Interferon regulatory factor 3 mediates Poly(I:C)-induced innate immune response and apoptosis in non-small cell lung cancer

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Abstract. Immunotherapy is considered one of the most promising treatments for lung cancer. The cell signalling molecules melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene I protein (RIG-I) are essential receptors that recognise intracellular pathogen-associated nucleic acids, whereas interferon regulatory factor 3 (IRF3) controls the expression of innate immunity-associated genes in macrophages. However, the innate immune response to polyinosinic:polycytidylic acid [Poly(I:C)] in lung cancer remains to be elucidated. In the present study, western blot analysis, reverse transcription-quantitative polymerase chain reaction, RNA interference, IRF3 plasmid construction, ELISA and apoptosis analysis were employed to study the innate immune response and apoptosis of non-small cell lung cancer (NSCLC) cells. Poly(I:C) transfection in NSCLC cells triggered apoptosis via the extrinsic apoptotic pathway, and activated the innate immune response by promoting interferon-β and C-X-C motif chemokine ligand 10 expression. Treatment with the IκB kinase ε/tumour necrosis factor receptor-associated factor family member-associated nuclear factor-κB activator-binding kinase 1 inhibitor BX795, which inhibits IRF3 phosphorylation, or transfection with small interfering RNA/short hairpin RNA to downregulate MDA5, RIG-I or IRF3, prior to Poly(I:C) transfection inhibited the innate immune response and apoptotic pathway. Conversely, IRF3 overexpression promoted activation of the apoptotic pathway, thus indicating that the MDA5/RIG-I/IRF3 axis may mediate responses to Poly(I:C) transfection. Furthermore, phosphorylation of the transcription factor signal transducer and activator of transcription 1 (STAT1) was associated with the alterations in IRF3 phosphorylation and apoptosis, thus suggesting that STAT1 may be involved in Poly(I:C)-induced apoptosis. In NSCLC surgical samples, MDA5, RIG-I and IRF3 were highly expressed, whereas the expression levels of phosphorylated-IRF3 were reduced. These findings indicated that the function of the MDA5/RIG-I/IRF3 axis may be impaired in some lung cancers. In conclusion, the present findings suggested that the MDA5/RIG-I/IRF3 axis, which is associated with innate immunity, is intact in NSCLC cells, and IRF3 is involved in regulating the apoptotic pathway in NSCLC cells.

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Abbreviations: MDA5, melanoma differentiation-associated protein 5; RIG-I, retinoic acid-inducible gene I protein; IRF3, interferon regulatory factor 3; IPS-1, interferon-β promoter stimulator 1; TBK1, IκB kinase ε/tumour necrosis factor receptor-associated factor family member-associated nuclear factor-κB activator-binding kinase 1; TLRs, Toll-like receptors

Key words: IRF3, apoptosis, lung cancer, Poly(I:C)

Introduction

Polyinosinic:polycytidylic acid [Poly(I:C)] is a synthetic analogue of double-stranded RNA (dsRNA), which is recognised by pattern recognition receptors, and elicits innate and adaptive immunity (1). Poly(I:C) exhibits potential by activating various types of immune cells, including dendritic cells, T helper (Th)1 cells and Th17 cells (2). The pathways that initiate immune activity mainly involve intact intracellular or extracellular signalling. Intracellularly, dsRNA is recognised by retinoic acid-inducible gene I protein (RIG-I)-like receptors [melanoma differentiation-associated protein 5 (MDA5) and RIG-I] that interact with the interferon (IFN)-β promoter stimulator 1 (IPS-1). Subsequently, IPS-1, which is a mitochondrial signalling adaptor protein, recruits and phosphorylates IκB kinase ε/tumour necrosis factor (TNF) receptor-associated factor family member-associated nuclear factor (NF)-κB activator-binding kinase 1 (TBK1), thus leading to the phosphorylation of interferon regulatory factor 3 (IRF3). Extracellularly, Toll-like receptors (TLRs) are the pivotal receptors. Upon Poly(I:C) binding to TLRs, the TLRs recruit adaptor proteins, including myeloid differentiation primary response 88, Toll/interlukin-1 receptor-domain-containing adapter-inducing IFN-β and translocating chain-associated membrane protein, and IRF3 is phosphorylated via NF-κB signalling followed by the activation of type I IFN signalling (3,4). A previous study revealed that Poly(I:C) exerts its antitumour properties in a type I IFN-dependent or IFN-independent manner in MCF10A, MDAMB-231, IMR32 and HEK293T cells (5).

MDA5 and RIG-I are cytoplasmic sensors of Poly(I:C), which participate in the Poly(I:C)-induced apoptosis of cancer cells by eliciting the canonical apoptotic signalling pathway, and may even boost innate immune responses (6). IRF3 is a downstream molecule of RIG-I/MDA5, which is usually located in the cytoplasm in its inactive form, and is a vital transcription factor for type I IFN. It is activated by phosphorylation at its carboxyl terminus in response to viral infection, DNA-damaging stress, ultraviolet light and chemical stimulation. Upon phosphorylation, IRF3 dimerizes and translocates into the nucleus, where its target genes are transcribed (7,8). IRF3 exerts dual roles in regulating the bioactivity of cancer. In previous studies, IRF3 has been reported to act as an antitumour factor due to its ability to control cancer cell progression (9,10). For example, IRF3 inhibits the growth of nasopharyngeal carcinoma cells, B16 melanoma cells, prostate cancer cells and malignant glioma cells via regulating the activation of natural killer cells or type I IFN signalling (9,11-13). In addition, it has previously been reported that Poly(I:C)-induced apoptosis of prostate cancer PC3 and DU145 cells is independent of IRF3 (14). Conversely, the mRNA and protein expression levels of IRF3 are upregulated in patients with acute myeloid leukaemia (AML), and IRF3 promotes the proliferation and survival of AML cells (15). In lung cancer tissues, IRF3 is aberrantly expressed in either the cytoplasm or nucleus, and its expression status is not associated with sex, histological grade, nodal metastasis, pathological stage or recurrence (16). Therefore, the present study aimed to explore the effects of Poly(I:C) on lung cancer cells and to reveal the probable role of IRF3 in these effects.

