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A novel cytokine profile associated with cancer metastasis to mediastinal and hilar lymph nodes identified using fine needle aspiration biopsy

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A NOVEL CYTOKINE PROFILE ASSOCIATED WITH CANCER METASTASIS TO MEDIASTINAL AND HILAR LYMPH NODES IDENTIFIED USING FINE NEEDLE ASPIRATION BIOPSY

by

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The author would like to thank Almighty for all the blessings and fullfillment of prayer, subHAnaka lA `ilma lanA illA mA `allamtanA innaka anta-l`aliymu-lHakiym (Sura Bukara: verse 31) *glory be to you, we have no knowledge except what you have taught us, you are the all-knowing, the all-wise.*

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A novel cytokine profile associated with cancer metastasis to mediastinal and hilar lymph nodes identified using fine needle aspiration biopsy

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Abstract:

Background: Cancer metastasis to mediastinal and hilar lymph nodes is indicative of a poor prognosis. Serum or sputum biomarkers provide limited information about cancer metastasis to lymph nodes. However, endobronchial ultrasound guided fine needle aspiration (EBUS-FNA) biopsy is increasingly used to sample lymph nodes for simultaneous cancer diagnosis and staging.

Objective: We compared cytokine expression in benign versus malignant mediastinal and hilar lymph nodes using EBUS-FNA biopsy specimen.

Methods: In this prospective, single center study, we collected EBUS-FNA biopsies from patients recruited using a convenience sampling approach. Cytokines were assayed using Bio-Plex Pro human cancer biomarker panels 1 and 2 in a Bio-Rad 200 suspension array system in two phases. The unique cytokine profile obtained from phase I for cancer metastasis was validated in phase II.

Results: In phase I, analysis was performed using 18 specimens obtained from 10 study participants. After collection of additional 36 specimens from 18 participants, phase II cytokine analysis was performed on 54 specimens collected from a total of 28 participants. Malignant and benign histologic

diagnoses were established in 16 participants (primarily non-small cell lung cancer) and 12 participants, respectively. Cytokine analysis showed repeated significant elevation of follistatin (p 0.001), uPA (p<0.001), osteopontin (p<0.001), HER2/neu (p<0.001), VEGFR-1 (p<0.001), HGF (p<0.001) and VEGF-A (p<0.001) in malignant samples when compared to benign lymph nodes.

Conclusion: We have identified and validated a novel cytokine profile of metastatic cancer in mediastinal and hilar lymph nodes. This will guide future studies to detect cancer biomarkers in lymph nodes with the goals to improve the diagnosis, treatment and prognosis of cancer metastasis.

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Introduction:

Cancer metastasis to lymph nodes is associated with poor prognosis, especially in patients with lung cancer (1). Despite significant improvements in the management and treatment of cancer patients, most deaths are due to chemo-resistant metastasis (2). The phenotypic drift (3, 4) and intrinsic heterogeneity of cancer cells (5, 6) contribute to the potential for metastasis and development of resistance to standard treatments. It is thus important to understand the pathogenesis of cancer metastasis on a cellular and molecular level with a goal to develop novel therapies directed towards prevention and treatment (7).

Increase in tumor size is associated with increased incidence of metastasis to the regional lymph nodes (8). Lymph nodes initially act as a barrier to the circulating cancer cells. Antigenicity of circulating cancer cells trigger the development of a complex immune reaction with histopathology indicative of follicular hyperplasia, sinus histiocytosis, lymph angiogenesis and foreign body granuloma formation (9). The cross talk between cancer cells trying to seed into the lymph nodes changes over time and a nurturing microenvironment in the lymph node allows for growth of tumor cells (9). The factors leading to this change are unknown.

Endobronchial ultrasound allows for real time fine needle aspiration (FNA) biopsy of mediastinal and hilar lymph nodes. Nakajima and colleagues reported expression of two cytokines in the FNA biopsy that correlated with ultrasound characteristics of the mediastinal lymph node (10). Their work illustrated that

