Article

The potential value of the PI3K/Akt/mTOR signaling pathway for assessing prognosis in cervical cancer and as a target for therapy†

Running title: PI3K/Akt in cervical cancer

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Abstract

Cervical cancer is a common gynecological cancer and a leading cause of cancer-related death in women globally. There is a need for the identification of prognostic and predictive biomarker for risk stratification. The phosphatidylinositol 3-kinase/ protein kinase B/ mammalian target of rapamycin (PI3K/ Akt/ mTOR) pathway is often dysregulated in cervical cancer, indicating that it may be a potential therapeutic target in the treatment of this malignancy, and could perhaps be used as a novel biomarker in the assessment of risk of developing cervical cancer. We aimed to provide an overview of the potential applications of the PI3K/Akt/mTOR pathway as biomarker for risk stratification, in predicting the prognosis of cervical cancer, and for developing new therapeutic approaches in patients with cervical cancer.

Keywords: PI3K/Akt/mTOR signaling pathway; human papillomaviruse; cervical cancer

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1. Introduction

Cervical cancer (CC) is one of the most common tumors in women globally; 530,000 new cases are diagnosed annually and approximately 275,000 patients die from the disease (Liao et al., 2014). There are several treatment modalities that are currently available for CC including, radiotherapy, chemotherapy and surgery. Surgical treatment is limited to women who have early stage disease and in those who have lost fertility (Piroozmand et al., 2016). CC is usually treated effectively with a combination of radiotherapy and platinum-based chemotherapy. However, these latter drugs may also damage normal cells (Jiang et al., 2015).

Human papilloma virus (HPV) is considered to be responsible for >90% of CC (de Sanjose et al., 2010). About 70–90% of viral infections are cleared by the immune system, but persistent HPV infections can cause a high-grade cervical intraepithelial neoplasia (CIN) and CC (Schiffman and Castle, 2005). HPV replicates episomally and can integrate into the genome. Integration is observed in late stage lesions or tumors, and is related to genome alteration and instability (Xu et al., 2013). The E6 and E7 viral oncoproteins inhibit TP53 and RB1 proteins altering cell cycle, apoptosis and DNA repair. The HPV16 and HPV18 types are the most oncogenic phenotypes; however at least 12 other high risk types have been identified (Guan et al., 2012).

HPV infects the genital areas of men and women, including the skin of the penis, vulva, and anus; the linings of the cervix, vagina, and rectum; and the linings of the throat and mouth. Because the signs and symptoms of HPV are often mild, most cases are unaware of the infection (Control and Prevention, 2011). Women who have sex at an early age, or who have

multiple sexual partners, or a have partner with multiple sexual partners are at greater risk of HPV infection and CC. However, HPV infections are often found in healthy female, who do not subsequently develop CC. The presence of immunosuppressive states may influence the rate of HPV. Women who are positive for HIV have an increased risk of HPV infection, and precancerous change may progress into invasive CC faster. Furthermore, infection with chlamydia has been considered to increase the risk for developing CC (Kessler, 2017). Infection with herpes simplex virus 2 (HSV2) may be linked with severe inflammation and microulcerative alterations of the cervical epithelium which may lead to the initiation and development of CC. Furthermore, long-term use of combined oral contraceptives has been related to an increased risk of CC; but, the risk falls after use is stopped (Kessler, 2017). Other potential risk factors for CC include more than 3 full-term pregnancies, cigarette smoking (Smith et al., 2015), treatment with immunosuppressive drugs, a first full-term pregnancy at age <17 years, a family history of CC, and a low socio-economic status (Kessler, 2017). General lifestyle factors that are associated with CC, include: a poor intake of fresh fruits and vegetable and being overweight.

Testing for HPV infection is now included as part of the screening protocol for CC, and HPV vaccines (bivalent and quadrivalent) are being used in routine clinical practice. With effective treatments such as surgery or concurrent chemoradiotherapy, the successful treatment rate of CC is over 80%–90% in the early and 60% in advanced stage. Although, the prognosis is still poor if cancer progresses to an advanced stage or there is relapse (Lim et al., 2017).

