Chapter 11

Modern Molecular and Clinical Approaches to Eradicate HPV-Mediated Cervical Cancer

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/55810

1. Introduction

Cervical cancer was formerly the second most common cancer killer of women worldwide. Following widespread adoption of Papanicolau cytologic screening (Pap test) for cervical cancer in the 1950s, this began to change. Today, advanced cervical cancer is rare in screened populations. Although an uncommon disease in developed nations, internationally about 500,000 women annually are diagnosed with cervical cancer, and about half of those women will die of their disease. In global terms, this ranks second only to breast cancer as a cause of cancer-specific mortality. Over the past three decades the scientific community has witnessed spectacular advances in the understanding of the underlying pathophysiology of cervical cancer, with the most profound discovery being in 1983 of the identification of the human papillomavirus (HPV) within cervical cancer (a discovery that earned Harold Zur-Hausen, M.D the Nobel prize for Medicine and Physiology in 2008). A viral etiology for cervical cancer implied that it may be possible to eradicate cervical cancer through vaccination. This promise was partially fulfilled in 2006 when the United States Food and Drug Administration approved an HPV vaccine for the prevention of HPV-induced cervical dysplasia and/or cancer. These advances, profound though they are, have yet to eradicate cervical cancer. Furthermore, due to the pervasiveness of HPV infection and the timeline of disease progression, it will be a few decades before we will be able to determine the impact preventive practices are having on cancer incidence and prevalence. In addition, those for whom preventative measures are not a solution, including HIV^+ individuals as well as women already infected with HR-HPV, await an answer.

Over the past several years, developments in innovative imaging, superior surgical technologies, immunotherapies, and molecular therapies have surfaced, making the eradication of

cervical cancer a much more achievable goal than in the past. Several areas of cervical cancer research continue to address the challenges posed by the need for appropriate therapeutic alternatives, and progress is occurring at each level of clinical management ranging from detection to the development of small molecule antiviral leads. Because the field is evolving rapidly in all directions and related disciplines, it is helpful to summarize the status of our growth, and to recognize those pioneering efforts that may ultimately contribute to achieving our goal of eliminating cervical cancer. This review seeks to survey the current understanding of cervical cancer etiology and treatment and to review areas requiring additional progress.

2. Prevention, interception and early detection

Disease Burden and Risk: Approximately 20 million Americans are currently infected with HPV, and another 6 million new infections occur annually. In about 90 percent of these cases, the infection is cleared by the immune system within two years [1, 2]. However, a relatively small subset of infections persists, sometimes resulting in viral oncoprotein-mediated perturbation of cell-cycle controls leading to cervical intraepithelial neoplasia (CIN). Approximately 5 percent of Pap specimens are classified as pre-cancerous (CIN 1, 2 or 3) while 0.3 - 0.5 percent are typically diagnosed as carcinoma *in situ* [3].

Occurring in men and women, HPV infection is most commonly transmitted by sexual contact. According to the National Cancer Institute (NCI), a woman's risk of acquiring HPV and subsequently developing cervical cancer is increased when the age of sexual debut occurs at a younger age and when the number of lifetime sexual partners is higher [4]. In addition, it has been shown that prevalence rates of infection are consistently higher by 70 percent in sexually experienced, low-income populations of racial and ethnic diversity compared to the general population [5-7]. Also, the risk of HPV infection progressing to cancer depends on lifestyle. For example [8], at a woman's first Pap test the risk that HPV16 infection is more likely to progress to carcinoma *in situ* is 70 percent higher in women who currently smoke (Relative Risk for current smokers is 1.9) [8-11].

Public Education: Media coverage over the last decade has increased general awareness of HPV infection. However, knowledge regarding how the virus is transmitted and the fact that HPV infection may cause cervical cancer is less well-known, particularly among vulnerable populations [12]. In one survey, when high school students were asked to name a few common sexually transmitted infections (STIs) only 17 percent of students mentioned HPV [13]. A general awareness about HPV infection leading to cervical cancer can be increased through education, but more needs to be done to influence the pre-conceived attitudes about prevention through vaccination and sexual behaviors [14]. For example, HPV infection, like most other STIs, are spread *via* bodily fluids that can be obstructed by condom use [15]. Consequently, this knowledge might heighten the risk-perception (a person's subjective appraisal of danger) of not using a condom during vaginal intercourse encounters and reduce sexually risky behaviors to some appreciable degree.

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UNESCO (United Nations Educational, Scientific, and Cultural Organization) has demonstrated the effectiveness of sex education in the global fight against HIV/AIDs. Although HPV does not currently compare to HIV/AIDs in terms of mortality and global magnitude, the need for HPV awareness has grown tremendously and it is speculated that such a plan may prove useful here as well. An increase in consciousness may decrease sexual risk behaviors if populations at high-risk for contracting HPV were actively targeted for education [16, 17]. However, it should be considered that gender inequalities experienced by women in locations like Sub-Saharan Africa, as reported by the Global Health Corps and UNESCO, may negatively impact these initiatives. Furthermore, the cost of such programs compared to other interventional methods has not been determined. But in general, education can be used as a powerful tool in preventing HPV-mediated diseases such as cervical cancer. Ideally, these programs would emphasize risk-perception in both men and women leading to lifestyle modifications, and a further reduction in the incidence of HPV-mediated carcinoma might be realized.

Prophylactic Immune Strategies: Presently, the most effective protective factor against the most prevalent and high-risk types of HPV infection is prophylactic vaccination [18]. It has been seven years since the introduction of the first HPV vaccine [19]. Since then, two vaccines, Gardasil and Cervarix, have been made available to the public to protect against the more common HPV strains [20]. The vaccines induce the production of neutralizing antibodies against HPV L1 capsid virus-like proteins (VLPs), which do not contain virus genetic material. The quadrivalent vaccine, Gardasil, protects against low- and high-risk HPV (LR- and HR-HPV, respectively) types 6, 11, 16, and 18 following full vaccination of all three doses at 0, 1, 2 and 6 months. Alternatively, the bivalent vaccine, Cervarix, prevents infection by HR-HPV types 16 and 18. Both vaccines have been documented to possess compelling prophylactic efficacy in preventing cervical, genital, and anal diseases. This protection is expected to persist for 7 years, or at least during the years of high infection risk for most individuals [20-22].

HPV infections of types other than the four mentioned above are not reliably prevented by vaccination. Also, studies are warranted regarding the vaccine's long-term effects and how they might impact the occurrence of infections by other HPV types. It has already been noted that quadrivalent and bivalent vaccines may exhibit cross-protection against HPV 31 and other types by 75 to 80 percent [23]. However, concerns are emerging that relate to HPV type-replacement. Type-replacement is an increased prevalence of other HPV strains that are not included in the vaccines, while vaccine-type HPV prevalence is decreased. It was recently reported that vaccine-type HPV has been reduced in vaccinated and nonvaccinated women, while nonvaccine-type HPV has slightly increased overall [24]. Researchers do not expect type-replacement to occur frequently. However, studies are becoming more attentive to changes in the prevalence of various HPV types, which are expected to surface first within the sexually experienced population. Such discoveries could encourage the research community to continue seeking multivalent solutions to as many HPV types as possible without eliciting additional harmful results. To date, clinical trials have revealed that the most common adverse response to both vaccines are injection site reactions, which occur more frequently in vaccine groups rather than in participants given placebos [25].

Though most industrialized countries, like Great Britain, have already implemented structured HPV immunization programs, well-functioning programs geared towards adults and young adolescents have yet to be seen in many developing countries. However, there are globally funded systems with strong infrastructure that support the immunization of infants in developing countries [26]. This anomaly is due to several challenges, which include the cost of the vaccines, though the biological, economical, and psychological disease burdens of HPV have also been considered [25, 26]. Therefore, it is no surprise that less developed countries have not made HPV vaccination programs a priority while other issues compete for the same limited governmental resources. The most apparent considerations regarding vaccine distribution in these countries relate to healthcare infrastructure, which can directly affect a country's ability to establish and maintain immunization programs that target the vaccine's intended population – adolescents. Other factors of significant importance comprise how to best promote these programs in a way that does not aggravate ethnic/cultural sensitivities and attitudes about vaccination against an STI [27, 28]. This would further include easing parental concerns about what an STI vaccine might imply if perceived as socially acceptable within the targeted age groups. Perhaps if immunization programs were set up in an educational setting, a stronger risk-perception might be instilled, which would encourage the formation of better habits of awareness. In years to come, these types of educational agendas might also improve adherence to the 3-dose regimen over the six-month vaccination period and increase compliance with screening routines throughout a woman's lifetime [29].

