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REVIEW

Recent advances in the study of HPV-associated carcinogenesis

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Human papillomaviruses (HPVs) cause virtually all cervical cancers, the second leading cause of death by cancer among women, as well as other anogenital cancers and a subset of head and neck cancers. Approximately half of women, who develop cervical cancer die from it. Despite the optimism that has accompanied the introduction of prophylactic vaccines to prevent some HPV infections, the relatively modest uptake of the vaccine, especially in the developing world, and the very high fraction of men and women who are already infected, means that HPV-associated disease will remain as a significant public health problem for decades. In this review, we summarize some recent findings on HPV-associated carcinogenesis, such as miRNAs in HPV-associated cancers, implication of stem cells in the biology and therapy of HPV-positive cancers, HPV vaccines, targeted therapy of cervical cancer, and drug treatment for HPV-induced intraepithelial neoplasias.

KEYWORDS human papillomavirus (HPV); carcinogenesis; vaccine; miRNA; cancer stem cell (CSC); cervical intraepithelial neoplasias (CIN); targeted therapy

INTRODUCTION

Human papillomaviruses (HPVs) are a group of small, non-enveloped, double-stranded DNA tumor viruses, categorized to the *papillomaviridae* family. Approximately 200 types of HPVs have been identified. HPVs are not only species specific but also display a tropism for squamous epithelia. A large number of HPVs infect cutaneous epithelia, whereas other groups infect mucosal epithelia (Bravo and Félez-Sánchez, 2015). The mucosal HPVs are classified as "high- risk" and "low-risk," depending on the capability of the viruses to cause the malignant progression of the lesions. Low-risk HPVs, such as HPV type 6 (HPV6) and HPV11, cause genital warts. High-risk HPVs, such as HPV16 and HPV18, trigger squamous intraepithelial lesions, which may progress to malignant foci (zur Hausen, 2009). High-risk HPVs are associated with greater than 99% of cervical carcinomas, a portion of additional anogenital tumors and approximately a quarter of oropharyngeal tumors (Walboomers et al., 1999; Mirghani et al., 2015). This is the combined action of two viral oncoproteins, E6 and E7, which subvert cellular regulatory pathways controlling cell cycle and cell survival (McLaughlin-Drubin and Munger, 2009; Moody and Laimins, 2010). The E7 oncoprotein binds to more than 20 cellular targets and interferes with numerous cellular processes, leading to deregulated cell cycle progression, malignant transformation, centrosome amplification, DNA damage, anoikis loss and anchorage-independent cell growth, immune surveillance evasion and persistent infection. The E6 oncoprotein abrogates cell growth arrest and apoptosis, induces genomic instability and somatic mutations, activates telomerase and the telomerase reverse transcriptase to promote immortalization, disrupts cell polarity, prevents anoikis, and allows cellular growth without attachment to extracellular matrix (ECM). Together, these observations demonstrate that E6 and E7 are multifunctional proteins driving oncogenic transformation and tumorigenicity of the HPVs.

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MOLECULAR ACTIVITIES OF HIGH-RISK HPV E6/7

The most prominent targets of high-risk E6 and E7 proteins are tumor suppressor genes p53 and pRb family pocket proteins (McLaughlin-Drubin and Munger, 2009; Moody and Laimins, 2010). The E7 interacts with a large number of host proteins (White et al., 2012b). In particular, the high-risk HPV E7 protein targets the pRb family proteins for degradation, thereby inhibiting pRb-mediated repression of E2F-responsive genes. In addition, it inhibits the cyclin-dependent kinase (CDK) inhibitors p21 and p27, and activates both cyclin A/CDK2 and cyclin E/CDK2 (Nguyen and Münger, 2008). E7 also interacts with histone deacetylases (HDACs) to affect cellular gene expression (Brehm et al., 1999; Longworth and Laimins, 2004). Therefore, E7 subverts cell cycle control and induces hyperproliferation. Overexpression of E7 stimulates centrosome amplification through the enhancement of CDK2 activity and interaction with γ-tubulin, contributing to the accumulation of chromosomal alterations and increased risk of genomic instability (Duensing et al., 2000). Moreover, E7 interacts with p600, preventing anoikis and rendering the cell anchorage-independent growth (Huh et al., 2005). Lastly, E7 inactivates interferon regulatory factor 1 (IRF1), contributing to the evasion of immune surveillance and the establishment of a persistent infection (Park et al., 2000).

