Published in final edited form as:

Gynecol Oncol. 2009 March ; 112(3): 583–589. doi:10.1016/j.ygyno.2008.11.013.

Markers of angiogenesis in high-risk, early-stage cervical cancer: A Gynecologic Oncology Group study[☆]

Leslie M. Randall^a, Bradley J. Monk^a, Kathleen M. Darcy^b, Chungqiao Tian^b, Robert A. Burger^a, Shu-Yuan Liao^c, William A. Peters^{d,1}, Richard J. Stock^{e,f,2}, and John P. Fruehauf^{a,*}

^aChao Family Comprehensive Cancer Center, University of California-Irvine Medical Center, Orange, CA, USA

^bGOG Statistical and Data Center, Roswell Park Cancer Institute, Buffalo, NY, USA

^cSt. Joseph's Hospital, Department of Pathology, 1100 W. Stewart Drive, Orange CA, USA

^dUniversity of Washington, Seattle, WA, USA

^eEastern Virginia Medical School, Norfolk, VA, USA

^fNaval Medical Center, Portsmouth, VA, USA

Abstract

Objectives—To determine whether markers of tumor angiogenesis were associated with progression-free survival (PFS) and overall survival (OS) in women with high-risk, early-stage cervical cancer treated on a phase III trial.

Methods—One hundred seventy-three tumor specimens were analyzed by semi-quantitative immunohistochemical (IHC) staining for vascular endothelial growth factor (VEGF, pro-angiogenesis factor), thrombospondin-1 (TSP-1, anti-angiogenesis factor), CD31 (non-specific endothelial marker), and CD105 (tumor-specific endothelial marker). Tumoral histoscores (HS) were calculated for VEGF using the formula: [% cells positive×(intensity+1)]. TSP-1 specimens were categorized as negative or positive. CD31 and CD105 microvessel density (MVD) “hotspots” were counted in three 20× high-power fields. Associations between angiogenesis markers and survival were evaluated.

Results—TSP-1 expression was observed in 65% of cases while 66% expressed high VEGF (≥200), 34% exhibited high CD31 (CD31≥110) and 66% displayed high CD105 (CD105≥28). In univariate analyses CD31 MVD, but not tumor TSP-1, was associated with improved PFS (HR=0.37; 95% CI=0.18–0.76; *p*=0.007) and OS (HR=0.37; 95% CI=0.17–0.79; *p*=0.010). After adjusting for prognostic clinical covariates, high CD31 MVD, but not TSP-1, VEGF or CD105 MVD, was an independent prognostic factor for PFS (HR=0.36; 95% CI=0.17–0.75; *p*=0.006) and OS (HR=0.36; 95% CI=0.17–0.79; *p*=0.010).

[☆]This original research was presented at the 2007 Annual ASCO Meeting (citation: *J Clin Oncol*, 2007 ASCO Annual Meeting Proceedings Part I. Vol 25, No. 18S (June 20 Supplement), 2007: 5536).

© 2008 Elsevier Inc. All rights reserved.

*Corresponding author. Division of Hematology/Oncology, Department of Medicine, Chao Family Comprehensive Cancer Center, University of California-Irvine Medical Center, Orange, CA 92868, USA. Fax: +1 714 456 2242. jfruehau@uci.edu (J.P. Fruehauf).

¹Currently at Pacific Gynecology Specialists, P.C.

²Retired MD, Capt, MC, Retired USN, 770 Pelican Bay Drive, Daytona Beach, FL 32119, USA.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Conclusions—Tumor angiogenesis measured by CD31 MVD is an independent prognostic factor for both PFS and OS in high-risk, early-stage cervical cancer. We hypothesize that this finding may be explained by improved treatment response in well-vascularized, well-oxygenated tumors.

Keywords

Angiogenesis; Cervical cancer; GOG; Angiogenic markers

Introduction

Cervical cancer is the second-most common cause of cancer-related deaths in women worldwide, causing an estimated 274,000 deaths annually [1]. Of the 19,339 cases registered with Surveillance Epidemiology and End Results (SEER) program between 1996 and 2003, 51% of cervical cancers were diagnosed as local disease, with a 5-year survival rate of 92% for these women [2]. Analysis of specimens obtained from women receiving surgical treatment for early-stage cervical cancer has identified several clinical and pathologic poor prognostic factors including increased age, African-American ethnicity, human papillomavirus (HPV) 18 infection, deep cervical stromal invasion, tumor size >2 cm, lymphovascular space invasion (LVSI), nodal metastases, microscopic tumor in uterine parametrial tissues, and positive surgical margins [3-6]. Although these prognostic factors have been well-established, the biologic factors associated with recurrence and survival remain largely unknown.

Angiogenesis, the development of new blood vessels in areas of new tissue growth, is not only central to normal physiologic processes such as embryogenesis, tissue remodeling, and wound healing, but is also one of the cardinal processes required for tumor growth, invasion, and metastasis. Malignant tumors with insufficient nutrient and oxygen delivery initiate angiogenesis by secreting growth factors that stimulate vascular sprouting from existing vessels and recruitment of circulating endothelial cells and marrow-derived endothelial progenitor cells.

