Original Article

Correlation of Angiogenic Biomarkers to Tumor Progression and Angiogenesis in Cervical Cancer Cell (CaSki)-implanted Nude Mice

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Abstract

Introduction: Several studies have indicated that microvascular density (MVD) and the expressions of angiogenic biomarkers were associated with tumor growth and angiogenesis in cervical cancer. However, the results were incomplete and inconsistent.

Objectives: To determine the correlation of the angiogenic biomarkers, including vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1-alpha (HIF-1α), cyclooxygenase-2 (COX-2), and epidermal growth factor receptor (EGFR), to tumor progression and angiogenesis in cervical cancer cell (CaSki)-implanted nude mice model.

Methods: 10×10^6 of CaSki cells were injected into the female nude mice (n = 50) to establish subcutaneous tumors. The tumor size was measured every 3 days for one month. The MVD was evaluated using the CD31 expression. VEGF, HIF-1α, COX-2, and EGFR expression were detected by immunohistochemistry.

Results: The results showed that HIF-1α (r = 0.979 and r = 0.942), VEGF (r = 0.972 and r = 0.929), COX-2 (r = 0.982 and r = 0.957), and EGFR (r = 0.993 and r = 0.971) closely correlated with tumor growth and tumor angiogenesis, respectively. Interestingly, EGFR was mostly involved in tumor growth and angiogenesis.

Conclusions: This study demonstrates that the HIF-1α, VEGF, COX-2, and EGFR are a set of biological markers which are strongly related to tumor progression and angiogenesis in cervical cancer.

Keywords: Angiogenic biomarkers, Cervical cancer, CaSki, Angiogenesis, Tumor progression

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Introduction

Cervical cancer and premalignant lesions constitute a major problem in women's health. In Thailand, cervical cancer is the most common cancer in women.[1] Current clinical data have indicated that angiogenesis has been associated with cervical cancer progression and an increase in microvascular density appeared to be a poor prognostic factor.[2,3] Understanding the molecular basis of the angiogenic pathways involved in cervical cancer can ultimately improve the diagnosis and management as well as facilitate the discovery of novel anticancer drugs or other therapeutic options for cancer. The angiogenesis process can be quantified by measuring intra-tumoral microvascular density or MVD, and the MVD can be assessed by measuring tissue expression of several representative molecules such as CD31, vascular endothelial growth factor (VEGF), hypoxia inducible factor-1α (HIF-1α), cyclooxygenase-2 (COX-2), and epidermal growth factor receptor (EGFR).

The degree of MVD in tumor can be assessed by CD31 protein expression. CD31 or platelet/endothelial cell adhesion molecule-1 (PECAM-1) is a 130 kDa type I transmembrane cellular adhesion and signaling receptor that strictly expresses on the surfaces of endothelial cells particularly at cell-cell borders.[4] It is expressed on endothelial and hematopoietic cells and plays important roles in angiogenesis, vascular permeability, and regulation of cellular responsiveness. The presence of CD31 on the membrane of malignant epithelial cells could also be explained in the context of vasculogenic mimicry.[5] Therefore, CD31 is considered as a representative for vascular differentiation and is used as a marker for the assessment of vascularization as well as tissue vessel density.

VEGF is the most important regulator of angiogenesis. It controls this process via detecting an oxygen tension.[6] Hypoxia induces VEGF expression and its receptor via HIF-1α.[7] Once induced, VEGF then promotes endothelial cell proliferation, migration and survival.[8] VEGF can also increase the leakage of blood vessels which induce edema around the tumor tissue,[9] leading to an incapability of blood vessels to provide effective blood flow, and subsequently creating the hypoxic condition to the tumor,[10] which in turn stimulates the continuous VEGF production. This continuous cycle leads to tumor angiogenesis and tumor progression under hypoxic conditions.[11] VEGF was thus found to be associated with a poor prognosis of cervical cancer.[12,13] Previous studies demonstrated that the VEGF level was higher in cervical cancer compared to normal cervical tissue, and the higher VEGF levels also correlated with the more advanced stages,[12] with increased risk of lymph nodes metastasis,[12] and with poorer outcomes.[14-16] Interestingly, it has been found that E6 and E7 oncoproteins produced by Human papillomavirus 16 could induce VEGF expression in a HIF-1α-dependent manner.[17] This growing evidence altogether supports that VEGF plays an important role in cancer angiogenesis and can be used as a potential angiogenic biomarker.

