RESEARCH ARTICLE

Down-regulation of Protease-activated Receptor 4 in Lung Adenocarcinoma is Associated with a More Aggressive Phenotype

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Abstract

The role of protease-activated receptors (PARs) in lung tumors is controversial. Although PAR4 is preferentially expressed in human lung tissues, its possible significance in lung cancer has not been defined. The studies reported herein used a combination of clinical observations and molecular methods. Surgically resected lung adenocarcinomas and associated adjacent normal lung tissues were collected and BEAS-2B and NCI-H157 cell lines were grown in tissue culture. PAR4 expression was evaluated by RT-PCR, RT-qPCR, Western blotting and immunohistochemistry analysis. The results showed that PAR4 mRNA expression was generally decreased in lung adenocarcinoma tissues as compared with matched noncancerous tissues (67.7%) and was associated with poor differentiation (*p*=0.017) and metastasis (*p*=0.04). Western blotting and immunohistochemical analysis also showed that PAR4 protein levels were mostly decreased in lung adenocarcinoma tissues (61.3%), and were also associated with poor differentiation (*p*=0.035) and clinical stage (*p*=0.027). Moreover, PAR4 expression was decreased in NCI-H157 cells as compared with BEAS-2B cells. In conclusion, PAR4 expression is significantly decreased in lung adenocarcinoma, and down-regulation of PAR4 is associated with a more clinically aggressive phenotype. PAR4 may acts as a tumor suppressor in lung adenocarcinoma.

Keywords: PAR4 - down-regulation - tumor suppressor - lung cancer - adenocarcinoma

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Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, causing more deaths than breast, prostate and colon cancers combined (Siegel et al., 2012). Furthermore, morbidity and mortality attributed to lung cancer is currently on the rise in China (Zhang et al., 2003). Because early screening strategies are not available for this malignancy, lung adenocarcinoma patients typically present at an advanced stage at the time of diagnosis, and there has been little improvement in lung cancer survival over the past 3 decades. There are 2 main types of lung cancer, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for approximately 85% of all lung cancer. Of the total, adenocarcinoma has remained the most prevalent lung cancer subtype among women over the past 3 decades, and has surpassed squamous cell carcinoma as the leading subtype of lung cancer in men in the world (Toh, 2009).

Protease-activated receptors (PARs) are multifunctional G protein–coupled receptors which are activated by specific proteolytic cleavage within the extracellular N terminus. The cleavage event unmasks a new N-terminal sequence, which acts as a peptide ligand by intramolecular binding to the body of the receptor, leading to the activation of transmembrane signaling (Trejo, 2003). Four PAR members (PAR1, PAR2, PAR3 and PAR4) have been cloned and characterized in many animal species. The chromosomal location of the PAR4 gene (19p12) is different from that of the PAR1 and PAR2 genes (5q13) (Kahn et al., 1996). Recently, several studies have suggested that PARs, especially PAR1 and PAR2, might exert a dual role in tumorigenesis (Vergnolle et al., 2001; Elste et al., 2010), as they were found to be overexpressed in several malignant tumors and implicated in tumor growth and cancer metastasis (Darmoul et al., 2001; Darmoul et al., 2003; DorsamGutkind 2007). However, other studies showed an opposite effect of PARs in breast, prostate and gastric cancers (Ahmad et al., 2000; Huang et al., 2000; Zhang et al., 2011). PAR3 and PAR4 have been less studied than PAR1 and PAR2.

PAR4 is preferentially expressed in human lung tissues (Lan et al., 2002), and mainly present in epithelium of lower bronchus, type II pneumocytes, endothelial cells

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and also in cultured lung epithelial cells (Nelken et al., 1992; Xu et al., 1998; Saifeddine et al., 2001). The physiological and potential pathological functions of PAR4 in lung epithelial cells have yet to been defined, and studies investigating the role of PAR4 in cancer have had conflicting results (Rullier et al., 2006; Kaufmann et al., 2007; Elste et al., 2010). Our research was aimed at resolving these issues, by investigating PAR4 expression in primary lung adenocarcinomas as well as cell culture models. The results suggest that PAR4 may act as a tumor suppressor in lung adenocarcinoma, and that the extent of down-regulation of PAR4 is correlated with cancer progression.

