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Evaluation of p53, p16^{INK4a} and E-Cadherin Status as Biomarkers for Cervical Cancer Diagnosis

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1. Introduction

Worldwide, carcinoma of the uterine cervix is one of the most common malignancies among women. Incidence rates of this disease vary from about 5 cases per 100 000 women per year in many industrialized countries to more than 50 per 100 000 in some developing nations. Approximately 80% of all cases occur in less-developed countries, because prevention programs are either non-existent or poorly conducted (WHO, 2009). Furthermore, in the less developed areas of the world, cervical cancer begins to strike significantly among women as young as 25-30 years of age, clearly identifying this disease as the cancer priority in women.

Clinical epidemiology have clearly identified that the association between infection with high-risk types of human papillomavirus (HPV) and high-grade cervical cancer precursors as well as cervical cancer is very strong (Wright Jr., 2006). However, even high-risk HPV infection are widespread in the world, the majority of HPV-associated lesions such as cervical intraepithelial neoplasia (CIN) will remain stable or spontaneously regress over time (zur Hausen, 2000; Ferenczy, 2001; Holowaty, 1999; Syrjanen, 1996), suggesting that other genetic and epigenetic events are likely to be involved in cervical carcinogenesis. Indeed, genomic alterations leading to tumor suppressor gene inactivation and/or oncogene activation are the critical pathways in the development and progression of cervical cancer as well as the other types of cancer. In this field, p53, p16 and E-cadherin are important proteins that play critical role in the development and the progression of cervical cancer.

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2. Human papillomavirus and cervical cancer

Association between HPV and cervical lesions and cancer has started in 1970s' years after the hypothesis that cervical cancer may arise from infections with the virus found in condylomata acuminata (zur Hausen, 1975; 1976). Then epidemiological and clinical studies have clearly demonstrated that HPV are the major etiologic agents of neoplasia of the cutaneous and mucosal epithelia; HPV positivity in cervical cancer is estimated to be between 90% and 95% (zur Hausen, 1991; Munoz, 2003). Currently, there is compelling evidence to indicate that the development of human cervical cancer without involvement of the specific HPV is exceptional.

Up to now, more than 200 HPV genotypes were recenssed, but the interest is focused only on 30 types that are closely associated to cervical lesions. Among them, 15 HPV types have been classified as high risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82); three have been classified as probable high risk types (26, 53, and 66); and 12 have been classified as low risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) (Munoz, 2003). Among the high risk HPV genotypes, HPV-16 is the most common in squamous cell carcinoma of the cervix (50–60%), followed by HPV-18, which is present in about 11–15% of cervical cancer cases (Munoz, 2003, Saranath, 2002).

HPV genomes code for at least six different early and two late proteins. The structure of the genome and the characteristic properties of individual viral proteins have been well reviewed (zur Hausen, 2000; Stanley, 2010; Moody & Laimins, 2010). High-risk HPVs code for at least three proteins with growthstimulating and transforming properties (E5, E6, and E7).

In the pathogenesis of cervical carcinoma we can identify three major factors. Two of them are related to the HPV presence, the effects of viral E6 and E7 proteins, and the consequences of HPV DNA integration in the cellular genome. The third factor is the accumulation of cellular genetic damage, not related to HPV, needed for tumour development (Lazo, 1999).

Viral DNA integrated into host genome is found in all cases of cervical carcinoma (Bosch, 1995), their metastasis and derivative cell lines (Cullen, 1991).

HPV DNA integration into host chromatin is usually a necessary event in the pathogenesis of HPV-related cervical cancer. It is one of the key stages in malignant progression and is therefore a potential biomarker that precedes invasive disease.

Many studies have demonstrated that the integrated HPV DNA is linearized between the E1 and L1 genes. Upon viral integration, variable parts of the HPV genome are disrupted; fragments containing E2 and E4 ORFs are missing whereas the entire E1, E6 and E7 ORFs are integrated and retained (Raybould, 2011).

HPV viral integration is made in such way that the viral regulatory region and the E6 and E7 genes are expressed from viral promoters, but with a different regulation, in which cellular factors might play an important role (Lazo, 1999; Raybould, 2011). In the normal HPV life-cycle expression of E5, E6 and E7 is tightly regulated within cells that are destined to be lost from surface epithelial layers, such that they do not pose a carcinogenic threat (Raybould, 2011)

E5 expression enhances oncogenic potential (Stoppler, 1996; Maufort, 2010) but the exact function of E5 remains poorly understood. E6 and E7 expression is essential for maintenance of the transformed state and malignant progression (Bosch, 1990; von Knebel Doeberitz, 1988).

The implication of E6 and E7 proteins in cervical cancer progression is mainly due through their interactions with hTERT, p53 and Retinoblastoma protein (pRB). hTERT is a catalytic subunit of Telomerase that acts to synthesise telomere ends of linear chromosomes during DNA replication. p53 is a transcription factor that regulates cell cycle arrest, apoptosis, senescence, DNA repair and cell metabolism; p53 activity is inhibited by ubiquitin ligase which also ubiquitinates p53 to initiate p53 degradation (Figure 1). In addition to inducing the rapid degradation of p53, E6 also binds to and degrades FADD, preventing the transmission of apoptotic signals via the Fas pathway (Filippova, 2004).

pRB is a tumour suppressor protein and interacts with transcription factor E2F to repress the transcription of genes required for the S phase of the cell cycle (Figure 1). E7 can also bind to other connected proteins such as p107 and p130 (Raybould, 2011).

