Immunohistochemical study of EGFR, HGF, c-MET, VEGF and CD34 in non small cell carcinomas of the lung

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Abstract

Aim: The aim of our study was the immunohistochemical investigation of the expression of Epidermal Growth Factor Receptor (EGFR), Hepatocyte Growth Factor (HGF), c-MET and Vascular Endothelial Growth Factor (VEGF) in non-small cell lung cancer (NSCLC) tissue samples.

Materials and Methods: Forty seven cases of NSCLC were selected from the archive material of the First Department of Pathology of National and Kapodistrian University of Athens. The immunohistochemical staining was performed using anti- EGFR/ HGF/c-MET/VEGF/ CD 34 antibodies. The immunoexpression was correlated with angiogenesis events, using the CD34 marker, and a qualitative and quantitative evaluation was performed. All data were statistically correlated with each other, so as with various clinicopathologic parameters too, in an attempt to draw conclusions about their prognostic significance.

Results: VEGF expression was found to be higher in squamous cell carcinomas and was negatively associated with the expression of CD34 and HGF. The last two were positively correlated with higher tumor grades and disease stage. CD34 and HGF expression was found to be higher in adenocarcinomas. Lastly, CD34 and HGF expression was found to be associated with lymph node infiltration.

Conclusions: The expression of the selected markers varies among different histological types of NSCLC.

Tumors with a positive expression of CD34 are more aggressive (high grade).

Tumors with positive expression of angiogenic factors are diagnosed at an advanced stage of the disease.

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Introduction

With more than 160,000 deaths worldwide annually, lung cancer is classified as the leading cause of death [1]. Non-small cell lung cancer (NSCLC) represents almost 75% of all lung cancer cases, with tobacco smoking as the main etiology factor in the Western world. The major representatives of NSCLC are adenocarcinoma, squamous cell carcinoma, giant/large cell carcinoma and adenosquamous carcinoma [2].

EGFR or ERBB 1 is the most important member of ERBB Family concerning lung cancer. The ERBB Family are transmembrane receptors of tyrosine kinase that act as positions - activators of significant pathways, and lead to millions of cellular response [3, 4].

HGF is a pluripotent growth factor, which derives from the mesenchyme. It is able to induce the mobilization and proliferation of epithelial cells, after binding with a transmembrane receptor that is a product of the c-MET proto-oncogene. The c-MET product is a protein which consists of an extracellular a-chain and a b-subunit. C-MET is activated by HGF, which induces phosphorylation at multiple sites [5, 6].

VEGF has a dominant role in physiological, pathological and therapeutic angiogenesis. It is a soluble dimeric glycoprotein weighing 34-45 KDa, which consists of five isoforms due to the splicing of eight exons. VEGF has a strong mitogenic action on endothelial cells which line the arteries, veins and lymphatic vessels [7].

CD34 concerns a human antigen that is expressed by the precursor forms of hematopoietic cells. It is a 110 KDa protein, which is expressed by the embryonic stem cells of the hematopoietic system, including lymphoid and myeloid cells, as well as by endothelia. It is used as an indicator of the vessels' density within a tumor, and as a direct indicator of the neoangiogenesis degree [8].

There are evidences that EGFR targeting induce resistant responses to chemotherapy schemes. These events are correlated with HGF/c-MET upregulation and overexpression of VEGF [9]. The selected markers were evaluated immunohistochemically, and we used CD34 to study the microvascular density (MVD) areas quantitavely.

Table 1. Clinicopathologic data of the study group of patients

Clinicopathologic data			%
Gender	Males	43	91.5
	Females	4	8.5
Age	73 years (SD ± 11.6)		
Histology	Adenocarcinoma	18	38.3
	Squamous cell carcinoma	23	49
	Adenosquamous carcinoma	3	6
	Giant/Large cell carcinoma	3	6
Differentiation	grade 1	2	4
	grade 2	14	30
	grade 3	31	66
TNM	Stage I	19	40
	Stage II	14	30
	Stage III	14	30
Lymph nodes	Lymph node (+)	11	23
	Lymph node (-)	36	77

Materials and methods

Forty seven cases of NSCLC were selected from the archive material of the First Department of Pathology of National and Kapodistrian University of Athens. The classification of tumor samples was made according to the WHO criteria and TNM classification system. The material consisted of 23 cases of squamous cell carcinoma, 18 cases of adenocarcinoma, 3 cases of adenosquamous carcinoma and 3 cases of giant-large cell carcinoma. None of our patients had undergone any chemotherapy or radiotherapy before the surgical resection of the tumors, in order to prevent tissue artifacts or possible immunostaining alterations. The main clinicopathologic data of our patients are presented in Table 1.

