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Exploiting HPV-Induced Carcinogenesis for a Rational Drug Development in Cervical Cancer

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Abstract: Cervical carcinomas are almost universally associated with high-risk human papillomavirus (HPV) infections, and are a leading cause of cancer death in women worldwide. Since the late 1990s, when a spate of studies reported the benefit of cisplatin-based chemotherapy, there had been a dearth of clinical trials in cervical cancer (CC). More effective therapies in locally advanced and recurrent or metastatic CC are an urgent clinical need. In the era of molecular oncology one should look beyond conventional chemoradiation and chemotherapy for locally advanced and advanced CC. The fact that the initiating oncogenic insult, infection with a high-risk HPV and viral oncoprotein expression is common to almost all CC offers unique opportunities for disease control. Diverse biologic pathways with an implication in the development and progression of CC are being explored. For the first time, increase in overall survival has recently been obtained for advanced CC patients with a target drug, the antiangiogenic agent bevacizumab, and durable complete responses after HPV-targeted adoptive T cell therapy in metastatic CC patients were achieved. In this review, we will summarize molecular aspects of HPV infection focusing on potential targets to stop the carcinogenic process, present updated drug development data, and discuss challenges and prospects for the future.

Keywords: Cancer, carcinogenesis, cervix, HPV, targeted therapies.

INTRODUCTION

Cervical cancer (CC) is one of the leading causes of cancer morbidity and mortality in women. Nearly half a million new cases occur each year, with the majority of them occurring in developing countries [1]. Despite the widespread use of screening programs and the recent advent of HPV vaccines, CC high incidence and advanced disease stage at diagnosis continue to represent an important public health problem worldwide. After the benefits obtained with the addition of platinum-based chemotherapy [2-8], cure of locally advanced cervical carcinoma (LACC) and disease control rate in metastatic patients have reached a plateau; therapeutic index is narrow, responses are unpredictable and often disappointingly brief in both scenarios. These circumstances highlight the limitations of traditional therapy and the need to explore new strategies to improve prognosis in these patients.

The molecular pathogenesis of CC involves several distinct pathways leading to tumorigenesis, starting from viral infection and leading to specific genetic and epigenetic alterations required in the carcinogenesis process. It is important to point out that carcinogenesis initiation and CC are likely to be separated by many years or even decades in individual patients. The fact that the initiating oncogenic insult, infection with a high-risk HPV and viral oncoprotein expression is common to almost all CC offers unique opportunities for disease control [9]. Increased understanding of the targets involved in the pathogenesis of CC and treatment resistance may help to develop better strategies for cancer and preneoplastic lesions control, the latter an unmet therapeutic need.

In this review, we will summarize molecular aspects of HPV infection focusing on potential targets to stop the carcinogenic process, present updated drug development data, and discuss challenges and prospects for the future.

MOLECULAR BIOLOGY

HPV and Cervical Cancer Carcinogenesis

Oncogenic viruses account for a considerable proportion of cancers in human. In CC, HPV is the single most important etiological agent, but HPV infection alone is insufficient for malignant transformation; rather, the virus provides host cells with additional growth stimuli, which extend the proliferative capacity of the infected cell. This implies that HPV oncogenes can override cellular control mechanisms, which in untransformed cells regulate cell cycle progression in response to various antiproliferative signals. Pathogenesis of CC is a multifactorial and multistage process, involving aberrant sequential expression of multiple sets of cellular and viral genes.

HPV infection is a common sexually transmitted infection, which a majority of infected women are able to clear by mounting an effective immune response. Almost 50% of women will be infected within four years after the

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onset of sexual activity, with prevalence peaking between 25 and 35 years of age. Despite a high prevalence of HPV, only 5% to 15% will develop cervical dysplasia. Over 40 types of HPV are known to infect the cervical mucosa, being either low-risk (including 6, 11, 40, 42, 54, and 57) or high-risk types (including 16, 18, 26, 31, 33, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68) for CC [10, 11]. HPV has a double-stranded circularized genome that can be divided into early (E1-E7) and late (L1, L2) open reading frames (ORF). High-risk HPV genotypes code for three early proteins (E5, E6, and E7) with cellular growth stimulating and transforming properties. In productive HPV infection, HPV DNA remains in an episomal state, and the E1/E2 ORFs repress expression of the two most important HPV oncoproteins, E6 and E7 [12]. In contrast, in CC, E1/E2 is frequently disrupted by integration of viral DNA into the host genome, resulting in upregulated overexpression of E6 and E7 [12, 13].

Both cell intrinsic and extrinsic phenomena work in concert to bring about oncogenesis. Cell extrinsic elements include factors contributing towards immune tolerance. Intrinsic factors include the integration of the viral genome into the genome of the host's cells which correlates with the progression of low grade lesions into high grade ones, inactivation of tumor suppressor genes like *p53* and *pRB* by HPV oncoproteins particularly E6 [10] and E7 [14], respectively, deregulation of cell cycle regulators, cell proliferation signaling pathway activation, host DNA synthesis and neoplasia progression.

The interaction of HPV oncoproteins with cell cycle regulators fall into three categories: viral oncoproteins modulation of the expression of key cell cycle regulatory genes, inactivation of growth-suppressive nuclear proteins by direct binding, or interference with various signal transduction pathways.

These three categories are pivotal steps for cervical carcinogenesis and potential targets to be drugged to reverse the process. The main focus of this review will be set to oncoproteins interference with signal transduction pathways, as it embraces most of the currently investigated targets for drug development.