Currently vaccination based strategy is an alternative method showed to be amore effective and practical approach than the implementation of regular and periodic cytological screening. Today it is very well established that intervention with vaccines permits already the statement that essential precursor lesions of this cancer are efficiently prevented (Zur Hausen, 2009).

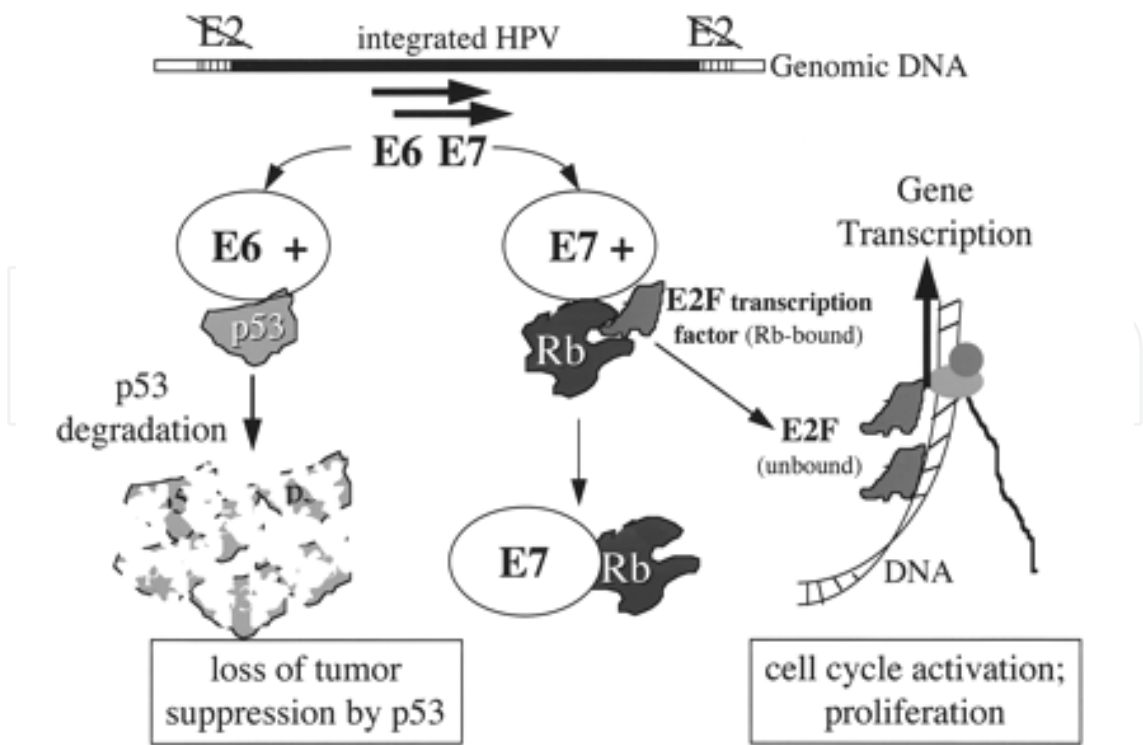


Fig. 1. Schematic representation of E6 and E7 activities in cervical cells

3. p53 expression and polymorphism in cervical cancer

The human tumour suppressor gene p53 plays a key role in the cell's response to genotoxic stress and loss of this 'guardian of the genome' is an important step in carcinogenesis. The highly significant tumour suppressor gene, p53, is implicated in a wide range of human cancers, and is a multifunctional protein that plays critical roles in cellular responses to DNA damage, cellular senescence and apoptosis to maintain genomic stability of a cell (Kashima, 2007). p53 encodes a transcription factor at the centre of a network that maintains cellular integrity by the inhibition of cell growth and stimulation of apoptosis in response to cellular stresses such as DNA damage (Scheffner, 1990).

Because mutation of the p53 gene is a relatively rare event in cervical cancer, p53 activity is mainly inhibited by the viral oncoprotein E6. It's clearly identified that abrogation of p53 function by the E6 protein of HPV is thought to be one of the major events in cervical carcinogenesis (Soussi, 2001). The viral E6 protein interacts with protein p53 and inhibits its activity, followed by proteolytic degradation through the ubiquitin pathway (Scheffner, 1990; Werness, 1990).

As shown if Figure 2, expression of p53 on cervical cancer biopsies showed that the p53 immunoreactivity is detected especially in nuclei. The expression is greater in the peripheral cells of tumours.

