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A promising role of interferon regulatory factor 5 as an early warning biomarker for the development of human non-small cell lung cancer

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ABSTRACT

Objectives: Non-small cell lung cancer (NSCLC) accounts for 85%–90% of lung cancer cases and is a covert disease lacking early symptoms. Since cancer is recognised as an inflammation-associated condition, we analysed the relationship between the expression of interferon regulatory factor 5 (IRF5), a key transcription factor controlling inflammatory responses, and NSCLC development with the aim of identifying a warning biomarker for early diagnosis of the disease.

Materials and methods: The expression of IRF5 and its associated inflammatory factors IL-6, IL-10, IP-10, and TNF-α in the peripheral blood of NSCLC patients (n = 66) and healthy controls (n = 42) was analysed by quantitative RT-PCR, flow cytometry, and a cytometric bead array. IRF5 protein expression in NSCLC tissues (n = 102) was detected by Western blotting. The diagnostic value of IRF5 expression was determined by a receiver-operating characteristic (ROC) curve analysis.

Results: The protein levels of IRF5, IL-6, and IP-10 were significantly higher in the peripheral blood of NSCLC patients than in that of healthy controls. IP-10 levels in plasma and IL-10 mRNA expression in white blood cells (WBCs) were significantly upregulated in early-stage NSCLC, whereas plasma IL-6 and IL-10 were elevated in the progressive stage. IRF5 protein levels in WBCs were positively correlated with plasma IP-10 but negatively correlated with plasma IL-10. Furthermore, the mRNA and protein levels of IRF5 in WBCs were significantly elevated in patients with early stage NSCLC compared to those in the progressive stage. Additionally, IRF5 protein levels were significantly lower in NSCLC tumour tissues than those in normal lung tissues.

Conclusions: IRF5 levels in WBCs can be significantly upregulated in early stage NSCLC and were shown to have diagnostic value as an early warning biomarker of NSCLC development.

1. Introduction

Lung cancer is the most frequently diagnosed cancer and the leading cause of cancer-related deaths worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for 85%–90% of lung cancer cases and includes two subtypes: lung squamous cell carcinoma and lung adenocarcinoma [2]. The survival of patients with lung cancer is strongly related to the cancer stage as evidenced by a steep decrease in the 5-year overall survival rate from 82% in stage IA to 6% in stage IV [3]. However, in routine clinical practise, many patients with early stage lung cancer are not diagnosed in due time because they are often asymptomatic [4]. As a result, at the time of diagnosis, approximately 70% of NSCLC patients already have locally advanced or metastatic tumours [5]. Reliable early warning indicators of NSCLC development should improve the survival of lung cancer patients; however, these indicators are not currently available and need to be identified.

Cancer cells evolve from normal cells altered by gene mutations. Normally, the body's surveillance system, the immune system, responds to the altered cells by initiating inflammation, which is a critical component of the innate immune response to harmful stimuli [6]. In

Abbreviations: IRF5, interferon regulatory factor 5; NSCLC, non-small cell lung cancer; AD, adenocarcinoma; SQ, squamous cell carcinoma; CT, cancer tissues; PCT, para-carcinoma tissues; ES, early stage; PS, progressive stage; HC, healthy controls; AUC, area under the receiver-operating characteristics curve; CI, confidence interval; OR, odds ratio; WBCs, white blood cells; NE, neutrophil; MO, monocyte; LY, lymphocyte; qRT-PCR, quantitative reverse transcription-polymerase chain reaction

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1863, Rudolf Virchow provided the first indication of a possible link between inflammation and cancer when he observed the presence of leucocytes in neoplastic tissues [7]. Research in recent decades provides abundant evidence of the role played by inflammation in tumorigenesis [8]. Inflammation supplies the tumour microenvironment with bioactive molecules activating epithelial-mesenchymal transition (EMT), growth factors and chemokines that support cell proliferation and inhibit death, and pro-angiogenic factors, extracellular matrix-modifying enzymes that facilitate tumour angiogenesis, invasion, and metastasis, thus promoting cancer progression [9–12]. The link between inflammation and cancer is confirmed by the activation of nuclear factor-κB (NF-κB), a key transcription factor. The activated NF-κB in inflammatory immune cells can lead to the production of inflammatory mediators that support the growth and survival of carcinoma cells. If NF-κB is activated in malignant cells, it can result in the elevated expression of cell-cycle genes, apoptosis inhibitors, and proteases that promote the invasive phenotype [13]. Furthermore, tumour cell-derived inflammatory mediators can activate pattern recognition receptors (PRRs), which are critical for triggering innate immune responses [10]. A PRR expressed on innate immune cells can also recognise its ligand expressed on tumour cells to trigger anti-tumour responses [14].

In innate immune cells, PRR activation upregulates the expression of many inflammatory genes, including those coding for NF-κB and interferon regulatory factors (IRFs). IRF5 is an IRF family member with diverse immunomodulatory activities [15]. IRF5 is regulated through the activation of Toll-like receptors (TLRs), a type of PRR that results in the formation of the MyD88-IRF5-TRAF6 complex [16] and the release of IRF5 from its C-terminal self-inhibition through phosphorylation [17]. The phosphorylated IRF5 is translocated from the cytoplasm into the nucleus, where it binds the IFN-stimulated response element (ISRE) and promotes transcription of the target genes [18]. Through such activity, IRF5 regulates type I IFNs and directly induces the expression of pro-inflammatory cytokines/chemokines such as IL-6, TNF-α, IL-12, macrophage inflammatory protein 1-alpha (MIP-1-α), and IFNγ-induced protein (IP)-10, while repressing the transcription of anti-inflammatory cytokines such as IL-10 [16,19–21]. IRF5, which is expressed in many immune cells including monocytes, macrophages, dendritic cells, B cells, and neutrophils [22,23], was shown to play a pivotal role in the inflammatory polarisation of monocytes/macrophages [21,24], regulation of B cell activation, proliferation, plasmablast differentiation [25,26], and in the recruitment of neutrophils to sites of inflammation [27].