2. The PI3K/Akt/mTOR pathway

Growth factor receptor tyrosine kinase (RTKs) ligation leads to the activation of PI3K and the PI3K/Akt/mTOR pathway (Figure 1). Activated PI3K converts phosphatidylinositol-4,5-bis-phosphate(PIP2) to phosphatidylinositol-3,4,5-triphosphate(PIP3). Accumulation of PIP3

at the cell surface causes the activation of Akt, a serine–threonine kinase. Phosphatase and tensin homolog (PTEN) is a tumor suppressor that can dephosphorylate PIP3, reversing Akt activation and preventing downstream signaling. But, in the absence of the inhibitory effect of PTEN, Akt is phosphorylated and leads to mTOR activation. Activated mTOR subsequently comprises two distinctive multi-protein complexes, mTOR complex 1(mTOR1) and mTOR complex 2 (mTOR2) (Avan et al., 2016). Finally, phosphorylation and activation of two downstream signaling molecules, ribosomal protein S6 kinase1 (S6k) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) results in an enhanced translation of proteins that contribute to cell growth, differentiation, proliferation, angiogenesis and metabolism, apoptosis, and survival (Figure 1) (Bahrami et al., 2017).

The PI3K/Akt/mTOR pathway regulates multiple cellular and molecular functions, which are crucial for tumor initiation, invasion and metastasis. Some studies have shown that activation of PI3K/Akt signaling pathway is critically involved in a wide spectrum of human cancers, that include malignancies of the breast, ovary, endometrium, and malignant glioma. Furthermore, activation of the PI3K/Akt pathway is related to incomplete response in CC (Schwarz et al., 2012).

Genomic expression analysis has confirmed the critical role of PI3K pathway in CC development. The expression and activating mutations of the PIK3CA gene have been found in 23 –36% of CC specimens (McIntyre et al., 2013). Data from high-throughput genotyping studies testing 80 cervical tumors in 139 cancer genes for 1250 mutations have shown the highest mutation rate in patients with CCs was in the PIK3CA gene (31.3%), with no major differences between adenocarcinoma (AD) and squamous cell carcinomas (SCC) (25% vs. 37.5%, $p = 0.33$) (Wright et al., 2013). In another report, analysis of 675 cervical tumors showed activation of PIK3CA and other PI3K/AKT pathway genes in 31% of SCC and 24%

of AD (Lou et al., 2015). Data from TCGA has shown that mutations in PTEN and PIK3CA are present in 6% and 14% of SCC, respectively (Gao et al., 2013). In comparison to SCC, a smaller number of ADs have been identified as having fewer PIK3CA/PTEN mutations (Ojesina et al., 2014). Retrospective data have showed a better prognosis in early stage CC, if a PIK3CA mutation is present (McIntyre et al., 2013). Although others have reported reduced survival across all cancer patients with invasive disease (Wright et al., 2013).

A further analysis of 60 biopsies samples of progressive SCCs, high-grade squamous intraepithelial lesions (HSIL), and normal cervix (n=20) showed an increased nuclear translocation of both pmTOR and p70S6K. These finding indicate that the constitutive activation and high-expression of the mTOR pathway in SCC and HSIL (Feng et al., 2009). Liao and colleagues found that PI3K, Akt and MDM2 were over-expressed but p53 down-expressed in CC (Liao et al., 2014).

Active HPV infection leads to an induction of benign and malignant neoplasms in the cervix via its interactions with different cellular pathways in host cells (Doorbar, 2005). In addition to the suppression of pRb and p53, HPVs interacts with at least four upstream pathways (Notch receptor, growth factor receptor, Ras and PI3KCA genes) to induce host cell proliferation and survival, leading to tumorgenesis by activation and alteration of the downstream compounds of the PI3K pathway (Keysar et al., 2013). It has been shown that PI3K is expressed and activated in HPV-induced CCs (Lee et al., 2006).

Taken together, the aberrant activation of the PI3K/Akt/mTOR pathway provides the rationale behind exploration of PI3K/Akt/mTOR pathway and its inhibitors in CC for potential use as prognostic and therapeutic purposes. PI3K and Akt specific inhibitors as well as dual PI3K/mTOR are currently being tested as non-hormonal targeted therapies in clinical trials (NCT01958112, NCT01217177, and NCT01026792).In this review, we summarize the

current knowledge of the roles of the PI3K/Akt/mTOR signaling pathway both as a biomarker and as a potential therapeutic target in cervical tumors.