Materials and Methods

Patients and samples

Ethical approval for this study was obtained by the Ethical Committee of Kunming Institute of Zoology, the Chinese Academy of Sciences. The lung cancer and adjacent normal lung tissues from 34 lung adenocarcinoma patients were received from the First Affiliated Hospital of Kunming Medical College in 2009 through 2011 and confirmed by pathological assessment by two trained pathologists. Of these patients, 23 were men and 11 were women; their ages ranged from 32 to 83 years. Immediately after removal, all tissues for molecular analysis were put in liquid nitrogen and stored at −80°C until use. Lung adenocarcinoma tissue microarrays representing 75 lung cancers with their non-neoplastic resection margins were constructed according Kononen et al. (1998), and were manufactured at Shanghai Outdo Biochip Center (Shanghai, China). The follow-up data including cancer-free survival was obtained for 56 patients.

Cell culture

BEAS-2B human lung epithelial cells and NCI-H157 lung adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). BEAS-2B cells were grown in GIBCO® LHC serum-free media. NCI-H157 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 containing 10% fetal calf serum (FCS). The cells were grown on plastic culture plates at 37°C in a humidified 5% CO_2 incubator until the cells reached confluence prior to analysis.

RNA extraction and polymerase chain reaction (PCR)

Total RNA from the tissue samples was extracted using the RNeasy Mini Kit (Qiagen; Hilden,Germany) with DNase I treatment. Total RNA from BEAS-2B and NCI-H157 cells was isolated using TRI Reagent (Molecular Research Center, Inc., USA) following the manufacturer's protocol. First-strand cDNA synthesis on the extracted RNAs was performed as previously described (Liu et al., 2008). For semi-quantitative reverse transcription PCR(RT-PCR), the primers used were as follows: PAR4:Forward: 5'-GGCAACCTCTATGGTGCCTA-3'; Reverse: 5'-TTCGACCCAGTACAGCC-TTC-3' (244 bp product); glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Forward: 5'-TCGGAGTCAACGGATTTGGTCGTA-3'; Reverse: 5'- AGCCTTCTCCATGGTGGTGAAGA-3' (321 bp product). Amplicons were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and viewed under ultraviolet illumination. The identity of the PCR product was confirmed by DNA sequencing. Real-time quantitative PCR (RT-qPCR) was performed with a continuous fluorescence detector (Opticon Monitor, Bio-Rad, iQ5, Hercules, CA, USA). The PCR reaction was carried out using a SYBR Green real-time PCR kit (TaKaRa, Dalian, China) with the following conditions: initial denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 15 s, 65°C for 15 s, 72°C for 20 s. Oligonucleotide primers used were forward 5'-CCTTCATCTACTACTACTACGTGTCG-3' and reverse 5'-ACTGGAGCAAAGAGGAGTGG-3' for PAR4 (147 bp); forward 5'-ATGGGGAAGGTGAAGGTCG-3', reverse 5'-GGGGTCATTGATGGCAACAATA-3' for GAPDH (107 bp) as internal standard.. The identity of the PCR products was confirmed by DNA sequencing. RT-qPCR for each gene was performed in triplicates in at least three separate amplifications. No template controls (no cDNA in PCR) were run to detect unspecific or genomic amplification and primer dimerization. Signal intensity of PAR4 was then quantitated by Optical systern software (Bio-Rad iQ5, Hercules, CA) and normalized to oragainst glyceraldehyde-3-phosphate dehydrogenase signal intensity. Fluorescence curve analysis was carried using Opticon Monitor software. Relative quantitative evaluation of PAR4 levels were performed using E-method and expressed as a ratio of the transcript of PAR4 to GAPDH in the tumor tissue divided by a similar ratio in the non-neoplastic tissue of the same patient.