One clear limitation of the HPV vaccines is their lack of efficacy for those who have already been exposed to the virus types included in the vaccines. Of course, this exposure is directly correlated to increasing age and sexual experience [30, 31]. For those who fall into this group, including older women regardless of vaccination status, it is important that screenings continue as outlined by the American Cancer Society (ACS) guidelines for Early Detection of Cervical Neoplasia and Cancer [5]. Therefore, integrated approaches of prevention and detection are required if efforts against HPV-mediated cervical cancer are to be maximized. Another important consideration for vaccination is the immune status of potential vaccine recipients; the immune system must be intact [32]. The immune system's ability to clear antigens depends largely on its strength and competence. Thus, immunocompromised women are especially in peril of HR-HPV infection progressing to cancer [33].

The most common scenarios for compromised immunity are seen in HIV^+ individuals and organ transplant beneficiaries. Those infected with HIV have a greater chance of HPV co-infection and progression to invasive cervical cancer as compared to those without HIV [34, 35]. The disparity observed here is most likely due to the immune system's inability to effectively clear virus among this subset due to decreased immune reactivity to HPV antigen [36, 37]. It was also found that HPV infection is prevalent among those receiving organ transplants [38], and that the infection increasingly persisted throughout immunosuppressive therapy to moderate graft rejection [39]. Despite these challenges, researchers agree that previous prophylactic HPV vaccination is still beneficial for organ transplant recipients as well as the HPV/HIV co-infected population who receive HPV vaccination before becoming HIV^+. In these cases, any future challenges of HPV infection following vaccination would be

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neutralized by an earlier developed immunity prior to the individual presenting as HIV^+. This is based on the premise that the protection conferred against the viral types represented in the vaccine is expected to last for the same time period as in others who are not infected with HIV. However, what remains unclear is whether the vaccine will prove effective for an individual already infected with HIV, or in any immunocompromised state [37]. The current understanding is that humoral immune responses remain relatively intact following HIV infection. However, in such a state of immune weakness, it is unknown whether protection against HPV can be sustained. Overall, individuals who have been vaccinated prior to becoming immune compromised are expected to benefit from vaccination by maintaining immune competency, because new HPV infections from these specific types and the risk of lesions reactivating from an ongoing, latent HPV infection would be reduced. Of course, they, like other individuals, will only be protected from the vaccine-type strains, and it is imperative to note that questions regarding long-term safety in such vulnerable groups remain unanswered [30, 31, 33, 40, 41].

To this end, it is important that innovative therapeutic approaches to improve immunological surveillance and clearance of HPV continue to develop. It is well-documented that cellular immune components contribute directly to natural clearance of the virus in most people. For instance, CD4+ and CD8+ cytotoxic T cells (CTLs) are thought to target HPV 16 early and late proteins, and active HPV-specific CTLs have been identified in patients with existing infections [42]. Furthermore, researchers have found that in response to a vaccine containing E6 and E7 oncoproteins, CD4+ and CD8+ CTLs were stimulated, thus inducing the regression of HPV-mediated vulvar intraepithelial neoplasia (VIN) in 50 percent of subjects [43]. A variety of other immunotherapy investigations are underway and will be discussed in more detail later in the review.

Physical Barriers: As noted above, the use of condoms can reduce the rate of HPV infection. One study showed that when condoms are used during all vaginal intercourse encounters, HPV transmission/infection is reduced by 70 percent. Even if a condom is used greater than half of the time, the risk of infection is still reduced by 50 percent [44]. Another study similarly reports that condoms benefit users by promoting viral clearance and possible regression of CIN [4]. The use of barrier protectors such as microbicidal and spermicidal gels can also reduce the risk of HPV infection [45, 46]. The recent utilization of pseudoviruses has proven helpful in better understanding HPV invasion into keratinocytes through cell surface proteins. In these pseudovirus studies, carrageenan exhibited a microbicidal function by blocking virus particle attachment to heparin sulphate proteoglycans on cell basement membranes. Beyond its function on tissue surfaces, carrageenan also exhibited post-attachment inhibitory actions. Carrageenan has been used as a thickening agent in sex lubricants and Pap smear gels, and has shown microbicidal function against a host of STI-causing microbes including HPV [46, 47]. Originally derived from red algae, it is structurally similar to heparin but several times more potent. Therefore, it is able to bind virus more effectively than host cells and thereby acts as a decoy receptor [45, 46].

Interception of HPV-Mediated Carcinogenesis: During the decades that frequently occur between HPV infection and cancer development, there exists a window of opportunity to intercept the process. Any interventions that increase viral clearance, for example, would fit into this

category. Another possible approach would be to target the process by which viral DNA integrates into the host chromosome, a relatively rare event that greatly increases the incidence of cancer. One study, for example, has postulated that chronic inflammation and the subsequent generation of reactive oxygen/nitrogen species (ROS/RNS) are harbingers of DNA damage, causing high HPV integration rates. Furthermore, smoking, cervical trauma induced by high parity, co-infection with other STDs, and long-term use of oral contraceptives have all been linked to cellular oxidative stress [48]. Thus, breaks in the DNA induced by this oxidative stress increase the probability of viral integration [49]. Interestingly, HR-HPV types integrate more frequently than do LR-HPV. The difference in integration occurrence suggests a distinction in the molecular variation and/or susceptibility between high- and low-grade lesions. Therefore, progression to cervical, and some anogenital, cancers is dependent on the presence of HR-HPV integration into the host genome. Furthermore, scientists are finding certain patterns in HPV integration events. In particular, the E2 ORF region of the viral genome is strongly preferred over other sites of integration, and integration at this site, with its accompanying loss of functional E2 protein, is linked to an increase in E6 and E7 oncogene expression [50, 51]. Consequentially, integration leads to uncontrolled expression of the oncogenes and ultimately to cellular transformation.

The role of chronic inflammation and its link to radical species production in cancer pathogenesis is widely recognized. If increased levels of oxidative stress and ROS do indeed increase the frequency of integration and cancer, one would predict that antioxidant mechanisms that counteract the generation of radical species could therefore exert chemopreventative and chemotherapeutic effects; such mechanisms have indeed been described [52, 53]. In contrast, other groups are studying ways to therapeutically harness the power of oxidative stress for actions against cancer cells. For example, the antimalarial drug, artemisin, was found to induce apoptosis in cervical cancer cells. The mechanism of action involves artemisin interacting with reduced iron to generate oxidative stress through ROS, as well as the destabilization of mitochondrial oxidative mechanisms [45, 54]. These discoveries merit further research that continues to seek ways of preventing HPV-mediated oncogenesis.

Screening for HPV and Cervical Cancer: The ultimate goal of cervical cancer screening, as outlined by the American Cancer Society (ACS) guidelines for the prevention and early detection of cervical cancer, is to prevent morbidity and mortality by determining appropriate treatment plans. In detecting the presence of HPV in the cervix, screening methods should serve to distinguish transient from persistent infections, and to effectively diagnose disease while minimizing or avoiding unnecessary complications induced by these techniques. Because 50 percent of women diagnosed with cervical cancer in the U.S. have never been screened, the importance of diligent watchfulness cannot be over-stated. Moreover, when HIV^+ women comply with regular detection methods and schedules, their otherwise 10-fold higher risk of progression to invasive cervical carcinoma is diminished [30, 31, 55]. Earlier detection corresponds to better prognosis [56]. Thus, it is ideal for precancerous women who are at significantly higher risk for invasive cervical carcinoma to be promptly identified and to undergo intervention. Generally, it is recommended that cervical cancer screening start no earlier than age 21 for both non-vaccinated and vaccinated women, implying that cervical monitoring is

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an integral component of preventing invasive disease at all stages [5]. Because cervical cancer detection programs are the most expensive preventive measure in developed countries, enhancements to existing screening techniques, or the development of completely innovative and economical methods would be beneficial.

