

Molecular Genetics of Cervical Precancerous Lesions: Implications to Prognostic Model Development

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INTRODUCTION

The genetic studies in cancer have provided important clues in our understanding of the molecular mechanisms of tumor development. Genetic mutations accumulate in a sequential manner during tumor progression. Thus, the dissection of molecular genetic changes that occur at various stages of tumor progression and invasion are of considerable importance in early diagnosis and prognosis. Squamous cell carcinoma of the cervix uteri (Cervical Carcinoma **(CC)**) provide a prototype system for genetic studies of progression because of its characteristic preinvasive stages that can be recognized by cytomorphologic techniques. Although much is known about the etiology and treatment of CC, the genetic basis of multi-step pathway in cervical tumorigenesis and the role of genetic alterations in invasion remains unknown. Evidence thus far accumulated does not support the role of inherited predisposing factors in CC development. The following review focuses on the early genetic and epigenetic events in precancerous cervical lesions.

EPIDEMIOLOGY AND NATURAL HISTORY OF PRECANCEROUS LESIONS

Carcinoma of the cervix uteri is second most common cancer among women worldwide [1]. It amounts to 500,000 new cases and 275,000 deaths per annum [2]. However, it is the most frequent cancer among women in India. According to a recent estimate 134,420 women are diagnosed with cervical cancer and 72,825 die among them every year [3]. While about 7.9% of women in general Indian population harbour HPV infection of which about 82.6% are attributed to HPVs 16 and 18, WHO also gives a projected number in 2025 in India of new cervical cancer cases to 203,757 and projected deaths due to it to 115,171. Infection of cervical epithelium to high-risk Human Papillomavirus (**HPV**) is prerequisite critical factor for the development of Cervical Cancer (**CC**) [4]. It evolves through well-defined noninvasive Cervical Intraepithelial Neoplasia (**CIN**) stages from normal to carcinoma through low-grade and high-grade Squamous Intraepithelial Lesions (**SILs**) that can be morphologically distinguished. However, only a small fraction of HPV infected SILs progress to invasive cancer [5-7] indicating that HPV infection alone is not sufficient and in addition other genetic and/or epigenetic alterations are required for progression of CC. Cytogenetic and molecular studies on early and invasive CC have identified several structural and functional alterations in oncogenes and candidate tumor suppressor gene site [8].

A number of comparative genomic hybridization studies have identified karyotypic alterations that include loss of 2q, 3p, 4p, 4q, 5q, 6q, 11q, 13q, and 18q regions and gain of 1q, 3q, 5p and 8q at different stages of CC [9-18]. Allelotype analysis has identified Loss Of Heterozygosity (**LOH**) at 2q, 3p, 4, 5p, 6p, 6q, 11q, and 13q [9-22]. Despite evidence of several of putative tumor suppressor gene sites (regions of LOH) and regional gains and amplifications only few genes have been identified so far to be critically involved in CC.

Promoter hypermethylation is one of the major mechanisms for gene inactivation in cancers [23-25]. Many studies have shown inactivation of putative tumor suppressor genes by promoter hypermethylation in a wide range of human cancers including CC [26-33]. Despite a wide range of studies, the mechanism of the progression of CC largely remains poorly understood.

Although there are several studies depicting genetic and epigenetic alterations in CC, most of these studies have addressed invasive tumors. Since the progression of CC is a multistep process, the understanding of the early changes will be of high prognostic value. This review is intended to give an overview of the studies done in preneoplastic lesions to understand the initiation and the gradual progression of the disease.

ROLE OF HPV IN CERVICAL CANCER PROGRESSION

Infection of Human Papillomaviruses (**HPV**) is primary and necessary etiologic factor in the development of the human cancer of the cervix uteri, more than 99% of which contain HPV

sequences [34-36]. HPVs are a group over 100 subtypes of small circular double-stranded DNA viruses containing approximately 8,000 base pairs and up to 10 open reading frames. They infect the basal epithelial cell and the viral genome integrates into the human genome. While high-risk subtypes (e.g. types HPV 16 and HPV 18) are shown to be causative factor in the cervical tumors, the low risk subtypes (e.g. types HPV 6 and HPV 11) cause benign lesions such as genital warts [37]. A frequent characteristic of Human Papillomavirus (**HPV**)-positive cervical cancers is the loss of viral E2 gene expression in HPV-infected cervical epithelial cells as a consequence of viral DNA integration into the cellular genome. The expression of E2 in HPV-positive cancer cells results in the repression of the viral E6/E7 oncogenes, activation of the p53 and pRB pathways, and a G1 cell cycle arrest, followed by induction of cellular senescence [38,39]. Persistent expression of two early viral proteins E6 and E7 are main contributing factors in the pathogenesis of cervical cancer and are capable of transforming human keratinocytes when expressed together [40,41]. Type-specific HPV infection and its associated cervical lesions tend to occur concurrently and Cervical Intraepithelial Neoplasia (**CIN**) is a common and apparently early manifestation of cervical infection by HPV, particularly types 16 and 18 [42,43].