Cyclooxygenase-2 or COX-2 is an inducible form of cyclooxygenase. It is unexpressed under normal conditions in most cells; however, it can be induced by oncogenes, growth factors and cytokines. COX-2-derived prostaglandins (PGs) activate cell proliferation, increase invasiveness, inhibit immune surveillance and apoptosis, and promote angiogenesis.[18-20] Furthermore, PGs have been shown to contribute to cancer progression and metastasis.[21] COX-2 expression can be associated with cervical cancer growth and progression; it is highly expressed in various types of cervical cancer including cervical intraepithelial neoplasia, squamous cell carcinoma and cervical adenocarcinoma.[22,23] Importantly, COX-2 has been shown to be involved in early cervical carcinogenesis and to accelerate tumor progression and angiogenesis by increasing VEGF expression.[13] Therefore, COX-2 is a candidate angiogenic biomarker for tumor angiogenesis and tumor progression/prognostic prediction of the cervical cancer.

The epidermal growth factor receptor (EGFR) is a member of the ErbB family, the tyrosine kinase receptors with growth promoting effects which include the tumor angiogenesis.[24] EGFR signaling pathways play roles in processes associated with carcinogenesis and tumor progression by inhibition of apoptosis, cell migration, cell growth, and angiogenesis.[25-27] Over-expression of EGFR signaling has been linked to carcinogenesis and angiogenesis. Activation of EGFR results in activation of MEK-extracellular signal-regulated kinase1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3K)-Akt pathways.[28] These two pathways regulate VEGF expression through change in VEGF

transcriptional activity. Therefore, EGFR signaling can also be one of the potential angiogenic biomarkers of cancer and angiogenesis.

Although extensive research has been carried out, currently there are only few studies that investigated multiple angiogenic biomarkers on tumor growth and angiogenesis in cervical tumor. Therefore, the purpose of the present study was to determine the correlation between aforementioned angiogenic biological markers (VEGF, HIF-1α, COX-2, and EGFR) and tumor progression and angiogenesis in cervical cancer cell (CaSki)-implanted nude mice model.

Methods

Cell Line and Cell Culture

CaSki cell was purchased from the American Type Culture Collection. The cell lines were cultured in medium (MEM) supplemented with 10% fetal bovine serum. All cultures were maintained in an incubator at 37°C with 5% CO_2 in a humidified atmosphere.

CaSki-induced Tumor Mice

The animal experiments were conducted according to the guidelines on experimental animals of The National Research Council of Thailand (ethical approval code 019/2558). Female BALB/c-nude mice (20-25 g) were used (n = 50). According to procedure reported previously,[29] a suspension of 10×10^6 CaSki cells in 0.2 mL of MEM[30] was injected into the dorsal area of mice at the proximal midline. After one month of the injection, the tumor size was measured with Vernier calipers, and when tumor volume reached 100-120 mm^3, it was counted as Day 0, and tumor size were continuously measured every 3 days for 27 days. Tumor volume was calculated by using the formula $a^2 \times b \times 0.52$ (where a = the shortest and b = the longest diameter). The tumor volume measured on each day is expressed as Relative Tumor Volume (RTV) and calculated according to the following formula: $RTV = TV_n/TV_0$, where TV_n is the tumor volume on measured day n and TV_0 is the tumor volume on day 0. Mice were sacrificed every three days, and the tumors were dissected and collected for immunohistochemistry analysis.

Immunohistochemical Technique

Paraffin section from tumor tissue was dewaxed and rehydrated through xylene and a graded alcohol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes at room temperature. After washing in water, nonspecific binding sites were blocked with 5% bovine serum in phosphate-buffered saline (PBS) for 30 minutes at room temperature. The tissue slide samples were incubated with primary monoclonal antibody targeting CD31 (Thermo Fischer Scientific., UK) (1:500), primary monoclonal antibody targeting VEGF (Thermo Fischer Scientific., UK) (1:75), primary monoclonal antibody targeting HIF-1α (1:1,000) (GeneTex Inc., USA), primary monoclonal antibody targeting COX-2 (Thermo Fischer Scientific., UK) (1:50) or primary monoclonal antibody targeting EGFR [VENTANA (ready to use), USA] at 4°C overnight. The slides were then gently rinsed with PBS and developed by the Envision system/HRP (DAKO cytomation, USA) for 30 minutes and substrate-chromogen for 10 minutes at room temperature. Finally, the sections were counterstained with hematoxylin, dehydrated and mounted.

