Research Article

Vascular Endothelial Growth Factor-C (VEGF-C) Expression Can Not Predict Pelvic Lymph Node Metastases and Response to Neo-adjuvant Chemotherapy in Bulky Cervical Cancer

Ekspresi Vascular Endothelial Growth Factor-C (VEGF-C) tidak dapat Memprediksi Metastasis ke Kelenjar Getah Bening Pelvis dan Respons terhadap Kemoterapi Neo Ajuvan pada Kanker Serviks Berukuran Besar

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Abstract

Objective: To assess whether VEGF-C expression can predict the response to neoadjuvant chemotherapy and pelvic lymphnode metastases in bulky cevical cancer.

Methods: Seventeen cervical cancer stage IB_2 and IIA_2 cases during the period of July 2009 until June 2010 were collected consecutively and given neoadjuvant chemotherapy (NAC) PVB prior radical surgery. Response to treatment was evaluated based on the change of tumour size. VEGF-C expression was examined immunohistochemically at tumour biopsy before chemotherapy. The presence of lymphnode metastases histopathologically were obtained from pelvic lymphnode dissection. The difference and correlation of response and metastases on VEGF-C expression were analized statistically. The validity of the cut off percentage of immunopositive cells to VEGF-C to identify non responding and metastatic cases was calculated with the ROC. Multivariate analysis were done to determine the predictor of no response to chemotherapy.

Results: Clinical response, using the RECIST version 1.1 criteria, was found in 41.18% cases and lymphnode metastases were found in 27.27% cases. VEGF-C was expressed in all cases. Statistically, there were no significant differences and correlation in response to treatment and pelvic lymphnode metastases on VEGF-C expression. At the cut off ≥ 76% immunopositivity to VEGF-C, the sensitivity to identify no response and the specificity to identify response to NAC are 70.00% and 71.43% respectively (LR+ 2.45 and LR- 0.42); whereas at the cut off ≥ 75% immunopositivity to VEGF-C, the sensitivity to identify lymphnode metastases and the specificity to identify no lymphnode metastases are 100.00% and 75.00% (LR+ 4.0 dan LR- 0). With multivariate analysis using logistic regression, the cut off ≥ 76% immunopositive cells to VEGF-C were found to have positive coefficient, largest OR and statistic score, 1.93, 6.88 (96% CI OR 0.45; 104.34) and 41 respectively, to predict non responders in a prediction score model.

Conclusion: VEGF-C expression on biopsy specimen bulky cervical cancers can not differentiate cases that respond to NAC and metastases to the pelvic lymphnode from that do not. The cut off ≥ 76% immunopositive cells to VEGF-C in a prediction model can be used as an alternative predictor to identify non responders.

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Keywords: bulky cervical cancer, neoadjuvant chemotherapy, response and metastases prediction, VEGF-C immunohistochemistry expression

Abstrak

***Tujuan**: Untuk menilai apakah ekspresi VEGF-C dapat memprediksi respons terhadap kemoterapi neoajuvan dan metastasis ke kelenjar getah bening pelvis pada kanker leher rahim lesi besar.*

***Metode**: Sebanyak 17 kasus kanker leher rahim stadium IB_2* dan IIA_2 dikumpulkan secara konsekutif selama periode Juli 2009-Juni 2010 dan diberi kemoterapi neoajuvan PVB sebelum bedah radikal. Penilaian respons terhadap kemoterapi didasarkan pada perubahan ukuran tumor. Ekspresi VEGF-C diperiksa secara imunohistokimia dari biopsi tumor sebelum pemberian kemoterapi. Adanya metastasis ke kelenjar getah bening pelvis diperoleh dari diseksi kelenjar getah bening pelvis. Uji beda dan korelasi respons terapi dan metastasis tumor pada ekspresi VEGF-C dianalisis secara statistik. Reliabilitas cut off persentase imunopositif VEGF-C untuk mengidentifikasi kasus tidak respons dan bermetastasis ditentukan dengan ROC. Analisis multivariat dilakukan untuk menentukan prediktor tidak adanya respons terhadap kemoterapi.

