Expression of Formyl-peptide Receptors in Human Lung Carcinoma

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Abstract. *Background/Aim: Formyl-peptide receptors (FPRs) are expressed in several tissues and cell types. The identification of markers involved in cell growth may further allow for molecular profiling of lung cancer. We investigated the possible role of FPRs as molecular markers in several types of lung carcinomas which is the main cause of cancer death worldwide. Materials and Methods: Tumor tissue samples were collected from six patients affected by lung cancer. Biopsies were analyzed for expression of FPR isoforms both in tumoral and peritumoral tissue by real-time polymerase chain reaction (PCR), western blot and immunofluorescence. Results: Real-time PCR, western blot and immunofluorescence analyses showed that FPR expression is lower in types of human lung cancer tissues when compared to the surrounding peritumoral tissues. Conclusion: The study of the mechanistic basis for the control of FPR expression in normal peritumoral versus tumoral tissues could provide the basis for new diagnostic and therapeutic interventions.*

The human formyl-peptide receptors FPR1, FPR2 and FPR3 belong to the G-protein-coupled receptor (GPCR) family (1) and were first detected in phagocytic leukocytes and in monocytes. Their expression has also been demonstrated in several tissues and cell types at the protein or mRNA levels (2, 3) and their relevant biological functions have emerged through identification of high affinity host-derived agonists. Such ligands trigger intracellular signaling cascades involving phosphatidylinositol 3-kinase, protein kinase C, mitogen-activated protein kinases, signal transducer and activator of transcription, cellular Src kinase, as well as several transcription factors (4-6).

Despite FPRs lack of intrinsic tyrosine kinase activity, tyrosine phosphorylation of a tyrosine kinase receptor (RTK) occurs in response to binding of agonists to such receptors, triggering mitogenic pathways. FPR-mediated RTK transactivation may occur by different molecular mechanisms, which include the activation of metalloproteases or the activation of membrane associated non-receptor tyrosine kinases (7). Further evidence supports the role of reactive oxygen species (ROS) in FPR-mediated RTK transactivation. For instance, in human lung cancer cells, FPR2 stimulation induces ROS-dependent Epidermal growth factor receptor (EGFR) tyrosine phosphorylation (8) and in human prostate cells, ROS mediate hepatocyte growth factor receptor transactivation by FPR2 (9).

Lung cancer remains the leading cause of cancer-related death worldwide. Tobacco smoking and air pollution exposure are mainly implicated in lung cancer development (10-13). Cytotoxic chemotherapy offers modest prolongation in survival, although the additional gain in terms of response rate and survival with combinations of chemotherapy and monoclonal antibodies have reached a plateau (14-16). The development of molecular profiling technologies to assess DNA, RNA, protein and metabolites have provided

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Table I. *Nucleotide sequences of FPR primers.*

FPR	Variant	Primer sequence
FPR1	Variant 1 (FPR1v1)	Sense: 5'-CCACATGTGGAGCAGACAAG-3' Antisense: 5'-CCGAGGACAAAGGTGACTG-3'
	Variant 2 (FPR1v2)	Sense: 5'-CCACATGTGGAGCAGACAAG-3' Antisense: 5'-GTCCAGGAGCAGACAAGATG-3'
FPR2	Variant 1 (FPR2v1)	Sense: 5'-GCAGCCTTGAGGTCATAAGC-3' Antisense: 5'-TGTAGCCAGCAGACTCATAGG-3'
FPR2	Variant 2 (FPR2v2)	Sense: 5'-CTGGTGCTGCTGGCAAGAT-3' Antisense: 5'-TCCGCAGAACAGTGTAGCCA-3'
FPR3		Sense: 5'-AGGATCTAAGCTGGTGGTGT-3' Antisense: 5'-GTGACTCCGTGGACTAGCA-3'

Table II. *Histological diagnosis of the 48 biopsies analyzed in this study.*

Histology	No. of samples
Squamous-cell carcinoma	8
Non-small cell lung cancer	8
Small cell lung cancer	8
Lung adenocarcinoma	8
Lung adenosquamous carcinoma	8
Typical lung carcinoid tumor	8

	Fold expression		
Sample	**FPR1**	**FPR2**	**FPR3**
I	0.173138684	0.168468929	0.104305138
II	0.487682678	0.221292332	0.143096892
III	0.054229821	0.05088025	0.035799424
IV	0.225133735	0.198547232	0.177249585
V	0.046548	0.0619785	0.0463935
VI	0.1185795	0.162925	0.1574305

