Original Article
Morphoproteomic Evidence of Constitutively Activated and Overexpressed mTOR Pathway in Cervical Squamous Carcinoma and High Grade Squamous Intraepithelial Lesions

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Abstract: Human papilloma virus (HPV) infection of the uterine cervix is linked to the pathogenesis of cervical cancer. Preclinical *in vitro* and *in vivo* studies using HPV-containing human cervical carcinoma cell lines have shown that the mammalian target of rapamycin (mTOR) inhibitor, rapamycin, and epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor, erlotinib, can induce growth delay of xenografts. Activation of Akt and mTOR are also observed in cervical squamous cell carcinoma and, the expression of phosphorylated mTOR was reported to serve as a marker to predict response to chemotherapy and survival of cervical cancer patients. Therefore, we investigated: a) the expression level of EGFR in cervical squamous cell carcinoma (SCC) and high-grade squamous intraepithelial lesions (HSIL) versus non-neoplastic cervical squamous epithelium; b) the state of activation of the mTOR pathway in these same tissues; and c) any impact of these signal transduction molecules on cell cycle. Formalin-fixed paraffin-embedded tissue microarray blocks containing 20 samples each of normal cervix, HSIL and invasive SCC, derived from a total of 60 cases of cervical biopsies and cervical conizations were examined. Immunohistochemistry was utilized to detect the following antigens: EGFR; mTOR pathway markers, phosphorylated (p)-mTOR (Ser2448) and p-p70S6K (Thr389); and cell cycle associated proteins, Ki-67 and S phase kinase-associated protein (Skp)2. Protein compartmentalization and expression were quantified in regard to proportion (0-100%) and intensity (0-3+). Mitotic index (MI) was also assessed. An expression index (EI) for p-mTOR, p-p70S6K and EGFR, respectively was calculated by taking the product of intensity score and proportion of positively staining cells. We found that plasmalemmal EGFR expression was limited to the basal/parabasal cells (2-3+, EI = 67) in normal cervical epithelium (NL), but was diffusely positive in all HSIL (EI = 237) and SCC (EI 226). The pattern of cytoplasmic p-mTOR and nuclear p-p70S6K expression was similar to that of EGFR; all showed a significantly increased EI in HSIL/SCC versus NL (p<0.02). Nuclear translocation of p-mTOR was observed in all SCC lesions (EI = 202) and was significantly increased versus both HSIL (EI = 89) and NL (EI = 54) with p<0.015 and p<0.0001, respectively. Concomitant increases in MI and proportion of nuclear Ki-67 and Skp2 expression were noted in HSIL and SCC. In conclusion, morphoproteomic analysis reveals constitutive activation and overexpression of the mTOR pathway in HSIL and SCC as evidenced by: increased nuclear translocation of p-mTOR and p-p70S6K, phosphorylated at putative sites of activation, Ser2448 and Thr389, respectively; correlative overexpression of the upstream signal transducer, EGFR, and increases in cell cycle correlates, Skp2 and mitotic indices. These results suggest that the mTOR pathway plays a key role in cervical carcinogenesis and targeted therapies may be developed for SCC as well as its precursor lesion, HSIL.

Key Words: mTOR pathway, cervical squamous carcinoma, high grade SIL, morphoproteomics

Introduction

Squamous cell carcinoma (SCC) of the uterine cervix is the most common malignant tumor of the female genital tract worldwide [1]. It is well known that the human papilloma virus (HPV), especially the high risk variants HPV 16 and 18, is the single most important etiologic agent in the pathogenesis of cervical carcinoma [1]. HPV-encoded oncoproteins, E5, E6 and E7 all possess immortalizing activity via their respective direct or indirect interactions with epidermal growth factor receptor (EGFR), p53 and retinoblastoma (Rb)

proteins. Past studies have shown that the expression of E7 is directly linked to the growth and survival of the HPV-associated cancer cells [2-5]. Furthermore, studies based on cell culture have also shown that the cellular translation machinery, namely the downstream effector of the mammalian target of rapamycin (mTOR) pathway, eukaryotic initiation factor (eIF) 4E via mTOR's interaction with translation inhibitor 4E-BP1, plays a significant role in maintaining a high level of E7 protein expression [6]. Additionally, E5 is shown to activate EGFR and delay its degradation to promote cellular proliferation [7].

The serine/threonine kinase mTOR pathway is an appealing therapeutic target because it has an identified inhibitor, rapamycin, an analogue of which (temsirolimus) has been utilized in phase II and III clinical trials for metastatic renal cell carcinoma [8, 9]. The mTOR pathway has been shown in preclinical studies to play a role in the carcinogenesis of breast, ovary and prostate as well as gastrointestinal malignancies. Studies have also demonstrated that the phosphorylative activation of p70S6K via the mTOR pathway resulted in increased G1 cell cycle progression, cell survival, and tumor cell proliferation [10-12]. A study from Japan showed that Akt and mTOR are activated in advanced stage of cervical squamous cell carcinoma and the activation was observed to serve as a marker to predict response to chemotherapy and survival of cervical cancer patients [13]. The receptor tyrosine kinase (RTK), epidermal growth factor receptor (EGFR) is involved in the regulation of cell proliferation, survival, mobility, and differentiation [14,15]. Its expression in a majority of cervical SCCs and encouraging results from preclinical trials in various tumors of the lung, pancreas, central nervous system and head and neck has made it an attractive target for targeted therapy using EGFR inhibitors, such as erlotinib, which also show synergistic response with the mTOR pathway inhibitors, rapamycin and temsirolimus [14-24].