The most utilized and successful of screening methods in lowering cervical cancer incidence rates (by 70 percent) is exfoliative cervicovaginal cytology, or the Pap test. The Pap test satisfies the aforementioned objectives for reducing the occurrence of squamous cervical carcinoma through appropriate screening [5]. Pap tests are recommended for all sexually active women and/or women ages 21 and older. Now, a modified liquid-based version of the Pap smear is available. In a liquid-based Pap test such as Cytoscreen or Thinprep, the cells are first filtered and fixed in preservative. Then the specimen is smeared on a glass slide, which is slightly in contrast to the conventional method of directly smearing a sample onto a microscope slide. Other tests such as visual inspection with acetic acid (VIA) are useful in resource-limited settings. Further modifications of VIA include magnified visual inspection with acetic acid (VIAM) and visual inspection with Lugol's iodine solution (VILI) [57]. Colposcopy, though considered more diagnostic, also allows a magnified visualization of abnormal cervical cells [56]. Other cervical cancer screening tests may also be applied: pelvic examination – involving internal palpation of the reproductive organs; automated cervical screening techniques – supplemental imaging that reduces false positives from the cytological tests; computer imaging; polar probe – measuring the differences in electrical stimulation between normal and abnormal cervical tissue; laser-induced fluorescence – measuring spectroscopic differences in florescence between normal and diseased cervix; speculoscopy – cervical inspection using acetic acid with chemiluminescent light; and cervicography – photo development while using acetic acid [56].

Complementing the Pap test is the detection of HPV DNA. The direct testing for HPV DNA is becoming standard in many cervical cancer screening regimens, as its combined use with liquid-based cytology has generated results with even better sensitivity (up to 100 percent) for predicting high-grade cervical dysplasia [58]. HPV DNA is usually obtained from cervical scrapes and/or biopsy specimens, and recent clinical studies continue to assert the unique value of HPV-DNA testing over cytology [59-61]. Nevertheless, only time will tell the extent to which the Pap test will be replaced by the more economically appealing HPV-DNA test. To date, the FDA has approved five HPV-DNA tests: the Hybrid Capture 2 HPV DNA test, the Cervista HPV HR test, the Cervista HPV16/18 test, the Cobas 4800 HPV test, and the Aptima HPV assay. Other commonly used assays not approved by the FDA include PCR and Southern Blot hybridization, the latter being the laboratory gold standard. Some other recent innovative HPV detection methods are complete HPV genotyping, HPV mRNA detection, HPV load quantitation, identifying HPV integration, p16 ELISA, methylation profiles, and the E6 Strip test [62, 63].

Cervical cancer incidence and mortality seem to be on a downward swing in the U.S., primarily due to cytological gynecologic screening through the Pap test. Nevertheless, the global burden of HPV infection remains. Because no single detection method is optimal for every situation, it is essential that novel techniques to identify cervical cancer and HPV infection be continuously developed. Ideally, these new procedures/assays would allow clinicians to easily

distinguish between low-risk and high-risk HPV status without causing undue concern in patients with transient infection. However, the cost of cervical cancer screening programs, even in developed countries, may hamper the implementation of these new advances.

3. Current clinical treatmemts

Staging and Treatment Options: Despite prevention initiatives to stay the tide of carcinogenesis caused by HR-HPV, thousands worldwide still require treatment. Currently, the most effective course of managing cervical carcinoma involves surgery and/or radiation. However, surgery provides the management team better insight into the extent of the disease because it allows the assessment of lymph node involvement [64, 65]. The surgical options available range from total removal of the cervix (radical hysterectomy) to less extreme options that preserve the fertility of the patient (radical trachelectomy), and are somewhat contingent upon disease progression. Other procedures such as chemotherapy, radiotherapy, or a combination thereof are routinely used, and their utilization depends on cancer stage as well. Factors further impacting the course of management include pregnancy; disease recurrence; fertility preservation; cervical location of the lesion; cancer type; age and general physical health. But the most important treatment determinant for cervical cancer is stage of disease, and years of clinical trials and case studies have formed the standard by which each stage is managed.

The staging of cervical cancer is based on the physical examination and is established by the International Federation of Gynecology and Obstetrics (FIGO). The World Health Organization reaffirms the FIGO organization of cervical carcinoma progression into four stages (I-IV):

Stage I: cancer found only in the cervix.

Stage II: cancer found beyond the cervix in the vagina, but has not spread to the pelvic wall and excludes the lower third of the vagina.

Stage III: cancer has spread to the lower third of the vagina and/or pelvic sidewall, and includes cases with kidney involvement.

Stage IV: cancer has grown beyond the pelvis and involves tissue of the rectum, bladder, and/or distal sites of metastasis.

The four main stages are then organized into sub-categories that further describe the extent of growth, adjacent tissue involvement, local organ participation, and metastasis to distal sites through the lymphatic system [66]. Present challenges to the optimal clinical staging of cervical cancer include complications associated with parametrial invasion, tumor location/size variation, and lymph node metastases, but developments in imaging are changing the tide [67, 68]. The ACS asserts that individuals diagnosed with cervical cancer from the early stages through late stage II actually have a survival rate greater than 50 percent. Cancers diagnosed at stage III and IV yield 30 and 15 percent survival rates, respectively. However, survival rates approach 100 percent if the cancer is caught early enough. The tools used in the process of staging and subsequent treatment of cervical cancer are numerous and will be mentioned only

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as they fit the scope of this review. Therefore, the sections below are not intended to represent a comprehensive discussion of these modalities.

Imaging: One of the most important aspects of cervical cancer treatment is identifying and evaluating abnormal tissue morphology using radiological technologies such as magnetic resonance imaging (MRI), x-ray computed tomography (CT scan), and positron emission tomography (PET). Because the effectiveness of these devices depends on clinical expertise and equipment, diagnostic imaging possesses several inherent discrepancies [64]. However, many researchers have begun studies that will help to improve these methods and/or to enable clinicians to draw better conclusions. Advancements in the functionality of diagnostic imaging are making it easier than ever to assess and exploit tumor parameters such as cellularity, blood flow, and glucose metabolism. Recent studies are showing that the glucose analogue, fluorine-labeled fluoro-2-deoxy-D-glucose (FDG), is particularly useful in gauging tumor metabolic activity. When combined with PET (FDG-PET), FDG is considered to have high sensitivity in detecting primary cervical tumors [69]. Additionally, combining PET and CT is becoming more acceptable as a way to eliminate the guesswork involved in evaluating metastasis to lymph nodes [70]. However, MRI is the preferred imaging method in managing cervical cancer due to the high quality of anatomic resolution it provides in the pelvis; this enhances its ability to evaluate primary tumor volume [67, 71]. New developments in MRI technology such as diffusion-weighted MRI (DWI) compare normal and abnormal tissues based on the Brownian motion of water molecules, the movement of which impacts cellular membrane integrity. However, these features may not be as distinct or reliable in excessively necrotic tumors. Another derivative of MRI is dynamic contrast-enhanced MRI (DCE-MRI), which provides an unprecedented appraisal of tumor vasculature through contrast distribution over time. Therefore, DCE-MRI may prove to be distinctly helpful in ascertaining a tumor's unique response to therapies [67, 72].

Surgery: Surgery is the advised treatment for cervical carcinoma at stages I and II. The preferred procedure, radical hysterectomy (RH), has a 75 to 80 percent cure rate according to the NCI and is the gold standard of treatment. RH is the complete removal of the uterus, cervix, and upper portion of the vagina, and involves measuring metastasis to the parametrial and pelvic lymph nodes [73, 74]. Other procedures, such as total and subtotal hysterectomies, do not require the removal of the vagina and cervix, respectively. At the early phase of stage I, less invasive techniques labeled *excisional* therapies selectively remove pathologic tissue [75, 76]. Large loop loop excision of the transformation zone (LLETZ) and cold knife conization procedures are classified as excisional therapies and are used without great risk of the cancer recurring. Conical biopsies, or the removal and microscopic examination of presumably abnormal tissue, may suffice in some situations. *Ablative* therapies such as laser and cryosurgery are utilized in expunging carcinomas *in situ* of lesser risk. This distinction between therapies is supported by studies showing that lesions from more progressed carcinomas return at a higher rate when treated with ablative techniques as compared to excisional ones [76]. In some instances, neither excision nor ablative therapies are suitable for the grade of disease, and hysterectomy is recommended. Indeed, patients not interested in fertility loss or those with lymph and vascular space involvement (LVSI) should elect for RH (RH). Nonethe-

less, for nulliparous women radical trachelectomy, or the removal of the uterine cervix only, with lymphadenectomy is always an option up to late stage I (Ib1) [77-79]. According to the ACS, RH is always optional for those diagnosed with early stage squamous cell cervical carcinoma, but it is strongly suggested for adenocarcinoma cervical cancer.