Immunohistochemical Analysis

The microvascular density (MVD) was evaluated on CD31 stained slides using the well-known "hot spot" method as previously described.[31] The sections were observed first under low power (×40), then the hot spot area of microvessel were identified and counted on three microscopic fields under the high power (×200, the surface area of every vision field being 0.4 mm^2).

The immunoassays for VEGF, HIF-1α, COX-2 and EGFR were quantitatively assessed. The percentage of positive areas of VEGF, HIF-1α, COX-2 and EGFR immune reactivated proportionate to the total area were analyzed by Image J 1.38 software (National Institutes of Health, USA).

Statistical Methods

Data were expressed as means with standard error (S.E.). SPSS.13 software was used for statistical analysis. The correlation between relative tumor volume, CD31, VEGF, HIF-1α, COX-2 and EGFR expressions was evaluated with Pearson Correlation (r) test. *P*-value less than .05 was considered significant.

Results

Tumor Progression

Tumor growth and relative tumor volume are shown in Figure 1 A and 1 B, respectively. At the end of the experiment, the relative tumor volume on day 3, 6, 9, 12, 15, 18, 21, 24, 27 were 2.01 ± 0.37, 3.23 ± 0.44, 4.88 ± 1.04, 5.90 ± 1.31, 7.92 ± 1.93, 9.82 ± 2.09, 12.09 ± 2.94, 13.79 ± 2.91, 15.81 ± 2.90, respectively. The tumors have been found to grow at constant rates for long periods of time. The result showed that relative tumor volume was significantly increased every 3 days from Day 15 to Day 27. Moreover, tumor volume on Day 27 showed significantly increased as compared to Day 9, 12, 15, 18, 21 (*P*-value < .05).