Tissue immunohistochemistry

Five micron thick sections were obtained from the lung adenocarcinoma tissue microarray, fixed on glass slides, dewaxed in xylene, dehydrated in ethanol, washed 3 times with distilled water, and then treated for 5 minutes in 10 mmol/L citrate buffer solution (pH 6) in an autoclave at 121°C, then cooled at room temperature and rinsed in phosphate-buffered saline (PBS). Sections were pre-incubated with 2% normal goat serum to block nonspecific binding of antibodies for 30 minutes and then incubated overnight with anti-human PAR4 antibody (C-20, 1:1200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C. Specific binding was detected using a streptavidin–biotin-peroxidase assay kit (Maxim, Fujian, China). The section was counterstained with Harris hematoxylin. Direct microscopic micrographs were captured using a Leica DFC320 camera controlled by Leica IM50 software (Leica, Germany). Sections incubated with normal goat IgG served as negative controls. Immunohistochemical staining was assessed semi-quantitatively by measuring both the intensity of the staining (0, 1, 2, or 3) and extent of staining (0, 0%; 1, 0–10%; 2, 10–50%; 3, 50–100%). The scores for the intensity and extent of staining were multiplied to give a weighted score for each case (maximum possible, 9). For the statistical analysis, the weighted scores were grouped into two categories where scores of 0–3 were considered negative and 4–9 positive (Moss et al., 2008).

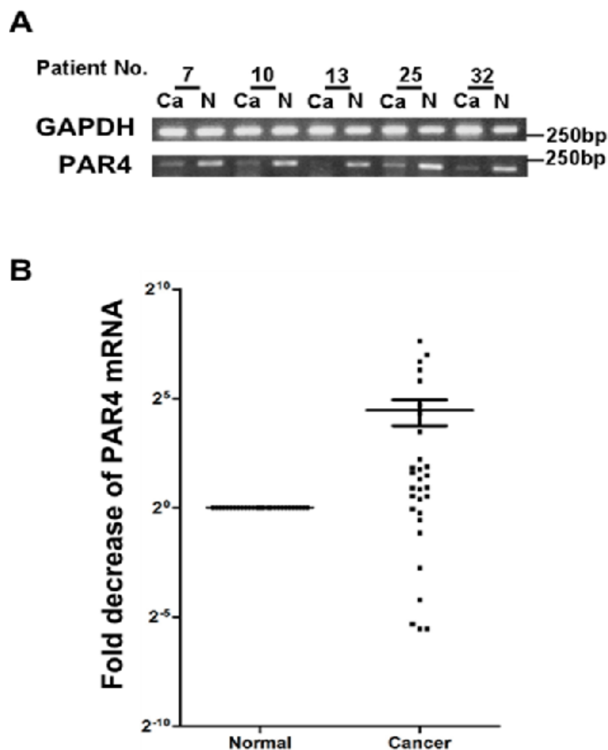

Figure 1. Expression of PAR4 mRNA in Lung Adenocarcinomas Compared with Non-neoplastic Lung Tissues. A. The matched normal (N) and cancerous (Ca) tissues from each patient were analyzed by RT-PCR using PAR4- and GAPDH- specific primers (n=5). Results showed significantly decreased mRNA levels of PAR4 in cancer compared to normal. B. PAR4 expression was measured in 34 lung adenocarcinoma patients by RT-qPCR. PAR4 mRNA was decreased in 67.7% (23 of 34) tumors. Mean fold decrease in the tumor tissue relative to non-neoplastic lung tissue was shown. Bars, SEM. The decreased folds of “>2” were defined as “decreased”, whereas decreased folds of “≤2” were defined as “not decreased”

Western blotting

Frozen tissues were pulverized in liquid nitrogen using mortar and pestle. The resulting powder was solubilized in RIPA lysis buffer. Cultured cells were harvested at 90-95% confluence, and washed twice in PBS. Cells were directly disrupted in the same lysis buffer. The protein concentration was determined using a protein assay kit (Bio-Rad). Samples were separated by electrophoresis on a 12% SDS-polyacrylamide gel (SDS-PAGE) gel with 50 μg total protein loaded per well and then electro-transferred onto a PVDF membrane (Millipore), then blocked with 3% bovine serum albumin (BSA) for 2 h prior to overnight incubation at 4°C with anti-human PAR4 polyclonal antibody (C-20, 1:3500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed with tris buffered saline (with 0.1% Tween-20, TBST), and then incubated with horseradish peroxidase-conjugated mouse anti-goat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. Protein bands were visualized with Super Signal reagents (Pierce, Rockford, IL, USA).

Statistical analysis

All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The Fisher’s exact test and chi squared test were used to determine the significance of correlations between PAR4 expression and clinicopathological parameters (Tables 1 and 2). Differences in the numerical data between the cancer and adjacent normal tissues were evaluated using the paired Wilcoxon test (Figure 1B). The cutoff value for statistical significance was set at the level of $p < 0.05$.