Surgery is immensely valuable for determining lymph node status, which is strongly correlated to survival [80]. The risk of lymph node metastasis is increased by 10 percent if tumor invasion reaches between 3 and 5 mm beyond the primary lesion. If this occurs, the NCI recommends that a modified RH comprising pelvic lymph node dissection be performed even in early disease stages. Furthermore, if metastasis to the lymph nodes or parametria is found, their removal is indicated as well as radiotherapy or chemo radiotherapy post-operatively. Post-surgery radiotherapy is also indicated if the tissue collected during surgery has a positive margin, which alludes to residual cancer and commonly occurs in late stage I. Though it is advisable to use radio and chemotherapies in stage II, some experts also support hysterectomy following these procedures. In summation, surgery yields its most potent benefits in the earlier stages of cervical carcinoma, though this fact can be viewed as a great limitation in the case of advanced disease. Other limitations of surgery include pelvic sepsis and thrombosis as well as vesicovaginal fistulas. However, opting for surgery in the management of cervical cancer may prevent vaginal stenosis, spare ovarian function, and protect local organs from future complications. There is no doubt that surgery is vital in the prospective treatment planning of the patient following operation because it allows the delineation of tumor metastasis [65]. However, surgical options are contingent upon early detection, and thus time will always be one of the most important factors in predicting a prognosis. Fortunately, advances in the field of surgery have given patients better alternatives that are less invasive (i.e. laparoscopic surgery), and these procedures, together with non-invasive therapies, will continue to benefit those for whom preventive measures have failed.

Radiotherapy: More advanced cervical cancer (stage IIb and higher) is treated with radiotherapy (RT), chemotherapy, or a combination of the two. However, surgery and RT both aim to completely eradicate malignancy and have equally positive results in attenuating disease in the initial phases. RT is generally substituted for surgery when circumstances render an operation less than optimal as in the case of elderly patients, obese patients, or patients with several co-morbidities [80, 81]. Usually younger patients elect for surgery in order to preserve sexual function and to avoid side effects such as vaginal dryness and narrowing caused by scar tissue (as described by the ACS). Elderly patients (65+ years), who account for 20 percent of cervical cancer cases, usually choose the less invasive RT [82]. Cancer cells are more susceptible to radiation than are normal cells because radiation uses high-energy particles to target and kill rapidly dividing cells through DNA damage. In general, there are two main types of radiation therapy: external beam radiation, and internal (implant) radiation usually referred to as intracavitary brachytherapy (BT). External beam radiation therapy (EBRT) is aimed wholly at the pelvis, much like a regular x-ray, and is often accompanied by cisplatin chemotherapy. The chemotherapy is added to enhance the effectiveness of the radiation and to treat metastasis to lymph nodes [83]. The selectivity of EBRT for cervical sites is enhanced by combining its use with a CT scan via 3-D conformal radiation therapy (3-D

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CRT), which better concentrates radiation to specific regions of interest. BT involves the placement of a radioactive device in the uterus or vagina. Low or high-doses of radiation are given over long or short periods of time, respectively, and in accordance with the Manchester triple source system. Also, BT is a highly specialized method of radiotherapy requiring costly equipment and extensive technical training, as device positioning is very important in treatment success [84].

Maximum dose toleration by adjacent tissues such as vaginal tissue is a major limiting factor in all radiotherapy procedures. Therefore, many strategies attempt to radiosensitize the appropriate tissues (*See Neoadjuvant and Combination therapies,* below) before exposing them to radiation. Intensity modulated radiation therapy (IMRT) offers a unique advantage through the virtual mapping of tumors so that the delivery of radiation is focused and minimal tissue damage occurs to the surrounding vital structures. Supplemental techniques to improve the localization of radiation such as hyperthermia, neutron therapy, and hypoxic cell sensitizers still need refinement, and are not routinely utilized [85, 86]. Despite potential high levels of toxicity necessitating close patient monitoring, RT is a powerful tool in managing cervical cancer. The side effects of RT that have caused the greatest concern include hematologic imbalances, GI distress, GU complications due to hydronephrosis, and secondary malignancy. Future improvements in the field of RT to treat cervical cancer must first rectify the issues of maximum dose tolerance by exploiting radiosensitizing methods in order to compensate for the systemic and potentially oncologic risks associated with radiation.

Chemotherapy: Chemotherapy is the principal treatment option for recurrent and metastatic cervical cancer, and it is recommended by the ACS for the management of late stage I of cervical cancer or higher. Chemotherapy can be curative or palliative. In early cervical disease, chemotherapy can be curative, but may also be given in more advanced stages (stage IV and recurrence) to alleviate the ravaging effects of the cancer itself and its related symptoms. Chemotherapy may also be given in the later stages to postpone the toxicities associated with it until absolutely necessary. Thus, designing optimal regimens suited for each case is essential to attaining both patient comfort and treatment success. Chemotherapy can also be given adjunctively to strengthen the effects of primary treatment, or provided post-operatively [87]. In fact, the ways in which chemotherapy can be administered are numerous, ranging from single, doublet, triplet, or quartlet-agent regimens to combined chemoradiation routines. However, a few agents such as cisplatin, paclitaxel, and ifosfamide are distinguished for being somewhat autonomously potent [88]. The success of single-agent chemotherapy generally depends on histology. For example, although cisplatin is most effective for treating squamous cell carcinomas (SCC), paclitaxel has been shown to improve the median survival of non-SCC type patients as compared to others who did not receive paclitaxel [89]. Furthermore, paclitaxel yielded a response rate (RR) of 33 percent, surpassing other single agents in non-SCC type cervical cancer treatment [88, 89].

Cisplatin, a platinum-based agent, is the accepted standard of chemotherapy for cervical cancer, and it improves survival in chemoradiation recipients as compared to the use of other chemotherapeutic drugs [90-94]. However, its adequacy in improving survival and quality of life in palliative management has been questioned. Some tumor cells acquire resistance to

cisplatin, and so non-platinum chemotherapy or higher doses of cisplatin, in these cases, are indicated [81, 91]. In the cases of cisplatin resistance or disease recurrence, non-platinum-based agents such as topotecan, vinorelbine, irinotecan, paclitaxel, mitomycin c, and ifosfamide are sometimes combined with cisplatin. Topotecan and 5-fluorouracil (5-FU), among other combinations, seem to produce an additive effect with cisplatin to reduce its toxicity, increasing its RR from 20 to 50 percent [90, 91, 95]. Similarly, when paclitaxel is combined with cisplatin, a high RR of 46 percent is reached for late stage IV cervical cancer and is accompanied by decreased hematologic complications. However, a Gynecologic Oncologic group study reported that consistent, weekly schedules of cisplatin alone are less toxic than cisplatin combined with other agents, particularly 5-FU [92, 96]. Sanazol and tirapazamine are relatively new chemotherapeutic agents that specifically target and destroy hypoxic tissue by dissociating into free radicals that cause DNA damage. Therefore, drug selectivity for hypoxic tissue will result in greater cytotoxicity among malignant cervical cells [81]. Multiple-agent regimens may also include the use of antibodies targeting a tumor's peculiar characteristics. For example, if a particular tumor markedly over-expresses EGFR-1, it would be appropriate to include Cetuximab in treatment, or Bevacizumab in the case of extreme vascularity [95].

