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p38 MAP kinases: plausible diagnostic and prognostic serum protein marker of non small cell lung cancer

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ABSTRACT

Introduction: p38 MAPK signaling molecules plays a dual role in cancer, both progression and suppression. Elevated expression of p38α was reported in lung cancer tissue in rat model. Our objective was to explore the concentration of all 4 isoforms of p38MAPK in serum of Non Small Cell Lung Cancer (NSCLC).
Material and methods: The blood samples were collected from 77 NSCLC patients, 52 ethically matched healthy controls and 18 follow up patients were collected as some patients expired and some discontinued the treatment. The concentration of all isoforms of p38 (p38α, p38β, p38γ, and p38δ) were evaluated by Surface Plasmon Resonance (SPR) technology.
Result: The levels of all isoforms of serum p38 were significantly elevated at pre-therapy compare to control. Only p38α expression was significantly associated with tumor stage and its expression reduced after treatment which is then validated by western blot. However, no changes were observed in other isoforms after therapy.
Conclusion: Our study revealed that, p38α is more efficient among all the isoform to predict the disease accurately and it can be concluded that p38 MAPK may be used as diagnostic as well as prognostic marker of NSCLC disease.

1. Introduction

Lung cancer remains at the top of the list of the world's deadliest cancers and is the second greatest cause of morbidity and mortality worldwide (Jemal et al., 2011). Despite, advances in early detection and standard treatment, non small cell lung cancer is often diagnosed at an advanced stage and has a poor prognosis. Although smoking remains the primary cause of most lung cancers, the mechanism by which cigarette smoke spurs cancer development are not fully understood. The treatment and prevention of lung cancer are major unmet needs that can probably be improved by a better understanding of the molecular origins and evolution of the disease.

MAPKinase pathway participates in various intracellular signaling pathways that control at all round of cellular processes that include growth, differentiation and stress responses which have a key role in cancer progression. The study of signaling molecule expression, functional and therapeutic response during the cancer progression and treatment are becoming important day by day. p38MAP kinases contains four isoforms, i.e., p38α, p38β, p38γ, and p38δ, their roles in several cancers identified based on expression patterns, substrate specificities and sensitivity to chemical inhibitors. p38MAPK can be further divided into two groups (Wong, Todd, Tsuji, and Donoff, 1996). One group includes p38α and p38β, which are homologus and have overlapping functions. In contrast p38γ and p38δ have restricted expression patterns and probably have specialized functions (Cobb, 1999). In earlier studies, researchers explained that human serum proteins originate from different tissues and enter the circulation as a result of secretion and leakage (Taylor, 1969). The study of serum protein marker is an important area of research. In case of the lung cancer, exact mechanism and expression pattern of all isoforms of p38MAPK still not known. The present study attempted to evaluate expression level of all four isoforms of p38 MAPK kinase signaling molecules in serum of NSCLC disease for the development of specific protein biomarker and accounting their therapeutic importance.

2. Materials and method

2.1. Patients and controls selection

All the 77 NSCLC patients were recruited from the department of Pulmonary Medicine and Sleep disorders at All India Institutes of Medical Sciences (AIIMS), New Delhi, India and 52 ethically matched

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healthy controls recruited were invited for participation in study through the advertisement or general announcement at the hospital. Patients undergoing fiber optic bronchoscopy for suspected lung cancer disease with confirmed cytological/histological diagnosis of Non Small Cell Lung Cancer (NSCLC) were selected for the study. The study was approved by an ethics subcommittee of AIIMS (Ref. No. IESC/T-364/28.09.2012). All patients were given informed consent. The cases with a history of surgery and/or any other major disease were excluded from the study group. Disease staging was done according to the criteria adopted by the American Joint Committee of Cancer (AJCC). Clinical assessment done after 4 cycles of chemotherapy by CT scan according to RECIST Guideline Version 1.1. The blood samples of the patients were collected twice; first time at the time of diagnosis and then after 4 cycles of chemotherapy given as three-weekly cycles. Among 77 patients, 54 patients expired and 5 discontinued the treatment during the study period hence only the blood sample of 18 follow up patients were collected.