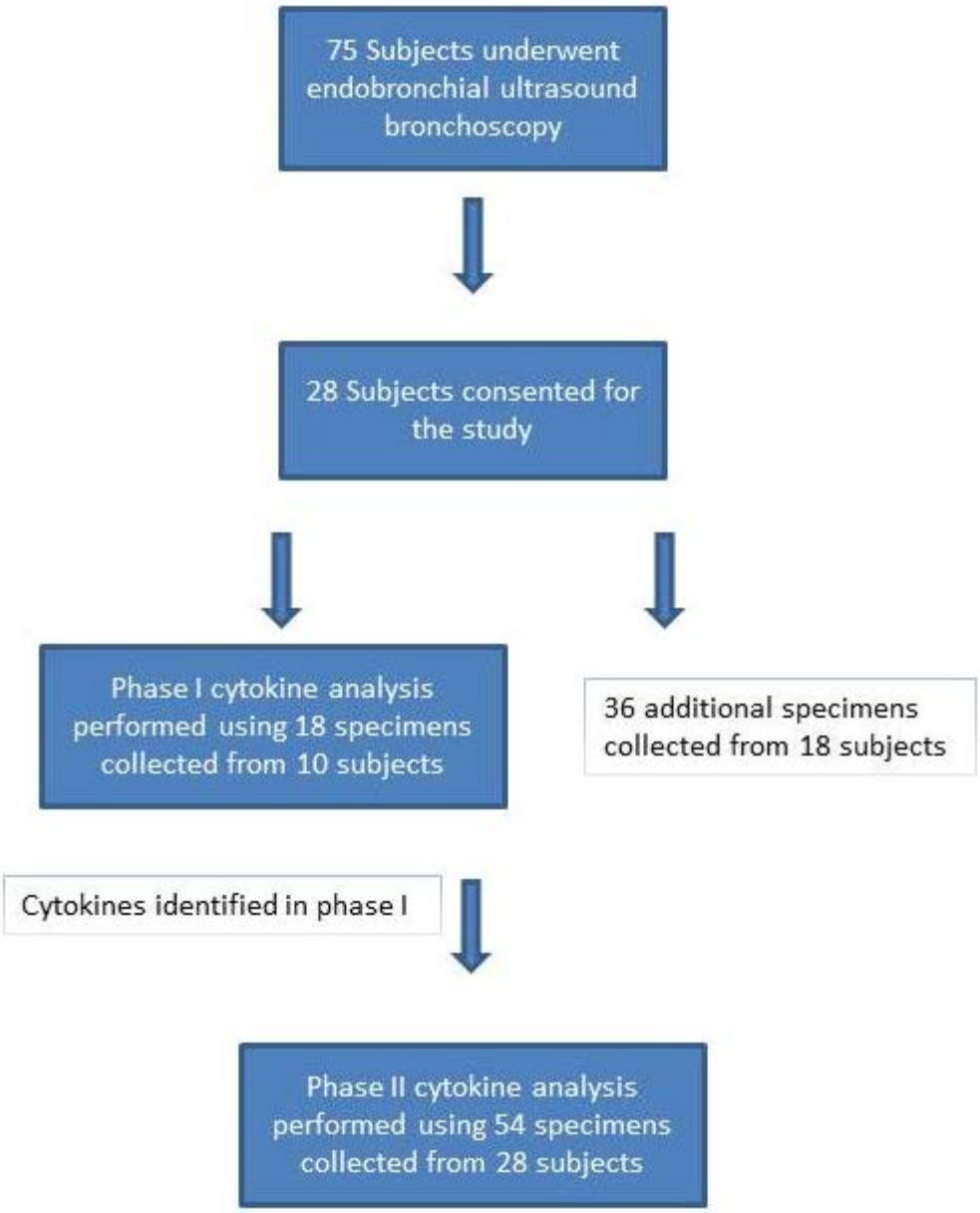
measuring cytokines in the mediastinal lymph node aspirate using EBUS is feasible. The current study is based on the hypothesis that a cytokine signature obtained from FNA biopsy of a mediastinal or hilar lymph node with cancer metastasis differs from that of a benign lymph node. We aim to determine if we can reliably identify a comprehensive panel of cytokines from lymph node aspirates that can explain the change in lymph node microenvironment with cancer metastasis. In addition, we outline a standardized approach for collection, storage and processing of lymph node aspiration biopsy using multiplex technology.

Methods

Study design. The study was conducted in two phases. Phase I was conducted to identify a unique cytokine panel and then validate the results in phase II using a narrower panel of significantly different cytokines identified in phase I (Fig.1). Fine needle aspiration biopsy specimens (third or fourth biopsy) were collected from mediastinal or hilar lymph nodes for cytokine analysis.

Subjects: Study participants were recruited from an outpatient specialized lung cancer clinic at a University Hospital in New Mexico using convenience sampling method. Eligibility criteria for inclusion were ability to communicate in English, at least 18 years of age, and enlarged mediastinal and hilar lymph nodes. Information reagarding: age, gender, race/ethnicity, and smoking history was obatined from each patient before the procedure. Informed consent was obtained from each subject by the physician performing the procedure. This study was approved by the Human Research Review Committee of the University of New Mexico (UNM) Health Science Center and TRICORE Reference Laboratory Research Review Committee.