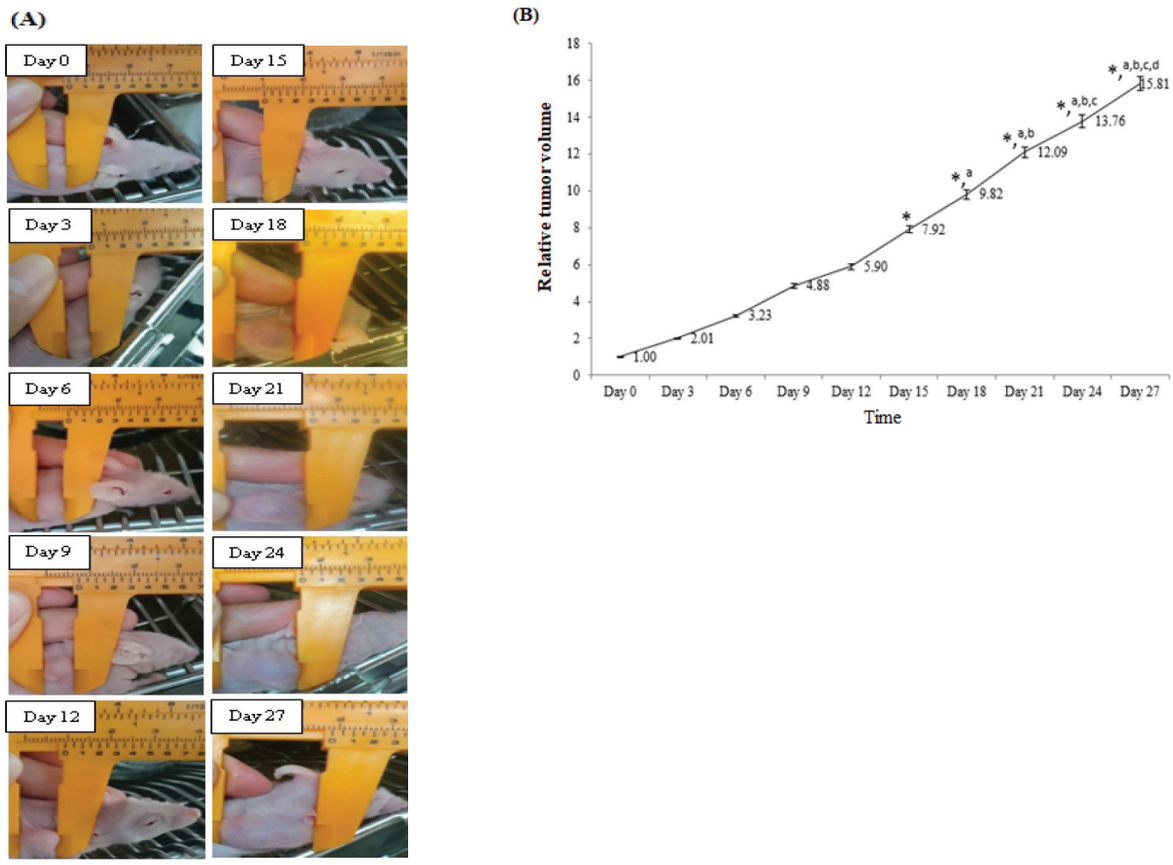

Figure 1 (A) Tumor size measurement. (B) Relative tumor volume (mm3) (Mean ± S.E.). **P*-value < .05 *vs.* Day 0, Day 3, and Day 6; [a]*P*-value < .05 *vs.* Day 9; [b]*P*-value < .05 *vs.* Day 12 and Day 15; [c]*P*-value < .05 *vs.* Day 18; [d]*P*-value < .05 *vs.* Day 21.

The Relationship Between the Expression of CD31 and Tumor Volume

Immunostaining for CD31 in tumor tissues from various time points is shown in Figure 2 A. We observed that the dilatation of blood vessels approximately appeared on day 3. At the early stage of tumor angiogenesis, the increment of tumor neocapillaries could be observed on day 6-day 9 followed by a gradual increase of CD31 expression for long periods of time.

Microvascular density (MVD) was determined by CD31 staining, to assess the quantity of the angiogenesis. The MVD in CaSki group on day 3 (8.50 ± 0.37) and 6 (8.60 ± 0.60) did not significantly different as compared with control group (7.40 ± 0.40). The MVD gradually increased on day 9 (11.80 ± 1.02), day 12 (16.50 ± 0.96), day 15 (19.60 ± 2.45), day 18 (23.34 ± 2.56), day 21 (26.10 ± 1.79), day 24 (32.45 ± 1.54), and day 27 (41.40 ± 1.80) (*P*-value < .05). Moreover, the greater

tumor volume showed the higher MVD. Correlation analysis showed that the relative tumor volume was positively correlated with MVD (*P*-value = .001, r = 0.983).