Combination Therapies: Multidisciplinary treatment might be indicated throughout any of the stages of cervical carcinoma, mainly depending on its aggressiveness. In fact, it is quite common for treatment schedules to include chemotherapy, radiation therapy, and surgery [81]. The concurrent use of chemotherapy and radiation therapy is reported by the NCI to reduce cervical cancer mortality by 30 to 50 percent, particularly in late stage II. Alternatively, neoadjuvant therapy, defined as a specific sequence for delivering any treatment before a definitive therapy such as surgery or radiotherapy, may be employed. Neoadjuvant therapy is intended to prime the target tissue, thus making it more susceptible to primary treatment [71, 93]. Neoadjuvant chemotherapy (NACT) is often administered before radiation in order to radiosensitize solid tumor cells and to decrease tumor size and hypoxic cell numbers. In few instances, NACT could potentially provide patients with the option of surgery even though it may have been unfeasible prior to NACT. Moreover, researchers are finding that patients who receive sequential NACT-RH have a 10 to 15 percent survival advantage five years after treatment [97]. In cases when surgery does not completely remove all traces of abnormal tissue as anticipated, chemotherapy or radiation must be given post-operatively to inhibit local and distal metastasis through the lymphatic system. Hence, there is no doubt that concurrent chemotherapy and radiation therapy can improve survival in women with locally advanced cervical cancer or recurrent cancer [93, 98]. Radiation treatment alone does not contain cancer in 35 to 90 percent of patients, but chemotherapy given with radiation treatment yields much higher survival rates. The chemotherapeutic drugs most commonly used with radiation are cisplatin, 5-FU, mitomycin C, and hydroxyurea, though cisplatin produces the largest increase in survival by reducing mortality and recurrence [93, 94]. Many times, the sensitizing effects of drugs are needed to accentuate the value of other treatment methods, as is the case with histone deacetylase inhibitors, decitabine and valproic acid, that radiosensitize tumors for RT [81]. Thus, researchers may build and forge new applications through trials that study combination therapies.

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4. Molecular therapies in development

Therapeutic Immune Strategies: The development of the prophylactic vaccine has forever changed the course of HPV-mediated cervical disease. Nevertheless, it is clear that there is still an immense need for therapeutic options, especially in developing countries where the positive, yet costly measures of preventative initiatives remain to be implemented. In contrast to prophylactic vaccines that target the L1 and L2 proteins and are protective against HPV infections, therapeutic vaccines would ideally target molecules such as E6 and/or E7 post-infection, which are directly linked to HPV-mediated carcinogenesis [99]. Therapeutic vaccines may be constructed in a variety of ways, as described below [100].

Live, vector-based vaccines, bacterial and viral, can generate very robust cell-mediated and adaptive immune responses, and because of this they are preferred over peptide/protein vaccines. Specifically, bacterial vectors function well when they are packaged with antigen (genes or proteins), thereby alerting antigen-presenting cells (APCs) to initiate an immune response. Though several bacterial vectors have been tested, *L. monocytogenes* is a prototypic example. Simply, *L. monocytogenes* stimulates antigen-specific CD8+ and CD4+ T cell responses following its evasion of immune destruction by releasing *Lm* toxin to avoid phagosomal lysis. However, the most appealing factor of the *L. monocytogenes* vector is that the immune response can be easily controlled by antibiotics should the body react adversely to *Lm* [101-104]. With regards to viral vectors, a few viruses, such as the vaccinia virus, adenovirus, vesicular stomatitis virus, and alphavirus, have distinguished themselves and show great promise. In fact, researchers have discovered that when an adenovirus vector is used to deliver calreticulin and HPV E7 antigens, the size of E7-expressing tumors in mice decrease [105]. A highly anticipated viral vaccine candidate is the TA-HPV vaccine, consisting of both HPV16 and HPV18 E6 and E7 antigens and a vaccinia virus vector. TA-HPV is safe and efficient in stimulating either a specific CTL response or a serological response, which might depend on the epigenetic patterns of each individual [106]. Similarly, the MVA E2 vaccine is also packaged with the vaccinia virus, and uses the bovine papillomavirus E2 protein to repress E6 and E7 transcription. MVA-HPV-IL2, currently undergoing a phase III clinical trial for CIN 2-3 treatment, utilizes a modified vaccinia Ankara viral vector, and uniquely contains HPV16 E6 and E7 DNA as well as IL-2 [99, 107]. The co-expression of a cytokine with HPV antigens induces a stronger immune response by stimulating dendritic cell maturation, though the refinement of viral vector tools must include solutions for overcoming pre-existing immunity. To rectify this, Cox-2 inhibitors are presently being tested to offset such immune interferences, thus allowing greater exploitation of a potentially powerful treatment. However, safety factors remain a high priority when viral vectors are considered, and these vectors must be properly constructed for use in both immunocompetent and immunocompromised individuals [100].

In peptide-based vaccines, antigens from HPV are directly administered to elicit a response from dendritic cells (DCs) *via* toll-like receptor (TLR) activation [108]. The peptide vaccine platform is ideal for mass production, but the breadth of its efficacy is limited by the expression of only one major histocompatibility complex I (MHC I) phenotype; protein-based vaccines are not encumbered in the same way. However, if the specific immunogenic epitopes on

peptides could be identified it would greatly remedy this difficulty. Some investigators are taking a different approach by overlapping peptides with a broad range of different epitopes to obtain a greater immune response [109]. Meanwhile, other researchers have focused on the development of vaccines that utilize a synthetic E7 peptide component to clear HPV-mediated tumors in mice, such as TriVax [110]. Unfortunately, a limitation of both peptide- and protein-based vaccines is low immunogenicity. This challenge, however, is no longer intractable with the advent of various immunomodulatory adjuvant agents such as TLR ligands, cytokines, and lipids, all of which help to stimulate a robust immune response. Another recently popular strategy thought to increase protein/peptide vaccine potency involves the Pan HLA-DR epitope peptide (PADRE), which binds MHC class II molecules with much stronger affinity [108]. Following the success of PADRE and other similar technologies, more potent enhancers of peptide vaccines such as 4-1BB ligand, CpG oligodeoxynucleotide, mutant cholera toxin, and lipopeptides are now emerging [111].

In general, protein-based therapeutic vaccines, like peptide-based vaccines, are advantageous for safety and tolerability. Although protein-based vaccines are not restricted by MHC compatibility, they cannot directly stimulate cytotoxic T lymphocytes. Protein vaccine adjuvants that are considered to compensate for this weakness in protein vector therapy include liposome-polycation-DNA and the saponin-based ISCOMATRIX. The ISCOMATRIX is an adjuvant complex consisting of phospholipids and cholesterols, and it causes a rapid innate immune cell response [112]. In general, any strategy that increases antigen uptake by APCs, antigen presentation, or the CTL response is expected to improve the immunogenicity of a protein. One protein-based therapeutic vaccine in clinical trials is TA-CIN. Essentially, TA-CIN is a mixture of L2, E6, and E7 proteins from HPV16. The L2 antigen launches a humoral response, and the E6 and E7 proteins induce T cell responses. However, further investigation revealed that TA-CIN is even more powerful when combined with the TA-HPV vaccine [113-115]. Another strong protein-based vaccine candidate, due to its safety and ability to induce lesion regression in various HPV-related diseases, is HspE7 [116]. HspE7 is a fusion product of HPV16 E7 and the *Mycobacterium bovis* Hsp65 proteins. Another potential strategy to improve immunogenicity in protein-based therapeutic vaccines is the use of the Fve adjuvant, which is derived from a fungal protein originating in the *Flammulina velutipes* species, and has been shown to produce potent humoral and cellular immune responses. The antitumor effects of Fve in HPV-mediated cancers are attributed to its ability to induce IFN-gamma secretion and to stimulate T helper and CTLs in tumor-bearing mice [117]. Our knowledge about how to apply protein and peptide therapeutic vaccines against cervical cancer is steadily increasing. The immediate next step is to follow up with successful clinical trials, and to implement the most useful of these methods, or a combination thereof.

One advantage of DNA-based vaccination is its capacity to increase immunological memory through constant antigen production. Because the immune response itself is not anti-vector, multiple vaccinations are possible. Moreover, the antigens produced by DNA vaccines can be delivered in a variety of ways, resulting in stimulation of both APCs and T lymphocyte immune defenses [118, 119]. However, DNA vaccines also present the challenge of overcoming low immunogenicity due to limited APC specificity. Therefore, future developments must

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focus on antigen modifications so as to elicit a stronger DC adaptive immune response. One such strategy increases the number of HPV DNA plasmid transfection events in DCs. These DCs will then present antigen to, and ultimately activate, naive CD4+ and CD8+ lymphocytes [119]. However, researchers still must determine the most efficient and effective way to deliver HPV DNA to DCs. A fairly recent investigation discusses a novel method to administer a dose-driven vaccine by gene gun technology, which forms a DNA-coated stream of gold particles targeting Langerhans cells in the skin [120]. Other studies justify the use of cell membrane permeabilization by electroporation, thereby causing cells to experience an electric shock and maximizing cellular uptake of DNA [121, 122].

