Lung Cancer 76 (2012) 183–190

Contents lists available at SciVerse ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

# Characterization of periostin isoform pattern in non-small cell lung cancer

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ARTICLE INFO

*Article history:*
Received 9 June 2011
Received in revised form
22 September 2011
Accepted 12 October 2011

*Keywords:*
Periostin protein
Isoforms
Non-small cell lung cancer
Epithelial–mesenchymal transition
Tissue microarray analysis
Laser capture microdissection

ABSTRACT

*Introduction:* The extracellular matrix N-glycoprotein periostin (OSF-2, *POSTN*) is a major constituent of the desmoplastic stroma around solid tumors. It promotes tumor invasion and metastasis via epithelial-mesenchymal transition (EMT). In this study we investigated periostin expression at both RNA and protein level as well as the expression pattern of its splice isoforms in non-small cell lung cancer (NSCLC).
*Methods:* Thirty fresh frozen and corresponding formalin-fixed NSCLC tissues (adeno- and squamous cell carcinoma subtype, each $n = 15$) and their matched non-neoplastic tissues were investigated. Periostin mRNA levels were analyzed by quantitative RT-PCR. The EMT-markers periostin and vimentin were analyzed by immunohistochemistry. Laser capture microdissection allowed for analysis of periostin expression in tumor epithelia and stroma, separately. Isoform patterns were investigated by isoform-specific PCR following sequencing in NSCLC, fetal and adult normal lung tissue.
*Results:* The qRT-PCR analysis showed periostin mRNA up-regulation in NSCLC tissue in relation to normal lung, with significantly higher levels in the adeno-compared to the squamous cell subtype ($p < 0.05$). However, protein levels in both tumor epithelia and stroma correlated with squamous cell carcinoma ($p < 0.001$) and larger tumor size ($p < 0.05$). Further, periostin tumor epithelia expression, correlated with higher tumor grade ($p < 0.05$). Sequence analysis detected eight periostin isoforms in fetal lung, but only five in both NSCLC and matched normal lung tissue. Among the eight isoforms, four are new and were labelled 5, 7, 8 and 9. The exclusive presence of isoforms 1 and 9 in fetal tissue suggests splice-specific regulation during lung embryogenesis. Finally, laser capture microdissection demonstrated that both tumor epithelia and stromal cells can be a source of periostin production in NSCLC.
*Conclusions:* This study represents the first analysis of periostin isoform expression patterns in NSCLC and a characterization of periostin expression in cancer versus stromal cells at both RNA and protein level.


## 1. Introduction

Epithelial cancers such as NSCLC are composed of malignant tumor cells embedded in a specialized form of supportive collagenous tissue, defined as desmoplastic stroma by surgical pathologists. More recently, this desmoplastic stroma has been called tumor microenvironment, inferring the principal idea that a carcinoma creates its own niche, in which the sum of all anti- and pro-tumoral factors favour a pro-tumoral environment, thereby enabling progressive tumor growth and invasion [1,2]. Invasive lung adenocarcinoma (AC) is differentiated from the non-invasive lepidic growth pattern (formerly called non-mucinous bronchiolo-alveolar carcinoma) by infiltration of small glands or single tumor cells into its own newly formed local stroma [3]. This newly formed stroma often forms a central scar [4], but there are also strands of desmoplasia between the tumor cells (intra-tumoral stroma) as well as at the tumor periphery abutting the lung parenchyma or the mediastinum (peri-tumoral stroma), particularly in case of squamous cell carcinoma (SCC). It has been suggested that epithelial–mesenchymal transition (EMT) of carcinoma cells, which confers increased invasion and metastasis properties, is relevant for NSCLC [5]. Thereby, loss of E-cadherin and increase of vimentin expression achieves a more migratory phenotype [6].

Periostin is a secreted matrix N-glycoprotein (*POSTN*, also known as OSF-2) of 93 kDa. It shares homology with the insect cell adhesion molecule fasciclin I and can be induced by TGF-β and BMP-2 [7–9]. It was first identified in osteoblast-like cells and was shown to be important for bone formation and maintenance [7,8] as well as for cardiac development and healing [10,11]. It is present in many healthy tissues such as periodontal ligament, periosteum, cardiac valves and placenta and in tissues under mechanical stress conditions [10–16]. Its up-regulation has been observed in a wide

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doi:10.1016/j.lungcan.2011.10.013

variety of cancerous tissues, such as ovarian, breast, pancreatic, head and neck carcinomas as well as in neuroblastoma [16–21]. Periostin is thought to promote EMT of carcinoma cells by targeting the crosstalk between the epidermal growth factor receptor (EGFR) and the integrins at the plasma membrane, with consecutive activation of the Akt/PKB (protein kinase B) and the FAK (focal adhesion kinase) pathway [5,17,19,22,23].

Thereby periostin upregulation in cancer is linked to a more invasive and metastatic phenotype because its fasciclin-like domains act as a ligand for the integrins αvβ3, αvβ5 and α6β4 [17,24]. Periostin activated signaling pathways are able to promote cellular survival, angiogenesis and resistance to hypoxia induced cell death [17–19,22,24,25]. As such, this protein is considered to be a potential cancer prognostic biomarker. Furthermore, it is considered to be a potential target for tumor-therapy, since the epitope is accessible via blood stream [26,27].