The present study demonstrated that Poly(I:C) triggered innate immunity via an intracellular signalling pathway, and induced apoptosis via the extrinsic apoptotic pathway in non-small cell lung cancer (NSCLC) cells. In addition, the results indicated that IRF3, activated by MDA5/RIG-I, may regulate the Poly(I:C)-induced apoptotic pathway. Finally, the results demonstrated that molecules in the innate immune pathway were synchronously upregulated; however, this was not accompanied by an increase in IRF3 phosphorylation, in tumour tissue samples obtained from patients with NSCLC compared with in matched adjacent tissues.

Materials and methods

Tissue specimen collection. NSCLC tissues and corresponding adjacent tissues were obtained from eight patients at Peking University First Hospital (Beijing, China). Patient characteristics are listed in Table I. The tissues were instantly frozen in liquid nitrogen once they were removed from the patients and were preserved at -80˚C. All of the subjects provided informed consent for their inclusion in the present study prior to participation. The present study was conducted in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of Peking University First Hospital.

Cell culture and drug pretreatment. The NSCLC cell line A549 was obtained from American Type Culture Collection (Manassas, VA, USA), and NCI-H1299 cells were obtained from Peking Union Medical College (Beijing, China). The A549 cells were cultured with Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the H1299 cells were cultured with Roswell Park Memorial Institute 1640 medium (Gibco; Thermo Fisher Scientific, Inc.). Both media were supplemented with 10% foetal bovine serum (Corning Incorporated, Corning, NY, USA) at 37˚C in a humidified incubator containing 5% CO_2.

Drug pretreatment and Poly(I:C) transfection. Once cell confluence reached 70-80% in the 6-well plates, the pan-caspase inhibitor Z-VAD-FKM (50 μM; InvivoGen, San Diego, CA, USA), the caspase-8 inhibitor Z-IETD-FKM (25 μM; EMD Millipore, Billerica, MA, USA) or the TBK1 inhibitor BX795 (1 μM); (InvivoGen) were used to treat the cells for 2 h at 37˚C. Subsequently, Poly(I:C) (0, 100, 200 and 400 ng/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was transfected into the cells (confluence, 70-80%) for 6 h using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, after which the cells were cultured for 24 h.

Western blot analysis. The cells/tissues were lysed in radioimmunoprecipitation assay buffer containing phenylmethylsulfonyl fluoride and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) for 5 min. The protein content in the cell lysates was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). The lysates, containing 40 µg protein, were separated by 12% SDS-PAGE, and proteins were transferred to a polyvinylidene fluoride membrane. Subsequently, the membrane was blocked with 5% blocking reagent (2 g skimmed milk power dissolved in 40 ml Tris-buffered saline with 1% Tween-20) for 1 h at room temperature. Then it was incubated with the following primary antibodies (1:1,000 dilutions) overnight at 4˚C: Anti-IRF3 (ab50772; Abcam, Cambridge, UK), anti-phosphorylated (p)-IRF3 (p-S386) (ab76493; Abcam), anti-MDA5 (ab126630; Abcam), anti-RIG-I (ab180675; Abcam), anti-NAK/TBK1 (ab109735; Abcam), anti-NAK/TBK1 (p-S172) (ab109272; Abcam), anti-GAPDH (ab9485; Abcam), anti-caspase-3 (622701; BioLegend, Inc., San Diego, CA, USA), anti-caspase-9 (621901; BioLegend, Inc.), anti-caspase-8 (645501; BioLegend, Inc.), anti-TNF-related apoptosis-inducing ligand (TRAIL) antibody (RLT4721; Suzhou Ruiying Biological Co., Ltd., Suzhou, China) and anti-p-STAT1 (p-Ser727) antibody (RLP0842; Suzhou Ruiying Biological Co., Ltd.). Subsequently, the membranes were incubated with goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) (sc-2005; 1:5,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and goat anti-rabbit IgG-HRP (sc2004; 1:5,000 dilution; Santa Cruz Biotechnology, Inc.) secondary antibodies for 2 h at room temperature. The blots were visualised using the Luminata Forte Western HRP Substrate (EMD Millipore).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells or tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (2 μg) was reverse transcribed to cDNA using a first-strand cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.), which was conducted according to the manufacturer's protocol. Subsequently, relative gene expression was determined by qPCR using the SYBR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and ABI 7500 real-time PCR detection system (Applied

Table I. Clinical information of patients with non-small cell lung cancer.

Patients	Sex (M/F)	Age (years)	Pathological type	Lymphatic metastasis
#1	F	58	Adenocarcinoma	No
#2	F	78	Squamous-cell carcinoma	Lobar bronchus
#3	M	59	Adenocarcinoma	No
#4	M	60	Adenocarcinoma	No
#5	F	80	Combined small cell carcinoma and adenocarcinoma	No
#6	F	53	Adenocarcinoma	No
#7	M	57	Adenocarcinoma	No
#8	F	57	Adenocarcinoma	No

F, female; M, male.

Biosystems; Thermo Fisher Scientific, Inc.). The amplification conditions were as follows: An initial denaturation step at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/extending at 60˚C for 1 min. The following primer sequences were used: IRF3 forward, 5'-TCA GGAGTTGGGGACTTTTC and reverse, 5'-GAATGTCTTCC TGGGTATCAG; and β-actin forward, 5'-ATATCGCCGCGC TCGTCGTC and reverse, 5'-CATGCCCACCATCACGCC CTG-3'. The MDA5 and RIG-1 primer sequences used are noted in previous studies (17,18), as are the sequences for C-X-C motif chemokine ligand 10 (CXCL-10) and IFN-β (19), and TRAIL (5). β-actin was used as an internal reference. The relative mRNA expression levels were normalised to β-actin mRNA. The results of the cell samples were calculated as $2^{-\Delta\Delta Cq}$ (20), whereas the $2^{-\Delta Cq}$ method was used to analyse the tissue samples.

ELISA. After the indicated treatments, the supernatants were collected and an ELISA analysis was performed to detect the expression levels of human CXCL-10 using an ELISA kit (555046; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol.

Apoptosis analysis. An equal number of A549 cells ($1.0x10^5$/ml) were seeded into a 6-well plate. Following transfection of A549 cells with Poly(I:C) for 24 h, with or without caspase inhibitor pretreatment, the cells were harvested ($1.0x10^6$). Subsequently, the apoptotic cells were stained using the Annexin V-fluorescein isothiocyanate/propidium iodide Cell Apoptosis Detection kit (Beijing Transgen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. Each sample was assessed by fluorescence-activated cell sorting using a flow cytometer (BD Biosciences) and the data were analyzed using FlowJo v10 software (FlowJo LLC, Ashland, OR, USA).

