SPANDIDOS PUBLICATIONS

Frequent down-regulation of *pim-1* mRNA expression in non-small cell lung cancer is associated with lymph node metastases

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Abstract. Pim kinases are emerging as important mediators of cytokine signalling pathways in hematopoietic cells, and contribute to the progression of certain leukemias and solid tumors. The mRNA expression of *pim-1*, a putative oncogenic serine-threonine kinase, was determined in non-small cell lung cancer (NSCLC) patients. Sixty-eight patients with potentially curative resections (R0 resections) for NSCLC in histopathological stages I-IIIA were included. An analysis of *pim-1* mRNA expression was performed on paired tumor and normal lung tissue samples by quantitative real-time reverse transcriptase-PCR (RT-PCR) standardized for ß-actin. *Pim-1* expression in the tumor (median 0.28) was significantly down-regulated ($p<0.0001$) compared to the paired normal tissue (median 4.96). Immunohistochemistry showed a strong expression of Pim-1 protein in the normal respiratory epithelium and a lower expression in the tumor cells. A significant association between the *pim-1* down-regulation and occurrence of lymph node metastases ($p=0.05$) was detected. The down-regulation of *pim-1* mRNA was demonstrated for 59 out of 68 lung cancer patients (86.8%). Down-regulation occurs already in the early stage of NSCLC and is either directly involved in the lymphatic progression in NSCLC or represents a surrogate marker.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide with an increasing incidence and poor prognosis. For the year 2006, 174,470 new cancer cases and 162,460 deceased resulting from lung cancer are expected in the USA, while an estimated 351,344 Americans are living with the disease (1). Despite improvements in its detection, surgical resection and systemic therapy, modest progress has been made in the outcome for patients diagnosed with lung cancer. The relative 5-year survival for all stages of lung cancer combined was 13% from 1974-1976 and 15% from 1995-2000 (2). The World Health Organization histological classification of lung cancer (3) describes three major subtypes of non-small cell lung cancer (NSCLC): squamous cell carcinoma, adenocarcinoma and large cell carcinoma, which represent ~75% of all lung cancer cases. Radical surgery (R0 resection) offers the best chance for cure in patients with NSCLC and survival probabilities are mainly dependent on tumor stage (4,5). Only 15% of lung cancer patients are diagnosed at an early stage (1). This places a high priority on elucidating the molecular mechanisms underlying the disease with the aim of ultimately developing novel and effective therapeutic strategies to target this malignancy. Substantial efforts have been made to identify prognostic factors in order to individualize treatment and improve prognosis (6,7). A variety of molecular markers have been implicated in the pathogenesis and prognosis of NSCLC (8,9).

Pim-1 was originally identified as a common integration site for moloney murine leukemia virus and appears to be involved in the control of cell growth, differentiation and apoptosis (10). It belongs to a family of serine/threonine protein kinases and is associated with the transcriptional regulation of cell cycle proteins (11). Two other proteins with significant sequence similarities exist, Pim-2 and -3. A report on its crystal structure indicates that Pim-1 is a constitutively active kinase. A number of cytoplasmic and nuclear proteins are phosphorylated by Pim kinases and may act as their effectors in normal physiology and in disease (12). Pim-1 associates with protein complexes necessary for mitosis (13). Two pim-1 proteins are produced from the same gene by using an alternative upstream CUG initiation codon, a 44 kDa and a shorter 34 kDa form that contain the characteristic kinase domain and play a role in drug resistance (14,15). Pim-1 is widely spread in tissues with the highest expression found in

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hematopoietic tissues and testes (16) enhancing cellular survival (17,18). Pim-1 also appears to be involved in the tumorigenesis of solid tumors. Microarray expression profiling identified Pim-1 overexpression in human prostate tumors. Expression of the Pim-1 protein significantly correlated with the survival of prostate (19) and gastric cancer patients (20). We analysed the expression of *pim-1* mRNA in NSCLC and its possible association with pathogenesis, histopathological parameters and prognosis.