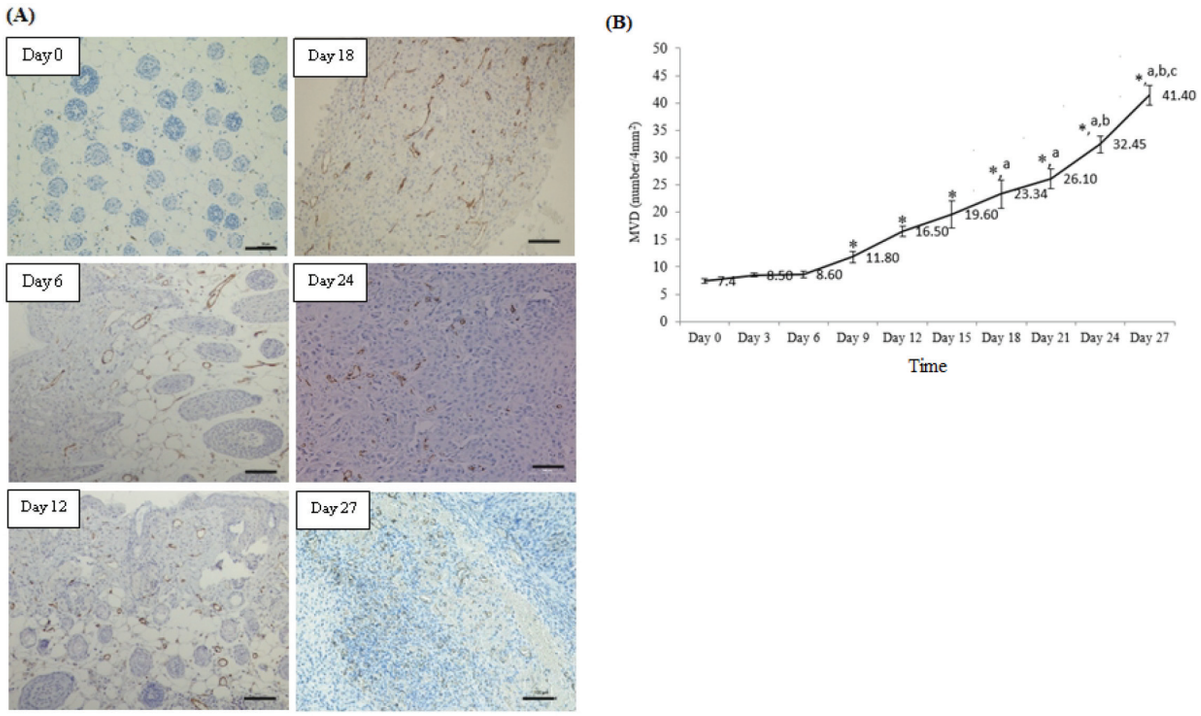

Figure 2 (A) CD31 expression. (B) MVD (number/4 mm^3) (Mean ± S.E.). **P*-value < .05 *vs*. Day 0, and Day 3; [a]*P*-value < .05 *vs*. Day 6 and Day 9; [b]*P*-value < .05 *vs*. Day 12, and Day 15; [c]*P*-value < .05 *vs*. Day 18 and Day 21. Bar = 100 μm, 200x.

The Relationship Between the Expression of HIF-1α, VEGF and Tumor Volume

Figures 3 A and 4 A show the immunohistochemical stained sections for HIF-1α and VEGF expression, respectively. The VEGF and HIF-1α expression gradually increased with time. The percentage positive staining of VEGF and HIF-1α are shown in Figures 3 B and 4 B, respectively. VEGF expression correlated with relative tumor volume (*P*-value = .001, r = 0.972) and MVD (*P*-value = .001, r = 0.929). Also, there was a positive correlation between HIF-1α and relative tumor volume (*P*-value = .001, r = 0.979) and MVD (*P*-value = .001, r = 0.942). Moreover, correlation analysis showed that the VEGF were positively correlated with HIF-1α (*P*-value = .001, r = 0.966).

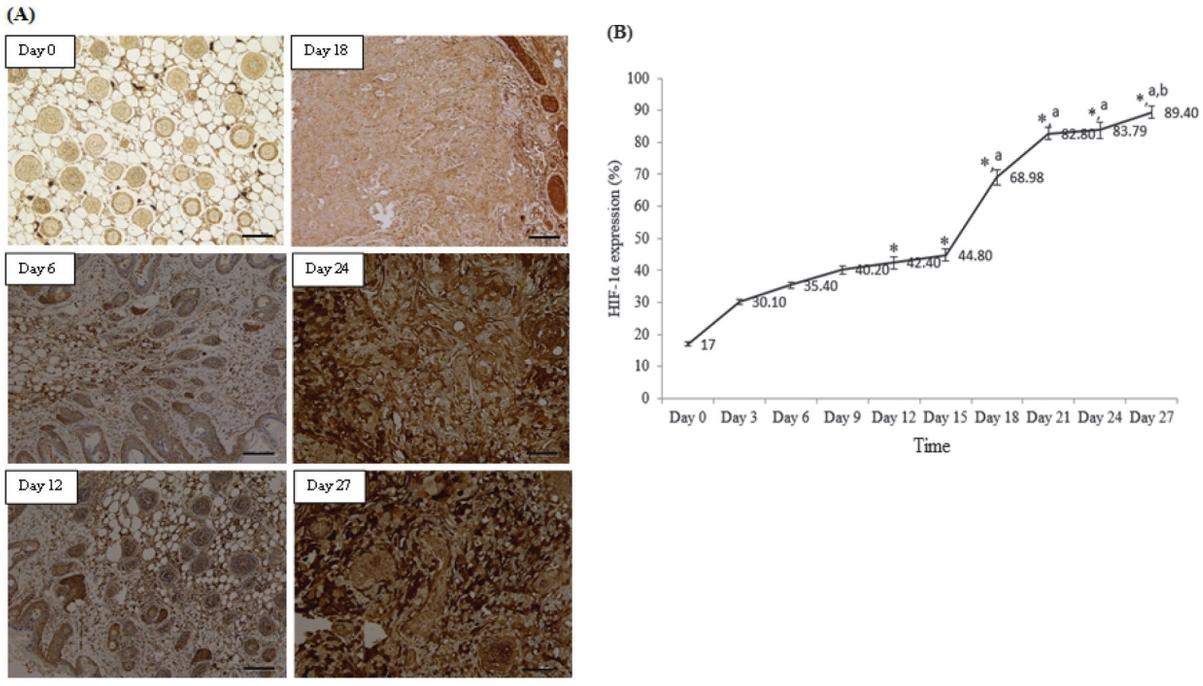

Figure 3 (A) HIF-1α expression. (B) HIF-1α expression ratio (%) (Mean ± S.E.). **P*-value < .05 *vs.* Day 0; [a]*P*-value < .05 *vs.* Day 3, Day 6, Day 9, Day 12 and Day 15; [b]*P*-value < .05 *vs.* Day 18. Bar = 100 µm, 200x.

