Expression of Circulating Microparticles for the Diagnosis of Non-small Cell Lung Cancer: Clinicopathological Correlations and Prognostic Value

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Abstract Increased values of circulating microparticles (MPs) have been reported in solid tumors including non-small cell lung cancer (NSCLC). We therefore investigated the utility of baseline MPs in the clinical setting of patients with NSCLC. Quantification of MPs in the plasma was performed by flowcytometry. Baseline MP values were correlated with clinical patients' characteristics, estimated tumor volume (ETV) and treatment response. Receiver operating characteristics (ROC) curves were plotted to discriminate between patients and controls in order to determine the diagnostic value of circulating MPs in NSCLC. Our prospective study included 134 NSCLC patients (98 at initial diagnosis, ID and 36 at relapse, R) and 30 healthy individuals. The mean of baseline MP numbers was significantly higher in patients presented either at ID or R than in controls ($p < 0.0001$). Basal MP numbers were inversely correlated with ETV values ($p = 0.04$). In addition, the difference in MP levels at diagnosis was significant according to tumor histology ($p = 0.02$) and primary tumor size ($p = 0.0007$). Using ROC analysis, the optimal cutoff value for baseline MPs was 1307 events/ μ L with a sensitivity and a specificity of 67.3% and 90.0%, respectively. High MPs expression was significantly associated with low-level smoking degree ($p = 0.001$), non-squamous cell types ($p = 0.017$) and decreased tumor size ($p = 0.003$). Our results suggest that high baseline MP values could be an indicator of tumor growth inhibition in NSCLC. Furthermore, high expression of circulating MPs at diagnosis might predict good prognosis in NSCLC patients.

Keywords Biomarkers, Clinicopathological Features, Microparticles, Non-small Cell Lung Cancer

1. Introduction

Non-small cell lung cancer (NSCLC) represents about 85% of lung cancer (LC) cases, and most patients have metastatic disease at their initial diagnosis [1]. However, NSCLC patients with locally-advanced stages are treated by surgery, chemotherapy and radiotherapy. It should be noted that surgical outcomes with lymph nodes dissection and intraoperative blood loss were significantly related to postoperative complications [2]. Adjuvant chemotherapy improved survival in patients with completely resected early-stage NSCLC, while standard chemotherapy relatively prolonged survival rate in NSCLC patients at advanced stages, but it is still low with 5-year survival rate of 2% [3-5]. Recently, molecularly targeted drugs have been introduced such as tyrosine kinase epidermal growth factor receptor (EGFR) inhibitors and anaplastic lymphoma tyrosine kinase (ALK) inhibitors [6]. These targeted drugs have become the treatment of choice for NSCLC patients with mutations in EGFR and ALK genes [7,8].

There is an urgent need for new diagnostic strategies to screen individuals at high risk for reducing LC mortality rate in NSCLC [9]. However, clinicopathological characteristics of patients have been previously proposed as prognostic factors in NSCLC. In fact, the determination of correct prognostic factors may predict disease outcome in LC. Therefore, the identification of an effective marker is essential for NSCLC to predict clinical outcome. Recently, better understanding of molecular biology of NSCLC have attracted attention for molecular-driven

targeted therapy. So it is warranted to develop new biomarkers that can be easily applied in the clinical setting in order to improve the therapeutic outcome of NSCLC [10]. Disappointingly, no effective biomarker has been yet clinically validated for the evaluation of newly diagnosed LC patients.