Substantial evidence has supported HPV as the causative agent in cervical cancer. The HPV genome encodes two oncogenes, E6 and E7, whose protein products inactivate p53 and pRb, respectively, impairing normal cell-cycle inhibition, and allowing proliferation of HPV-infected cells. In addition to this classically described mechanism of malignant transformation, the loss, or inactivation, of wild-type p53 has been found to indirectly promote tumor angiogenesis by up-regulation of angiogenesis-promoting protein, vascular endothelial growth factor (VEGF) [7-10], and down-regulation of a potent angiogenesis inhibitor, thrombospondin-1 (TSP-1) [10-12], providing rationale for the contribution of angiogenesis to cervical cancer early in carcinogenesis. Furthermore, because cancer development, metastasis and progression are critically dependent on vasculogenesis and angiogenesis, we hypothesize that markers of tumor angiogenesis or angiogenic potential in early stage cervical carcinogenesis not only influence the biology of this disease but also response to treatment and patient outcome.

The majority of studies attempting to demonstrate a correlation between angiogenesis and outcome in cervical cancer report conflicting results [13-21], but most have concluded that more extensive tumor angiogenesis is associated with higher rates of tumor recurrence following treatment and poorer survival in cervical cancer [13-17]. However, results of these retrospective studies are likely influenced by inclusion of women at different stages of disease who were treated with various, non-standardized modalities.

The purpose of this study was to determine whether markers of tumor angiogenesis, as measured by microvessel density of CD31- or CD105-positive endothelial cells in addition to VEGF and TSP-1, were associated with progression-free survival (PFS) or overall survival (OS) in women with high-risk, early-stage cervical cancer treated on an intergroup Phase III trial.

Materials and methods

Cervical cancer specimens

Primary tumor specimens were obtained between 1991 and 1996 from women undergoing type III radical hysterectomy and pelvic lymphadenectomy for International Federation of Gynecology and Obstetrics (FIGO) stages IA2, IB, and IIA cervical cancer with pathologic findings of lymph node metastases, parametrial involvement, or positive surgical margins prior to treatment with adjuvant pelvic radiotherapy (RT) with or without radiosensitizing chemotherapy on a multi-center randomized phase III trial (SWOG 8797/GOG 109/RTOG 91-12) [22]. Squamous carcinoma, adenosquamous carcinoma, and adenocarcinoma histologic subtypes were included. Eligible women were randomized within six weeks of surgery to receive 49.3 Gy±45 Gray delivered via a standard four-field box to the para-aortic nodes in women with positive high common iliac nodes with or without 70 mg/m² cisplatin given as a 2-hour intravenous infusion on day 1 and 1000 mg/m² 5-FU per day for 4 consecutive days delivered as a 96-hour infusion every 21 days for a total of 4 cycles. Follow-up consisted of physical examinations performed quarterly for 2 years, semiannually for 3 years, and annually thereafter. Histologic eligibility was verified by the GOG Pathology Committee. All women provided written informed consent in accordance with federal, state, and local requirements for both the treatment protocol, and approval for immunohistochemistry assays was obtained from the University of California, Irvine institutional review board.

Immunohistochemistry (IHC)

Unstained sections on positively charged slides were prepared from formalin-fixed and paraffin-embedded (FFPE) pre-treatment radical hysterectomy or excisional biopsy specimens. Hematoxylin and eosin-stained specimens were examined by the study pathologist (SYL) to confirm that at least 50% of each section consisted of malignant tissue. Specimens were de-paraffinized in xylene, rehydrated in graded alcohols, and rinsed in distilled, deionized water. Automated IHC was performed using a DAKO Autostainer (Dako, Carpinteria, CA). Specimens were incubated at ambient room temperature with 300 µL primary mouse monoclonal antibodies against CD31 (JC70A, 6.9 µg/mL, Dako, Carpinteria, CA), CD105/endoglin (SN6, 1:500, Dako, Carpinteria, CA) or TSP-1 (8A6b, 0.1 µg/mL, Vision BioSystems, Norwell, MA,) for 1 h, or VEGF (VG-1, 2.5 µg/mL, LabVision, Fremont, CA) for 30 min. Secondary antibody incubation was performed with either Envision Plus anti-mouse and horseradish peroxidase (HRP) labeled polymer (Dako) or, for CD-105, Catalyzed Signal Amplification II (CSA II, Dako), for 30 min, followed by 3',3'-diaminobenzidine (DAB) for 5 min. Specimens were counter-stained with hematoxylin (Dako, Carpinteria, CA), rinsed in double distilled water, dehydrated in graded alcohols, cleared in Clear-Rite (Dako), and mounted. The following formalin-fixed and paraffin-embedded cells lines or tissues were used as positive or negative controls. MCF7-WT cells were the positive control and UCI-101 cells were the negative control for TSP-1. HT1080 and MCF7-WT cells were the positive control and RC-786-0 cells were the negative control for VEGF. Doxorubicin-resistant derivative MCF7-40F cells were the positive control and MCF7-WT cells were the negative control for p53. An invasive breast carcinoma specimen with high micro-vascular staining was the positive control, and the non-staining areas in the same tumor tissue were used for the negative control for CD31 and CD105. Human vascular

endothelial cells (HVEC) cells were another positive control for CD31 and CD105. Non-specific reactivity was examined by replacing the primary antibody with mouse IgG.