Accumulating evidence indicates that IRF5 negatively affects the growth of many types of cancer cells. Thus, it was shown that ectopic expression of IRF5 reduced proliferation of chronic myeloid leukaemia cells in vitro [28], whereas IRF5 loss stimulated tumorigenesis of HCV-associated hepatocellular carcinoma and promoted the progression and metastasis of breast cancer [29,30]. IRF5 deficiency resulted in tumorigenic transformation of murine embryonic fibroblasts in vitro and in athymic nude mice [31]. In IRF5$^{-/-}$ mice, massive tumour growth and marked metastasis of B16F1 melanoma cells to the lungs were observed after the subcutaneous injection of B16 cells [14]. Furthermore, IRF5 knockdown promoted invasion and metastasis of NSCLC cells [32]. In contrast, IRF5 was shown to induce tumorigenesis in thyroid cancer and classical Hodgkin's lymphoma [33,34], indicating that the effects of IRF5 can be cancer type-specific.

To date, there have been no reports on the association between IRF5 activity in innate immune cells and NSCLC development. In this study, we analysed IRF5 gene polymorphisms and the expression of IRF5 and its target cytokines TNF-α, IL-6, and IL-10, as well as the chemokine IP-10 in the peripheral blood of NSCLC patients to determine whether IRF5 and downstream inflammatory mediators could be used as biomarkers for assisting in early NSCLC diagnosis.

2. Materials and methods

2.1. Collection and preparation of human specimens

Using anticoagulant-containing tubes, peripheral blood specimens were collected from NSCLC patients (43 male and 23 female) prior to surgery or chemoradiotherapy in the Department of Respiratory Medicine and from healthy controls in the Health Checkup Centre of the First Hospital of Jilin University, China. White blood cells (WBCs) were used directly and the plasma was stored at −70 °C until use. The inclusion criteria for the NSCLC patients were as follows: primary NSCLC was diagnosed for the first time and there were no other tumours, acute inflammation, autoimmune diseases, or bronchial/lung diseases such as COPD, bronchiectasis, asthma, and interstitial lung disease. The healthy controls were recruited based on the following criteria: no cancer, autoimmune, infectious, or other lung diseases or pregnancy. Paired samples of lung cancer tissues and adjacent non-tumour lung tissues (> 5 cm from the edge of the tumour) from 69 male and 33 female NSCLC patients were provided by the Research Centre of Clinical Medicine, the First Hospital of Jilin University. According to the tumour lymph node metastasis (TNM) classification (Eighth Edition, International Association for the Study of Lung Cancer, 2015) of lung cancer, we defined NSCLC stages I-IIA and IIB-IV as early and progressive, respectively. The general characteristics of the NSCLC patients in the two cohorts are presented in Table 1. None of the patients received chemoradiotherapy prior to surgery. The present study was approved by the Ethics Committee of the First Hospital of Jilin University

Table 1
General characteristics of the NSCLC patients in this study.

Variable	Blood specimen				*p* value		Tissue specimen			*p* value
	HC(%) (N = 42)	NSCLC patients			HC vs. NSCLC	ES vs.PS	NSCLC patients			
		All (%) (N = 66)	ES(%) (N = 34)	PS(%) (N = 32)			All (N = 102)	ES(%) (N = 32)	PS(%) (N = 70)	
Age	57.21 ± 11.98	59.7 ± 7.899	59.68 ± 8.831	59.72 ± 6.915	0.2390[a]	0.9829[a]	58.12 ± 10.27	58.88 ± 5.820	57.77 ± 10.52	0.617[a]
Gender										
male	22(52.4%)	43(65.2%)	21(48.8%)	22(51.2%)	0.186[b]	0.552[b]	69	22(31.9%)	47(68.1%)	0.872[b]
female	20(47.6%)	23(34.8%)	13(56.5%)	10(43.5%)			33	10(30.3%)	23(69.7%)	
Smoking	ND									
Yes		41	20(48.8%)	21(51.2%)		0.569[b]	74	22(29.7%)	52(73.3%)	0.561[b]
No		25	14(56%)	11(44%)			28	10(35.7%)	18(64.3%)	
Histological type										
AD		35	19(54.3%)	16(45.7%)		0.632[b]	60	20(33.3%)	40(66.7%)	0.61[b]
SQ		31	15(48.4%)	16(51.6%)			42	12(28.6%)	30(71.4%)	

Note: (1) Abbreviations: NSCLC, non-small cell lung cancer; HC, healthy controls; AD, adenocarcinoma; SQ, squamous cell carcinoma; ES, early stage; PS, progressive stage; ND, not detected; N, number of individuals. (2) [a] Student's t test; [b] Chi-squared test.

(Ethical Approved No. 2016-406). All of the participants signed informed consent forms and their privacy rights were always observed.

2.2. RNA isolation and quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from the peripheral WBCs with TRIzol reagent (CWBIO, Beijing, China) and reverse-transcribed into cDNA using a cDNA synthesis kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. A quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed in a Light Cycler 480 system (Roche, Shanghai, China) with SYBR Green and the following gene-specific primers: GAPDH: 5′-ACTGGCGTCTTCACC ACCAT-3′ and 5′-GCAGGAGGCATTGCTGATGA-3′; IRF5: 5′-GGGAAAT ACACCGAAGGCG-3′ and 5′-TCCTGCACCAAAAGAGTAATCCT-3′; IL-6: 5′-CTGGCAGAAAACAACCTGAAC-3′ and 5′-ATGATTTTCACCAGGCAA GTC-3′; TNF-α: 5′-CATCTTCTCGAACCCCGAGT-3′ and 5′-GGATACCA CTCCCAACAGACC-3′; IP-10: 5′-AACTGTACGCTGTACCTGCAT-3′ and 5′-GCATCGATTTTGCTCCCCTC-3′; IL-10: 5′-GGCTTGTCACTCGGGGT TCG-3′ and 5′-GCCAAGCCTTGTCTGAGATGA-3′. The cycling conditions were 40 cycles of 95℃ for 15 s and 60℃ for 1 min. The relative transcription levels of the target genes were calculated via the $2^{-\Delta\Delta Ct}$ method using GAPDH mRNA levels as in reference [35].