Circulating microparticles (MPs), which are small vesicles (0.1-1µm) in diameter, can be released in the peripheral blood by activated or apoptotic cells [11,12]. They are resulting from vesiculation of platelet plasma membrane and other blood cells and could be detected in fresh-frozen plasma as circulating MPs [13]. The exposure of phospholipids and phosphatidyl serine on the surface of MPs could be derived from platelets and endothelial cells. These MPs may also most likely express tissue factor (TF) and be potential inducer of procoagulation [14]. Accordingly, it has been demonstrated that MPs expressing phosphatidylserine have an important role in blood coagulation and thrombosis [15,16]. In fact, TF activity on tumor cells could be potentially mediated by the expression of anionic phospholipids on the outer leaflet of cell membrane [17,18]. In addition, it has been found that MPs especially those derived from platelets play an important role in angiogenesis [16]. Actually, they promote capillary like structures and production of proangiogenic factor [19,20]. Thus, increased MP values have been reported in many tumor types including hematological malignancies and breast cancer [21,22]. However, the clinical utility of MPs in NSCLC is still undefined. We therefore investigated the potential clinical value of circulating MPs as diagnostic and prognostic tool in NSCLC.

2. Patients and Methods

2.1. Cohort Selection

Between August 2015 and February 2017, we restricted our analyses to patients diagnosed with histologically confirmed NSCLC of more than 18 years-old and Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≤ 2 . Our patients presented at the department of Thoracic Oncology in Albairouni University Hospital either at initial diagnosis (ID) or at relapse (R). Detailed clinical characteristics and follow-up data including age, gender, pathological, computed tomography (CT) and bone scans findings, were prospectively collected for analysis. To circumvent adverse influence on MPs measurement, patients with recent surgery or trauma during the preceding 2 months, history of other malignancy or febrile disease, chronic kidney failure, heart disease, liver cirrhosis, hematologic disorders, current use of anti-platelet agents, acute or chronic inflammatory, and autoimmune disease were excluded from the study.

2.2. Data Collection

Blood samples were collected from patients and healthy

individuals. Smoking degree was considered according to the number of packs per year. A pack-year (P/Y) is defined as the number of packs of cigarettes a person smoked every day multiplied by the number of years the patient has smoked. With regard to smoking degree, the patients being considered as heavy smokers for those who smoked more than 15 P/Y, while low-level smoking degree was considered for those who smoked less than 15 P/Y.

Recent weight loss was considered for patients who had lost during the last three months more than 5% of their body weight or less than 2% for those with body mass index (BMI) less than 20. The remaining patients were considered without recent weight loss.

According to tumor histology, our patients were divided into two groups: patients with squamous cell type and those with non-squamous cell types. Our population were also classified according to tumor histological grade into patients with well-moderately differentiated grade and those with poorly differentiated grade.

The included patients were either at initial diagnosis (ID) or at relapse (R). Then, all NSCLC cases were staged by chest and abdominal computed tomography (CT). According to radiological findings based on the American Joint Committee on Union for International Cancer Control Criteria, our population was divided into two groups: patients with early stages (stage I to IIIA) and those with locally-advanced and metastatic stages (stage IIIB, IV).

Estimated tumor volume (ETV) was determined according to CT findings by measuring the major axis (a) and the minor axis (b) of the primary tumor using the following formula:

$$ETV = \frac{4}{3} \times \pi \left[\left(\frac{a}{2} \times \frac{b}{2} \right) \times \left(\frac{a}{2} + \frac{b}{2} \right) \right] \div 2$$

Follow-up data were collected until August 2018. The therapeutic efficacy was evaluated according to response evaluation criteria for solid tumors (RECIST) [23]. An objective response (OR) was identified in patients who had partial response (PR) or stable disease (SD) after treatment. However, progression disease (PD) was defined when the patient had an increase of more than 20% in the longest diameter of the tumor mass or the appearance of new metastases on CT after chemotherapy.

Pretreatment MP values were correlated with clinical characteristics of NSCLC patients including gender, age, smoking history, BMI, clinical presentation, histological type, ETV, staging and treatment response. According to their cutoff value, MP levels were correlated with clinicopathological parameters of our patients including gender, age, smoking degree, recent weight loss, clinical presentation, tumor histology, pathological grade, primary tumor size, clinical stage and treatment response.

