Role of circulating cytokeratin fragments and angiogenic factors in NSCLC patients stage IIIa–IIIb receiving curatively intended treatment

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Non-small cell lung cancer (NSCLC) is derived from epithelial cells and accounts for approximately 80% of all lungcancers. Cytokeratins are specific for epithelial cells and during malignant transformation the cytokeratin profile usually remains constant. Angiogenesis is the formation of new blood vessels out of the existing vascular bed. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are potent circulating angiogenic factors. The aim of the present study was to determine if increased levels of a new cytokeratin assay (MonoTotal, which in comparison with TPAcyk detects not only fragments of cytokeratins 8 and 18 but also of cytokeratin 19) is correlated with circulating angiogenic factors (VEGF and bFGF) and the secondary aim was to investigate if increased levels of these circulating markers are associated with survival.

In the present study, a total of 45 NSCLC patients (26 patients stage IIIa and 19 patients stage IIIb) receiving only curatively intended treatment for advanced NSCLC were included. These patients donated a total of 291 serum samples during follow-up which was investigated for the presence of MonoTotal, VEGF and bFGF. MonoTotal was statistically significantly correlated with bFGF (R=0.26, p=0.00049) and VEGF (R=0.26, p=0.00007). From the time of histological diagnosis until time of death, MonoTotal increased by 603 U/l (p<0.0001). VEGF increased by 430 pg/ml (p=0.0004) whereas the corresponding value for bFGF was 5.93 pg/ml (p=0.018).

MonoTotal, a newly developed commercial cytokeratin assay, seems to be a potentially very interesting serum marker that, in conjunction with other clinical data, might be used for monitoring of patients with NSCLC.

Key words: non-small cell lung cancer, survival, cytokeratins 8, 18, 19, angiogenesis, VEGF, bFGF, MonoTotal

Non-small cell lung cancer is derived from epithelial cells and accounts for approximately 80% of all lung cancers [1]. Cytokeratins are intermediate filament proteins specific for epithelial cells and cells of epithelial origin. The expression profile of cytokeratins is stable and usually remains constant even during malignant transformation [2]. As a consequence, this biological feature is utilized in routine pathology, where cytokeratin antibodies are used in immunohistochemistry to separate lung carcinomas from metastatic carcinomas to the lung [3, 4]. Specific plasma or serum cytokeratin assays are routinely used as prognostic and monitoring markers in several types of malignancies [5–7].

Cytokines and growth factors that mediate biological activities are essential for growth and maintenance of biology of the organism. Vascular endothelial growth factor (VEGF) is expressed in tumor cells, T-cells and macrophages, as well as in astrocytes and keratinocytes [8]. VEGF regulates both vascular permeability and proliferation, and is believed to interfere with apoptosis [9].

The fibroblast growth factor family (FGF) includes several members that are of importance for growth and differentiation of cells of mesodermal and neuroectodermal origin [10]. The FGFs have been shown to be mitogenic and are involved in inflammatory processes, haematopoiesis, wound healing and angiogenesis.

During malignant progression, tumors are known to release cytokines that facilitate growth and development of metastasis [11], as well as stimulating further neovascu-

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larization of the tumor. Since NSCLC tumors are of epithelial origin, this process theoretically would result in increasing amounts of detectable cytokeratin fragments and other proteolytic material in the blood circulation, thereby providing an interesting early diagnostic tool during malignant progression of NSCLC. In the present study, a new cytokeratin-based tumor marker utilizing a combination of three monoclonal antibodies directed against soluble fragments of cytokeratin 8, 18 and 19, respectively, has been evaluated in conjunction with the angiogenic cytokines VEGF and bFGF in stage IIIa–IIIb NSCLC patients.

Patients and methods

Patients. The study was approved by the Regional Ethics Committee of Uppsala University. Serum samples were collected from patients receiving treatment for NSCLC stages IIIa and IIIb, at the Department of Oncology, Uppsala University Hospital, Uppsala, Sweden. Blood samples were collected before initiation of treatment, during treatment and until death. The blood samples were collected in 7 ml serum tubes without additives (Becton Dickinson, Rutherford, NJ), and the samples were stored at –70 °C until analyzed.