Studies have shown that HPV integration is random throughout the genome, however, 48% of HPV 16 and 63% of HPV 18 integrations occurred within common fragile sites [44,45]. The integration of the viral genome at or near tumor suppressor genes/oncogenes may disrupt (inactivate) or upregulate expression respectively. Five HPV16 integration site were detected in the FHIT/FRA3B region at 3p14.2 and three integrations into promoter of TERT gene at 5p15 while HPV18 had high propensity of integration near the MYC locus [46-48]. Since the HPVs do not express all the enzymes needed for viral genome replication, they reprogram the host cell's replication machinery to replicate their own genome. To facilitate the viral genome replication the two viral early oncoproteins E6 and E7 target critical negative regulatory signaling pathways and thus alter pathways involved in cell cycle regulation.

While E6 and E7 proteins of high-risk HPVs are essential for cellular transformation, E6 and E7 from low risk HPVs do not interact or less efficiently interact with these targets, hence, are weaker transforming oncogenes [reviewed in 49]. The high-risk oncoprotein E7 interacts and subsequently degrades the retinoblastoma tumor suppressor family proteins (pRB, p107, p130), and also inactivates Histone Deacetylase (**HDAC**), AP-1 transcription factors, TATA Box Binding Protein (**TBP**), Cyclin Dependent Kinases (**CDKs**) and CDK inhibitors (p21^{Cip1} and p27^{Kip1}) and M2 Pyruvate Kinase (**M2-PK**) [50-54]. The hypophosphorylated Rb protein binds to E2F family of transcription factors thereby repressing the cell cycle genes [55]. E7 oncoprotein binds to hypophosphorylated Rb protein and disrupts Rb-E2F complexes [56-58]. In addition to its well known effects on pRb, E7 significantly blocks both Smad transcriptional activity and the ability of TGF-beta to inhibit DNA synthesis. E7 interacts constitutively with Smad2, Smad3, and Smad4 suggesting the suppression of Smad-mediated signaling by E7 may contribute to HPV-associated carcinogenesis [59]. The dysregulation of cell cycle control upregulates E2F mediated

transcription of cyclin A and cyclin E and inhibition of CDK2 activity [60,61]. On the other hand binding of E7 to AP-1 transcription factors like c-Jun transactivates transcription from c-Jun promoter that potentiates in turn transactivation of genes involved in early cell cycle progression [54]. Binding of E7 to the transcriptional coactivator p300 results in inhibition of p300 function and can abolish p300-mediated E2 transactivation function [62]. E7 also associates with Mi2 β a component of NURD histone acetylase complex indicating its targeting of deacetylation pathways [63].

HPV viral oncoprotein E6 targets tumor suppressor p53 pathway. Being a transcription factor p53 upregulates the expression of the genes responsible in cell cycle arrest and apoptosis. It is also important to note here that stimulation of E2F by HPV E7 oncoprotein can activate p14^{ARF} that stabilizes p53 through MDM2 [64]. Binding of E6 to E6-associated protein (E6-AP, a cellular protein) to form E6-E6-AP complex, a HECT domain ubiquitin ligase, that specifically interacts with p53 results in rapid ubiquitin-dependent degradation of p53 [65, 66]. Similarly, E6 by binding to Bak, a proapoptotic member of Bcl-2 family, stimulates its degradation and reduce Bak-induced apoptosis [67]. E6 increases telomerase hTERT gene transcription coordinately stimulating and maintaining high levels of E6-induced telomerase activity, causing immortalization [68,69]. E6 protein from HPV-16 have been shown to bind to three regions (C/H1, C/H3 and the C-terminus) of both co-activators CBP and p300, involved in cell differentiation and cell cycle progression inhibiting the intrinsic transcriptional activity of CBP/p300 and decreases the ability of p300 to activate p53- and NF-kappaB-responsive promoter elements which in turn controls IL-6 and IL-8 promoters [70,71]. E6 is also shown to bind and degrade human homologues of Drosophila tumor suppressor genes hDLG and hScribb [72-74].