2.3. Isolation and Quantification of Circulating MPs by Flow Cytometry

Peripheral blood samples were collected in vacutainer

tubes containing acid citrate dextrose (ACD) as anti-coagulant. Blood samples were analyzed using protocol outlined previously [24]. Then, they were kept at (4-8)°C and processed within 24 hours after collection. Whole-blood was centrifuged at 2500xg for 15 minutes at 4°C without acceleration to prepare platelet-rich plasma. Then, plasma samples were centrifuged at 19800g for 10 min. The supernatant was transferred to a new tube and centrifuged at 13000g for 5 min at room temperature in order to obtain platelet-free plasma (PFP), which was divided into two aliquots of 200 µl. If needed, aliquots of PFP were stored at -80°C for later batch analysis. According to the manufacturer's instructions, standard fluorescent beads (Megamix, BioCytex, Marseille, France) of different diameters (0.5–3 µm) were used for size calibration and to set the gate for MP detection. Then 10-µL pellet of MPs was diluted with 490 µL of AnnexinV binding buffer, and incubated in a TruCOUNT tube with FITC anti-AnnexinV (BioVision, Milpitas, CA, USA). Isolated MPs were identified as particles <1.0 µm in diameter and positively stained with FITC-Annexin V. The amount of MPs was expressed as events/µL of plasma.

3. Statistical Analysis

Data were expressed as means ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to determine the differences in MP levels. Continuous variables were analyzed by independent *t*-test, while Pearson's chi-square test was used to analyze categorical variables. The diagnostic accuracy of circulating MP levels to distinguish between patients and controls was assessed using receiver operating characteristic (ROC) curves. Sensitivity and specificity were calculated for different cut-off values, and the optimal cut-off value for MP

numbers were determined using ROC analysis. The overall performance of ROC analysis was quantified by computing the area under curve (AUC). All statistical analyses were conducted using statistical package social sciences software for windows, version 15.0 (SPSS Inc., Chicago, IL, USA). All statistical tests were two-tailed and *p* value ≤ 0.05 were considered statistically significant.

4. Results

4.1. Patients' Characteristics and Baseline MP Values

A total of 134 NSCLC patients (113 men, 21 women), presented at ID (n=98) or at R (n=36) and 30 healthy volunteers (25 men, 5 women), were prospectively included in this study. The main clinical characteristics of patients were correlated with baseline MP values as shown in the Table 1. The mean age of patients was significantly higher than that in healthy subjects (mean ± SEM: 55.42 ± 8.99 vs. 37.13 ± 6.89 years, *p*<0.0001). However, basal MP numbers were not significantly correlated with increasing age in patients (*p*=0.81). The mean of baseline MP numbers was significantly higher in patients either at ID or R than in controls (mean ± SEM: 2902 ± 264 and 2753 ± 442, respectively vs. 780 ± 106, *p*<0.0001), figure (1). However, the difference in their numbers was not significantly different between patients at ID and those at R (mean ± SEM: 2902 ± 264 vs. 2753 ± 442, *p*=0.75). Additionally, no significant difference in circulating MP values at diagnosis was obtained according to patients' characteristics including gender (*p*=0.96), age (*p*=0.53), BMI (*p*=0.56), and smoking status (*p*=0.73). Moreover, there was no significant difference in basal MP numbers between patients at early stages and those at advanced stages (mean ± SEM: 2704 ± 451 vs. 2916 ± 298, *p*=0.47).

Table 1. Baseline MP values and clinical characteristics of non-small cell lung cancer patients

Clinical characteristics		N° (%)	MPs value Mean ± SEM	<i>p</i> - value
Patients		134	2862±372	
Healthy controls	Number	30	780±106	<i>p</i> < 0.0001*
Gender	Male	113 (84.3)	2863±220	
	Female	21 (15.7)	2835±616	0.96
Age	≤60 years	92 (68.7)	2835±616	
	>60 years	42 (31.3)	3074±480	0.53
Clinical presentation	ID	98 (73.1)	2902±293	
	R	36 (26.9)	2689±464	0.75
Histological type	SCC	55 (46.2)	2281±308	
	Non-SCC	64 (53.8)	3355±419	0.02*
Pathological grade	Well/moderate	52 (53.6)	2515±349	
	Poor	45 (46.4)	2435±363	0.86
Primary tumor size (major axis)	> 4 cm	50 (60.7)	3611±509	
	≤ 4 cm	33 (39.3)	1846±324	0.0007*
TMN Staging	Early	36 (26.9)	2704±450	
	Advanced	98 (73.1)	1916±298	0.68
Response to chemotherapy	PR/SD	77 (66.4)	2886±328	
	PD	39 (33.6)	2959±477	0.85