HPV Interaction with Critical Signaling Cascades in Cervical Cancer

In the absence of viral gene products, elaborate signaling cascades serve to integrate various positive and negative environmental signals, which determine the proliferation rate of a given cell. Typically, binding of a ligand, *e.g.* a growth factor, to its receptor at the cell membrane activates a kinase in the cytoplasmic compartment. The signal is then transduced by a cascade of phosphorylation events involving cytoplasmic and nuclear substrates. At the receiving end of such signaling cascades stands the modulation of cellular gene expression. HPV oncoproteins were shown to intervene at specific points in various signaling cascades, giving rise to specific downstream signals, which mimic physiological activation of a given pathway. Alternatively cellular transcription factors, which under physiological conditions are activated indirectly through signal transduction, can be directly activated by the binding of viral oncoproteins without any intermediate signaling event required [15].

BRIDGING THE GAP BETWEEN TUMOR BIOLOGY AND THE DEVELOPMENT OF AGENTS THAT CAN PROVIDE CLINICAL BENEFIT

Epidermal Growth Factor (EGF) Pathway and EGFR/HER2 Inhibitors

The HER family of receptor proteins plays a key role in tumorigenesis and disease progression. HER molecules are cell membrane-bound proteins comprising of four distinct receptors: HER1/EGF receptor (EGFR), HER2, HER3, and HER4 [16-19].

Immunohistochemical analyses have identified all members of the HER family in cervical neoplasia. EGFR is frequently overexpressed in HPV-associated dysplasias and carcinomas, implying that it is important for the progression of keratinocytes to malignancy. Around 80% of squamous cell cervical carcinoma (SCCC) tumors express EGFR [20, 21] and cell lines from recurrent and metastatic sites of disease tend to express higher levels of EGFR when compared to those obtained from primary sites [21]. Arias-Pulido and co-investigators analysed 89 samples for *EGFR* mutations in exons 19–21 [22], and nine CC cell lines were evaluated for mutations in exons 18–21: no mutations were detected in any sample in either group. In a separate study, no amplification of the *EGFR* gene was detected [23]. In a recent study using a high-throughput genotyping platform exploring differences between adenocarcinoma and SCC histologic subtype, a novel *EGFR* mutation was detected only in SCC (0% vs 7.5%; p=0.24) [24]. HER2 expressed strongly (3+) in 6% and moderately (2+) in 20% of the specimens; amplification of the gene (>4 copies) was observed in overall 21%, with 80% of the 3+ (4/5), but only 19% of the 2+ (3/16) cases being positive [21]. Overexpression was also found in 74.4% for HER3 and in 79.5% for HER4 [21]. Survival analysis revealed a significant association of HER2 and HER3 overexpression with poor prognosis (p=0.006; p=0.05, respectively), and most data also associates HER1 overexpression with poor outcome [25], although some controversies exist [21]. The finding of immunohistochemistry test conversion to 2+ and 3+ in recurrent tumors in approximately 50% of the patients indicates the opportunity of HER2 inhibition in metastatic and recurrent settings [26].

Locally Advanced Disease

Studies combining EGFR inhibitors with chemotherapy and/or radiotherapy (RT) have shown that these compounds increase the RT sensitiveness *in vitro* and *in vivo* in different models, including CC [27-29]. The addition of EGFR inhibitors to RT and cisplatin in LACC patients has been explored in two phase I trials. One trial showed that the combination of the chimeric anti-EGFR monoclonal antibody cetuximab, cisplatin and radiotherapy was feasible, except for patients receiving extended field RT [30]. Exploring the strategy of increasing radiosensitivity in LACC, our group has initially performed a phase I trial adding the anti EGFR TKI (tyrosine kinase inhibitor) erlotinib to chemoradiotherapy; erlotinib dose has been defined as 150mg and the combination has shown a favorable toxicity profile [31]. Following on from this, we conducted a phase II trial with 36 patients which has shown that the combination has

promising activity. The therapy was well tolerated, and 34 (94.4%) patients achieved a complete response (CR). Two- and 3-year cumulative overall (OS) and progression-free survival (PFS) rates were 91.7% and 80.6% and 80% and 73.8%, respectively [32] Table **1**.

Persistent, Recurrent or Metastatic Disease

In general, EGFR inhibitors presented limited activity as single agents in advanced disease in different tumor types. In CC, in phase II studies, erlotinib and cetuximab did not show any activity in terms of response in patients with recurrent or advanced disease [33, 34]. The EGFR TKI inhibitor gefitinib has shown around 20% of stable disease (SD) as best response in two phase II studies [35, 36].

The combination of chemotherapy and EGFR inhibitors did not show promising results in recurrent or advanced disease either. In a phase II trial, the combination of cetuximab with cisplatin was adequately tolerated but did not indicate additional benefit beyond cisplatin therapy [37]. In another phase II trial, the combination of cetuximab, cisplatin and topotecan, despite demonstrating some response, induced a high rate of serious adverse events and deaths (28%) that lead to the premature closure of the study [38].

The low frequency of HER2 expression in primary cases suggests that HER2 inhibitors could have a limited value for the primary management of CC patients. In a preclinical model, the anti HER2 monoclonal antibody trastuzumab combined to cetuximab reduced cell survival and MAPK phosphorylation, independently of EGFR expression [29]. Lapatinib, a HER2 TKI, showed only 5% overall response rate (ORR) and inferior PFS than pazopanib (an anti-angiogenic multikinase inhibitor) in a phase 2 study in advanced CC unselected for HER2 expression. The combination of lapatinib and pazopanib in this study crossed the futility boundary for efficacy and showed an increase toxicity that lead combination discontinuation [39, 40]. Table **2**.