Lentivirus short hairpin (sh)RNA infection. The lentivirus shRNA pLent-U6-shIRF3-GFP-Puro and the empty vector pLent-U6-GFP-Puro were purchased from Vigene Biosciences, Inc. (Jinan, China). The sequence of IRF3 shRNA was as follows: 5'-CCGGCCCTTCATTGTAGATCTGATTCTCGAGAATCA GATCTACAATGAAGGGTTTTT-3'. A549 cells were infected with the lentiviruses once cell confluence reached 70-80%. A total of 48 h postinfection, a low concentration of puromycin (5 mg/ml) was used to select lentivirus-infected cells. After 14 days of screening, the stably infected cells were obtained.

Plasmid construction. IRF3 cDNA was obtained from peripheral blood cells collected from a healthy human volunteer, and PCR was performed with primers tagged with restriction endonuclease sequences (*Xho*I and *Age*I; Thermo Fisher Scientific, Inc.). Subsequently, the PCR products, or the vector plasmid pEGFP-N1 (Clontech Laboratories, Inc., Mountainview, CA, USA), were incubated with *Xho*I or *Age*I for 1.5 h and were ligated with T4 ligase for 1.5 h (Thermo Fisher Scientific, Inc.) at room temperature. Following ligation, the mix was transformed into competent *Escherichia coli* (Beijing Transgen Biotech Co., Ltd.) cells by heat shock treatment (42˚C for 90 sec in a water bath). Finally, the transformed bacteria were cultured with Lennox L Broth Base (Invitrogen; Thermo Fisher Scientific, Inc.) containing kanamycin (30 μg/ml; Gibco; Thermo Fisher Scientific, Inc.) and the recombinant plasmid IRF3-pEGFP-N1 was identified by PCR using the IRF3 primer.

Plasmid and small interfering (si)RNA transfection. Once cell confluence reached 80-90%, the expression plasmid IRF3-pEGFP-N1 and the empty vector were transfected into A549 cells, whereas the IRF3-pEnter with His-tag (Vigene Biosciences, Inc.) and the empty vector were transfected into H1299 cells using Lipofectamine® 3000. The culture medium was changed after 6 h, and IRF3 expression was assessed after 48 h of transfection. For siRNA transfection, the RIG-I- and MDA5-specific siRNAs, and the scrambled sequence were prepared (Suzhou GenePharma, LLC, Suzhou, China). The sequences used were as follows: Scrambled, 5'-CAUAGCGU CCUUGAUCACAUU-3';RIG-I,5'-AACGAUUCCAUCACUA UCCAUdTdT-3' (21); MDA5, 5'-GGUGUAAGAGAGCUA CUAAtt-3' (22). siRNA transfection was performed using Lipofectamine® 3000 once cell confluence reached 70-80%, and the concentration of siRNAs used was 50 nM. Subsequently, cells were analysed after 36 h of transfection.

Statistical analysis. Statistical analyses were performed using SPSS 20 (IBM Corporation, Armonk, NY, USA). The data were presented as the means ± standard error of the mean. Semi-quantification of western blotting was analysed using ImageJ2 software (National Institutes of Health, Bethesda, MD, USA). For comparisons between two groups, Student's t-test was used, whereas comparisons among multiple groups were made using univariate analysis of variance, followed by least significant difference post hoc test. $P<0.05$ was