2.2. Treatment details

All study participants underwent a wide assessment that included a disease, medical history and complete physical examination. Staging and possible treatment options were decided by clinician in Lung cancer clinic of department of Pulmonary Medicine and Sleep disorder, AIIMS. Most of the NSCLC patients presented in advanced stage of the disease for 4 cycles of chemotherapy (Carboplatin/Paclitaxel) was given to each patient by intravenous route at three weekly intervals following standard protocol and blood sample was collected after two month of 4th cycle of chemotherapy.

2.3. Estimation of p38 MAPkinase concentration by surface plasmon resonance (SPR)

Label free real-time target bimolecular interactions were monitored by using BIAcore 3000 (Pharmacia Biosensor AB, Sweden). Mouse anti-human p38 monoclonal IgG, Goat anti-human p38β monoclonal IgG, mouse anti-human p38γ monoclonal IgG and mouse anti-human p38δ monoclonal IgG (Santa Cruz Biotechnology, CA, USA) were immobilized on the CM5 sensor chip by using an amine coupling kit (Wipro GE healthcare AB, Sweden) on four different flow cells by amine coupling method (Gill, Mohanti, Ashraf, Singh, and Dey, 2012). To generate standard curve, pure recombinant proteins p38α, p38β, p38γ and p38δ were cloned, expressed, purified and characterized for this study.

Standard graphs were prepared by passing different known concentration of pure p38α (0.1, 0.2, 0.3, 0.6, 1, 1.8 ng/μL), p38β (0, 1.6, 4, 8, 16, 24 and 48 ng/μL), p38γ (0.30, 0.76, 1.52, 3.05, 4.58, 6.1, 9.17 ng/μL) and p38δ (0.48, 0.97, 2.42, 4.8, 5.8, 9.7, 14.5 and 19.4 ng/μL) over respective flow cells of sensor chip.

Serum samples of all the participants were diluted (1.4:98.6 μl) with HBS-EP buffer and allowed to run over immobilized antibodies. The concentration of p38α, p38β, p38γ and p38δ of study groups were determined from respective standard curves.

2.4. Estimation of p38 level in serum by Western blot

Western blot experiment was done for p38α protein. Serum samples collected from 3 control, 3 NSCLC pre chemo, 3 post chemo patients were subjected to removal of albumin using Albumin out kit (G-Biosciences USA). Total protein concentration was determined using a Bicinchoninic acid assay (BCA) using bovine serum albumin as standards. Western blot experiment was done by using primary and secondary antibodies by standard protocol (Gill, Mohanti, Ashraf, Singh, and Dey, 2012). The PVDF membrane was developed by Enhanced Chemiluminescent System (PierceECL Western Blotting Substrate, Thermo Scientific, Rockford, IL) and density of band obtained were determined using myImageAnalysis™ Software(Thermo fisher scientific).

2.5. Statistical analysis

For Statistical analysis Graph Pad Prism version 5.0 (La Jolla, CA, USA) was used. Descriptive analysis was done for all variables and with percentages or mean (95% confidence interval) as appropriate. Baseline comparison between NSCLC and control population was done using appropriate statistical tests; Student unpaired *t*-test for comparison of 2 categories. ROC curves were constructed to determine cut-off value for proteins. Statistical significance p value of < 0.05 was considered.

3. Results

3.1. Demographic data of study groups

The demographic data of 77 NSCLC patients are illustrated among the NSCLC patients. Majority NSCLC patients were male (83.1%) as compared to female (16.9%). Fifty two healthy subjects participated in this study, in which 43 (82.7%) were males and female were 9 (17.3%). The majority (75.3%) of patients were active tobacco chewers or smokers. The major site affected in NSCLC was right upper lobe (33.7%). Majority of patient had stage IV disease (63.6%), followed by stage III (31.2%) and stage II (5.2%).

3.2. Immobilization of p38 MAP kinase antibody

Standard curve were s plotted with RU obtained by passing different concentrations of purified p38α, p38β, p38γ and p38δ proteins over the sensor chip. The binding of the proteins; p38 MAPK, was in the linear range.

The response unit (RU) of immobilized antibodies; p38α, p38β, p38γ and p38δ were 4468, 5728, 2354, and 7169 RU, respectively; where as 1 RU corresponds to 1 pg.