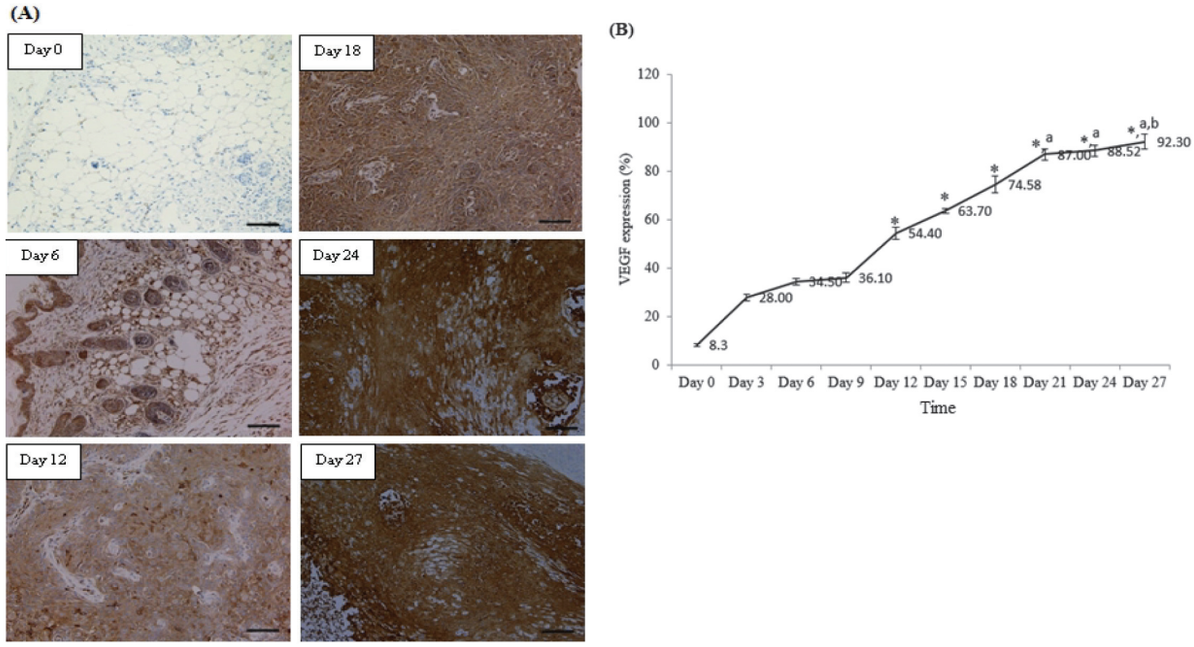

Figure 4 (A) VEGF expression. (B) VEGF expression ratio (%) (Mean ± S.E.). **P*-value < .05 *vs*. Day 0, Day 3, Day 6, and Day 9; [a]*P*-value < .05 *vs*. Day 12; [b]*P*-value < .05 *vs*. Day 15. Bar = 100 µm, 200x.

The Relationship Between the Expression of COX-2, EGFR and Tumor Volume

Figures 5 and 6 demonstrate the expression of COX-2 and EGFR, respectively. The COX-2 expression has higher correlation with VEGF (*P*-value = .001, r = 0.981) and MVD (*P*-value = .001, r = 0.957) and relative tumor volume (*P*-value = .001, r = 0.982). Likewise, the EGFR expression

has higher correlation with COX-2 (*P*-value = .001, r = 0.988), VEGF (*P*-value = .001, r = 0.973), and MVD (*P*-value = .001, r = 0.971) and relative tumor volume (*P*-value = .001, r = 0.993).