3. The PI3K/Akt/mTOR pathway as a prognostic marker in cervical cancer

In vitro studies have shown that PI3K is over-expressed in CC cell lines, and treatment with PI3K inhibitors prevents cell growth (Zhang et al., 2008). Faried *et al*. evaluated the predictive and prognostic value of molecular over-regulation in CC treated with cisplatin-based chemotherapy (Faried et al., 2006). They report that activated AKT and mTOR are indicators of poor prognosis. Furthermore, expression analysis of activated AKT and mTOR in patients with AD of the cervix showed that p-AKT and p-mTOR were present in 50% and 53% of AD, respectively (Faried et al., 2008). Overall the expression level p-mTOR was a significant independent prognostic biomarker in AD of the cervix. Recent data from CC cells lines have demonstrated that activation of the PI3K pathway triggers the PTEN ubiquitination, leading to uncontrolled positive feedback (Lee et al., 2015). These mechanistic studies indicate acquired PI3K pathway activation. Investigators have examined downstream mTOR inhibition and observed potent *in vivo* activity using cell line derived xenografts (Molinolo et al., 2012).

HPV regulates E2F and thereby modulates the Akt/mTOR signaling pathway (Banister et al., 2017). In line with these data another study has shown that oncogenic HPV E6 causes activation of the mTOR pathway in CC cell lines (Molinolo et al., 2012). The PI3K/Akt /mTOR pathway is associated with the up-regulation of tumor suppressor p16INK4a by HPVs. A high-expression of p16INK4a is common in CC where there is a high-risk of HPVs inactivating pRb protein (Lee et al., 2006). S6K phosphorylation is related to HPV16 infection in CC (Zhou et al., 2007). Immunohistochemical analysis of p-S6K and p-S6 in 140

CC specimens has shown that both p-S6K and p-S6 were significantly more common in the HPV16 (+) CC compared to those in the HPV16 (-) samples. The oncoprotein, HPV16 E6 activates S6K through Akt signaling, which increases S6K phosphorylation and maintains the activity of the both mTORC1 and mTORC2 signaling pathway (Figure 1) (Zhou et al., 2007).

It has been shown that HPV E7 is associated with a markedly overexpressed Akt activity, which relies on the ability of E7 to inactivate the pRb family of proteins (Menges et al., 2006). Up-regulation of AKT activity and loss of pRb were found in HPV (+) cervical advanced squamous intraepithelial lesions compared to normal cervical tissue. Thus, pRb expression is negatively related with Akt activity in HPV(+) cervical advanced squamous intraepithelial lesions (Menges et al., 2006). E7 directly activates Akt through phosphorylation which subsequently causes phosphorylation of BAD (a downstream target of Akt). Akt phosphorylation is related to activated Notch1 signaling pathway which regulates the PI3K pathway (Wu et al., 2013). It has been found that protein phosphatase 2 (PP2A), a serine/threonine phosphatase, joins with the structural subunits of p-Akt in order to dephosphorylate Akt (Li et al., 2003). Akt dephosphorylation reduces its activity in inhibiting cell apoptosis. E7 binds to the PP2A subunits to inhibit their interactions with p-Akt, thus maintaining Akt signal activation (Pim et al., 2005). These preclinical studies support an important role for PI3K pathway inhibition in the clinic.

4. PI3k/Akt/mTOR inhibitors as a therapeutic target in cervical cancer

CCs are usually treated with a combination of chemotherapy (platinum-based) and radiation; however, a number of tumors acquire resistance to chemotherapeutic compounds, resulting in treatment failure. There are also a number of natural products derived from plants that have been shown to prevent cell proliferation, induce apoptosis, restore metastasis and inhibit angiogenesis in CC by regulating the PI3K/AKT/mTOR signaling pathway. It has been

suggested that these compounds have potential for cancer treatment. Several in vitro studies have assessed the effects of natural compounds in CC cell lines (summarized in Table1).

A tissue microarray analysis showed that 52% of CC patients express p-mTOR in the cytoplasm and on the membrane of tumor cells (Faried et al., 2006). Lee and colleagues have reported that pretreatment of CC cell lines with the PI3K/Akt inhibitor LY294002 promoted radiation sensitivity *in vitro* (Lee et al., 2006). Furthermore, LY294002, significantly reduced the cisplatin-mediated viability reduction of Caski and HeLa cells, indicating the contribution of PI3K/Akt pathway in Cisplatin resistant CC cells (Shu et al., 2015). Rapamycin and other rapalogs disrupt the PI3K/Akt/ mTOR pathway primarily by blocking mTOR1, inhibiting phosphorylation of S6 kinase1, 4E-BP1, and other proteins causing cell cycle arrest and reducing angiogenesis (Diaz-Padilla et al., 2012).

