary human foreskin keratinocytes transfected with episomal form of HPV16 (Leonard *et al.*, 2013).

This study shows, for the first time, that an extensive methylation defects, i.e. hypermethylation, occur at the RARB tumor suppressor gene promoter in naturally HPV16-infected CIN2 and CIN3 keratinocytes. Similar data were obtained data were showed in HPV16-positive exfoliated cervical CIN2 and CIN3 samples (Chang *et al.*, 2011), and in cervical SCC samples (Narayan *et al.*, 2003). In the present study, hypermethylation of RARB gene promoter was significantly higher in CIN23 keratinocytes compared to CIN23 keratinocytes ($p < 0.05$). Therefore Furthermore, these results indicate that methylation defects at the RARB locus frequently occur in CIN keratinocytes, suggesting that increased methylation of the gene promoter is predictive of with CIN2 progression towards >CIN3 progression. It can be hypothesized that the presence of HPV16 could be involved in this improper promoter methylation, as previously reported

(Leonard *et al.*, 2012). In my study, in CIN2 and CIN3 keratinocytes ~~were~~ found a progressive down-regulation of RARB and a progressive up-regulation of the proto-oncogene c-Jun ~~transcript, respectively~~ ($p < 0.0001$). This negative correlation between RARB and c-JUN was previously reported in Cervical Carcinoma HeLa Cells (De-Castro *et al.*, 2004). It is conceivable that the progressive RARB hypermethylation may cause a consequent RARB mRNA down-regulation and this reduction of expression could enhance c-JUN expression (De-Castro *et al.*, 2004). As previously mentioned, c-JUN expression is increased in SV40 immortalized human epidermal keratinocytes, transfected with HPV-16 E5 coding sequence (Chen *et al.*, 1996). Moreover, it is known that HPV-16 E5 suppresses the expression of tumor suppressor gene p21 by c-JUN activation inducing human keratinocytes immortalization. (Tsao *et al.*, 1996). This might be one of the mechanisms by which HPV-16 stimulates cell proliferation by c-Jun activation and p21 inactivation. It is conceivable that the same molecular effect could occur in naturally HPV-16-positive CIN keratinocytes used in my study.

As for RARB, this study shows that extensive methylation defects, i.e. hypermethylation, occur also at the IRF6 gene promoter in HPV16-positive CIN2 and CIN3 keratinocytes. This gene encodes for a member of the interferon regulatory transcription factor (IRF) family. The encoded IRF6 protein regulates craniofacial development and epidermal proliferation ~~and may be a transcriptional activator.~~ ReFurthermore, recently findings suggest ~~have suppose~~ that IRF6 exhibits tumor suppressor activity ~~IRF6~~ (Botti *et al.*, 2011). Despite the epigenetics data of the present study show extensive DNA hypermethylation in IRF6 gene promoter region in CIN keratinocytes compared to normal, this trend was not in progression between CIN2 and CIN3 keratinocytes. However, it is appreciable a methylation increase, even though not statistically significant ($p > 0.05$), between these two groups. These epigenetics data are consistent with a work conducted by Botti and colleagues, where IRF6 gene promoter region was aberrantly methylated in primary SCCs cultures and SCC cell lines (Botti *et al.*, 2011). Furthermore, in the present study was observed a reduced IRF6 expression in the same cell cultures in association with hypermethylation of whose its IRF6 promoter region is hypermethylated. Accordingly, in this work, ~~In my work, was detected a similar~~ IRF6 expression was detected to be reduced ~~trend~~ in HPV16-positive CIN2 and CIN3 keratinocytes ~~were IRF6 promoter resulted hypermethylated.~~ Specifically, was evaluated Indeed, it was observed a progressively down-regulation of IRF6 transcript from normal to CIN2 as well as from CIN2 to CIN3 ($p < 0.0001$) in HPV16-positive

keratinocytes ~~with progressive CIN2 to CIN3 IRF6 promoter region hypermethylation.~~ Therefore, it is conceivable that the hypermethylation of IRF6 promoter region may cause a consequent IRF6 transcript reduction in CIN keratinocytes. It is known that IRF6 is involved in squamous differentiation (Biggs *et al.*, 2012). This biological effect is limited by p63, a protein present in squamous epithelia that promotes renewal of basal keratinocytes (Okuyama *et al.*, 2007). Furthermore, IRF6 is a p63 transcriptional target that limits keratinocyte proliferation by inducing p63 proteasome-mediated degradation (Moretti *et al.*, 2010). In the present work ~~an increased~~ of p63 expression was detected ~~an increased p63 expression~~ in HPV16-positive CIN2 and CIN3 keratinocytes compared to normal. This expression was ~~confirmed~~ evaluated also by immunofluorescence, where p63 resulted highly expressed in CIN3 keratinocytes with nuclear localization. ~~Moreover~~ Furthermore, p63 transcript up-regulation resulted in progression from CIN2 to CIN3 and in progressive negative correlation with IRF6 expression ($p < 0.0001$). It is conceivable that the deregulation of this molecular loop, altering the critical balance between differentiation and proliferation during CIN progression, as previously reported in Neck Squamous Cell carcinoma (Nicolas Stransky *et al.*, 2011), ectodermal dysplasias (Moretti *et al.*, 2010) and cleft palate (Thomason *et al.*, 2010). These findings led us to hypothesize that RARB and IRF6 gene promoter hypermethylation could be a potential prognostic epigenetic marker for HPV16-positive CIN progression. Such changes might therefore be used as markers of cervical neoplasia, either alone or in conjunction with other molecular prognostic markers. Furthermore, RARB, c-Jun, IRF6 and p63 deregulation may contribute to progression of CIN pre-cancerous lesions.

CONCLUSIONS

In conclusion, in my study the molecular mechanisms occurring in CIN lesions progression were investigated. In particular, it was investigated gene expression profiles and methylation status of gene promoters in a novel study model, i.e. primary colonies of CIN2 and CIN3 keratinocytes derived from HPV16-CIN2 and CIN3 lesions. Gene expression analysis revealed 37 down-expressed or over-expressed genes which may contribute to CIN progression. One of these genes, the phosphoglycerate dehydrogenase, which resulted over-expressed at both mRNA and protein level in CIN2 and CIN3

keratinocytes and in CIN2, CIN3 and cancer tissues, respectively, is likely to be associated with tumorigenesis and may be a potential prognostic marker for CIN progression. Aberrant promoter hypermethylation of RARB and IRF6 genes also may be a potential epigenetic prognostic marker for CIN progression.

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During my doctoral period, conducted at the laboratories of Professors Mauro Tognon and Fernanda Martini, in addition to the work presented in this thesis, I have published or contributed significantly to the following publications:

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