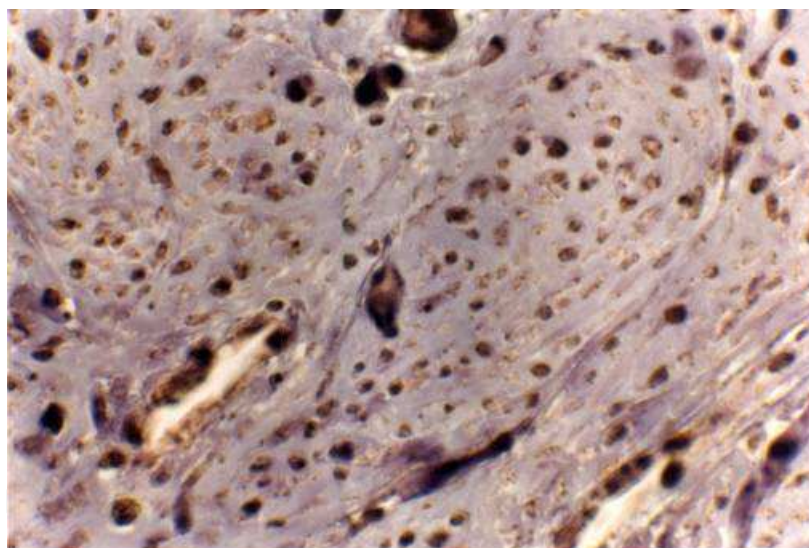


Fig. 2. Representative p53 immunohistochemical staining in epidermoid carcinoma

Different studies showed that p53 expression did not correlate with tumour recurrence demonstrating that immunohistochemistry for p53 protein appears to provide no prognostic information for all patients with cervical cancer (Abd El All, 1999; Vasilescu, 2009; Abrahao, 2011). However, it still remains a prognostic factor for the aggressive behavior of the tumour, when it exceeds more than 30% positivity in tumour cells nuclei (Vasilescu, 2009). Many studies, using different p53 monoclonal antibodies, have reported the lack of any association between p53 IHC expression and staging (Abd El All, 1999). Indeed, in HPV positive cervical carcinoma, the wild type p53 complex with E6 of HPV 16 or 18, is degraded and cannot be detected by IHC. In other cancers, Overexpression of p53 in tissues has generally been assumed to reflect accumulation of p53 mutations.

Several polymorphisms have been identified within the p53 gene, both in non-coding and coding regions and may represent an important contribution to cancer susceptibility and tumour behaviour (Costa, 2008). The common polymorphism is known at codon 72, with two alleles encoding either arginine (p53 Arg) or proline (p53 Pro) (Matlashewski, 1987). The genotype of p53 gene at codon 72 is detected by PCR with allele specific primers “ASP” that especially detects either the p53Pro or p53Arg allele. DNA is amplified in separate reactions with p53 Pro and p53 Arg primers. Example of resulting amplifications is reported in Figure 3.

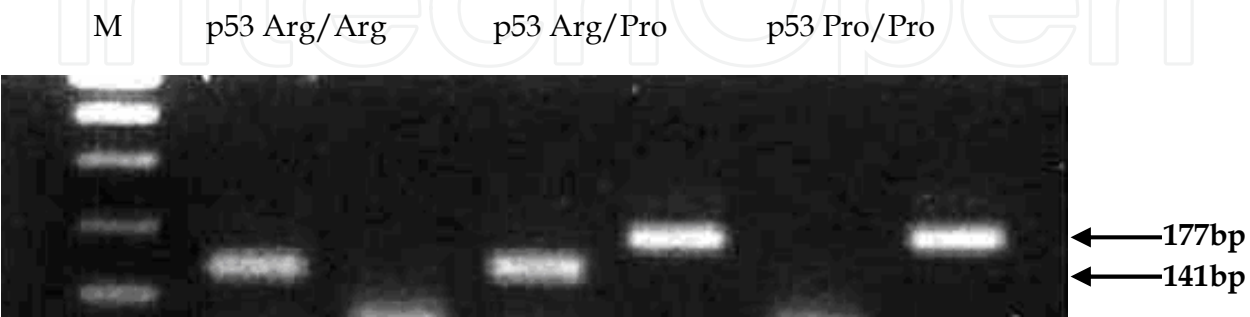


Fig. 3. Analysis of p53 codon 72 polymorphism by PCR using allele specific primers Arg allele specific primers: 141 bp and Pro allele specific primers: 177 bp. M: 100 bp ladder.

The Pro/Pro, Pro/Arg, and Arg/Arg frequencies have been reported in human cancers including lung, colorectal, breast, stomach, bladder, head and neck and oral, for their association with predisposition and subsequent increased susceptibility to the cancer. These studies have shown that the presence of Arg/Arg genotype has been associated with increased susceptibility to cervical cancer and Pro/Pro genotype is more frequent in lung cancers (Storey, 1998; Hamel, 2000; Tandle, 2001).

This polymorphism occurs in the proline-rich domain of the p53 protein, which is necessary for the protein to fully induce apoptosis (Zhu, 2007). The functional difference between the 2 alleles of this polymorphism is that the Arg/Arg genotype induces apoptosis with faster kinetics and suppresses transformation more efficiently than the Pro/Pro genotype (Kuroda, 2007). In cervical cancer, different studies have investigated the effect of the codon 72 polymorphism of p53 on the susceptibility to E6-mediated degradation. They reported that individuals homozygous for p53 Arg are more susceptible to HPV-associated carcinogenesis of the cervix than heterozygotes (Storey, 1998). However the relationship between the p53 polymorphism and susceptibility of HPV infection as well as cervical cancer development is still unclear.

Storey *et al.* (1998) showed that the codon 72 arginine variant of p53 encodes a protein that is more sensitive to HPV16 and HPV18 degradation than the proline variant. The biological and biochemical differences between the two p53 genotypes at codon 72 were demonstrated by a study showing that the arginine form of the protein was much more susceptible to HPV E6 mediated degradation than the proline form (Mitra, 2005; Oliveira, 2008). Moreover, Thomas *et al.* (1999), presented evidence that, *in vitro*, the p53 arginine variant induces apoptosis with faster kinetics and suppresses transformation more efficiently than the p53 proline variant. These observations may have implications for the development of cancer in subjects harbouring p53 modified sequences and for the responsiveness of tumours to therapy.

The case-control study conducted in 113 cancerous lesions and 100 healthy women from Morocco highlighted the absence of any association between p53 polymorphism at codon 72 and cervical cancer development (Meftah El Khair, 2009). However reported data on the prevalence of p53 polymorphism in cervical cancer patients are controversial and the ethnic group characteristics seem to be an important reason for discrepancies in the frequency of this polymorphism (Brenna, 2004; Wang, 1999; Wu, 2004; Pegoraro, 2002; Klug, 2001; Szarka, 2000; Agorastos, 2000; Hildesheim, 1998; Bhattacharya, 2002). Moreover, other potential confounding factors should be also considered including the sample size, the source of DNA and the detection techniques used. Another important reason for these discrepant results could be misclassification of the p53 polymorphism, due to inter-laboratory variations in protocols, affecting the ability to detect p53 polymorphisms (Brenna, 2004; Govan, 2007; Sousa, 2007).