Immunohistochemistry scoring

Intracellular VEGF, nuclear p53 and stromal TSP-1 intensity equaled 0–4 relative to the positive control assigned as intensity of 3. Relative intensity of non-specific staining was subtracted from test specimen intensity values as a normalization method. Percent of positively-stained intracellular VEGF or nuclear p53 was also quantified. VEGF and p53 were quantified by histoscore (*H*-Score) as previously published [$H\text{-Score} = \% \text{ positive} \times (\text{intensity} + 1)$] [23]. CD31 and CD105 microvessel density (MVD) were quantified by counting the number of vessels plus immunoreactive endothelial cells per 200× high power field in three vascular “hot spots” within the malignant tumor and reporting the highest number as the final MVD. An independent reviewer who had no previous knowledge of the clinical outcome data (LR) performed IHC staining and scoring.

Statistical analysis

Biomarker and clinical data for this ancillary study were analyzed using SPSS version 14 (SPSS Inc., Chicago, IL) or SAS version 9.1 (SAS Institute, Inc., Cary, NC). Biomarkers were evaluated as continuous variables and dichotomous categorical variables. Cut points were identified based on visual inspection of individual histograms for each biomarker. Correlations between categorized biomarkers were examined using Kendall's tau-b. Associations with PFS and OS were examined using the Kaplan–Meier product limit method [24] with the log–rank test [25] and Cox proportional hazard regression analysis unadjusted or adjusted for major clinical prognostic variables [26]. PFS was calculated as the time in months from date of enrollment to disease progression or death, or date of last contact. OS was calculated as the time in months from date of enrollment to death, or date of last contact.

Results

Of the 243 eligible and evaluable women who participated in the multi-center randomized phase III trial, SWOG 8797/GOG 109/RTOG 91-12, 173 of the women enrolled at GOG institutions provided FFPE primary tumor tissue and were eligible for this study. One hundred and fifty nine of the specimens (92%) were from radical hysterectomies and 14 (8%) were from large excisional biopsies. Seven were excluded for the following reasons: one had benign disease, two were low-risk, early stage disease, and there was no FFPE tumor for testing for four cases. The patient characteristics for the 173 women in this cohort are summarized in Table 1 and are representative of that observed in the entire cohort of women who participated in the phase III intergroup trial, SWOG 8797/GOG 109/RTOG 91-12. At the time of the final analyses, 113 women were alive with no evidence of disease, 5 were alive with disease progression, 47 died due to disease progression, 5 died due to a reason other than disease progression or treatment, and three died of unknown cause. Median follow-up for the 118 women who were still alive at the time of the final analysis was 105.9 (range: 2.7 to 184.8) months. Equal numbers of women from each treatment group were included.

Table 2 displays the categorized expression for CD31 MVD, TSP-1 intensity, VEGF histoscore and CD105 MVD, and significant associations between these biomarkers and with p53. TSP-1 expression was detected in 112/166 (65%) cases with intensity ranging from very light to dark. A majority of the cases exhibited low TSP-1 expression (score of 1). Of the 161 cases evaluated for VEGF, high expression defined as a histoscore ≥ 200 was observed in 106 (66%) cases. Of the 163 cases evaluated for CD31 MVD, 56 (34%) cases

exhibited high CD31 ($CD31 \geq 110$). There were 143 cases that underwent CD105 immunostaining, and 95 (66%) cases displayed high CD105 ($CD105 \geq 28$). The reasons for missing biomarker data included: limited tumor cells, no tumor tissue, limited tissue, or no tissue. A modest positive correlation was observed between categorized CD31 MVD and p53 ($r=0.193$, $p=0.013$) or CD105 ($r=0.169$, $p=0.034$), and TSP-1 and CD105 ($r=0.185$, $p=0.032$). There was no evidence to suggest a relationship between (a) CD31 and TSP-1 or VEGF, (b) TSP-1 and VEGF or p53, (c) VEGF and CD105 or p53 or (d) CD105 and p53 (data not shown).

The Kaplan–Meier method was used to estimate PFS and OS by each biomarker (Figs. 1A–G). The five-year PFS and OS between respective categories are shown in Table 3. Women whose tumors expressed high CD31 MVD had a statistically significant improvement in both PFS (Fig. 1A, $p=0.003$) and OS (Fig. 1B, $p=0.005$). In contrast, women whose tumors expressed high CD105 MVD demonstrated worse PFS (Fig. 1G, $p=0.057$) and OS (Fig. 1H, $p=0.076$), but this was not statistically significant. Neither VEGF nor TSP-1 expression was significantly associated with PFS (Figs. 1C and E, respectively) or OS (Figs. 1D and F, respectively).