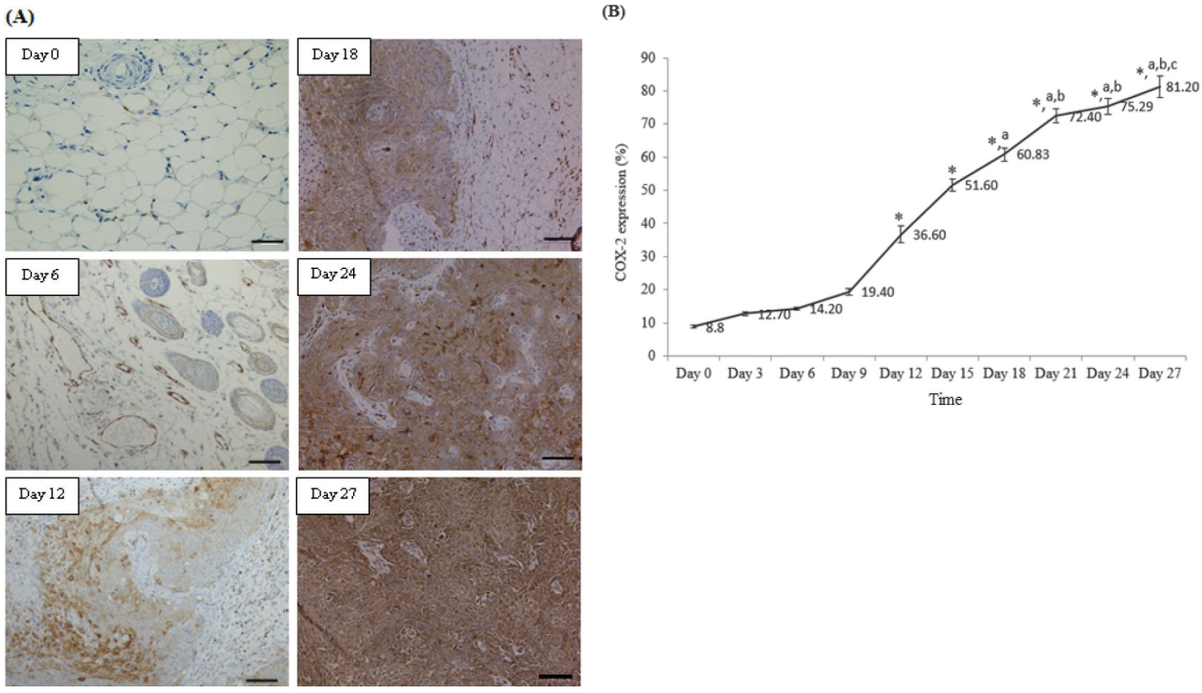

Figure 5 (A) COX-2 expression. (B) COX-2 expression ratio (%) (Mean ± S.E.). **P*-value < .05 *vs.* Day 0, Day 3, Day 6 and Day 9; [a]*P*-value < .05 *vs.* Day 12; [b]*P*-value < .05 *vs.* Day 15; [c]*P*-value < .05 *vs.* Day 18. Bar = 100 μm, 200x.