3.3. Quantitative analysis of p38 MAPK isoforms in study group by SPR

Levels of p38α, p38β, p38γ and p38δ were significantly lower in controls subjects as compared to the NSCLC as shown in Table 1.

The concentration of p38α, p38β, p38γ, p38δ protein in serum were significantly ($p < .0001$) higher at pre therapy of patients 0.4625 ± 0.1567 ng/μl, 3.819 ± 0.5965 ng/μl, 1.844 ± 0.6156 ng/μl and 2.911 ± 1.141 ng/μl as compared to controls 0.1718 ± 0.09440 ng/μl, 3.331 ± 0.3401 ng/μl, 1.422 ± 0.3233 ng/μl and 2.147 ± 0.66 ng/μl, respectively (Fig. 1).

The concentration of p38α was increased significantly with the stage of disease stage II, III, IV (Fig. 2). However, no significant difference appeared in case of other isoforms with cancer stage.

ROC curves were generated for each protein between control and patient. Based on the SPR data, the area under the ROC curves was calculated to measure the utility of protein for detecting NSCLC based on the distribution of specificities and sensitivities. It can be stated that at a cut off value of ≥0.29 ng/μl can detect NSCLC with sensitivity of 89.6% and specificity of 90.4%. Similarly, for p38β, a threshold value of ≥3.50 ng/μl (sensitivity of 63.6% and specificity of 69.2%), for p38γ and p38δ ≥1.55 ng/μl, ≥2.54 ng/μl with sensitivity and specificity were 57.9%, 59.7% and 62.3%, 65.4%, respectively (Fig. 3) However; the diagnostic efficiency of p38α to detect NSCLC was higher among all other p38 MAPK isoform.

3.4. Estimation of expression level of p38 MAPK protein after therapy

Clinical and experimental assessment was performed after 4 cycles of chemotherapy. Response evaluation was done by repeating CT or PET scan at the end of four chemotherapy cycles. Response was

Table 1
Concentration of p38α, β, γ and δ protein represented mean and standard deviation with different attributes.

Attributes	p38 α(ng/μl)	p	p38β (ng/μl)	p	p38γ (ng/μl)	p	p38δ (ng/μl)	p
Controls (n = 52)	0.1718 ± 0.09440	0.0001	3.331 ± 0.3401	0.0001	1.422 ± 0.3233	0.0001	2.147 ± 0.66	0.0001
NSCLC(n = 77)	0.4625 ± 0.1567		3.819 ± 0.5965		1.844 ± 0.6156		2.911 ± 1.141	
Males (64)	0.4599 ± 0. 1575	0.7484	3.850 ± 0.5841	0.319	1.873 ± 0.7042	0.357	2.972 ± 1.081	0.306
Females(13)	0.4753 ± 0.1584		3.668 ± 0.6577		1.700 ± 0.5979		2.614 ± 1.412	
Age								
30–50(18)	0.4998 ± 0.2028	0.353	4.174 ± 0.6367	0.009	1.974 ± 0.7122	0.591	3.144 ± 1.220	0.591
51–70(47)	0.4415 ± 0.1446		3.677 ± 0.5332		1.797 ± 0.5884		2.812 ± 1.176	
> 70(12)	0.4848 ± 0.1211		3.831 ± 0.5885		1.829 ± 0.5908		2.94 ± 0.9093	
Habits								
Smoker(58)	0.4475 ± 0.1634	0.142	3.760 ± 0.5891	0.129	1.822 ± 0.6211	0.592	2.875 ± 1.118	0.633
Non Smoker(19)	0.5083 ± 0.1273		4.000 ± 0.5978		1.910 ± 0.6101		3.020 ± 1.232	
Tumor size								
T1 + T2(16)	0.4739 ± 0.1677	0.610	3.862 ± 0.6468	0.667	1.872 ± 0.7412	0.482	3.039 ± 1.366	0.875
T3(18)	0.4301 ± 0.1924		3.708 ± 0.5458		1.690 ± 0.5420		2.845 ± 1.077	
T4(43)	0.4718 ± 0.1373		3.850 ± 0.6057		1.898 ± 0.5976		2.892 ± 1.100	
Stage								
II(4)	0.3227 ± 0.1235	0.003	3.369 ± 0.2386	0.246	1.568 ± 0.4720	0.412	2.829 ± 0.8553	0.412
III (24)	0.3993 ± 0.1626		3.780 ± 0.5828		1.759 ± 0.6803		2.671 ± 1.156	
IV(49)	0.5048 ± 0.1408		3.876 ± 0.6133		1.908 ± 0.5917		3.035 ± 1.153	
Nodes								
N0(24)	0.4644 ± 0.1769	0.943	3.878 ± 0.6790	0.561	1.909 ± 0.7092	0.539	2.986 ± 1.404	0.702
N+ (53)	0.4616 ± 0.1485		3.793 ± 0.5601		1.815 ± 0.5732		2.878 ± 1.013	
Histopath								
SCC (48)	0.4303 ± 0.1670	0.019	3.699 ± 0.4812 8	0.021	1.771 ± 0.6298	0.180	2.709 ± 1.108	0.022
Adeno(29)	0.5158 ± 0.1425		4.018 ± 0.7150		1.965 ± 0.5816		3.247 ± 1.133	
Metastasis								
M0(34)	0.4182 ± 0.1507	0.008	3.737 ± 0.5820	0.317	1.744 ± 0.6073	0.219	2.657 ± 1.064	0.0891
M+(39)	0.5145 ± 0.1507		3.880 ± 0.6290		1.925 ± 0.6325		3.119 ± 1.203	
Mx(4)	0.33218724		3.930249172		1.909599075		3.047118785	