In the present study, a total of 45 NSCLC patients (26 patients stage IIIa and 19 patients stage IIIb) receiving only curatively intended treatment for advanced NSCLC are included. Treatment of patients with stage IIIa included solely curatively intended radiation treatment (>50 Gy) in 15 patients, 5 patients received Cisplatin + Vepesid prior to curatively intended radiation treatment, whereas 2 patients received mitomycin C/ifosfamide/cisplatin regime prior to initiation of curatively intended radiation treatment. Concomitant radiation treatment implying Bleomycin concomitant with curatively intended radiation treatment was delivered in 3 patients, whereas one patient received 5-Fu concomitant with curatively intended radiation treatment.

Tumor burden was re-evaluated in those patients in whom the chest x-rays were located. A total of 23 patients tumor burden was re-evaluated using the response evaluation criteria in solid tumors (RECIST) [12] and this was performed by two independent clinicians.

MonoTotal. MonoTotal (IDL Biotech, Bromma, Sweden) utilizes three monoclonal catcher antibodies 6D7, 3F3 and IDLC4. These antibodies have specificity for cytokeratin 8, 18 and 19, respectively. 6D7 is specific for an epitope on cytokeratin 8 located at amino acid residues 340-365. The antibody 3F3 is specific for an epitope on cytokeratin 18, located at amino acid residues 270-429, and IDLC4 is specific for an epitope on cytokeratin 19 located in the region of amino acid residues 340-370 [13]. MonoTotal is based on an immunoradiometric standard principle, where the sample initially is incubated with a monoclonal antibody-coated plastic bead (containing the three monoclonal catcher antibodies described above) simultaneously with ^{125}I-labeled polyclonal antibody (tracer). After washing, the bound radioactivity is measured using a gamma counter. MonoTotal is calculated and expressed as Units per litre (U/l) of serum.

To investigate the 95% lognormal distribution of MonoTotal, blood samples from 300 healthy blood donor individuals were analyzed. The 95th lognormal distribution values of this cytokeratin 8, 18 and 19 assay from the healthy blood donors were 75 U/l.

Analyses of VEGF and bFGF. Serum levels of VEGF and bFGF were measured using commercially available enzyme-linked immunosorbent assays (R&D Systems Inc., Minneapolis, MN, USA). The VEGF immunoassay was designed to measure $VEGF_{165}$ levels. Briefly, an immobilized murine monoclonal antibody specific for either bFGF or VEGF was coated to microtiter plates. Standards and samples were pipetted into the wells. After incubation and washing, the addition of a second alkaline phosphatase-conjugated polyclonal antibody enabled quantification of bound bFGF or VEGF by measurements of absorbance at 450 nm in a Titertek Multiscan. Calculation of results was performed according to the manufacturer's recommendations. Elevated bFGF and VEGF levels were defined as being greater than the 95th percentile value in a normal control subject group described by the manufacturer. The described cut-off values obtained from the manufacturer was used, and for bFGF elevated levels were >7.25 pg/ml and for VEGF >500 pg/ml.

Statistics. Survival was estimated using the Kaplan-Meier product limit method, where univariate analysis was performed using a log-rank test. Cox regression analysis was performed to see if certain continuous factors had a significant effect on survival or to perform multivariate survival analyses. Spearman's rank order correlation was utilized for tests of associations between factors. The survival analysis together with the descriptive statistics is based on the first serum sample collected from each patient, whereas the correlation analyses were performed using all serum samples.

In order to investigate if the levels of VEGF, bFGF and MonoTotal increased during the progression of the disease, a statistical model was designed. Time zero in the model was set to be the date of histopathological diagnosis and time one was set to time of death, i.e. the time to death was standardized for all patients. Using a fixed effect leased square estimator to allow for individually different starting values, the effect from diagnosis to death was studied. In the descriptive statistics, range is defined as the minimum and maximum. Throughout the paper, a 5% significance level was used.