Thus the small activity of these agents and the lack of predictive factors, the understanding of the relative contribution of individual members of the ErbB receptor family and activated downstream pathways in CC cell proliferation needs further investigation.

Angiogenesis and Antiangiogenic Therapy

HPV infection may promote the "angiogenic switch" - the loss, or inactivation, of wild-type p53 by HPV oncoprotein has been found to up-regulate vascular endothelial growth factor (VEGF) [41-43], and down-regulate a potent angiogenesis inhibitor, thrombospondin-1 (TSP–1) [43-45], providing rationale for the contribution of angiogenesis to CC. In addition, the angiogenesis-promoting protein, eukaryotic translation initiation factor 4E (eIF4E), is increased in cervical neoplasia, as well as in cervical dysplasia. HPV-infected cell lines transfected with eIF4E produce increased amounts of E7 oncoprotein as compared with nontransfected cell lines, suggesting that HPV may also play a role in angiogenesis in cervical neoplasia by E7 protein role in the elF4E/c-myc cascade, and eIF4E may be a marker for early CC [46].

VEGF and platelet-derived growth factor (PDGF) are highly expressed in CC and there is a stepwise increase in expression of VEGF and PDGF from normal cervix to CC [47, 48]. Increased VEGF expression is also correlated with higher stage, increased risk of lymphovascular space invasion, greater likelihood of parametrial spread, and lymph node metastasis [49, 50]. It is also known that PDGF receptors determine high interstitial fluid pressure found in cervical tumors [51, 52], which is related to resistance to treatment and an obstacle in cancer therapy [53].

Locally Advanced Disease

A phase II study, RTOG 0417 (N=49), explored the safety and efficacy of the addition of the anti-angiogenic monoclonal antibody bevacizumab to chemoradiation. Treatment was well tolerated with 26.5% of grade 3 and 10.2% of grade 4 toxicity, mostly hematological. 94% had received planned radiotherapy and 76% had both their cisplatin and bevacizumab administered. In 3.8 years of follow-up, there was 20% locoregional failures and the 3-year OS and DFS were 81.3% and 68.7%, respectively [54]. Despite all limitations of comparing historical data, the results of RTOG 0417 is similar in terms of DFS rates and slightly better for OS when compared to RTOG 90-01 chemoradiation arm [5] Table **1**.

Given the positive results of Gynecology Oncologic Group (GOG) 240 trial [55], it is reasonable to assume that bevacizumab may have a role in the early stage disease and further investigation of the combination in a phase III trial is warranted.

Persistent, Recurrent or Metastatic Disease

A retrospective study (N=6) of bevacizumab combined with 5-fluorouracil or capecitabine in heavily pretreated CC

Table 1. Phase II clinical trials of targeted agents in locally advanced cervical cancer.

Author	Phase (Setting)	Patient (N)	Schedule	ORR (%)	3 Year PFS (%)	3 Year OS (%)
EGFR inhibitors						
Nogueira-Rodrigues, 2014	II	36	Erlotinib 150 mg/day combined to cisplatin and radiotherapy	94.4	73.8	80.6
Anti Angiogenic						
Schefter, 2014	II	49	Bevacizumab (10mg/kg every 2 week for 3 cycles) combined to cisplatin and radiotherapy	NR	68	80.

Legend: ORR: objective response rate; PFS: Progression free survival; OS: Overall survival; NR: not reported.

found 67% clinical benefit rate with tolerable safety profile [56]. In addition, a case report of one patient with recurrent CC treated with carboplatin and low-dose bevacizumab 7.5mg/kg showed a "delay in terms of progression" [57].

The GOG 204 evaluated single-agent bevacizumab in a phase II trial with 46 patients with recurrent or metastatic CC patients who had experienced progression after 1 or 2 cytotoxic chemotherapy regimens. Bevacizumab was well tolerated. The most important grade 3 or 4 toxicities included thromboembolism (10%), fistula (2%) and one (2%) death due to infection. Five patients (10%) had partial responses with duration of 6.2 and 24% of the patients were free of progression after 6 months, which compared favorably with historical data of GOG trials in this setting [58].

Another phase II study (N=27) tested the triplex combination of topotecan, cisplatin and bevacizumab in first-line treatment for recurrent or persistent CC [59]. One (4%) patient presented CR, 8 (31%) PR and 10 (39%) SD; the probability of surviving free of progression at 6 months was 59%. However, toxicity was a concern in this trial: grade 3 and 4 hematologic toxicity was frequent (thrombocytopenia in 82%, leukopenia in 74%, anemia in 63% and neutropenia in 56% of the patients) and 78% of the patients had unanticipated hospitalizations.

Following this, a phase 3 randomized trial (GOG 240) investigated the addition of bevacizumab to combination chemotherapy (paclitaxel combined to cisplatin or topotecan). The incorporation of bevacizumab significantly improved the median OS as compared with chemotherapy alone (17.0 *vs*. 13.3 months; hazard ratio (HR) 0.71; 98%CI,0.54-0.95). Also, patients that received bevacizumab had a higher ORR, CR (13% *vs*. 6%) and 33% improvement in PFS. Bevacizumab, as compared with chemotherapy alone, was associated with an increased incidence of hypertension grade 2 or higher (25% *vs*. 2%), thromboembolic events grade 3 or higher (8% *vs*. 1%), and gastrointestinal fistula grade 3 or higher (3% *vs*. 0%) [55].

