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Review

Evaluation of Detection Methods and Values of Circulating Vascular Endothelial Growth Factor in Lung Cancer

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Abstract

Lung cancer is the deadliest cancer in the world. Angiogenesis plays a crucial role of the incidence, progression, and metastasis in lung cancer. Angiogenesis inhibitors are used to treat non-small cell lung cancer (NSCLC) patients, and the molecular biomarkers are also being assessed to predict treatment response/therapeutic response and patients' prognosis. Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that stimulates angiogenesis. Due to its predictive values of prognosis on NSCLC, a large number of methods have been developed and evaluated to detect VEGF levels in a variety of studies. In this article, we review the detection methods designed to measure the VEGF levels in different body fluids and prognosticate the value of VEGF in treatment, diagnosis and survival in lung cancer.

Key words: NSCLC; small cell lung cancer (SCLC); VEGF; evaluation; angiogenesis; biomarker

Introduction

Lung cancer has been the most common cancer in the world. In China, there were approximately 4,292,000 people to be diagnosed with lung cancer in 2015 and around 2,814,000 people died from this disease. Because lung cancer is the most fatal cancer, it is a serious public health problem [1]. As such, considerable research on the disease mechanism, drug development, and therapeutic application has been done.

In the past decade tremendous advances have been made in minimally invasive operation, chemotherapy response rate, advanced radiotherapy technology, progression-free survival (PFS) and overall survival (OS) in NSCLC and SCLC. Molecular marker for tumor progression under chemotherapy has been one of the focused field for the study. In particular, VEGF, an angiogenic factor, has been studied widely. Angiogenesis plays a vital role in the incidence, progression, and metastasis in lung cancer. Many trials displayed that a poor or less favorable prognosis for patients with lung cancer was indicated by VEGF over-expression in tumor cells; however, VEGF is not an independent prognostic factor in some multivariate analyses [2-5]. On the contrary, some attempts do not show any correlation between VEGF expression and survival [6,7]. Delmotte et al. [8] made a meta-analysis studying the prognostic impact of VEGF expression in patients with NSCLC. A meta-analysis with fifteen trials (1549 patients) found that VEGF is not a favorable prognostic factor in NSCLC (hazard ratio (HR): 1.48; 95% confidence interval (CI): 1.27–1.72). Recently, another

meta-analysis included 4499 patients with NSCLC from 38 studies suggested that VEGF over-expression, regardless of its isoform, displayed a poor prognosis for patients (HR = 1.46; 95% CI 1.38–1.54). However, VEGF-C over-expression was not significantly associated with patients' survival (HR = 1.22; 95% CI 0.96–1.47) [9]. Presently, most trials supported that VEGF expression in NSCLC tumor tissue has an impact on patient clinical prognosis and VEGF over-expression correlated with a poor prognosis, although this failed to reach a complete consensus. These studies indicated the importance of evaluation of VEGF in lung cancer development and need in understanding the role of VEGF in lung cancer prognosis.

A critical step in evaluation of the role of VEGF is the accurately measurement of the VEGF expression. A large number of methods have been used; however, detection of VEGF in tumor tissue was invasive, complex and expensive compared with measuring circulating VEGF level [2-9]. In recent years, increasing studies on expression of circulating VEGF level have been published. Among these publications, a considerable number are clinical trials that involved in evolution of detection methodology and in prognosis values of VEGF. To detect VEGF level noninvasively and cost-effectively, to improve patient compliance for obtaining samples easily, and to look for a stable, accurate and reliable method of measuring VEGF level for clinical physicians, we have written this review to analyze the value of circulating VEGF in lung cancer for clinical physicians and to support researchers in future.

Role and mechanism of angiogenesis

Angiogenesis plays a vital role under not only physiological conditions but also many kinds of disease conditions, such as rheumatoid arthritis, diabetic retinopathy, and tumor [10]. The process is very important for the development of new vessels during fetal growth and tissue repair; whereas uncontrolled angiogenesis promotes some disorders and neoplastic diseases. Normal regulation of the process depends upon the balance between growth inhibitory factors and promoting factors [11, 12]. Some angiogenical molecules can induce the process while some inhibitory molecules can cease it [13]. The balance will be broken under some abnormal conditions. Angiogenesis relies on the balance between different molecules released by the host and tumor cells, while the process consists of a series of steps including separation of endotheliocyts from pericytes and the basement membrane, invasion and migration across basement membranes, and eventual becoming into a tumor body [14, 15]. A trial indicated that the balance of circulating angiogenic leaded to serum TSP1/VEGF value being significantly higher in the control group than in patients with NSCLC (P = 0.039) [16].

Evidence has displayed that angiogenesis is a pathogenesis at early stage lung cancer. Angiogenic squamous dysplasia represents slight lesions where capillary loops into abnormal bronchial epithelium. They observed the expression in preneoplastic lesions from individuals with high risk of developing lung cancer and it related to VEGF over-expression [17, 18]. Uncontrolled angiogenesis result in tumor progression and metastasis. The different processes of angiogenesis are controlled by a large number of other mediators including the vascular endothelial growth factor receptors (VEGFRs), the basic fibroblast growth factor (bFGF), the matrix metalloproteinases (MMPs) and their inhibitors (MMPIs), the platelet-derived growth factor (PDGF), the plasminogen activators (PAs), and the transforming growth factor-βs (TGF-β) [19], among many others. One of the most key and specific regulators of angiogenesis is VEGF [20, 21].

VEGF gene, VEGF family and VEGF receptor

VEGF gene

The VEGF gene is located on the short arm on chromosome 6 and it is composed of six exons. It has differently spliced to yield four isoforms (VEGF121, VEGF165, VEGF189, VEGF206) [22]. In addition, some uncommonly expressed isoforms are also identified (VEGF145 and VEGF183) [23].

VEGF family

The VEGF family contains 7 secreted glycoproteins, which are designated respectively by VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor (PlGF), and VEGF-F [24-26].

VEGF-A (also known as VEGF) was firstly identified by Senger group in 1983, as a vascular permeability factor secreted by tumor cells [27]. It, a tumor-secreted cytokine, plays an important role in both normal- and tumor-associated angiogenesis [28].

VEGF-B links to VEGFR-1 not VEGFR-2 or VEGFR-3, while the glycoprotein activates a poor mitogenic signal for endothelial cells, suggesting that its receptor VEGFR-3 is mainly in regions with developed lymphatic vessels [29]. By now, we have known little about the molecular mechanism regulating VEGF-B expression.

VEGF-C can induce selective lymphangiogenesis without accompanying angiogenesis [30], and it also plays a positive role in lymphatic invasion, lymphatic metastasis and patient survival [31].

VEGF-D, expressed in the majority of human tissues, especially in the lungs and skin during embryogenesis it is the most abundant [32]. To date, it is one of the key members identified in lymphangiogenesis. In experimental studies, VEGF-D played a vital role in inducing lymphangiogenesis and lymphatic metastasis [33]. It stimulated growth of vascular and lymphatic endotheliocytes through signaling by the tyrosine kinase receptors KDR (VEGFR-2) and Flt-4 (VEGFR-3) [34].

VEGF-E is a strong angiogenic factor, the study displayed that VEGFR-2 alone can induce angiogenesis efficiently [35]. VEGF-$_{ENZ-7}$ is a new isoform of VEGF-E encoded by the *orf* virus genome [36]. VEGF-E family members are potential angiogenic factors in clinical proangiogenic therapy for VEGF-E_{NZ-7} inducing significant angiogenesis *in vivo* with few side effects.

PlGF discovered in the placenta is expressed in the placenta, heart and lungs [37]. A trial showed that PlGF was associated with disease progression and survival in colorectal cancer [38]. PlGF is also implicated in other diseases, being studied in leukemia [88] and Ewing's sarcoma [39] as a therapeutic target.

VEGF-F identified from snake (viper) venom recently is the seventh member of the VEGF family and has unique properties. Through the compare with VEGF165, SutoK group found that it showed potent biological activity both *in vitro* and *in vivo* [40].

VEGF receptors

The VEGFs express present their biological activity through interaction with their receptors. After the dimerization and autophosphorylation of the intracellular receptor tyrosine kinases, the receptors, transmembrane tyrosine kinases, bind their ligands to the extracellular domain of the receptor and activate a cascade of downstream proteins. The receptors identified are designated respectively VEGFR-1, VEGFR-2, VEGFR-3 and the neuropilins (NP-1 and NP-2) so far.

VEGFR-1 binds VEGF-A, VEGF-B and PlGF with high affinity. VEGFR-1 is first expressed in angioblasts and endothelium, although its expression is less strongly than VEGFR-2. The soluble receptor could act as a specific antagonist of PlGF or VEGF-A. Soluble VEGFR-1 is expressed in some tumors, such as astrocytic tumor and breast cancer. Its actual effect in these tumors remains to be investigated [41, 42].

VEGFR-2 binds VEGF-A, VEGF-C, VEGF-D and VEGF-E and it represents a key molecular target for antiangiogenic intervention for its integral involvement in endotheliocytes proliferation and migration. Study displayed that the inhibition of VEGFR-2 can improve tumor response by molecular targeting at tumor vasculature [43].