Immunohistochemistry: All 47 cases were analyzed immunohistochemically using sections at 4μm thick and stained with the indirect method of biotin-streptavidin-peroxidase with the use of DAB as chromogenic. We used the following antibodies: anti c-MET: Invitrogen Rabbit antibody (United Kingdom), anti-HGF: Becton Dickinson (Switzerland), anti-EGFR: Dako M 3563 clone H11 Mouse (Denmark), anti-VEGF: Becton Dickinson (Switzerland) and anti-CD34: Dako clone QBEND 10 M 7165(Denmark) - (dilution 1:100). Some indicative images of the immunohistochemical staining are listed (Figure 1).

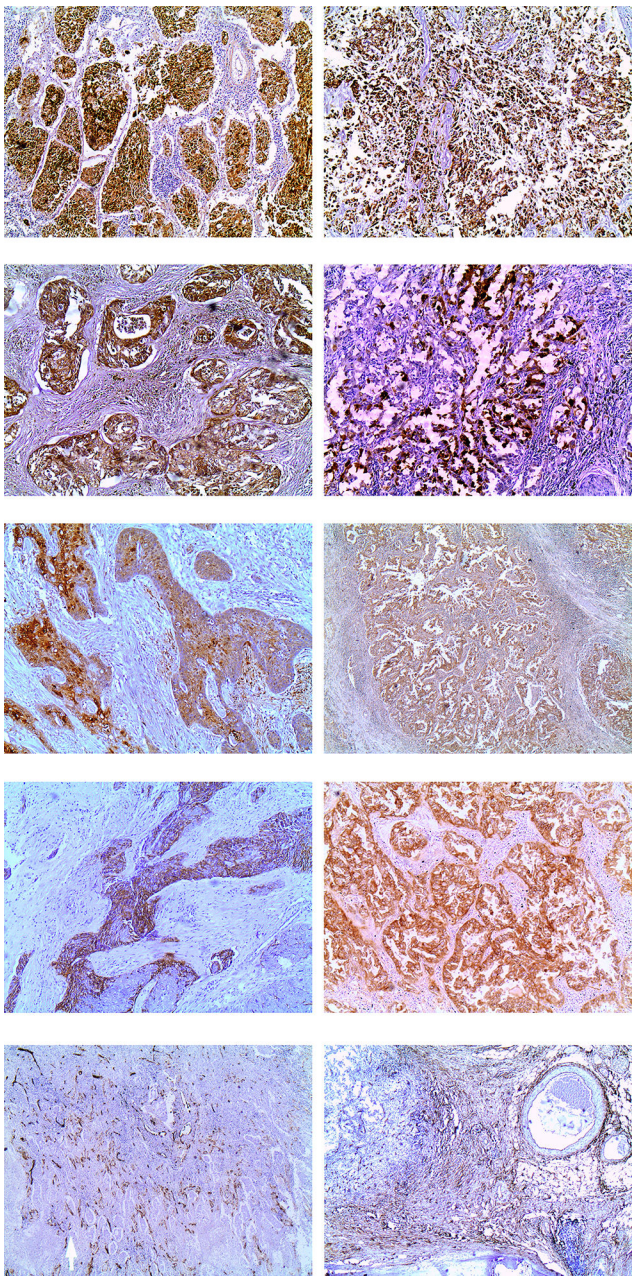


Figure 1 Top to bottom, left to right (a-j):

a. squamous carcinoma HGF(+), b. adenocarcinoma HGF(+), c. squamous carcinoma c-MET(+), d. adenocarcinoma c-MET(+), e. squamous carcinoma VEGF(+), f. adenocarcinoma VEGF(+), g. squamous carcinoma EGFR(+), h. adenocarcinoma EGFR(+), i. squamous carcinoma CD34(+), j. adenocarcinoma CD34 (+)