***Hasil**: Respon klinis, menggunakan kriteria RECIST 1.1, dijumpai pada 41,18% kasus dan metastasis kelenjar dijumpai pada 27,27% kasus. VEGF-C diekspresikan pada seluruh kasus. Secara statistik tidak dijumpai perbedaan dan korelasi respons terapi dan metastasis kelenjar yang bermakna pada ekspresi VEGF-C. Pada cut off ≥ 76% imunopositif terhadap VEGF-C, sensitivitas untuk mengidentifikasi kasus tidak respons dan spesifisitas untuk mengidentifikasi kasus respons terhadap kemoterapi neoajuvan masing-masing adalah 70,00% dan 71,43% (LR+ 2.45 dan LR- 0.42); sementara pada cut off ≥ 75% imunopositif terhadap VEGF-C, sensitivitas untuk mengidentifikasi metastasis kelenjar dan spesifisitas untuk mengidentifikasi tidak ada metastasis kelenjar adalah 100,00% dan 75,00% (LR+ 4,0 dan LR- 0). Dengan analisis multivariat menggunakan regressi logistik, cut off ≥ 76% imunopositif terhadap VEGF-C dijumpai mempunyai koefisien positif, OR dan skor statistik terbesar, masing-masing 1,928 dan 6,88 (96% CI OR 0,45; 104,34) dan 41, untuk memprediksi kasus tidak respons di dalam suatu model skor prediksi.*

***Kesimpulan**: Ekspresi VEGF-C pada spesimen biopsi kanker leher rahim stadium IB_2 dan IIA_2 tidak dapat membedakan kasus yang berespons terhadap kemoterapi neoajuvan maupun yang bermetastasis dari yang tidak. Cut off ≥ 76% imunopositif terhadap VEGF-C di dalam suatu model skor prediksi dapat digunakan sebagai alat prediktor alternatif untuk menemukan kasus tidak respons.*

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***Kata kunci**: ekspresi VEGF-C secara imunohistokimia, kanker leher rahim lesi besar, kemoterapi neoajuvan, prediksi respons dan metastasis*

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INTRODUCTION

Cervical cancer is the 3rd most common cancer in women, and the seventh overall, with an estimated 530,000 new cases in 2008.[1] In Indonesia, based on the National Cancer Registry on 2010, the incidence rate of cervical cancer accounts for 27.89% of cancer in women and from 509 clinical staging done on 2009, there were 18 and 36 cases with stage IB_2 and IIA disease.[2]

Caused by the high incidence of pelvic lymphnodes metastases and disease recurrence in bulky tumour, FIGO (Federation of International Gynecologists and Obstetricians) on 1995 divided cervical cancer stage IB into 2 sub-stages, and also stage IIA on 2008 (Revised FIGO staging for cervical cancer). The sub-stages represents lesion size ≤ 4 cm and > 4 cm (bulky tumour).[3-4]

Although chemoradiation has already been accepted as standard therapy for cervical cancer patients with bulky tumour size, the role of neoadjuvant chemotherapy (NAC) before radical surgery need to be explored further because it can reduce tumour size and improve overall survival.[5-6] On the other hand, delay of definitive therapy, acceleration of tumour growth, and even metastases can be expected in patients who do not shows any response to this approach. A convenient predictor that can predict response to neoadjuvant chemotherapy and metastases to the pelvic lymphnodes before choosing an available therapy modality, has become an important issue.[7]

A biomarker is still controversial as a predictor of response to chemotherapy and in testing metastases.[8-9] But for Indonesia, where sophisticated imaging techniques are not always available and considered expensive, Vascular Endothelial Growth Factor-C (VEGF-C) that has been shown to have a role in the development and progression of cancer could be a valuable alternative predictor.[10]

In cervical cancer, including bulky lesion, VEGF-A on previous studies has been shown to have a role in prediction of response to neoadjuvant chemotherapy[11-13] and also VEGF-C serum level in prediction of metastases.[14] In this study we want to know whether VEGF-C expression can predict response and metastases to pelvic lymphnodes before neoadjuvant chemotherapy in order to improve patient management.