Results

MonoTotal. The mean overall value for MonoTotal levels prior to therapy was 376 U/l with a median value of 207 U/l (31-3809). Further descriptive data for the individual stages prior to therapy are shown in Tables 1, 2. MonoTotal was correlated with tumor volume (R=0.6; p=0.004). Multivariate analysis for investigated parameters and stages IIIa + IIIB is shown in Table 3.

Table 1. Descriptive data for patients stage IIIa concerning investigated parameters and survival as well as median (range) for investigated circulating markers

Variables	Patients	Median Survival (days)	VEGF (pg/ml) Median/Range	bFGF (pg/ml) Median/Range	MonoTotal (U/L) Median/Range
Gender					
Male	21	306	524 (47– 2003)	6 (1–32)	202 (50–1916)
Female	5	306	343 (65–792)	7 (0–10)	317 (127–376)
Performance status					
0	13	295	409 (65– 1152)	6 (0–32)	200 (82–514)
1	11	352	580 (237–1634)	10 (2–30)	317 (52–1916)
2	2	286	195 (47–343)	5 (3–8)	232 (125–304)
Histology					
Squamous	20	306	461 (47– 2002)	5 (2–29)	317 (50–1916)
Adenocarcinoma	6	330	723 (65–1634)	14 (1–32)	184 (92–376)
Treatment					
Solely radiation(>50 Gy)	15	306	409 (47– 1634)	7 (0–32)	200 (52–407)
Chemoradiation	7	287	385 (149–2002)	6 (2–20)	328 (50–792)
Concomitant	4	381	686 (548–1036)	7 (4–10)	254 (168–1916)

Table 2. Descriptive data for patients stage IIIb concerning investigated parameters and survival as well as median (range) for investigated circulating markers

Variables	No. of patients	Median Survival (days)	VEGF (pg/ml) Median/Range	bFGF (pg/ml) Median/Range	MonoTotal (U/L) Median/Range
Gender					
Male	15	308	551 (132–1149)	9 (0–64)	417 (45–1785)
Female	4	516	808 (200–1642)	10 (8–16)	155 (74–249)
Performance status					
0	12	298	498 (132–1401)	10 (0–64)	447 (74–1052)
1	5	372	442 (264–1084)	3 (1–15)	163 (45–1785)
2	1	332	731 (–)	8 (–)	417 (–)
Histology					
Squamous	14	249	497 (132–1642)	8 (0–64)	334 (45–1785)
Adenocarcinoma	5	389	727 (428–1401)	12 (8–13)	249 (163–513)
Treatment					
Solely radiation (>50 Gy)	15	344	551 (170–1642)	9 (0–26)	250 (45–1785)
Chemoradiation	1	332	731 (–)	8 (–)	417 (–)
Concomitant	3	210	201 (132–1401)	12 (8–64)	249 (74–1052)

From the time of histological diagnosis until time of death, a statistically significant increase in the levels of MonoTotal was found, p<0.0001. On average, this assay increased by 603 U/l. A descriptive figure showing the relation between MonoTotal in NSCLC patients versus healthy blood donors is shown in Figure 1.

VEGF. The mean value for VEGF levels prior to therapy in the total population was 740 pg/ml, with a median value of 595 pg/ml (47-7918). Further descriptive data for the individual stages prior to therapy are shown in Tables 1, 2. VEGF was not correlated with tumor volume (p=0.77), neither with platelets (p=0.12).

Multivariate analysis for investigated parameters and stages IIIa + IIIB is shown in Table 3.

From the time of pathological diagnosis until time of death, a statistically significant increase in the levels of VEGF was found, p=0.0004. On average, this assay increased by 430 pg/ml.

bFGF. The mean value for bFGF levels prior to therapy in the total population was 11 pg/ml, with a median value of 8 pg/ml (0-64). Further descriptive data for the individual stages prior to therapy are shown in Tables 1–2. bFGF was correlated with platelet counts (R=0.43, p=0.03), though not correlated with tumor volume (p=0.06).

Multivariate analysis for investigated parameters and stages IIIa + IIIB is shown in Table 3.