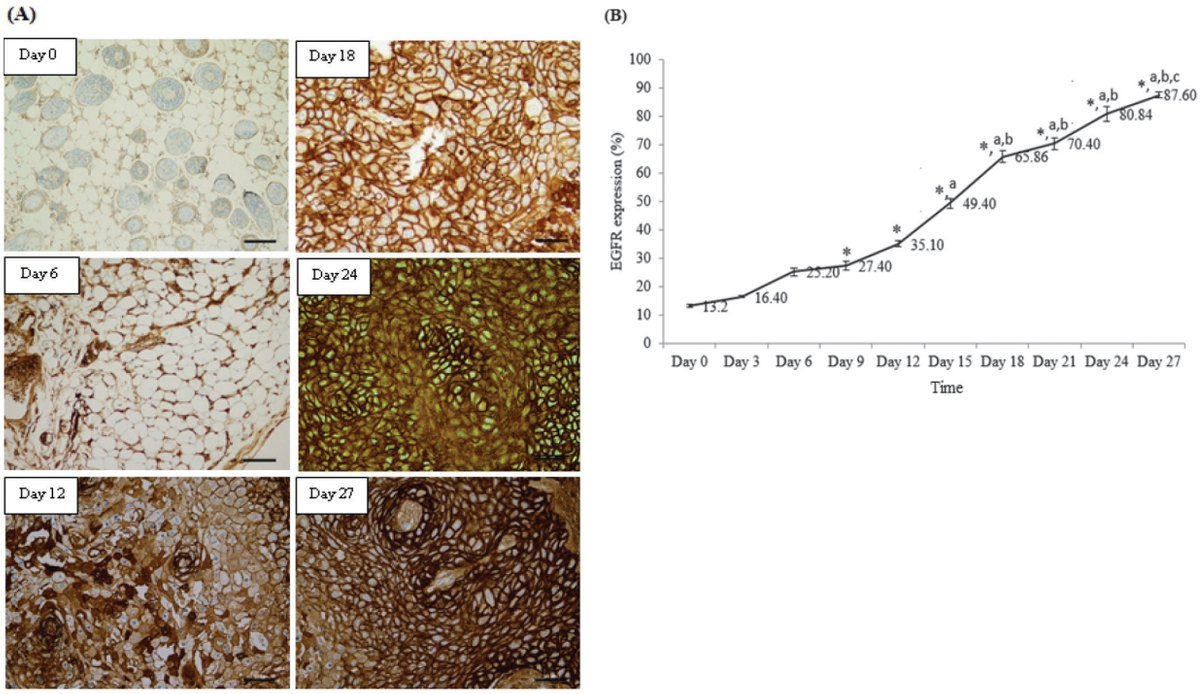

Figure 6 (A) EGFR expression. (B) EGFR expression ratio (%) (Mean ± S.E.). **P*-value < .05 *vs.* Day 0, Day 3, Day 6; [a]*P*-value < .05 *vs.* Day 9, and Day 12; [b]*P*-value < .05 *vs.* Day 15; [c]*P*-value < .05 *vs.* Day 18. Bar = 100 μm, 200x.

Discussion

Tumor progression and metastasis rely partly on the proliferation of blood vessel networks that supply tumor. This angiogenesis process is more-or-less linked with the poor outcomes of cancer sufferers. It is thus important to elucidate the relationship between tumor growth and the angiogenesis. The present study determined the correlation of biological markers including VEGF, HIF-1α, COX-2, and EGFR to the tumor progression and angiogenesis in CaSki-implanted nude mice. We demonstrated that MVD, as measured by CD31 expression, positively correlated with the tumor size in the cervical cancer cell-implanted nude mice. This result in consistent with an experimental study in an orthotopic murine model of human cervical carcinoma which also showed the positive correlation between CD31 expression and the tumor size.[32] Overall, this finding suggests that large tumors have a proportionally greater number of pre-existing vessels entrapped by the tumor which can be stained by pan-endothelial markers.

HIF-1 is a dimeric protein complex that plays an integral role in the body's response to hypoxia. It is among the primary genes involved in the homeostatic process, which can increase vascularization in hypoxic areas such as localized ischemia and tumors.[33] The alpha subunit (HIF-1α) is stabilized under hypoxic conditions and, therefore, might represent an intrinsic marker for tissue hypoxia. The HIF-1 is known to induce the expression of several proteins such as VEGF, a prime inducer of angiogenesis, linked to the maintenance of oxygen homeostasis, cellular energy metabolism, and tumor progression. Our findings showing relationship between VEGF expression and HIF-1α as measured by immunohistochemical staining in cervical tumors are in accordance with the positive correlation between hypoxia and VEGF expression. Moreover, we demonstrated that both VEGF and HIF-1α expression correlated with MVD and relative tumor volume. These results indicate that the angiogenesis through the expression of HIF-1α, VEGF and MVD plays an important role in supporting the tumor progression as seen in our cervical cancer model.

It is known that VEGF stimulates COX-2 expression and prostaglandin synthesis, which in turn induces VEGF production.[34] Kulkarni *et al* found that EGFR signaling markedly induced COX-2 in a cervical cancer cell line, suggesting that deregulated signaling through EGFR is at least in part through increased expression of COX-2.[23] The present study found that COX-2 expression has higher correlation with VEGF and MVD. Interestingly, our data demonstrated that EGFR was mostly involved in tumor growth and angiogenesis. Furthermore, the EGFR expression showed positively correlated with the expression of COX-2. These results were supported by our previous study that VEGF, COX-2, and EGFR over-expressed in cervical cancer-implanted mice.[25] Moreover, VEGF expression was strongly correlated with both COX-2 and EGFR expression. These findings together with the positive correlation between COX-2 and EGFR expression suggest that EGFR-COX-2–PGE2 pathway plays a part in the acquisition of tumor growth and angiogenesis in cervical cancer. Collectively, these data suggest that VEGF, COX-2, and EGFR expressions highly correlated with tumor growth and angiogenesis in cervical cancer.

In conclusion, angiogenesis is an important process for tissue survival. Tumor growth and development as well as tumor metastasis are dependent on this process. The present study demonstrates that the angiogenic biomarkers including HIF-1α, VEGF, COX-2, and EGFR have a strong correlation with the tumor progression and angiogenesis in cervical cancer cell (CaSki)-implanted nude mice. Any agents that target these angiogenic biomarkers could potentially become cancer therapeutic options.

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Potential conflicts of interest. All authors report no conflicts of interest relevant to this article.

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