METHODS

From 17 cervical cancer patients stage IB_2 and IIA_2, studied in Dr. Cipto Mangunkusumo Jakarta and Dr. Hasan Sadikin Bandung referral hospital (Indonesia) from July 2009 until June 2010 and approved by the tumour board to give neoadjuvant chemotherapy, were collected data about tumour size, histopathology, tumour VEGF-C expression and pelvic lymphnode status. All subjects, that had no history of therapy and been diagnosed histopathologically, had given written informed consent.

Chemotherapy in this study consists of cisplatin 50 mg/m^2, vincristine 2 mg/m^2 and bleomycin 15 mg at 7 days intervals for 3 cycles. After receiving complete chemotherapy, patients considered suitable for surgery, had been performed radical hysterectomy and pelvic ≤ lymphnode dissection in 6 weeks. Metastases to the lymphnodes and other high risk factors were evaluated histopathologically. Patients who did not respond or not suitable for surgery received radiation.

Response criteria to therapy was based on revised RECIST (Response Evaluation Criteria in Solid Tumours) (1.1). Measurement of the tumour diameter, was done in 4 weeks before and in 3 weeks after chemotherapy, through pelvic examination using a tampon forcep measured with a ruler (cm) in 3 dimensions. If the stage of the disease clinically progressed the response was considered progressive. If the response was clinically complete, with no evidence of primary tumour, pelvic lymphnode and parametrial involvement histopathologically, the response was considered as complete pathologic response.[15-16]

Cervical biopsy specimen, preserved with 10% formalin and embedded in paraffin to build tissue blocks, were processed for histologic examination with hematoxylin and eosin (H&E) staining and immunohistochemistry analysis using avidin-biotin-peroxidase method. VEGF-C protein expression was detected with mouse anti-human monoclonal VEGF (C-1) antibody using Star Trek Universal HRP Detection System (Biocare Medical, LLC, Concord CA, USA) as a detector. Paraffin blocks were sectioned 4μm thick, developed in waterbath, adhered to object glasses and incubated at 60°C for 30 minutes. The slides were deparaffinized in xylene, dehydrated through graded alcohol concentrations, incubated at 94°C with 0.5% methanol-H_2O_2 for 30 minutes, and finally soaked in Tris EDTA (TE) so-

lution as pretreatment for antigen retrieval. The slides were then incubated using microwave at 90-100°C followed at 50-60°C for 5 minutes respectively. After cooling at room temperature and washed with Phosphate-Buffer Saline (PBS) at pH 7.4, to the slides were added a protein blocking agent to block background sniper. The primary antibody was then added and incubated for 30-45 minutes at room temperature and washed with PBS. After adding the secondary antibody, biotinylated secondary antibody (Trekkie Universal Link), and bound with Horseradish peroxidase (HRP) labeled with septravidine, the antigen antibodi complex was visualized with diaminobenzidine that is brown stained. Positive controls originates from colon tissue that express VEGF-C and negative controls originates from the study cervical cancer tissue without giving primary antibody.

VEGF immunoreactivity was scored based on brown staining of cytoplasm of tumour cells at immunohistochemistry slides semiquantitatively using the IRS (Immunoreactive Scoring System).[17] (Figure 1).