Table 1. Association Between the mRNA Levels of PAR4 and Clinic-pathologic Data in Lung Adenocarcinoma Patients

	Total	PAR4 mRNA levels				*P*
	(n=34)	Decreased* (n=23)		Not decreased* (n=11)		
	No.	No.	%	No.	%	
Gender						
Male	23	14	60.87	9	39.13	0.222
Female	11	9	81.82	2	18.18	
Age						
≤60	20	14	70.00	6	30.00	0.113
>60	14	9	64.29	5	35.71	
Differentiation						
Well	12	5	41.67	7	58.33	0.017
Poor and moderated	22	18	81.82	4	18.18	
Lymph node metastasis						
Positive	15	10	66.67	5	33.33	0.914
Negative	19	13	68.42	6	31.58	
Distant metastasis						
Positive	7	7	100.00	0	0.00	0.04
Negative	27	16	59.26	11	40.74	
Tumor size						
≤3cm	17	12	70.59	5	29.41	0.714
>3cm	17	11	64.71	6	35.29	

*The decreased folds of “>2” were defined as “decreased”, while the decreased folds of “≤2” were defined as “not decreased”

Results

PAR4 mRNA expression down-regulation in lung adenocarcinomas

Northern blot analysis has shown that the PAR4 gene is expressed at especially high levels in normal lung (Xu et al., 1998). In the present study, RT-PCR was performed on matched normal and lung cancer tissue specimens from 5 randomly selected patients and the samples were normalized to GAPDH levels. The RT-PCR analysis showed significantly decreased mRNA levels of PAR4 in all 5 lung cancer tissue samples compared to the corresponding normal tissues (Figure 1A). Differential expression of PAR4 mRNA was further examined by RT-qPCR in all 34 lung adenocarcinoma samples, comparing primary tumors with matched normal adjacent tissue. Down-regulated PAR4 expression was observed in 67.7% (23 of 34) of these lung adenocarcinoma tissues (Table 1). PAR4 mRNA was reduced 21.09±8.5 fold (mean ± SEM) in tumors (p= 0.0198, Figure 1B). We then investigated the clinical significance of loss of PAR4 expression using available clinicopathological data. There were significant differences in PAR4 mRNA expression in well-differentiated tumors compared to poorly and

100.0
75.0
50.0
25.0
0

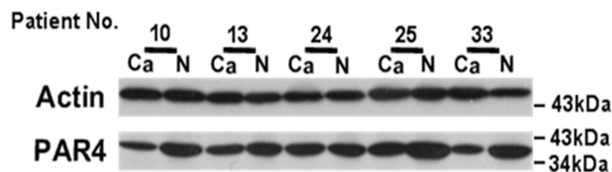

Figure 2. Western Blot Analysis of Normal and Cancerous Lung Tissues. Western blotting of tissue lysates from 5 cases of lung adenocarcinoma (Ca) and relevant adjacent non-neoplastic tissues (N). The expression of actin was served as a control. The evident decrease of PAR4 (4 of 5) was observed in the cancerous tissues in contrast to their matched normal tissues

Table 2. Association Between the Protein Levels of PAR4 with Histopathologic Features of Lung Adenocarcinoma

	Total (n=75)	PAR4 expression				P
		Decreased (n=46)		Not decreased (n=29)		
	No.	No.	%	No.	%	
Gender						
Male	41	22	53.66	19	46.34	0.134
Female	34	24	70.59	10	29.41	
Age						
≤60	36	20	55.56	16	44.44	0.324
>60	39	26	66.67	13	33.33	
Differentiation						
Well	10	3	30.00	7	70.00	0.035
Poor and moderated	65	43	66.15	22	33.85	
TNM stage						
T1+T2	55	33	60.00	22	40.00	0.694
T3+T4	20	13	65.00	7	35.00	
Clinical stage						
I~II	32	15	46.88	17	53.13	0.027
III~IV	43	31	72.09	12	27.91	
Lymph node metastasis						
Positive	41	25	60.98	16	39.02	0.944
Negative	34	21	61.76	13	38.24	
Distant metastasis						
Positive	5	2	40.00	3	60.00	0.311
Negative	70	44	62.86	26	37.14	
Tumour size						
≤3cm	29	18	62.07	11	37.93	0.917
>3cm	46	28	60.87	18	39.13	

moderately differentiation tumors (p= 0.017). We also observed differences in PAR4 expression between tumors showing distant metastases compared to non- metastatic tumors (p= 0.04) (Table 1).