4. Epigenetic alteration and cervical cancer

Cancer is a multi-factor process. Molecular analysis of tumours reveals genetic and epigenetic abnormalities. Genetic mutations, which alter DNA sequence, lead to constitute activation of some oncogenes (as RAS and RAF genes) and inhibition of some tumour suppressors (as p53 gene). Epigenetic mutations (epimutations) lead often to gene silencing without altering the DNA sequence. Alteration in expression of key genes through aberrant epigenetic regulation can lead to initiation, promotion and maintenance of carcinogenesis, and is even implicated in the generation of drug resistance. The significance of epigenetic alterations is used as predictive biomarkers and as new targets of anticancer therapy.

Genetic information may not be the only relevant source of information in order to understand the molecular basis of disease. Epigenetic information may hold the key to a better understanding of various pathological conditions (Chahwan, 2011).

Epigenetic mechanisms are essential for normal development and maintenance of tissue-specific gene expression patterns in mammals. These include embryonic development, transcription, chromatin structure, X-chromosome inactivation, genomic imprinting, and chromosome stability (Watanabe, 2010). Epigenetic dysregulation, which may be passed from one generation to the next, are believed to be implicated in the promotion of tumorigenesis in cancers through the downregulation of tumour repressor genes (Hidemi, 2011). Epigenetic aberrations, often observed in tumours, include changes in DNA methylation and histone modifications that influence the chromatin states and impact gene expression patterns. Methylation of cytosine bases in DNA is the main epigenetic event and is involved in most cellular physiopathological processes (Watanabe, 2010).

The CpG islands, where Cytosine and Guanine are connected by a phosphodiester bond, are short stretches of DNA in which the frequency of the CG sequence is higher than other regions. CpG islands are mostly located in the upstream promoter and exon 1 region of over half of human genes (Lo, 2008). In mammals, the methylation process is achieved by the DNA methyltransferase (DNMT), that catalyses the transfer of the methyl group from S-adenosyl L-methionine (SAM) to the cytosine in 5'-CG-3' sequence.

In cancer, abnormal hypermethylation of gene promoter CpG islands induces transcriptional silencing of tumour suppressor genes (Lo, 2008) and is by far the best-categorised epigenetic change.

Inactivation of tumour suppressor genes by promoter hypermethylation has been recognized to be at least as common as gene disruption by mutation in tumorigenesis (Jones, 2002).

Multiple genes are hypermethylated in primary carcinomas as well as in carcinoma cell line and the methylation profile has strong associations with genetic and clinicopathological features (Lind, 2004).

HPV regulates the methylation status of genes involved in the cell cycle regulation, apoptosis, DNA repair, cell adhesion and migration, development and differentiation, cellular signalling and metabolism (Anita Szalmás, 2009).

The HPV oncoprotein E7 control cellular proliferation pathways through epigenetic mechanisms. Recently, it has been shown that E7 bind directly to the DNMT1 and activate its DNA methyltransferase activity. This direct association may lead to aberrant methylation of the genome followed by cellular transformation as a result of tumour suppressor gene silencing (Burgers, 2007). E7 can induce viral replication also through epigenetic changes. E7 inhibits HDAC (histone deacetylase) binding to the E2F promoter resulting in activation of expression and facilitates HPV replication (Longworth, 2005).

HPV can epigenetically regulate cell cycle via down regulation of p16^{INK4A}, an inhibitor of cyclin dependent phosphorylation and inactivation of Rb (retinoblastoma) tumour suppressor protein. The occurrence of p16^{INK4A} promoter hypermethylation is very low in low grade of cervical cancer and it increases moderately with the severity of the carcinogenetic stages (Wong, 1999; Gustafson, 2004).

Cyclin A1 (CCNA1) is involved in cell cycle regulation and in repair of DNA doublestrand breaks. Kitkumthorn *et al.* (2006) have evaluated the epigenetic status of cyclin A1 in HPV-associated cervical cancer. The authors demonstrated that cyclin A1 methylation is common in cervical cancer and is specific to the invasive phenotype indicating that hypermethylation of promoter of cyclin A1 is a potential tumour marker for early diagnosis of invasive cervical cancer (Kitkumthorn, 2006).

E-cadherin is a transmembrane glycoprotein that mediates interactions between adjacent epithelial cells. Hypermethylation of CpG islands in E-cadherin promoter regions is associated with suppressed transcriptional activity and it is particularly found in various in invasive cervical cancers (Kang S 2006).

5. Study of gene promoter methylation

For gene-specific methylation analysis, a large number of techniques have been developed. Most early studies used methylation sensitive restriction enzymes to digest DNA followed by Southern detection or PCR amplification. Recently, bisulfite reaction based methods have become very popular. In these techniques, analysis of DNA methylation is based on bisulfite treatment of genomic DNA, which converts cytosine to uracil, but methylated cytosines remain unaltered in this process. Several techniques have been applied to analyse bisulfite-modified DNA, with variation in sensitivity, amount of DNA needed. However, PCR based techniques are the fast, simple and reliable methods to assess the methylation status of DNA. After PCR amplification, uracil will be converted to thymidine, which will be determined by direct PCR sequencing (bisulfite sequencing) or methylation specific PCR

(MSP-PCR) (Figure 4). In bisulfite sequencing, primers are designed not to contain any CpGs to avoid discrimination against methylated or unmethylated DNA. In MSP-PCR, two pairs of primers are designed, one of which is specific for methylated DNA (M) and the other for unmethylated DNA (U).