While the periostin N-terminal region (up to exon 16) is conserved, the C-terminal region (comprising exon 17–23) gives rise to different splice isoforms upon alternative splicing. The isoforms differ in the cassette exons 17–21 (from UniProtKB/Swiss-Prot database) and have a molecular weight range from 83 kDa to 93 kDa. Six different periostin splice isoforms have been reported, but only four of them were sequenced and annotated [26,28]. In bladder carcinoma, increased invasiveness has been associated with variant I [29] (corresponding to isoform 4 in UniProtKB/Swiss-Prot database), but their global functional significance as well as their expression patterns in the various types of cancer is still unknown.

Previously, we identified periostin in a proteomics screen of malignant pleural effusions from patients with lung adenocarcinoma by shotgun mass-spectrometry [30] and assessed its importance as a prognostic factor for decreased progression-free survival in NSCLC [5]. Additionally, we characterized the periostin isoform pattern in renal cell carcinoma (RCC), identifying a new, tumor-associated isoform [31]. We also found upregulation and correlation with poor survival of periostin in RCC, malignant pleural mesothelioma, bile duct carcinoma and prostate cancer [31–35]. Consequently, the aim of this study was to characterize periostin isoform patterns in NSCLC and to analyze periostin RNA and protein levels in both tumor epithelia and desmoplastic stroma, separately.

## 2. Methods

### 2.1. Patients

In this study we assessed periostin RNA and protein expression levels in surgically resected and fresh frozen as well as formalin-fixed NSCLC tissue samples from a cohort of 30 patients. Tumors were classified according to the current WHO classification (2004) and the 7th TNM edition (2010) of the UICC [36]. This cohort included 15 adenocarcinoma (AC) and 15 squamous cell carcinoma (SCC) subtypes. The age range was 41–84 years and the tumor size ranged between 1.2 cm and 13.0 cm. Eight patients were diagnosed as stage pT1, 16 as pT2, and 6 as pT3-4. Three patients were diagnosed as grade 1, 10 as grade 2, and 17 as grade 3.

Selection criteria for the frozen samples were (a) sufficient tissue quantity available and (b) preserved morphology. Intra-tumoral stroma was defined as areas consisting mostly of sheets or glands of carcinoma, with interspersed fibrillary extracellular matrix (ECM), sometimes containing single tumor cells. In contrast, peri-tumoral stroma was by definition around the tumor, without intermingled single tumor cells, and mostly representing a central scar or a peripheral capsule.

All tumors were entirely reviewed on haematoxylin–eosin (H&E) and alcian-blue periodic acid Schiff (AB-PAS) staining of whole sections by L.M. and A.S. The study was approved by the institutional ethical review board of the canton of Zurich under ref. nr. StV 29 – 2009.

### 2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from 10 to 15 mg of frozen tissue using the RNeasy mini kit (Qiagen, Hombrechtikon, Switzerland). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Rotkreuz, Switzerland) following the manufacturer's protocol. RNA yield and purity were analyzed by NanoDrop 1000 (Thermo scientific, Wohlen, Switzerland).

### 2.3. Quantitative RT-PCR analysis

Quantitative RT-PCR was performed using ABI 7900HT Fast RealTime PCR apparatus (Applied Biosystems). cDNA synthesised as described above was used, together with the following two PCR systems for gene expression assays (Applied Biosystems): Hs01566748 for human periostin (GenBank accession no NM_006475) and Hs99999905 for human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (GenBank accession no NM_002046.3) [37]. The cycle threshold values for periostin were first normalized against GAPDH as endogenous control and, second, against normalized matched normal lung tissue. Further, the relative expression levels were normalized against percentage of tumor (with its associated intra-tumoral stroma) per total surface on the corresponding H&E-stained frozen section, thereby excluding attached normal lung tissue.

### 2.4. Semi-quantitative RT-PCR analysis

For semi-quantitative RT-PCR we used the primer pairs previously described by Castronovo et al. [26] which detect all isoform transcripts for human *POSTN*, according to Gene Bank accession number NM_006475. The primer sequences for periostin are as follows: Per-F2, 5′GCTGATAGATCTTATCCAGCAGACACACCTGTTG3 and Per-R2, 5′CGATTCAAGCTTAGTGATGGTGATGGTGATGCTGAGAACGACCTTCCCTTAATC3′. Control GAPDH primers were: GAPDH-F 5′TGGAAGGACTCATGACCACA3′ and GAPDH-R 5′TGCTGTAGCCAAATTCGTTG3′.

Periostin PCR was performed with 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 50 s, followed by a final extension of 7 min. GAPDH PCR was similarly performed, with annealing at 50 °C. The PCR products were separated by electrophoresis on 1.5% agarose gels.

### 2.5. Laser capture microdissection (LCM) of fresh frozen SCC tissue sample

In order to collect cells from different tissue compartments separately, we performed LCM and collected tumor epithelia, intra- and peri-tumoral stroma. Tissue from a SCC frozen section was cut, 8 μm thick, and mounted on a MMI-membrane slide (MMI, Glattbrugg, Switzerland). The tissue was immediately stained using the H&E staining kit for LCM (MMI) and air dried with desiccant granular silica gel for 15 min. Subsequently, the tissue was microdissected using the CellCut Plus 3.47 apparatus (MMI) and collected in 500 μl tubes with adhesive lid and diffuser (MMI). About 6 $mm^2$ of tissue were collected from each sample. Tumor epithelia, intra- and peri-tumoral stroma were separated on morphologic basis by L.M. and A.S. After microdissection, total RNA was immediately extracted using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA). RNA integrity was analyzed by capillary electrophoresis using 2100 bioanalyzer