The goal of this study is to characterize the expression levels of EGFR, the activation of the mTOR pathway and the proliferative cell cycle markers in the same tissues of cervical SCC and high-grade squamous intraepithelial lesions (HSIL) versus non-neoplastic cervical squamous epithelium.

Materials and Methods

Study Group

The pathology database at Lyndon B. Johnson Hospital was retrospectively reviewed and a total of 60 cases of normal cervix, HSIL or cervical dysplasia and invasive cervical SCC with adequate tissue were selected with IRB approval. All identifiers were removed to protect patient's confidentiality. Three tissue microarray blocks containing 20 tissue samples each of normal cervix, HSIL and invasive cervical SCC were created by manually re-embedding archived paraffin-embedded tissues.

Immunohistochemical Staining

Tissue microarray sections, 4 µm thick, were deparaffinized in xylene and rehydrated in a graded series of ethanols. Heat-induced epitope retrieval was performed. Phospho-specific probes include antibodies directed against mTOR and p70S6K, phosphorylated at putative sites of activation, serine (Ser) 2448 and threonine (Thr) 389, respectively (Cell Signaling Technology, Inc., Beverly, MA, U.S.A.). The tissue was then treated with 3% H_2O_2 and rinsed with Tween-20 (TBST) buffer. A few drops of diluted normal blocking serum were placed on the tissue and incubated at room temperature. The serum was then blotted off and the slides were incubated with primary antibody overnight at 4°C. The rest of the staining procedure took place on a DAKO Autostainer programmed to treat each slide with diluted biotinylated secondary antibody for 30 minutes. The slides were then rinsed and incubated with DAB (3, 3'-diamino-benzidine chromogen solution, EnVision+ System Kit, DAKO) for 10 minutes. The slides were rinsed again and counterstained with Gill II hematoxylin, treated with xylene and cover slipped. The remaining immunohistochemical staining for EGFR (monoclonal, 1:50, 30 minute incubation at room temperature, 28-8005, Zymed, San Francisco, CA, U.S.A.), Ki-67 (1:500, 30 minute incubation at room temperature, M7240, DAKO, Denmark) and Skp2 (1:50, 45 minute incubation at room temperature, sc-7164, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) were performed as described above with DAKO Autostainer. Positive and negative controls were noted to react appropriately.
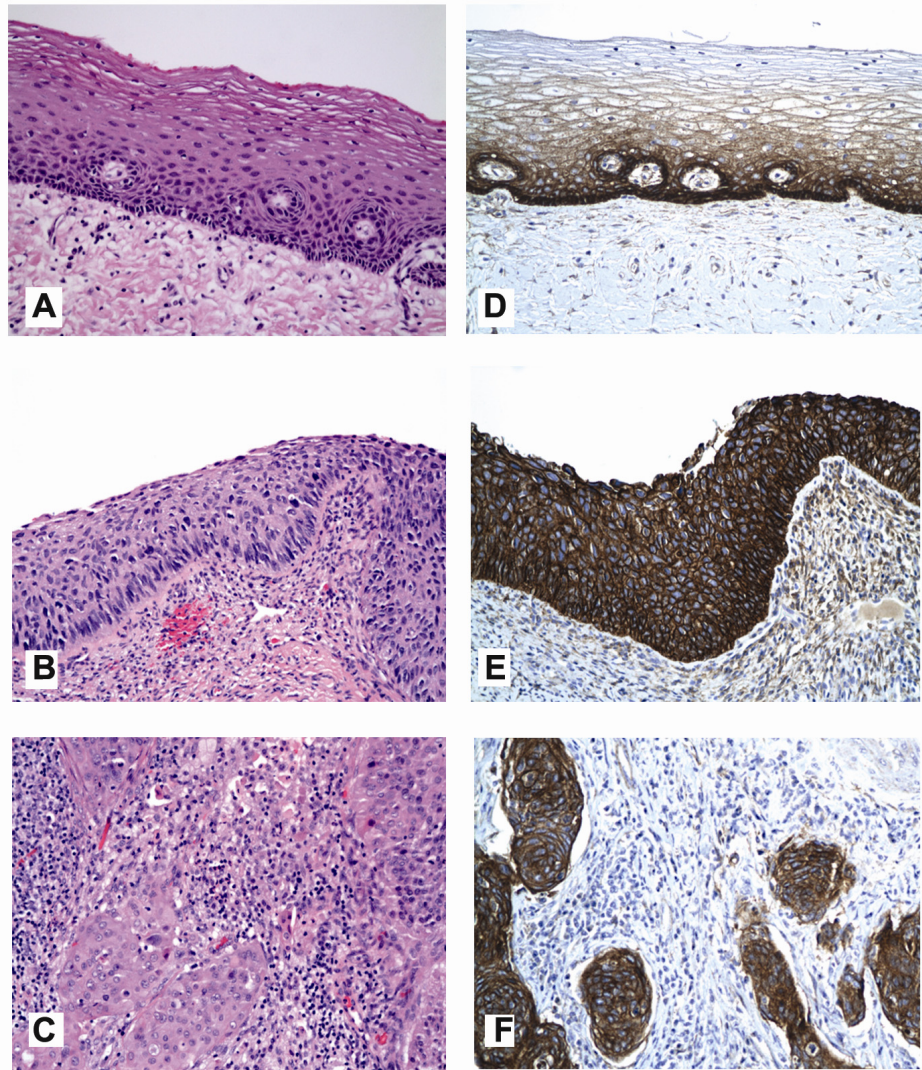
Figure 1 Representative hematoxylin-eosin (H&E) images of uterine cervical mucosa illustrating non-neoplastic (normal) squamous epithelium (frame **A**), high grade squamous intraepithelial lesion (HSIL; frame **B**) and invasive cervical squamous cell carcinoma (SCC, frame **C**). Companion images depicting the level of expression of epidermal growth factor receptor (EGFR) on the plasmalemmal (cell membranous) aspect of the epithelial cells reveal: moderate to strong (2 to 3 +) DAB [brown] chromogenic signal limited to the basal and parabasal cells of normal epithelium (frame **D**); and overexpression of EGFR protein in HSIL and SCC evident by strong (3+) plasmalemmal expression that decorates virtually all lesional cells (frames **E** and **F**, respectively) (DAB [brown] chromogen; original magnification x200).