Materials and methods

Patients. Paired normal and tumor tissues from 68 patients with histopathologically confirmed NSCLC, available from a previous study, were included in this study (21). Patients who did not receive pre-treatment therapy with completely resected (R0 resection) tumors were chosen. There were 56 (82.4%) men and 12 (17.6%) women, with a median age of 65 years (range 34-79). Thirty-six (52.9%) patients had squamous cell carcinomas; 22 (32.4%), adenocarcinomas and 10 (14.7%), large-cell carcinomas. The primary tumors were graded histopathologically as moderately (G2, 15 patients) or poorly differentiated (G3, 53 patients). Tumor staging was performed according to the International Union Against Cancer (UICC) TNM classification: 34 (50.0%) had stage I; 14 (20.6%), stage II and 20 (29.4%), stage IIIa tumors. Tumors were radically removed by lobectomy (n=42), bilobectomy (n=11), pneumonectomy (n=8) or extended pneumonectomy (n=70), including mediastinal lymphadenectomy. Patients with histopathological stage IIIa tumors received post-operative radiotherapy. Informed consent was obtained from all patients. The median follow-up was 85.9 months (range 63-105) and no patient was lost to follow-up.

Tissue acquisition. For the evaluation of the gene expression, tumor samples from lung cancers and corresponding normal tissues were obtained immediately after lung resection before starting radical mediastinal lymphadenectomy, snap-frozen in liquid nitrogen and then stored at -80˚C until further processing. The histologically normal-appearing lung tissues were taken from the greatest distance to the tumor. Samples were carefully selected after control staining with hematoxylin and eosin of individual biopsies and evaluation by a pathologist (S.E.B.).

RNA isolation. Total cellular RNA was isolated using TRIzol reagent (Life Technologies/Gibco, Grand Island, NY) according to the manufacturer's recommendation and quantitated at A260/280 nm (Smart Spec, BioRad, Hercules, CA).

Quantitative real-time reverse transcriptase (RT)-PCR. Total cellular RNA (0.5 μg) was reverse-transcribed as already reported (22) using MMLV (molony murine leukaemia virus) reverse transcriptase kit (Clontech Lab., Palo Alto, USA). Real-time RT-PCR was performed by applying 25 ng of cDNA (TaqMan ABI PRISM 7900HT sequence detection system, Applied Biosystems, Darmstadt, Germany). To normalize the amount of total RNA, the housekeeping gene ß-actin was amplified as described (23). Customized primers and probes were obtained from Applied Biosystems (assay on demand Hs00171473_m1). The reliability of PCR amplification and detection was verified on serial dilutions of standard cDNAs, RNA as well as genomic DNA prior to analyses of the patient samples as reported. Briefly, the PCR reaction mixture contained 300 nM of each primer and 200 nM probe in a final volume of 20 μl. PCR conditions were 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Gene expression levels were calculated using standard curves generated by serial dilutions of placenta cDNA (Clontech Lab.). All reactions were performed in triplicate.

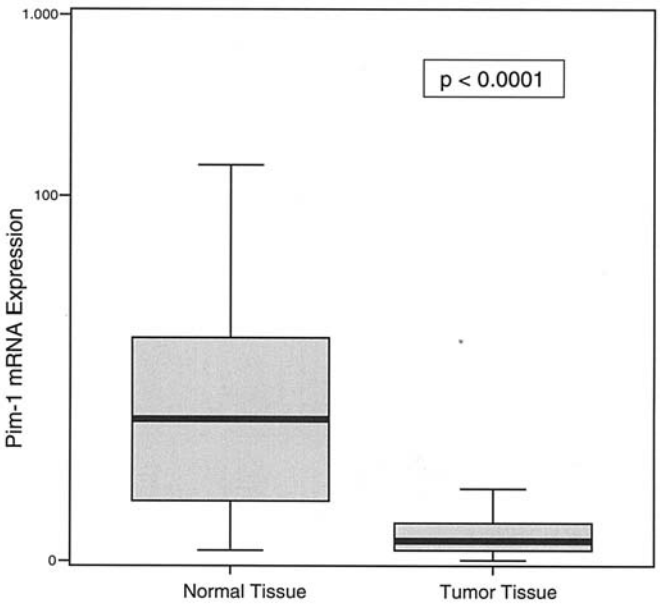

Figure 1. *Pim-1* mRNA expression in paired NSCLC and uninvolved lung tissues. Gene expression levels (mRNA) are reported using the median as a point estimator and the range of values. *Pim-1* mRNA levels are significantly down-regulated in the tumor compared to paired normal tissues; Wilcoxon rank test, p<0.0001.

Immunohistochemistry. Pim-1 protein was detected by the polyclonal antibody anti-Pim-1 (Abgent Company, San Diego, USA) raised against a synthetic peptide selected from C-term amino acids 298-313 of the protein.