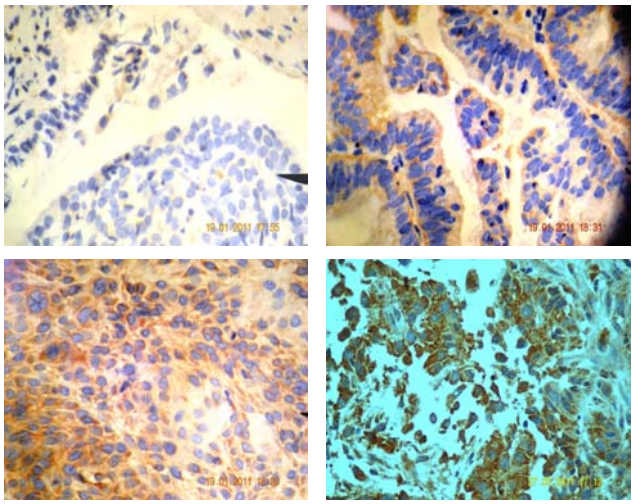


Figure 1. VEGF-C expression (magnified 400x) at IHC slides.
A. Negative expression; B. Weak expression;
C. Moderate expression; D. Strong expression.

Assesments were done independently by two investigators, who were not aware of the clinical outcome, and then evaluated together until a consensus reached.

Statistical analysis were done using SPSS 15. Bivariate analysis were calculated using Fisher exact, Pearson chi square and Wilcoxon signed-rank tests. Probability was considered significant if p value <0.05 with confidence interval 95%. Correlation beween variables were evaluated with Spearman test. A ROC (Receiver Operating Characteristic) analysis and a multivariate analysis using logistic regression, were done to determine a reliable cut off point of the percentage immunopositive cells to VEGF-C and a predictive score.

RESULTS

Thirty cases were collected consecutively. As many as 13 cases (43%) had to excluded, primarily caused by delay in chemotherapy schedule.

The average age of the cases is 45.20 years and cummulatively were more at the age <50 years. Other clinicopathologic characteristics are as listed in Table 1.

Table 1. Clinicopathologic characteristics

Variables	n	%	Avg	SD	95% CI	p
Age (year)						
< 50	17	56.7	45.2	9.26		
≥ 50	13	43.3				
BMI						
18.5-24.99	23	76.7	23.1	3.16		
≥ 25	7	23.3				
T shape						
Exophytic	26	86.7				
Endophyt.	4	13.3				
Stage disease						
IB_2	20	66.7				
IIA_2	10	33.3				
Tumour size						
Before NAC	17	100	4.76	0.41	4.50-5.05	0.008*
After NAC	11	64.7	2.9	1.69	1.76-4.04	
Histologic type						
Squamous	12	70.6				
Adenoca	3	17.6				
Adenosq	1	5.9				
Others	1	5.9				
Cell diff						
G1-G2	11	64.7				
G3	6	35.3				
LVSI						
Negative	16	94.1				
Positive	1	5.9				

** Wilcoxon signed-rank test*
Avg=average

All cases expressed VEGF-C. After neoadjuvant chemotherapy there was a significant decrease in tumour size (p = 0.0087). From 7 responding cases (41.18%), 2 (11.76%) showed clinical (CR) and pathological complete response (CPR). To all cases that were considered respectable were performed radical hysterectomy and pelvic lymphnode dissec-

tion (10 cases, 58.82%), while cases that were considered too large or disease progressed were irradiated (7 cases, 41.18%). To one irradiated case, was performed also lymphnode dissection. Tumour cells at surgical margins after radical surgery were found only in 1 of 10 cases (10%).

The p values of response to NAC at clinicopathologic characteristics were not significantly different. Also at VEGF-C expression, respectively at every grade, there were no statistical differences (p = 0.65, 0.69 dan 0.67) (Table 2).