Table 1 Average expression index (EI) of EGFR and mTOR pathway markers: p-mTOR & p-p70S6K; percent cells with positive expression of proliferation markers: Ki-67 & Skp-2; and average mitotic index per ten (10) high power fields.

Tissue type	Cytoplasmic p-mTOR	Nuclear p-mTOR	Nuclear p-p70S6K	Cytoplasmic/ membranous EGFR	Mitotic index	Ki-67	Skp-2
Normal	42	54	197	67*	0	2%*	4%*
HSIL	123	89	251	237	13	70%	25%
SCC	94	202	251	226	14	72%	54%

*Basal/parabasal cell staining only. Each tissue type consists of 20 specimens
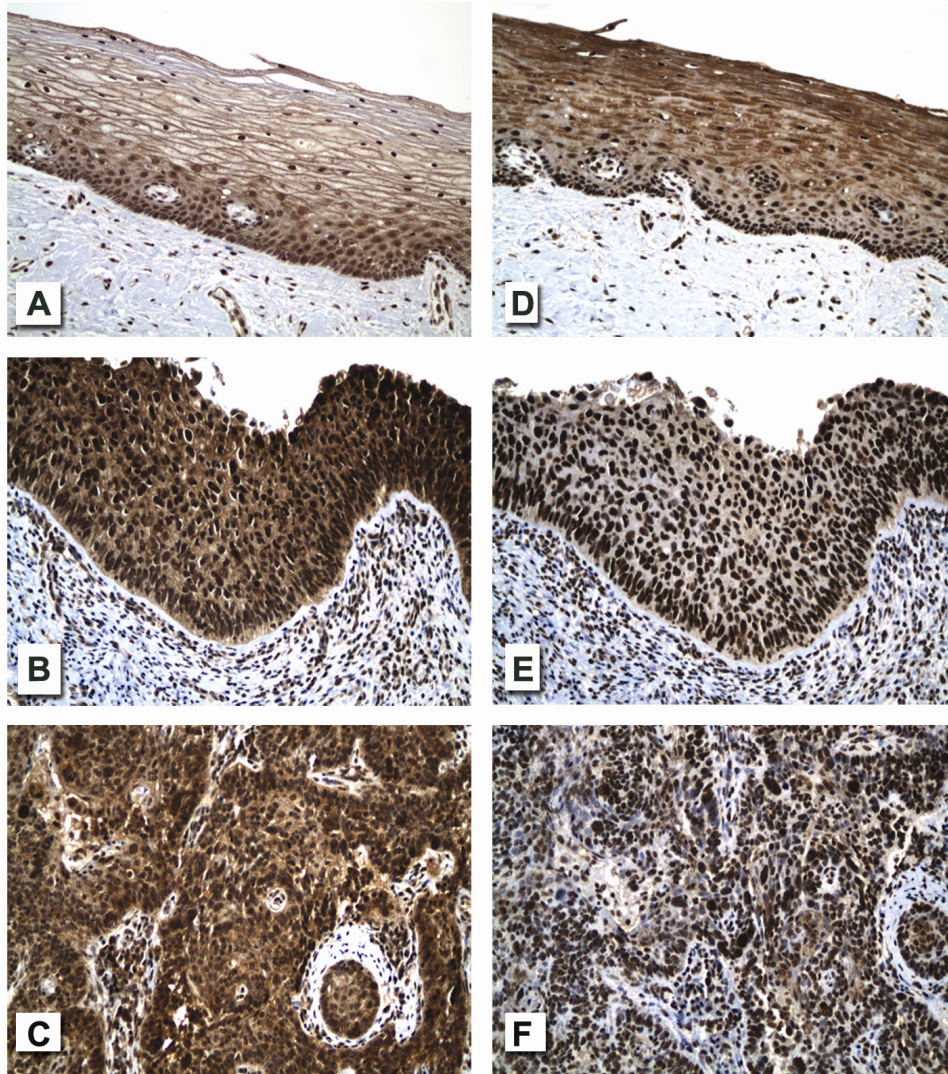
Figure 2 Relative expression levels for mTOR pathway components, mTOR (phosphorylated on serine 2448) and p70S6K (phosphorylated on threonine 389) reveal: weak to moderate (1-2+) cytoplasmic and nuclear immunoreactivity (brown [DAB] chromogenic signal) for p-mTOR (Ser 2448) in the normal cervical squamous epithelium, particularly in the basal and parabasal cells (frame **A**) but with moderate to strong (2-3+) cytoplasmic and nuclear expression of this analyte in both HSIL and invasive squamous cell carcinoma involving virtually all of the lesional cells (frames **B** and **C**, respectively); and with diffuse and qualitatively similar nuclear expression levels for p-p70S6K (Thr 389) in normal cervical squamous epithelium, HSIL and invasive squamous cell carcinoma (frames **D**, **E** and **F** respectively; also see quantitative Expression Index [EI] for each of these analytes in **Figure 4** and statistical differences in **Table 2**. Note the moderate cytoplasmic signal for p-p70S6K in supra basal zones of normal cervical epithelium (frame **D**) vis-à-vis that seen in HSIL and invasive squamous cell carcinoma (frames **E** and **F**). Subepithelial and interstitial inflammatory cell infiltrate present in HSIL and invasive squamous cell carcinoma shows variable nuclear signals for p-mTOR (frames **B** and **C**) and p-p70S6K (frames **E** and **F**) (original magnification x200).