Abbreviation: NSCLC: non small cell lung cancer, N0: node absent, N+: node present. SCC: squamouse cell carcinoma, Adeno: adenocarcinoma, M0: no metastasis, M +: metastasis present, Mx: not known.

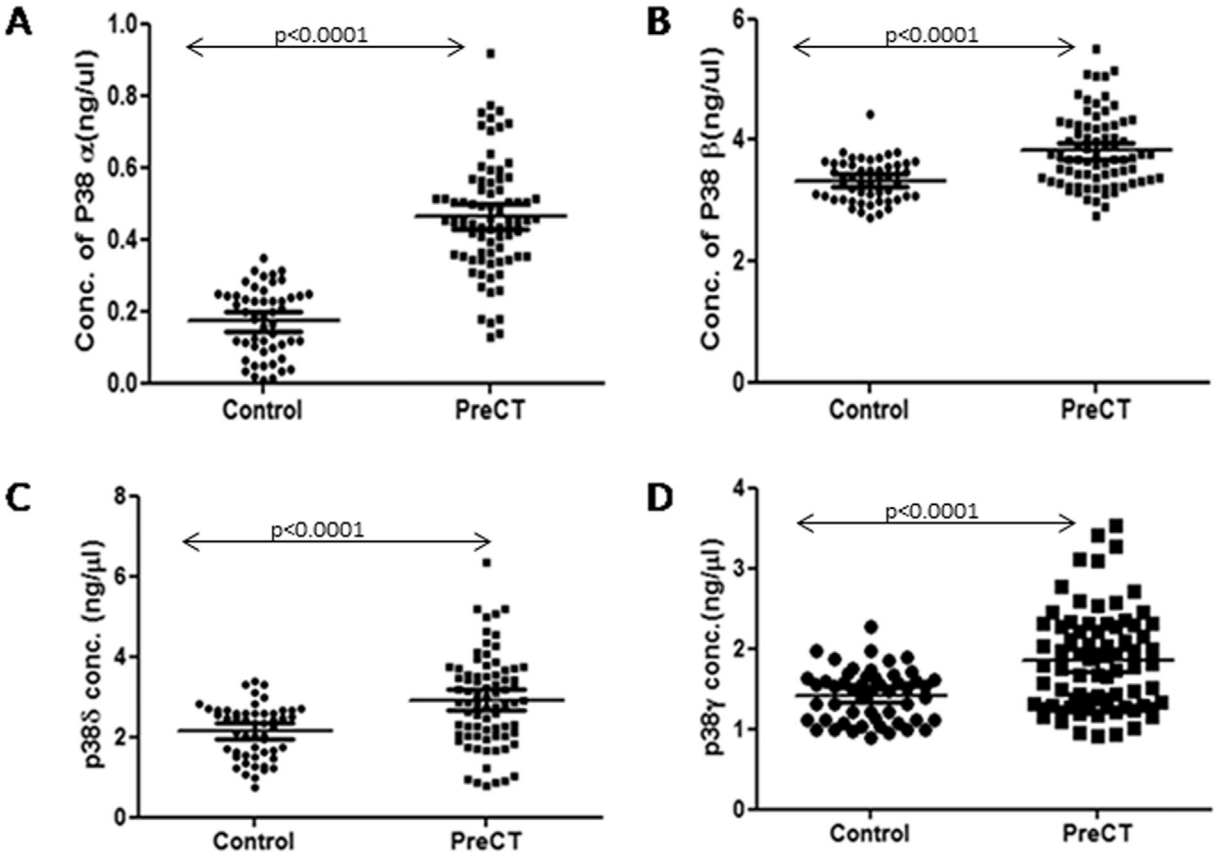

Fig. 1. Scatter graph showing the concentration of serum p38 α (A) p38β (B) p38γ (C) p38δ (D) in healthy controls and NSCLC subjects at the time of pre chemotherapy(Pre CT).

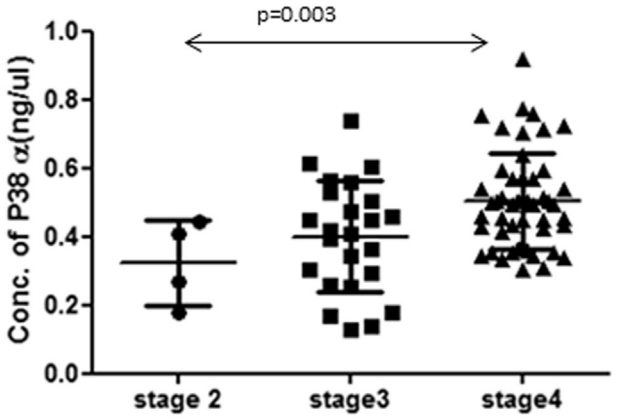

Fig. 2. Scatter graph showing the concentration of serum p38 α at different stage of Non Small Cell Lung Cancer.

classified as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) based on the basis of RECIST 1.1 criteria. Among the 18 follow up patients, the concentration of p38α was 0.4218 ± 0.1387 ng/μl at the time of pre chemotherapy and decreased significantly, 0.2643 ± 0.1138 ng/μl after chemotherapy. Out of 18 post therapy patients, 11 in the group of CR + PR, 7 in SD + PD. The mean value of p38α at pre chemo and post chemo of CR + PR group of patients were 0.3894 ± 0.1637 ng/μl and 0.2311 ± 0.1192 ng/μl, respectively. While in case of other isoform p38 β, γ, δ no significant changes appear after therapy as mentioned in Table 2.

3.5. By Western blot

SPR data clearly shows that p38α is one of the important molecule of NSCLC disease which elevated with the disease and its expression gets down regulated along with chemotherapy. The Western blot experiment in serum samples was performed to validate the differential expression of p38α in healthy controls, pre-chemotherapy and post-chemotherapy patients. The result was clearly consistent with SPR result; band densities in pre therapy patients were more prominent as compared to post therapy and healthy control individuals (Fig. 4).

4. Discussion

Lung cancer is one of the prominent cancer deaths in India. Almost 75% of the lung cancer patients are detected in the late stage. Better understanding of the molecular pathology of lung cancer may help in the improvement of diagnosis and development of targeted therapies. Presently used biomarkers for lung cancer for clinical diagnosis are not very efficient biomarker due to their poor sensitivity and specificity. The potent sensitive and specific molecular markers related to the pathophysiology with the disease are essential for early detection of lung cancer.