VEGFR-3 activation and its ligands upregulation are found in some neoplastic conditions, including breast cancer and melanoma [44,45]. Moreover, its blockades obviously inhibited lymph node metastasis and lymphangiogenesis [46]. So, blocking VEGFR-3 with specific inhibitors may control new lymphatic growth.

NP-1 and NP-2

In humans, NP-1 and NP-2 are located respectively on chromosome 10 and 2. Both of them are composed of 17 exons [47, 48]. NP-1 is expressed in the cardiovascular, skeletal, and nervous systems during embryonic development [49, 50], but it is also expressed in tumor cells, heart, lungs, pancreas, liver, osteoblasts, kidney, and bone marrow stromal cells in adults [51,52]. NP-2 expression is similar to NP-1.

Detection methods of circulating VEGF in lung cancer

We found 71 studies by searching for key words "lung" "cancer" "VEGF" in: "title" or "Title/Abstract" fields, with "clinical trial" as the limited condition through PubMed. At the same time, similar searching from PMC did not reveal any additional publication. Within these studies, 32 had been reported with data that related to VEGF. Three of the 32 studies were further excluded. Two were lung metastatic tumor from primary head and neck cancer and leukemia. Another one was about cell testing, not an *in vivo* study. Therefore, a total of 29 clinical trials were utilized in this review.

Mattern et al. first reported the VEGF expression in NSCLC in 1996 [53] and they illustrated a negative prognostic role of VEGF expression in lung cancer tissue [54, 55]. Since then, a large number of studies in early stage NSCLC have reported the over-expression of VEGF and its association with disease progression or poor survival [56–65]. However, some studies did not display any correlation between VEGF expression and outcome [6, 7]. So far, a complete consensus of the association between response of treatment and survival has not been reached, while the detecting method was invasive, expensive, and inconvenient. Recently, increasingly more trials have been conducted to detect the VEGF level in body fluid, but not in tumor tissue, in NSCLC and SCLC. The majority of them detected VEGF levels from plasma and others from serum, a few ones from sputum, exhaled breath condensate (EBC), and malignant pleural effusion (MPE). We analyzed ten studies on detecting VEGF from serum, sixteen from plasma, two from pleural fluid, one from EBC and one from sputum in this review.

Table 1. The information of ten studies detecting VEGF level in serum.

Ref/First Author	Ethnic	No. of cases	Character	Method (kits)	Kit's Sensitivity	Biomarkers	Detecting time	Stage/ type	Treatment/ diagnosis
[71] A.M. C. Dingemans	Dutch	223	Multicenter Random, protective	ELISA(R&D Systems Minneapolis, MN)	Standard curve 15-2000pg/ml	VEGF	0, 3w, 6w, PD	IV/ NSCLC	Chemotherapy
[66] Andrea Camerini	Italian	43	protective	ELISA (-)	-	VEGF, TSP1	0,3w,6w,9w,3m, PD	IIIB-IV /NSCLC	Chemotherapy
[73] Faruk Tas	Turk	40	protective	ELISA(R&D Systems Minneapolis, MN)	Reader at 450nm（China)	VEGF, TSP1, VEGFR-1	0,1w,2w,3w	III-IV /NSCLC	Chemotherapy
[72] Martin J. Edelman	White85% black11% other3%	140	Protective Random	ELISA(R&D Systems Minneapolis, MN)	Lower Limited detection 9.0pg/ml	VEGF, COX-2 5-LOX	0, 1cycle, 2cycle	IIIB-IV /NSCLC	Chemotherapy
[68] Petri SALVEN	Finnish	68	Protective Random	ELISA(R&D Systems Minneapolis, MN)	Microtitre plate reader at 450nm (50-1000pg/ml)	VEGF	0(pretreatment)	Limited-extensive /SCLC	Chemotherapy
[76] Peng Zhao	Chinese	50	Protective nonrandom	ELISA(R&D Systems Minneapolis, MN)	Microplate reader at 450nm	IL-4, IL-10, IFN-γ	0, after treatment	I-III/ NSCLC	Immunotherapy
[67] Junbao Liu,	Chinese	60	Protective Random	American GB company（San Francisco)	--	VEGF, bFGF, TNF-α	0, after 2m	IIIB-IV /NSCLC	Traditional Chinese medicine
[74] Eleftherios Dalaveris	Greek	30	Protective	ELISA(R&D Systems Minneapolis, MN)	Lower Limited detection 0.9pg/ml	VEGF, TNF-α, 8-ISO	0(pretreatment)	IIIB-IV /NSCLC	Diagnose
[70] Masaya Tamura,	Japanese	78	Protective	ELISA(R&D Systems Minneapolis, MN)	Lower Limited detection 9.0pg/ml	VEGF, VEGF-C, MMP-9	0(pretreatment)	I-III / NSCLC	Diagnose
[98] Songwen Zhou	Chinese	112	Protective	ELISA(R&D Systems Minneapolis, MN, USA)	Microplate reader	VEGF, TGF-α	0, 1m	IIIB-IV /NSCLC	Targeted therapy

Assessment of serum VEGF level

In detecting the serum VEGF levels, all of the ten studies used the enzyme linked immunosorbent assay (ELISA) method. Most of them collected blood specimens in tubes without anticoagulant, then lightly inverted to mix completely. Within half an hour, the tubes were centrifuged for 10 minutes at 1100 to 2000 rpm. After centrifugation, serum was removed into a polypropylene tube and frozen to -20°C [66-68] or –80°C [69, 70] until analysis. The investigators were blinded to the whole process, including the identities, treatment allocation, and outcome. VEGF was detected using a commercially available ELISA kit (Table 1).

Five of them were about chemotherapy, others were on targeted therapy, traditional Chinese medicine therapy, immunotherapy, and diagnosis of NSCLC or SCLC. All of the five studies on chemotherapy were prospective and three studies with more than 50 cases were randomized [68, 71, 72]. All of the three studies had a similar result: the baseline VEGF level was associated with survival and two of them measured the VEGF level in duplicate. NVALT12 [71] was a multicenter, randomized, open-label parallel group phase II trial conducted by the Dutch Lung Physician Society (NVALT) and a total 223 patients were recruited in 17 centers across the Netherlands. Pretreatment, week 3 and week 6 serum levels of VEGF were assessed through ELSIA and they found that the baseline median serum VEGF (n = 178) was 111 pg/ml (interquartile range 55–218pg/ml) and equal in both arms. A higher baseline VEGF level was associated with poor PFS and OS, while VEGF became undetectable or very low at 3-week and 6-week in both groups. Another randomized study [68] enrolled 68 patients with histologically proven SCLC diagnosed and treated in the Helsinki University Central Hospital, Finland. They only detected the pretreatment VEGF level and their findings showed that pretreatment serum VEGF concentration of patients with SCLC ranged from 70 to 1738 pg/ml (mean, 527 pg/ml). Higher baseline serum VEGF was significantly associated with poor response to therapy (p =0.0083, CR +PR vs. NC+PD). Patients with lower serum VEGF level had longer survival than those with higher pretreatment value. The 1-year and 2-year survival rates of the patients with lower VEGF were 54% and 24% respectively, but patients with higher VEGF level were 33 and 7% respectively (p = 0.012). In the analysis, only pretreatment VEGF [relative risk (RR)=1.5; 95% CI 1.0–2.3; p=0.050] and clinical stage (RR=2.2; 95% CI 1.4–3.4; p=0.0006) had independent influence on survival through the multivariate analysis. Another randomized trial [72] recruiting 140 patients with NSCLC (stage IIIB /IV) and VEGF was measured in duplicate. The median baseline VEGF level was 502 pg/ml (range 55–3453 pg/ml). Baseline VEGF levels were strongly related to OS when dichotomized at the median (p= 0.008). Higher baseline VEGF levels as a continuous variable also significantly correlated to worse OS after log transformation. There was a decrease in VEGF level after treatment, compared

with pretreatment. However the reduction in VEGF from baseline did not significantly relate to OS (p = 0.730), failure-free survival (p = 0.722), or treatment response. During the rest of two non-randomized trials, one [66] with 28 patients, illustrated the baseline serum VEGF concentration was associated with the response to therapy. Another one [73] with 40 cases found that the baseline VEGF level was not any associated with response, moreover, there was no change during treatment. From the above data, we saw that the vast majority of (85.0%) cases revealed that the pretreatment serum VEGF level was associated with survival [68, 71, 72], and the baseline serum VEGF level also correlated with response to treatment [66, 68], while there was a decrease after therapy [68, 71, 72], only one study showed that there was not any correlation with response to treatment and no change during treatment.