Tissue Microarray Analysis

Two pathologists visually read the tissue array under the microscope and scored each core individually. Cases in which no epithelial cells were found or no core was available were excluded. Protein expression was semiquantified in regard to the intensity of cell staining graded as 0 to 3+ ("0" for negative staining if there is total absence; score of "1", "2", or "3" in order of ascending strength of positive staining), as well as the percentage (ranging from 0 to 100%) of lesion with positive staining of any intensity ranging from

1 to 3. Resulting staining intensity scores and percentage of positive staining were averaged between the two pathologists. Mitotic index (MI) was also assessed on routine H&E stained microarray slides as number of mitotic figures per ten (10) high power fields. An expression index (EI) for p-mTOR, p-p70S6K and EGFR, respectively was calculated by taking the product of intensity score and proportion of positively staining cells.

Statistical Analysis

Student t-test was used to determine significance of differences in staining expression between normal cervix, HSIL and SCC. Excel was used for statistical analysis and 2-tailed p values were used to assess statistical significance.

Results

Plasmalemmal (membranous) EGFR expression was strongly positive (2-3+) in the majority (65%) of the normal cervical epithelium (NL) cases but is partial and limited to the basal/parabasal cells with an EI of 67, and more diffusely and strongly positive in all HSIL (2-3+ in 95% of cases, EI = 237) and SCC (2-3+ in 90% of cases, EI 226) (**Figures 1** and **4** and **Table 1**).

The pattern of cytoplasmic p-mTOR and nuclear p-p70S6K expression was similar to that of EGFR. Cytoplasmic p-mTOR expression was intermediate positive (1-2+) in most (100% of cases) of NL cervical epithelium but is partial and limited to the basal/parabasal cells with an EI of 67, and more diffusely and strongly positive in all HSIL (2-3+ in 95% of cases, EI = 237) and SCC (2-3+ in 90% of cases, EI 226). Nuclear p-p70S6K expression was strongly positive (2-3+) in majority (80% of cases) of NL cervical epithelium but was partial and generally limited to the basal half with an EI of 197, and more diffusely and strongly positive in all HSIL (2-3+ in 85% of cases, EI = 251) and SCC (2-3+ in 80% of cases, EI = 251) (**Figures 2** and **4** and **Table 1**). Moderately increased cytoplasmic retention of p-p70SbK was evident in the NL epithelium *Vis-a-Vis* that in HSIL/SCC.

Nuclear p-mTOR expression was intermediate positive (1-2+) in most (95%) of NL cervical epithelium but is partial and limited to the basal/parabasal cells with an EI of 54, and more diffusely and strongly positive in all HSIL (2-3+ in 30% of cases, EI = 89) and SCC (2-3+ in 45% of cases, EI = 202) (**Figures 2** and **4** and **Table 1**).

The proliferation markers Ki-67 and Skp-2 showed similar stepwise increase in percent of positive cells as in the average mitotic index in NL cervical epithelium (limited to the basal/parabasal layer), HSIL (lower two-thirds in majority of cases) and SCC (diffuse). Skp2 showed positive staining in a smaller percent of cells compared to Ki-67 in NL, HSIL and SCC (**Figure 3** and **Table 1**).