The importance of the p38 MAPK signaling pathway involved in cancer has been increasingly accepted (Liu, He, and Yang, 2015; Gonzalez-Villasana et al., 2015). Its diverse role is still contradictory, in cancers (Cuenda and Rousseau, 2007; Rennefahrt, Janakiraman, Ollinger, and Troppmair, 2005; Loesch and Chen, 2008). p38 MAPK has been reported to induce apoptosis in some cells, but prevent apoptosis in others (Chen et al., 2009). Conversely, a number of studies implicated p38 MAPK play a negative regulator for cell proliferation (Grossi, Peserico, Tezil, and Simone, 2014; Tai, Su, Chen, Jou, and Lin,

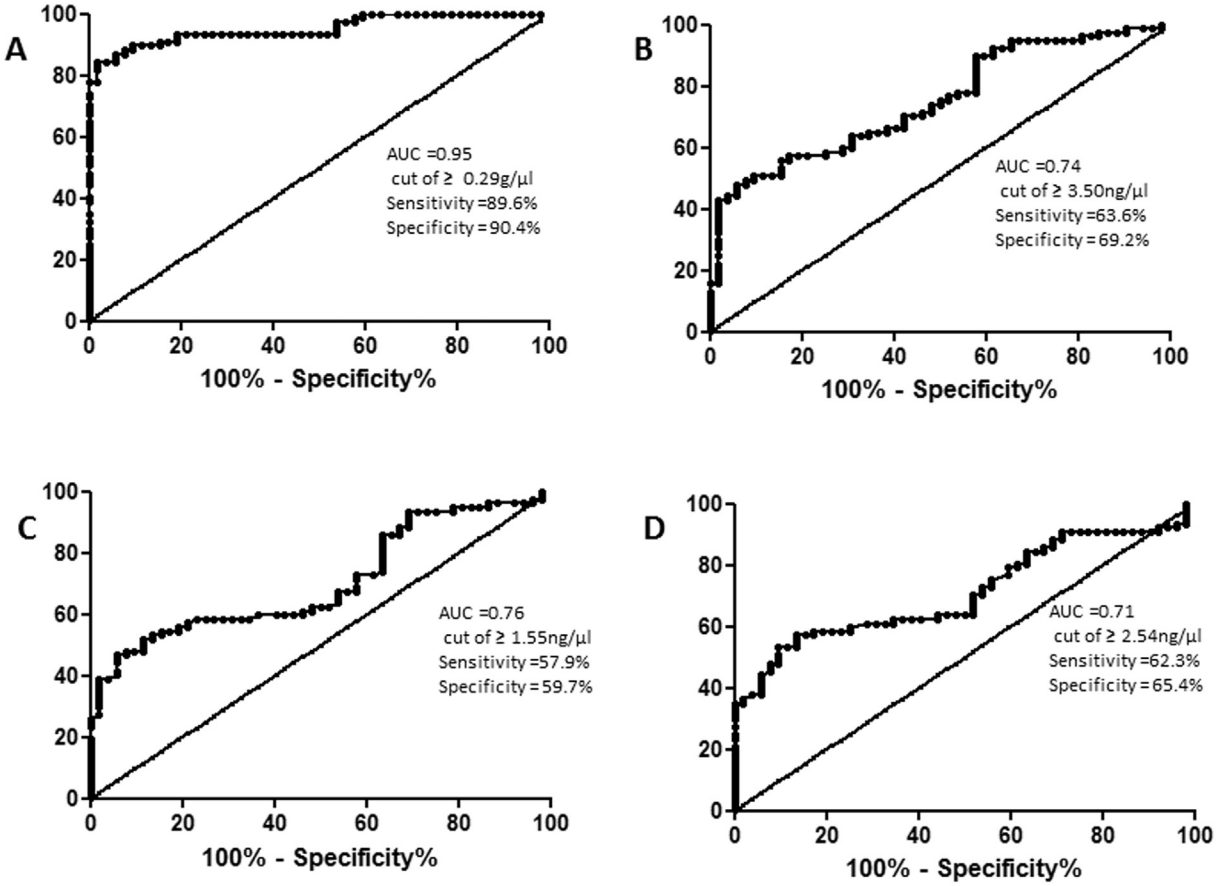

Fig. 3. ROC analysis showing the area under curve for p38α (A) p38β (B) p38γ (C) p38δ (D) to distinguish NSCLC to healthy control subjects.

Table 2
Concentration of p38α, β, γ and δ protein represented as mean and standard deviation of post treatment patients with different attributes.