Unadjusted and adjusted Cox regression analyses were performed evaluating the angiogenic markers individually for associations with PFS or OS (Table 4). When expressed as continuous variables, none of the markers were associated with PFS or OS for this cohort. Unadjusted Cox modeling (Table 4) demonstrated that high CD31 MVD was significantly associated with improved PFS ($HR=0.37$; $p=0.007$) and OS ($HR=0.36$; $p=0.006$). High CD105 MVD, again, demonstrated a trend toward diminished PFS and OS. After adjusting for stage, depth of cervical invasion, parametrial extensions, positive lymph nodes and treatment, only CD31 MVD, but not TSP-1, VEGF or CD105 MVD, was an independent prognostic factor for PFS ($HR=0.36$; 95% $CI=0.17–0.75$; $p=0.006$) and OS ($HR=0.36$; 95% $CI=0.17–0.79$; $p=0.010$).

Discussion

Irregularly-shaped, atypical blood vessels found on colposcopic examination are well-documented as a finding consistent with invasive cancer of the uterine cervix. Furthermore, highly vascular tumors (i.e. tumors that bleed when biopsied) are generally considered by some to have an aggressive clinical phenotype. Though insightful, these observations cannot characterize the biologic relationship between angiogenesis and outcome in cervical cancer. HPV-E6-mediated inactivation of p53 has been shown to both up-regulate angiogenesis-promoting VEGF expression and also down-regulate angiogenesis-inhibiting TSP-1 expression, formulating a biologic basis for the role of angiogenesis in early cervical carcinogenesis. To our knowledge, this is the first translational study to report a correlation between CD31, a marker of angiogenesis, and outcome in cervical cancer using specimens collected on a Phase III protocol with defined inclusion criteria, central pathologic review, standardized treatment regimens, and standardized follow-up criteria in addition to rigorous validation and quality control of IHC detection and evaluation methods. This study provides compelling evidence that high CD31 MVD is not only associated with prolonged PFS and OS but is an independent prognostic factor in women with high-risk, early stage cervical cancer treated with radiation therapy alone or chemoradiation.

Previous studies in cervical cancer evaluating the prognostic significance of MVD as measured by IHC-detection of non-specific vascular markers (Factor 8, CD34, and CD31) have reported conflicting results. In the largest of these studies, Obermair, et al, reported worse outcome in women with high tumor MVD. In this study of stage IB patients, the five-year survival rate for women with high MVD (>20 vessels/hpf) was 90% versus 63% for

women with low MVD [13]. Additional studies have supported these findings [14-17]; however, high MVD has also been shown to be associated with improved survival [18,19] or to not correlate with outcome at all [20,21]. These inconsistencies are most likely explained by heterogeneity in the populations studied, the antibody detection method, quantification methods, and the treatment modalities employed. In this study, we report the simultaneous quantification and analysis of two separate markers of MVD in the same patient cohort. Our data show that CD31 MVD was significantly associated with an improvement in both PFS and OS independent of prognostic clinical covariates. We hypothesize that high CD31 MVD is a surrogate marker for improved tumor blood flow and oxygenation, resulting in a better response to adjuvant RT±CT.

CD105, an accessory component of the transforming growth factor beta (TGF- β) receptor system [27], has been shown to elicit an anti-apoptotic effect in endothelial cells under hypoxic stress [28]. High CD105+MVD counts have been associated with worse survival in squamous cell head and neck cancer [29], and with deep stromal invasion and nodal metastases in cervical cancer [30]. In this study, high CD105 MVD was associated with a trend suggesting worse PFS and OS (Figs. 1G and H), though this was not statistically significant. Given the modest effect size associated between this angiogenic marker and outcome, a larger study will be required to determine if CD105 MVD has clinical relevance in this patient setting. We hypothesize that the difference in HR direction between CD31 and CD105 in this study reflects the biologic difference between CD31-positive endothelial cells in more stable blood vessels and CD105-positive endothelial cells in newly formed vasculature that tends to be more disorganized and leaky and may result in less efficient delivery of oxygen, nutrients and chemotherapy as well as a more efficient route for metastatic spread. The potential inverse prognostic relevance of CD31 and CD105 MVD merits further investigation. A modest positive correlation was observed between CD31 and CD105 which is consistent with that reported by Mazibrada et al. [31] but the magnitude of the association was not as strong. We hypothesize that the disparity in these observation reflects the distinction in the patient populations at least in part. Our study included 173 women with highrisk, early-stage disease whereas the study by Mazibrada et al. included 50 women with cervical dysplasia and eight with invasive cervical cancer.