In the other five studies, two of them [70, 74] showed that serum VEGF also played an important role in the diagnosis of NSCLC. Tamura's group [70] devised a prospective trial to enroll 78 patients with NSCLC in Japan who underwent surgery and measure their VEGF, VEGF-C, and matrix metalloproteinase-9 (MMP-9). They found that patients with lymph node metastasis had higher serum VEGF, VEGF-C, and plasma MMP-9 concentrations than those without metastasis (VEGF-C, P =0.0004; VEGF, P = 0.001). Serum VEGF, VEGF-C, and MMP-9 reached a sensitivity of 80%, 85%, and 63% respectively and a specificity of 59%, 68%, and 75% respectively, when a cutoff value was 316.8 pg/ml, 1762.0 pg/ml, and 51.4 ng/ml respectively. VEGF-C (AUC =0.761) had the biggest area under the curve in the ROC curve analysis, followed by MMP-9 (AUC =0.723) and VEGF (AUC =0.694). A combination assay of three markers had higher sensitivity and specificity (AUC =0.837) for prediction than single-marker assays. Their conclusion displayed that a combination assay of these three markers expression in circulation could assess lymph node metastasis in NSCLC patients with higher accuracy than single-marker assays. Another trial revealed that increased serum VEGF and 8-isoprostane levels related to advanced lung cancer and that increased TNF-α levels were observed in lung cancer patients, whereas increased VEGF levels were observed in advanced T-stage in exhaled breath condensate (EBC). Authors observed VEGF, TNF-α and 8-isoprostane levels in EBC and serum of patients with lung cancer [74]. Peng Zhao et al. [75] found overproduction of VEGF in tumor-bearing patients revealed a statistically positive correlation with IL-4 by detecting serum VEGF level before and after immunotherapy in patients with NSCLC. A control trial [67] enrolled 60 patients discovered that the serum VEGF levels decreased after traditional Chinese medicine treatment compared with pretreatment. Another study [69] (112 cases) found that the baseline serum VEGF level was associated with response to erlotinib-targeted therapy. From the data above, we saw VEGF also had an impact on lymph node metastasis diagnosis. The baseline VEGF level also decreased after traditional Chinese medicine treatment [67] and correlated with response to targeted therapy [69].

Evaluation of VEGF plasma level

Sixteen studies measured VEGF from plasma, which included nine studies on chemotherapy [76-84], six in the targeted therapy field [85-90], and one was on surgery [91].

A total of 3ml-5ml of peripheral vein blood was drawn into a citrated Vacutainer tube and was mixed immediately for measuring plasma VEGF level. Then, samples were centrifuged for 10 minutes at 4°C at 3000 rpm within 30 minutes. Plasma was removed into cryogenic storage tubes, stored immediately at -70°C to -80°C [77, 80-82, 87-91], and only one stored at -20°C [76]. In some multicenter trials, these specimens were shipped on dry ice to another center for analysis. Most of the studies detected the plasma VEGF by using commercially available enzyme-linked immunosorbent assay (ELISA) kits and two did not share details (Table 2).

In the sixteen prospective studies on detecting plasma VEGF level, six [78-80, 86, 89-90] of them were multicenter trials and seven [78, 80, 82, 84, 89-91] were randomized studies. The numbers of patient cases were from 10 to 878, and there were 6 studies that had more than 100 patients [78, 80, 82, 88, 90-91]. All of the three multicenter randomized studies [78, 80, 90] with more than 100 cases (from a total of 742 cases) showed that higher pretreatment plasma VEGF levels were associated with worse outcome, and that a Swedish double-blind study also displayed the result that higher baseline VEGF level correlated with the response to treatment [91]. Some studies demonstrated that the baseline plasma VEGF level did not predict survival [76-77, 79, 81-82, 88-89]. Other studies also supported that baseline plasma VEGF levels were associated with their response to treatment [76, 82-83, 87, 89-90], but just two trials [81, 86] revealed that the pretreatment VEGF levels did not show any correlation with response to therapy. Many researchers observed that there was a significant decrease after treatment than before [77, 79, 83, 87-88, 90-91], but one [90] showed that an increase of VEGF level was seen after treatment, while some trials suggested that no change was observed

during treatment [76, 81, 86]. In addition, some trials revealed that higher baseline levels of VCAM [81], VEGFR1 [77] and ICAM [82] before combination therapy were associated with worse survival. Mack's group [88] also described that osteopontin (OPN) plasma level had a significant association with patient OS and PFS, but not response to chemotherapy. As a result, all of the three multicenter randomized trials with more than 100 cases had the same finding, that lower pretreatment plasma level of VEGF gave a benefit to survival prognosis [78, 80, 90], moreover most trials agreed that the baseline plasma VEGF concentration decreased during treatment, and was also associated with the response to therapy, although there were a few trials with a different finding. Most studies [76-78, 81] that found the baseline VEGF level did not predict survival were nonrandomized and enrolled fewer cases in their trials, except two random ones [82, 89] and two with more than 100 cases [82, 88]. Their findings might be biased or be affected by fewer cases or being non-randomized. Overall, there was a trend that the baseline plasma VEGF level affected prognosis outcome and was associated with response to treatment, although there was not complete consensus. In future, researchers need to devise large-scale, multicenter, randomized trials under the same conditions to confirm the value of VEGF level. In another aspect, we found some potentially useful biomarkers from these studies and we suggest that researchers can conduct further research focusing on the VEGF-A, VEGFR-1, SP-1, ICAM, OPN and VCAM.

Assessment of VEGF concentration in malignant pleural effusion (MPE)

There were two studies on detecting VEGF level in MPE. One study [84] was a randomized prospective protocol, recruiting 72 advanced NSCLC patients with MPE. MPE was centrifuged for 10 minutes at 4,000 rpm at 4°C, then the supernatant was collected and assessed by ELISA using the VEGF-A ELISA kits (USCN), according to the manufacturer's instructions. Using a Microplate Reader (Bio-Rad, model 550) read the assay plates. Compared with the cisplatin-only group, VEGF levels in the MPE significantly decreased in the treatment group with bevacizumab and cisplatin before and after treatments ($p<0.01$). The VEGF-positive condition was confirmed when the pleural VEGF value was higher than the normal maximum value (300.6 pg/l). Bevacizumab was significant efficient in the treatment of study subjects with VEGF-positive ($p<0.01$); this suggested that bevacizumab was specific to patients with VEGF-positive but CEA was not a specific marker for this treatment. Another study was a multicenter trial [79] in Japan, enrolling 23 NSCLC patients with MPE. They measured the baseline VEGF level in MPE and assessed by ELISA using the Alpha LISA Human VEGF kit (PerkinElmer Japan). The median baseline VEGF value in MPE was 1798.6 (range 223.4–35,633.4) pg/ml, which was higher than the average VEGF level in plasma (513.6 ± 326.4 pg/ml). The mRNA expression of VEGF-A decreased sharply ($p<0.01$) in both group. However, the VEGF-A mRNA decrease was much greater in the combination therapy arm than in cisplatin monotherapy arm ($p<0.01$). The observation suggested that bevacizumab could reduce significantly the levels of VEGF-A mRNA. The former trial supported that the baseline VEGF-A level in MPE was associated with the response to therapy and the finding in latter one was similar, both with a significant decrease of VEGF circulating level after treatment($p<0.01$).

VEGF level in exhaled breath condensate (EBC)

A Greek study [74] recruited prospectively 30 lung cancer patients and 15 age and gender-matched healthy smokers as control group. The trial analyzed the levels of VEGF, TNF-γ, and 8-isoprostane in EBC and serum by an immunoenzymatic method (ELISA). VEGF levels were measured with commercially available ELISA kits (Table 3). All of the serum and EBC samples were obtained prior treatment. EBC was collected noninvasively into a condenser with nongaseous components of the expiratory air (Ecoscreen, Jaeger, Wurzburg, Germany). All of the EBC and blood sample were collected in the early morning (8:00-9:00 am.) in order to avoid any influences of the circadian rhythm on the measured biomarkers. After supervised abstinence from smoking for two hours, patients rinsed their mouth with distilled water. Then, they performed tidal breathing for 15 minutes through a mouthpiece and a two-way non-rebreathing valve which also served as a saliva trap wearing a nose clip. All patients were guided to swallow saliva. At least 1ml of EBC was collected and transferred to Eppendorf tubes and frozen immediately at −80°C. All EBC collections were executed according to the ERS/ATS Task Force on EBC [92]. The reproducibility of the detecting of VEGF, TNF-γ, and 8-isoprostane in EBC was tested on EBC samples obtained on two continuous days in a total of 12 people (8 patients with lung cancer and 4 controls).

VEGF levels of lung cancer patients in EBC did not differ significantly from control group. However, levels of VEGF were higher in patients with T3–T4 tumor stage than in those with T1–T2 ($p = 0.047$). A statistically significant association was found between VEGF levels in EBC and in serum ($r = 0.52$, $p = 0.019$).

In EBC, TNF-γ levels increased in patients with lung cancer, whereas VEGF levels increased in advanced T-stage. These results displayed that the VEGF level was less sensitive in EBC than in serum or plasma. Accordingly, it is not advised for patients with early-stage lung cancer to check VEGF level from EBC.