Treatment response	p38α	PCT	p	p38β	PCT	p
p38(18)	0.4218 ± 0.1387	0.2643 ± 0.1138	< 0.0001	3.998 ± 0.5353	3.732 ± 0.3962	0.0344
CR + PR (11)	0.3894 ± 0.1637	0.2311 ± 0.1192	0.0004	4.088 ± 0.6022	3.730 ± 0.4522	0.0511
SD + PD (7)	0.4727 ± 0.07020	0.3165 ± 0.08839	0.0017	3.858 ± 0.4121	3.736 ± 0.3223	0.4563
Stage						
3 (8)	0.3497 ± 0.1615	0.1897 ± 0.1048	0.0156	3.906 ± 0.6790	3.579 ± 0.4049	0.1733
4 (10)	0.4795 ± 0.08808	0.3239 ± 0.08382	0.0039	4.072 ± 0.4112	3.855 ± 0.3622	0.1055
Tumor						
T2 (5)	0.3868 ± 0.1509	0.2354 ± 0.07645	0.125	3.722 ± 0.3818	3.618 ± 0.2989	0.625
T3 (4)	0.3973 ± 0.1769	0.2031 ± 0.1140	0.0156	3.970 ± 0.5179	3.377 ± 0.4613	0.25
T4 (9)	0.4522 ± 0.1252	0.3075 ± 0.1234	0.0078	4.164 ± 0.5973	3.954 ± 0.2877	0.25
Node						
N0 (6)	0.4167 ± 0.06341	0.2664 ± 0.1167	0.0625	4.058 ± 0.6263	3.871 ± 0.4420	0.6875
N+ (12)	0.4244 ± 0.1670	0.2632 ± 0.1176	0.001	3.968 ± 0.5115	3.663 ± 0.3716	0.0522
Metastasis						
M0 (10)	0.4281 ± 0.1764	0.2565 ± 0.1209	0.0039	4.118 ± 0.6315	3.701 ± 0.4171	0.0371
M+ (6)	0.4404 ± 0.05829	0.3086 ± 0.09557	0.0625	3.857 ± 0.3635	3.694 ± 0.3578	0.3125
Habit						
Non smoker (4)	0.4333 ± 0.01990	0.2730 ± 0.04620	0.125	3.986 ± 0.3510	3.835 ± 0.3782	0.625
Smoker (12)	0.4185 ± 0.1582	0.2618 ± 0.1281	0.0005	4.002 ± 0.5885	3.703 ± 0.4100	0.0676

Treatment response	p38γ	PCT	p	p38δ	PCT	p
p38 (18)	2.005 ± 0.5940	1.948 ± 0.2487	0.6877	3.046 ± 1.164	2.869 ± 1.121	0.571
CR + PR (11)	1.977 ± 0.5989	1.829 ± 0.2378	0.4329	3.352 ± 0.8339	2.652 ± 0.9797	0.0615
SD + PD (7)	2.050 ± 0.6311	2.135 ± 0.1204	0.7221	2.566 ± 1.497	3.209 ± 1.318	0.2178
Stage						
3 (8)	1.780 ± 0.6761	1.833 ± 0.2405	0.8438	2.959 ± 1.297	2.434 ± 1.138	0.3125
4 (10)	2.186 ± 0.4792	2.040 ± 0.2250	0.4922	3.116 ± 1.113	3.216 ± 1.031	0.7695
Tumor						
T2 (5)	1.967 ± 0.8943	1.967 ± 0.3216	1	3.150 ± 1.285	3.412 ± 0.8858	0.8125
T3 (4)	1.829 ± 0.6641	2.035 ± 0.1319	0.625	2.856 ± 1.701	2.404 ± 1.540	0.7076
T4 (9)	2.105 ± 0.3960	1.934 ± 0.2605	0.3008	3.074 ± 0.9708	2.773 ± 1.041	0.6523
Node						
N0 (6)	1.998 ± 0.5196	1.913 ± 0.2738	0.8438	3.024 ± 1.157	3.135 ± 1.299	0.6875
N+ (12)	2.009 ± 0.6500	1.965 ± 0.2460	0.8501	3.058 ± 1.219	2.735 ± 1.057	0.4697
Metastasis						
M0 (10)	1.967 ± 0.5871	1.897 ± 0.2020	0.8457	3.165 ± 1.121	2.567 ± 1.128	0.2324
M+ (6)	2.157 ± 0.6380	2.084 ± 0.2368	1	2.926 ± 1.440	3.373 ± 1.028	0.5625
Habit						
Non smoker (4)	1.995 ± 0.4869	2.008 ± 0.1677	1	2.547 ± 1.359	2.861 ± 1.500	0.625
Smoker (12)	2.008 ± 0.6377	1.931 ± 0.2700	0.6698	3.189 ± 1.117	2.871 ± 1.060	0.391

Abbreviation CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease, PCT: post chemotherapy.