Quantitative estimation of immunohistochemical stains: All immunohistochemical markers were estimated quantitatively using the Image Analyzing System. Sigma Scan Pro Software (Jandel Scientific, Erkrach Germany) and Color Estimator Software were used. The initial estimation of the tumor vascularity was made by searching highly vascularized areas i.e. hot spots. Each section was studied field by field at x 100 magnification to identify highly vascularized areas (MicroVascularDensity i.e. MVD), as it was revealed through the immunostaining of CD34 antigen. The selection of hot spots was also conducted by two pathologists (NK and AL) at x 200 magnification choosing areas situated within tumor. Visibly stained micro-vessel endothelial cells, clearly located apart from the adjacent cells were considered as individually evaluable micro-vascular structures, regardless the presence of lumen. Regarding the rest of immunohistochemical markers (VEGF, c-MET, HGF, EGFR) Color Estimator Image analyzing software was used. Ten optical fields at a magnification x 200 were selected for each section and a semi-automated measurement of positive stained nuclei or tumor cells (expressed in %) was made out of a total of 500 at least tumor cells or nuclei (stained or not).

Statistical analysis: All data analysis was conducted with IBM SPSS Statistics 21 software. The level of statistical significance was set at 5% ($p \leq 0.05$). The comparison between histological type, degree of differentiation and stage of the disease was made with X2 Pearson's control. The different percentage of immunoexpression among the tested antibodies was calculated with Pearson's r coefficient. The Student's t-test was used to measure various independent variables like histological type, grade and lymph node metastasis. The expression of the markers selected and the stage of the disease was analyzed with ANOVA method, one way analysis of variance.

Results

The majority of the total 47 cases were males (43/91.5%). The average age of males was 71.3 years and of females 65.8 years. The correlation between the histological type, differentiation grade and stage of the disease is presented in Table 2. A statistically significant correlation between histological type, differentiation grade and stage was observed ($p = 0.007$).

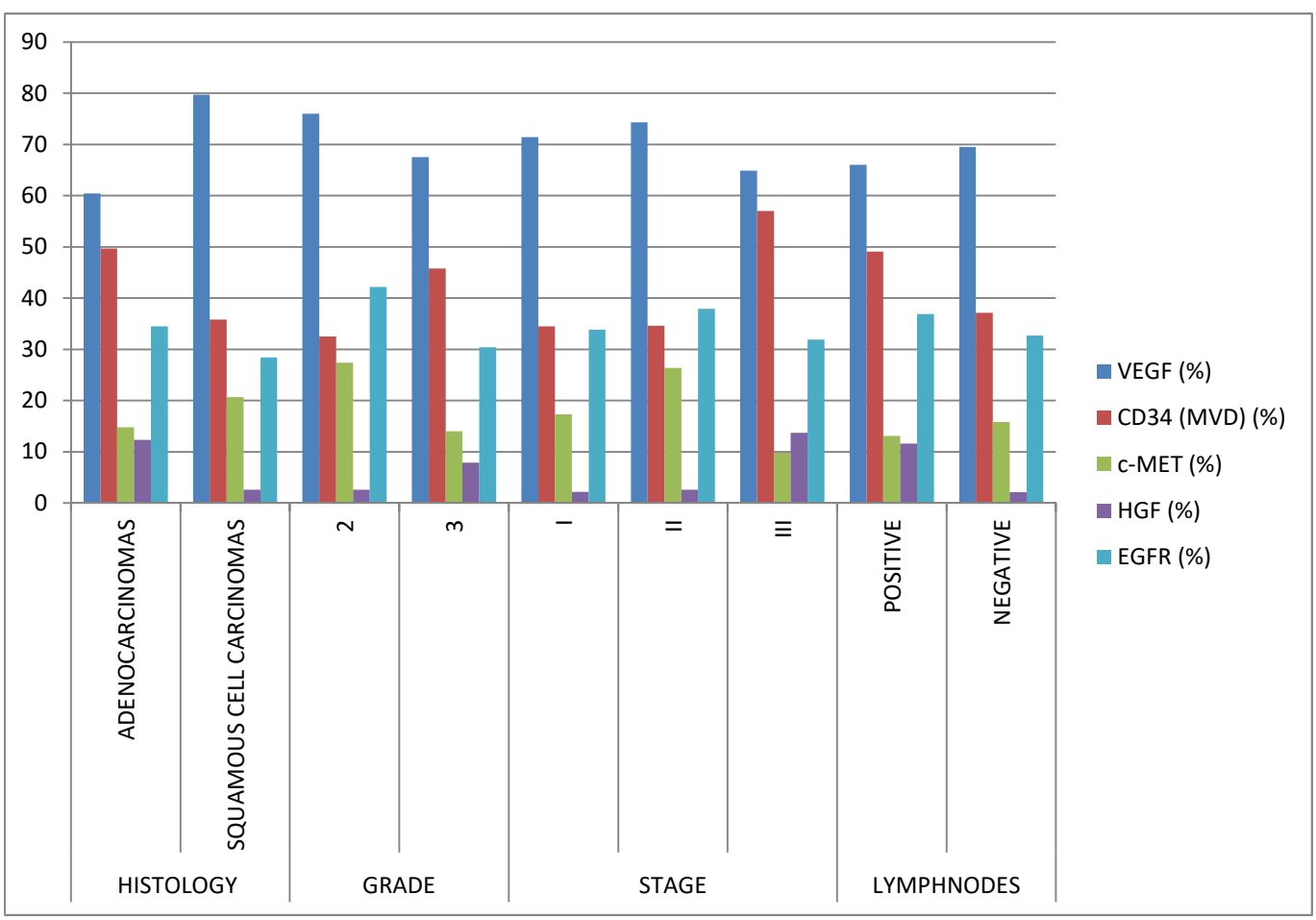
Table 2. Correlation of the histological type with the differentiation grade and the stage