Detecting VEGF level in sputum

Rovina's group devised a prospective control study [93] with 76 patients with lung cancer in Greece. They induced and collected sputum from all patients in the early morning. Patients inhaled 3% saline for 15–20 minutes. Sputum was disposed according to a modification method described by Pin et al. [94]. The samples were shacked for 30 seconds and then lightly mixed in a bench rocker for 20 min. An equal to sputum volume of phosphated buffered saline (PBS)added and followed by vortex for 15 s and mixing at the bench rocker for another 5 min. Homogenized sputum was filtered through a 70 μm cell strainer and then centrifuged for 10 min at 1600 rpm at 20°C. The supernatant was collected and stored at -70°C. IL-18 and VEGF in sputum supernatants were detected using an ELISA kit (Table 3).

Table 2. The information of sixteen studies detecting VEGF level in plasma.

Ref/First Author	Ethnic	No. of cases	Character	Method (kits)	Kit's sensitivity	Biomarkers	Detecting time	Stage/ type	Treatment /diagnosis
[77] M. Shingyoji	Japanese	18	Protective	ELISA(R&D Systems Minneapolis, MN)	Lower Limited detection 15.6pg/ml (vegf-121,165)	VEGF	0, 2cycle	III-IV /NSCLC	Chemotherapy
[78] Rebecca S. Heista	Americans	36	Protective	ELISA(R&D Systems Minneapolis, MN)	-	VEGF, VEGFR-1, VEGF-C, PlGF	0, d7, d14, 3cycle, 5cycle	IIIB-IV /NSCLC	Chemotherapy
[79] Tony Mok	Asia Caucasian	303	Protective Multicenter Randomized	ELISA(R&D Systems Minneapolis, MN)	Microplate reader (Bio-Tek Elx 800)	VEGF,VEGFR-1,VEGFR-2,bFGF,ICAM,PlGF, E-selection	0, every 6week	IIIB-IV /NSCLC	Chemotherapy
[80] Motohiro Tamiya	Japanese	23	Protective Multicenter Non-Randomized	ELISA(-)	-	VEGF	0 (pleural effusion) 0, 3cycle (plasma)	NSCLC with MPE	Chemotherapy
[81] Emer O. Hanrahan	White (112) Black (3) Asia (2) Other(6)	123	Protective Multicenter Randomized	---	---	VEGF, VEGFR-2, MMP-9, ...	d-7, d8+-1, d22+-3, d43+-3	IIIB-IV /NSCLC	Chemotherapy
[82] Leora Horn	White (60) Non-white (3)	63	Protective	ELISA(R&D Systems Minneapolis, MN)	Lower Limited detection 6.4pg/ml	VEGF, VCAM, ICAM, bFGF, E-selection	0, 2cycle	Extensive /SCLC	Chemotherapy
[83] Afshin Dowlati	-	878	Protective Randomized	ELISA(R&D Systems Minneapolis, MN)	Lower Limited detection 12.5pg/ml	VEGF, ICAM, bFGF, E-selection	0(VEGF) 0, 7w(others)	IIIB-IV /NSCLC with MPE	Chemotherapy
[84] Hiroyasu Yasuda	Japanese	17	Protective	ELISA(R&D Systems Minneapolis, MN, UK)	-	VEGF,HIF-1α,P53	0, after treatment	IIIB-IV /NSCLC	Chemotherapy
[91] Sverre Sorenson	Swiss	316	Protective Multicenter Randomized	ELISA(R&D Systems Minneapolis, MN)	Lower Limited detection 9.0pg/ml	VEGF-165, VEGF-121	0, 6w, 12w, 20w	IIIB-IV /NSCLC	Chemotherapy
[86] Astrid A.M. Van der Veldt	Swiss	10	Protective	ELISA(R&D Systems Minneapolis, MN)	-	11C, 15O	3h, 4d	Advanced NSCLC	Chemotherapy
[87] Petros G. Nikolinakos	Irish Spanish American	33	Protective Multicenter Non-randomized	ELISA(R&D Systems Minneapolis, MN)	-	VEGF, VEGFR-2, PlGF	Before and after of operation	I-III /NSCLC	Chemotherapy
[88] Eric B. Haura	White (33) Hispanic (1)	34	Protective	ELISA(Bio source international, Inc (Camarillo, CA))	-	VEGF, bFGF, IL-8	0, d15, d29	III-IV /NSCLC	Targeted therapy
[89] Philip C. Mack	American	172	Protective	ELISA(R&D Systems Minneapolis, USA)	-	VEGF, OPN, PAI-1	Pretreatment	III-IV /NSCLC	Targeted therapy
[90] Katsuyuki Kiura	Japanese	53	Protective Multicenter randomized	ELISA(R&D Systems, Abingdon, UK)	-	VEGF, VEGFR-2, Tie-2	0, d29, d57	IIIB-IV /NSCLC	Targeted therapy
[85] Nan Du	Chinese	72	Protective Randomized	ELISA(USCN)	Microplate reader(Bio-Rad, model 550)	VEGF, CEA	0, 3cycle	III-IV /NSCLC	Chemotherapy
[92] Lianbin Zhang	Chinese	122	Protective Randomized	-	-	VEGF, IL-6, IGFBP-1	0, d1, d3, d5	I-II /NSCLC	operation

Table 3. The information of studies detecting VEGF level in sputum, MPE, and EBC.

Ref/First Authors	Ethnic	No. of cases	Character	Method (kits)	Kit's sensitivity	Biomarkers	Detecting time	Stage/ type	Treatment /diagnosis
[80]Tony Mok	Asia Causasian	303	Protective Multicenter Randomized	ELSIA(R&D Systems Minneapolis, MN)	Microplate reader (Bio-Tek Elx 800)	VEGF,VEGFR-1,VEGFR-2,bFGF,ICAM,PlGF, E-selection	0, every 6week	IIIB-IV /NSCLC	Chemotherapy
[85]Nan Du	Chinese	72	Protective Randomized	ELSIA(USCN)	Microplate reader(Bio-Rad, model 550)	VEGF, CEA	0, 3cycle	III-IV /NSCLC	Chemotherapy
[74]Eleftherios Dalaveris	Greek	30	Protective	ELSIA(R&D Systems Minneapolis, MN)	Lower Limited detection 0.9pg/ml	VEGF, TNF-γ, 8-ISO	0(Pretreatment)	NSCLC	diagnosis
[94]Nikoletta Rovina	Greek	76	Protective	ELSIA(R&D Systems Minneapolis, MN, USA)	Lower Limited detection 20.0pg/ml	VEGF, IL-18	0(Pretreatment)	NSCLC/ SCLC	diagnosis

Patients with NSCLC and SCLC had statistically higher levels of VEGF than healthy nonsmokers (p = 0.002 and 0.03, respectively), but did not significantly differ from healthy smokers. No statistical difference was observed in the levels of VEGF between NSCLC and SCLC patients. There was no difference in VEGF levels between different stages in NSCLC or SCLC patients. However, higher VEGF levels of baseline sputum were associated with worse survival (p = 0.034) in extensive stage SCLC. The expected mortality risk was 1.14 (95% CI 1.006–1.283) for every increase of 100 pg/ml in VEGF level. No association was observed between baseline sputum VEGF levels and OS in limited SCLC and in any stage of NSCLC. From this study we saw there was no difference between patients and healthy smokers, and different stage NSCLC or SCLC patients. But they found a correlation between the baseline sputum VEGF level and survival in extensive SCLC, not NSCLC, and limited SCLC. We need further research if we want to confirm the value of the sputum VEGF level, because there were only data reported from one trial. But the authors provided a research direction in evaluation of the VEGF value in future.

Advantages and disadvantages of these different methods

We compared the five different detecting methods of body fluid VEGF and found that there were some benefits and drawbacks from these methods. Firstly, to collect these samples, it was easier to draw from the peripheral vein than to drain from pleural effusion, and easier to induce sputum or to collect EBC. In addition, a simple method of obtaining samples, like drawing from a peripheral vein, needs only a short time to perform. But other methods, like draining pleural effusion, inducing sputum, or collecting EBC, need a longer time (Table 4). So, measuring the VEGF level in serum or plasma was relatively easier and saved time generally than the other methods, but the simplest method was to collect sputum when wanting to study advanced patients with expectoration symptoms. Second, almost all of the studies measured VEGF level using ELISA kits were from R&D Systems, Minneapolis, MN, and just a few trials used kits from other brands like American GB company (San Francisco) [67], Biosource International, Inc. (Camarillo, CA) [88], and R&D Systems, Minneapolis, USA [89, 93]. Different brands of kits and different catalog kits from the same company had different sensitivity (Table 4). Also, the lower limit for detecting of VEGF was from 0.9 pg/ml to 20 pg/ml, and the highest sensitivity was reported at 0.9 pg/ml, used by only one trial [74]. For the calculating method in studies, some used a microplate reader at 450 nm and the rest did not publish the details (Table 4). Finally, for the stability of the VEGF level, we did not find that there was a significant difference between serum and plasma, nor other body fluid. But Hyodo et al. [96] reported on the stability of VEGF levels in plasma, in contrast to its instability in serum. The levels of serum VEGF in drawn blood samples were also found to increase during clot formation, and this increase may be caused by the release from platelets. We also need to study further research and to confirm it. Briefly, we suggest that detecting VEGF levels in plasma was relatively simple, took a short time, and was an economical method using the high sensitivity kit, compared with the factors discussed above.