The GOG 240 trial established bevacizumab as the new standard of care in advanced CC. Improvements in OS and PFS attributed to the incorporation of bevacizumab into the treatment of advanced CC were not accompanied by any significant deterioration in health-related quality of life [60]. The US Food and Drug Administration (FDA) approved bevacizumab for the treatment of advanced CC in August 2014, however its cost effectiveness is doubtful, especially in developing countries whereas restrict budget for cancer care is a reality [61].

Another class of antiangiogenic agents is the TKIs, small molecules able to block the activation of various downstream signaling pathways intracellularly. Pazopanib showed a slightly better activity over lapatinib in advanced CC as described above [40]. Sunitinib, a multi-targeted TKI, was evaluated in a phase II trial, with insufficient activity as a single agent: 84% patients had SD as their best response with median duration of 4.4 months, in patients with advanced CC who had received up to one prior line of chemotherapy for advanced disease. Toxicity was a concern as 31% had experienced a SAEs, as well as a higher rate of fistula formation (26.3%) than would be expected [62].

In a recent phase II study (CIRCCa), 69 patients with metastatic or relapsed CC were randomized to cediranib (a vascular endothelial growth factor TKI) or placebo combined to chemotherapy with carboplatin and paclitaxel for a maximum of six cycles [63]. Around 83% had received one line of previous treatment. Cediranib improved PFS and significantly increased ORR when added to conventional chemotherapy. More grade 2-4 adverse events was observed in cediranib arm than placebo (19% *vs*. 9% $p = 0.240$), as well as grade 3/4 neutropenia (31% *vs* 9%; $p = 0.019$) Table **2**.

PI3K/AKT/mTOR Pathway and Inhibitors

The mammalian Target of Rapamycin (mTOR) is a protein kinase that regulates cell growth, protein translation, autophagy and metabolism [64]. Upstream, phosphatidylinositol3'-kinase (PI3K)/AKT signaling is deregulated through a variety of mechanisms, including overexpression or activation of growth factor receptors such as HER family and insulin-like growth factor receptor (IGFR), *PI3K* mutations and *AKT* mutations/amplifications [65]. The tumor suppressor phosphatase and tensin homolog (*PTEN*) deleted from chromosome 10 is a negative regulator of PI3K signaling. PTEN expression is decreased in many cancers, including breast, endometrial, thyroid, and prostate cancers; melanoma; and glioblastoma. PTEN may be downregulated through several mechanisms, including mutations, loss of heterozygosity, methylation, aberrant expression of regulatory micro RNA, and protein instability.

Evidence of involvement of this signaling pathway in HPV cervical carcinogenesis has been reported. HPV18 E6 variants are able to upregulate phospho-PI3K protein, strongly correlating with activated Mitogen-Activated Protein Kinase (MAPK) and cell proliferation [66]. In addition, the E7 oncoprotein from HPV16 enhance both the cytoplasmic retention of p27 and the migration of human foreskin keratinocytes, positive regulators of cellular motility and markers of poor prognosis in several forms of cancer, in a PI3K/AKT-dependent manner [67]. E7 protein from HPV-16 can modulate the cytoplasmic localization of p27 and may in turn regulate tumor metastasis/aggressiveness through the PI3K/AKT pathway. Human papillomavirus virus-like particles (VLPs) are also able to activate the RAS/MAPK pathway and RAS can also elicit an anti-apoptotic signal *via* PI3K. Binding of VLPs from HPV types 6,18, 31, 35 results in activation of PI3K. Activation is achieved by either L1 or L1/L2 VLPs and is dependent on both VLP-cell interaction and correct conformation of the virus particle. VLP-induced PI3K activity results in efficient downstream signaling to AKT. Bertelsen *et al.* have demonstrated that PI3K-AKT pathway is constitutively activated in CC, but *PTEN* mutation or loss of heterozigosity is not frequent [68, 69]. PTEN promoter methylation has been detected in up to 40% of cervical dysplasia patients and up to 58% of CC specimens [69]. A significantly higher *PTEN* methylation rate in late compared to early disease was not verified, which suggests *PTEN* methylation may be an early event in cervical carcinogenesis [69].

In vitro studies have shown that mTOR inhibition enhances chemosensitivity of CC cells. It has been demonstrated that paclitaxel, an effective antineoplastic

agent in CC, down-regulates the phosphorylation of AKT in CC cell lines [70]. In CaSki cells, which showed the lowest sensitivity to paclitaxel, suggesting a resistant phenotype, the chemotherapeutic drug induced the activation of mTOR as a downstream target of AKT. Pre-treatment with rapamycin inhibited activation of mTOR signaling and significantly enhanced the sensitivity of CaSki cells to paclitaxel by increasing apoptotic cell death. Other studies showed that everolimus dramatically enhanced cisplatin-induced apoptosis in wild-type p53-harboring tumor cells *in vitro* [71], suggesting that it may play a role in reversing drug resistance to this important chemotherapeutic agent in CC.

The observation by Oh *et al.* that rapamycin blocked phosphorylation of the protein 4E-BP1 and significantly decreased the level of E7 protein in cellular studies also sparks interest in exploring earlier mTOR inhibition to stop cervical carcinogenesis [72].

Moreover, high expression of p-mTOR has been associated with radiation resistance, therefore, p-mTOR may be a prognostic marker for response to radiotherapy in patients with CC [73], and its inhibition may enhance radiosensitivity. Preclinical data by Lee *et al.* shows that PI3K inhibition with LY294002 alone did not produce CC cell cytotoxic effects. However, treatment with LY294002 significantly radiosensitized HeLa and CaSki cell lines, a finding that supports future investigation of PI3K inhibitors in combination with radiation therapy for CC [74].