MPs: microparticles; SCC: squamous cell carcinoma; P/Y: pack-years; ID: initial diagnosis; R: relapse; dif: differentiated, TNM: tumor-node-metastases; PR: partial response; SD: stable disease; PD: progression disease; *: *p* ≤ 0.05

Figure 1. Baseline microparticles (MP) levels (events/μL) in patients with non-small cell lung cancer (NSCLC) and in healthy subjects. Basal MP values were significantly higher in patients at initial diagnosis (ID) and relapse (R) than in healthy subjects ($p=0.007$ and $p<0.0001$, respectively). There was no significant difference in basal MP numbers between patients at ID and those at R ($p=0.75$)

Although no significant difference in basal MP values was observed according to histological grade ($p=0.86$), higher pretreatment MP numbers were obtained in patients with non-squamous cell types compared to those with squamous cell carcinoma ($p=0.02$), figure (2).

Pretreatment MP values were inversely correlated with ETV numbers in 83 assessable patients ($p=0.002$), figure (3). Moreover, baseline MP values were significantly higher in patients with primary tumor size less than 4 cm

than in those with primary tumor size more than 4 cm (mean $\pm$ SEM: 2611 ± 509 vs. 1846 ± 324 , $p=0.0007$).

On the other hand, clinical follow-up data were available in 116 patients for the evaluation treatment response showing an OR in 77 patients and PD in 39 patients. Data analysis revealed that there was no significant difference in the mean of baseline MP numbers between patients with OR and those with PD after treatment (mean $\pm$ SEM: 2856 ± 328 vs. 2959 ± 477 , $p=0.85$).

Figure 2. Pretreatment microparticles (MP) levels in non-small cell lung cancer (NSCLC) patients according to histological subtypes. Circulating MP levels were significantly higher in patients with non-squamous cell types than those in patients with squamous cell type ($p=0.02$)

Figure 3. Scatter plot analysis to correlate basal microparticles (MP) numbers with estimated tumor volume (ETV) values in patients with non-small cell lung cancer (NSCLC). Basal MP numbers were inversely correlated with ETV values ($r^2 = 0.07$, $p=0.002$)

4.2. Circulating MPs Expression and Clinicopathological Parameters

As illustrated in figure (4), ROC curves analysis showed that AUC was 0.847 which significantly differs from 0.5 ($p < 0.002$). The best efficacy for baseline MP numbers was observed at 1307 events/ μ L with a sensitivity and a specificity of 67.3% and 90.0%, respectively. According to this value, 92 and 42 patients showed high and low MPs expression, respectively. As shown in Table (2), high expression of circulating MPs was significantly associated with low-level smoking degree ($p = 0.001$), non-squamous cell histological types ($p = 0.017$), and decreased primary tumor size ($p = 0.003$). However, MPs expression was not significantly correlated with the other clinicopathological parameters including gender, age, estimated weight loss, clinical presentation, histological grade, staging, and treatment response (all $p > 0.05$).