2.3. Genotyping

Genomic DNA was isolated from the peripheral WBCs using the BloodGen Mini kit (Genomic DNA Isolation Mini Kit; CW0540, CWBIO, Beijing, China). Genotyping for IRF5 rs77571059 and rs2004640 single nucleotide polymorphisms (SNPs) was performed by polymerase chain reaction (PCR) using the Verity 96-well PCR system (Applied Biosystems, Foster City, CA, USA). The identified PCR products were sequenced in a 3730XL sequencer (Applied Biosystems, Foster City, CA, USA) using the following primers: rs77571059: 5′-CATTCCAGATTGC CAAAAGAGC-3′ and 5′-CAACCTGCCGGGCATTC-3′; rs2004640: 5′-CAAGACGCGGAAGTGCCC-3′ and 5′-GCGAGTGCATCGAAAGTAA GGA-3′.

2.4. Blood processing and flow cytometry

Whole blood samples (30 µl each) were stained with PerCPCy5.5-anti-CD45, FITC-anti-CD14, and PE-anti-CD19 (BD Pharmingen, San Diego, CA, USA) for 30 min at room temperature in the dark and red blood cells were lysed. The WBCs were fixed, permeabilised with Transcription Factor Buffer (BD Biosciences, San Jose, CA, USA) for 50 min, stained with APC-labelled anti-IRF5 antibody (Novus, Littleton, CO, USA) for 50 min at 4 ˚C, and subjected to flow cytometry using the FACSCalibur platform (BD Biosciences). The data were analysed with FlowJo software (v.7.6.1) (Tree Star, Ashland, OR, USA).

2.5. Cytometric bead array (CBA) to measure plasma cytokine/chemokine levels

Plasma levels of TNF-α, IL-6, IL-10, and IP-10 were analysed using the CBA Flex Set system (BD Biosciences) according to the manufacturer's protocol. Flow cytometry was conducted using FACSCalibur (BD Biosciences). Quantitative analysis was performed with CellQuestPro and CBA software (BD Biosciences).

2.6. Western blotting

Total protein was extracted from paired samples of NSCLC and adjacent non-tumour lung tissues using the ProteinExt Mammalian Total Protein Extraction Kit (Transgen, Beijing, China) according to the manufacturer's instructions; the protein concentration was measured via the bicinchoninic acid method. Total proteins (20 µg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gels and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 2 h at room temperature with Tris-buffered saline containing 5% dried non-fat milk and incubated with primary antibodies against IRF5 (ab33478; Abcam, Cambridge, MA, USA) or β-actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4℃. After washing with TBST (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], and 0.5% Tween-20) three times for 10 min, the membranes were incubated with horseradish peroxidase-labelled goat anti-rabbit IgG (sc-2004; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature, then washed and incubated with electrochemiluminescence agent for 1 min. Protein-specific signals were detected using X-ray films; the signal intensity was determined by densitometry using the Image Gauge V3.12 program (Fuji Photo Film, Tokyo, Japan) and expressed as the grey value.

2.7. Statistical analysis

Differences between the normally distributed and non-normally distributed variables were analysed via Student's *t*-test and the Mann-Whitney U test, respectively. The genotype and allele frequencies of each SNP were compared using the Chi-squared test or Fisher's exact test. Hardy-Weinberg equilibrium was observed in the patient group. Spearman's test was used for the correlation analysis. Kaplan-Meier survival curves were compared using the log-rank test. Two-tailed p values < 0.05 were considered statistically significant. The sensitivity and specificity of the IRF5 mRNA levels were evaluated by receiver-operating characteristic (ROC) curve analysis and their diagnostic value was assessed based on the area under the ROC curve (AUC). The diagnostic value was considered low at AUC values of 0.5-0.7, moderate at AUC values of 0.7-0.9, and high at AUC values above 0.9. Statistical analyses were performed using Prism 5.0 (GraphPad Software, San Diego, CA, USA) and SPSS 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Association between IRF5 expression in WBCs and NSCLC stage

Since peripheral WBCs are closely involved in the immune response and IRF5 is an inflammatory response-regulated transcription factor, we addressed the question of whether IRF5 could be an early biomarker of peripheral WBC response to the development of NSCLC by analysing the IRF5 expression in the WBCs of the NSCLC patients and the healthy control individuals. The results indicated that the IRF5 transcription in the WBCs of the NSCLC patients was significantly upregulated (approximately 5-fold) compared to the healthy controls ($p < 0.0001$; Fig. 1A). Furthermore, the IRF5 mRNA levels were significantly higher in the WBCs of the patients with early stage NSCLC compared to those with progressive NSCLC ($p = 0.0337$). Thus, the relative IRF5 mRNA expression was > 10 in 55.8% of the early stage NSCLC patients, but only in 37.5% of the progressive stage NSCLC patients (Fig. 1B). ROC analysis revealed that the IRF5 expression in the WBCs had significant diagnostic value for NSCLC ($p < 0.0001$), which was higher for early stage disease (AUC = 0.904) than for progressive disease (AUC = 0.81) (Fig. 1C). Because some of the NSCLC patients had low levels of IRF5 mRNA, we examined whether the difference in the IRF5 expression was related to polymorphisms in the IRF5 gene. Genotyping for polymorphisms at the rs77571059 and rs2004640 loci revealed that the distribution of the rs77571059 SNP in the NSCLC patients was significantly different from that in the healthy controls. Thus, the frequencies of both the -/CGGGG genotype and CGGGG allele were higher in the NSCLC patients than in the healthy controls ($p = 0.026$ and $p = 0.036$, respectively) (Table 2). Further analysis demonstrated that although the distribution of the -/CGGGG genotype was not different between the early stage and progressive stage NSCLC patients (Fig. 1D),