In a recent study, treatment of CC cell lines with LY294002 resulted in reduced AKT1 activity. The authors found that treatment with LY294002 led to G1 cycle arrest in cervix cancer-derived cells (Prasad et al., 2015).

The differences between HPV-inactive and HPV-active tumors have supported the potential use of various targeted therapeutic approaches. For instance, an EGF-receptor inhibitor, gefiinib, is more effective in treating HPV-active CCs, while dasatinib is more efficient in treating HPV-inactive tumors. Somatic gene mutations upstream of AKT are more frequent in HPV-inactive cancers, suggesting that dual PI3K/mTOR inhibitors may be more potent in these patients (Banister et al., 2017). Genetic polymorphisms in the PI3K/ Akt pathway are related to the sensitivity to platinum-based neoadjuvant chemotherapy in cervical squamous cervical cancer (CSCC) patients (Guo et al., 2015).

It has also been shown that treatment with a combination of PI3K inhibitors and NaBT significantly reduces the viability of HeLa cells. Suppression of PI3K promotes NaBT-

mediated apoptosis via activation of caspase-3 and -9 and the cleavage of poly (ADP-ribose) polymerase in human CC cells(Park et al., 2006).

Several studies show that mTOR activation is present in at least 60% of the HPV-related cancer patients, suggesting that mTOR activation plays an important role in HPV-induced carcinogenesis. Simultaneous use of mTOR inhibitors such as RAD001and rapamycin added to standard-of-care cisplatin/radiation therapy has been investigated in HPV(+) cervical cancer squamous cell carcinomas (CCSCC) tumor xenografts and mouse models for examining the preclinical efficacy of mTOR inhibitors (Coppock et al., 2013). Both inhibitors reduce mTOR activity, and significantly reduce tumor burden (Molinolo et al., 2012) and improve survival in immune-compromised mice (Coppock et al., 2013). As well the effect of oncogenic HPV infection on mTOR signaling, a high prevalence of PIK3CA mutations have been found in CC, providing further support for a possible role of mTOR inhibitors in this disease. Taken together, these studies provide evidence for the clinical application of PI3K/Akt/mTOR inhibitors as a molecular targeted therapy approach for HPV-related cancers.

4.1.Clinical trials with PI3K targeted agents

The data on the application of targeted therapy affect the PI3K pathway in CC patients is limited (Table 2). In a Phase I study of 15 CC patients have PIK3CA mutations, among 5 cases were treated with compounds targeting the PI3K/AKT/mTOR pathway, 2 had a partial response (Janku et al., 2012). Another phase I study included two women with advanced or recurrent CSCC. Patients were allocated with weekly temsirolimus and topotecan and one of them experienced stable disease for 3 months. The combined regimen was not well-tolerated in patients who previously received pelvic radiation therapy (Temkin et al., 2010).

Preliminary results of a phase II study using temsirolimus (CCI-779) monotherapy in patients with metastatic, recurrent, locally advanced CC showed that among 38 enrolled patients; 33 were evaluated for response and 37 for toxicity. One patient had a partial response for 7.2 months, and 19 patients experienced stable disease with a median 6.5 months (range 2.4–12.0 months). The median PFS was 3.52 months (95% confidence Interval (CI): 1.81–4.7%) and 6-month PFS was 28% (95% CI:14–43%).No grade 4–5 adverse events (AEs) were found(Tinker et al., 2013).

NVP-BEZ235 is a novel dual PI3K/mTOR inhibitor that has dramatic effects on many neoplasms. Recently the effects of NVP-BEZ235 has been investigated in the proliferation and invasion of CC cells. NVP-BEZ235 effectively inhibited dysfunctional PI3K/mTOR pathway activation, blocked cell growth in a concentration - and time -dependent manner, caused G1 cycle arrest, and stimulated apoptosis in CC cell lines. Furthermore, NVP-BEZ235 treatment in combination with cisplatin/ carboplatin led to synergistic anti-tumor response in CC cells(Xie et al., 2017).