Figure 1: Study Outline



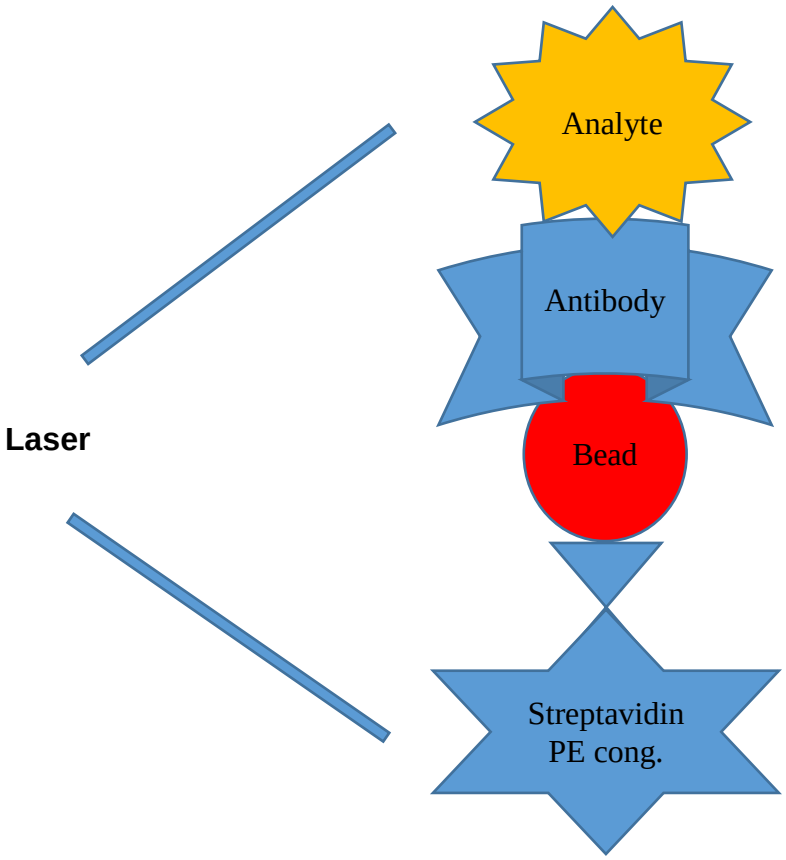
Collection of aspiration biopsy samples: The aspiration biopsy specimens were collected in sterilized vials (2ml). The collection protocol required use of air in a 5 milliliter syringe to push the specimen out of the endobronchial aspiration biopsy needle, so as to avoid excessive dilution. If necessary, a metal stylet was used to push the specimen out of the biopsy needle. The specimens were immediately placed on ice and delivered within two hours to the T1 laboratory of the UNM Clinical and Translational Science Center (CTSC). Samples were stored at -72°C until cytokine analysis was performed.

Histopathology of aspiration biopsy: The histologic diagnosis of the lymph node aspiration biopsy was made by a staff pathologist at the University of New Mexico. Patients with FNA biopsy showing polymorphous population of lymphocytes or inadequate samples were referred for mediastinoscopy. If mediastinoscopy was not done, or offered, then these patients were followed with repeat contrast enhanced computed tomography scan of the chest in 3 to 6 months interval to confirm stability or resolution of mediastinal and hilar lymph nodes. If the histopathology diagnosis was other than a malignancy, the respective sample was included for cytokine and data analysis as a benign lymph node. For lymph nodes with cancer metastasis, the primary sites of cancer were recorded for descriptive analysis.

Analysis of lymph node aspirates: A Bio-Rad Bioplex 200 suspension array system (Bio-Rad Laboratories, Inc., 171-000201) was used for both phases of the study. The bead based Luminex technology allows for multiple protein analysis in a single sample. The data are reported as median fluorescence

intensity (MFI) and concentration (pg/ml). The concentration of unknown analytes bound to fluorescent bead is proportional to the MFI of the reporter signal. The experimental protocol allows for simultaneous reporting of standards, controls, blanks and cytokines of interest in duplicate.

Figure 2: Streptavidin phyco-erytherin conjugate bead based “sandwich” essay



Phase 1 study was conducted using the Bio-Plex Pro human cancer biomarker panels 1 and 2 that included 16 and 18 cytokines, respectively (Table1). Efforts were made to process all specimens simultaneously according to the Bio-Plex human cancer biomarker assay instructions and experiment protocol. Bio-Rad Bioplex Data Pro software ® (Bio-Rad Laboratories, Inc., 171-001513) was used for data analysis to identify the extreme values, data distribution and selection of range. Statistical analysis: Descriptive statistics for patient specific variables and diagnostic variables were obtained using the STATA 12 software® (StataCorp, 12.1, College Station, Texas 77845) and are expressed as mean ± standard deviations. The analytical data for lung cancer cytokines was performed by using both Bio-Rad Bioplex Data Pro software ® and STATA 12 software. Using histopathological diagnosis of cancer as an outcome variable, data for each cytokine was analyzed for normality of distribution. Non parametric analysis was performed using the Wilcoxon rank-sum test for non-normally distributed variables. Data for variables that were not normally distributed were expressed as median and interquartile range. Benferroni correction was used to address multiple comparisons. Phase II was conducted using the protocol described above to replicate results in a narrower panel of cytokines identified to have a significantly different expression with cancer metastasis when compared to benign histology, in phase I