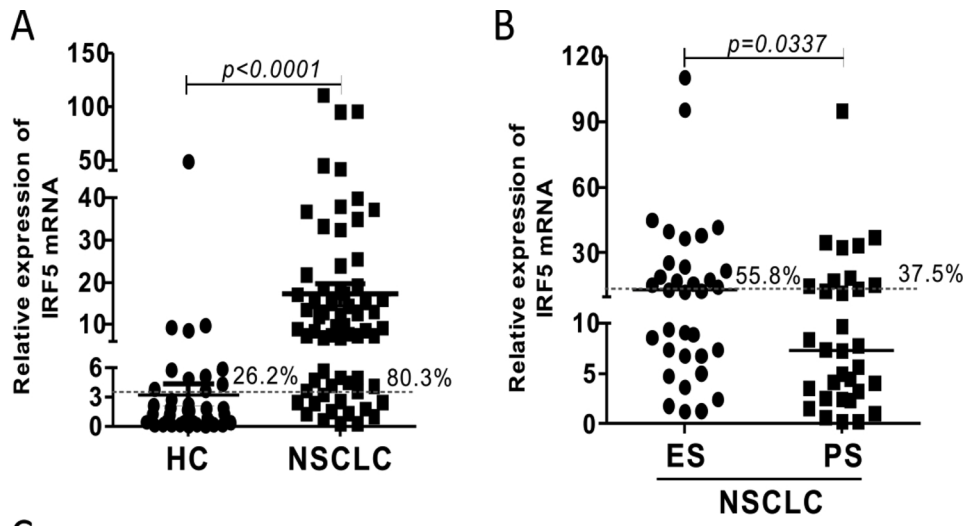

Fig. 1. IRF5 mRNA levels in the WBCs of the NSCLC patients. The IRF5 mRNA was amplified from the WBCs of the NSCLC patients or the healthy donors by qRT-PCR and analysed. **(A)** IRF5 mRNA levels in the NSCLC patients (NSCLC) and the healthy controls (HC). **(B)** IRF5 mRNA levels in the NSCLC patients at either early stage (ES) or progressive stage (PS). **(C)** Diagnostic value of IRF5 mRNA levels in the WBCs for NSCLC. **(D)** Genotype frequency distribution of IRF5 rs77571059 polymorphism at the NSCLC stages. **(E)** Correlation of IRF5 rs77571059 polymorphism with IRF5 mRNA levels in the WBCs of the NSCLC patients. NSCLC, non-small cell lung cancer; AUC, area under the receiver-operating characteristics curve; CI, confidence interval.

C

NSCLC	Target gene	Sensitivity	Specificity	Criterion	AUC	95%CI	*p* value
All	IRF5	87.90%	71.40%	>2.3244	0.858	0.785-0.931	<0.0001
ES	IRF5	85.30%	81%	>4.560847	0.904	0.836-0.972	<0.0001
PS	IRF5	84.40%	71.40%	>2.32443	0.81	0.71-0.909	<0.0001

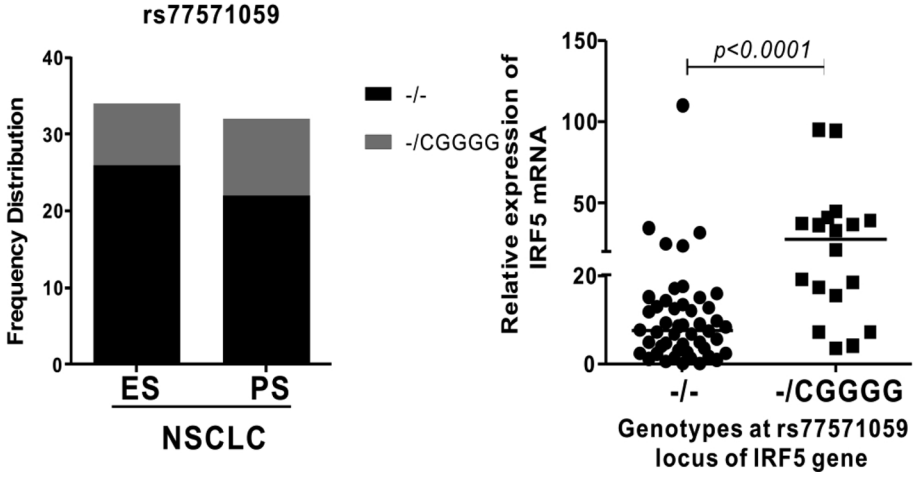

Table 2
Genotypes and allele frequencies of IRF5 gene polymorphisms at rs77571059 and rs2004640 loci in the NSCLC patients in this study.

	Genotypes	NSCLC N (%)	HC N (%)	OR (95%CI)	*p*	χ2, *p* for all	Allele	NSCLC N (%)	HC N (%)	OR (95%CI)	χ2, *p value*
rs77571059	-/-	48 (72.7%)	38 (90.5%)	reference			–	114 (86.4%)	80 (95.2%)	reference	
	-/CGGGG	18 (27.3%)	4 (9.5%)	3.653 (1.112–11.410)		4.985, **0.026***	CGGGG	18 (13.6%)	4 (4.8%)	3.158 (1.030–9.683)	4.419, **0.036***
rs2004640	G/G	39 (59.1%)	28 (66.7%)	reference			G	101 (76.5%)	67 (79.8%)	reference	
	G/T	23 (34.8%)	11 (26.2%)	1.501 (0.631–3.573)	0.357	0.972, 0.652[a]	T	31 (23.5%)	17 (20.2%)	1.21 (0.621–2.358)	0.313, 0.576
	T/T	4 (6.1%)	3 (7.1%)	0.957 (0.198–4.618)	1[a]						

Note: (1) Abbreviations: NSCLC, non-small cell lung cancer; HC, healthy controls; OR, odds ratio; CI, confidence interval; N, number of genotype or allele gene. (2) [a] Fisher's exact test; * $p < 0.05$.