In summary, the PI3K/AKT/mTOR signaling pathway is a promising targeted approach for CC treatment and further clinical trials are required. At present, there are ongoing trials with temsirolimus alone or in combination with chemo- and radiation therapy with or without natural as a sensitizer (Wu et al., 2013).

4.2. Biomarkers related with response to PI3K pathway inhibitors

Although preclinical evidence suggests that alterations in PI3K pathway lead to an improved response to well-matched targeted biologic drugs, data from phase I /II trials have been inconsistent. Reliable biomarkers have yet to be identified. A retrospective pooled analysis of 140 patients with ovarian, cervical, endometrial and breast cancer treated with PI3K/ AKT/ mTOR inhibitors demonstrated that patients with PIK3CA mutations had a higher response

rate than those without mutations (partial response 30% vs. 10%, p = 0.04) (Janku et al., 2012). Also, the association between alterations in the PI3K signal path and targeted therapy has been evaluated in metastatic or recurrent CC. Targeted therapies directly against the PI3K pathway in patients who had tumors containing PIK3CA mutations or PTEN loss led to a favorable rate of stable disease over 6 months or complete response or partial response (53%) and significantly lasting PFS than non-matched therapy (Hou et al., 2014). In addition, in patients with SCC PIK3CA mutation was linked with significantly longer OS than for those tumors without a PIK3CA mutation (median 9.4 vs. 4.2 months, p = 0.019, respectively).

Moreover, the expression of p-mTOR may be implicated as an indicator to predict survival and response to chemotherapy of CC patients (Feng et al., 2009). Indeed, Kim *et al.* have expressed that cytoplasmic expression of p-mTOR was related with poorer response to radiotherapy (Kim et al., 2010).These data require confirmation but indicate that alterations in the PI3K pathway may possibly affect response.

5. E6/E7 inhibitors in cervical cancer

RNA interference (RNAi) has been found to be an effective tool to selectively silence gene expression and has great promise for the treatment of viral diseases, genetic disorders and cancer (Bitko et al., 2005). Previously it has been shown that small interference RNA (siRNA) can prevent HPV E6/ E7 expression in different cancer cell lines (Butz et al., 2003; Jiang and Milner, 2002; Yamato et al., 2006). However in these studies suppression was either transient, inconsistent,or did not affect all cells, leading to the potential of escape. Gu et al. reported that short hairpin RNA which were delivered using a lentiviral vector (LV-shRNA) was an effective way to get stable inhibition of E6/E7 oncogene expression and stimulate suppression of tumor growth either in vitro or in vivo(Gu et al., 2006)

6. Conclusion:

Essentially all cervical cancers contain human papillomavirus (HPV) DNA suggesting that HPV infection is necessary for cervical cancer initiation (Walboomers et al., 1999).Women with CC commonly display alterations in the PI3K signaling pathway, and biological agents have shown the potential, albeit modest, to impact on the treatment of advanced and metastatic disease. The data provide a proof of concept of its potential value as a prognostic biomarker, although further studies with larger sample population in multi-center setting are needed to explore its value as a prognosticate marker. Indeed, the careful selection of patients based on predictive biomarkers, the utilization of novel combinations of chemotherapeutic, hormonal and immunomodulatory agents, and a greater understanding of resistance pathways and metabolic alterations will allow PI3K pathway inhibitors to be used to their greatest effect. Results from ongoing trials in selected populations with translational endpoints will guide our use of PI3K pathway inhibitors in the future. Future clinical trial designs that select patients based on biomarkers or characterized preclinical molecular interactions will be crucial to identifying populations of women with CCs most likely to respond to PI3K pathway inhibition.