Cytoplasmic/plasmalemmal or membranous EGFR, cytoplasmic p-mTOR and nuclear p-p70S6K all show significantly increased EI in HSIL/SCC compared to NL (p<0.02). The nuclear p-mTOR EI in SCC lesions was significantly increased compared to both HSIL and NL (p<0.015 and p<0.0001, respectively). Similarly, the proliferation markers Ki-67, Skp2 and mitotic index were significantly increased in HSIL and SCC compared to NL (**Table 2**).

These comparative results for normal cervical epithelium (NL), HSIL and SCC are graphically depicted in **Figure 4** and **Table 1**, and significant mean differences are summarized in **Table 2**.

Discussion

This is a morphoproteomic study of the mTOR pathway in HSIL and invasive cervical SCC in relation to non-neoplastic (normal) uterine cervical epithelium. Morphoproteomic analysis utilizes phosphospecific probes directed against putative sites of activation in a given protein to characterize its expression level and subcellular translocation within a signal transduction pathway. It also incorporates expression levels of other protein analytes such as upstream transducers and downstream effectors from a signal transduction pathway to affirm the state of constitutive activation. Ultimately, comparative quantitation of signaling pathway activation between normal and neoplastic or non-neoplastic diseased tissues provides insight into the potential role of a signaling pathway in the etiopathogenesis of a disease process and/or the histogenesis of a tumor [25].

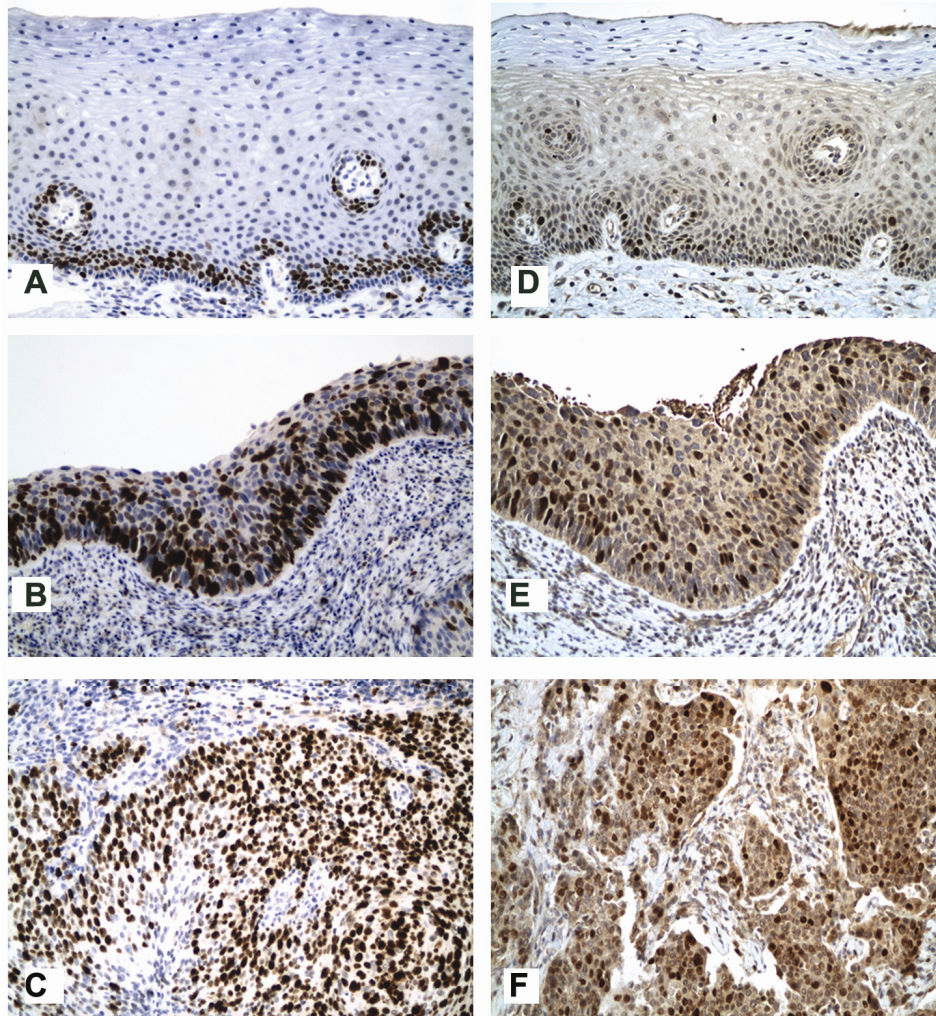The morphoproteomic analysis in this study reveals the constitutive activation and

Figure 3 Analysis of nuclear expression levels for cell cycle–associated markers, Ki-67 (G1, S, G2 and M phases of cell cycle) and Skp2 (S phase kinase-associated protein) reveals limited (parabasal only) expressions for both analytes in normal cervical squamous epithelium (frames **A** and **B**, respectively) but with strong and frequent expression involving middle to full thickness epithelium in HSIL and strong and diffuse expressions in invasive squamous cell carcinoma (frames **B** and **D**, and **E** and **F** for Ki-67 and Skp2, respectively) (original magnification x 200; also see numerical data and statistical comparisons in **Tables 1** and **2**).