Table 1: Bioplex human cancer panel 1 and 2

HCP 1	HCP 2
sEGFR	Angiopoientin -2
FGF-basic	sCD40L
Follistin	EGF
G-CSF	Endoglin
HGF	sFASL
sHER-2/neu	HB-EGF
sIL-6α	IGFBP-1
Leptin	IL-6
Osteopontin	IL-8
PECAM-1	IL-18
PDFG-AB/BA	PAI-1
Prolactin	PLGF
SCF	TGF-α
sTIE-2	TNF-α
sVEGFR-1	uPA
sVEGFR-2	VEGF-A
	VEGF-C
	VEGF-D

Results.

Cytokine expression in FNA biopsies from metastatic lymph nodes compared to benign lymph nodes.

Phase I: Cytokine analysis was performed in 18 FNA biopsy specimens collected from 10 patients that comprised seven FNA biopsies with polymorphous population of lymphocytes and one right paratracheal mass with histologic diagnosis of hamartoma. Ten mediastinal and hilar lymph node FNA biopsies were positive for cancer metastasis from lung, esophagus and skin (Table 2). Patients with cancer diagnosis were of older age compared to patients with a benign diagnosis. There were no stastical significant differences of smoking or abstinence between the two groups (Table3). The mean sample volume was 166 micro liters (µl). The volume of benign lymph node aspirates was greater than the volume from lymph nodes with metastasis, but did not reach stastical significance.

The concentrations of 10 of the 34 cytokines measured, using human cancer panel 1 and 2 were significantly elevated in malignant samples versus benign samples: osteopontin, prolactin, vascular endothelial growth factor receptor 1 and granulocyte- colony stimulating factor ($p < 0.01$). In addition, vascular endothelial growth factor-A, urokinase plasminogen activator, follistatin, hepatocyte growth factor, stem cell factor and human epidermal growth factor receptor 2 concentrations were significantly elevated in samples with cancer metastasis ($p<0.05$) when compared to benign samples (Fig 3 and 4)

The concentrations of prolactin in three samples and concentrations of VEGFR-1 in two samples were found to be out of range and were rejected for stastical analysis in phase I. (Table 4)

Table 2: Phase I detail of lymph node station and histologic diagnosis

Patients	**Lymph node station**	**Histological diagnosis**	**Cancer Stage**
Patient 1	R4	Squamous cell Carcinoma	IIIA (lung)
Patient 2	R4 and 7	Adenocarcinoma	IIIA (esophageal)
Patient 3	L4	Adenocarcinoma (lung)	IIIA (lung)
Patient 4	R10, 7 and R11	Benign	
Patient 5	R4 L4	Benign Adenocarcinoma	IIIB →IV (lung)
Patient 6	R10 and R11	Squamous CA	(skin)
Patient 7	R12, R4 and L4	Benign	CT chest with resolution at 6 months
Patient 8	R4	Adenocarcinoma	IV (lung)
Patient 9	R4	Benign	(hamartoma)
Patient 10	7	Squamous cell Carcinoma	IIIB (lung)

Table 3: Phase I patient characteristics, based on histologic diagnosis

Variables	Patients	Diagnosis		p value
		Benign	Metastasis	
Total	10	3	7	
Male	7	2	5	
Female	3	1	2	
Age, years*	68.7 (9.8)	63.5 (10.4)	73 (7.3)	0.03
Smoking, years *	31.2 (20.45)	21.8 (23.7)	38.7 (14.6)	0.07
Quit Smoking, years*	28 (20.1)	31.5 (21.2)	25.2 (19.9)	0.52

* Mean and standard deviation

Table 4: Significant cytokines identified on analysis of 18 initial specimens (8 benign and 10 malignant)

Analytes	Benign, pg/ml (n=8)	Malignant, pg/ml (n=10)	p value**
uPA	*64 (42 - 96)	360 (85 -1117)	0.013
VEGF-A	1063 (732 – 1927)	7049 (1540 – 20863)	0.026
Follistatin	438 (304 – 563)	1213 (558 – 2110)	0.013
G-CSF	12.5 (9.3 – 20.6)	38.2 (27 – 54.3)	0.0077
HGF	1540 (1172 – 1826)	3670 (1842 – 11480)	0.016
Osteopontin	1704 (790 – 3754)	5344 (2558 – 38952)	0.0099
Prolactin[#]	1262 (932 – 1664)	2732 (2028 – 4467)	0.0038
SCF	75 (51 -103)	113 (89 – 192)	0.033
HER2/neu	2577 (1873 – 3292)	9036 (3362 – 13466)	0.013
VEGFR-1[$]	866 (520 – 1378)	9498 (2946 -18532)	0.0045