In addition to the above mentioned targets E6 also interacts with other proteins contributing to the progression of the disease by disrupting actin cytoskeleton, abrogating JAK-STAT signaling pathway, and activating various signaling pathways to inhibit apoptosis and promote cell proliferation [reviewed in 54]. Like E7, binding of low risk HPV E6 to at least E6-AP, Bak and CBP/p300 is inefficient. Thus, the HPV early oncoproteins E7 and E6 essentially target and abrogate various cellular functions including tumor suppressor genes to facilitate cell proliferation and hence initiation and progression of CC.

GENETIC ALTERATIONS IN CINS

Cancer of the cervix uteri is an ideal tumor system to study the accumulation of genetic aberrations during disease progression since the histomorphologic subtypes are defined for multiple stages of the tumor progression. In this section we will discuss the results of cytogenetic (numerical and structural chromosome aberrations, chromosomal instability, and aneuploidy), and molecular (loss of heterozygosity, microsatellite instability, and gene mutation,) studies.

Regional and whole chromosome dosage change (aneuploidy and aneusomy) is characteristics of tumor cells. Cancer of the cervix uteri was one of the first types of human cancer to be studied cytogenetically [75] and there have been a number of cytogenetic studies on preinvasive CC.

Aneuploidy in preinvasive CC has been shown to be a marker for prospective malignancy [76,77]. Only few studies have been done utilizing chromosome banding techniques among which three have shown chromosome 1 aberration [reviewed in 75]. Most of the reports come from DNA cytometry. Cortes-Gutierrez et al. [78] have shown increasing aneusomy of chromosome 1 from 7.44% in CIN 1 to 25.65% in invasive CC indicating the numerical change as an early and progressive event. Similarly, another study showed increasing aneusomy of chromosome 1 from CIN 1 through invasive cancer [79]. A cytogenetical analysis in lymphocytes of 30 patients with precancerous lesions of the uterine cervix and C-band heteromorphisms of chromosomes 1,9 and 16 in 100 women with various grades of precancerous lesions [80,81] revealed increased frequency of chromosomal breaks, SCEs and C-band heteromorphisms of chromosome 1 and decreased activity of ribosomal genes in progressed compared to the patients with nonprogressed lesions in a period of 60 months follow-up. In polyploid HSILs (determined by DNA content) Mehes et al. [82] observed polysomy of chromosome 3 in 70% of polyploidy cells of three cases and a similar accumulation of aneusomy 17 was also observed by FISH. A gain of 3q has been shown in severe dysplasia/CIS [83]. In a similar study numerical aberration of chromosome 17 has been shown to be significant by FISH in CINs through early invasive and invasive SCC suggesting numerical aberrations on chromosome 17 to be useful as an additional method for the differential diagnosis of these lesions [84]. In a DNA cytometry study comprising 151 Pap smears with follow up while an aneuploid DNA stemline was found in CINs that progressed, in the group without progression, the number of aneuploid stemlines was 2% [76]. Using Centromeric FISH probes (**CEP**) Marzano et al. [85] demonstrated in a series of 19 LSILs and 28 HSILs, that the monosomy and polysomy of chromosomes 3, 7 and X are frequent aberrations in LSILs and HSILs. Chromosomal instability occurs together with the centrosome defect in preinvasive CC and it may be the early change that increases with the progression [86-88] and may contribute directly to chromosome missegregation and genetic instability.

With the advent of high-resolution cytogenetic methodologies like Comparative Genomic Hybridization (**CGH**) it has been possible to observe chromosomal aberrations in detail. Umayahara et al. [16] has identified gain of 3q, 1p and 1q and deletions on 11q, 4p, 4q, 6p, 2q, 3p, 11p and 17p in various stages of CIN. The overall frequency of genetic alterations in this study was 1.1 in CIN1, 2.7 in CIN2 and 4.1 in CIN3 lesions. In another study the CGH profiles from dysplastic cells showed various chromosomal imbalances affecting six to nine different chromosomes, the most frequent gains in DNA being on chromosomes 1p, 2q, 4, and 5, whereas losses were found on chromosomes 6q and 13q [89]. Similarly, Kirchhoff et al. [12] detected aberrations in 61 and 55 chromosomal arms respectively in two different studies involving chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 16, 18, 19, 20, and X in 13/17 preinvasive cases by CGH, the most frequent being losses of 5p and Xq [90].