Table 4. The comparison of method of detecting VEGF level in different sample.

Sample	Simple/ complex	Invasive/ noninvasive	Prepared time before detecting	Number of studies
serum	simple	noninvasive	20-30 minutes	9
plasma	simple	noninvasive	20-30 minutes	16
sputum	complex	noninvasive	50-60 minutes	1
MPE	complex	invasive	50-60 minutes	2
EBC	complex	noninvasive	60-70minutes	1

Affecting factors of VEGF level

Study showed that there was not a significant association between the patients' age, sex, histological

type, disease stage, ECOG performance status, or serum CEA levels and VEGF levels at baseline (Spearman's rank correlation coefficient, −0.172, / P-value=0.5001) [76]. All of the studies did not stratify through these factors and just a few trials considered different disease stage and type. So, the effects of ethnic group, detecting time, disease stage, subtype, and other biomarkers measured were analyzed together in this review.

Ethnic group

There were nine of 29 trials on Asians with the VEGF level including four papers from China and five from Japan. Six [91, 75, 67, 79, 83-84] showed that the baseline VEGF level expressed had a significant decrease after treatment; of the other three, one [70] was about lymph node metastasis diagnosis, another one [89] showed an increasing trend of the VEGF level during treatment, and the last one [76] showed no change during therapy, while the VEGF baseline level was associated with response to therapy [76, 89]. In addition, the baseline level of VEGF does not predict the outcome of patients with lung cancer [76, 79]. Around 82.9% cases suggested that the pretreatment level of VEGF can predict patient response to treatment in Asians, and there was a change during therapy, but the baseline level of VEGF cannot provide a prognosis for patient survival.

Five trials [77, 87-88, 82, 80] enrolled Americans, but one of these was about diagnosis [72]. The majority of the American studies also revealed a decrease during the treatment [77, 87-88] and these pretreatment VEGF levels partly related to response to therapy [83, 88], but were not associated with patient outcome [76, 82, 88]. Only one study showed that the baseline level of VEGF correlated with outcome [80]. In another aspect, some studies showed that VEGFR-1, ICAM, and OPN were correlated with patient survival [76, 82, 88], so we suggest that further research on these biomarkers in other races should be conducted in future.

Eight studies were conducted using Europeans, including two about diagnosis and six on treatment. Dada showed that the baseline VEGF levels correlated with survival in four of six studies (cases account for 91.3%) [90, 71, 93, 68] and response to treatment during three of four articles (cases account for 92.6%) [90, 70, 68], and only one study showed that the pretreatment VEGF level was not associated with response to therapy [86]. In addition, two papers indicated that a decrease of the circulating VEGF level was seen during treatment [90, 71].

In summary, there was some distinction in different ethnic populations. The majority of Asian patients revealed a decrease of circulating VEGF level after treatment, and baseline VEGF level partly correlated with the response to treatment, but most of them were not associated with patient outcome. Similarly, the baseline circulating VEGF level also decreased after therapy in American studies and was also partly associated with response to treatment, but not correlated with patient survival. Compared with Asian and American research, most of the European studies showed that the baseline VEGF level predicted the outcomes of patients and was related to response to therapy. About half of the studies with Caucasian showed that the level of VEGF decreased after treatment. There was some distinction among different racial populations. From the above data, we could see that, to some extent, the circulating baseline level of VEGF can predict the response to treatment in Asian, American and European patients, and it was also associated with European patient survival. We will have a benefit trend to design some international multicenter (different races) trials to confirm the result under the same experimental conditions and also can devise some studies on VEGFR-1, VEGFR-2, ICAM, and OPN to find more prediction value markers in future.

Detecting time of VEGF level

We found six of 18 trials (detecting VEGF levels in before and after treatment) with no change of VEGF level during treatment, detected at 3-week, 6-week, 12-week and 1- or 2-chemotherapy cycle(s) respectively. Also, we reviewed ten trials with decreasing VEGF levels after treatment, measured at 3- or 5- cycle, 6- or 12-week, and 2 months after treatment. In addition, there were 2 studies involving baseline VEGF level increases after operation [91] and vandetanib treatment, d29 and d57 [89]. These results showed that the detection time of VEGF in most studies with the VEGF level unchanging was shorter than that of studies with VEGF levels decreasing generally, although the measuring time extended to 12-week or 2-chemotherapy cycle in a few studies. The detection time of VEGF level was more than 6- or 12-week, 3-cycle or longer among most studies with a VEGF level decrease. On the other hand, the time point of detecting VEGF level was another aspect. It was more possible to change detecting time before the next chemotherapy cycle rather than just finishing chemotherapy. Maybe there was a change process during different time points of treatment. In future, we need to design measurement at different time points as a control in the same trial.

As for the two studies [89, 91] with VEGF levels increasing after therapy, one [91] was about operating treatment of early stage patients and another [89] was about targeted therapy. The former hypothesized that

the reasons for VEGF level increase may be related to harmful patient body and immune system sensitivity after a traditional operation because it did not change after a minimally invasive thoracoscope operation. The latter was regarding a vandetanib phase II trial. A potential reason for the increase of VEGF level may be associated with bias or some other reason. It needs further research in future.

Lung cancer stage

There was no study observing the VEGF level in different cancer stage cases. Almost all trials were on advanced NSCLC, metastatic tumor, or relapse. A few studies were on early stage (I-II) NSCLC [91], stage I-III NSCLC [70, 75], stage I-IV NSCLC [71], NSCLC and SCLC [93]. None of them compared different stages among the patients. Some researchers did not [76] observe an association between the patient disease stage and VEGF levels at baseline (Spearman's rank correlation coefficient, −0.172, / P-value=0.5001). One result [74] showed that higher levels of VEGF were found in patients with T3–T4 tumor stage than in those with T1–T2 (9.3±2.8 pg/ml vs. 2.3±0.7 pg/ml, respectively; p = 0.047). It appeared that it was difficult to analyze stage effect on the VEGF level. Well-controlled study among patients at different disease stages may be able to detect an accurate relationship among them in future.

Subtype effect to VEGF level

Most of the studies were about advanced NSCLC, while just three of 29 trials were about SCLC, which enrolled American and European patients. They did not analyze the differences in VEGF levels between the different subtypes of NSCLC. The one American study [81] with 63 SCLC patients revealed that baseline level was not only associated with patient survival but also related to response to chemotherapy, and the level of VEGF had not changed during chemotherapy. In contrast, the Finland prospective study [68] showed that serum VEGF ranged from 70 to 1738 pg/ml (mean, 527 pg/ml), similar to NSCLC. They found that the baseline VEGF level was associated with response to treatment (p = 0.0083) and high (>527 pg/ml) serum VEGF also related to poor survival (p = 0.012, Log Rank Test), and all 3-year survivors had lower than mean pretreatment serum VEGF. The Greek study [93], enrolling both SCLC and NSCLC patients and detecting sputum VEGF, showed that the VEGF levels were both higher in NSCLC and SCLC compared with the control group, while higher baseline levels of VEGF in extensive SCLC were associated with worse survival similar to that in NSCLC. These data suggested that there was no substantial distinction between NSCLC and SCLC.

Detecting potential value of other biomarkers

Researchers found some other biomarkers had correlation with survival or response to treatment during these studies. We describe and discuss them in this review for further trials in future.

VEGFR-1

Four of 29 studies measured VEGFR-1 with VEGF together. One international multicenter trial [78] enrolling 303 patients found that both baseline and dynamic levels of VEGFR-1 were not associated with patient outcome. Another international multicenter study [96] using specific individual genotype sequencing assays (kinetic thermocycling polymerase chain reaction [PCR], Sanger sequencing and fragment analysis assays) to identify the single nucleotide polymorphisms (SNPs), also recruiting 303 patients, revealed that one variant in VEGFR-1 was associated with worse PFS/OS (not statistically significant after correction for multiple testing). The result showed that four genetic variants in VEGF-A and VEGFR-1 related to treatment outcome. Three VEGF-A variants correlated with best overall response (BOR), one variant in VEGFR-1 related to poor PFS/OS (not statistically significant after correction for multiple testing). In the other two studies, a Turkish study [73] illustrated that decrease of VEGFR-1 level persisted for at least 3 weeks after the chemotherapy initiation, and another study [77] showed that the baseline higher level of VEGFR-1 was associated with worse survival, although there was a transient decrease in plasma VEGFR-1 concentration at day 7. We could see that three of the four studies showed that the circulating level of VEGFR-1 decreased after therapy or correlated with patient outcome, and only one found that it was not associated with survival. Although further research studies on VEGFR-1 may be needed to confirm more accurate results, circulating level of VEGFR-1 is most likely important in the prediction of therapy or correlated with patient outcome.