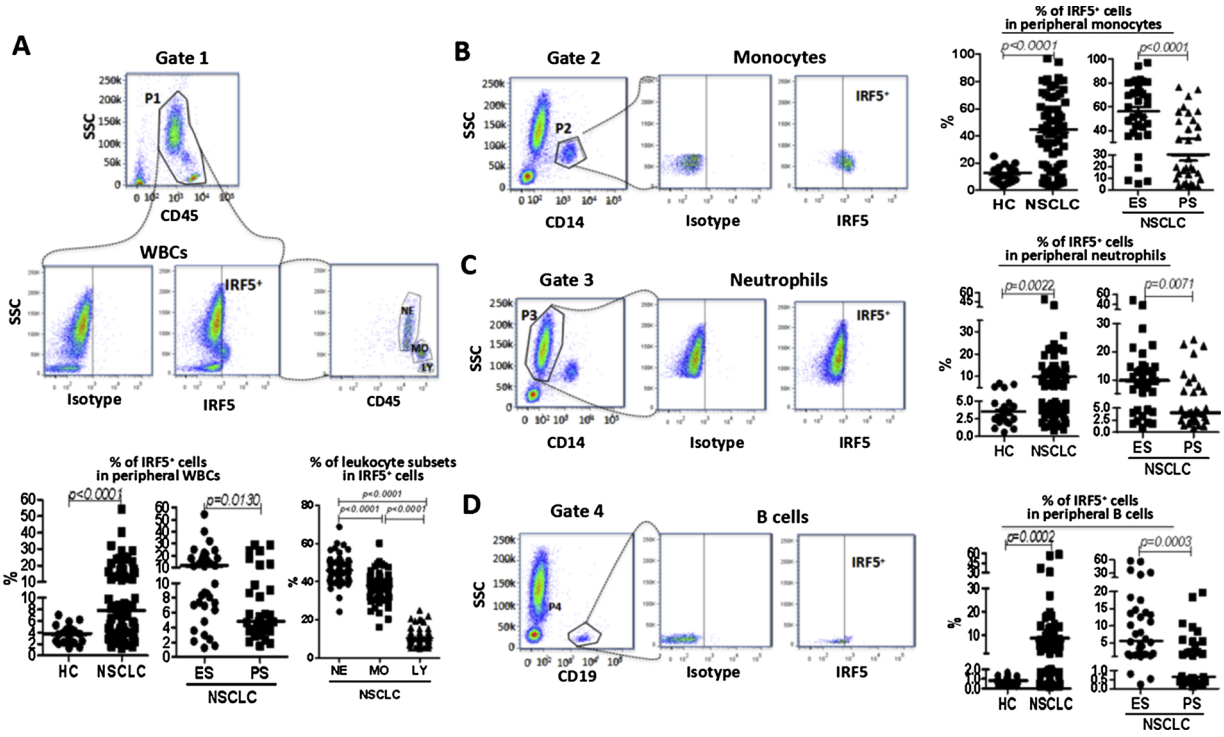

Fig. 2. IRF5 protein levels in the WBCs of the NSCLC patients. Peripheral WBCs from the NSCLC patients (NSCLC) or the healthy controls (HC) were collected and stained with surface or intracellular fluorescence-labelled anti-CD45, CD14, CD19, and IRF5. After gating for different group cells, the percentages of IRF5$^+$ cells were analysed. **(A)** % of IRF5$^+$ cells in the peripheral WBCs and % of leukocyte subsets including neutrophil (NE), monocyte (MO), and lymphocyte (LY) in the IRF5$^+$ cells. **(B)** % of IRF5$^+$ cells in the peripheral monocytes. **(C)** % of IRF5$^+$ cells in the peripheral neutrophils. **(D)** % of IRF5$^+$ cells in the peripheral B cells. NSCLC, non-small cell lung cancer; ES, early stage; PS, progressive stage.

this genotype was significantly positively associated with higher levels of IRF5 mRNA ($p < 0.0001$) (Fig. 1E), indicating that the genetic background influenced the IRF5 response. Consistent with the mRNA expression, the IRF5 protein levels in the WBCs of the NSCLC patients were significantly higher compared to those in the healthy controls ($p < 0.0001$); furthermore, they were much higher in the patients with early stage NSCLC than in those with progressive stage NSCLC ($p = 0.0130$); in the IRF5$^+$ WBCs, neutrophils accounted for the highest proportion, followed by monocytes, while the proportion of lymphocytes was the lowest ($p < 0.0001$) (Fig. 2A). Analysis of the WBC subpopulations indicated that the IRF5 expression was elevated in the neutrophils, B cells, and especially in the NSCLC patient monocytes, particularly in those at the early cancer stage (Fig. 2B-D). These results suggest that increased IRF5 expression in peripheral WBCs may be an early warning sign of NSCLC development.

3.2. Correlation between IRF5 and inflammatory cytokine/chemokine expression in peripheral circulation of NSCLC patients

To determine whether IRF5 upregulation in the peripheral WBCs of the NSCLC patients affected the expression of inflammatory cytokines/chemokines downstream of IRF5, we examined the levels of IL-6, IP-10, TNF-α, and IL-10 in the peripheral WBCs and plasma. The mRNA levels of TNF-α and IL-10 were significantly elevated in the NSCLC patients compared to those in the healthy controls ($p = 0.0177$ and $p < 0.0001$, respectively), but those of IL-6 and IP-10 did not show significant differences between the two groups (Fig. 3A-D). However, stratification according to the NSCLC stage revealed that the IP-10 mRNA levels were higher in the patients with progressive NSCLC ($p = 0.0199$; Fig. 3C), whereas the IL-10 mRNA levels were higher in those with early stage NSCLC ($p = 0.0016$; Fig. 3D). The expression of the other cytokines, TNF-α and IL-6, was similar in the patients with early and progressive NSCLC (Fig. 3A-B). Because mRNA levels are not always the same as protein levels, we detected the plasma cytokines/chemokines of the same NSCLC patients and healthy donors using a CBA assay and found that IL-6 and IP-10 were significantly increased, whereas TNF-α levels were significantly decreased in the NSCLC patients compared to the healthy controls ($p < 0.0001$; Fig. 3E-G). The higher IL-6 levels correlated with progressive NSCLC (56.3%) rather than with early NSCLC (20.6%) ($p = 0.0066$; Fig. 3F), whereas the higher IP-10 levels were more characteristic for early stage NSCLC (52.9%) than for progressive disease (28.1%) ($p = 0.0063$; Fig. 3G). Although the IL-10 levels were not significantly different between the NSCLC patients and the healthy controls, they were higher in the progressive NSCLC patients (56.3%) compared to those in early disease (29.4%) ($p = 0.0263$; Fig. 3H). We next analysed the correlation between the IRF5 expression in the WBCs and plasma levels of the inflammatory cytokines/chemokines in the NSCLC patients. The results revealed that the plasma levels of IP-10 positively correlated with the IRF5 protein expression in the WBCs ($p < 0.0001$, $r = 0.7221$; Fig. 3I) and monocytes ($p < 0.0001$, $r = 0.7345$; Fig. 3J), whereas those of IL-10 negatively correlated with the IRF5 protein expression in the WBCs ($p = 0.0028$, $r = -0.3618$; Fig. 3K) and monocytes ($p = 0.0033$, $r = -0.3568$; Fig. 3L), indicating that IRF5 can directly affect the expression of downstream inflammatory cytokines and chemokines. Overall, these findings indicate that the levels of IRF5 and its downstream targets IL-10 and IP-10 in the peripheral circulation may be valuable early warning signs of NSCLC development.