Janku and colleagues have investigated *PI3KCA* and MAPK pathway mutation status in patients with advanced breast and gynecological (cervical, endometrial and ovarian) cancer and detected 18% of the tumors with *PI3KCA* mutations, being as high as 36% in CC. Patients harboring *PI3KCA* mutations and refractory to a median of two prior therapies were treated with PI3K/AKT/mTOR pathway inhibitors and a RR of 30% was observed [75].

Hou and colleagues [76] analyzed the outcomes of patients with metastatic or recurrent CC who had a test for *PIK3CA* mutation and/or *PTEN* loss/mutation, and received ≥1 phase I therapeutic regimen. Patients with adenocarcinoma had fewer *PIK3CA* mutations (14%), and survived longer (median 14.2 months) than those with SCC (48% and 7.2 months; p = 0.016, and 0.001, respectively). Matched therapy targeting the activated PI3K/AKT/mTOR pathway led to a favorable rate of SD ≥ 6 months/CR/PR (53%) and significantly longer PFS (median 6.0 months) than non-matched therapy (11% and 1.5 months; p = 0.08 and 0.026; respectively). In patients with SCC the presence of *PIK3CA* mutations was associated with a significantly longer OS (median 9.4 months) than the absence of PIK3CA mutations (median 4.2 months; p = 0.019).

A phase I study has evaluated the combination of the mTOR inhibitor temsirolimus with topotecan in the treatment of advanced and/or recurrent gynecologic malignancies, however, only 2 patients with CC were included in this study and detailed data pertaining to RR, OS, PFS, efficacy and safety were not reported [77].

Tinker and colleagues [78] have published a 2-stage phase II study assessing the activity of the mTOR inhibitor temsirolimus (25 mg intravenously, weekly in 4 week cycles), in patients with measurable metastatic and/or locally advanced, recurrent CC. Thirty-eight patients were enrolled and 37 were evaluable for toxicity and 33 for response. One patient experienced a partial response (3.0%), 19 patients had SD (57.6%) with median duration of 6.5 months (range 2.4–12.0 months). The 6-month progression free survival rate was 28%(95% CI: 14–43%) and the median progression free survival was 3.52 months (95% CI 1.81–4.70). Adverse events were mild to moderate in most cases and similar to other temsirolimus studies. No toxicity higher than grade 3 was observed. Correlative molecular studies were performed on archival tumor tissue and assessment of PTEN and PIK3 by immunohistochemistry, copy number analyses and PTEN promoter methylation status did not reveal subsets associated with disease stability Table **2**.

Melo *et al.* [79] presented in the last International Gynecologic Cancer Society world conference the results of a phase I study of the mTOR inhibitor everolimus in association with cisplatin and radiotherapy for the treatment of locally advanced CC. In a modified Fibonacci design the trial aimed to treat 3 cohorts of at least 3 patients with daily escalating doses of everolimus (2.5/5/10mg), cisplatin (40 mg/m^2 per week) and radiotherapy (teletherapy - 4.500 cGy plus brachytehrapy - 4 fractions of 600 cGy) in CC patients, stage IIB-IIIB. Patients received everolimus from day -7 up to the last day of brachytherapy. Thirteen patients were enrolled, 6 in the cohort #1, 3 in #2 and 4 in #3. Four patients did not complete the planned schedule, 1 at 2,5mg presented grade 4 acute renal failure interpreted as dose limiting toxicity (DLT) and 3 at 10mg: 1 with disease progression, and 2 with DLT – grade 3 rash and grade 4 neutropenia. The maximum tolerated dose has been defined as 5mg. Details regarding toxicity and RR are still pending.

MAPK/ERK Pathway and Inhibitors

The pivotal role of the RAS/RAF/MEK/ERK MAPK pathway in multiple cellular functions underlies the importance of the cascade in oncogenesis and growth of transformed cells [80]. As such, the MAPK pathway has been a focus of intense investigation for therapeutic targeting. Many receptor tyrosine kinases are capable of initiating MAPK signaling including receptors important in cancer biology, such as the HER family, PDGF and VEGF. The cellular functions of ERK are diverse and include regulation of cell proliferation, survival, mitosis, and migration.

Chen *et al.* studied 23 samples of normal cervical epithelium, 25 of low-grade squamous intraepithelial lesions, 19 high-grade squamous intraepithelial lesions and 31 SCCC. The expression of phospho-MAPK/ERK1/2 were strongly associated with cervical neoplastic progression [81]. VLPs are also able to activate the RAS/MAP kinase pathway. RAS can also elicit an anti-apoptotic signal *via* PI3K, as described above. These data suggest that papillomaviruses use a common receptor that is able to signal through to RAS. Combined activation of the RAS/MAPK and PI3K pathways may be beneficial for the virus by increasing cell numbers and producing an environment more conducive to infection [82].

Table 2. Phase II and III clinical trials of targeted agents in advanced or metastatic cervical cancer.