Previous studies have consistently reported an increase in VEGF expression with increasing severity of dysplasia and advancing cervical cancer stage [32-34], in addition to correlation with increased tumor MVD [10,33-36]. In a study of 117 women with FIGO Stage IB2 cervical cancer treated with surgery, Lee, et al, reported a significant correlation between VEGF expression and deep cervical invasion, lymph node metastases, and large tumor size [36]. While the intensity of VEGF signal correlated negatively with OS in this study, the percentage of tumor cells expressing VEGF did not correlate with OS. In 55 women with locally advanced cervical cancer treated primarily with pelvic radiotherapy, Gaffney, et al, demonstrated a statistically significant association between increasing VEGF expression and diminished PFS and OS [37]. In our cohort, VEGF expression was evaluated semiquantitatively using a Histoscore (*H*-score) that incorporates data regarding the percentage of positively stained cells and staining intensity using the formula % cells positive $\times$ [intensity+1]. Statistical analyses demonstrated that when evaluated as a continuous variable (data not shown) or dichotomized using an *H*-score of 200 (Tables 3 and 4), VEGF expression was not associated with PFS or OS

Previous data on TSP-1 in cervical cancer is limited, but decreased TSP-1 expression has correlated inversely with microvessel density [10,38] and low levels of TSP-1 expression have been shown to be associated with advancing FIGO stage, parametrial extension, and diminished survival [38]. Furthermore, TSP-1 expression demonstrates prognostic significance in squamous cancers of the vulva [39], esophagus [40], and lung [41]. Our

study did not detect an association between TSP-1 expression and PFS or OS, and this lack of association might be secondary to a disproportionate number of specimens in this cohort demonstrating negative or low TSP-1 expression.

In addition to providing prognostic information, biologic characterization of angiogenesis may also guide the application of anti-angiogenic therapies in cervical cancer. Current evidence supports that anti-angiogenic therapies target erratic angiogenic vessels with abnormally shaped, leaky endothelial cells that produce high interstitial fluid pressures, poor oxygen and drug diffusion, and express anti-apoptotic signals [42], promoting normalization of tumor vasculature which restores blood flow and oxygen supply to the hypoxic tumor fraction.

These data represent the first systematic study of angiogenesis markers in women undergoing radical surgery for early-stage cervical cancer followed by adjuvant radiation with or without radiosensitizing chemotherapy. This study provides strong evidence that CD31 MVD is not only associated with PFS and OS but is an independent prognostic factor in women with high-risk, early stage cervical cancer treated with radiation therapy alone or chemoradiation. Independent studies, however, will be required to validate these findings and to determine if CD31 MVD has prognostic significance in women with locally-advanced disease. If validated, CD31 MVD has the potential to identify a group of women who is less likely to benefit from standard adjuvant chemoradiotherapy and might be better served with neoadjuvant chemotherapy and surgery, a new radiosensitizing regimen like cisplatin and tirapazamine or an anti-angiogenesis drug like bevacizumab which exhibited single-agent activity in recurrent cervical cancer [43].

Acknowledgments

This study was supported by National Cancer Institute grants to the Gynecologic Oncology Group (GOG) and the GOG Tissue Bank (CA 27469), the GOG Statistical and Data Center (CA 37517), and Dr. Monk (K23 CA 087558). The following Gynecologic Oncology Group member institutions participated in the primary treatment studies: University of Alabama at Birmingham, Oregon Health Sciences University, Duke University Medical Center, Walter Reed Medical Center, Wayne State University, University of Southern California at Los Angeles, University of Pennsylvania Cancer Center, University of Miami School of Medicine, Milton S. Hershey Medical Center, Georgetown University Hospital, University of Cincinnati, University of North Carolina School of Medicine, University of Iowa Hospitals and Clinics, University of Texas Southwestern Medical Center at Dallas, Indiana University Medical Center, Wake Forest University School of Medicine, University of California Medical Center at Irvine, Tufts-New England Medical Center, Rush University Medical Center, SUNY Downstate Medical Center, Eastern Virginia Medical School, Johns Hopkins Cancer Center, State University of New York at Stony Brook, Washington University School of Medicine, Cooper Hospital/University Medical Center, Columbus Cancer Council, M. D. Anderson Cancer Center, University of Massachusetts Medical School, Fox Chase Cancer Center, Women's Cancer Center, University of Oklahoma, University of Virginia Health Sciences Center, University of Chicago, University of Arizona and Case Western Reserve University.

References

- [1]. Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;55:4–10.
- [2]. Ries, LAG.; Melbert, D.; Krapcho, M. SEER cancer statistics review, 1975–2004. National Cancer Institute; Bethesda, MD: (http://seer.cancer.gov/csr/1975_2004/), based on November 2006 SEER data submission, posted to the SEER web site, 2007)
- [3]. Delgado G, Bundy B, Zaino R, et al. Prospective surgical–pathological study of disease-free interval in patients with stage IB squamous cell carcinoma of the cervix: a Gynecologic Oncology Group study. *Gynecol Oncol* 1990;38:352–7. [PubMed: 2227547]
- [4]. Monk BJ, Wang J, Im S, et al. Rethinking the use of radiation and chemotherapy after radical hysterectomy: a clinical-pathologic analysis of a Gynecologic Oncology Group/Southwest Oncology Group/Radiation Therapy Oncology Group trial. *Gynecol Oncol* 2005;96:721–8. [PubMed: 15721417]