After pyrosequencing or MSP-PCR, amplified fragments are usually cloned to determine the degree of methylation (Herman, 1996).

Additionally, in order to identify unknown methylation hot-spots or methylated CpG islands in the genome, several of genome-wide screen methods have been invented such as Restriction Landmark Genomic Scanning for Methylation (RLGS-M), and CpG island microarray.

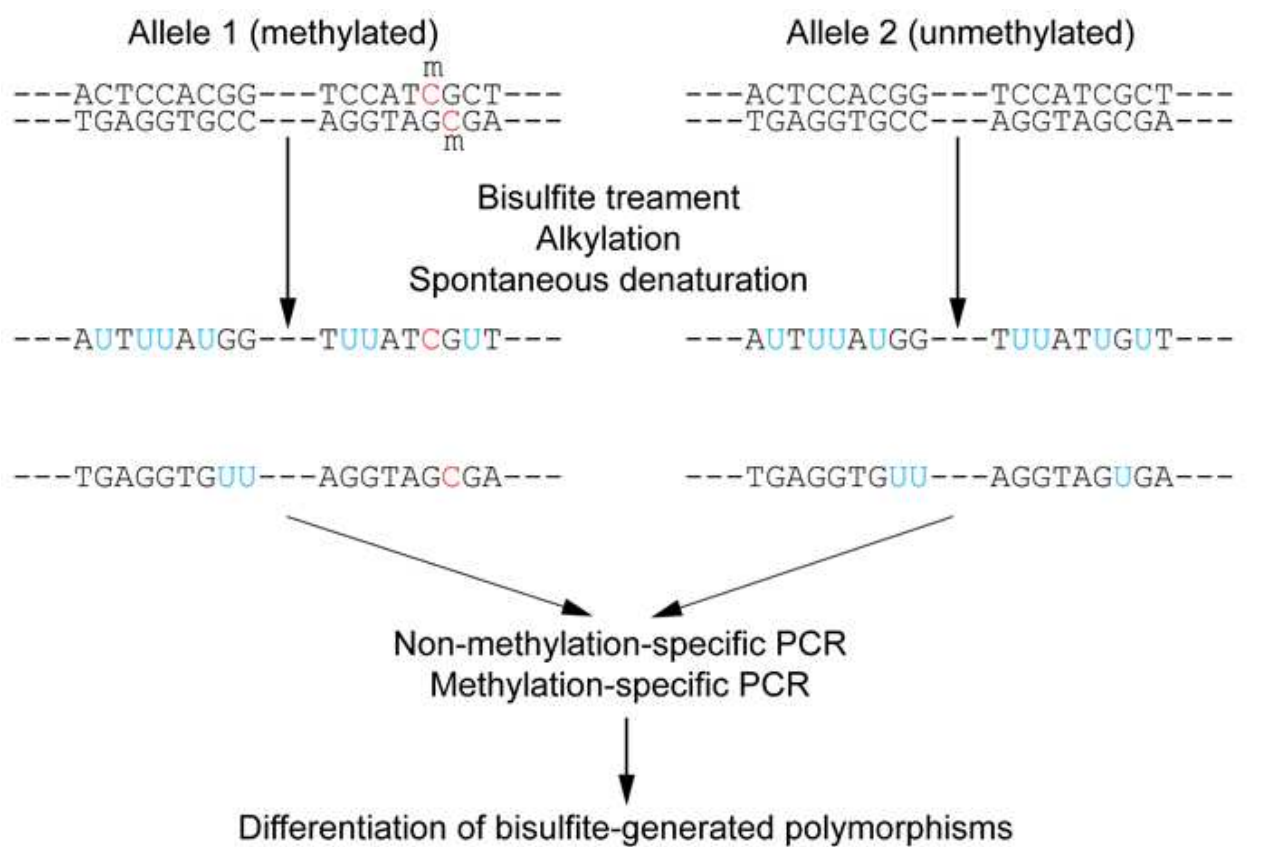


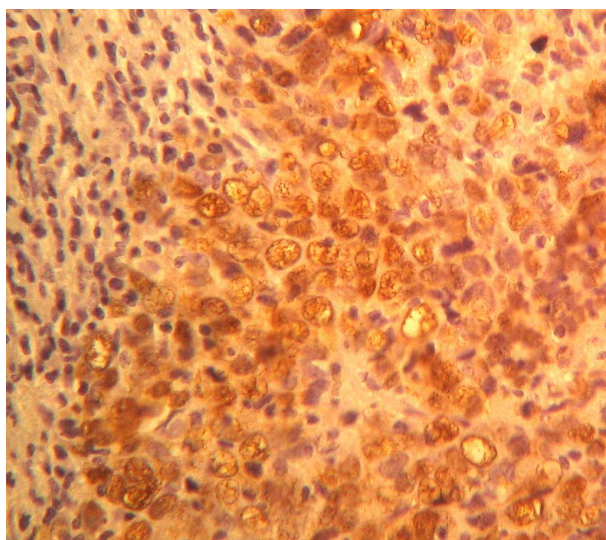
Fig. 4. Schematic representation of bisulfite based modifications