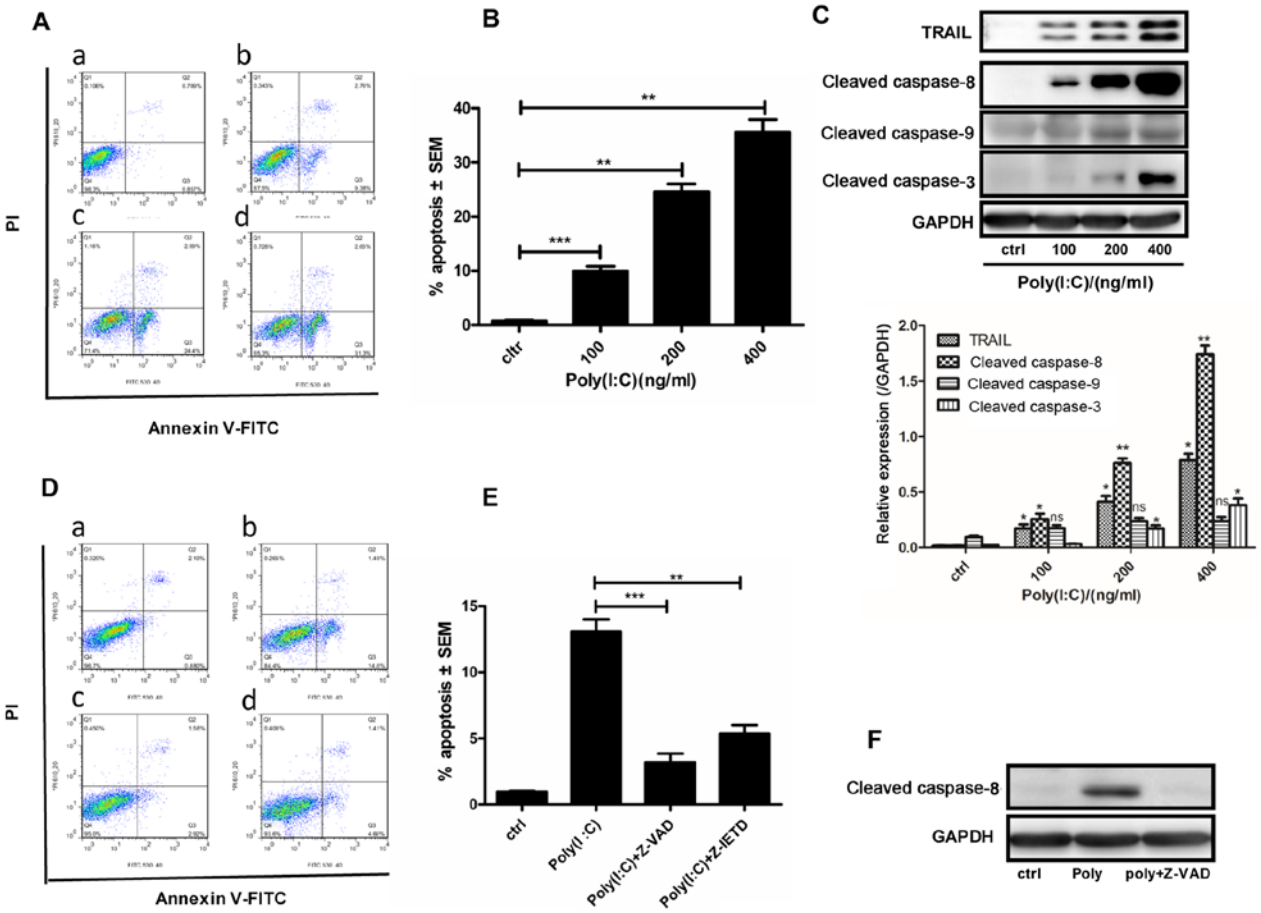

Figure 1. Poly(I:C) induces apoptosis of A549 cells via the extrinsic apoptotic pathway. (A-D) A549 cells were transfected with 0, 100, 200 or 400 ng/ml Poly(I:C) for 24 h; the control group was treated with Lipofectamine® 3000 alone. (A) Flow cytometry was used to detect apoptotic cells. (B) Histogram of the percentage of apoptotic cells in panel (A). (C) Western blotting was used to examine the protein expression levels of TRAIL, cleaved caspase-8, -9 and -3. (D) Flow cytometry was used to detect apoptosis of cells pretreated with the pan-caspase inhibitor Z-VAD or the caspase-8 inhibitor Z-IETD for 2 h prior to 100 ng/ml Poly(I:C) transfection for 24 h. (E) Histogram of the percentage of apoptotic cells in panel (E). (F) Cleaved caspase-8 expression was detected by western blotting in A549 cells pretreated with the pan-caspase inhibitor Z-VAD. *P<0.02; **P<0.005; ***P<0.001. FITC, fluorescein isothiocyanate; ns, not significant; PI, propidium iodide; Poly(I:C), polyinosinic:polycytidylic acid; SEM, standard error of the mean; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand.

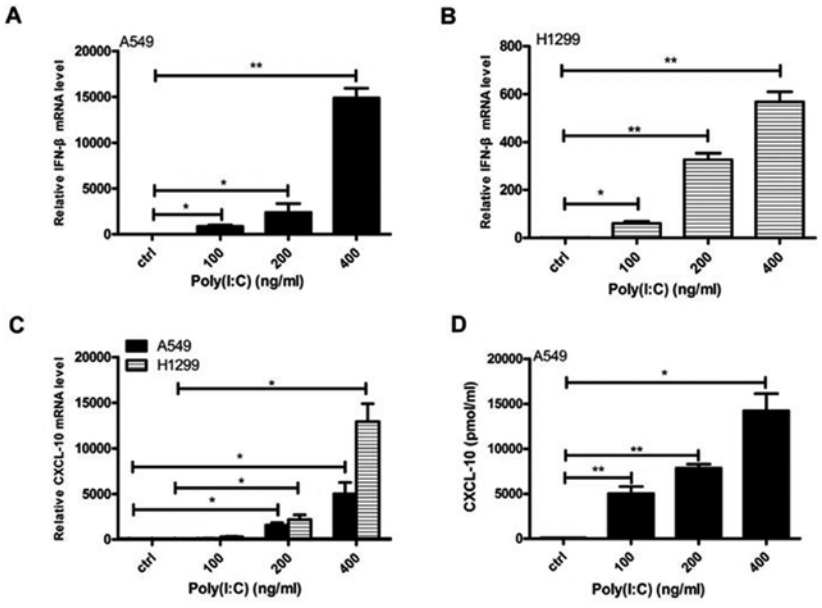

Figure 2. Poly(I:C) initiates upregulation of IFN-β and CXCL-10 in non-small cell lung cancer cell lines A549 and H1299. NSCLC cell lines A549 and H1299 were transfected with 100, 200 and 400 ng/ml Poly(I:C) for 24 h. (A-C) IFN-β and CXCL-10 mRNA expression was detected by quantitative polymerase chain reaction. (D) Protein expression levels of CXCL-10 were measured by ELISA in A549 cells. *P<0.05; **P<0.005. CXCL-10, C-X-C motif chemokine ligand 10; IFN-β, interferon-β; Poly(I:C), polyinosinic:polycytidylic acid.