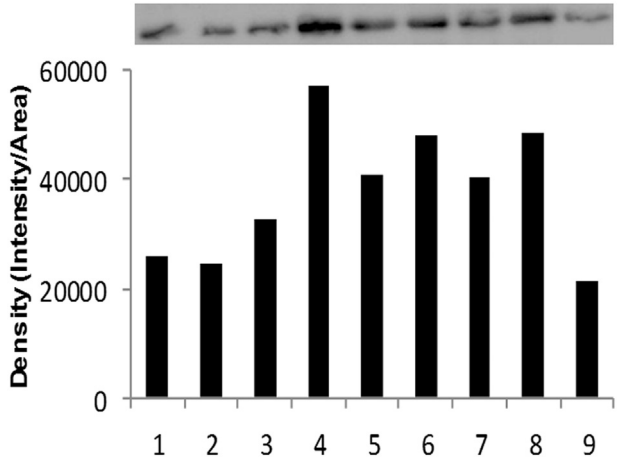

Fig. 4. Western blot and density analysis showing levels of p38α in the study group of patients and controls. Lanes (1–3) controls, lanes (4–6) pre chemo patients, lanes (7–9) post chemo patients.

2014).

p38 α is abundant in most cell types and is the best-described isoform in the literature and reported to be over-expressed in many cancers like oral (Riebe, Pries, Kemkers, and Wollenberg, 2007) breast (Davidson et al., 2006), gastric (Liang et al., 2005) and non small lung cancer (Greenberg et al., 2002).

The expression of p38β, γ and δ were also appeared to be higher in specific tissues. p38β expression was abundant in the brain tissue. The elevated level of p38β has been reported in serum of human pancreatic cancer patients and also enhanced bone metastasis in breast cancer (Singh et al., 2012; He et al., 2014).

The expression of p38γ was found to be more in skeletal muscle tissues (Cuenda and Rousseau, 2007). Over expressions of p38γ were also detected in colon and breast cancer (Meng and Wu, 2013).

In cancers, increased p38δ expression has been detected in human primary cutaneous SCCs, (Haider et al., 2006) and enhanced activation of p38δ has been observed in NSCLC cells lines as well as in HNSCC tumors (Junttila et al., 2007). The role of p38δ was found in cholangiocarcinoma (CC) and Liver malignancy (Tan et al., 2010). P38δ has been reported to regulate HNSCC and CC invasion (Junttila et al., 2007; Tan et al., 2010). Therefore, specifying and to know the contributions of each specific p38 isoform would allow more precise targeting of specific subsets of cancer. Previously our group has reported the elevated p38α protein levels in the serum of HNSCC patients, which decline after radiotherapy (Gill, Mohanti, Ashraf, Singh, and Dey, 2012). In the present study, we first time found significant overexpression of serum p38α, p38β, p38γ, and p38δ in NSCLC patients comparison to normal controls, in which p38α expression was significantly associated with tumor stage and its expression reduced after

treatment.

However, other isoforms p38β, p38δ, and p38γ significant elevated level were also associated with NSCLC disease as compared to controls but did not find any correlation with stage, tumor and treatment response.

This study being the first time to comprehensively provided evidences concerning the role of p38α, p38β, p38γ, and p38δ in the NSCLC. Our data may have important implications for understanding the significant role of all different isoforms of p38 MAPK in NSCLC for early stage detection and therapeutic target.

In conclusion, among all four p38 MAPK, p38α was found to be associated with overall stage specific and prognosis of NSCLC disease and higher levels were associated with disease condition. The area under curve obtained from ROC.

analysis for p38α is more efficient among all the isoform to predict the disease accurately. The present study may open up the view to develop these signaling proteins as serum marker for NSCLC for diagnostic, prognostic and therapeutic value for cancer prevention.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgements

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