Table 2. Differences in response to NAC at VEGF-C expression

VEGF-C expression	Response	No Response	Total	p
Weak	4 (23.53%)	6 (35.29%)	10 (58.82%)	0.65*
Moderate	2 (11.76%)	3 (17.65%)	5 (29.41%)	0.69*
Strong	1 (5.88%)	1 (5.88%)	2 (11.76%)	0.67*
Total	7 (41.18%)	10 (58.82%)	17 (100.0%)	

** Fisher exact test*

Metastases to the pelvic lymphnodes were found in 3 cases (27.27%). Metastatic tumour cells, respectively were found at 1 of 3 pelvic lymphnodes with infiltration to the right parametrium, at 1 of 5 obturator lymphnodes, and at 3 pelvic lymphnodes each side of 19 lymphnodes dissected. There were no statistically significant differences in metastases to the pelvic lymphnodes at VEGF-C expression (p=0.44). (Table 3)

Table 3. Differences in lymphnode metastases at VEGF-C expression

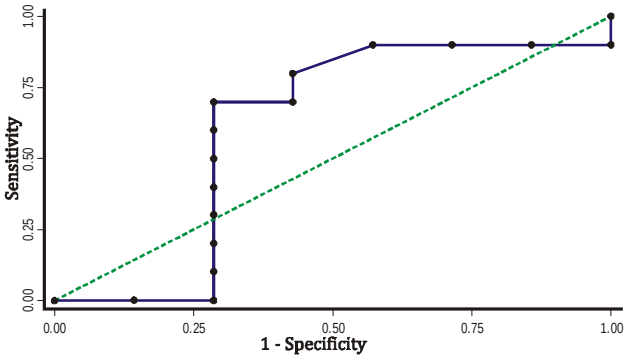
VEGF-C expression	Positive	Negative	Total	p
Weak	1 (9.09%)	6 (54.55%)	7 (63.64%)	0.44*
Moderate	1 (9.09%)	1 (9.09%)	2 (18.18%)	
Strong	1 (9.09%)	1 (9.09%)	2 (18.18%)	
Total	3 (27.27%)	8 (72.73%)	11 (100.0%)	

** Pearson chi square*

With Spearman test, a correlation could not be demonstrated between VEGF-C expression and response; neither also between VEGF-C expression and metastases (Spearman's rho = -0.0286, 0.3858 and p = 0.9133, 0.2413 respectively).

Because bivariate analysis and Spearman test could not answer the hypothesis of this study, we decide to determine a reliable cut off point at the percentage of immunopositive cells to VEGF-C to identify non responding and metastatic cases with the ROC analysis. The rationality to use the percentage of immunopositive cells to VEGF-C, because this continuous numeric data were not categorized like the IRS that could hide the differences. On each percentage, we tested the sensitivity and specificity, positive likelyhood ratio (LR+) and negative likelyhood ratio (LR-).

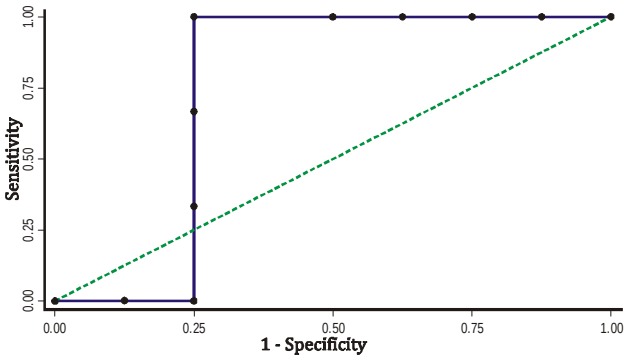
The reliable cut off point to identify non responder was at ≥ 76% immunopositive cells (sensitivity 70% and specificity 71.43%, correctly classified 70.59%, with LR+ 2.45 and LR- 0.42), although not statistically different (p=0.09). The area under the curve (AUC) at the ROC analysis was 0.61 with SE 0.17 (95% CI 0.27 - 0.95). (Figure 2)



Area under ROC curve = 0.6071

Figure 2. ROC analysis curve of response at VEGF-C immunopositivity

Whereas the reliable cut off point to identify metastatic cases, was at ≥ 75% immunopositive cells (sensitivity 100% and specificity 75.00%, correctly classified 81.82%, with LR+ 4.0 and LR- 0). The AUC at the ROC analysis was 0.75 with SE 0.16 (95% CI 0.43-1.00). (Figure 3)