VEGFR-2

There were four studies detecting VEGFR-2 level and three of them were multicenter trials. Two of the multicenter trials [86, 89] found that the plasma level of VEGFR-2 expressed a decreasing trend during treatment, and one [86] showed the baseline VEGFR-2 level correlated with the response to treatment, while the other did not [89]. Another multicenter trial [78] demonstrated that baseline level of VEGFR-2 was not significantly associated with patient survival and there was also no change during the treatment. One

American study [80] with 123 patients showed plasma VEGFR-2 level decreased after treatment but had no relation to outcome. From the above data, we could see that the majority of studies suggested there was a decrease after treatment, but the baseline level of VEGFR-2 was not associated with patient survival. Two multicenter trials found the baseline VEGFR-2 level correlated with response to therapy but the other two did not. We need further research to confirm whether or not a relation exists between the pretreatment level of VEGFR-2 and the response to treatment.

VEGF-C

Just two studies were about the detection of VEGF-C levels. One study [70] was the diagnosis of the circulating level of VEGF-C enrolled 78 patients with NSCLC. The findings showed that lymph node metastasis patients had higher serum VEGF, VEGF-C, and plasma MMP-9 concentrations than did those without metastasis. Combination assay of three markers had higher specificity and sensitivity for prediction (AUC =0.837) compared with single-marker assays. It suggested that VEGF-C played a more important role than VEGF in lymph node metastasis. Studies displayed that VEGF-C induced selective lymphangiogenesis without the accompanying angiogenesis [30], and it had a positive role in lymphatic invasion and metastasis and patient survival [31]. The other study [77] illustrated that the plasma VEGF-C level had a transient decrease during chemotherapy plus bevacizumab. Briefly, VEGF-C played a key role in the diagnosis of lymph node metastasis, especially with MMP-9 and VEGF together.

Osteopontin (OPN)

An American study (SWOG0003) [88], enrolling 172 patients with advanced NSCLC, found that baseline plasma level of OPN was significantly associated with response to treatment, PFS, and OS in NSCLC. Patients with lower OPN level had a significantly better OS and PFS than patients with higher level (HR= 0.60, P=0.002, HR= 0.69, P= 0.02, respectively). When examined as a continuous variable, OPN maintained its obvious correlation with PFS (HR= 1.05, P= 0.01) and OS (HR= 1.09, P= 0.0001). Moreover, patients with lower plasma OPN levels were also more likely to have tumor response (P=0.03). No differences were observed between treatment arms. But tumor OPN level did not correlate with patient outcome or with plasma level. It suggested that OPN was a potentially useful biomarker while assessing patient outcome. So the OPN level in plasma, not in tumor tissue, need to be studied in future.

IL-12

There were two studies about the circulating level of IL-12. Both of them found that the plasma IL-12 level decreased during treatment, and one [80] revealed that the baseline level was associated with PFS and the other one [86] suggested that the change level related to tumor shrinkage with an 81% accuracy rate. So, IL-12 also plays an important role in estimating the response to treatment and patient outcome.

In summary

Angiogenesis plays a key role in lung cancer, including tumor incidence, progression, and metastasis. Evidence displays that angiogenesis is a relatively early event in lung cancer pathogenesis. Uncontrolled angiogenesis result in tumor progression and metastasis. The different processes of angiogenesis are controlled by a large number of mediators. VEGF is one of the most specific and key regulators of angiogenesis, among many regulators [20, 21].

Most large-sample, multicenter, randomized control trials [68, 71, 72, 78, 80, 90] in these studies（1172 cases ）demonstrated a similar result, that higher baseline circulating VEGF level predicted a poor outcome, although some studies did not show any association between VEGF expression and survival（305 cases ）[81, 97, 76-77, 79, 89]. The result was not different from other studies showing that a worse prognostic significance of VEGF over-expression in tumor cells; however, VEGF was not an independent prognostic factor in multivariate analysis [59, 60, 64, 71]. By now, it has not reached a complete consensus in tumor. Therefore, we need to devise future studies to answer whether higher circulating VEGF levels predict worse outcomes or not.

Compared with detection from tumor tissue, detecting the circulating level of VEGF was noninvasive, simple, and cost-effective. Increasing studies showed that the circulating VEGF level was useful and stable during treatment and diagnosis, especially in plasma. Hyodo's group reported on the stability of VEGF level in plasma, in contrast to its instability in serum. The levels of serum VEGF in drawn blood specimens were also observed to increase during clot formation, and this increase may be caused by the release from platelets [95]. However, from the data reported, we found that the changes in VEGF serum levels were not more than that in the plasma concentrations of VEGF. The data showed that baseline serum level related to outcome or response to treatment was not more than the plasma level, compared with those articles measuring plasma

concentration of VEGF. Presently, more studies regarding plasma level of VEGF were more than those regarding serum trials that we engaged to analyze. To affirm the difference in stability between these two different circulating concentrations, additional studies to directly compare them is necessary in future.

Recent data suggested that a novel ELISA method that preferentially detects short VEGF-A isoforms, including VEGF-110 and VEGF-121, may have more promising predictive value [98]. One international multicenter randomized study [96] detected SNPs using this method. DNA analysis for 12 SNPs across three genes was reported: VEGF-A (five SNPs), VEGFR-1 (three SNPs), and VEGFR-2 (four SNPs). The results showed that four genetic variants in VEGF-A and VEGFR-1 related to treatment outcome. A Swedish study [90], recruiting 316 patients with NSCLC, also used this method to measure VEGF-165 and VEGF-121. These two isoforms basically represent the majority of VEGF structures. Results showed that the value at 6-week and 12-week was significantly lower than before treatment and lower baseline plasma level of VEGF predicted a benefit for survival. The result agreed with the above result, thus, consolidated our analysis.

From these studies we also found some potentially useful biomarkers like VEGFR-1, VEGF-C, OPN, and IL-12, and they may play a more important role during prediction of patient outcome or response to treatment. The importance of these is as follows:

VEGFR-1 played a crucial role in deciding patients survival; 2. VEGF-C had a significant usefulness in the diagnosis of lymph node metastasis, especially with MMP-9 and VEGF together; 3. OPN was a very useful biomarker, so we can study the OPN level further in plasma, but not in tissue, in future; 4.IL-12 also played an important role during estimating the response to treatment and patient outcome. So we suggest that researchers devise further studies to confirm the effect of OPN, VEGFR-1, and IL-12 in assessing lung cancer patient survival or response to therapy, and promote the role and understanding of VEGF in future. We also recommend the design of further studies to verify VEGF-C usefulness in the diagnosis of lymph node metastasis, especially with MMP-9 and VEGF together. As for VEGFR-2, a significant impact was not found in these studies. Accordingly, researchers ought to conduct further studies to confirm or find a new role for this biomarker in NSCLC patients.

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Competing Interests

The authors have declared that no competing interest exists.