		Histology					
		Adenocarcinoma	Squamous cell carcinoma	Adenosquamous carcinoma	Giant/Large cell carcinoma	χ^2	p-value
Grade	1	2 (11.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	17.717	0.007
	2	1 (5.6%)	10 (43.5%)	3 (100%)	0 (0.0%)		
	3	15 (83.3%)	13 (56.5%)	0 (0.0%)	3 (100%)		
Stage	I	3 (16.7%)	14 (60.9%)	2 (66.7%)	0 (0.0%)	30.924	<0.0001
	II	2 (11.1%)	8 (34.8%)	0 (0.0%)	3 (100%)		
	III	13 (72.2%)	1 (4.3%)	1 (33.3%)	0 (0.0%)		

Our findings according to the immunostaining of the markers were analyzed using Pearson's correlation (r) coefficient. Positive expression rates of VEGF is associated with negative rates of CD34 (MVD) and HGF (r = -0.507/p = 0.003 and r = -0,516/p = 0.003 respectively). That means that as VEGF expression increases, CD34 and HGF expression tends to decrease. Furthermore, CD34 (MVD) is positively associated with HGF (r = 0.472/p = 0.001), and HGF is negatively associated with the expression of EGFR (r = -0.414/p = 0.006).

The study of the immunoexpression of the selected markers and their correlation with various clinicopathologic parameters is illustrated on Table 3. VEGF expression is positively associated with the histological type (p = 0.033). In particular, squamous cell carcinomas present a higher percentage of VEGF positive nuclei compared to adenocarcinomas (79.7 ± 4.4 and 60.4 ± 8.12 respectively). Also, the histological type is significantly correlated with the expression of CD34 (MVD) (p = 0.015), with adenocarcinomas showing a higher rate (49.7 ± 4.4) compared to squamous cell carcinomas (35.8 ± 3.1). The HGF marker expression is positively correlated with histological type, too (p = 0.047); adenocarcinomas show a higher average rate (12.33 ± 4.7) compared to squamous cell carcinomas (2.6 ± 1.8). C-MET and EGFR expression didn't show significant correlation with the histological type (p = 0.45/0.5 respectively).

Table 3. Immunoexpression rates in relation with the parameters studied



Concerning to the relationship between immunohistochemical indicators and the infiltration of lymph nodes, our research showed a positive statistical correlation between lymph node infiltration and CD34 expression (MVD) ($p = 0.04$), with the cases with infiltrated lymph nodes having a higher average rate compared to those that have no infiltration (49.05 ± 4.21 and 37.05 ± 3.81 respectively). Similarly, the expression of HGF ($p = 0.05$) is related to the infiltration of lymph nodes, with the cases with infiltrated lymph nodes having a higher average rate in relation to those that do not (11.55 ± 4.34 and 2.09 ± 1.43 respectively).