One hundred and seventeen surgical specimens were available. Sections of the paraffin-embedded tissues (5 μm) were cut and deparaffinized according to standard histological techniques. Subsequently, a high-sensitivity immunohistochemical staining was performed by applying the Dako EnVision System (DakoCytomation, Hamburg, Germany) according to the manufacturer's instructions. In brief, endogenous peroxidase activity was blocked by immersing the slides in 0.3% hydrogen peroxide in methanol for 5 min. Pretreatment was performed in a microwave using 10 mM citrate buffer, pH 6.0, for 3x5 min at 700 W. Subsequently, sections were incubated with primary antibody anti-Pim-1 (polyclonal ab, Abgent Company), diluted 1:50 with 10 mM phosphate-buffered saline (PBS), pH 7.4, at 4˚C overnight. After washing twice in Tris-buffered saline (TBS), sections were incubated with HRP (avidin-horseradish) peroxidase conjugated secondary antibody (goat anti-rabbit) and the Envision kit, for 30 min at room temperature. After washing

urvival in NSCLC based on clinical parameters.

Parameter	N=68	5-year survival (%) ±SD	Median survival (months)	P-value
UICC stage				
I	34	70.6±7.8	n.r.	<0.0001
II	14	50.0±13.4	34.0	
IIIA	20	10.0±6.7	16.7	
pT				
pT1	12	75.0±12.5	n.r.	n.s.
pT2	47	46.8±7.3	51.4	
pT3	9	22.2±13.9	26.7	
pN				
pN0	37	70.3±7.5	n.r.	<0.0001
pN1	18	38.9±11.5	29.0	
pN2	13	0	16.7	
Histology				
SCC	36	58.3± 8.2	n.r.	n.s.
AC	22	40.9±10.5	46.8	
LC	10	30.0±1.5	20.8	
Grading				
G2	15	46.7±1.3	59.7	n.s.
G3	53	49.1±0.7	51.8	

N, number of patients; n.r., not reached; n.s., not significant; UICC, Union Internationale Contre Le Cancer (5th edition, 1997); pT, histopathological tumor category according to UICC; pN, histopathological lymph node category according to UICC; SCC, squamous cell carcinoma; AC, adenocarcinoma and LC, large cell carcinoma.

(2x5 min in TBS), the sections were stained by AEC (chromogen-3-amino-9-ethylcarbazol) for 30 min and rinsed with H_2O. The nuclei were counterstained with hematoxylin. The staining procedure without a primary antibody was used as a negative control. The staining results were monitored by concurrently treating the negative and positive controls.

Statistical analysis. The gene expression analyses yield values which are expressed as ratios between two absolute measurements: the gene of interest and the internal reference gene ß-actin. Associations between the gene expression levels and clinicopathological parameters were evaluated using the χ^2 test for dichotomized variables, the Wilcoxon rank test for paired variables and the Mann-Whitney U test for independent variables by applying Fisher's exact testing for significance. Partitioning of gene expression levels to construct prognostic groups was performed according to LeBlanc and colleagues (24). Kaplan-Meier plots (25) were used to describe the survival distribution and the log-rank test was used to evaluate for survival differences (26). The level of significance was set to $p<0.05$ unless otherwise specified, p-values were given for two-sided testing. Statistical tests were performed using the software package SPSS for Windows, version 12.0, Chicago, IL, USA.

Results

Clinical data. Median survival rates depending on various clinical variables are summarized in (Table I). The histopathological UICC tumor stage ($p<0.0001$) and pN category ($p<0.0001$) were of significant prognostic importance using the log-rank test. Gender, age and grading or histology of the primary tumor, as well as the pT category, had no prognostic impact on survival.

Pim-1 mRNA expression in tumor and histologically normal-appearing lung tissues and its correlation to clinicopathological data in NSCLC. The expression of *pim-1* mRNA was detected by quantitative real-time PCR in 67 out of 68 tumor specimens and all of the corresponding normal epithelia. The median *pim-1* mRNA expression levels standardized for ß-actin were 0.28 (min. 0, max. 40.69) in tumor and 4.96 (min. 0.14, max. 3380.95) in normal tissues, respectively. The median relative expression level (ratio tumor:normal) was 0.06 (min. 0, max. 195.67). *Pim-1* mRNA levels were lower in the tumor compared to the corresponding normal tissues in 59 out of 68 patients (86.8%) and this difference was highly significant (Wilcoxon signed-rank test: $p<0.0001$) as shown in Fig. 1. *Pim-1* mRNA expression was not associated with