3.3. Association of IRF5 expression in cancer tissues with the NSCLC stage

To understand the role of IRF5 in NSCLC development, we analysed the IRF5 expression in the cancer tissues (CT) and para-carcinoma tissues (PCT) of the NSCLC patients (n = 102) using Western blotting. The results showed that in most of the NSCLC cases, the protein levels of IRF5 in the CT were lower than those in the PCT of the same patients (Fig. 4A, left). Comparing the data from all of the NSCLC patients, the

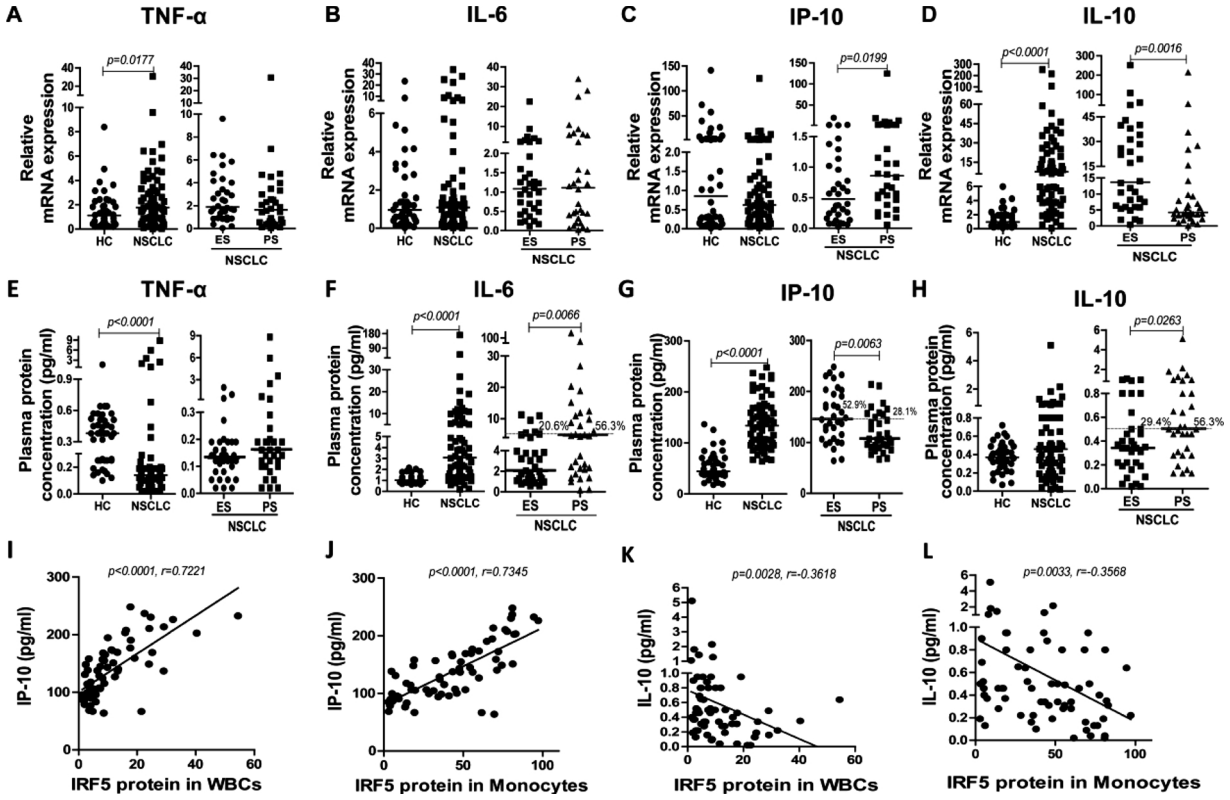

Fig. 3. Association of IRF5 with inflammatory cytokine/chemokine in the peripheral circulation of the NSCLC patients. **(A–D)** The mRNA levels of TNF-α, IL-6, IP-10, and IL-10 in the WBCs were detected by qRT-PCR. **(E–H)** The protein levels of TNF-α, IL-6, IP-10, and IL-10 in the plasma were analysed by cytometric bead array. **(I–L)** Correlation of the $IRF5^+$ WBCs or monocytes with the protein levels of IP-10 and IL-10 in the plasma of the NSCLC patients. NSCLC, non-small cell lung cancer; HC, healthy controls; ES, early stage; PS, progressive stage.

IRF5 levels in the CT were significantly lower than those in the PCT ($p = 0.0345$; Fig. 4A, right), and there was no difference between adenocarcinoma and squamous cell carcinoma (Fig. 4B). Analysis of the IRF5 expression according to the NSCLC stage indicated that it was significantly higher in the CT at the early stage than at the progressive stage ($p = 0.0205$). The average level (grey value) of the IRF5 expression in the CT at the progressive stage was approximately 0.5, whereas it was 0.7 at the early stage. The ratio of NSCLC with an IRF5 level > 0.7 was 56.3% at the early stage and 25.7% at the progressive stage (Fig. 4C). To analyse the correlation between the IRF5 expression in the CT and NSCLC patient survival, we divided the NSCLC patients into two groups based on their average IRF5 level (0.5430 ± 0.3303): $IRF5^{high}$ (IRF5 levels > 0.5430) and $IRF5^{low}$ (IRF5 levels < 0.5430). The results revealed that the NSCLC patients in the $IRF5^{high}$ group tended to have longer survival times than those in the $IRF5^{low}$ group, although the difference was not significant (Fig. 4D). Further analysis demonstrated that the IRF5 expression was significantly increased in the PCT compared to the CT in the NSCLC patients younger than 45 years ($p = 0.0227$) or older than 60 years ($p = 0.0195$), but not in those between 45 and 59 years old (Fig. 4E), whereas the IRF5 levels in the CT were similar in the three age groups (Fig. 4F). Furthermore, only in the older NSCLC patients (≥ 60 years), the IRF5 levels in the CT were significantly higher at the early stage than at the progressive stage ($p = 0.0457$; Fig. 4G). These results suggest that higher IRF5 levels in the CT are associated with early stage NSCLC and that the IRF5 expression in the PCT is upregulated sooner than in the CT.