6. p16^{INK4a} expression in cervical cancer

The cellular tumour suppressor protein p16 has a central function in the regulation of cell cycle activation. The p16^{INK4a}, the product of *CDKN2A* gene is a negative regulatory protein that regulates the progression of eukaryotic cells through the G1 phase of the cell cycle (Serrano, 1997). p16^{INK4a} is a component of p16^{INK4a}-Cdk4-6/CyclinD-pRb signalling pathway and is perturbed in many cancers. In these tumours, the functions of p16^{INK4a} may be lost due to mutations or suppression of its transcription by promoter methylation (Gonzalgo, 1998). In high risk-HPV positive cervical cancer, the oncogene E7 disrupts pRb/E2F interaction, releases active E2F and induces the pRb degradation (Liu et al, 2006). The

existence of the regulatory feedback in the pRb/p16 pathway leads to an overexpression of p16^{INK4a} in cervical tumours (Ivanova, 2007). Klaes *et al.* (2001) have shown that overexpression of p16^{INK4a} is a specific marker for dysplastic and neoplastic epithelial cells in the cervix. They have clearly demonstrated that use of p16^{INK4a} immunostaining allows precise identification of cervical lesions and significantly reduce false-negative and -positive interpretation in cervical cancer screening. For these reasons, p16^{INK4a} expression is usually used in cervical neoplasia diagnosis (Klaes, 2001).

Many studies have analyzed the presence of p16^{INK4a} in cervical neoplasia and have found a relationship between p16^{INK4a} expression and cervical neoplasia; raising hope that p16^{INK4a} could represent a specific and sensitive marker for cervical neoplasia (Klaes, 2001; Milde-Langosch, 2001; Riethdorf, 2004). A representative p16^{INK4a} immunohistochemical staining in epidermoid carcinoma is given in Figure 5. It is generally believed that p16^{INK4a} functions as Cdk-inhibitor in the nucleus. Klaes *et al.* have showed that 58 of 60 invasive cervical carcinomas expressed p16^{INK4a} both in nuclei and cytoplasm (Klaes, 2001). These findings were corroborated with published data from Moroccan cases. Indeed, p16^{INK4a} staining by IHC in 53 cervical cancer biopsies from Morocco showed that 92.4% had high level of p16^{INK4a} expression with a predominance of both nuclear and cytoplasmic staining (El Hamdani, 2010).



(Intense Nuclear staining of p16^{INK4a})

Fig. 5. Representative p16 immunohistochemical staining in epidermoid carcinoma sample

On the other hand, Ivanova *et al.* (2007) have clearly demonstrated that in normal cells p16^{INK4a} localizes mainly in nuclei, the loss of p16^{INK4a} nuclear staining in favour of cytoplasmic staining have been observed earlier in different tumours including cervical carcinomas and cervical cancer cell lines.

Loss of p16^{INK4a} protein expression, leading to overcome cell cycle arrest at senescence and immortalization, could be studied by evaluating the methylation status of its promoter (Ivanova, 2007).

Indeed, hypermethylation of the promoter region of a tumour suppressor gene has been increasingly recognized as an alternative mechanism for inactivation of function of a tumour

suppressor gene. In this topic, hypermethylation of the *p16* gene has been suggested to be a shared epigenetic alteration in multiple human cancers, including cervical cancer (Esteller, 2001).

An example of MSP analysis of the promoter regions of *p16*^{INK4a} after bisulfite treatment is given in Figure 6. Using MSP and/or bisulfite sequencing, hypermethylation of *p16*^{INK4a} gene was observed in 19 to 61% of invasive cervical carcinoma (Ivanova, 2007; Nehls, 2008; Attaleb, 2009). However, hypermethylation of *p16*^{INK4a} promoter region is absent in DNA specimens from normal cervical swabs as well as cervical cell lines such as SiHa, HeLa, C33A and Caski (Attaleb, 2009).

Moreover, the increased risk for disease progression was independent from clinical and pathological factors, suggesting that *p16*^{INK4a} gene promoter methylation is an early event in cervical cancer development.

Moreover, in HPV induced cervical cancer, the cell cycle activation is not mediated by Cdks but by E7-related Rb disruption. The *p16* inactivation would not confer any further growth promoting effect, because in this cancer the HR-HPV oncogene E7 induces a permanent release of E2F from its binding to pRb, leading to continuous cell cycle activation (Nehls, 2008).

Thus, hypermethylation of *p16*^{INK4a} promoter gene may be a result of genetic and epigenetic events produced during the carcinogenesis steps of cervical cancer development, and when occurs, it did not affect the regulation of *p16*^{INK4a} expression. Moreover, the high *p16*^{INK4a} immunoreactivity with partial promoter hypermethylation needs to be further investigated.

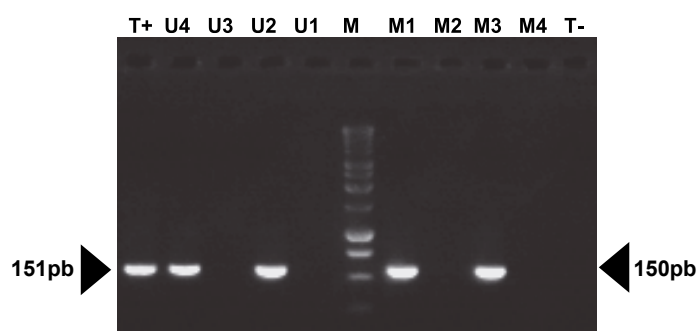


Fig. 6. MSP analysis of the promoter regions of *p16*^{INK4a}

The presence of a visible PCR product in lane U indicates the presence of unmethylated genes; the presence of a PCR product in lane M indicates the presence of methylated genes. Normal lymphocytes DNA (T) was used as a negative control for methylation. Cases 1 and 3 were methylated at *p16*^{INK4a}. M: 100 bp ladder.