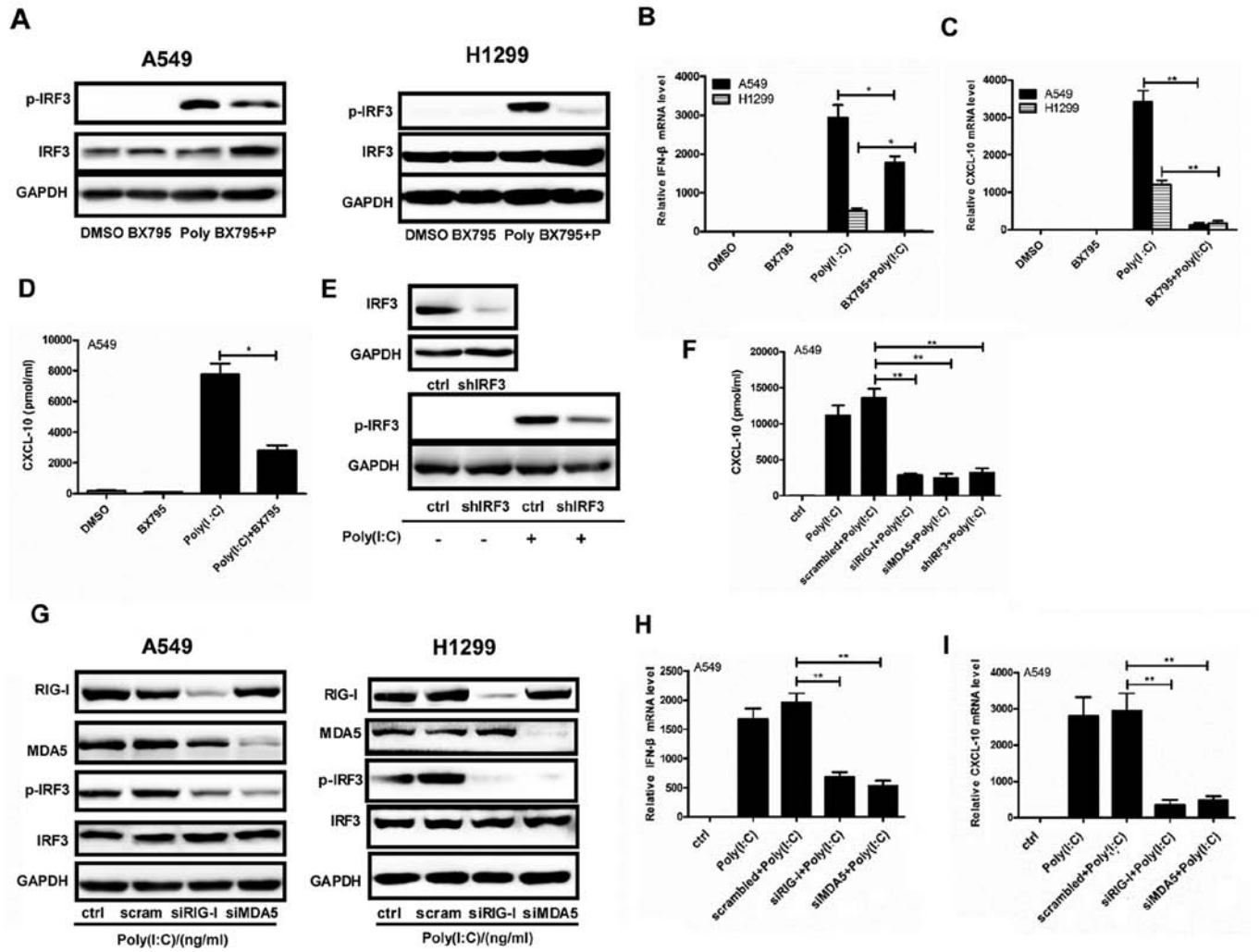

Figure 3. MDA5/RIG-I/IRF3 axis mediates the responses of NSCLC cell lines to Poly(I:C) transfection. (A-D) Poly(I:C) (200 ng/ml) was transfected into A549 and H1299 cells for 24 h following 2 h pretreatment with BX795. DMSO was used as a control. (A) Protein expression levels of IRF3 and p-IRF3 were examined by western blotting. (B and C) IFN-β and CXCL-10 expression was quantified by qPCR. (D) CXCL-10 secretion was determined in A549 cell supernatants by ELISA. (E) IRF3 was stably downregulated by infection with a lentivirus containing shIFR3, the protein expression levels of p-IRF3 were detected by western blotting after Poly(I:C) transfection for 24 h. (F-I) siRNAs against RIG-I or MDA5 were transfected into cells for 36 h prior to Poly(I:C) transfection (200 ng/ml) for 24 h. Scrambled sequences were used as a negative control. (F) CXCL-10 was detected by ELISA in A549 cells with RIG-I or MDA5 or IRF3 depletion after Poly(I:C) transfection for 24 h. (G) Western blotting was used to examine the protein expression levels of RIG-I, MDA5, IRF3 and p-IRF3. (H and I) IFN-β and CXCL-10 mRNA expression was assessed by qPCR in A549 cells with RIG-I or MDA5 depletion following Poly(I:C) transfection for 24 h. *P<0.05; **P<0.005. CXCL-10, C-X-C motif chemokine ligand 10; DMSO, dimethyl sulfoxide; IFN-β, interferon-β; IRF3, interferon regulatory factor 3; MDA5, melanoma differentiation-associated protein 5; p-IRF3, phosphorylated-IRF3; Poly(I:C), polyinosinic:polycytidylic acid; qPCR, quantitative polymerase chain reaction; RIG-I, retinoic acid-inducible gene I protein; sh, short hairpin RNA; si, small interfering RNA.

considered to indicate a statistically significant difference. Three independent experiments were conducted.

Results

Poly(I:C) induces apoptosis via the extrinsic apoptotic pathway in A549 cells. It was hypothesised that Poly(I:C) may induce apoptosis of NSCLC cells via the canonical apoptotic pathway. The present study demonstrated that the number of apoptotic A549 cells increased in a concentration-dependent manner following transfection with Poly(I:C) for 24 h (Fig. 1A and B). The mRNA expression levels of TRAIL were significantly increased (data not shown), and the protein expression levels of TRAIL, and cleaved caspase-3 and -8, were markedly increased. Conversely, cleaved caspase-9 expression was slightly increased post-transfection (Fig. 1C). To further determine the role of caspases in Poly(I:C)-induced apoptosis of A549 cells, the cells were treated with the pan-caspase inhibitor Z-VAD-FMK and the caspase-8 inhibitor Z-IETD-FMK for 2 h prior to transfection with 100 ng/ml Poly(I:C) for 24 h. As expected, apoptosis was significantly inhibited (Fig. 1D and E) and cleaved caspase-8 expression was decreased following Z-VAD-FMK pretreatment (Fig. 1F). These findings indicated that Poly(I:C) may induce apoptosis of A549 cells via activation of an extrinsic apoptotic pathway.

Poly(I:C) transfection activates innate immunity in NSCLC cells. The present study transfected Poly(I:C) into the NSCLC cell lines A549 and H1299. Subsequently, the mRNA expression levels of IFN-β and CXCL-10 were increased in both cell lines, and the secretion of CXCL-10 was increased in A549 cells in a concentration-dependent manner (Fig. 2).