4. Discussion

Inflammation plays an important role in cancer initiation, promotion, progression, and metastasis [10,36] and the inflammatory microenvironment is an essential component of tumour niches [37]. For an established tumour, blood circulation is a link between the local tissues and the entire body: on the one hand, immune cells in the blood can be recruited to the tumour site; on the other hand, tumour-derived exosomes and inflammatory mediators can enter the circulation and activate innate immune cells. This study found that IRF5 expression was significantly higher in the peripheral blood cells of the NSCLC patients compared to the healthy controls, suggesting the activation of the IRF5 signalling pathway in the WBCs in response to NSCLC development. The activation manifested as the increased protein expression of IL-6 and IP-10, two downstream inflammatory factors of the IRF5 pathway, and the positive correlation between the IRF5 and IP-10 protein expression in the peripheral blood of the NSCLC patients.

Research has found that N-glycan on lung carcinoma cells activates innate immune cells with C-type lectin receptors (CLRs) [14]. Moreover, N-glycan is a ligand of Dectin-1, a CLR family member, and Dectin-1 activation triggers the expression of IRF5 and its downstream cytokines [38]. Based upon these findings, we speculate that NSCLC cells activate innate immune cells through the Dectin-1 pathway. Notably, the IL-6 levels were elevated in the plasma of the NSCLC patients. Alternatively, the IRF5 and IL-6 protein expression levels were reversed in NSCLC development and there was no correlation between them, which could be partially due to IL-6 secretion from the cancer cells [39]. The maintenance of high-\IL-6 levels in the peripheral blood resulted in monocyte polarisation to M2 macrophages [40], which rarely express IRF5, but rather produce IL-10 [21]. In this study, the elevated IP-10 protein expression was also detected in the peripheral blood of the NSCLC patients, especially in the early stage patients. In mice, IP-10 can inhibit tumour growth by acting to inhibit angiogenesis and limit tumour metastasis by activating NK cells [41]. In addition, the NSCLC severity was positively correlated with the plasma IL-10 levels, but negatively correlated with the IL-10 mRNA expression in the WBCs. This discrepancy could be due to the feedback effects of IL-10, which