overexpression of the mTOR pathway in HSIL and invasive cervical SCC vis-à-vis non-neoplastic (normal) uterine cervical epithelium by virtue of the following findings: the phosphorylation of mTOR at serine 2448 with nuclear translocation [25-29] and an increased EI in both HSIL and invasive SCC for cytoplasmic and nuclear p-mTOR (Ser 2448) as compared with normal cervical squamous epithelium; the correlative downstream overexpression of nuclear p-p70S6K (Thr 389) [27, 28, 30-36]; consistent cell cycle promoting effects of p-p70S6K in terms of the percent of neoplastic epithelial nuclei expressing the cell cycle protein analytes, Ki-

67 and Skp2 [11, 12, 37-41], as well as increased mitotic activity compared with the normal epithelial cells; and finally, correlative overexpression of EGFR, an upstream tyrosine kinase that transduces signals through both the PI3'-K/Akt/mTOR and ras/Raf kinase/extracellular signal-regulated kinase (ERK) 1/2 pathways, thereby contributing to the constitutive activation of mTOR kinase and p70S6K in HSIL and invasive SCC (see **Figure 5**). Parenthetically, signaling of EGFR through both pathways is consistent with the finding by Bertelsen and colleagues that there is frequent PI3KCA amplification and Akt phosphorylation in uterine cervical neoplasia
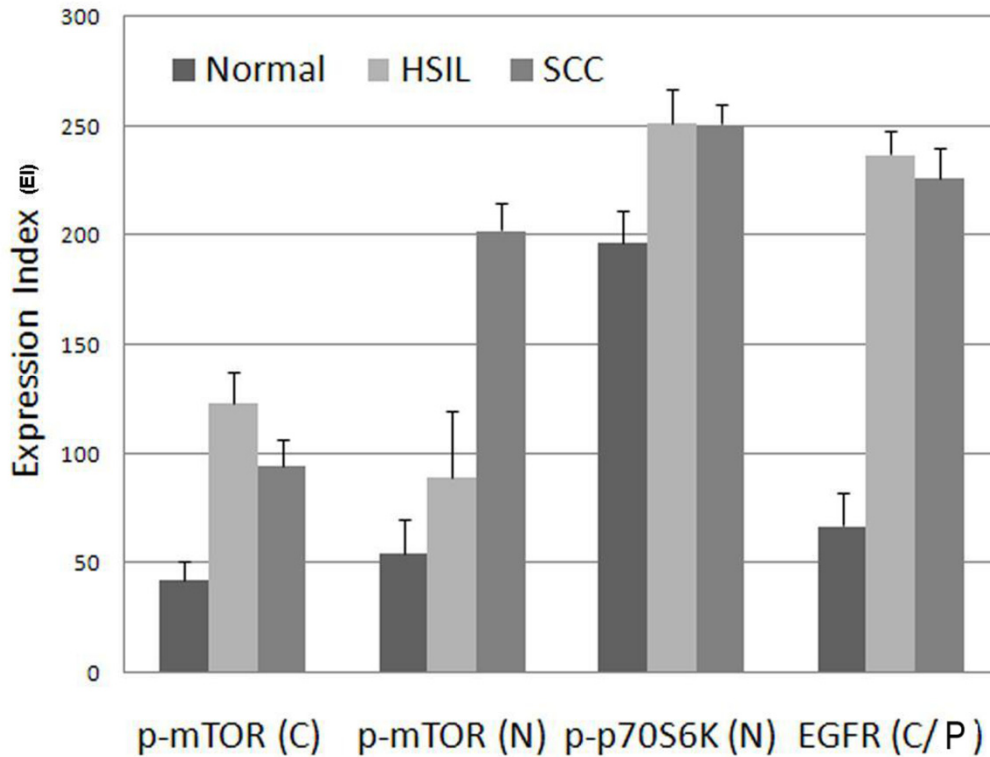
Figure 4 Graphic representation of expression index (EI) for EGFR and mTOR pathway markers, p-mTOR (Ser 2448) and p-p70S6K (Thr 389) comparing and contrasting normal (uterine cervical squamous epithelium), HSIL and invasive SCC. EI is the product of staining intensity (0-3+) and percent of positive staining cells (0-100%). C, cytoplasmic; N, nuclear; C/P, cytoplasmic/plasmalemmal (membranous).

[42] and with the finding by Chen and associates of increased expression of phospho-ERK1/2 and Skp2 during tumor progression of cervical neoplasms [43].