Protein levels of PAR4 are frequently decreased in cancerous lung tissues and are correlated with histopathologic features

The presence of PAR4 protein in normal lung tissue was verified using Western blotting (Figure 2). In lung adenocarcinoma tissues, a marked reduction of PAR4 expression was observed in 4 of 5 cases, compared with the matched nonmalignant lung tissue (Figure 2). As determined using immunohistochemical analysis, we found that PAR4 was mainly expressed in non-neoplastic lung epithelial cells (A, B, arrow), macrophagocytes in the alveolar space (C, arrow) and epithelium of lower bronchus (C, D) (Figure 3). These observations are in agreement with those previously reported (Fujiwara et al., 2005). PAR4 staining was diffused throughout the cytoplasm of the epithelial cells, and some nuclear staining was also observed. Regarding the intensity of staining, the PAR4 staining was reduced in well-differentiated lung adenocarcinoma tissues (F), significantly reduced and even almost absent in moderated and poorly-differentiated lung adenocarcinomas (G, H, I) (Figure 3). The overall PAR4 expression was decreased in 61.3% (46 of 75) of lung adenocarcinoma tissues (Table 2). Immunohistochemical staining of PAR4 in lung adenocarcinoma tissues was graded and classified according to the criteria described in Section 2. The correlation between reduced PAR4 expression and clinicopathological parameters was then investigated further (Table 2).

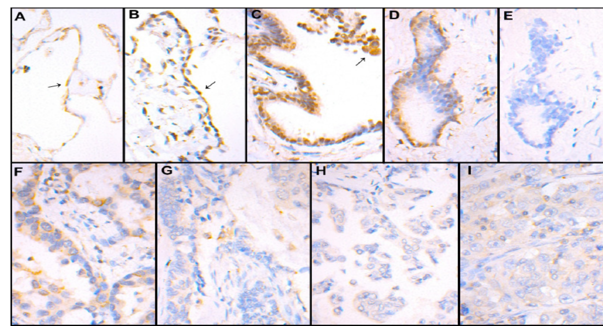

Figure 3. Photomicrographs of Immunohistochemical Staining for PAR4 in Paraffin Sections of Lung Tissues. PAR4 expression was mainly in epithelial cells (A, B, arrow), macrophagocytes in alveolar space (C, arrow) and epithelium of lower bronchus (C, D). PAR4 staining was diffuse throughout the cytoplasm membrane of the lung epithelial cells and part of nucleus. E: the absence of immunolabeling using anti-PAR4 antibody (C20) blocked with PAR4 antigen. PAR4 expression was reduced in well-differentiated lung adenocarcinoma tissues (F: Acinar adenocarcinoma), significantly reduced and even almost absent in moderated and poorly-differentiated lung adenocarcinomas (G: Mucinous adenocarcinoma; H: Micropapillary adenocarcinoma; I: Entity type adenocarcinoma). (400×)

Statistical analysis showed that decreased PAR4 expression was strongly associated with tumor differentiation and clinical stage. PAR4 expression was decreased in 30.0% of well differentiated cancers, 66.2% of poor and moderated differentiated cancers (p= 0.035) and decreased in 46.9% of clinical I-II stage cancers and in 72.1% of clinical III-IV stage cancers (p = 0.027) (Table 2).

PAR4 mRNA and protein levels are down-regulated in NCI-H157 lung adenocarcinoma cells compared with BEAS-2B non-malignant lung cells

The normal human bronchial epithelial cell line BEAS-2B and the human lung adenocarcinoma cell line NCI-H157 were selected to further explore the roles of PAR4 in lung adenocarcinoma.

RT-PCR was performed on BEAS-2B and NCI-H157 cell lines and expression levels of PAR4 were normalized to GAPDH levels. The results showed that PAR4 mRNA expression is decreased in NCI-H157 cells when compared with BEAS-2B cells (Figure 4A). RT-qPCR results showed that mRNA expression of PAR4 in NCI-H157 cells is only 58% of that in BEAS-2B cells (Figure 4B).