The E6 proteins of various HPV types interact with a variety of host proteins (Howie et al., 2009). p53 is among the most important targets. The high-risk HPV E6 protein forms a trimeric complex with E6-associated protein (E6AP) and p53 resulting in the degradation of p53 (Scheffner et al., 1993; McLaughlin-Drubin and Munger, 2009; Moody and Laimins, 2010). E6 interacts with the histone acetyl transferases p300, CREB binding protein (CBP), and alteration/deficiency in activation-3 (ADA3) to prevent p53 acetylation, hence suppressing the transcription of p53-responsive genes (Thomas and Chiang, 2005). Thus, p53-dependent cellular responses to aberrant proliferation, genomic instability, and mutations are suppressed by E6. Through the interaction with IRF3 (Ronco et al., 1998), E6 would interrupt the interferon response. E6/E7 inhibits growth-suppressive cytokines-induced apoptosis by interaction with and suppression of the TNF-α-FADD (FAS-associated protein with death domain)-caspase 8 signaling, and by the degradation of BAX and BAK (Boccardo et al., 2004; Garnett et al., 2006; Liu et al., 2008; Underbrink et al., 2008). In addition, E6 activates the telomerase reverse transcriptase (TERT) and telomerase and hence prevents the telomere shortening in response to persistent proliferation and in turn promoting immortalization (Klingelhutz et al., 1996; Xu et al., 2013). Moreover, E6 mediates the degradation of several PDZ domain containing host proteins, leading to the loss of cell polarity and inducing hyperplasia (Pim et al., 2012).

In a recent report, White *et al*. (2012a) applied a mass spectrometry-based platform to systematically identify and characterize interactions between HPV oncoproteins and host cellular proteins. They found that HPV E6 interacts with host proteins in a genus and species common or specific manner. The E6 interaction data set not only contains previously reported interaction proteins of E6, such as p300/CBP, E6AP, and p53, but also newly identified proteins that bind to and interact with E6, such as Ccr4-Not complex. Ccr4-Not acts as a deadenylase conserved from yeast to humans, and affects mRNA metabolism (Collart and Panasenko, 2012). In addition, it possesses an ubiquitin ligase function linking to ubiquitylation and the proteasome. These findings, on the one hand, provide a comprehensive database for studies on the diverse biology of the HPVs. On the other hand, they suggest that current understanding for the functions of HPV oncoproteins is incomplete. Thus, a continued exploration for the multifaceted roles of HPV oncoproteins in driving proliferation and carcinogenesis is necessary.

MIRNAS IN HPV-ASSOCAITED CANCERS

microRNAs (miRNAs) are noncoding regulatory RNAs of 18–25 nucleotides in size. They are derived from RNA polymerase II transcripts of coding or noncoding genes (Bartel, 2004). miRNAs expression is tissue- or differentiation-specific. They modulate gene expression at the posttranscriptional level by base-pairing with complementary nucleotide sequences of target mRNAs, leading to the degradation of mRNA or translational suppression (Lewis et al., 2005; Bueno et al., 2010). As part of the transcriptome, the small non-coding RNA species have attracted much attention due to both their genesis and their ability to regulate gene expression.

miRNA expressions have been frequently found near fragile sites in chromosomes or integration sites of high-risk HPVs (Georgakilas et al., 2014). Integration of HPV oncogenes may alter miRNA expression via deletion, amplification, or genomic rearrangement (TCGA, 2015). A large number of miRNA genes are regulated by transcription factors c-Myc, p53, and E2F, which are targeted by oncogenic HPV E6 and E7 (Zheng and Wang, 2011). For example, E6-mediated degradation of p53 reduces the expression of miR-34a (Wang et al., 2009; Li et al., 2010) and miR-23b (Au Yeung et al., 2011) at the transcriptional level. In addition, E6 and E7 oncoproteins interact with multiple cellular factors, and these interactions could lead to increased or decreased expression of cellular miRNAs. In a recent report, 13 host miRNAs were found to be specifically regulated by HPV16 and HPV18 in organotypic raft cultures of foreskin and vag-

inal keratinocytes with a miRNA array assay in combination with small RNA sequencing (Wang et al., 2014b). The increase of miR-16, miR-25, miR-92a, and miR-378 and the decrease of miR-22, miR-27a, miR-29a, and miR-100 could be attributed to and mediate the functions of viral oncoprotein E6, E7, or both. The authors suggest that an expression ratio ≥ 1.5 of miR-25/92a group over miR-22/29a group might be used to distinguish cervical cancers from normal cervix (Wang et al., 2014b).