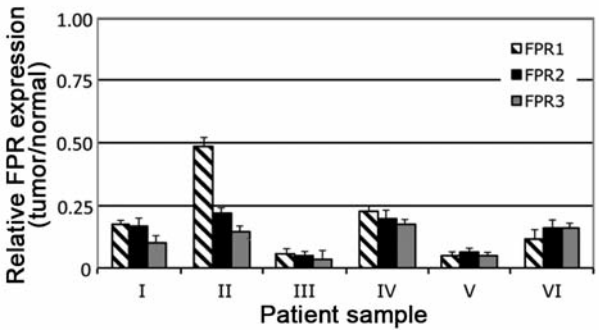

Figure 1. *Expression of FPRs isoforms in lung cancer tissues. Total RNA was isolated from tumoral and normal peritumoral area of lung cancer biopsies. cDNAs analysis was performed by real time-PCR. The fold of increase of FPRs isoforms, expressed as* $2^{(Ct\ test\ gene - Ct\ \beta\text{-}Actin)}$*, was obtained as mean value and reported in the Table. The graphic shows the relative gene expression of FPRs isoforms (mean value) as ratio of tumor versus adjacent tissue.*

substantial advances in our understanding over the molecular basis of cancer (17-20), leading to the potential development of more effective targeted therapies (21-23).

Identification and characterization of oncogenic drivers involved in sustained cell growth may further delineate the molecular profiling of lung cancer. Despite the increasing number of potentially targetable molecular alterations in non-small-cell lung cancer (NSCLC), the oncogenic drivers with approved targeted agents remain the EGFR mutations and the anaplastic lymphoma kinase gene re-arrangements (24). Novel biomolecular markers in NSCLC also include metabolic pathways (25, 26), DNA damage-repair genes (Excision Repair Cross-Complementation 1, Ribonucleotide Reductase M1), Breast Cancer Type 1 susceptibility protein (27) and more recently BRCA1-associated RING domain protein 1 (BARD1) (28, 29).

We investigated the possible role of FPR in lung carcinoma by assessing their isoforms in neoplastic and surrounding peritumoral tissues of NSCLC as well as other types of lung carcinoma.

Materials and Methods

Patients. Patients enrolled in this study were required to fulfil the following eligibility criteria: a) cytological or histological confirmation of lung cancer; b) surgically resectable disease; c) no previous or concomitant malignancy; d) chemotherapy naive. Biopsies were analyzed for the expression of FPR isoforms both in tumoral and adjacent tissues. All participants gave written informed consent, approved by Institutional Ethic Committees (approval number 528/2007). The study was carried out in accordance with the principles of the Declaration of Helsinki.

RNA purification and real-time PCR analysis. Total RNA from lung tissue specimens was purified with TRIZOL® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNAs were synthesized in a GeneAMP-PCR system 9700 (Applied Biosystem, Carlsbad, CA, USA). Two micrograms of total RNA were reverse-transcribed by using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) in a final volume of 20 μl. The samples were incubated at 25˚C for 10 min followed by 42˚C for 60 min and 85˚C for 5 min. Aliquots of cDNA (1⁄20 of reverse transcription reactions) were used in real-time PCR experiments. SYBR Green-based real-time PCR was used to determine cDNA levels. The sequence of the FPR

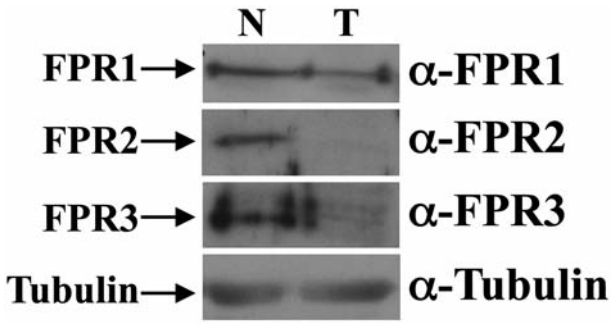

Figure 2. *Western blot analysis of FPRs isoforms were performed on whole lysates purified from lung cancer specimens (T) and normal surrounding tissue (N). Same amounts of proteins were resolved on 10% SDS-PAGE and FPR1, FPR2 and FPR3 isoforms were identified with specific antibodies (α-FPR1, α-FPR2, α-FPR3). Filters were normalized with an anti-Tubulin antibody (α-Tubulin). The experiment is representative of the mean level of expression of FPRs isoforms.*

primers is reported in Table I. Expression levels were calculated relative to those of β-actin as an endogenous control. The primers for β-actin were: 5'-AGAGCTACGAGCTGCC TGAC-3' (sense) and 5'-AGCACTGTGTTGGCGTACAG-3' (antisense). Relative expression was calculated as 2(Cttest gene-Ctβ-actin). Each cDNA sample was analyzed in triplicate, and the corresponding no-RT mRNA sample was included as a negative control.