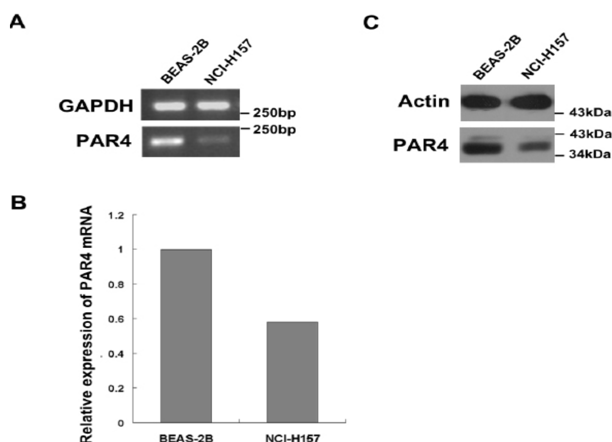

Figure 4. Expression of PAR4 in NCI-H157 and BEAS-2B Cell Lines. The significantly decreased expression of PAR4 mRNA (A: RT-PCR) and protein (C: Western blot analysis) was observed in NCI-H157 cells in contrast to BEAS-2B cells. RT-qPCR results showed the expression of PAR4 mRNA in NCI-H157 cells is only 58% of that in BEAS-2B cells (B)

Western blot analysis also revealed that PAR4 expression was significantly decreased in NCI-H157 cells compared to BEAS-2B cells (Figure 4C).

Discussion

Although ectopic expression of PARs has been reported in some epithelial cancers, including breast, renal, gastric, colon, ovarian, laryngeal and lung (Rudroff et al., 2002; DorsamGutkind 2007; Zhang et al., 2011), the roles of PARs in tumorigenesis and the possible molecular mechanism are not well understood.

The human respiratory tract appears to express all four PAR subtypes and PARs may play roles in the development of inflammation, asthma, chronic obstructive pulmonary disease (COPD) and cancer (Sokolova et al., 2007; Ramachandran et al., 2008; Peters et al., 2009). Abundant expression of PAR4 in normal human lung tissues has been found by Northern blot (Xu et al., 1998), but the function of PAR4 in lung cancer is not clear.

In the present studies, using RT-PCR, RT-qPCR, Western blotting, and immunohistochemical assays, we present strong evidence that PAR4 is frequently down-regulated in lung adenocarcinoma tissues and in the NCI-H157 lung adenocarcinoma cell line. Consistent with previous studies, we found that in normal lung tissue, PAR4 is mainly present in epithelium of lower bronchus, type II pneumocytes, endothelial cells, and alveolar macrophages (Nelken et al., 1992; Xu et al., 1998; Saifeddine et al., 2001). We also report that expression of PAR4 in most lung adenocarcinoma tissues examined was decreased when compared with adjacent noncancerous tissues, and that PAR4 expression was undetectable in some poor-differentiated lung adenocarcinomas.

Positive expression of PAR4 in some tumors have been found in previous studies and is thought to be involved in hepatocellular carcinoma cell migration and colon cancer progression (Kaufmann et al., 2007; Gratio et al., 2009). It was also reported that PAR4 activation contributed to the hematogenous metastasis of melanoma using the knock-out mice (Camerer et al., 2004). But some datas are quite heterogeneous and there are contradictory results. For instance, Primary hepatic cell cancer (HCC) was reported to be negative in one study (Rullier et al., 2006). While another reported a 100% positivity on 2 primary HCC cells and two cancer cell lines (Kaufmann et al., 2007). RT-PCR revealed no PAR4 mRNA in the LnCaP prostate cell line and MDA-MB-231 breast cell line while by FACS analysis they were shown to be positive (Elste et al., 2010). Furthermore, these studies focus only on cancerous tissue, and pay little attention to adjacent normal tissues.

In our previous studies, we found that PAR4 mRNA and protein were frequently down-regulated in gastric cancer and that down-regulation were closely associated with tumor differentiation and metastasis (Ahmad et al., 2000; Huang et al., 2000; Zhang et al., 2011). The present studies extend these finding to lung adenocarcinoma. We observed that while PAR4 mRNA and protein are detectable in most lung adenocarcinoma tissues, the levels of both are decreased when compared with adjacent noncancerous lung tissues and that reduced levels are positively correlated with tumor differentiation, metastasis and clinical stage. Immunohistochemistry results of 56 patients with follow-up data showed that decreased PAR4 expression was associated with shortened survival time, but the difference did not reach statistically significant levels ($p = 0.348$, data not show). Our results are inconsistent with those about stage IB non-small cell lung cancer previously reported by Ghio et al, who found that PAR4 protein was expressed in 39 of all 60 cases (65%) and 26 of 30 adenocacinoma cases (87%), and 3-year survival was shorter in patients expressing PAR4 versus negative cases (Ghio et al., 2006). The contrast maybe related to differences in patient demographics, immunoreagents or methodology etc, and Ghio's study just only look at protein, not mention PAR4 mRNA.