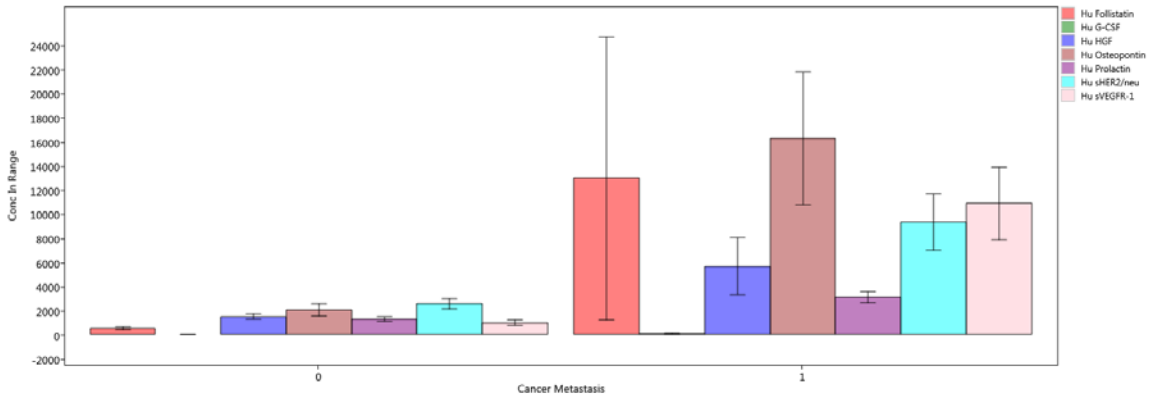
* Median and interquartile range (25th and 75th percentiles)

** Benferroni correction for multiple comparisons with statically significant p value of 0.0017 (34 cytokines)

[#] Prolactin, valid benign specimen (n=7) and valid malignant specimens (n=8)

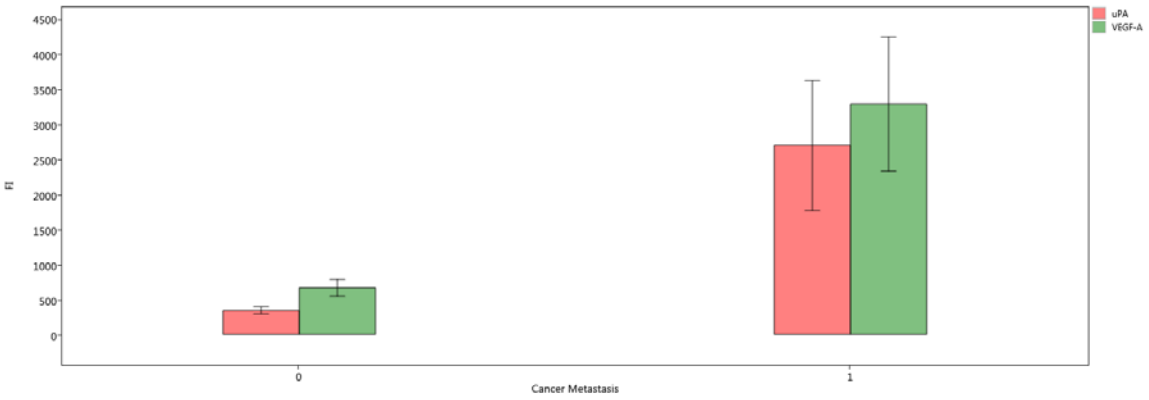
[$] VEGFR-1, valid malignant samples (n=8)

Figure 3: Phase I analysis with significant elevation of cytokines in human cancer panel 1*



*Standard error of the mean

Figure 4: Phase I analysis with significant elevation of cytokines in human cancer panel 2*



*Standard error of the mean

Phase II: After collection of an additional 36 FNA biopsy specimens, final analysis was performed using 54 mediastinal and hilar lymph node aspiration biopsy specimens collected from 28 eligible patients (19 men and 9 women, Table 1). Histologic diagnosis of metastatic cancer was established in 25 specimens (9 adenocarcinoma lung, 5 squamous cell carcinoma lung, 4 small cell carcinoma lung, 1 adenocarcinoma breast, and 2 each of adenocarcinoma colorectal and esophagus, and squamous cell cancer from skin). Twenty nine FNA biopsies were found to have a benign histologic diagnosis (1 hamartoma, 1 sarcoid, 2 granulomas and 8 mature lymphocytes). Patients with cancer had a higher pack year history of smoking than those with benign diagnoses (Table 5). The distribution of age and years of abstinence from smoking did not differ between the two groups.