Losses and gains of chromosomal segments and microsatellite instability have been widely studied in invasive cancer using microsatellite markers, however, reports in preinvasive cancer

are few. We have reported that 89% of high-grade (CIN2 and CIN3) and 40% of low-grade (CIN1) CINs exhibited Loss Of Heterozygosity (**LOH**) at 2q [22]. LOH at 3p25 and 3p14 as frequent events have also been described [91]. A high frequency of deletion of 3p as an early event on one or more 3p loci were recorded in CIN2 and CIN3 lesions without co-existing invasive cancer, whereas an increasing percentage of LOH was observed in the precancerous lesions synchronous with invasive cancer, with 71% CIN2 and 76% CIN3 lesions [92,93], 3p22-21.3 and 3p21.1 being the most frequently deleted. Precancerous lesions and CIS showed deletions and microsatellite instability at various loci of 5p [94-95]. The high-grade CINs exhibited 91.7% LOH, and low-grade CINs had 50% LOH on 6p [18]. Rha et al. [96] in a study with microsatellite markers of multiple chromosomes has shown LOH at 6p21.3, 7q31, 9q22, and 11q24 in HSILs with overall microsatellite instability of 63%. In an effort to study the genomic deletions in CINs we have shown deletions on 2q and 5p as early events in the pathogenesis of the CC [22, 95].

EPIGENETIC ALTERATIONS IN PRECANCEROUS LESIONS

DNA methylation is a frequent epigenetic alteration in many human tumors [23,97]. Methylation of cytosine residues at CpG dinucleotide pairs is a widely studied mechanism of gene silencing. Hypermethylation of CpG dinucleotides at the promoter region (CpG islands) of several tumor suppressor genes have been well characterized in cancers including CC. Although progressive global hypomethylation in cervical cancers [98] may serve as a biochemical marker for cervical neoplasia, hypermethylation of CpG islands at promoters of several genes in various stages of CC has been well documented. However, there are few reports on the promoter hypermethylation in precancerous lesions of the cervix.

One of the promoter hypermethylation studies on precancerous cervical lesions was done on a panel of 6 genes (p16, RAR β , FHIT, GSTP1, MGMT, hMLH1) [99]. They demonstrated the low frequency of promoter hypermethylation of these genes individually and a high frequency of at least one of these genes being methylated collectively. Although these genes were rarely methylated in low grade CINs, p16, RAR β , and MGMT were more frequent in high grade CINs, while hMLH1 was the least frequent. Steenbergen et al. [100] detected promoter hypermethylation in Tumor Suppressor in Lung Cancer 1 (**TSLC1**) gene in 35% of the high-grade CIN lesions (CIN2 and CIN3). Loss of protein expression of tumor suppressor gene PTEN is associated with its promoter methylation that showed hypermethylation in 40% of high grade CINs [101]. In a recent report, in a panel of 20 genes known to be involved in oncogenesis as potential biomarkers for CIN3/CIS or invasive cervical cancer, 69.6% of samples with CIN3/CIS, and 28.9% of cervical biopsies without neoplasia, one or more of 10 genes (CDH13, DAPK1, RAR β , TWIST1, SYK, MGMT, FHIT, ASC, CCND2, and MLH1) were methylated [102]. Among these DAPK1, RAR β , and TWIST1, that were predictive of ICC showed methylation in 56.5% of CIN3/CIS samples and were negative in 95% in samples with CIN1 or less.

We have analysed the promoter hypermethylation of 20 genes (that showed hypermethylation in invasive CC in low and high grade CINs and have found that promoter hypermethylation of 12

genes (CDH1, DAPK, HIC1, RAR β , RASSF1A, five SLIT-ROBO pathway genes, AHRR, and PCDH10) was an early event during the progression [30, 103-105].