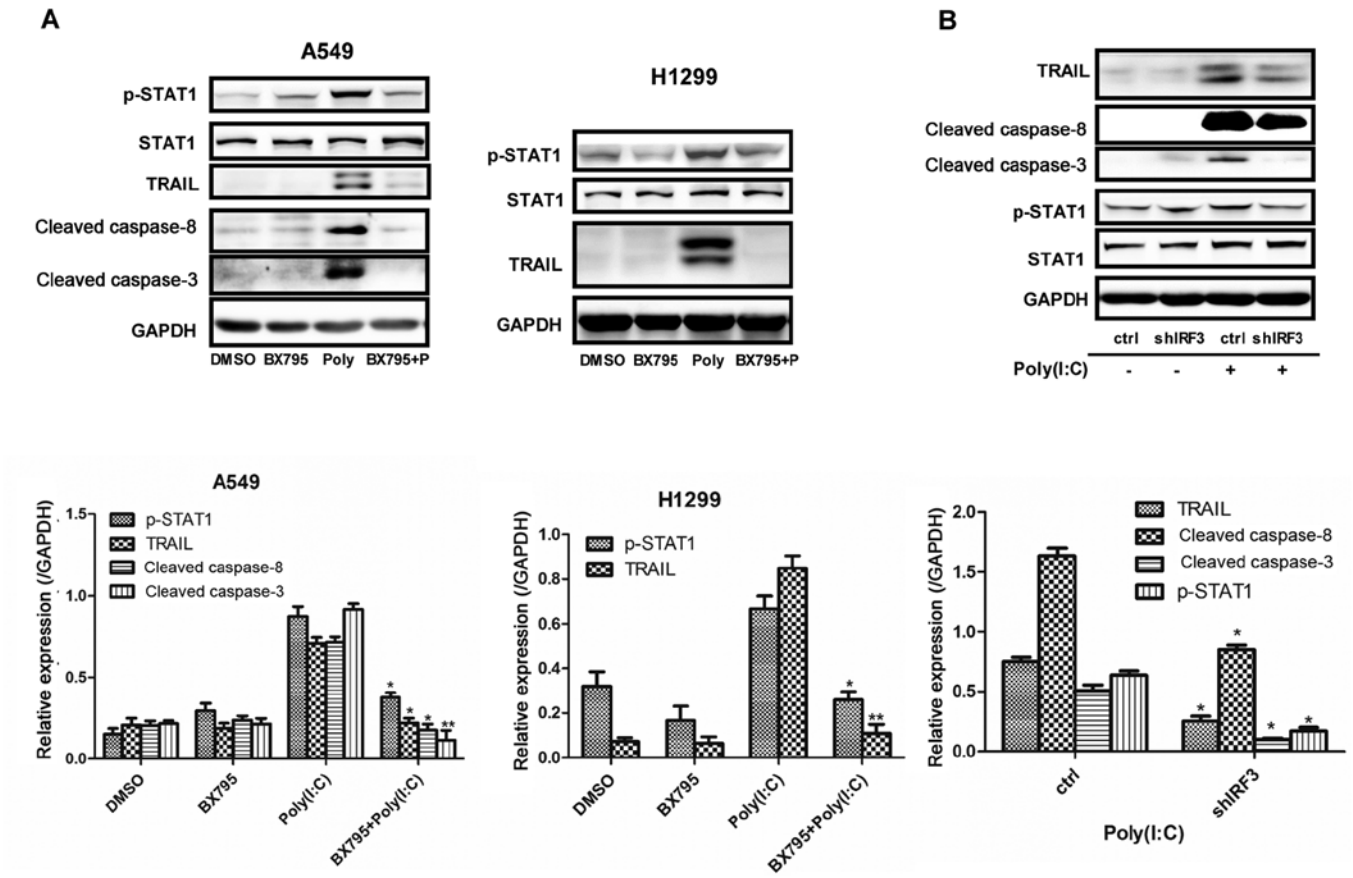

Figure 4. Apoptosis of non-small cell lung cancer cells was inhibited by the suppression of IRF3 activity. (A) Expression levels of p-STAT1, TRAIL, and cleaved caspase-3 and -8, were detected by western blotting in A549 and H1299 cells pretreated with BX795 for 2 h prior to Poly(I:C) transfection for 24 h. *P<0.01; **P<0.005 vs. Poly(I:C) group. (B) In A549 cells with stable downregulation of IRF3, western blotting was conducted to detect TRAIL, p-STAT1, and cleaved caspase-3 and -8 expression, following Poly(I:C) transfection for 24 h. *P<0.01; **P<0.005 vs. ctrl group. IRF3, interferon regulatory factor 3; p-IRF3, phosphorylated-IRF3; Poly(I:C), polyinosinic:polycytidylic acid; p-STAT1, phosphorylated signal transducer and activator of transcription 1; sh, short hairpin RNA; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand.

Furthermore, MDA5/RIG-I expression was upregulated, and their downstream molecules, TBK1 and IRF3, were activated (p-TBK1, p-IRF3) (data not shown). However, these alterations were not observed when Poly(I:C) was directly added to the medium (data not shown).

MDA5/RIG-I/IRF3 axis is associated with the effects of Poly(I:C) transfection on NSCLC cell lines. When the NSCLC A549 and H1299 cell lines were pretreated with BX795 for 2 h, and were then transfected with Poly(I:C) for 24 h, p-IRF3 was inhibited, whereas no changes in total IFR3 were detected (Fig. 3A). In addition, poly(I:C)-induced increases in IFN-β and CXCL-10 mRNA expression in both cell lines, and CXCL-10 secretion in A549 cells, were abrogated by BX795 (Fig. 3B-D). Furthermore, IRF3 was stably knocked down by shRNA in A549 cells, which inhibited the increase in p-IFR3 levels and CXCL-10 secretion following Poly(I:C) transfection (Fig. 3E and F). Furthermore, both cell lines were transfected with Poly(I:C) for 24 h following transfection with RIG-I and MDA5 siRNAs. Notably, the expression levels of p-IRF3 were decreased (Fig. 3G), which was accompanied by a decrease in IFN-β and CXCL-10 mRNA expression, and CXCL-10 secretion in A549 cells (Fig. 3F, H and I). These results indicated that responses to Poly(I:C) transfection were regulated by the MDA5/RIG-I/IRF3 axis in NSCLC cells.

IRF3 mediates apoptosis of NSCLC cells via STAT1 phosphorylation. The present study initially treated NSCLC cells with BX795 or shRNA to downregulate IRF3; the results demonstrated that inhibition of IRF3 suppressed Poly(I:C)-induced TRAIL, and cleaved caspase-3 and -8 expression, and decreased p-STAT1 (Fig. 4A and B). Conversely, IRF3 overexpression in NSCLC cells induced upregulation of TRAIL, cleaved caspase-3 and -8, and p-STAT1 (Fig. 5). Furthermore, knockdown of MDA5 or RIG-1 reduced TRAIL expression in both cell lines (data not shown). These results suggested that Poly(I:C) activated the apoptotic pathway in NSCLC cells via the MDA5/RIG-I/IRF3 pathway. In addition, STAT1 phosphorylation may also engage with IRF3-mediated apoptosis of NSCLC cells.