Electroporation also leads to inflammation and cytokine recruitment, thus enhancing the immune environment. Additionally, electroporation was found to be particularly effective against E7-expressing tumors. The VGX-3100 plasmid DNA vaccine, targeting E6 and E7 antigens of HPV16 and 18, seemed to have great efficacy when it was combined with electroporation administration [123]. Furthermore, clinical trials attest to the value of this particular method of treatment delivery in CIN 2 and 3 lesions. Strategies that increase transfection efficiency are continuously being sought through experimentation with diverse routes of vaccine administration, such as intramuscular *versus* intradermal techniques. The efficient intramuscular administration of DNA is achieved *via* microencapsulation, which uses a biopolymer that surrounds the plasmid to prevent degradation by nucleases. Conversely, intradermal administration involves skin patch tattooing using microneedles [124]. One encapsulated DNA-based vaccine that has been tested with both administrative routes is the amolimogene bepiplasmid (also known as ZYC101a), which contains T cell epitopes and HPV16 and 18 E6/E7 viral protein fragments [125, 126]. In this study, it was concluded that intramuscular methods were more effective [127, 128].

Another strategy to strengthen DNA-based vaccines focuses on improving DC antigen processing. Those cells that have become transfected with HPV DNA material may be prompted to generate a more potent immune response through codon optimization or demethylation techniques that will increase gene translation efficiency [100]. These methods work to improve antigen translation and expression in cells with HPV DNA. Additionally, DNA vaccination with the MHC class I chaperone molecule, calreticulin, was shown to increase the CD8+ immune response, thereby leading to an antitumor effect [129]. It is also possible to improve antigen processing through the MHC class II pathway. For instance, the E7/LAMP-1 vaccine allows antigen to be further sorted in endosomal and lysosomal compartments, thus priming CD4+ and CD8+ lymphocytes for a greater response as compared to the administration of E7 alone [130]. Substitution of the MHC class II peptide, CLIP (Class II-associated peptide), for the PADRE peptide in the invariant chain is a promising strategy to not only increase antigen presentation, but also to secrete cytokines that stimulate T cell proliferation, thus resulting in greater CD4+ lymphocyte activity [131, 132]. Other methods of improving antigen presentation include cross-presentation by extracellular proteins like HSP 70, up-regulation of MHC II expression on the surface of DCs, and single chain trimer technology (SCT). SCT involves the fusion of HPV antigen to the MHC class I molecule, beta-2 microglobulin, resulting in the appropriate recognition of antigen and action against an E6-expressing tumor [133, 134].

RNA replicon-based vaccines have some advantages over DNA vaccines: 1) they are less likely to integrate into the host genome, thus decreasing the risk of cell transformation and 2) they can potentially generate more protein than can DNA methods. Of course, RNA replicon-based vaccines may be introduced into the host as DNA. From here, the cell can then transcribe the DNA molecule into RNA, but without the structural genes needed to construct viral particles. Therefore, no antibodies are produced against viral immunologic molecules and administration can be repeated. One significant limitation of using replicons is that RNA is inherently unstable. However, the use of a DNA-launched RNA replicon could surmount this difficulty, and concerns of gene integration could be addressed by designing the DNA to self-destruct following gene expression. Because immunologic cells undergo apoptosis in this process, it is necessary to fuse the HPV antigen to an anti-apoptotic protein, otherwise DC numbers will be drastically reduced [100, 135, 136]. The Kunjin flavivirus has the potential to accomplish the same goal by delivering the desired antigens into cells without immediately inducing apoptosis, thus prolonging the window of time for antigen presentation by transfected cells and improving overall immunogenicity [137-139].

Dendritic cell-based vaccines can be prepared in several ways: by introducing exogenous HPV antigen via endocytosis in to DCs; by infusing DCs with E6/E7 DNA or RNA through electroporation; or, the antigen may be packaged together with liposomes or nanoparticles to be delivered into DCs [140]. DC interactions with T cells and the subsequent perpetuation of the immune signal are essential features that determine whether an organism will demonstrate a strong immune response, or whether it will exhibit immune tolerance (e.g. if the DCs are immature) [141, 142]. Essentially, DCs activate T cells and T cells, in turn, mediate DC apoptosis. Therefore, it has been proposed that prolonging DC survival may strengthen and lengthen the initial T cell stimulation [143]. However, because the idea of combining HPV vaccines with anti-apoptotic proteins has not gained much popularity due to the possibility of cellular transformation, other approaches such as co-administering vaccines with siRNAs targeting pro-apoptotic proteins are gaining traction. Designing shRNAs directed at FasL produced by DCs to promote T cell apoptosis, for example, could increase the number of T cells stimulated [144]. DC activation can also be prolonged by deactivating the negative regulation of cytokine signaling through SOCS-1, which acts on the Jak-Stat pathway [132, 145, 146].

Because tumor cell-based vaccines have shown promise in malignancies like melanoma, colon and prostate cancers, many subscribe to this paradigm as the key to solving the cervical carcinoma dilemma. The idea of manipulating tumor cells into becoming more discernible by the immune system is based on their expression of immunomodulatory cytokines like IL-2 and IL-12 [147]. Other studies have found that engineering tumor cells to secrete pro-immune cytokines such as GM-CSF produces antitumor immunity as well [148]. The advantage of using tumor cell-based vaccines is that multiple antigens can be targeted on the surface of a tumor, thus increasing the chance that a single cell or group of cells expressing those antigens will be eliminated by the immune system. As can be expected, such an individualized treatment is costly and may border on the impractical as compared to other recent advances in the field of cervical cancer vaccination. Furthermore, patients who qualify for tumor cell-based vaccination would be at greater risk in the receipt of new cancer cells than if they were to employ a treatment plan composed of existing therapies [149].

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Of course, every approach has its advantages and disadvantages, but combining several therapeutic vaccines into a single regimen may offer synergy and thus strengthen treatment efficacy. For example, one preclinical study tested a prime-boost vaccination model. The immune system was first primed with a DNA vaccine consisting of HPV16 E7 and LAMP-1 (Sig/E7/LAMP-1). Then, a booster dose of Sig/E7/LAMP-1 was given again to maintain and increase the T-cell response over a longer period [150]. Because several prime-boost studies have yielded continuous positive results in safety and efficacy, we can expect to see similar combinatory therapeutic trials in the future.

RNA-based Therapy: RNAs have the unique ability to form double-stranded molecules by hybridizing with complementary antisense RNA. It was established years ago that antisense E6/E7 RNAs in a plasmid could stagnate cellular growth [151]. More recently, the ability to design antisense oligodeoxynucleotides (ODNs) that specifically bind E6/E7 RNA molecules at the translation initiation region with high affinity have made the use of translation inhibition more feasible for achieving cervical carcinoma treatment goals. Later studies have confirmed these results, and suggest that stronger, additive effects may also occur when antisense ODNs are designed with adjacent mRNA targets in mind [152, 153].

Tristetrapolin (TTP) is an RNA-binding protein with anti-cancer properties, and yields its effects by binding to AU rich regions of mRNA and promoting their destruction. These AU-rich elements (AREs) of mRNA, located in the untranslated region of the strand, are naturally involved in regulating cellular growth and inflammation *via* mediators such as TNF-alpha and COX-2. Therefore, the affinity with which TTP binds these elements makes this interaction an attractive point of intervention. For example, HPV^+ HeLa cells exposed to TTP demonstrated higher levels of p53 as compared to untreated cells. Additionally, in the presence of TTP, these same cells acquired the ability to inhibit E6-AP expression, suggesting a possible mechanism for the rescue of p53 [154]. Other RNA-based methods targeting HPV include the use of siRNAs directed against E6 and E7. siRNAs cause the cleavage and degradation of homologous sequences through their participation in the RNA-induced Silencing Complex (RISC). Researchers are now investigating the use of vehicles such as shRNAs to target and destroy RNAs of interest. However, better systems for delivering shRNAs to the nuclei, and better ways to access cellular uptake mechanisms for siRNAs, are needed [155].