The primary purpose of this approach was to validate the results of initially identified 10 cytokines in phase I. uPA, VEGF- A, follistatin, HGF, osteopontin, Her2/neu and VEGFR-1 cytokine concentration were again significantly elevated in metastatic versus benign lymph nodes (Table 6). Using a Benferroni correction value of 0.005 for 10 repeated comparisons; all 7 out of 10 cytokine concentrations were significantly different between the two groups with the greater concentrations found in the cancer group.

Table 5: Phase II patient characteristics, based on histologic diagnosis

Variables	Patients	Diagnosis		P value
		Benign	Metastasis	
Total	28	12	16	
Male	19	11	8	
Female	9	1	8	
Age, years*	64.2 (9.6)	62.4 (9.2)	65.6 (10.0)	0.39
Smoking, years *	25.8 (19.9)	17 (19.6)	32.3 (18.0)	0.04
Quit Smoking, years*	12.81 (18.23)	13.3 (18.1)	12.4 (18.8)	0.89

* Mean ± standard deviation

Table 6: Significant cytokines identified in 54 specimens (29 benign and 25 malignant)

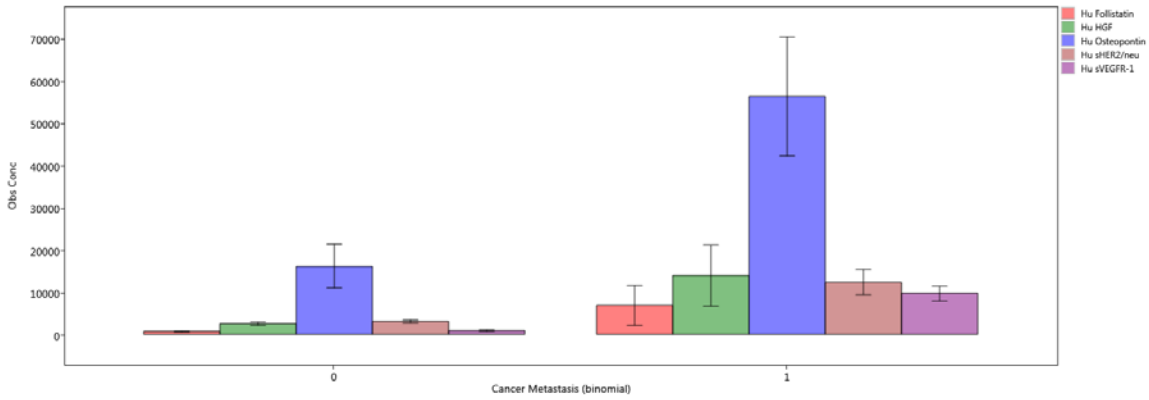
Analytes	Benign, pg/ml (n=29)	Malignant, pg/ml (n=25)	p value**
uPA	*158 (73 – 188)	442 (195 – 5091)	0.0000068
VEGF-A	488 (204 – 914)	12941 (1562 – 26720)	0.000000055
Follistatin	723 (438 - 937)	1216 (660 - 3961)	0.0012
HGF	2339 (1358 - 3742)	6190 (2298 – 10014)	0.00032
Osteopontin	5548 (1783 – 19186)	386230 (5344 – 70901)	0.00079
HER2/neu	3242 (1950 – 4007)	8198 (4130 – 13693)	0.0000040
VEGFR-1[#]	766 (445 – 1276)	9678 (2004 – 15120)	0.00000021

* Median and interquartile range (25^{th} and 75^{th} percentile)

** Benferroni correction for multiple comparisons with statically significant p value of 0.005 (ten cytokines identified on initial analysis)

[#] VEGFR-1, valid benign samples (n=25) and valid malignant samples (n=23)

Figure 5: Phase II analysis with significant elevation of cytokines in human cancer panel 1*



*Standard error of the mean

Figure 6: Phase II analysis with significant elevation of cytokines in human cancer panel 2*

*Standard error of the mean

Cytokine analysis in FNA biopsies non-small cell lung cancer metastasis (adenocarcinoma and squamous cell carcinoma) compared to benign lymph nodes.

To test an additional hypothesis that non-small cell lung cancer (NSCLC) metastasis to mediastinal and hilar lymph nodes express a unique cytokine signature, different from benign lymph nodes, a subgroup analysis was performed using data with NSCLC metastasis. Fourteen specimens with histology proven NSCLC diagnosis obtained from eight patients were compared to 29 benign lymph node aspiration biopsy samples, using the same approach as outlined for all types of cancer. Except for follistatin, which was no longer significant, the cytokine expression in lymph nodes with non-small cell lung cancer was identical to all cancer metastases after correction for Benferroni error (Table 7).