- [5]. Im SS, Wilczynski SP, Burger RA, et al. Early stage cervical cancers containing human papillomavirus type 18 DNA have more nodal metastasis and deeper stromal invasion. *Clin Cancer Res* 2003;9:4145–50. [PubMed: 14519638]
- [6]. Burger RA, Monk BJ, Kurosaki T, et al. Human papillomavirus type 18: association with poor prognosis in early stage cervical cancer. *J Natl Cancer Inst* 1996;88:1361–8. [PubMed: 8827013]
- [7]. Lopez-Ocejo O, Vilorio-Petit A, Bequet-Romero M, et al. Oncogenes and tumor angiogenesis: the HPV-16 E6 oncoprotein activates the vascular endothelial growth factor (VEGF) gene promoter in a p53 independent manner. *Oncogene* 2000;19:4611–20. [PubMed: 11030150]
- [8]. Lee JS, Kim HS, Jung JJ, et al. Expression of vascular endothelial growth factor in adenocarcinomas of the uterine cervix and its relation to angiogenesis and p53 and c-erbB-2 protein expression. *Gynecol Oncol* 2002;85:469–75. [PubMed: 12051876]
- [9]. Lee JS, Kim HS, Park JT, et al. Expression of vascular endothelial growth factor in the progression of cervical neoplasia and its relation to angiogenesis and p53 status. *Anal Quant Cytol Histol* 2003;25:303–11. [PubMed: 14714296]
- [10]. Toussaint-Smith E, Donner DB, Roman A. Expression of human papillomavirus type 16 E6 and E7 oncoproteins in primary foreskin keratinocytes is sufficient to alter the expression of angiogenic factors. *Oncogene* 2004;23:2988–95. [PubMed: 14968115]
- [11]. Wu MP, Tzeng CC, Wu LW, et al. Thrombospondin-1 acts as a fence to inhibit angiogenesis that occurs during cervical carcinogenesis. *Cancer J* 2004;10:27–32. [PubMed: 15000492]
- [12]. Dameron KM, Volpert OV, Tainsky MA, et al. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. *Science* 1994;265:1582–4. [PubMed: 7521539]
- [13]. Obermair A, Wanner C, Bilgi S, et al. Tumor angiogenesis in stage IB cervical cancer: correlation of microvessel density with survival. *Am J Obstet Gynecol* 1998;178:314–9. [PubMed: 9500492]
- [14]. Bremer GL, Tiebosch AT, van der Putten HW, et al. Tumor angiogenesis: an independent prognostic parameter in cervical cancer. *Am J Obstet Gynecol* 1996;174(1 Pt 1):126–31. [PubMed: 8571995]
- [15]. Dellas A, Moch H, Schultheiss E, et al. Angiogenesis in cervical neoplasia: microvessel quantitation in precancerous lesions and invasive carcinomas with clinicopathological correlations. *Gynecol Oncol* 1997;67:27–33. [PubMed: 9345352]
- [16]. Cooper RA, Wilks DP, Logue JP, et al. High tumor angiogenesis is associated with poorer survival in carcinoma of the cervix treated with radiotherapy. *Clin Cancer Res* 1998;4:2795–800. [PubMed: 9829744]
- [17]. Cooper RA, West CM, Wilks DP, et al. Tumour vascularity is a significant prognostic factor for cervix carcinoma treated with radiotherapy: independence from tumour radiosensitivity. *Br J Cancer* 1999;81:354–8. [PubMed: 10496365]
- [18]. West CM, Cooper RA, Loncaster JA, et al. Tumor vascularity: a histological measure of angiogenesis and hypoxia. *Cancer Res* 2001;61:2907–10. [PubMed: 11306466]
- [19]. Kainz C, Speiser P, Wanner C, et al. Prognostic value of tumour microvessel density in cancer of the uterine cervix stage IB to IIB. *Anticancer Res* 1995;15:1549–51. [PubMed: 7544572]
- [20]. Rutgers JL, Mattox TF, Vargas MP. Angiogenesis in uterine cervical squamous cell carcinoma. *Int J Gynecol Pathol* 1995;14:114–8. [PubMed: 8601522]
- [21]. Hutchison GJ, Valentine HR, Loncaster JA, et al. Hypoxia-inducible factor 1alpha expression as an intrinsic marker of hypoxia: correlation with tumor oxygen, pimonidazole measurements, and outcome in locally advanced carcinoma of the cervix. *Clin Cancer Res* 2004;10:8405–12. [PubMed: 15623619]
- [22]. Peters WA 3rd, Liu PY, Barrett RJ 2nd, et al. Concurrent chemotherapy and pelvic radiation therapy compared with pelvic radiation therapy alone as adjuvant therapy after radical surgery in high-risk early-stage cancer of the cervix. *J Clin Oncol* 2000;18:1606–13. [PubMed: 10764420]
- [23]. Mehta R, Kyshtoobayeva A, Kurosaki T, et al. Independent association of angiogenesis index with outcome in prostate cancer. *Clin Cancer Res* 2001;7:81–8. [PubMed: 11205922]
- [24]. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1974;53:457–81.