Antibody-based Therapy: The use of monoclonal antibodies in cancer treatment is an appealing concept due to the selectivity and specificity with which an antibody can bind to the molecule of interest. Molecules participating in tumor progression can be targeted by antibodies through three general mechanisms: 1) Recognition of specific tumor-associated receptors, such as EGFR; 2) Binding to immune effector cells, and 3) Binding to tumor-promoting molecules such as VEGF. Though no monoclonal antibodies have been approved for the treatment of cervical cancer, researchers are accruing more convincing evidence of their value [156]. As with most cancers, cervical carcinomas possess a dynamic vascular network. Thus, much investigation has gone into developing biologic agents that target molecular pathways associated with vascularization, such as those involving vascular endothelial growth factor (VEGF). Bevacizumab is an angiogenesis inhibitor that ultimately delays vasculogenic processes, and has long been used effectively in the treatment of other malignancies such as colorectal cancer. The

discovery of Bevacizumab's anti-angiogenic properties in recurrent cervical cancer during a phase II clinical trial has now warranted further investigation within the context of both single-agent and combination therapies [157]. Another antibody, Cetuximab, has a high affinity for epidermal growth factor receptor (EGFR), which is influential in cell differentiation processes. However, Cetuximab has distinguished itself as effective only against growths of squamous cell origin. Thus, other EGFR inhibitors such as gefitinib, erlotinib, and lapatinib are being investigated [158, 159]. In one research model, Cetuximab was shown to inhibit tumor cell growth following exposure to ionizing radiation, which induced EGFR pathway activation and VEGF over-expression [160]. Though others have reported Cetuximab to be more limited in activity in some populations [158], the roles of both VEGF and EGFR in cervical cancer remain under intense study. Another unique approach in helping to further define molecular targets using antibodies is to structure them against a particular domain of an HPV oncoprotein. By designing mAbs against HPV16 E6 zinc-binding domains researchers are able to map key peptide sequences, and potentially interfere with cell transformation mechanisms affecting p53 tumor suppressor levels [161].

Small Molecule Inhibitors and Antiviral Leads: As the field of prevention continues to advance (e.g. through the development of prophylactic vaccines), one might ask why additional resources should be directed towards the discovery of small molecule HPV inhibitors. The short answer is that due to the timeline of disease progression, it will be a few decades before these preventative measures will make a significant impact on the disease burden. In the meantime, infected women and others who do not benefit from these approaches have access to only a limited, and frequently inadequate, set of options such as lesion removal. In addition to the obvious drawbacks of surgical treatment, such as invasiveness and cytodestruction, it is well established that viral persistence is mainly responsible for disease, especially among the elderly and the immunodeficient [162]. Hence, lesions do frequently recur. In addition to these concerns, as previously stated, a low risk perception might further short circuit preventive measures, thus increasing the need to contain HPV therapeutically. Moreover, the fact that one-third of all cervical cancers are caused by types of HPV that are not included in the current vaccines [163] should maintain a sense of urgency with regards to developing more comprehensive and long-lasting approaches. Though no small molecule inhibitor of HPV has yet been approved, a significant amount of antiviral agent research has focused on five major potential targets for intervention: 1) Inhibition of E1/E2 interactions, 2) E6 and E7 oncoprotein blockade, 3) Direct interference with E6AP-mediated p53 degradation, 4) Interference with interactions between HPV and other apoptotic factors (i.e. Bax and FADD), and 5) Stalling the ubiquitin proteasome system to reduce the degradation of anti-tumor proteins. The following sections will discuss these topics in greater detail.

Studies have revealed that certain host proteins are co-opted by the virus and used to carry out viral functions. For instance, the bromodomain protein, Brd4, which normally serves as a regulator of cell growth and transcription, has been implicated in the tethering of bovine papillomavirus (BPV) episomes to chromosomes in dividing cells [164, 165]. Also, it was recently published that Brd4 not only binds to the HPV regulatory protein, E2, aiding in many of its functions, but also stabilizes it [166, 167]. Although the ways in which Brd4 can interact

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with the bovine and human papillomaviruses may differ, the concern that Brd4 may play a key role in viral replication appears to be substantiated. Regarding PV E2, research has indicated that its N-terminal transactivation domain is quite conserved among the papillomaviruses [168]. Thus, many of the properties of the PV E2 protein are likely to be shared between many PVs [166]. Origin-specific viral DNA replication is overseen by E2 once the viral helicase, E1, has been loaded successfully onto the origin of replication by E2. E2 also represses the expression of E6/E7 oncoproteins at the transcriptional level, in addition to performing other regulatory tasks. Therefore, it could be quite detrimental to the intracellular establishment of the virus, its subsequent replication and cellular transformation if the interactions between E2 and its cellular partners could be targeted. For example, while Brd4 is bound to E2, E2 is unable to engage P-TEFB, a transcription elongation factor, and this affects the expression of downstream genes such as E6 and E7 from the integrated viral genome [169]. Future studies are expected to provide more conclusive data regarding P-TEFB, the roles of Brd4, and their association with HPV proteins. But as a key regulatory protein, the importance of E2 on HPV viability and replication makes it a prime target for intervention.

E1 is the only enzymatic product of the viral genome, coding for an ATPase, and is thus an appealing target for molecular intervention. Indeed, if E1's binding and helicase properties could be blocked, DNA replication would be halted. Inevitably, impeding this process would also thwart the hijacking of cellular replication machinery for viral genome multiplication. Because the virus uses cellular replication factors derived from the host, current antiviral agents that block viral proteases and polymerases are ineffective in opposing HPV. DNA helicase unwinding is powered by the energy provided through ATP hydrolysis *via* the ATPase. ATP acts not only as the substrate, but also as an E1-E2 allosteric modulator [170]. As such, inhibitors sensitive to ATP concentrations, such as biphenylsulfonacetic acid, seem quite promising, in part because this agent does not directly bind ATP. Adding amides to certain positions of the biphenyl group enhances the compound's affinity for HPV6 E1, increasing its specificity [171]. However, whether biphenyl inhibitors can be applied to other HPV types remains undetermined. Furthermore, researchers have struggled to demonstrate inhibitory activity in cell-based assays [172]. In summary, future small inhibitors of E1 must directly target the enzyme's binding pocket, thereby conferring greater binding strength and specificity to E1-inhibitor complexes.

Small molecular inhibitors called indandiones are recognized as the first class of molecules to block HPV DNA replication by interrupting E1-E2 binding. The presence of indandiones induces conformational changes in E2, forming a deep binding pocket through which the small molecule modifies protein activity [173]. The success of preliminary trials attests to the great potential and need of inhibitors intended for binding pockets. Repaglinides operate similarly to indandiones in disrupting E1-E2 binding, though their effect is reversible, and they are reported to occupy a larger area of the binding pocket than do their indandione counterparts. One limitation for these classes of compounds is the poor binding frequently observed between small molecules and a large protein interface. However, these studies have demonstrated that designing small molecules to target large protein interfaces might actually be necessary in order to disclose pockets thought not to exist, or to create new ones. Another factor that must

be considered is the fact that viral integration into the host genome frequently leads to loss of E1/E2 gene expression, meaning that established cancers are likely to have lost the molecules targeted by inhibitors of E1 and/or E2, thereby limiting their usefulness [174, 175].