From the time of pathological diagnosis until time of death, a statistically significant increase in the levels of bFGF was found, p=0.018. On average, this assay increased by 5.93 pg/ml.

MonoTotal vs. bFGF and VEGF. Correlation analysis showed that serum MonoTotal was correlated with circulating levels of bFGF (R=0.3; p=0.03). MonoTotal was correlated with bFGF (R=0.26, p=0.00049) and VEGF (R=0.26, p=0.00007). VEGF was further significantly correlated with bFGF (R =0.4; p<0.001). In Figure 2 the relations between MonoTotal, bFGF and VEGF are shown in a representative patient.

Discussion

During malignant progression, tumors release cytokines that facilitate growth as well as degradation of interstitial connective tissue and basement membranes, facilitating the development of metastasis [11] and neovascularization of the tumor. Since NSCLC tumors are of epithelial origin, cells known to generally maintain their original cytokeratin expression pattern during malignant progression, in theory detection of increasing amounts of cytokeratin fragments in the blood circulation, should be of diagnostic importance. The two most potent angiogenic cytokines are VEGF and bFGF, having been shown to stimulate tumor vascularization [14, 15]. Furthermore, bFGF has also been shown to be involved in cell differentiation and proliferation [16]. In the present study, a statistically significant correlation was found between MonoTotal and bFGF (R=0.26, p=0.00049) as well as between MonoTotal and VEGF (R=0.26, p=0.00007).

The cytokeratin markers have been shown to be associated

Table 3. Multivariate analysis performed for stage IIIa and stage IIIb

	IIIa			IIIb		
	Parameter	Relative Risk	p–value	Parameter	Relative Risk	p–value
MonoTotal	0.000322	1.000322	0.817465	0.002848	1.002852	0.022063
bFGF	–0.000062	0.999938	0.998947	–0.016288	0.983843	0.357718
VEGF	–0.000377	0.999624	0.631549	–0.001441	0.998560	0.113853
Gender:						
Man	*Reference*			*Reference*		
Woman	0.327100	1.386940	0.645401	–0.037196	0.963487	0.964486
Treatment intention:						
Curative	*Reference*					
Palliative	0.772238	2.164606	0.635103	–	–	–
Performance status:						
0	*Reference*			*Reference*		
1	–0.212592	0.808486	0.708477	0.297308	1.346229	0.686527
2	0.437253	1.548447	0.727390	0.688040	1.989812	0.568441
Histology:						
Squamous	*Reference*			*Reference*		
Adeno	0.599997	1.822113	0.397210	–0.026442	0.973904	0.968234

with increased cell turnover. In previous studies with patients subjected to surgical intervention due to gastrointestinal cancer or lung cancer, these patients were shown to have increased amounts of circulating cytokeratins during the first weeks after surgery [17]. Furthermore, in an *in vitro* study, in which fragments of cytokeratins 8 and 18 were measured in the supernatants from cells exposed to irradiation, a trend towards increased amounts of cytokeratin fragments was found in the supernatant of irradiated cells [18]. These data suggest that assays measuring circulating cytokeratins are so-called activity markers or, which has been postulated, tumor activity markers [7]. A large tumor would theoretically have an increased cell turnover, releasing more proteolytic debris into the circulation than a small tumor. In the present study, MonoTotal was correlated with tumor volume (p=0.004), which was not found for either VEGF (p=0.77) or bFGF (p=0.06). These data further support the role of cytokeratins as activity markers.

Follow-up of cytokeratin markers during management of various carcinomas have been shown to correlate both to therapy effectiveness and clinical outcome, and this commonly with considerable lead times in comparison to clinical diagnosis [19].

In the present study, circulating markers were investigated before as well as throughout treatment and during follow-up (Tab. 1–2). The clinical significance of circulating levels of cytokeratins in patients with malignancies might cause confusion since several commercially available assays exist. In NSCLC the most frequently studied cytokeratin assay is CYFRA 21-1, measuring soluble fragments of cytokeratin 19 [20]. Several studies have shown that increasing levels of this assay is associated with worse prognosis for patients with NSCLC [21–27]. In the present study, MonoTotal increased with 603 U/L from time of admittance until time of death, and for stage IIIb MonoTotal was statistically associated with survival (p=0.02) as seen in the multivariate analysis.