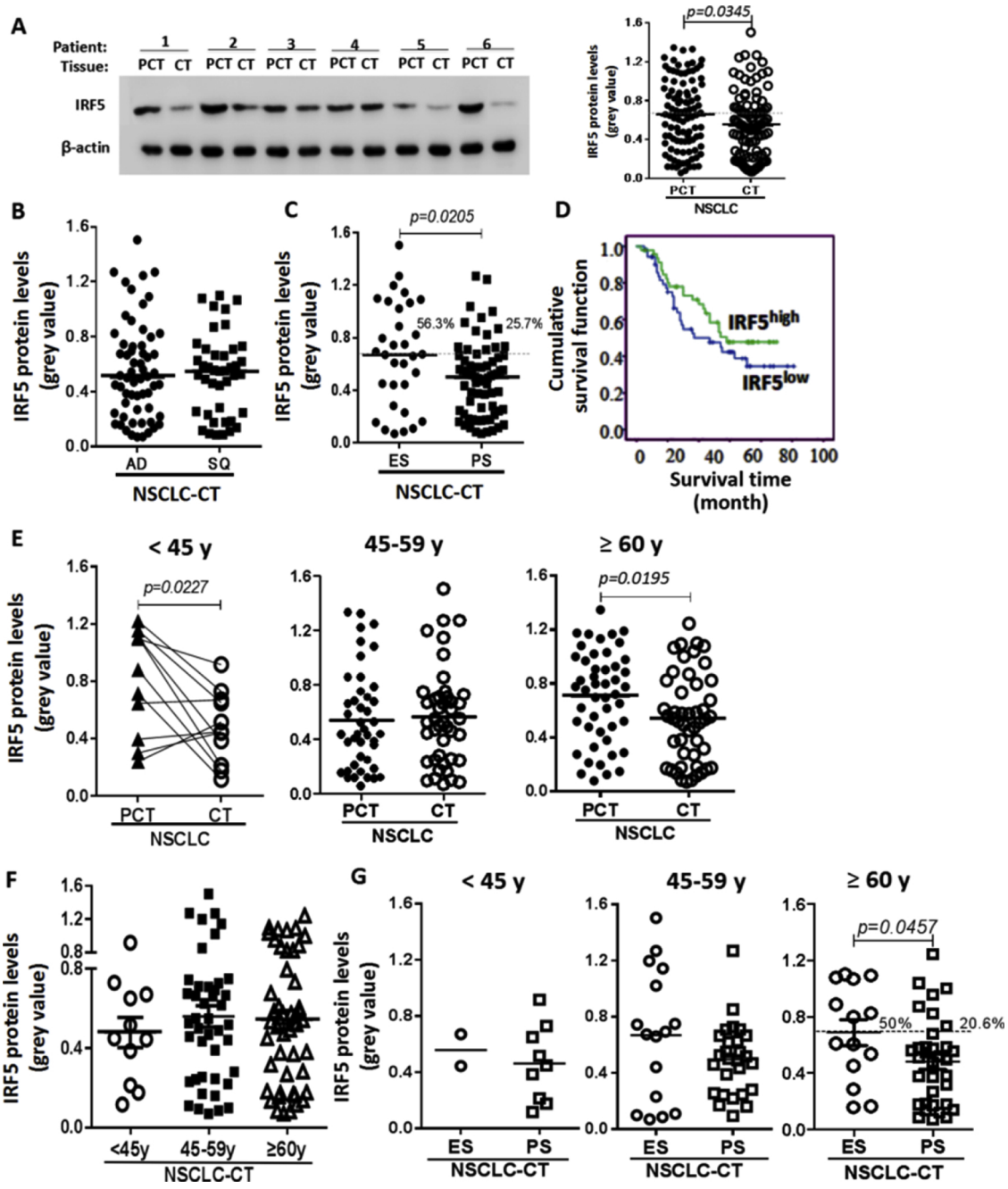

Fig. 4. Expression of IRF5 protein in the tissues of non-small cell lung cancer (NSCLC). Cancer tissues (CT) and para-carcinoma tissues (PCT) derived from the NSCLC patients were analysed for IRF5 protein levels by Western blotting and relative quantitative analysis based on the grey values of the blotting. Based on the grey values, the IRF5 protein levels in the PCT and CT were analysed, which were used to analyse the relationship with stages of the disease, survival rates, and ages of the patients. **(A)** Expression levels of IRF5 protein in the PCT and CT of the NSCLC patients. **(B)** IRF5 protein levels in the CT of the NSCLC patients at different histological types. **(C)** IRF5 protein levels in the CT of the NSCLC patients at different stages. **(D)** Survival rates of the total NSCLC patients with high or low levels of IRF5 protein. The average IRF5 level in the CT of the NSCLC patients, 0.5430, was set as the cutting edge: $IRF5^{high}$ (IRF5 levels > 0.5430) and $IRF5^{low}$ (IRF5 levels < 0.5430). **(E)** IRF5 protein levels in the CT and PCT of the NSCLC patients in the different age groups. **(F)** Expression levels of IRF5 protein in the CT of the NSCLC patients in the different age groups. **(G)** Relationship between IRF5 protein levels in the CT and the NSCLC stage in the different age groups. AD, adenocarcinoma; SQ, squamous cell carcinoma; ES, early stage; PS, progressive stage.

was shown to significantly inhibit its own mRNA expression [42]. High expression of IL-10 in the sera of the NSCLC patients was associated with lower survival rates [43]. Overall, our results indicate that the IRF5 expression in the WBCs and the levels of its downstream cytokines, IL-10 in the WBCs and IL-6 and IP-10 in the plasma, could be used as candidate biomarkers of the possible presence of NSCLC, which is in agreement with a previous report that IL-6, IL-10, and IP-10 have a diagnostic value in lung cancer [44,45]. However, since IRF5 is an upstream regulator of IL-6, IL-10, and IP-10, IRF5 expression could be a more important diagnostic biomarker for early stage NSCLC.

In the present study, higher IRF5 levels were detected in the WBCs of the NSCLC patients than in those of the healthy controls, and in early stage NSCLC than in progressive NSCLC, suggesting that IRF5 expression in the peripheral blood cells could be used as a diagnostic biomarker of NSCLC in general and early stage NSCLC in particular. However, it should be noted that patients with other diseases such as

influenza virus infection, community-acquired pneumonia, systemic lupus erythematosus (SLE), and hepatitis C may also have high IRF5 expression in their peripheral blood cells [46–49]. Patients infected with influenza virus often have a high fever, body aches, and conjunctivitis, with influenza virus particles detected in the mucosal epithelial cells in nasal washes. In SLE patients, butterfly-like erythema often appears on the cheeks, and abnormal clinical manifestations can occur in the kidneys, lungs, central nervous system, and/or heart with disease progression. In addition, positive anti ds-DNA antibody in the serum is conducive to SLE diagnosis. In patients with community-acquired pneumonia, elevated IRF5 levels could be significantly reduced by effective pathogen-targeting therapy, as reported by our group [47]. Additionally, elevated IRF5 in patients with HCV-1 is reduced after effective antiviral therapy [49]. In contrast, IRF5 upregulation in the peripheral blood cells of NSCLC patients persists, which could be primarily from the response of innate immune cells to tumour cells and should not be affected by pathogen-targeting diagnostic treatment before definitive diagnosis. Therefore, increased IRF5 expression in peripheral blood cells could be used as a biomarker of early stage NSCLC after the exclusion of the other diseases associated with IRF5 upregulation. In particular, IRF5 monitoring may improve diagnostic accuracy when pulmonary nodules are detected by low-dose computed tomography (LDCT), because only 60% of the observed nodules larger than 4 mm appear to be malignant [50]. In such cases, the cancerous nature of the nodules should be confirmed based on lung cancer-associated molecular changes. Currently, a panel of seven tumour-associated antigens, including p53, NY-ESO-1, CAGE, GBU4-5, SOX2, HuD, and MAGE A4, is used to detect respective autoantibodies indicative of anticancer humoral immune response, with an overall specificity of 91% in NSCLC cases [51]. The measurement of IRF5 expression in peripheral blood cells could be complementary to this assay and improve its diagnostic specificity for early stage NSCLC. In addition, bronchial gene expression biomarkers could be added to provide a comprehensive lung cancer risk assessment [52].

Although the IRF5 expression was significantly higher in the WBCs of the NSCLC patients compared to those of the healthy controls, the IRF5 levels varied among the patients, even in those at the same clinical stage, which could be associated with single nucleotide polymorphisms (SNPs) in the IRF5 gene. An SNP at the rs77571059 locus, which was detected in 18 out of the 66 NSCLC patients, was positively associated with high IRF5 mRNA expression in the WBCs. The rs77571059 SNP represents a 5-bp (CGGGG) insertion/deletion at 64 bp upstream of the transcription start site for exon 1A of the IRF5 gene. Each IRF5 allele contains either three (3×) or four (4×) CGGGG repeats; the fourth copy presents an additional binding site for transcription factor Sp1, thus enhancing the IRF5 expression [53]. In systemic lupus erythematosus, the rs77571059 SNP was included in risk haplotypes and was associated with high levels of IRF5 expression [48]. Our data suggest that the rs77571059 SNP could also be a risk factor of NSCLC development, and together with pulmonary nodules and increased IRF5 expression in the peripheral blood cells, should alert clinicians to the possibility of NSCLC. However, it should be noted that in this study, we also observed NSCLC patients with the wild-type sequence at the rs77571059 locus and low IRF5 mRNA expression in the WBCs who obviously would not be identified based on IRF5 genetic or transcriptional changes.

5. Conclusions

In summary, dynamic monitoring of IRF5 and its downstream cytokines/chemokines in the peripheral blood could be used to assist in the early discovery and diagnosis of NSCLC. The possibility of developing NSCLC should be considered if: 1) pulmonary nodules are noticed by LDCT scanning during physical examination, 2) IRF5 expression in the peripheral WBCs is persistently increased, 3) IL-6 and IP-10 levels in the plasma and IL-10 mRNA levels in the peripheral WBCs are elevated, 4) IRF5 rs77571059 polymorphism is detected, and 5) other IRF5 elevation-associated diseases are excluded.

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