Western blot analysis. Lung biopsies were washed in phosphate-buffered saline (PBS) solution and about 100 mg of tissue were homogenized. Total proteins were purified and separated by electrophoresis on SDS PAGE; western blot analysis was then performed according to procedures, as previously described (30-33). Antibodies to FPR1 and FPR3 were purchased from Abcam (Cambridge, UK). Anti-FPR2, anti-tubulin, anti-goat and anti-mouse antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunofluorescence. Immunofluorescence assay was performed on fixed-embedded lung biopsies. After de-paraffinization in xylene

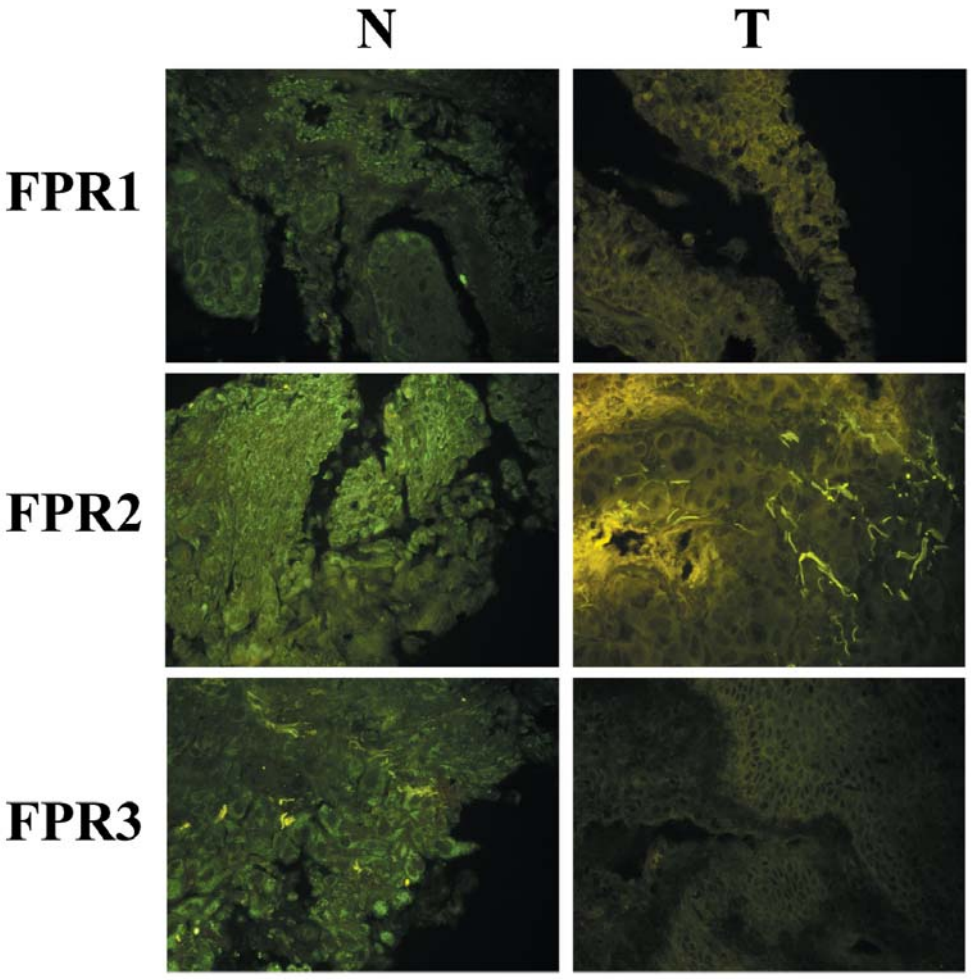

Figure 3. *Confocal images of lung biopsies isolated from lung cancer specimens (T) and normal surrounding tissue (N). The slides were stained with selective antibodies for the different FPR isoforms. The experiment is representative of the mean level of expression of FPRs isoforms in tumoral and normal portion.*