- [25]. Crowley J. A note on some recent likelihoods leading to the log rank test. *Biometrika* 1974;61:533–8.
- [26]. Cox DR. Regression models and life tables. *Journal of Royal Statistical Society* 1972;34:187–220.
- [27]. Cheifetz S, Bellón T, Calés C, et al. Endoglin is a component of the transforming growth factor- β receptor system in human endothelial cells. *J Biol Chem* 1992;267:19027–30. [PubMed: 1326540]
- [28]. Li C, Issa R, Kumar P, et al. CD105 prevents apoptosis in hypoxic endothelial cells. *J Cell Sci* 2003;116:2677–85. [PubMed: 12746487]
- [29]. Martone T, Rosso P, Albera R, et al. Prognostic relevance of CD105+ microvessel density in HNSCC patient outcome. *Oral Oncol* 2005;41:147–55. [PubMed: 15695116]
- [30]. Brewer CA, Setterdahl JJ, Li MJ, et al. Endoglin expression as a measure of microvessel density in cervical cancer. *Obstet Gynecol* 2000;96:224–8. [PubMed: 10908767]
- [31]. Mazibrada J, Ritta M, Mondini M, et al. Interaction between inflammation and angiogenesis during different stages of cervical carcinogenesis. *Gynecol Oncol* 2008;108:112–20. [PubMed: 17936343]
- [32]. Dobbs SP, Hewett PW, Johnson IR, et al. Angiogenesis is associated with vascular endothelial growth factor expression in cervical intraepithelial neoplasia. *Br J Cancer* 1997;76:1410–5. [PubMed: 9400935]
- [33]. Fujiwaki R, Hata K, Iida K, et al. Vascular endothelial growth factor expression in progression of cervical cancer: correlation with thymidine phosphorylase expression, angiogenesis, tumor cell proliferation, and apoptosis. *Anticancer Res* 2000;20:1317–22. [PubMed: 10810442]
- [34]. Kodama J, Seki N, Tokumo K, et al. Vascular endothelial growth factor is implicated in early invasion in cervical cancer. *Eur J Cancer* 1999;35:485–9. [PubMed: 10448304]
- [35]. Tanaka H, Yasuda Y, Musha T, et al. Hypoxia correlates with angiogenesis in cervical cancers. *Int J Clin Oncol* 2005;10:35–9. [PubMed: 15729599]
- [36]. Lee IJ, Park KR, Lee KK, et al. Prognostic value of vascular endothelial growth factor in Stage IB carcinoma of the uterine cervix. *Int J Radiat Oncol Biol Phys* 2002;54:768–79. [PubMed: 12377329]
- [37]. Gaffney DK, Haslam D, Tsodikov A, et al. Epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) negatively affect overall survival in carcinoma of the cervix treated with radiotherapy. *Int J Radiat Oncol Biol Phys* 2003;56:922–8. [PubMed: 12829126]
- [38]. Kodama J, Hashimoto I, Seki N, et al. Thrombospondin-1 and -2 messenger RNA expression in invasive cervical cancer: correlation with angiogenesis and prognosis. *Clin Cancer Res* 2001;7:2826–31. [PubMed: 11555600]
- [39]. Tringler B, Grimm C, Sliutz G, et al. Immunohistochemical expression of thrombospondin-1 in invasive vulvar squamous cell carcinoma. *Gynecol Oncol* 2005;99:80–3. [PubMed: 16009408]
- [40]. Oshiba G, Kijima H, Himeno S, et al. Stromal thrombospondin-1 expression is correlated with progression of esophageal squamous cell carcinomas. *Anticancer Res* 1999;19(5C):4375–8. [PubMed: 10650779]
- [41]. Yamaguchi M, Sugio K, Ondo K, et al. Reduced expression of thrombospondin-1 correlates with a poor prognosis in patients with non-small cell lung cancer. *Lung Cancer* 2002;36:143–50. [PubMed: 11955648]
- [42]. Fukumura D, Jain RK. Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization. *Microvasc Res* 2007;74(2-3):72–84. [PubMed: 17560615]
- [43]. Monk BJ, Sill MW, Burger RA. Phase II trial of bevacizumab in the treatment of recurrent squamous cell carcinoma of the cervix: a Gynecologic Oncology Group study. *Gynecologic Oncology* 2008;108:S23.

Fig. 1. Kaplan-Meier plots for progression-free survival (A, C, E, G) and overall survival (B, D, F, H) for women categorized by CD31 MVD (A, B), stromal TSP-1 expression (C, D), VEGF histoscore (E, F) or CD105 MVD (G, H).