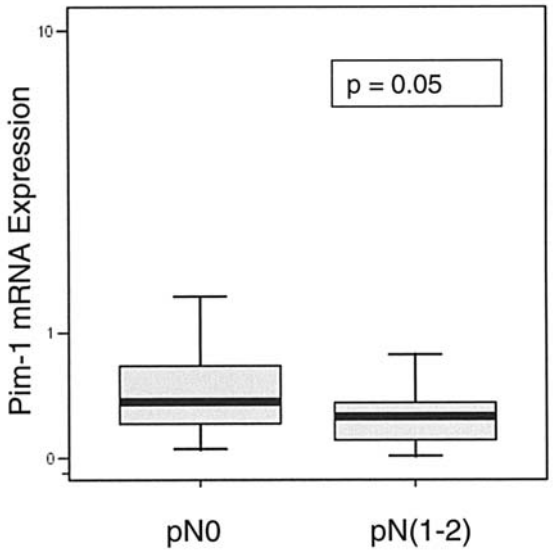

Figure 2. Association of *pim-1* mRNA expression with lymph node metastases. A significant association (p=0.05) between the down-regulation of *pim-1* expression was identified in patients with (pN1-2) compared to patients without lymph node metastases (pN0). A Mann-Whitney U test was applied for statistical analysis.

the pT category, histological subtype or the grading of the primary tumor. However, the down-regulation of *pim-1* expression was associated with the presence of lymph node metastases (pN1-2 median rank 29.4 versus pN0 median rank 38.7, Mann-Whitney U test, p=0.05), as shown in Fig. 2.

Immunohistochemical detection of Pim-1 protein expression in tumor and normal cells. Immunohistochemistry confirmed the down-regulation of Pim-1 protein expression in NSCLC. Fig. 3 demonstrates strong protein staining in normal respiratory bronchioles, artery walls and macrophages, whereas normal alveoles did not express Pim-1. Tumor cells were positive for Pim-1 protein, although at a lower level as compared to normal bronchioles.

Discussion

Our data present a study of the *pim-1* gene expression in 68 patients with curatively resected NSCLC. We demonstrate a frequent down-regulation of this gene in 59 out of 68 tumor patients by comparing tumor tissues to the uninvolved lung tissues. The *pim-1* gene was described as being expressed at high levels in hematolymphoid tissues and testes (15). Recent analyses indicated that pim-1 is overexpressed in head and neck squamous cell carcinomas (27). The expression of the pim-1 protein kinase is also elevated in prostate tumors (28,29). Dhanasekaran *et al* (19) reported that *Pim-1* has emerged as a potential prognostic biomarker since they found it was frequently up-regulated in prostate cancer and demonstrated a correlation with clinical outcome. It is assumed that pim kinases contribute to cell proliferation and survival, thereby providing a selective advantage in tumorigenesis. These observations are in agreement with the findings of Shay *et al* (17) that Pim-1 may act as a cell survival factor and prevent apoptosis in malignant cells. Our data, however, clearly demonstrate that the *pim-1* mRNA expression is significantly ($p<0.0001$) down-regulated in lung cancer, compared to the matching normal tissues from a median of 4.96 to one of 0.28 (nearly 18-fold).

Chen *et al* (20) characterized the gene expression profile of gastric cancer patients by microarray-based analysis. A prediction model was developed consisting of *CD36*, *SLAM* (signalling lymphocyte activation molecule) and *pim-1* expression discriminating between good and poor survival probabilities. In their report, tumor samples with clearly down-regulated *pim-1* mRNA expression are shown although data about the frequency of this observation are lacking. In our study, in 59 (86.8%) NSCLC patients *pim-1* was down-regulated as compared to the corresponding normal tissues, whereas in 9 (13.2%) tumors, the expression of *pim-1* was up-regulated.

In NSCLC *pim-1* expression is down-regulated compared to the normal bronchial epithelium. We were able to demonstrate by immunohistochemistry that the down-regulation