Moreover, these morphoproteomic findings coincide with the effects of HPV, the principal etiologic agent associated with HSIL and invasive SCC of the cervix, in providing an etiopathogenic sequence that enables the renewal and proliferation of the dysplastic process and the maintenance of growth of the

Table 2 Statistically significant differences among morphoproteomic analytes expression in normal cervical squamous epithelium (NL), HSIL and SCC

| Significant mean differences	Student's t-test, 2 tailed p-value
Nuclear p-mTOR in HSIL vs NL	0.049
Nuclear p-mTOR in SCC vs NL	<0.0001
Nuclear p-mTOR in SCC vs HSIL	0.015
Cytoplasmic p-mTOR in HSIL vs NL	0.0001
Cytoplasmic p-mTOR in SCC vs NL	0.0026
Nuclear p-p70S6K in HSIL vs NL	0.019
Nuclear p-p70S6K in SCC vs NL	0.0042
Cytoplasmic/membranous EGFR in HSIL/SCC vs NL	<0.0001
Nuclear Ki-67 in HSIL/SCC vs NL	<0.0001
Nuclear Skp2 in SCC vs NL	<0.0001
Nuclear Skp2 in SCC vs HSIL	<0.0001
Mitotic Index in HSIL/SCC vs NL	<0.0001
Mitotic Index in NL vs SCC	0.0003
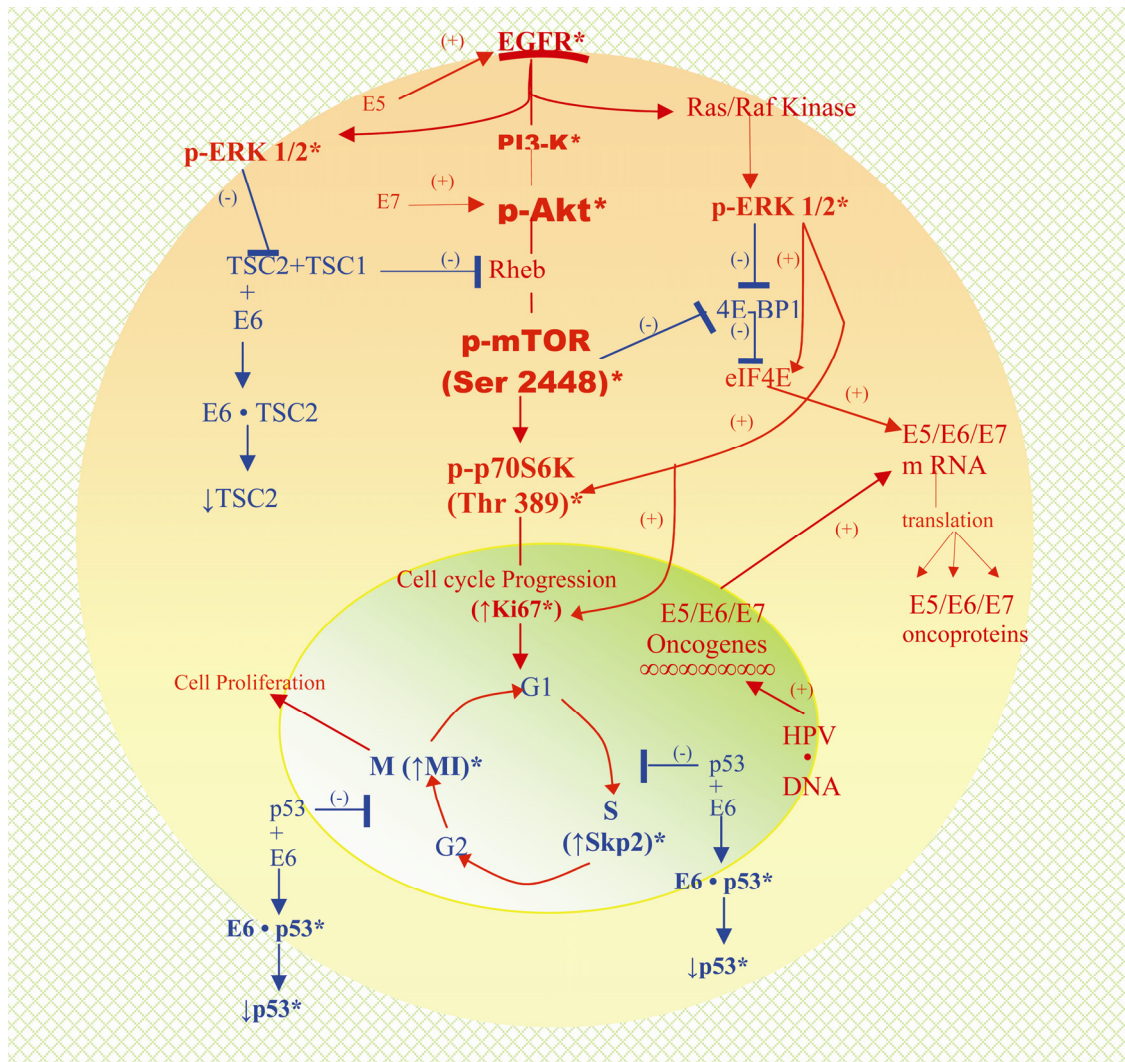
Figure 5 A schematic composite of the complex protein circuitries identified by the authors and others utilizing immunohistochemistry in cases of HSIL and/or invasive squamous cell carcinoma of the uterine cervix [42, 43, 48] and depicting established interrelationships with both HPV-encoded oncoproteins - E5, E6, and E7 [2-5, 7, 45, 46] and with the mTOR pathway. HPV infection of cervical squamous epithelium leads to the incorporation of HPV-oncogenes, E5, E6 and E7 into the genome resulting in the production of the corresponding oncoproteins. E5 activates EGFR and delays its degradation resulting in increased EGFR expression and then its signaling through the PI3'-K/Akt and ras/Raf kinase/ERK pathway, thereby contributing to phosphorylative activation of Akt and ERK1/2, respectively. This accounts for the expression of p-Akt and p-ERK1/2 in HSIL and invasive squamous cell carcinoma. Downstream signaling by p-Akt results in the constitutive activation and expression of p-mTOR (Ser 2448). Interaction of E6 oncoprotein with TSC2 (tuberin) contributes to signaling through the mTOR and probably ERK pathways [43, 45, 49, 50]. The former accounts for increased expression of p-p70S6K (Thr 389) and its downstream effector p-pS6 kinase and the latter collaborates with p-mTOR and contributes to activation of eIF4E through the phosphorylative inactivation of 4E-BP1. Additionally, p-ERK1/2 could contribute to mTOR pathway signaling by also contributing to the inactivation of TSC2 [51]. Translational synthesis of proteins including E7 oncoprotein results from the activation of eIF4E. E7 oncoprotein has been linked to growth and survival (immortalization) of HPV-associated, transformed cancer cells and to the signaling *via* the Akt/mTOR pathway by upregulating Akt activity. Cell cycle progression associated with mTOR and ERK pathway downstream signaling and aided by destabilization of wild type p53 by E6 oncoprotein is consistent with the increased expressions of Ki-67 and S phase kinase-associated protein (Skp) 2 and mitotic indices in HSIL and invasive squamous cell carcinoma. * indicates established immunohistochemical findings with respect to protein analytes; HSIL: high grade squamous intraepithelial lesion; HPV, human papilloma virus; mTOR, mammalian target of rapamycin; EGFR, epidermal growth factor receptor; PI3'-K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; eIF4E, eukaryotic initiation factor 4E; 4E-BP1, 4E-binding protein-1.