Regarding to the differentiation grade and the expression rate of the antibodies, CD34 (MVD) expression was positively correlated ($p = 0.04$) to grade, with grade 3 tumors having a higher rate compared to grade 2 cases (45.8 ± 3.7 and 32.5 ± 4.2 respectively). Finally, concerning the stage of the disease, a positive correlation between CD34 and HGF expression was found ($p = 0.001$ and $p = 0.041$ respectively). CD34 was expressed at higher rates at stage III compared to stages I and II.

Discussion

The importance of angiogenesis in the neoplastic manifestation of malignant neoplasms' aggressive behavior clearly shows the need for its qualitative and quantitative valuation [8, 10-13]. As in our Laboratory, accordingly to others too, our results were obtained through immunohistochemical study, rather than a molecular one. Even though the molecular techniques can detect mutations, i.e. the chargeable event of oncogenesis [14], the immunohistochemical study often complements them, because it focuses on a protein's expression, which constitutes a consequence of the mutation and simultaneously a proof of malfunction, since a lot of mutations are 'silent' and don't affect the cell's phenotype [15].

According to the literature, the multivariate analysis of Tanaka et al. [10] about NSCLC showed a significant correlation between VEGF's over-expression ($p = 0.00029$) and a high density of vessels (Microvascular Density MVD), evaluated with the expression of CD34 ($p = 0.0081$), CD105 ($p = 0.0261$) and vessels' infiltration ($p = 0.0245$). The same analysis concludes that the presence of two risk factors (CD34 and infiltration of vessels) is indicative of a poor prognosis for NSCLC. The above are also confirmed by the study of Mineo et al. [11] that attempted to identify the most promising indicators of neo-angiogenesis. Interestingly, CD105 was proved to be an important indicator of neo-angiogenesis; however it has been excluded from the multivariate analysis. This contradicts the study of Tanaka et al., who concluded that CD105 is the best indicator of angiogenesis and a predictor of survival superior than CD34. This contradiction is explained by the fact that CD34 and vascular infiltration are directly related to the metastatic process than the neo-angiogenesis itself. Recently, Yano et al. [16] reported a high correlation between distant metastases and poor prognosis, with MVD evaluated by the CD34 expression and the degree of vessels' infiltration.

In 2007, Inda et al. [17] studied angiogenesis in poorly differentiated NSCLC of 72 patients, by using immunohistochemical anti VEGF and anti CD34 indicators. In adenocarcinomas VEGF is strongly expressed, compared to squamous cell carcinomas ($p = < 0.001$), while MVD estimated with CD34 showed no differences between tumors. These observations are consistent with Ghio et al. [18], but not with Nakashima et al. [19], who argue that VEGF is one of the most important prognostic factors in squamous cell carcinomas of the lung.

Zhang et al. [20], in a study of 113 patients, concluded that the expression of HGF-a, c-MET and VEGF-C was higher in tumor tissue compared to the healthy one ($p < 0.001$) HGF-a: (67.3 vs. 20.4%) c-MET: (74.3% vs. 23.0%) and VEGF-C: (65.5 vs. 23.9%). A strong correlation of the expression of HGF with VEGF-C ($r = 0.234$, $p = < 0.012$) and c-MET ($r = 0.648$, $p = < 0.001$) was also found.

Regarding to the relation between the expression of markers, grade and stage of the disease, we showed a significant positive correlation between CD34 (MVD) and grade ($p = 0.04$), as well as a strong positive correlation between CD34/HGF expression and the stage of the disease ($p = 0.001$ and 0.041 respectively). There are few reports in literature that correlate the expression of immunohistochemical markers with the grade and the stage. Indicatively, we mention the study of Ding et al. [12], which shows a positive correlation of VEGF with the grade and the stage, and the study of Bing et al. [13], which shows a positive correlation of CD34 (MVD) with the stage of the disease ($p < 0.05$).

Conclusions

Our study confirms that the expression of the selected markers varies among different histological types of NSCLC. Particularly, VEGF tends to have a higher expression in squamous cell carcinomas, where as CD34 and HGF tend to have a higher expression in adenocarcinomas.

We also confirm that tumors with a positive expression of CD34, and thus a greater ability of angiogenesis are more aggressive (high grade).

Furthermore, CD34 and HGF expression was found to be associated with lymph node infiltration. Tumors with positive expression of angiogenic factors (CD34 and HGF) are diagnosed at an advanced stage of the disease, and therefore have a poor prognosis, as it has been expected.

Unfortunately, there are no statistically significant results, in order to reach a meaningful conclusion on distant metastases.