PAR4 has been shown in some studies to be involved in angiogenesis (Italiano et al., 2008). Ma et al. demonstrated that PAR4 can play a crucial role in regulating angiogenesis, which may influence the processes of tumor growth and metastasis (Ma et al., 2005). Our observations that PAR4 expression in 7 lung adenocarcinoma tissues with distant metastasis are all decreased (100%) support the idea that reduction of PAR4 in lung adenocarcinoma tumors increases the likelihood of hematogenous metastasis.

The causes and mechanisms of PAR4 down-regulation in cancer are not yet clear. Down-regulation of PAR4 expression in gastric cancer was previously found to be related to gene promoter hypermethylation (Zhang et al., 2011). But our unpublished studies have shown that the methylation level of CpG sites in the PAR4 promoter region have minimal difference between NCI-H157 and BEAS-2B cell lines (data not shown). MicroRNAs have recently emerged as important regulators of gene expression,and their main function is to decrease target mRNA levels (Guo et al., 2010). MicroRNAs are abundant in the lung and several microRNAs such as let-7, miR-155, miR-17~92 cluster, miR-34 families, miR-212, miR-210 and miR-218 have been shown to be involved in lung carcinogenesis (Tomankova et al., 2010). Maybe dysregulation of microRNAs play a possible role in the

downregulation of PAR4 mRNA. The results suggest that multiple modes of PAR regulation may exist in cancer. Further studies will be needed to elucidate the role of PAR4 as a possible tumor suppressor.

Our findings indicate that dysregulation of PAR4 may play a role in tumorigenesis, progression and metastasis of lung adenocarcinoma. In addition, our observations of reduced PAR4 expression in lung cancer patients for whom follow-up data is available suggest that PAR4 might also play a negative role in lung cancer progression. Further studies will be required to resolve the reasons for inconsistencies in reports of PAR4 expression in lung cancer. Better understanding of the molecular mechanisms of PAR4 action may lead to the identification of new targets for cancer prevention and treatment.

Acknowledgements

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References

Ahmad R, Knafo L, Xu J, et al (2000). Thrombin induces apoptosis in human tumor cells. *Int J Cancer*, **87**, 707-15.

Camerer E, Qazi AA, Duong DN, et al (2004). Platelets, protease-activated receptors, and fibrinogen in hematogenous metastasis. *Blood*, **104**, 397-401.

Darmoul D, Gratio V, Devaud H, et al (2003). Aberrant expression and activation of the thrombin receptor protease-activated receptor-1 induces cell proliferation and motility in human colon cancer cells. *Am J Pathol*, **162**, 1503-13.

Darmoul D, Marie JC, Devaud H, et al (2001). Initiation of human colon cancer cell proliferation by trypsin acting at protease-activated receptor-2. *Br J Cancer*, **85**, 772-9.

Dorsam RT, Gutkind JS (2007). G-protein-coupled receptors and cancer. *Nat Rev Cancer*, **7**, 79-94.

Elste AP, Petersen I (2010). Expression of proteinase-activated receptor 1-4 (PAR 1-4) in human cancer. *J Mol Histol*, **41**, 89-99.

Fujiwara M, Jin E, Ghazizadeh MK, awanami O (2005). Activation of PAR4 induces a distinct actin fiber formation via p38 MAPK in human lung endothelial cells. *J Histochem Cytochem*, **53**, 1121-9.

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

Gratio V, Walker F, Lehy T, et al (2009). Aberrant expression of proteinase-activated receptor 4 promotes colon cancer cell proliferation through a persistent signaling that involves Src and ErbB-2 kinase. *Int J Cancer*, **124**, 1517-25.

Guo H, Ingolia NT, Weissman JS, Bartel DP (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*, **466**, 835-40.

Huang YQ, Li JJ, Karpatkin S (2000). Thrombin inhibits tumor cell growth in association with up-regulation of p21(waf/cip1) and caspases via a p53-independent, STAT-1-dependent pathway. *J Biol Chem*, **275**, 6462-8.