The clinical utility of serum VEGF is unclear. Some studies have shown that circulating levels of VEGF are not associated with disease stage [28, 29], whereas others have re-

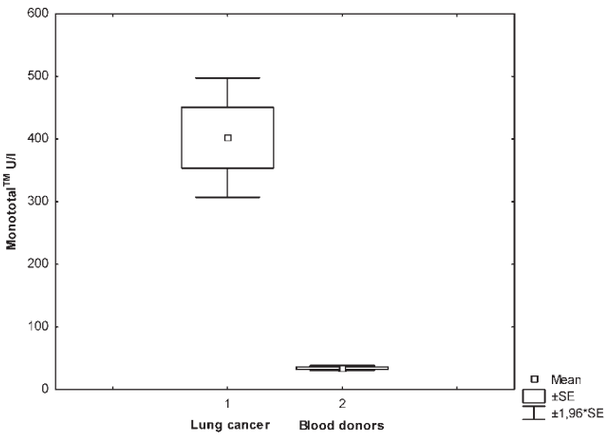

Figure 1. Analysis of MonoTotal from the first serum sample donated from each participating lung cancer patient in the present study in comparison with healthy blood donors.

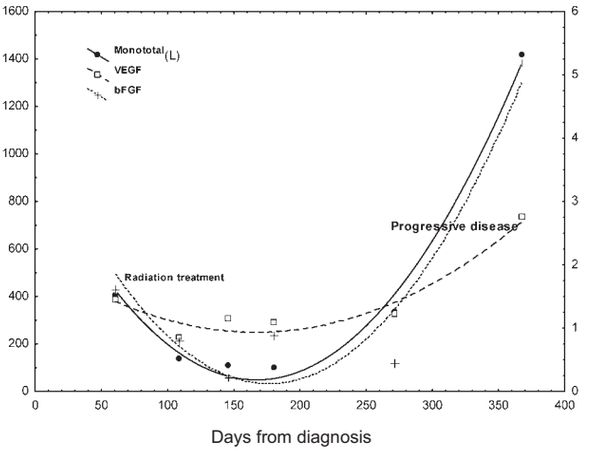

Figure 2. Descriptive figure of MonoTotal, VEGF and bFGF for one representative patient during treatment and until time of death.

ported that circulating VEGF increases significantly according to disease stage progression [14, 30–32]. Furthermore, circulating VEGF has been shown not to be associated with survival [30], while others have reported a prognostic importance for patients with NSCLC [14]. In the present study, VEGF increased with 430 pg/ml from time of admittance until time of death and for each of the individual stages in the multivariate analysis, VEGF was not statistically associated with survival (Tab. 3).

The clinical role of bFGF is also controversial. Several studies have reported that high levels of serum bFGF are found in patients with NSCLC [14, 28, 33–35]. However, according to our knowledge there has been only one study in which the authors show that bFGF was not correlated with the response to chemotherapy [36]. In the present study, bFGF increased with 5.9 pg/ml from time of admittance until time of death, and for each of the individual stages in the multivariate analysis bFGF was not statistically associated with survival (Tab. 3).

A retrospective study should always be interpreted with caution. However, since the majority of investigated patients have been followed consistently, and since the majority of the investigated patients have had advanced disease which ultimately progressed, significant biological changes must have occurred that were reflected even in sera.

We therefore conclude that MonoTotal, measuring soluble fragments of cytokeratins 8, 18 and 19, may function as a tumor activity marker as indicated by increased levels in NSCLC patients and its correlation with tumor volume. Reflecting the tumor activity, MonoTotal was also found to be correlated with both VEGF and bFGF, further supporting the role of cytokeratins as activity markers. This newly developed cytokeratin assay seems to be a potentially very interesting serum marker that, in conjunction with other clinical data, might be used during monitoring of patients with NSCLC.

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