Figure 4. Receiver operating characteristic (ROC) curve of circulating microparticles (MPs) for the discrimination between patients and healthy controls. AUC: area under curve

Table 2. Relationship between circulating MPs expression and clinicopathological features of NSCLC patients

Clinicopathological features		N°	Low MPs expression N° (%)	High MPs expression N° (%)	Chi-squared	p-value
Gender	Male	113	32 (28.3)	81 (71.7)	0.166	0.96
	Female	21	10 (47.6)	11 (52.4)		
Age (years)	≤ 60	54	18 (33.3)	36 (66.7)	0.166	0.71
	> 60	80	24 (30)	56 (70)		
Smoking degree	≤15 P/Y	24	17 (70.8)	7 (29.2)	12.144	0.001
	>15 P/Y	110	36 (32.7)	74 (67.3)		
Estimated weight loss	None	47	14 (29.8)	33 (70.2)	0.739	0.42
	Yes	61	23 (37.7)	38 (62.3)		
Clinical presentation	ID	98	32 (32.7)	66 (67.3)	0.291	0.68
	R	36	10 (27.8)	26 (72.2)		
Tumor histology	SCC	54	22 (40.7)	32 (59.3)	6.120	0.017*
	Non-SCC	69	14 (20.3)	55 (79.7)		
Pathological grade	Well dif.	52	16 (30.8)	36 (79.2)	0.528	0.52
	Poorly dif.	45	17 (37.8)	28 (62.2)		
Primary tumor size (major axis)	≤ 4 cm	33	19 (57.6)	14 (42.4)	9.577	0.003*
	>4 cm	50	12 (24)	38 (76)		
Staging	I-III A	36	13 (36.1)	23 (63.9)	0.520	0.52
	IIIB, IV	98	29 (29.6)	69 (70.4)		
Treatment response	PR/SD	77	24 (31.2)	53 (68.8)	0.176	0.68
	PD	39	13 (33.3)	26 (66.7)		

MPs: microparticles; SCC: squamous cell carcinoma; P/Y: pack-years; ID: initial diagnosis; R: relapse; dif: differentiated; PR: partial response; SD: stable disease; PD: progression disease; *: $p < 0.05$

5. Discussion

Angiogenesis is the formation of new blood vessels originating from an existing microvasculature [25,26]. This process occurs during pathological conditions such as cancer. However, tumor angiogenesis occurs when there is a local imbalance between proangiogenic and antiangiogenic factors [26]. The pathogenesis of blood coagulation activation in cancer is complex. However, thrombosis could be the first sign of malignant disease, preceding the clinical detection of cancer by months or even years [27]. Nevertheless, TF regulates tumor growth through angiogenesis and coagulation. The relationship between angiogenesis and TF expression has been previously reported in human NSCLC carcinoma [28]. However, the procoagulant activity of MPs expressing TF did not change. The process of fibrin formation and fibrinolysis parallels the development of malignancy, increasingly in those with metastases [29]. Clinicians tried to discover a useful biomarker for early detection of LC [30,31]. Actually, circulating MPs had differential roles in angiogenesis [16,20]. Alterations in subtle hemostatic, such as high levels of circulating MPs shed by tumor cells and platelets, are detected [32]. Furthermore, plasma MP numbers have been suggested as potential mediated signals for stimulating angiogenesis in solid tumors including LC [33].

It has been also proposed that the concentration of platelets-derived MPs, which represent the majority of MPs, may be an indicator of progression in NSCLC [34]. However, it has been found that MP numbers increased in NSCLC patients at early operable stages, but the difference in their levels was not significant between NSCLC patients three months after surgery and healthy controls [35]. These findings may explain why increased baseline MP values were observed in NSCLC patients either at ID or R in our study.

It has been demonstrated that circulating MPs increased in NSCLC patients diagnosed in end-stage compared with controls [36,37]. By contrast, higher baseline MP values have been found in NSCLC patients at early and advanced stages compared with those in healthy subjects in our study. Similar results have been also observed in a heterogeneous group of NSCLC and small-cell lung cancer (SCLC) patients [34,38]. Moreover, high MPs expression was also found in SCLC patients either at limited and or at extensive stages [39]. However, it has been suggested that cancer metastasis may be due to an effective gateway through lymphatic circulatory system [40]. This path way may be more efficient than direct metastatic dissemination from the primary tumor. These findings might explain why baseline MP values increased in LC patients either at early stages or at advanced stages.