IMPLICATION OF STEM CELLS IN THE BIOLOGY AND THERAPY OF HPV-ASSOCIATED CANCERS

Stem cells are a group of undifferentiated cells that act as a reservoir for new cells in order to replace defective or necrotic cells. An essential characteristic of stem cells is the ability to self-renew and to differentiate into diverse types of cells. Current researches regarding cancer stem cells (CSCs) demonstrate that these cells maintain the capabilities of normal tissue stem cells, such as unlimited self-regeneration, and differentiation into various cell types. CSCs initiate malignant tumors on a single cell basis via symmetric proliferation, and express cancer stem cell specific markers. These characteristics of CSCs are responsible for tumor maintenance and metastasis and possibly also for the resistance toward chemotherapy and radiation therapy.

Researchers have characterized the surface markers for cervical cancer stem cells, *e.g.* p63, cytokeratin 17 (CK17), Nanog, Musashi-1 (Msi1), Nucleostemin (NS), CD49f, ALDH1, CD44, and a few more others. Nanog, NS, and Msi1 were found to be highly expressed in cervical carcinomas relative to normal cervix and were proposed to be involved in carcinogenesis of the cervix (Ye et al., 2008). Embryonic stem cells (ESCs) markers (Sox2, Oct4) and the Wnt signal pathway (β-catenin) are crucial for the progression of various human malignancies. Ji *et al*. (2014) showed that Sox2 and Oct4 are highly expressed in cervical squamous cell carcinomas and that Wnt signal (beta-catenin) is activated. Thus, Sox2 could be a novel predictor for poor prognosis of such lesions (Ji et al., 2014). Using a different approach, Lopez *et al*. (2012) investigated the tumor initiating cells in cervical carcinoma cell lines, HeLa and SiHa, and found a high level of CD49f in these cells, whereas Gu *et al*. (2011) showed that cancer initiating cells in HeLa cell line exhibit a CD44 (high) / CD24 (low) expression pattern.

HPVs infect stem cells, which are speculated to be the origination of the cervical epithelial cancers. Stem cells could also be sources for latently infected cells that can persist for a long period. The HPV oncogenic E6 and E7 modulate multiple cellular pathways with parallel roles in the carcinogenic process. Michael *et al*. (2013) examined the effects of HPV16 E6/E7 on stem cells located in the bulge of hair follicles. The results showed that expression of HPV16 oncogenes reduces the number of quiescent cells, a typical feature of stem cells within hair follicles, whereas stem cell markers in the follicles are also decreased. The authors suggest that HPV infection may induce aberrant mobilization of the stem cells. These effects may play a role in viral life cycle and/or ensuing carcinogenesis.

Tang *et al*. (2013) applied *in vitro* and *in vivo* analysis to determine if CSCs of head and neck squamous cell carcinoma (HNSCC) are affected by HPVs. It shows that HPV status does not correlate with the proportion of CSCs present in HNSCC. The HPV positive cells and those transduced with HPV E6/E7 possess a greater clonogenicity than HPV-negative cells. CSCs of the HNSCC are more resistant to cisplatin than non-CSCs. Consistently, HPVs do not affect the response of CSCs to the treatment of cisplatin, further supporting the notion that the functions of HPV are not overlapped with CSCs.

Taken together, characterization of stem cells in cervix provides new insights into the mechanisms by which cervical cancer is developed. However, it remains largely unknown what the role of HPV oncogenic E6/E7 may play in the formation of cervix CSCs and the potential therapeutic significance of CSCs may exist in HPV positive cancers.