In contrast, E6 and E7 are frequently over-expressed in established cancers, making these two proteins quite attractive as targets. E6 and E7 are the zinc finger-containing proteins primarily responsible for the malignant alterations and de-differentiation of keratinocytes observed during cell transformation. These changes occur following integration of the HPV genome into host DNA [163, 176]. During this process, the regulators of viral replication, E1 and E2, are frequently disrupted, allowing over-expression of E6 and E7. HR-HPV types induce cell immortalization and transformation primarily through the over-expression of E6 and/or E7, which are best known for their ability to accelerate the degradation of the p53 and retinoblastoma proteins (pRB), respectively. The E6-mediated loss of p53 function leads to an insensitivity to apoptotic signals as well as to a loss of cell cycle regulation at the G1/S checkpoint in response to DNA damage. E7 contributes to the hyperplasia crisis by accelerating the degradation of pRB and thereby stimulating cells in Interphase to re-enter the cell cycle at S phase [177-179]. Together, over-expression of the E6 and E7 oncoproteins, decrease apoptosis and increase cell division, setting the stage for cancer [180]. Antiviral agents that can partially, if not fully, inhibit E6 and/or E7 functions clearly have the potential to negatively impact the carcinogenic process. One group, for example, proposed such a strategy in their study of the HPV16 E7-antagonizing peptide, Pep-7 [181]. Pep-7 was originally introduced as a short peptide component of the vacuole/lysosomal pathway [182]. However, Pep-7 was later shown not only to reduce the viability of HPV-positive cells *in vitro*, but it also decreased expression of E7 in SiHa cells in a xenograft model. It is conjectured that the selective mechanism Pep-7 uses to suppress cell proliferation may hinge on its ability to obstruct E7-pRB associations, even releasing pRB from E7 [181].

In contrast to E7, which appears to act primarily by increasing the ability of expressing cells to replicate, E6 acts by reducing the ability of expressing cells to undergo apoptosis. Apoptosis is a natural, cell-mediated death response to irreparable DNA damage. One target of E6 is the p53 tumor suppressor, which is degraded following association of E6 with the ubiquitin protein ligase, E6AP. The E6/E6AP complex binds to p53 and initiates its ubiquitination and consequent proteolytic destruction [183]. This means that the downstream targets of p53, which mediate cell cycle arrest and apoptosis, are not activated. Therefore, interference with the E6/E6AP-mediated proteasomal degradation of p53 has been seen as another possible strategy for treatment. The ubiquitination proteasome system (UPS) begins with the ubiquitin activating E1 molecules interacting with E2 conjugating enzymes, followed by catalyzation of the polyubiquitination cascade onto target proteins by E3 enzymes [184]. A subset of E3s, called RING-finger E3s, are a group of ubiquitin ligases that have domains to which ubiquitination substrates bind, and it is thought that by inhibiting this interaction, p53 might be preserved. One prominent p53-related RING-finger ubiquitin ligase is MDM2. MDM2 is normally expressed in a negative feedback manner to regulate p53 levels. Three dominant trains of thought have guided approaches seeking ways in which the negative effects of MDM2 might be neutralized: 1) Blocking activation domains on p53, 2) Increasing nuclear export of p53 so as not to activate MDM2 transcription, and 3) Inhibiting MDM2. Of these, the third approach has received the

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most attention. In one such study, small molecules were screened and selected based on their MDM2 inhibitory properties, and a class called the Nutlins was discovered. Nutlins competitively bind MDM2 at the same site typically occupied by p53, and structurally interpose themselves between p53 and MDM2 [185]. In contrast, another molecule labeled RITA actually binds to p53 and stabilizes it against degradation by inhibiting p53 from interacting with most of its binding partners, including MDM2 [186]. A more recent addition to the MDM2 inhibitor group is TRIAD1, a RING-finger bearing molecule, that functions similarly to RITA in that it binds p53 (at the C-terminus), and also intercepts ubiquitination triggered by MDM2 [187].

One well-established inhibitor of the UPS is Bortezomib. Bortezomib targets and reversibly blocks 26S proteasome activity, and has already been FDA-approved for the treatment of multiple myeloma and lymphoma [188]. Though its use has been proposed for the treatment of many diseases, from non-small cell lung cancer to pancreatic cancer, an equivalent and thorough exploration in the context of cervical carcinoma is still needed [189]. This suggestion is solidly founded on the observed sensitization of cervical cancer cells to apoptosis by another protease inhibitor (PI), MG132 [190]. A final set of PIs are those that inhibit the HIV protease. The anti-oncogenic properties of HIV PIs were first noted with respect to the 20S proteasome, and further investigation explicitly demonstrated Lopinavir active against E6-induced p53 degradation. Though Lopinavir also stabilizes p53, it exhibits low potency and virus is not fully cleared. The value of HIV PIs in cervical cancer treatment could be potentiated by its current availability as an antiviral agent, which might expedite the clinical trial process [191-193].

While p53 and the proteins to which it is connected are clearly targets worth exploring, other pro-apoptotic targets could prove just as important in halting the progression of HPV-mediated disease. HPV16 E6 binds to several additional signaling molecules in the intrinsic and extrinsic apoptotic pathways, including Bax, FADD, and procaspase-8, thus blocking their ability to interact with their normal partners and leading to their premature disposal by the proteasome. Not only does HPV16 E6 indirectly affect Bax *via* the degradation of p53, but Bax mRNA levels are decreased and the protein itself is destabilized in the presence of E6. Therefore, apoptotic cascades involving Bax and p53 represent a compelling site at which antiviral therapy could be targeted [194, 195]. It has also been reported that HPV16 E6 binds to both FADD and caspase 8 *via* DED residues, and a peptide corresponding to the binding site of FADD blocked both of these interactions. Expression of this peptide in HPV+ cells was able to re-sensitize those cells to apoptosis triggered through the extrinsic pathway [196, 197]. A search for small molecules capable of interfering with these interactions was conducted and several candidates were identified, primarily among the flavones and flavonols. Of these compounds, myricetin generated the lowest IC_{50} in assays designed to detect inhibition of E6-procaspase 8 binding [198]. More research is needed to optimize and test these small molecule leads.

5. Final remarks

In summary, the scientific community has witnessed tremendous progress in the recent years towards the goal of eradicating HPV-mediated cervical carcinoma. Of these endeavors, routine Pap testing and the prophylactic vaccines, Gardasil and Cervarix, are particularly noteworthy

for their documented and anticipated progress in decreasing the burden of this disease. Improved vaccines are under development, as are better methods for early detection. Additionally, recent discoveries pertaining to the HPV life cycle, viral infection, and immune clearance have provided guidance toward educating the public about the biological and behavioral risk factors linked to cervical cancer. However, awareness among the populations of greatest risk, in both developed and underdeveloped countries, is lacking. Although high-risk individuals may belong to diverse ethnic groups and/or have lower socioeconomic standing, they may not all benefit equally from any single approach, necessitating the importance of targeted education and intervention. Thus, future initiatives for the prevention of cervical cancer must aim to decrease existing inequalities, with a strong emphasis on educating about HPV transmission and screening throughout a woman's lifetime, particularly in groups where incidence and death rates are disproportionate. The hope is that these preventive methods – and in particular, the vaccine – will significantly reduce the HPV disease burden for future generations.

While progress in prevention must continue, complementary approaches that can provide better treatment options to populations that cannot directly benefit from vaccine-associated therapies must also be developed. These groups include women who are already infected with HPV, immunocompromised individuals such as those with HPV/HIV co-infections, and organ transplant patients. In treating these individuals, the prognosis and treatment of cervical cancer depends on our ability to medically diagnose and assign a disease stage. Therefore, improvements in diagnostic imaging, surgery, radiation therapy, chemotherapy, or a combination thereof are being studied to give women more options and to enhance each patient's ability to make better-informed decisions.

Along with clinical treatment, molecular therapies that target cervical cancer processes are also anticipated to contribute to the elimination of cervical cancer. Research focusing on HPV early proteins will continue to provide insights regarding the viral mechanisms used to take control over cellular processes. Of these viral components, the E6 and E7 oncoproteins have long been recognized as the main mediators of HPV-associated malignancies. Therefore, the idea that approaches targeting these two oncoproteins are likely to act in an anti-oncogenic manner is quite reasonable. Such discoveries have the potential to exert a broad impact in the field of virology, as they will enable researchers to more fully understand virus-host interactions and how to better equip the body to respond to or even prevent infection.

In conclusion, cervical cancer research has come a long way, but there is still much more to be done to ensure that our accomplishments are not overshadowed by failures to educate, vaccinate, improve clinical management, and strengthen our knowledge about HPV. Indeed, it is quite possible that the challenge of HPV-mediated cervical cancer can be overcome in this generation, given the abundance of advancements, ideas and potential avenues that have been discussed here.

Acknowledgements

This work was partially supported by a grant from the National Institutes of Health 5R25GM060507, which provided support to WE.

http://dx.doi.org/10.5772/55810

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