Table 1

Clinical characteristics and end points

Clinical characteristics	Cases (%)
Age (median=39.1 years)	
<30	23 (13.3)
30–39	71 (41.0)
40–49	39 (22.5)
50–59	25 (14.5)
60–69	12 (6.9)
70–79	3 (1.7)
Race and ethnicity	
Caucasian	106 (61.3)
African American	29 (16.8)
Hispanic	29 (16.8)
Other ^a	9 (5.2)
Stage	
IB	165 (95.4)
IIA	8 (4.6)
Cell type	
Squamous carcinoma	139 (80.3)
Adenocarcinoma	22 (12.7)
Adenosquamous carcinoma	12 (6.9)
Grade	
1	14 (8.1)
2	83 (48.0)
3	76 (43.9)
Tumor size	
<2 cm	48 (27.9)
2–2.9 cm	83 (48.3)
≥3 cm	41 (23.8)
Missing	1
Depth of cervical invasion	
Inner third	6 (3.5)
Middle third	28 (16.5)
Outer third	136 (80.0)
Missing	3
Positive margin	
No	163 (94.2)
Yes	10 (5.8)
Parametrial extensions	
No	109 (63.0)
Yes	64 (37.0)

Clinical characteristics	Cases (%)
Positive nodes	
No	27 (15.6)
Yes	146 (84.4)
Number of positive lymph nodes	
None	27 (15.6)
One	71 (41.0)
Two	74 (42.8)
Three	1 (0.6)
Lymphovascular space invasion	
No	43 (25.0)
Yes	129 (75.0)
Missing	1
Treatment regimens	
Radiation therapy	89 (51.4)
Chemoradiation	84 (48.6)

Seven women were excluded from the study for the following reasons: benign disease (1), low-risk, early stage disease (2), and no FFPE tumor for testing (4).

^a Other includes Asian/Pacific Islander (5), Filipino (2), Native American (1), and not specified (1).

Table 2

Marker expression and significant marker associations

Cases		Significant marker associations		
<i>CD31 MVD</i>		p53 Histoscore		
Low<110	107 (65.6)	CD31 MVD	Negative	Positive
High≥110	56 (34.4)	Low<110	57 (54.8)	47 (45.2) <i>r</i> =0.193
Missing §	10	High≥110	19 (34.5)	36 (65.5) <i>p</i> =0.013
<i>TSP1 stromal intensity</i>		CD31MVD		
Negative	54 (33.3)			
Positive	112 (64.5)			
Missing ^a	7	CD105 MVD	Low<110	High≥110
<i>VEGF Histoscore</i>		Low<28	37 (77.1)	11 (22.9) <i>r</i> =0.169
Low<200	55 (34.2)	High≥28	56 (60.2)	37 (39.8) <i>p</i> =0.034
High≥200	106 (65.8)			
Missing ^a	12			
<i>CD105 MVD</i>		TSP-1 intensity		
Low<28	48 (33.6)	CD105 MVD	Negative	Positive
High≥28	95 (66.4)	Low<28	21 (43.8)	27 (56.3) <i>r</i> =0.185
Missing ^a	30	High≥28	24 (25.5)	70 (74.5) <i>p</i> =0.032

Kendall's tau-b correlation coefficient (*r*).

^aThe reasons for missing biomarker data included: limited tumor cells, no tumor tissue, limited tissue, or no tissue.

Table 3

Five-year progression-free survival and overall survival by angiogenic markers

	<u>Progression-free survival</u>		<u>Overall survival</u>	
	Five-year (%)	p^a	Five-year (%)	p^a
CD31				
Low<110	63.6	0.003	67.0	0.005
High $\geq$ 110	83.6		85.2	
TSP-1				
Negative	76.5	0.201	80.3	0.131
Positive	69.5		71.7	
VEGF				
Low<200	64.7	0.193	69.5	0.223
High $\geq$ 200	73.3		75.1	
CD105				
Low<28	74.5	0.057	77.7	0.076
High $\geq$ 28	66.0		69.2	

^a Logrank test to compare the survival functions.

Table 4
Associations between angiogenic markers and progression-free survival or overall survival

	Progression-free survival					Overall survival				
	Unadjusted Cox model			Adjusted Cox model ^a		Unadjusted Cox model			Adjusted Cox model ^a	
	HR	95% CI	p	HR	95% CI	HR	95% CI	p	HR	95% CI
CD31										
Low<110	1.0			1.0		1.0			1.0	
High≥110	0.37	0.18–0.76	0.007	0.36	0.17–0.75	0.006	0.17–0.79	0.010	0.36	0.17–0.79
TSP-1										
Negative	1.0			1.0		1.0			1.0	
Positive	1.50	0.80–2.82	0.204	1.30	0.67–2.54	0.444	0.85–3.33	0.132	1.44	0.70–2.75
VEGF										
Low<200	1.0			1.0		1.0			1.0	
High≥200	0.61	0.34–1.08	0.088	0.62	0.34–1.11	0.106	0.402–1.240	0.225	0.639	0.350–1.167
CD105										
Low<28	1.0			1.0		1.0			1.0	
High≥28	1.80	0.94–3.44	0.077	1.56	0.78–3.12	0.205	0.89–3.48	0.103	1.62	0.79–3.33

HR: hazard ratio; 95% CI: 95% confidence interval.

^aStratified by stage, depth of cervical invasion, parametrial extensions, positive lymph nodes and treatment.