ROLE OF SPECIFIC GENETIC PATHWAYS IN CERVICAL CANCER PROGRESSION

As discussed above, the major critical pathways leading to cervical oncogenesis are initiated by interaction of high-risk human papillomavirus types (including HPV 16 and 18) early oncoproteins E6 and E7. In this section we will discuss additional genetic pathways that are thought to be responsible for development and progression of CC. There is evidence of possible interaction between genetic and epigenetic factors in cervical cancer. The C677T polymorphism of MTHFR (methylene-tetrahydrofolate reductase) was significantly associated with the frequency of MGMT promoter hypermethylation [106]. Abnormal expression (over-/under-expression) of oncogenes and tumor suppressor genes are hallmark of several cancers including CC. As discussed above genetic analysis of cervical cancer has demonstrated frequent allelic loss in the 3p chromosomal region. A putative tumor suppressor gene FHIT (fragile histidine triad) is located at chromosome region 3p14.2. Reduced or absent expression of this has been described to be an early event in CC and its frequency increases with the progression of cervical dysplasia through CIN stages and has been linked to the poor prognosis [107-109]. The progression of CIN grade 1 to grade 3 has been associated with reduced expression of p150 [110] and Syndecan-1 [111]. Mutation of tumor suppressor gene BRCA1 in exon 11 was detected in a very high frequency (>76%) of cases with precancerous lesions of the cervix. The mutations were either complete deletion or deletion of one or more nucleotides, leading to frame shifts [112]. Immunohistochemical analysis of cervical tissue specimens showed downregulated expression of GATA-3 (harboring 10p which shows allelic loss [12]) in 11% (1 of 9) of CIN3 lesions compared to CIN1 and 2 where normal expression was recorded whereas expression is lost in 67% of invasive CC [113]. CD44, CD44-4v, and CD44-6v showed reduced expression in precancerous lesions [114]. In an effort to identify biomarkers in CIN lesions that can be modulated by retinoids *in vitro* Xu et al. [115] demonstrated downregulated expression of retinoic acid receptors-RAR- α , RAR- β , and RAR- γ , and differentiation markers-IVL (involucrin), SPRR1B (cornifin), TGF- β 1 and TGF- β 2. All CIN lesions including CIN1, CIN2 and CIN3 exhibited decreased expression of more than one of these genes either by FISH or immune histochemistry.

Over-expressions of certain genes in preinvasive CINs/SILs have been well documented. Aminopeptidase A expression was found on dysplastic cells and increased with severity of the precancerous lesions [116]. Proto-oncogene C-myc and bcl-2 expression is elevated with the progression of low grade CINs to high grade [117,118]. Detry et al. [119] demonstrated that the BSP expression was a common feature in high grade SILs and invasive CC. Immunosuppressive cytokines IL-10 and IL-12 expression increased within both low- and high grade SILs and the increase was parallel to the severity of the lesion to a maximum level in high grade SILs [120].

Both IL-10 and IL-12 were localized in the lesion-associated stroma, providing microenvironment for the tumor progression. The transforming zone, region of the cervix most sensitive to lesion development, was associated with higher average levels of the cytokines IL-10 and TGF-beta1. Patients with either high or low grade SILs had significantly higher serum levels of IGF-I and IGFBP-3 [121]. HSP60 expression was gradually increased from undetectable in normal to higher levels in LSILs and HSILs showed markedly high immunostaining [122]. Progressive increased expression of nm23-H1 was evident from low grade CIN/SIL to high grade CIN/SIL [123,124]. The progressive overexpression of p53 may be involved at an early stage in cervical carcinogenesis [125,126]. Several studies have shown the overexpression of the cyclin-dependent kinase inhibitor, p16INK4a in precancerous lesions and the level increased with the progression from low CIN to high CIN grades [127-129] while its expression was undetectable in normal cervical squamous epithelium. Telomerase activity increased with the progression of CIN/SIL grades [130,131]. While there is no expression of MT1-MMP (MMP14) in normal and LSILs, it is moderately or strongly expressed in HSILs and invasive CCs [132]. Increased expression of p16INK4A, p14ARF, p53, and PCNA has been linked to the shorter progression period of CIN3 to ICC [133].

PROGNOSTIC MODEL OF PROGRESSION

The biological behavior of cervical precursor lesions, as pointed out previously, is clinically highly unpredictable. The underlying genetic basis of the progression of CIN lesions is not known. The process of tumor evolution represents acquisition of mutations during the progression. Cervical tumorigenesis, histopathologically progresses through multiple stages exhibiting distinct clinical behavior of precancerous lesions during progression. Fewer molecular genetic changes are known in CC, and the role of specific gene alterations during the progression is unclear. The molecular genetic studies reported in invasive CC have shown several consistent genetic alterations including regions of allelic deletions and amplification of genes. However, characterization of these changes in precancerous stages is rather rare and fragmentary. Thus, the genetic alterations in CC progression should occur in some sequence depending on the stage of their requirement. Our understanding of a particular stage with specific genetic alterations in CC progression is very limited. To date we can only correlate isolated genetic alterations with specific stage in progression, e.g. 3q gain at CIN3/CIS stage [9]. The molecular definition of the timing of a specific set of genetic mutations at each stage of progression likely predicts the risk for progression and has implications in the management of the disease.

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