Table 7: Subgroup analysis, in specimens with non- small cell lung cancer

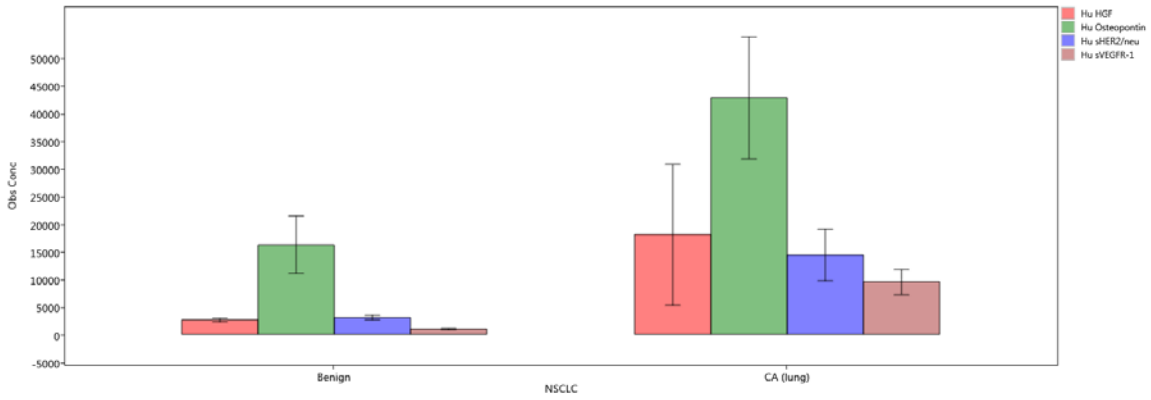
Analytes	Benign, pg/ml (n=29)	Malignant, pg/ml (n=14)	p value**
uPA	*158 (73 – 188)	625 (311 – 15415)	0.000000064
VEGF-A	488 (204 – 914)	15980 (1570 – 29991)	0.00000082
HGF	2339 (1358 – 3742)	5251 (2134 – 7241)	0.0034
Osteopontin	5548 (1783– 19186)	33669 (17844 -50215)	0.0014
HER2/neu	3242 (1950 – 4007)	10012 (4475 – 16765)	0.00062
VEGFR-1[#]	766 (445 – 1276)	8495 (2232 – 14904)	0.00022

* Median and interquartile range (25^{th} and 75^{th} percentiles)

** Benferroni correction for multiple comparisons with statically significant p value of 0.005

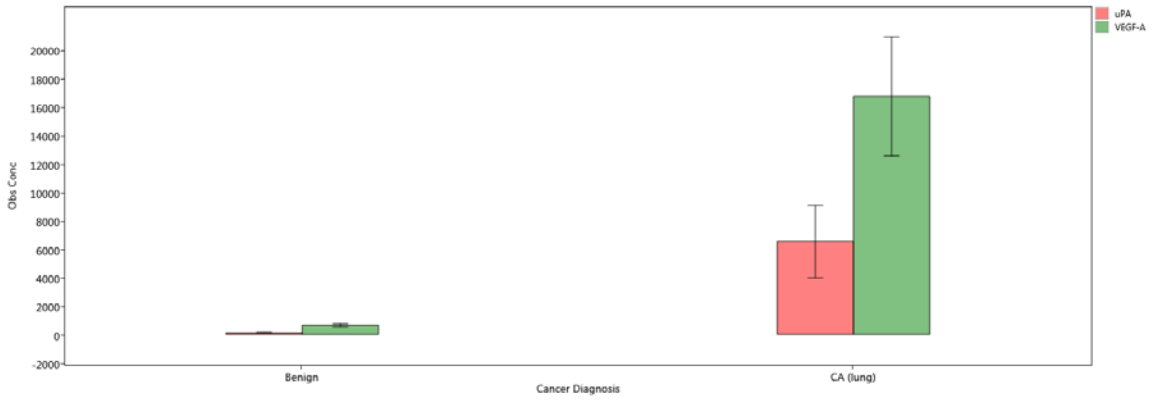
[#] VEGFR-1, valid benign samples (n=25) and valid malignant samples (n=12)

Figure 7: Phase II subgroup analysis with significant elevation of cytokines in human cancer panel 1*



*Standard error of the mean

Figure 8: Phase II subgroup analysis with significant elevation of cytokines in human cancer panel 2*



* Standard error of the mean

Discussion:

The data presented in the current study shows that the cytokine expression of lymph nodes with metastasis differs from a benign lymph node. It is important to understand the change in the microenvironment of a regional lymph node upon exposure to circulating cancer cells with varying metastasis potential. It is unknown why a lymph node initially acts as a barrier by providing a repulsive, antigenic response to the circulating cancer cells and then transitions by providing a nurturing microenvironment to another cancer cell from the same site allowing establishment of metastasis.