7. Expression of E-cadherin in cervical cancer

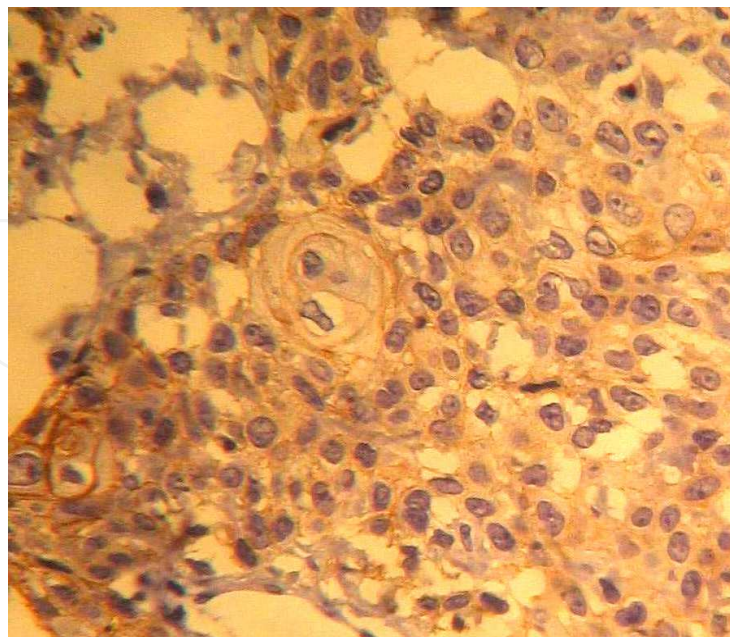
Cadherins are a family of cell-cell adhesion molecules which can modulate epithelial phenotype and morphogenesis in a variety of tissues. E-cadherin is the major cadherin expressed on the surface of normal epithelial cells and plays a pivotal role in maintenance of normal adhesion in epithelial cells but has also been shown to suppress tumour invasion and participate in cell signalling (Chen, 2003; Virmani, 2001; Ziober, 2001). Cell adhesion is mediated through Ca^{2+} -dependent homotypic binding. This transmembrane glycoprotein is encoded by *CDH1* gene.

Based on its biological functions, E-cadherin is regarded as an invasion and metastasis suppressor. Loss of E-cadherin expression or function correlates with increased invasiveness and metastasis in carcinomas of several anatomical sites (Chen, 2003; Virmani, 2001). E-cadherin-mediated cell adhesion system is inactivated by multiple mechanisms. It may be inactivated as a result of genetic alteration, reduced gene expression, changes of other cadherin-catenin complexes or posttranslational modification of the protein leading to cytoplasmic delocalization (Widschwendter, 2004; Oki, 2007).

The expression of E-cadherin is impaired as squamous intraepithelial lesions progress to squamous cervical carcinoma (Laird, 2003). In cervical cancer, the presence and localisation of cytoplasmic E-cadherin were significantly correlated with CIN grade. In invasive types, the expression of E-cadherin was significantly reduced (Hirohashi, 1998) and this is mainly due to gene silencing by methylation processes (Nehls, 2008).

E-cadherin expression, as well as p16^{INK4a} expression, is usually used in cervical neoplasia diagnosis. In squamous cervical epithelium, E-cadherin is predominantly found at the cell-to-cell borders in the basal and parabasal cell layers (Laird, 2003). However, E-cadherin expression is reduced during tumour progression and metastasis, and associated with poor prognosis in a variety of cancers (Karayiannakis, 1998; Sulzer, 1998; Zheng, 1999). A representative E-cadherin immunohistochemical staining in epidermoid carcinoma is given in Figure 7.

Reported data showed that E-cadherin is moderately expressed in about 85% of informative cases with a main localisation at the cell membrane and cytoplasm (El Hamdani, 2010). Decrease or loss of E-cadherin expression is a common feature of many human epithelial cancers, including cervical cancer, although a decreased expression of this molecule has been described in metastasis, but not primary tumours (Carico, 2001).



Moderate membranous staining of E-cadherin

Fig. 7. Representative E-cadherin immunohistochemical staining in epidermoid carcinoma sample

It's widely accepted that hypermethylation plays a critical role in gene silencing. Promoter hypermethylation has been proposed as an explanation for the decrease of *E-cadherin* expression (Chen, 2003, Graff, 2000) and was even suggested as a potential marker for identifying cervical cancer patients at high risk for relapse (Widschwendter, 2004).

Thus, methylation status of E-cadherin promoter has been studied to understand the implication of this gene silencing in cervical cancer development. Methylation status was mainly studied using MSP analysis, as shown in Figure 8. Reported data showed that less than 50% of cervical cancer cases exhibited E-cadherin promoter hypermethylation at their CpG islands (Chen, 2003, Dong, 2001; Narayan, 2003; Attaleb, 2009). Moreover, E-cadherin promoter was also hypermethylated in 3 cervical cell lines (HeLa, SiHa and C33A) (Attaleb, 2009). Thus, partial methylation of the E-cadherin gene promoter leads to down-regulate the gene expression.