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Table 1. Natural products as potential therapeutic agents in the treatment of cervical cancer

Natural products	Origin	Cervical cancer cell line	results	Author. year
Dehydrocostus lactone (DHC)	a main sesquiterpene lactone ingredient of natural Chinese herb medicine Saussurea lappa	Hela and C33a	reduced the level of p-Akt phosphorylation, independently of HPV infection	(Jiang et al., 2015)
RCE-4	most abundant and bioactive member in R. carnea ethyl acetate fraction	HeLa	reduced PI3K/Akt/mTOR signaling pathway	(Bai et al., 2016)
4-Omethylhonokiol (MH)	bioactive compound of the Magnolia bark derived from Magnolia officinalis	SiHa	decreased p-Akt in the apoptotic process	(Hyun et al., 2015)
Butein	isolated in various plants including Toxicodendron vernicifluum (Rhus verniciflua), Semecarpus anacardium and Dalbergia odorifera	HeLa	reduced the phosphorylation of PI3K, AKT and mTOR expression	(Bai et al., 2015)
Fucoxanthin	as a carotenoid pigment that commonly found in marine algae	HeLa	inhibited Akt phosphorylation	(Ye et al., 2014)
Concanavalin A (Con A)	mannose or glucose specific legume lectin	HeLa	suppresses the PI3K/Akt/mTOR pathway	(Roy et al., 2014)
Kaempferol	a flavonoid	HeLa	inhibiting PI3K/AKT pathways	(Kashafi et al., 2017)

Table 2. Cervical cancer clinical trials using PI3K/Akt/ mTOR inhibitors

Cancer type	*Intervention*	*Phase*	*Status*	***References***
Metastatic or Locally Advanced Cervical Cancer	BKM120(PI3K inhibitor)	II	withdrawn prior to enrollment	**NCT01613677**
Solid Tumors	ARQ092 (AKT inhibitor) Carboplatin paclitaxe	I	recruiting participants	**NCT02476955**
Advanced Solid Malignancies	AZD5363 (AKT inhibitor)	I	currently recruiting participants	**NCT01226316**
Cervical Cancer	GSK1120212 (MEK inhibitor) GSK2141795 (AKT inhibitor)	II	terminated	**NCT01958112**
cervical cancer that is recurrent, locally advanced, metastatic, or cannot be removed by surgery	Temsirolimus (mTOR inhibitor)	II	completed	**NCT01026792**
Unresectable or Metastatic Solid Tumors	Temsirolimus (mTOR Inhibitor) Vinorelbine ditartrate	I	completed	**NCT01155258**
locally advanced cervix cancer	Everolimus (mTOR inhibitor) + radiotherapy + cisplatin	I	completed	**NCT01217177**

Figure 1 .Role of the PI3K/Akt/mTOR pathway in cervical cancer. E6, E7 HPV oncoproteins inducing the PI3K/Akt/mToR signalling pathway. PI3K=phosphoinositide 3 kinase; PTEN= phosphatase and tensin homologue deleted on chromosome ten; Akt=protein kinase B; mTORC= mammalian target of rapamycin complex; ; 4EBP1=eukaryotic initiation factor 4E-binding protein 1; S6K=s6 kinase

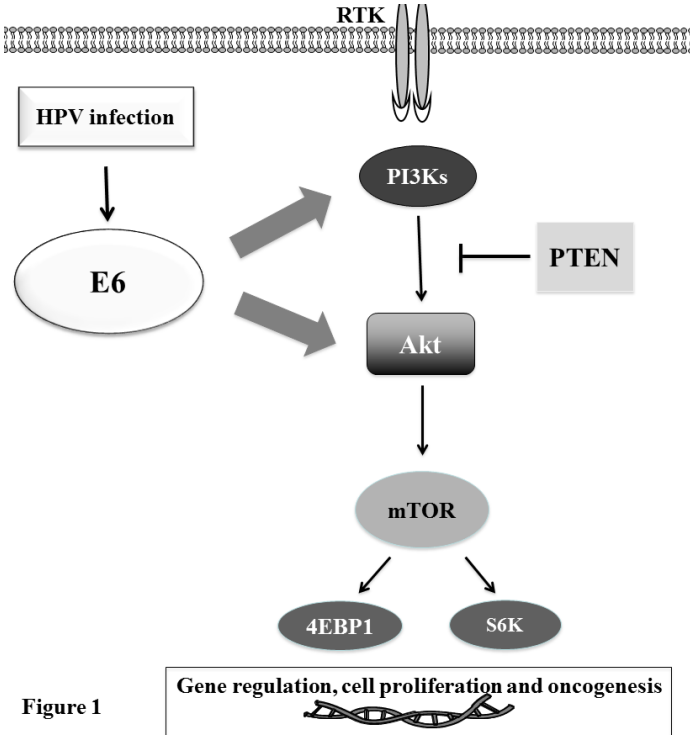

Figure 1