Area under ROC curve = 0.7500

Figure 3. ROC analysis curve of metastases at VEGF-C immunopositivity

Table 4. Prediction model of no response to NAC with statistical score

Variable	Coefficient	SE coefficient	z	OR	95% CI OR	p	Score
% immunopositive (≥ 76)	1.93	1.39	1.39	6.88	0.45; 104.34	0.17	41
Hard lymphocyte reaction	0.59	1.73	0.34	1.81	0.06; 53.83	0.73	10
Moderate diff.	1.57	1.98	0.79	4.79	0.10; 233.61	0.43	23
Poor diff.	–.005	1.77	–.003	1.00	0.03; 32.00	0.10	0
BMI < 23.1	0.55	1.45	.378	1.73	0.10; 29.66	0.70	11
Constanta	–2.28	2.16	–1.06				

At the multivariate analysis with logistic regression, the variables that included in the prediction model of no response are as listed in Table 4. A positive coefficient, highest OR (Odds ratio) and statistical score were found at the variable ≥ 76 percentage immunopositivity to VEGF-C to predict no response, respectively 1.93, 6.88 (96% CI OR 0.45; 104.34) and 41.

If we compared the AUC from the mathematical model with the AUC from the model that has been transformed into statistical score, there were no statistical differences (p=0.3068). The statistical score with cut off ≥ 52 can be used as a predictor of no response (sensitivity 60%, specificity 85.71% and accuracy 70.59%).

DISCUSSION

The number of complete clinical response in this study are smaller compared with the results in Modarress et al and Benedetti-Panici P et al studies (respectively 16.7% and 78%); either is complete pathologic response compared with the studies of Choi CH et al (27.6%), but similar with the studies of Modarress et al and Benedetti-Panici P et al (respectively 10 and 13%).

In this study, despite VEGF-C was expressed in all cases, it could not differentiate responder from non-responder neither also metastatic from non-metastatic cases. These observations are different with that reported by Choi CH et al and Cheng WF et al at VEGF expression,[11-13] and Andrijono dan Priyanto H at VEGF-C serum level in their studies.[14]

The differences of the results reported in the studies mentioned above at response and metastases, is probably caused by the sample size needed to see differences in a relatif homogen percentage of responder and non responder population (41.18% vs 58.82%) is larger, although the minimum sample size to see differences in response and metastases at VEGF-C expression has been reached. This difference can be referred as a statistical bias by chance.

At the ROC analysis, the percentage =76 and =75 immunopositive cells to VEGF-C, respectively for no response and metastases, are reliable and the AUC of both ROC curves in this analysis (0.61 and 0.75 respectively) were considered to have fairly and good reliability. With multivariate analysis, although statistically not significant (p = 0.17), the variable percentage ≥ 76 immunopositive cells to VEGF-C can be demonstrated as the strongest predictor of no response (Table 4).

The variables included in the prediction model, with cut off ≥ 52 prediction score, can further be used as a tool to predict non responding cases; although this conclusion should be further studied with more samples. If the prediction score found for a certain cervical cancer with bulky lesion is ≥ 52, chemoradiaton would be more reasonable than neoadjuvant chemotherapy followed with radical surgery as the definite therapy. Alternatively, if the choice is radical surgery anti VEGF should be added, although this approach in cervical cancer need to be explored.

CONCLUSION

VEGF-C expression in this study can not differentiate non responding from responding and metastatic from non-metastatic cases in stage IB_2 and IIA_2 cervical cancer given neoadjuvant chemotherapy. The prediction score, using ≥ 76 percentage immunopositive cells, with cut off ≥ 52 could be a valuable alternative tool in this approach.

In the immunohistochemistry report of VEGF-C expression, the percentage of immunopositive cells to VEGF-C need to be included in order to have a more clearer impression of the immunopathology of the disease.

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