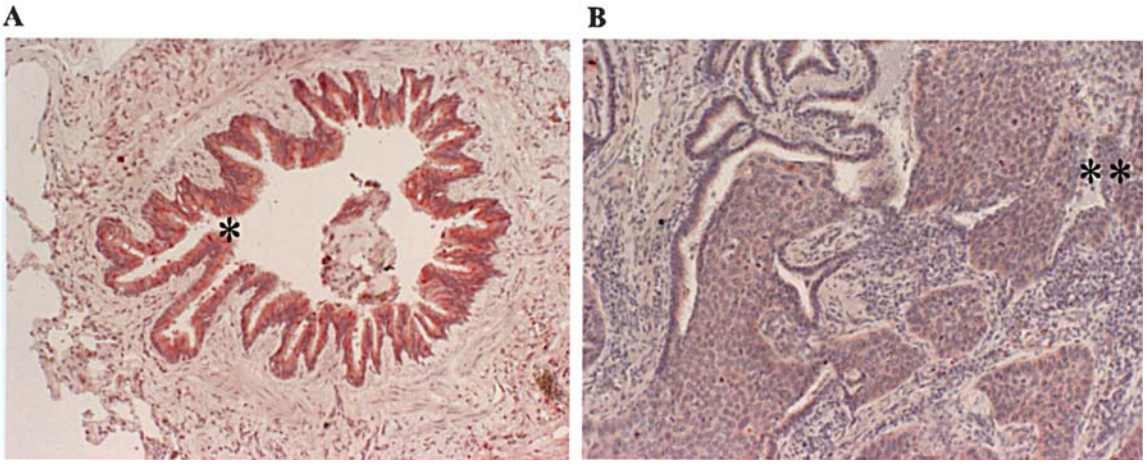

Figure 3. Immunohistochemical localization of the Pim-1 protein expression in NSCLC and the normal respiratory epithelium. (A) Normal respiratory epithelium (*): bronchioles, macrophages and artery walls reveal a strong positive brownish stain for Pim-1 protein. Normal aveoles do not express Pim-1. (B) Tumor cells are stained for Pim-1 at a lower level (**). Immunohistochemistry confirms the down-regulation of Pim-1 in the tumor compared to the normal respiratory epithelium.

by RT-PCR is due to strong Pim-1 protein expression normal respiratory bronchioles, artery walls and macrophages. Although Pim-1 staining was detected in lung cancer cells this expression level was lower than in the normal respiratory epithelium. Normal alveolar cells did not express Pim-1 protein. Thus, we were able to clarify the controversy between our data of the down-regulation of *pim-1* mRNA expression in NSCLC and the up-regulation of pim-1 published for several other carcinomas (20,27,29). During tumorigenesis the respiratory epithelial cells obviously lose the ability to express Pim-1 protein and Pim-1 mRNA transcripts, indicated by the presented immunohistochemical and RT-PCR data.

The down-regulation of *pim-1* expression was not associated with the pT category and had already occurred in early stage lung cancer (pT1). However, it was associated with the presence of lymph node metastases which represents a novel finding.

Rahman *et al* (30) observed the down-regulation of pim-1 and Bcl-2 in IL-6-depleted 7TD1 cells. These mouse hybridoma cells were dependent on IL-6 and underwent apoptosis following starvation of IL-6. The down-regulation of *pim-1* may induce apoptosis since pim-1 has been shown to prevent apoptosis in malignant cells (16).

Mutations in the *pim-1* gene have been found in diffuse large cell lymphomas in the 1.2 kb stretch of the first 2 kb from the transcription initiation site, resulting in an altered structure and function of pim-1 (31). Since the mRNA expression of *pim-1* is predominantly lower in NSCLC, mutations appear to be very unlikely as they will lead to overexpression. Large areas of deletions (e.g. chromosome 3p9p) were commonly seen in the genome of NSCLC (32,33) preferentially in regions coding for tumor suppressor genes. *Pim-1* is located at 6p21.2. However, no deletions were reported for NSCLC patients within this area thus far. It is therefore unlikely that the down-regulation of *pim-1* expression is due to a deletion process pointing to a potential tumor-suppressor function of pim-1 in NSCLC.

The oncogenic potential of the *pim-1* gene was suggested by its first identification as a common proviral integration site in MMLV-induced murine T-cell lymphomas (34). Transgenic mice overexpressing pim-1 in their lymphoid compartment show a low but significant increase in spontaneous lymphomagenesis (35) indicating that overexpression of pim-1 contributes to the tumorigenic process. By itself, *pim-1* has a low oncogenic potential but cooperates strongly with other oncogenes, such as *c-myc*, *N-myc* and *gfi-1*, in T-lymphomagenesis (36).

In conclusion, our analyses indicate a highly significant down-regulation of *pim-1* mRNA expression in NSCLC. The mechanism leading to the down-regulation of *pim-1* in NSCLC has yet to be identified. Down-regulation already occurred in early stage NSCLC (pT1) and was associated with the occurrence of lymph node metastases (pN1-2). The down-regulation of *pim-1* may therefore either be involved in the process of lymphatic invasion or merely represent a surrogate marker for the lymphatic progression of NSCLC.

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