Author	Phase (Setting)	Patients (N)	Schedule	ORR (%)	PFS (Months)	OS (Months)
EGFR inhibitors						
Schilder *et al*. 2009	II (2nd or 3rd-line)	28	Erlotinib 150 mg/day	0	1.9	5
Santin *et al*. 2011	II (2nd or 3rd-line)	35	Cetuximab 400 mg/m^2 followed by 250 mg/m^{2}weekly	0	2	6.7
Farley *et al*. 2011	II (1st-line)	69	Cisplatin 30 mg/ m^2, D1 and 8 + Cetuximab 400 mg/m^2 followed by 250 mg/m2 D1, 8 and 15, q3w	12	3.9	8.8
Kurtz *et al*. 2009	II (1st-line)	19	Cisplatin 50 mg/m^2, D1 + Topotecan 0.75 mg/m^2/day,D1 to 3, q3w + Cetuximab 400 mg/m^2 followed by 250 mg/m^2, weekly	32	5.6	7.2
Goncalves *et al*. 2008	II (2nd or 3rd-line)	30	Gefitinib500 mg/day	0	1.2	3.5
Sharma *et al*. 2013	II (2nd)	20	Gefitinib 250 mg/day	10	4	5
Anti Angiogenic						
Monk *et al*. 2009	II (2nd or 3rd-line)	46	Bevacizumab 15 mg/kg q3w	10.9	3.4	7.2
Zighelboim *et al*. 2013	II (1st-line)	27	Topotecan 0.75 mg/m^2, D1-3 + Cisplatin 50 mg/m^{2}D1 +bevacizumab 15 mg/kg, D1, q3w	35	7.1	13.2
Tewara KS *et al*. 2014	III (1st-line)	452	Cisplatin50 mg/m^2+ Paclitaxel 135 or 175 mg/m^2, D1 or Topotecan0.75 mg/m^2, D1 to 3 + Paclitaxel 175 mg/m^2, D1without or withBevacizumab15 mg/kg	48% vs. 36% (*P*=0.008)	8.2 vs. 5.9 (*P* = 0.002)	17 vs. 13.3 (*P*=0.004)
Mackay HJ *et al*. 2010	II (1st or 2nd line)	19	Sunitinib 50 mg/day, 6 week cycle (4 weeks on and 2 weeks off)	0	3.5	NR
Monk BJ *et al*. 2010 and 2011	II (2nd-line or more)	152	Pazopanib 800 mg daily vs. Lapatinib 1,500 mg daily	9 vs. 5	4.1 vs. 3.9 (*P*<.01)	11.4 vs. 10.1 (*P* =.40)
Symonds P *et al*. 2014	II (1st line)	69	Carboplatin AUC5 + Paclitaxel 175 mg/m^2, q3w for 6 cycles with or without Cediranib 20 mg daily	66 vs. 42 (*P* =.03)	8 vs. 6.8 (*P* =.046)	13.5 vs. 14.4 (*P* =.401)
mTOR inhibitors						
Tinker A *et al*. 2013	II (2nd-line or more)	38	Temsirolimus 25 mg weekly in 4 week cycles	60.6%	3.52	NR
Immunotherapy						
Sugiyama T *et al*. 2014	III (1st-line)	249	Z-100 (0.2 μg) or placebo twice a week during the radiotherapy and once every 2 weeks during the follow-up period. Z-100 or placebo was administered until progression or recurrence	99 vs 99%	No difference *(P* =.46)	75.7% vs 65.8% (5-year survival rate, *P* =.07)

Legend: ORR: objective response rate PFS: Progression free survival; OS: Overall survival; NR: not reported; q3w: every 3 week
Ref.
Schilderhttp://www.ncbi.nlm.nih.gov/pubmed/19574787
Santinhttp://www.ncbi.nlm.nih.gov/pubmed/21684583
Farleyhttp://www.ncbi.nlm.nih.gov/pubmed/21329967
Monk BJ *et al*. 2010http://www.ncbi.nlm.nih.gov/pubmed/20606083 e http://www.ncbi.nlm.nih.gov/pubmed/22084371

Preclinical studies are evaluating combination therapy with standard radiation plus cisplatin in combination with cetuximab and trastuzumab or a MEK1/2 inhibitor (PD98059). Cetuximab with trastuzumab or PD98059 reduced survival and MAPK phosphorylation of different CC cell lines. These data propose that MAPK inhibitors could have useful applications for CC treatment [83].

Ongoing phase 2 study is evaluating the combination of two drugs called Trametinib (MEK inhibitor) and GSK2141795 (AKT inhibitor) as a possible treatment for recurrent or persistent cervical cancer [84].

DNA Damaging Anticancer Agents

PARP Inhibitors

Poly (ADP-ribose) polymerase (PARP) is a family of nuclear proteins with enzymatic properties and recruiting ability for DNA repair proteins. The most important member of the PARP family is PARP1, which is involved in the base excision repair system that repairs DNA damage induced by radiation and alkylating agents [85]. Inhibitors of PARP (iPARP) were shown to be highly selective for cancer cells that harbor homologous recombination (HR) deficiencies, such as those harboring mutations in *BRCA1 or BRCA2* genes [86]. iPARP belong to a family of multifunctional enzymes with ability to block base excision repair and lead to accumulation of DNA single-strand breaks (SSB). The latter cause DNA double-strand breaks at replication forks [87]. In normal cells, these breaks are repaired in the presence of the tumor suppressor proteins BRCA1 and 2. On the other hand, in the absence of these proteins, the lesions cannot be repaired, resulting in cell death [88].

The use of iPARP in CC has been recently preclinically explored. Michels *et al.* created CC HeLa cell lines resistant to cisplatin [89] and exposed those cells to pharmacologic PARP inhibition, which resulted in cell death. Therefore, it is suggested another role for PARP inhibition as a treatment for cisplatin-resistant CC. Currently, an ongoing phase I trial is evaluating the combination of olaparib, a iPARP, associated with carboplatin in refractory or recurrent disease enrolling patients with CC along with other gynecological malignancies (NCT01237067). Another phase I/II trial is investigating the use of veliparib with cisplatin and paclitaxel in advanced, persistent, or recurrent CC (NCT01281852).