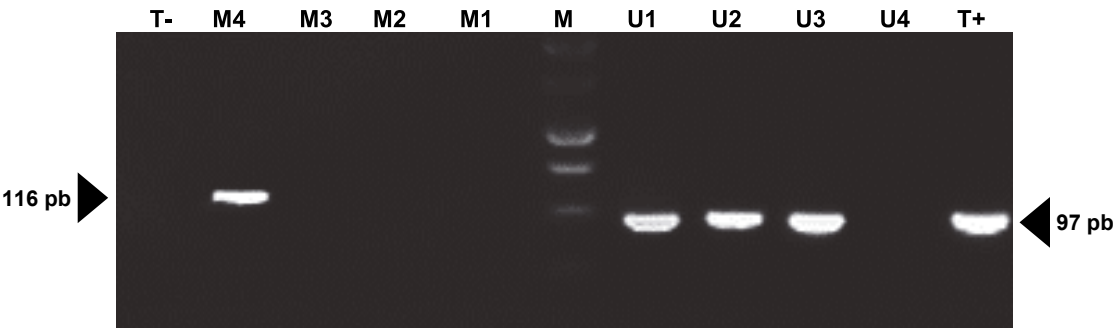


Fig. 8. MSP analysis of the promoter regions of p16^{INK4a}
The presence of a visible PCR product in lane U indicates the presence of unmethylated genes; the presence of a PCR product in lane M indicates the presence of methylated genes. Normal lymphocytes DNA (T) was used as a negative control for methylation. Case 4 was unmethylated at E-cadherin. M: 100 bp ladder.

8. Epigenetic based therapy

In tumours, epigenetic silencing of genes involved in DNA damage response pathways, such as cell cycle control, apoptosis signalling and DNA repair, has the potential to influence drug resistance and clinical outcome following therapy (Teodoridis, 2004).

Promoter hypermethylation and histone hypoacetylation contribute to this transcriptional inactivation. It is possible to reverse silencing using small molecule inhibitors. Such compounds, that can reverse this epigenetic inactivation, show anti tumour activity and can increase the sensitivity of drug resistant preclinical tumour models (Teodoridis, 2004). Hypomethylating and hyperacetylating drugs, HDAC (histone deacetylase) and DNMT inhibitors, can reverse epigenetic silencing and improve the cancer therapy (Hidemi, 2011).

The targeting of epigenetic pathways is an attractive therapeutic strategy and current clinical trials aim to improve efficacy of DNA hypomethylating drugs for e.g. by combination with standard chemotherapy. Key components in the regulation of DNA

methylation are DNA methyltransferases (DNMT1, 2, 3A and 3B) and methyl CpG-binding proteins, which recognize methyl cytosine residues and recruit transcriptional repressor complexes, including histone deacetylases (HDAC). Because of the interdependence of epigenetic processes, combinations of these approaches may have maximum clinical efficacy (Ferguson, 2011).

Epigenetic therapy leads to gene reactivation in primary tumours of cervical cancer patients. A number of these reactivated genes have a definitive role as tumour suppressors (De la Cruz-Hernández, 2011).

Hydralazine, a demethylating agent, was administrated in different doses to cohorts of previously untreated patients with histological diagnosis of cervical cancer in a phase I study. Hydralazine at doses between 50 and 150 mg/day is well tolerated and effective to demethylate and reactivate the expression of tumour suppressor genes without affecting global DNA methylation (Zambrano, 2005).

Valproic acid, an HDAC inhibitor, exerted a growth inhibitory effect on cervical cancer cell line: HeLa, SiHa and CaSki (De la Cruz-Hernandez, 2007; Chen, 2006). These drugs led to an increase of p53 transcription, and increase its stabilisation due to acetylation at lysines 273 and 282, protecting it from degradation by E6 (CruzHernandez, 2007). Valproic acid impede Akt1 and Akt2 expression, which leads to Akt deactivation and apoptotic cell death mediated through the caspase dependent pathway (Chen, 2006).

The combined antineoplastic effect of the DNA methylation inhibitor hydralazine and the histone deacetylase inhibitor valproic acid leads to increase in the cytotoxicity of cisplatin, adriamycin or gemcitabine in human cervical cancer cell lines (ChavezBlanco, 2006).

Also, epigenetic profiling using DNA methylation and histone analysis, can provide useful information for translational purposes, with a special emphasis on the potential use of DNA methylation marks for early disease detection and prognosis (Park, 2011) and for effective treatment strategies (Watanabe, 2010).

9. Conclusion

Identification of relevant biomarkers for early and specific diagnosis as well as the identification of promising therapeutic targets for molecular targeted therapy is a key role to improve cancer management worldwide.

In cervical cancer, even the importance of combined cytology and HPV testing, the use of epigenetic alterations as biomarkers will be of a great interest to enhance cervical diagnosis. Since alterations of the cellular epigenome usually precede morphologic changes and genetic alterations, identification of related aberrant DNA methylation profiles according to specific anatomopathologic status may serve as a reasonable early diagnostic marker for cervical cancer diagnosis.

Moreover, the crucial role of epigenetic alterations at an early stage in the carcinogenesis may be promising targets for the prevention or treatment of cancer. Thus, understanding the epigenetic derepression of oncogenes, or cancer-promoting genes, would be important for the development of epigenetic-based therapies used in combination with other therapies for cervical cancer treatment.

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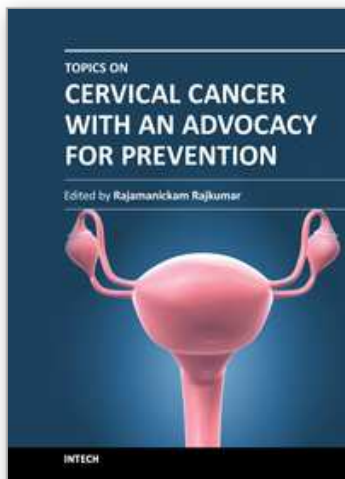
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Cervical Cancer is one of the leading cancers among women, especially in developing countries. Prevention and control are the most important public health strategies. Empowerment of women, education, "earlier" screening by affordable technologies like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

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