References

1. Cancer Statistics in China. Ca Cancer J Clin. 2016; 66: 115-132.
2. Volm M, Koomägi R, Mattern J. Prognostic value of vascular endothelial growth factor and its receptor Flt-1 in squamous cell lung cancer. Int J Cancer. 1997; 74: 64–68.
3. Mattern J, Koomägi R, Volm M. Coexpression of VEGF and bFGF in human epidermoid lung carcinoma is associated with increased vessel density. Anticancer Res. 1997; 17: 2249–2252.
4. Imoto H, Osaki T, Taga S, et al. Vascular endothelial growth factor expression in non-small-cell lung cancer: prognostic significance in squamous cell carcinoma. J Thorac Cardiovasc Surg. 1998; 115:1007–1014.
5. O'Byrne KJ, Koukourakis MI, Giatromanolaki A, et al. Vascular endothelial growth factor, platelet-derived endothelial cell growth factor andangiogenesis in non-small-cell lung cancer. Br J Cancer. 2000; 82: 1427–1432.
6. Liao M, Wang H, Lin Z, et al. Vascular endothelial growth factor and other biological predictors related to the postoperative survival rate on non-small cell lung cancer. Lung Cancer. 2001; 33: 125–132.
7. Baillie R, Carlile J, Pendleton N, et al. Prognostic value of vascularity and vascular endothelial growth factor expression in non-small cell lung cancer. J Clin Pathol. 2001; 54: 116–120.
8. Delmotte P, Martin B, Paesmans M, et al. VEGF and survival of patients with lung cancer: a systematic literature review and meta-analysis. Rev Mal Respir. 2002; 19: 577–584.
9. Zhan P, Wang J, Lv XJ, et al. Prognostic value of vascular endothelial growth factor expression in patients with lung cancer: a systematic review with meta-analysis. J Thorac Oncol. 2009; 4: 1094–1103.
10. Risau W. Mechanisms of angiogenesis. Nature. 1997; 386: 671–674.
11. Otrock ZK, Makarem JA, Shamseddine AI. Vascular endothelial growth factor family of ligands and receptors: review. Blood Cells Mol Dis. 2007; 38: 258–268.
12. Pandya NM, Dhalla NS, Santani DD. Angiogenesis—a new target for future therapy. Vascul Pharmacol. 2006; 44: 265–274.
13. Folkman J, Shing Y. Angiogenesis. J Biol Chem. 1992; 267: 10931–10934.
14 Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med. 2000; 6: 389–395.
15. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996; 86: 353–364.
16. Faruk Tas, Derya D, Hilal OS, et al. EVect of maximum-tolerated doses and low-dose metronomic chemotherapy on serum vascular endothelial growth factor and thrombospondin-1 levels in patients with advanced non-small-cell lung cancer. Cancer Chemother Pharmacol. 2008; 61: 721–725.
17. Keith RL, Miller YE, Gemmill RM, et al. Angiogenic squamous dysplasia in bronchi of individuals at high risk for lung cancer. Clin Cancer Res. 2000; 6: 1616–1625.
18. Fontanini G, Calcinai A, Boldrini L. Modulation of neoangiogenesis in bronchial preneoplastic lesions. Oncol Rep. 1999; 6: 813–817.
19. Otrock ZK, Mahfouz RA, Makarem JA, et al. Understanding the biology of angiogenesis: review of the mostimportant molecular mechanisms. Blood Cells Mol Dis. 2007; 39: 212–220.
20. Ferrara N. VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer. 2002; 2: 795–803.
21. Poon RT, Fan ST, Wong J. Clinical implications of circulating angiogenic factors in cancer patients. J Clin Oncol. 2001; 19: 1207–1225.
22. Tischer E, Mitchell R, Hartman T, et al. The human gene for vascular endothelial growth factor: Multiple protein forms are encoded through alternative exon splicing. J Biol Chem. 1991; 266: 11947–11954.
23. Neufeld G, Cohen T, Gengrinovitch S, et al. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 1999; 13: 9–22.
24. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003; 9: 669–676.
25. Houck KA, Ferrara N, Winer J, et al. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol. 1991; 5: 1806–1814.
26. Suto K, Yamazaki Y, Morita T, et al. Crystal structures of novel vascular endothelial growth factors (VEGF) from snake venoms: insight into selective VEGF binding to kinase insert domain containing receptor but not to fms-like tyrosine kinase-1. J Biol Chem. 2005; 280: 2126–2131.
27. Senger DR, Galli SJ, Dvorak AM, et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of as cites fluid. Science. 1983; 219: 983–985.

28. Rini BI, Small EJ. Biology and clinical development of vascular endothelial growth factor-targeted therapy in renal cell carcinoma. J Clin Oncol. 2005; 23: 1028–1043.
29. Karkkainen MJ, Haiko P, Sainio K, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol. 2004; 5: 74–80.
30. Jeltsch M, Kaipainen A, Joukov V, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science. 1997; 276: 1423–1425.
31. Fujimoto J, Toyoki H, Sato E, et al. Clinical implication of expression of vascular endothelial growth factor-C in metastatic lymph nodes of uterine cervical cancers. Br J Cancer. 2004; 91: 466–469.
32. FarneboF, Piehl F, Lagercrantz J. Restricted expression pattern of vegfd in the adult and fetal mouse: high expression in the embryonic lung. Biochem Biophys Res Commun. 1999; 257: 891–894.
33. Stacker SA, Caesar C, Baldwin ME, et al. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med. 2001; 7: 186–191.
34. Akahane M, Akahane T, Matheny SL, et al. Vascular endothelial growth factor-D is a survival factor for human breast carcinoma cells. Int J Cancer. 2006; 118: 841–849.
35. Meyer M, Clauss M, Lepple-Wienhues A, et al. A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. EMBO J. 1999; 18: 363–374.
36. Lyttle DJ, Fraser KM, Fleming SB, et al. Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. J Virol. 1994; 68: 84–92.
37. Persico MG, Vincenti V, DiPalma T. Structure, expression and receptor-binding properties of placenta growth factor (PlGF). Curr Top Microbiol Immunol. 1999; 237: 31-40.
38. Wei SC, Tsao PN, Yu SC, et al. Placenta growth factor expression is correlated with survival of patients with colorectal cancer. Gut. 2005; 54: 666–672.
39. Dalal S, Berry AM, Cullinane CJ, et al. Vascular endothelial growth factor: a therapeutic target for tumors of the Ewing's sarcoma family. Clin Cancer Res. 2005; 11: 2364–2378.
40. Suto K, Yamazaki Y, Morita T, et al. Crystal structures of novel vascular endothelial growth factors (VEGF) from snake venoms: insight into selective VEGF binding to kinase insert domain-containing receptor but not to fms-like tyrosine kinase-1. J Biol Chem. 2005; 280: 2126–2131.
41. Lamszus K, Ulbricht U, Matschke J, et al. Levels of soluble vascular endothelial growth factor (VEGF) receptor 1 in astrocytic tumors and its relation to malignancy, vascularity, and VEGF-A. Clin Cancer Res. 2003; 9: 1399–1405.
42. Toi M, Bando H, Ogawa T, et al. Significance of vascular endothelial growth factor (VEGF) / soluble VEGF receptor-1 relationship in breast cancer. Int J Cancer. 2002; 98: 14–18.
43. Li J, Huang S, Armstrong EA, et al. Angiogenesis and radiation response modulation after vascular endothelial growth factor receptor-2 (VEGFR2) blockade. Int J Radiat Oncol Biol Phys. 2005; 62: 1477–1485.
44. Achen MG, Williams RA, Minekus MP, et al. Localization of vascular endothelial growth factor-D in malignant melanoma suggests a role in tumour angiogenesis. J Pathol. 2001; 193: 147–154.
45. Valtola R, Salven P, Heikkila P, et al. VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. Am J Pathol. 1999; 154: 1381–1390.
46. He Y, Rajantie I, Pajusola K, et al. Vascular endothelial cell growth factor receptor 3-mediated activation of lymphatic endothelium is crucial for tumor cell entry and spread via lymphatic vessels. Cancer Res. 2005; 65: 4739–4746.
47. Klagsbrun M, Takashima S, Mamluk R. The role of neuropilin in vascular and tumor biology. Adv Exp Med Biol. 2002; 515: 33–48.
48. Bielenberg DR, Pettaway CA, Takashima S, et al. Neuropilins in neoplasms: expression, regulation, and function. Exp Cell Res. 2006; 312: 584–593.
49. Miao HQ, Klagsbrun M. Neuropilin is a mediator of angiogenesis. Cancer Metastasis Rev. 2000; 19: 29–37.
50. Kitsukawa T, Shimono A, Kawakami A, et al. Ove-rexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. Development. 1995; 121: 4309–4318.
51. Soker S, Takashima S, Miao HQ, et al. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell. 1998; 92: 735–745.
52. Tordjman R, Ortega N, Coulombe IL, et al. Neuropilin-1 is expressed on bone marrow stromal cells: a novel interaction with hematopoietic cells. Blood. 1999; 94: 2301–2309.
53. Mattern J, Koomägi R, Volm M. Association of vascular endothelial growth factor expression with intratumoral microvessel density and tumour cell proliferation in human epidermoid lung carcinoma. Br J Cancer. 1996; 73: 931–934.
54. Volm M, Koomägi R, Mattern J. Prognostic value of vascular endothelial growth factor and its receptor Flt-1 in squamous cell lung cancer. Int J Cancer. 1997; 74: 64–68.
55. Mattern J, Koomägi R, Volm M. Coexpression of VEGF and bFGF in human epidermoid lung carcinoma is associated with increased vessel density. Anticancer Res. 1997; 17: 2249–2252.
56. Ohta Y, Endo Y, Tanaka M, et al. Significance of vascular endothelial growth factor messenger RNA expression in primary lung cancer. Clin Cancer Res. 1996; 2: 1411–1416.
57. Mountzios G, Dimopoulos MA, Soria JC, et al. Histopathologic and genetic alterations as predictors of response to treatment and survival in lung cancer: a review of published data. Crit Rev Oncol Hematol. 2010; 75: 94–109.
58. Ohta Y, Tanaka Y, Watanabe G, Minato H. Predicting recurrence following curative surgery in stage I non-small cell lung cancer patients using an angiogenesis-associated factor. J Exp Clin Cancer Res. 2007; 26: 301–305.
59. Giatromanolaki A, Koukourakis MI, Kakolyris S, et al. Vascular endothelial growth factor, wild-type p53 and angiogenesis in early operable non-small cell lung cancer. Clin Cancer Res. 1998; 4: 3017–3024.
60. Yuan A, Yu CJ, Luh KT, et al. Aberrant p53 expression correlates with expression of vascular endothelial growth factor mRNA and interleukin-8 mRNA and neoangiogenesis in non-small-cell lung cancer. J Clin Oncol. 2002; 20: 900–910.
61. Yuan A, Yu CJ, Shun CT, et al. Total cyclooxygenase-2 mRNA levels correlate with vascular endothelial growth factor mRNA levels, tumor angiogenesis and prognosis in non-small cell lung cancer patients. Int J Cancer. 2005; 115: 545–555.
62. Nakashima T, Huang CL, Liu D, et al. Expression of vascular endothelial growth factor-A and vascular endothelial growth factor-C as prognostic factors for non-small-cell lung cancer. Med Sci Monit. 2004; 10: 157–165.
63. Huang C, Liu D, Masuya D, et al. Clinical application of biological markers for treatments of resectable non-small-cell lung cancers. Br J Cancer. 2005; 92: 1231–1239.
64. Tanaka F, Ishikawa S, Yanagihara K, et al. Expression of angiopoietins and its clinical significance in nonsmall cell lung cancer. Cancer Res. 2002; 62: 7124–7129.
65. O'Byrne KJ, Koukourakis MI, Giatromanolaki A, et al. Vascular endothelial growth factor, platelet-derived endothelial cell growth factor and angiogenesis in non-small-cell lung cancer. Br J Cancer. 2000; 82: 1427–1432.
66. A. Camerini, C. Puccetti, S. Donati, et al. Metronomic oral vinorelbine as first-line treatment in elderly patients with advanced non-small-cell lung cancer: results of a phase II trial (MOVE trial). BMC Cancer. 2015; 15: 359-359.
67. Liu JB, Liu YQ. Influence of Erbanxiao solution on inhibiting angiogenesis in stasis toxin stagnation of non-small-cell lung cancer. J Tradit Chin Med. 2013; 33: 303-306.
68. Salven P, Ruotsalainen T, Mattson K, et al. High pre-treatment serum level of Vascular Endothelial Growth Factor (VEGF) is associated with poor outcome in small-cell-lung cancer. Int J Cancer. 1998; 79: 144–146.
69. Zhou SW, Ren SX, Yan LH, et al. Clinical efficacy of erlotinib in patients previously treated for advanced non-small-cell lung cancer. Respirology. 2009; 14: 709–715.
70. Tamura M, Oda M, Matsumoto I, et al. The combination assay with circulating Vascular Endothelial Growth Factor (VEGF)-C, Matrix Metalloproteinase-9, and VEGF for diagnosing lymph node metastasis in patients with non–small cell lung cancer. Annals of Surgical Oncology. 2004; 11: 928–933.
71. Dingemans AMC, Groen HJM, Herder GJM, et al. A randomized phase II study comparing paclitaxel-carboplatin–bevacizumab with or without nitroglycerin patches in patients with stage IV nonsquamous nonsmall-cell-lung cancer: NVALT12 (NCT01171170). Annals of Oncology. 2015; 26: 2286–2293.
72. Edelman MJ, Hodgson L, Wang XF, et al. Serum vascular endothelial growth factor and COX-2/5-LOX inhibition in advanced non-small cell lung cancer and leukemia group B 150304. J Thorac Oncol. 2011; 6: 1902–1906.
73. Tas F, Duranyildiz D, Soydinc HO, et al. EVect of maximum-tolerated doses and low-dose metronomic chemotherapy on serum vascular endothelial growth factor and thrombospondin-1 levels in patients with advanced non-small-cell lung cancer. Cancer Chemother Pharmacol. 2008; 61: 721–725.
74. Dalaveris E, Kerenidi T, Katsafli AK, et al. VEGF, TNF-γ and 8-isoprostane levels in exhaled breath condensate and serum of patients with lung cancer. Lung Cancer. 2009; 64: 219–225.
75. Zhao P, Bu XC, Wei XF, et al. Dendritic cell immunotherapy combined with cytokine-induced killer cells promotes skewing toward Th2 cytokine profile in patients with metastatic non-small cell lung cancer. International Immunopharmacology. 2015; 25: 450-456.
76. Shingyoji M, Ando S, Nishimura H, et al. VEGF in patients with non-small cell lung cancer during combination chemotherapy of carboplatin and paclitaxel. anticancer research. 2009; 29: 2635-2640.
77. Heista RS, Dudab DG, Sahani DV, et al. Improved tumor vascularization after anti-VEGF therapy with carboplatin and nab-paclitaxel associates with survival in lung cancer. PNAS. 2015; 112: 1547–1552.
78. Mok T, Gorbunova V, Juhasz E, et al. A Correlative biomarker analysis of the combination of bevacizumab and carboplatin-based chemotherapy for advanced nonsquamous nonive biomarker results of the phase II randomized ABIGAIL study (BO21015). J Thorac Oncol. 2014; 9: 848-855.
79. Tamiya M, Tamiya A, Yamadori T, et al. Phase 2 study of bevacizumab with carboplatin–paclitaxel for non-small cell lung cancer with malignant pleural effusion. Med Oncol. 2013; 30: 676.
80. Hanrahan EO, Lin HY, Kim ES, et al. Distinct patterns of cytokine and angiogenic factor modulation and markers of benefit for vandetanib and/or chemotherapy in patients with non–small-cell lung cancer. Journal of Clinical Oncology. 2010; 28: 193-201.
81. Horn L, Dahlberg SE, Sandler AB, et al. Phase II study of cisplatin plus etoposide and bevacizumab for previously untreated, extensive-stage small-cell lung cancer: Eastern Cooperative Oncology Group Study E3501. J Clin Oncol. 2009; 27: 6006-6011.