Activation of IRF3 is inhibited in NSCLC surgical samples. The present study collected surgical NSCLC samples from eight patients. The results of qPCR revealed that the mRNA expression levels of MDA5 were increased by ~3-fold in NSCLC tissues compared with in adjacent normal tissues (Fig. 6A). Conversely, RIG-1 mRNA expression exhibited

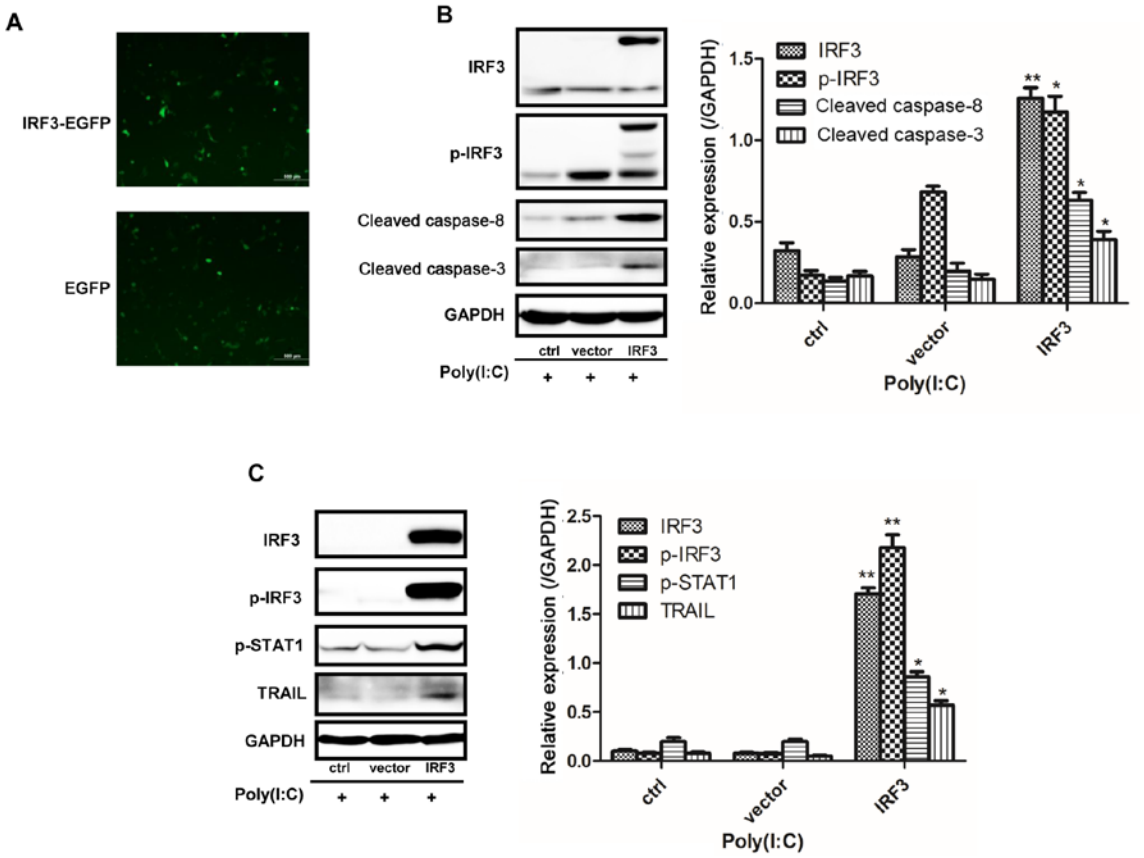

Figure 5. IRF3 overexpression promotes the apoptosis of NSCLC cells. (A) Successful infection with the p-EGFP-N1 plasmid in A549 cells. (B) A549 cells overexpressed IRF3 following infection with an IRF3-EGFP plasmid, and exhibited increased expression of p-IRF3, cleaved caspase-3 and -8 following Poly(I:C) transfection. (C) H1299 cells overexpressed IRF3 following infection with an IRF3-pEnter with His-tag plasmid, and exhibited increased expression of p-IRF3, p-STAT3 and TRAIL. *P<0.02; **P<0.005 vs. vector group. EGFP, enhanced green fluorescent protein; IRF3, interferon regulatory factor 3; p-IRF3, phosphorylated-IRF3; Poly(I:C), polyinosinic:polycytidylic acid; p-STAT1, phosphorylated signal transducer and activator of transcription 1; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand.

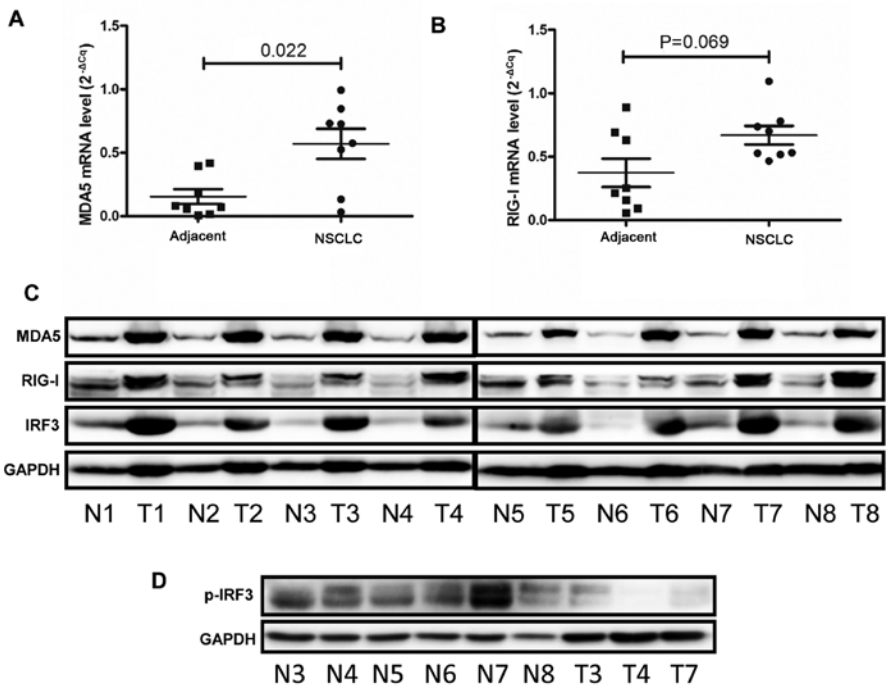

Figure 6. Activation of IRF3 is inhibited in NSCLC tissues. (A and B) Quantitative polymerase chain reaction demonstrated that MDA5 mRNA was significantly increased and RIG-I mRNA was slightly increased in eight NSCLC tumour samples compared with in adjacent tissues. (C and D) MDA5, RIG-1, IRF3 and p-IRF3 were examined by western blotting. IRF3, interferon regulatory factor 3; MDA5, melanoma differentiation-associated protein 5; N, adjacent tissue; NSCLC, non-small cell lung cancer; p-IRF3, phosphorylated-IRF3; RIG-I, retinoic acid-inducible gene I protein; T, tumour tissue.

no marked alterations in NSCLC tissues compared with in adjacent normal tissues (Fig. 6B). Western blotting demonstrated that MDA5, RIG-1 and IRF3 were increased in tumour tissues (Fig. 6C). However, p-IRF3 was decreased in the three tumour tissues compared with in the six normal samples (Fig. 6D). These results suggested that innate immunity may be impaired in NSCLC.