References

1. Dela Cruz CS, Tanoue LT, Matthau RA. Lung cancer: epidemiology, etiology and prevention. Clin Chest Med 2011; 32(4): 605-644.

2. Travis WD, Brambilla E, Burke AP, Marx A, Nicholson AG eds., WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart, 4th Edition, WHO Press; 2015

3. Gullick WJ. Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. Br Med Bull 1991; 47(1): 87-98.

4. Lin SY, Makino K, Xia W, Matin A, Wen Y, Kwong KY, et al. Nuclear localization of EGF receptor and its potential new role as a transcription factor. Nat Cell Biol 2001; 3(9): 802-808.

5. Naldini L, Vigna E, Narsimhan RP, Gaudino G, Zarnegar R, Michalopoulos GK, et al. Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET. Oncogene 1991; 6(4): 501-504.

6. Watermann I, Schmitt B, Stellmacher F, Müller J, Gaber R, Kugler Ch, et al. Improved diagnostics targeting c-MET in non-small cell lung cancer: expression, amplification and activation? Diagn Pathol 2015; 10: 130.

7. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997; 18(1): 4-25.

8. Vermeulen PB, Gasparini G, Fox SB, Toi M, Martin L, McCulloch P, et al. Quantification of angiogenesis in solid human tumors: an international consensus on the methodology and criteria of evaluation. Eur J Cancer 1996; 32A(14): 2474-2484.

9. Nakade J, Takeuchi S, Nakagawa T, Ishikawa D, Sano T, Nanjo S, et al. Triple inhibition of EGFR, Met, and VEGF suppresses regrowth of HGF-triggered, erlotinib-resistant lung cancer harboring an EGFR mutation. J Thorac Oncol 2014; 9(6): 775-783.

10. Tanaka F, Otake Y, Yanagihara K, Kawano Y, Miyahara R, Li M, et al. Evaluation of angiogenesis in non-small cell lung cancer: comparison between anti-CD34 antibody and anti-CD105 antibody. Clin Cancer Res 2001; 7(11): 3410-3415.

11. Mineo TC, Ambrogi V, Baldi A, Rabitti C, Bollero P, Vincenzi B, et al. Prognostic impact of VEGF, CD31, CD34, and CD105 expression and tumour vessel invasion after radical surgery for IB–IIA non-small cell lung cancer. J Clin Pathol 2004; 57(6): 591-597.

12. Ding M, Liu L, Hu C, Liu Y, Qiao Y, Jiang X. Expression of VEGFR2 and NRP-1 in non-small cell lung cancer and their clinical significance. Chin J Cancer Res 2014; 26(6): 669–677.

13. Bing Z, Jian-ru Y, Yao-quan J, Shi-feng C. Evaluation of angiogenesis in non-small cell lung carcinoma by CD34 immunohistochemistry. Cell Biochem Biophys 2014; 70(1): 327-331.

14. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994; 54(18): 4855-4878.

15. Tsao MS, Aviel-Ronen S, Ding K, Lau D, Liu N, Sakurada A, et al. Prognostic and predictive importance of p53 and RAS for adjuvant chemotherapy in non-small-cell cancer. J Clin Oncol 2007; 25(33): 5240-5247.

16. Yano T, Tanikawa S, Fujie T, Masutani M, Horie T. Vascular endothelial growth factor expression and neovascularisation in non-small cell lung cancer. Eur J Cancer 2000; 36(5): 601-609.

17. Inda AM, Andrini LB, Garcia MN, Garcia AL, Fernandez A, Furnus CC, et al. Evaluation of angiogenesis with the expression of VEGF and CD34 in human non-small cell lung cancer. J Exp Clin Cancer Res 2007; 26(3): 375-378.

18. Ghio P, Cappia S, Selvaggi G, Novello S, Lausi P, Zecchina G, et al. Prognostic role of protease-activated receptors 1 and 4 in resected stage IB non-small-cell lung cancer. Clin Lung Cancer 2006; 7(6): 395-400.

19. Nakashima T, Huang CL, Liu D, Kameyama K, Masuya D, Ueno M, 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(6): BR157-165.

20. Zhang N, Xie F, Gao W, Yu S, Qiu L, Lin W, et al. Expression of hepatocyte growth factor and c-Met in non-small-cell lung cancer and association with lymphangiogenesis. Mol Med Rep 2015; 11(4): 2797-2804.