and rehydratation in PBS, 4 μm-thick sections were prepared and mounted on glass slides. Sections were fixed in 4% paraformaldehyde (PFA) at 4˚C for 15 min and permeabilized in 0.1% TritonX-100 (Sigma, Saint Louis, MO, USA) in PBS at room temperature for 3 min. Sections were then blocked by 1% bovine serum albumin (BSA) at room temperature for 30 min followed by incubation with antibodies against FPR1, FPR2 or FPR3 overnight at 4˚C. Sections were then washed in PBS and stained with secondary antibody Alexa Fluor 488 (1:200; Invitrogen) for 1 h at room temperature. Images were captured by using a Zeiss LSM 510 meta confocal microscope equipped with an oil immersion plan Apochromat 63×objective 1.4 NA, using the following settings: green channel for detecting FITC, excitation 488 nm argon laser.

Results

Previously, we analyzed FPR expression in human lung cancer cells and we characterized their ability to transactivate EGFR. By RT-PCR and western blot analysis, we observed that FPR2, but not FPR1 or FPR3, is expressed in these cells (8). The unexpected expression of FPR2 in CaLu6 cells prompted us to investigate the expression of FPRs in human lung cancer tissues, as well as in the surrounding peritumoral tissues. Histological reports of the 48 samples that we analyzed showed different diagnoses of lung carcinoma (Table II). Total RNAs from normal peritumoral and tumor tissues were purified and analyzed by real-time PCR using specific primers for *FPR1, FPR2, FPR3* and the respective variants of the receptors. Oligonucleotide sequences are summarized in Table I.

The results show that all human lung cancer tissues significantly underexpressed the three FPR family members when compared to the surrounding peritumoral tissues, independently of the tumor classification (Figure 1). We also analyzed FPR protein expression by western blotting. Specific antibodies recognized the three isoforms of the receptor in protein extracts purified from tumoral and adjacent tissues, showing a reduced expression of the proteins in all types of lung carcinoma (Figure 2). The same antibodies were used in immunofluorescence experiments performed on slides of lung cancer and surrounding tissues. According to the real-time PCR data, FPR1, FPR2 and FPR3 expression was lower in tumoral tissues (Figure 3). All the results are expressed as mean value of three independent experiments performed on eight samples of each histological subgroup, as reported in Table II.

Discussion

The results demonstrate that FPRs are expressed in normal human lung tissues and the FPR antigens are present in normal lung sections. We have not examined a large number of samples and the sections that we analyzed do not represent all the different types of structures and cells in the lung. Nevertheless, our results clearly show that FPR expression decreases in lung tumoral tissues. Since FPRs are involved in EGFR transactivation (8), these results suggest a potential role of the cross-talk mechanisms in tyrosine kinase inhibitors resistance observed in lung cancer treatment. On the other hand, it has been shown that FPR1 is expressed by highly malignant human glioma cells and appears to mediate motility, growth and angiogenesis of human glioblastoma by interacting with host-derived agonists (34). In addition, the human glioblastoma cell line U-87 expresses high levels of FPR1, which upon activation by N-formyl-methionyl-leucyl-phenylalanine or by an agonist released by necrotic tumor cells, promotes the directional migration, survival and production of angiogenic factors by tumor cells (35). In these cells, FPR1 activates EGFR by increasing the phosphorylation of a selected tyrosine residue in the intracellular tail of EGFR (36). FPR1 and FPR2 are overexpressed in primary melanoma and correlate with aggressive tumor characteristics, underscoring them as potential therapeutic targets (37), whereas in laryngeal carcinoma cells, FPR2 expression is down-regulated (38).

Tumor cells often produce aberrant levels of growth factors that stimulate cell surface receptors to increase cell proliferation in an autocrine manner. In addition, malignant tumor cells express receptors that interact with agonists that are present in the vicinity of the tumor or produced by distant organs to increase tumor cell motility and thus to favor tumor cell invasion and metastasis. Our analysis suggests that receptors other than FPRs, such as C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 7 (CCR7), might be involved in the pathogenesis of different types of lung carcinoma examined in this study. Further studies are required to define the relationship between FPR expression and the progression of human lung cancer and to identify the mechanistic basis for the control of FPR expression in peritumoral *versus* tumoral tissues. In addition, the relationship between FPR expression and the survival of patients with lung carcinoma after treatment needs to be established. The promiscuity of these receptors in binding different ligands, coupled with their presence in different cells and tissues, indicates a diverse role in multiple biological settings. A better understanding of these fundamental functions could lead to the identification of new therapeutic targets for drug development.

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