82. Dowlati A, Gray R, Sandler AB, et al. Cell adhesion molecules, vascular endothelial growth factor, and basic fibroblast growth factor in patients with non-small-cell lung cancer treated with chemotherapy with or without bevacizumab -- an Eastern Cooperative Oncology Group Study. Clin Cancer Res. 2008; 14: 1407-1412.
83. Yasuda H, Nakayama K, Watanabe M, et al. Nitroglycerin treatment may enhance chemosensitivity to docetaxel and carboplatin in patients with lung adenocarcinoma. Clin Cancer Res. 2006; 12: 6748-6757.
84. Du N, Li XS, Li F. Intrapleural combination therapy with bevacizumab and cisplatin for non-small cell lung cancer-mediated malignant pleural effusion. Oncology Reports. 2013; 29: 2332-2340.
85. Vander Veldt AAM, Lubberink M, Bahce I, et al. Rapid decrease in delivery of chemotherapy to tumors after anti-VEGF therapy: implications for scheduling of anti-angiogenic drugs. Cancer Cell. 2012; 21: 82–91.
86. Nikolinakos PG, Altorki N, Yankelevitz D, et al. Plasma cytokine and angiogenic factor profiling identifies markers associated with tumor shrinkage in early-stage non-small cell lung cancer patients treated with pazopanib. Cancer Res. 2010; 70: 2171–2179.
87. Haura EB, Tanvetyanon T, Chiappori A, et al. Phase I/II study of the Src inhibitor dasatinib in combination with erlotinib in advanced non–small-cell lung cancer. J Clin Oncol. 2010; 28: 1387-1394.
88. Mack PC, Redman MW, Chansky K, et al. Lower osteopontin plasma levels are associated with superior outcomes in advanced non–small-cell lung cancer patients receiving platinum-based chemotherapy: SWOG Study S0003. J Clin Oncol. 2008; 26: 4771-4776.
89. Kiura K, Nakagawa K, Shinkai T, et al. A randomized, double-blind, phase IIa dose-finding study of vandetanib (ZD6474) in Japanese patients with non-small cell lung cancer. J Thorac Oncol. 2008; 3: 386–393.
90. Sorenson S, Fohlin H, Lindgren A, et al. Predictive role of plasma vascular endothelial growth factor for the effect of celecoxib in advanced non-small cell lung cancer treated with chemotherapy. European Journal of Cancer. 2013; 49: 115–120.
91. Zhang LB, Wang B, Wang XY, et al. Influence of video-assisted thoracoscopic lobectomy on immunological functions in non-small cell lung cancer patients. Med Oncol. 2015; 32: 201.
92. Horvath I, Hunt J, Barnes PJ, et al. Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J. 2005; 26: 523-526:
93. Rovina N, Hillas G, Dima E, et al. VEGF and IL-18 in induced sputum of lung cancer patients. Cytokine. 2011; 54: 277-281.
94. Pin I, Gibson PG, Kolendowicz R, et al. Use of induced sputum cell counts to investigate airway inflammation in asthma. Thorax.1992; 47: 25-29.
95. Hyodo I, Doi T, Endo H, et al. Clinical significance of plasma vascular endothelial growth factor in gastrointestinal cancer. Eur J Cancer. 1998; 34: 2041-2045.
96. Pallauda C, Reckb M, Juhasz E, et al. Clinical genotyping and efficacy outcomes: Exploratory biomarkerdata from the phase II ABIGAIL study of first-line bevacizumab plus chemotherapy in non-squamous non-small-cell lung cancer. Lung Cancer. 2014; 86: 67–72.
97. Zhou SW, Ren SX, Yan LH, et al. Clinical efficacy of erlotinib in patients previously treated for advanced non-small cell lung cancer. Respirology. 2009; 14: 709–715.
98. Jayson GC, Haas S, Delmar P, et al. Evaluation of plasma VEGF-A as a potential predictive pantumour biomarker for bevacizumab. Eur J Cancer. 2011; 47: 96.