Discussion

Poly(I:C) is a synthetic analogue of dsRNA, which may be used as a vaccine adjuvant to boost innate and adaptive immunity (6,23,24). A vast array of genes are not only induced but also repressed by dsRNA. The inducible genes include interferon-stimulated genes, apoptosis-associated genes, cytokines and growth factor genes, and genes associated with metabolism and biosynthesis, RNA synthesis, protein synthesis and degradation, cytoskeletal components, transporters and the extracellular matrix. The repressed genes include those involved in metabolism, cell cycle regulation and cell adhesion (25). In the present study, the results indicated that the MDA5/RIG-I innate immunity pathway was activated and that type I IFN was induced when Poly(I:C) was transfected into NSCLC cells, which indicated that intracellular Poly(I:C) elicited innate immune responses by activating the MDA5/RIG-I pathway (24). However, when Poly(I:C) was added to the medium directly, it did not induce the innate immune response in NSCLC cells, which may result from a deficiency of TLR3 expression, which is the mainstay sensor for extracellular Poly(I:C) (3). However, the exact mechanism requires further investigation. The TBK1 inhibitor BX795 inhibits phosphoinositide-dependent kinase-1 and TBK1 and, subsequently blocks phosphorylation of IRF3 (26). Treatment with BX795 or knockdown of IRF3 prior to Poly(I:C) transfection led to the inhibition of innate immunity. These findings suggested that the MDA5/RIG-I/TBK1/IRF3 signalling pathway was initiated by intracellular Poly(I:C), and was intact in NSCLC cells, as in other cancer cells (14,27,28).

Previous studies have revealed that Poly(I:C) induces apoptosis of prostate cancer cells, pancreatic cancer cells and other cells (27,28). Furthermore, the canonical apoptotic pathways, including the caspase-9-dependent intrinsic pathway and the caspase-8-dependent extrinsic pathway, engage Poly(I:C)-induced apoptosis to some degree (14,27). In the present study, Poly(I:C)-induced apoptosis of A549 cells mainly via the extrinsic pathway, as determined by the slight change in activated caspase-9 expression, and this was associated with an upregulation of TRAIL. TRAIL belongs to the TNF family and induces apoptosis by binding death receptor (DR)4 or DR5, in order to activate the extrinsic apoptotic pathway (29,30). Furthermore, IRF3 serves a crucial role in TLR3-mediated apoptosis of androgen-sensitive prostate cancer LNCaP cells (12), whereas it is not implicated in androgen-resistant prostate cancer cells (14). In the present study, downregulation of IRF3 in NSCLC cells resulted in a decline in the expression of apoptosis-related proteins, and vice versa. A previous study revealed that IRF3 binds and transactivates the TRAIL promoter in the nucleus where the IRF3 response element is located (31). Therefore, active IRF3 may shuttle into the nucleus of NSCLC cells and directly target TRAIL to activate extrinsic apoptosis. However, another study elucidated the concept of RIG-I-induced IRF3-mediated apoptosis, revealing that IRF3 in the cytoplasm interacts with B-cell lymphoma 2-associated X protein and causes activation of caspase-9 and -3 (24), which mainly activate intrinsic apoptosis. Therefore, in the present study, it is likely that the results are consistent with the first mechanism. Notably, the present study demonstrated that variation of STAT1 phosphorylation was in accordance with the alterations in the apoptotic pathway. This finding suggested that activation of STAT1 by type I IFN may affect DNA damage and adaptive immunity (32), and may modulate the expression of FAS, FAS ligand and casapse-1, and the function of p53 to accelerate apoptosis (33). In addition, STAT1 suppresses the cell cycle by inducing p53, NF-κB p65, cyclin A, cyclin D1, cyclin E, F-box and WD repeat domain containing 7, Hes family BHLH transcription factor 1 and cyclin-dependent kinase 2 expression. Conversely, the Janus kinase (JAK)2/STAT1 pathway participates in the upregulation of programmed death-ligand 1 and may serve a pivotal role in inducing tumour immune escape (34). Generally, IRF3 is indeed involved in Poly(I:C)-induced apoptosis of NSCLC cells; however, the exact mechanism is ambiguous, and whether the JAK/STAT1 pathway orchestrates IRF3-mediated apoptosis in NSCLC remains to be determined.

In human tissue samples, the present study demonstrated that MDA5, RIG-I and IRF3 were increased in NSCLC samples, thus indicating that the innate immune pathway was intact in NSCLC. Notably, p-IRF3 expression was decreased, which was inconsistent with the increase of IRF3, in three samples. These findings suggested that MDA5 and RIG-I may be positively correlated with the overall survival of patients with NSCLC, similar to in patients with hepatocellular carcinoma (35). However, IRF3 may also be favourable to the survival of cancer cells via producing various inflammatory factors into the tumour microenvironment (36). In patients with herpes simplex encephalitis, a G-A mutation at the 854 base-pair region in exon 6 prevents IRF3 from forming homodimers and being phosphorylated at S286, which disrupts its transcriptional activity and the innate immune response (37). Whether the same mutation occurs in patients with NSCLC is currently unknown.

In conclusion, the present study revealed that the innate immune pathway was intact in NSCLC cells, and IRF3 was involved in regulating the apoptotic pathway. However, in NSCLC tissue samples, the innate immunity pathway may be disrupted. These findings may provide novel insights into the role of IRF3 in innate immunity and apoptosis in NSCLC. In addition, IRF3 may be considered a target to induce apoptosis in NSCLC therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XL conceived and designed the experiments; LY performed the experiments, analysed the data and wrote the paper; DS ZL and ZZ helped collect the tissue specimens; QH, YW and XC conducted statistical analysis.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Peking University First Hospital (Beijing, China). All of the subjects gave their informed consent for inclusion before they participated in the study. The peripheral blood cells were collected from a healthy volunteer and informed consent was obtained as well.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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