Wee1

Wee1 is another protein kinase involved with terminal phosphorylation and inactivation of cyclin-dependent kinase 1-bound cyclin B. This process results in G2 cell cycle arrest in response to DNA damage [90]. Wee1 also plays a critical role in cell division – it modulates the activity of cyclin-dependent kinases 1 and 2 through inhibitory phosphorylation of conserved tyrosine15 residues on both kinases, controlling mitosis and DNA replication during S phase. Overexpression of Wee1 has been observed in several malignancies, including hepatocellular carcinoma, breast cancers, glioblastoma, and malignant melanoma, where high expression has been shown to correlate with poor DFS. Inhibition of Wee1 either by the pyrido-pyrimidine derivative (PD0166285) or *via* siRNA gene knockdown has been shown to sensitize ovarian, colon, cervical, osteosarcoma, glioblastoma, and lung cancer cells to DNA damage by irradiation and topoisomerase inhibition.

MK-1775, a recently developed pyrazolo-pyrimidine derivative, is a potent and selective small-molecule inhibitor of Wee1. The studies enrolled until now demonstrate that chemical inhibition of Wee1 with MK-1775 allows potentiation of cytotoxicity of various DNA damaging agents with different modes of action, and that this effect is more pronounced in p53-deficient cancers, supporting the knowledge that tumors with defective G1checkpoint are more dependent from G2 checkpoint for escape from mitotic lethality [90].

There is a phase I/II study evaluating the combination of MK-1775 with cisplatin and topotecan in patients with advanced CC (NCT01076400). Results are expected. Another phase I study is currently evaluating the safety and maximum tolerated dose of MK-1775 as a single-agent in advanced refractory solid tumors (NCT01748825).

Development of MK-1775 reveals a novel therapeutic approach to enhance the antitumor efficacy of traditional chemotherapeutic agents. Recent evidence implicates greater role for Wee1 in DNA repair, highlighting the potential combination with other targeted agents, although no specific role in the treatment of CC has been defined.

Immunotherapy

The objective of immune checkpoint inhibitors is to reverse the immune privileged state exhibited by malignant microenvironment. Nowadays, the categories of therapeutic immune modulators are mainly represented by cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death receptor-1 (PD-1).

CTLA-4, an immune inhibitory molecule expressed in activated T cells and suppressor T-regulatory cells, develops a role with its lymphocytes ligands CD80 and CD86, based on early attenuation of naïve and memory cells. Ipilimumab, a fully human monoclonal antibody directed against CTLA-4, stimulates the immune response breaking immune tolerance by surpassing immune suppression. The use of ipilimumab almost doubled the patients survival in comparison with control arm [91]. Currently, there is a phase one trial evaluating the use of ipilimumab after chemorradiation in locally advancer CC patients.

PD-1, a cell surface protein expressed on T cells following T-cell receptor activation, is involved in modulating T-cell activity in peripheral tissues. PD1 binds to programmed death ligand-1 (PDL-1) and PDL-2, causing down-regulation of T-cell receptor signaling. This results in T-cell anergy, apoptosis and immune suppression [92]. A rational for PD-1 therapeutic blockade in patients with HPV-related disease was established by Lyford-Pike *et al.* when they studied head and neck squamous cell cancer immune resistance [93]. Pembrolizumab was the first PD-1 inhibitor approved by FDA, for treatment of patients with advanced or unresectable melanoma. The PD-1 blockade was effective in patients with disease progression after ipilimumab therapy. This response shows the complementary effects of the different kinds of checkpoint inhibitors. Nivolumab, a fully human IgG4 programmed death 1 (PD-1) immune-checkpoint–inhibitor antibody, similarly to Pembrolizumab, selectively blocks the interaction of the PD-1 receptor with PD-L1 and PD-L2. An open-label, randomized, phase 3 study with ipilimumab-refractory melanoma patients associated nivolumab with higher objective response rate than chemotherapy (32% *vs* 11%) [94].

Z-100, a hot-water extract from human bacillus tuberculosis, is a carcinostatic immunomodulatory agent [95]. A double-blinded placebo-controlled phase III trial was conducted comparing the efficacy of Z-100 and placebo in patients with LACC (stages IIB-IVA). The 5-year OS rate was 75.7% for Z-100 arm and 65.8% for placebo arm (P= 0.07, HR 0.65 (95% CI 0.40–1.04). This not statistically significant difference in OS improvement was justified by the authors by the occurrence of fewer events than expected. Therefore, further evaluation is necessary to confirm the benefit of this treatment.

A diversity of therapeutic HPV vaccines targeting HPV E6 and E7 antigens have been tested – live vector-based, protein-based, peptide-based, nucleic acid-based and whole cell-based vaccines. Their role in CC treatment is still under evaluation. Adoptive T-cell therapy (ACT) is a promising cancer treatment modality with potentially broad application with tumor-infiltrating lymphocytes (TIL) selected for HPV E6- and E7-reactivity (HPV-TIL). In a recent trial, nine CC patients were treated with a median of 81 x 109 T cells (range 33 to 159 x 109) as a single infusion. The infused cells possessed reactivity against high-risk HPV E6 and/or E7 in 6/8 patients. The two patients with no HPV reactivity did not respond to treatment. 3/6 patients with HPV reactivity demonstrated objective tumor responses by RECIST (1 PR and 2 CR). One patient had a 39% best response. Two patients with widespread metastases had complete tumor responses. One patient with a complete response had a chemotherapy-refractory HPV-16+ SCCC and the other a chemoradiation-refractory HPV-18+ adenocarcinoma. Both patients demonstrated prolonged repopulation with HPV-reactive T cells following treatment. Increased frequencies of HPV-specific T cells were detectable after 13 months in one patient and 6 months in the other. Two patients with HPV-reactive TIL that did not respond to treatment did not display repopulation with HPV-reactive T cells. Continued investigation of HPV-TIL for CC, and possibly other HPV-induced malignancies, is warranted [96].