However, it is well known that a primary cancer site has a heterogeneous population of malignant cells (11), with different metastatic potentials dependent upon the mutations (deletions, translocations, and amplifications) (12, 13). Over the last two decades, scientists have investigated the role of cytokines expressed by cancer cells especially for angiogenesis, lymphangiogenesis but more importantly understand the cytokine interaction of cancer cells with surrounding mesenchymal cells, dendritic cells, macrophages, fibroblast and extracellular matrix (14). In this study we demonstrate that the inflammatory microenvironment, in terms of cytokine expression of lymph node with cancer metastasis, is very different from a benign lymph node. This allows for the opportunity to understand the pathophysiology at a molecular level and to develop novel cytokine-based strategies in preventing, delaying and even reversing metastasis.

The seed and soil theory initially proposed by Stephen Paget's in 1889 encompasses crosstalk between the circulating cancer cell and site (organ) of metastasis. Rodent models of cancer have revealed that certain tumor types tend to metastasize to specific organs independently of number of cancer cells, vascularity and blood flow. This finding confirms that site of metastasis is dependent not only on the metastatic potential of cancer cells but also the microenvironment of host tissue (15). Several clinical studies have shown that In certain tumors spread preferentially via the lymphatic route into the draining lymph nodes including malignant melanoma of skin, lung cancer, head and neck, breast, colorectal and squamous cell carcinoma of uterine cervix (14).

Modulations of certain cytokines are well understood in cancer metastasis. A cancer cell requires down regulation of e-cadherin in the primary tumor site to decrease cohesiveness with surrounding cells (16). It then abuts the lining of capillaries and lymphatics to gain entry into the circulation. Once in the lymphatic circulation, it enters into the medullary sinus, medulla and cortex through the afferent lymphatic of the regional lymph nodes (9). At this point the lymph node can mount a barrier response to the antigenicity of the cancer cell. Reactive follicular hyperplasia, sinus histiocytosis and even granuloma formation is commonly seen in the regional draining lymph nodes (9).

Up regulation of certain cytokines is associated with increased metastasis. Over expression of VEGF-C in patients with breast cancer is associated with enhanced metastasis into the draining lymph nodes and lung (17). VEGF-D induces angiogenesis and lymphangiogenesis in primary tumor. VEGF-D

expression on tumor cells enhances metastasis potential (18). Role of VEGF-A in promotion of lymph node angiogenesis remain unclear (19). Angiopoientin 1 and 2 expression is important in angiogenesis and are also found to play an important role in lymphangiogenesis and metastasis (20). Chemokine expression is often associated in enhanced lymphatic metastasis (21) and possibly acts as homing signal causing increased metastasis (22-25).

The current study focused on understanding cytokine expression in cancer metastasis in the lymph node (lung, breast, colon, skin and esophageal). In a subgroup analysis comparing cytokine expression of different cancers with metastasis to lymph nodes, we did not find significant difference in cytokine expression. This allows for generation of hypothesis that possibly lymph node behaves similarly for any cancer cell presenting to the lymph node, irrespective of the primary site of origin. On key limitation of the above stated hypothesis is the limited number of non-lung cancer biopsy specimens, as we also did not find statistical significant difference in cytokine expression of all cancer metastasis when compared to lung cancer. It is also unclear if the cytokines expression is from cancer cells or lymphoid cells. In the current study we have used the number of lymph node aspiration biopsy specimens “n” to perform stastical analysis. On an average we collected FNA- biopsy specimens from two different lymph node stations per patient while performing endobronchial ultrasound to diagnose or stage cancer. It is an ongoing debate if the number for analysis “n” should be patients and not lymph node station, there is mostly a correlation of histologic results. In the current study we identified two patents, one with breast

cancer and another with NSCLC, one lymph node station histology was positive for malignancy on FNA biopsy and other lymph node station (contralateral) histology without metastasis. On review of literature most cytokine analysis were performed using collected specimens as number of specimens for stastical analysis. The strengths of our study include a two phased approach that included validation, strict quality control, and application of Benferroni correction.

We anticipate that the above findings will help researchers to use FNA biopsy obtained using endobronchial ultrasound as a feasible research specimen to conduct cytokine expression studies to predict the lymph node's malignant potential. This will also provide opportunities for development of novel biomarkers for selection and response to current treatment regimens and possible development of novel drugs in prevention, delay and even reversal of metastasis in hilar and mediastinal lymph nodes.

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