Understanding the fundamental role of angiogenesis in cancer growth has led to a special interest regarding regulatory mechanisms for better management of cancer

patients. Interestingly, we found that baseline MP numbers were inversely correlated with ETV values. Moreover, high basal MP values were associated with a decrease in the primary tumor size. However, it has been suggested that increased levels of monocyte-derived MPs might be an indicator of vascular complication in LC patients [34]. It has been also found that tumor size and neovascularization decreased after the injection of MPs in mice with lung carcinoma [41]. These results may explain why higher baseline MP levels were associated with a decrease in ETV values in our study.

It has been demonstrated that MPs may stimulate cellular proliferation and the adhesion of cancer cells that can affect vascular angiogenesis through the release of proangiogenic factors [42], suggesting that circulating MPs may predict treatment response in solid tumors including LC. By contrast, we found that there was no significant difference in the mean of baseline MP values between patients with OR and those with PD after chemotherapy. This discordance may due to the heterogeneity of our population, in which variable tumor response was obtained according to individual risk factors and clinical stages.

Finally, patients with non-squamous cell types and low-level smoking degree had high MPs expression in our study. However, high rate of positive EGFR mutation has been reported in these patients [43]. In fact, the thyroid transcription factor 1 (TTF-1) plays a physiologic role in the development and the morphogenesis of the thyroid and the lung during embryogenesis. It has been found that high expression of TTF-1 promote cancerization by regulating the activity of proliferating cells and new vessels formation, at least in lung adenocarcinoma [44]. This mechanism might be due the fact that TTF-1 was more frequently expressed in patients with adenocarcinoma compared to those with squamous cell lung carcinoma [45]. Moreover, it has been recently found that metabolic tumor volume demonstrated a significant difference between patients with wild-type and those with mutant EGFR especially when combined with smoking status [46]. We also found that high pretreatment MP numbers were associated with a decrease in ETV values. However, NSCLC patients with low-level smoking, high expression of TTF-1 and positive EGFR mutation showed good prognosis. Similarly, patients with non-squamous cell types and low-level smoking showed high baseline MP values in our study. These findings suggest that high MP values at diagnosis could be an indicator of good prognosis in NSCLC.

6. Summary

Taken together, our results not only extended the findings outlined in previous reports, but also provide a clinically relevant information suggesting baseline MPs as a useful potential biomarker in the clinical setting of NSCLC. We found that high basal MP values were

associated with tumor growth inhibition in NSCLC patients. Moreover, high expression of circulating MPs was observed in patients with low-level smoking degree and non-squamous cell types, suggesting MPs expression at diagnosis as predictive tool of prognosis in NSCLC. Further studies are warranted to investigate the utility of MPs variation after treatment for the prediction of tumor response, prognostic outcome and survivals in NSCLC.

Abbreviations

ACD: acid citrate dextrose
 ALK: anaplastic lymphoma kinase
 AUC: area under curve
 BMI: body mass index
 CT: computed tomography
 ECOG: eastern cooperative oncology group
 EGFR: epidermal growth factor receptor
 ETV: estimated tumor volume
 ID: initial diagnosis
 LC: lung cancer
 MPs: microparticles
 NSCLC: non-small cell lung cancer
 OR: objective response
 PD: progression disease
 PFP: platelet free plasma
 PR: partial response
 PS: performance status
 P/Y: pack-year
 R: relapse
 RECIST: response evaluation criteria in solid tumors
 ROC: receiver operating characteristic
 SCC: squamous cell carcinoma
 SCLC: small-cell lung cancer
 SD: Stable disease
 SEM: standard error of mean
 TF: tissue factor
 TKIs: tyrosine kinase inhibitors
 TTF-1: thyroid transcription factor 1
 VEGF: vascular endothelial growth factor

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Conflict of Interest

The authors state that there is no potential conflict of

interest.

Ethics Clearance

Taken from the independent Ethics Committee on Human Research at Albairouni University Hospital. All procedures performed in the study involving human participants were in accordance with the principles of the Declaration of Helsinki.

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