CONCLUSION

CC are almost universally associated with high-risk HPV infections, and are a leading cause of cancer death in women worldwide. Although CC is often curable if detected early, a significant number of patients present with locally advanced disease at diagnosis, a clinical scenario associated with suboptimal therapeutic benefits. For these patients the initial treatment by far offers the best chance of cure. Conversely, persistent or recurrent disease carries a poor prognosis and leads to death in more than 85% patients. More effective therapies in locally advanced and recurrent or metastatic CC are an urgent clinical need.

In the era of molecular oncology one should look beyond conventional chemoradiation and chemotherapy for locally advanced and advanced CC. A scenario filled with a better understanding of the HPV carcinogenic process and availability of a range of novel pathway and microenvironment targets inhibitors under development may provide innovative, less toxic and more rational future directions for clinical trials in CC.

Assuming that infection by high-risk HPV is a necessary step to CC initiation, progression and maintenance, the possibility of identifying druggable targets in the HPV-induced carcinogenic process may be reasonable. As a result of the HPV genome integration into the host genome, alterations such as inactivation of tumor suppressor genes *TP53* and *RB* by E6 and E7 may occur. Although these pivotal steps for cervical carcinogenesis, as well as E6 and E7 proteins themselves are potential targets, they are not easily druggable. Hinrichs *et al.* have recently reported CC control through an adoptive T-cell therapy targeting HPV [96]: two metastatic patients (2/9) achieved durable CRs. Customized treatment was created for each patient by culturing T cells harvested from the patient's tumor, testing the cells for reactivity against the HPV E6 and E7 antigens. It was the first clinical evidence that HPV-targeted adoptive T cell therapy is feasible and may provide a new strategy for CC treatment [96].

Conversely HPV oncoproteins interference with signal transduction pathways embraces most of the currently promising druggable targets in CC. Those oncoproteins have been shown to intervene directly at specific points in various signaling cascades, giving rise to specific downstream signals, which mimic physiological activation of a given pathway.

Diverse biologic pathways with an implication in the development and progression of CC have been explored and, for the first time, increase in OS has recently been obtained for advanced CC patients with a target agent. The incorporation of bevacizumab significantly improved the median OS as compared with chemotherapy alone [55]. In LACC, bevacizumab [54] and erlotinib [32] also presented promising results when combined to chemoradiation, but further confirmation in phase III trials is necessary. In the palliative scenario, EGFR inhibitors have undergone extensive clinical testing in CC, unfortunately with no very much success. The radiosensitising effects may explain the discrepancy between the findings in LACC and the lack of an objective response in advanced CC patients treated with EGFR inhibitors without radiotherapy.

A plethora of new targeted agents are in their early stages of development for CC (*e.g.* mTOR/ MEK/AKT/PARP inhibitors, DNA damaging anticancer agents). In order to successfully integrate targeted drugs into CC treatment, it is necessary to fully understand the functional consequences of a given alteration within a druggable pathway. A way to conquer it is to test biomarkers and targeted agents at the same time. Furthermore, the clinical and molecular heterogeneity of CC must be taken into account for future studies with targeted agents. Recent whole-exome, transcriptome and whole-genome sequencing have shown previously unknown somatic mutations in primary CC, including recurrent E322K substitutions in the *MAPK1* gene (8%), inactivating mutations in the *HLA-B* gene (9%), mutations in *EP300* (16%), *FBXW7* (15%), *NFE2L2* (4%), *TP53* (5%), *ERBB2* (6%), *ELF3* (13%) *CBFB* (8%) [97]. These data demonstrate several recurrent genomic alterations in CC that suggest new strategies to combat this disease. The comprehensive genomic characterization of adenocarcinoma and SCCC by the Cancer Genome Atlas will certainly support advances in

developing more specific and effective ways to prevent, diagnose and treat CC.

Another key challenge to CC control in the era of molecularly targeted therapies is access to the new technologies. 80% of CC cases occur in the developing world where the limited resources available for treatment are not enough to provide effective surgical, radiotherapy and chemotherapeutic services; not much of the palliative care needed is available either. The advances in CC prevention and treatment will need to be extended to countries with limited resources to have a real impact in global mortality.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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LIST OF ABBREVIATIONS

CC	=	Cervical cancer
CTLA-4	=	Cytotoxic T-lymphocyte-associated protein 4
DLT	=	Dose limiting toxicity
EGF	=	Epidermal growth factor
EGFR	=	Epidermal growth factor receptor
FDA	=	Food and drug administration
HPV	=	Human papillomavirus
iPARP	=	Poly (ADP-ribose) polymerase inhibitor
LACC	=	Locally advanced cervical cancer
MAPK	=	Mitogen-Activated Protein Kinase
mTOR	=	Mammalian Target of Rapamycin
ORF	=	Open reading frame
ORR	=	Overall response rate
OS	=	Overall survival
PARP	=	Poly (ADP-ribose) polymerase
PD-1	=	Programmed death receptor-1
PFS	=	Progression free survival
PI3K	=	Phosphatidylinositol3'-kinase
PTEN	=	Phosphatase and tensin homolog
RT	=	Radiotherapy
SCCC	=	Squamous cell cervical carcinoma
SD	=	Stable disease
TKI	=	Tyrosine kinase inhibitor
VLPs	=	Virus-like particles

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