Italiano JE, Jr., Richardson JL, Patel-Hett S, et al (2008). Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood*, **111**, 1227-33.

Kahn M, Ishii K, Kuo WL, et al (1996). Conserved structure and adjacent location of the thrombin receptor and protease-activated receptor 2 genes define a protease-activated receptor gene cluster. *Mol Med*, **2**, 349-57.

Kaufmann R, Rahn S, Pollrich K, et al (2007). Thrombin-mediated hepatocellular carcinoma cell migration: cooperative action via proteinase-activated receptors 1 and 4. *J Cell Physiol,* **211**, 699-707.

Kononen J, Bubendorf L, Kallioniemi A, et al (1998). Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med*, **4**, 844-7.

Lan RS, Stewart G AHenry PJ (2002). Role of protease-activated receptors in airway function: a target for therapeutic intervention? *Pharmacol Ther*, **95**, 239-57.

Liu SB, He YY, Zhang Y, et al (2008). A novel non-lens betagamma-crystallin and trefoil factor complex from amphibian skin and its functional implications. *PLoS One*, **3**, e1770.

Ma L, Perini R, McKnight W, et al (2005). Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets. *Proc Natl Acad Sci U S A*, **102**, 216-20.

Moss SF, Lee JW, Sabo E, et al (2008). Decreased expression of gastrokine 1 and the trefoil factor interacting protein TFIZ1/GKN2 in gastric cancer: influence of tumor histology and relationship to prognosis. *Clin Cancer Res*, **14**, 4161-7.

Nelken NA, Soifer SJ, O'Keefe J, et al (1992). Thrombin receptor expression in normal and atherosclerotic human arteries. *J Clin Invest*, **90**, 1614-21.

Peters T, Henry PJ (2009). Protease-activated receptors and prostaglandins in inflammatory lung disease. *Br J Pharmacol*, **158**, 1017-33.

Ramachandran R, Hollenberg MD (2008). Proteinases and signalling: pathophysiological and therapeutic implications via PARs and more. *Br J Pharmacol*, **153**, S263-82.

Rudroff C, Seibold S, Kaufmann R, et al (2002). Expression of the thrombin receptor PAR-1 correlates with tumour cell differentiation of pancreatic adenocarcinoma in vitro. *Clin Exp Metastasis*, **19**, 181-9.

Rullier A, Senant N, Kisiel W, et al (2006). Expression of protease-activated receptors and tissue factor in human liver. *Virchows Arch*, **448**, 46-51.

Saifeddine M, Al-Ani B, Sandhu S, et al (2001). Contractile actions of proteinase-activated receptor-derived polypeptides in guinea-pig gastric and lung parenchymal strips: evidence for distinct receptor systems. *Br J Pharmacol*, **132**, 556-66.

Siegel R, Naishadham D, Jemal A (2012). Cancer statistics, 2012. *CA Cancer J Clin*, **62**, 10-29.

Sokolova E, Reiser G (2007). A novel therapeutic target in various lung diseases: airway proteases and protease-activated receptors. *Pharmacol Ther,* **115**, 70-83.

Toh CK (2009). The changing epidemiology of lung cancer. *Methods Mol Biol*, **472**, 397-411.

Tomankova T, Petrek M, Kriegova E (2010). Involvement of microRNAs in physiological and pathological processes in the lung. *Respir Res*, **11**, 159.

Trejo J (2003). Protease-activated receptors: new concepts in regulation of G protein-coupled receptor signaling and trafficking. *J Pharmacol Exp Ther*, **307**, 437-42.

Vergnolle N, Wallace JL, Bunnett NW, Hollenberg MD (2001). Protease-activated receptors in inflammation, neuronal signaling and pain. *Trends Pharmacol Sci*, **22**, 146-52.

Xu WF, Andersen H, Whitmore TE, et al (1998). Cloning and characterization of human protease-activated receptor 4. *Proc Natl Acad Sci U S A*, **95**, 6642-6.

Zhang H, Cai B (2003). The impact of tobacco on lung health in China. *Respirology*, **8**, 17-21.

Zhang Y, Yu G, Jiang P, et al (2011). Decreased expression of protease-activated receptor 4 in human gastric cancer. *Int J Biochem Cell Biol*, **43**, 1277-83.