invasive SCC, when it develops. Specifically, HPV has been implicated in high risk cervical cancers as well as its precursor lesion, HSIL, reportedly accounting for some 80% of cervical cancers [1, 44]. Furthermore, as noted previously, the HPV-encoded oncoproteins, E5, E6 and especially, E7 confer replicative and immortalizing activities in cervical neoplasms. Such effects are achieved, in part, by virtue of their interactions with or impact upon EGFR, TSC2, p53 and Rb proteins, respectively [2-5, 7, 45, 46]. E5 activates EGFR and delays its degradation [7] and E6 interacts with and degrades the mTOR pathway inhibitor, tuberous sclerosis complex (TSC) 2 [45]. These effects, in turn, would lead to increased signaling through the PI3'-K/Akt/mTOR and ras/Raf kinase/ERK 1/2 pathways [25], eventuating in cellular proliferation. The binding of E6 with p53 has been shown to destabilize wild type p53 [5], which should also facilitate cell cycle progression (see **Figure 5**). Similarly, the binding of viral oncoprotein, E7 to Rb has been linked to its transforming capacity [5], possibly related in part to its upregulation of Akt activity [46]. This sequence accords with our morphoproteomic findings of increased plasmalemmal expression of EGFR in HSIL and invasive SCC of the uterine cervix and with the correspondingly increased cell cycle activity, as described above. In addition, the constitutive activation and overexpression of the mTOR pathway in HSIL and invasive SCC in this study should result in the phosphorylative inactivation of 4E-BP1 and thereby, the release of eIF-4E to effect translational synthesis of proteins to include viral oncoprotein E7 from their corresponding messenger RNA [6]. As noted earlier, E7 oncoprotein has been directly linked to the growth and survival (immortalization) of the HPV-associated cancer cells [2-5]. A schematic depicting the role of the HPV-encoded oncoproteins and incorporating our observations and those of others from the literature regarding EGFR overexpression and the activation and overexpression of the mTOR pathway with cell cycle correlates in HSIL and invasive cervical squamous cell carcinoma is represented in **Figure 5**.

In summary, our morphoproteomic analysis of high grade squamous intraepithelial lesions and invasive squamous cell carcinoma of the uterine cervix strongly suggests that a constitutively activated and overexpressed mTOR pathway, acting in concert with overexpressed EGFR and in collaboration with viral oncoproteins, plays a key role in both neoplastic processes. Furthermore in those cases associated with HPV infection, which represent the historical majority, the mTOR pathway may play a role in the translational synthesis of the encoded viral oncogene protein products, leading to the perpetuation of HSIL and the development of invasive SCC in some cases. As shown in previous studies, HSIL has a greater risk of persistent/recurrent disease after excision, and is the precursor lesion to invasive SCC of the uterine cervix [1, 44, 47]. Therefore, targeting the mTOR pathway and EGFR signaling with small molecule inhibitors to include a rapamycin and a tyrosine kinase inhibitor such as erlotinib [17-25] in patients with persistent or recurrent HSIL may be a reasonable therapeutic strategy. This combinatorial approach could obviate recurrence and reduce the risk of developing invasive SCC. Such an approach is consistent with certain stated objectives of the U.S. National Cancer Institute, namely to apply treatments or interventions at the individual level that keep cancers from starting or progressing.

Acknowledgement

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