HPV VACCINES

Vaccines have been developed to prevent infections with certain types of HPVs. Currently, there are a quadrivalent HPV vaccine and a bivalent HPV vaccine licensed in the United States. The former produces immunity against HPV 6, 11, 16, and 18, whereas the latter is administrated to prevent infections of HPV 16 and 18. Both vaccines are based on virus-like particles of the L1 capsid protein. The vaccines are highly efficient and immunogenic if given before exposure to these types of HPVs (Schiller and Lowy, 2012). Both vaccines are administered in a 3-dose series. The protective duration of the bivalent vaccine is over 10 years and counting. Recent studies suggest that 2-dose provide effective protection as well (Romanowski et al., 2014; Dobson et al., 2013). To increase the protection, a VLP based nine-valent prophylactic HPV vaccine (HPV 6/11/16/18/31/33/45/52/58) (Serrano et al., 2012) has been approved by FDA. A cross-reactive prophylactic L2-based vaccine is also under development (Wang et al., 2104 a). Several therapeutic vaccines are undergoing clinical trials in women with cervical cancers (Hibbitts et al., 2012; Tran et al., 2014). These vaccines are developed to produce an immune reaction against HPV16 E6 or E7 protein. Thus, the en-

hanced immunity might kill the cancer cells or stop them from growing.

TARGET THERAPY

Based on international guidelines, treatment of cervical cancer (CC) consists of surgery in early stages and of chemoradiation in locally advanced disease. Metastatic disease is usually treated with palliative chemotherapeutic regimens. Cytostatic drugs exhibit substantial side effects and limited efficacy. Thus, the discovery of new anticancer agents, interfering with molecular targets expressed in the microenvironment of the cancer or by the tumor cell itself, represents an opportunity challenging the cancer. Vascular endothelial growth factor (VEGF) promotes angiogenesis in cervical cancer, leading to disease progression. Bevacizumab, a humanized anti-VEGF monoclonal antibody, was tested in patients with recurrent, persistent, or metastatic cervical cancer in conjunction with chemotherapies. The increase in time to progression or survival was 3 to 4 months relative to chemotherapy alone (Wright et al., 2006; Tewari et al., 2014). As another example, pazopanib, a multi-tyrosine kinase inhibitor (multi TKI), has been used to treat kidney and ovarian cancer. Recent clinical trials confirmed the activity of the antiangiogenesis agents in advanced and recurrent cervical cancer. However, the benefits in progression free survival only amounted to 3 months (Monk et al., 2010).

DRUG TREATMENT FOR HPV-INDUCED INTRAEPITHELIAL NEOPLASIAS

Standard treatment of cervical pre-cancer (such as cervical intraepithelial neoplasia; CIN) includes cryotherapy, laser treatment, and conization. Researchers are asking whether CIN could be treated with pharmaceutical agents. Del Priore *et al*. (2010) treated CIN2 and CIN3 patients for 12 weeks with diindolylmethane (DIM), a component of indole-3-carbinol (I3C) found in Brassica vegetables. The subjects were evaluated every 3–4 months with Pap smear, HPV, colposcopy, biopsy and physical examination at follow-up. The results showed that oral administration of DIM leads to a high rate of clinically significant improvement in confirmed CIN 2 or 3 lesions in a one-year follow-up. However, there is no statistically significant difference between DIM- and placebo-treated groups. In another study, a randomized controlled trial was applied to evaluate a topical treatment for CIN 2+ using anti-viral drug cidofovir (Van Pachterbeke et al., 2009). Although regression was more frequently achieved than the placebo group as judged by histology and by *in situ* hybridization, the more sensitive Hybrid Capture 2 assay did not reveal significant difference between the two groups of patients. The authors, therefore, concluded that it is a promising candidate for topical chemotherapy but it cannot replace conization at this juncture.

The most promising agent appears to be imiquimod, an immune response modifier. It is a patient-applied cream used to treat certain types of skin diseases, superficial malignant melanomas, as well as genital warts (*condylomata acuminata*). Several groups applied imiquimod to subjects with CIN 2 and 3 and showed a higher histologic regression as compared with placebo treatment subjects (Grimm et al., 2012). More importantly, HPV clearance rates are increased in the imiquimod group (60%) compared with the placebo group (14%). In patients with HPV-16 infection, complete remission rates are 47% in the imiquimod group compared with 0% in the placebo group. Consistently, administrations of imiquimod alone or in combination with HPV therapeutic vaccination in patients with anal and vulval intraepithelial neoplasia produced similar results (Hibbitts et al., 2010; Daayana et al., 2010). There was a substantially increased local infiltration of CD8 and CD4 T cells in lesion responders. Taken together, current clinical trials suggest that topical imiquimod 5% cream may be beneficial in anogenital intraepithelial neoplasias.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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