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Prognostic value of serum soluble interleukin-23 receptor and related T-helper 17 cell cytokines in non-small cell lung carcinoma

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Abstract

The signaling of interleukin (IL)-23 and its receptor (IL-23R) play a crucial role in the development of cancers. However, the clinical significance of human serum soluble IL-23R (sIL-23R) and its relationship with IL-23 are still not explored in non-small cell lung cancer (NSCLC). In our study, sIL-23R was first identified in the serum of NSCLC patients, but not in healthy controls, by proteomics. The IL-23R mRNA and protein were upregulated in NSCLC cell lines and tissues tested by quantitative PCR, western blot analysis and immunohistochemistry. The levels of sIL-23R, IL-23, and IL-17 in 195 NSCLC patients' serum were determined by ELISA, and high levels of sIL-23R were significantly associated with advanced N stage (P = .039), clinical stage (P = .007), and poor 5-year survival rate. In vitro, sIL-23R was shown binding to IL-23 and the balance could affect patients' N and T stage, overall survival, and downstream cytokine IL-17 in a potential antagonistic relationship. Although sIL-23R, IL-23, and IL-17 were all associated with poor prognosis, only the sIL-23R/IL-23 ratio (hazard ratio, 1.945; 95% confidence interval, 1.147-3.299; P = .014) was found to be an independent factor for prognosis. Therefore, we identified fragments of soluble cytokine receptor of IL-23R with affinity ability to its natural ligand IL-23 in NSCLC patients' serum. The balance between the 2 antagonists can work as a potential prognostic serum marker.

KEYWORDS
combination, IL-23, NSCLC, prognosis, sIL-23R

Liu, Xing and Wang contributed equally to this work.

1 | INTRODUCTION

Due to its high morbidity and mortality rates, lung cancer (LC) is currently one of the most common malignant cancers and has become a "deadly disease" among malignant carcinomas in China and the rest of the world.[1] The link between chronic immune activation and tumorigenesis is well established, and LC is a typical inflammation-associated carcinoma.[2] Compelling evidence has accumulated that different host immune response components in LC help identify different prognostic patient subgroups.[3] Precise prediction of the prognosis certainly leads to the better treatment of LC cases and provides many new avenues for therapeutic research. Therefore, there is an urgent need to explore valuable diagnosis and prognostic biomarkers related to tumor inflammation for LC patients. Non-small cell lung cancer (NSCLC) accounts for more than 80% of all LC cases, and it includes 2 major types: squamous cell (epidermoid) carcinoma and nonsquamous, including adenocarcinoma, large cell carcinoma, and other cell types.[4]

Interleukin (IL)-23, a member of the IL-12 family, is known to play an important role for the induction and maintenance of T-helper cell 17 (Th17), the production of the downstream IL-17 by the Janus kinase signal transducers 2 and activator of transcription 3 (JAK2/STAT3) signaling pathway.[5-8] Its receptor, the IL-23 receptor (IL-23R) complex, is a heterodimer composed of the shared IL-12R β1 subunit and a specific IL-23R subunit. Interleukin-23R mRNA is 2.8 kb in length and contains 12 exons (NM_144701). The translated protein includes 629 amino acids, which is a type I penetrating protein comprising a signal peptide, an N-terminal fibronectin III-like domain, and an intracellular residue containing 3 potential tyrosine phosphorylation domains.[5] Multiple variable splicing versions of IL-23R have been reported in up to 24 forms in mitogen-activated leukocytes.[9] A nonsynonymous single nucleotide polymorphisms in the IL-23R was found in the binding domain to IL-23 and could affect the activation of Th17.[10] There was a variant that lacked the transmembrane and intracellular portions but only the extracellular portion, known as serum soluble IL-23R (sIL-23R) △9. It can compete with IL-23R on membrane to bind IL-23, regulate Th17 cell pathway and participate in inflammation and immune function.[11]

The IL-23 axis has been implicated in the regulation of LC promotion. Interleukin-23 was found to induce LC proliferation, and given its potential roles in cancer persistence, survival, and/or proliferation it can promote tumor growth and development.[12] Interleukin-23R was reported to express in lung adenocarcinoma and small cell carcinoma but not in lung squamous cell carcinoma tissue cells, and interacted with IL-23 to regulate the proliferation of tumor cells.[13] The polymorphism of the *IL-23R* gene was associated with susceptibility to breast cancer, LC, and nasopharyngeal carcinoma in Asia.[14]

The role of sIL-23R in serum of NSCLC patients remains largely unknown. In this study, we found sIL-23R binding with its ligand IL-23 in the serum of NSCLC patients, and their balance was involved in the tumor characteristics and prognosis. The ratio of sIL-23R/IL-23 serves as a useful biomarker for evaluating tumor progression and prognosis of NSCLC.

2 | MATERIALS AND METHODS

2.1 | Patients, sera, and tissues

All the tissues and serum samples were collected from the Sun Yat-sen University Cancer Center (SYUCC, Guangzhou, China). All of the patients were classified according to the TNM classification system and the resected specimens underwent pathological examination. Sera were collected at the time of diagnosis before treatment.

Twenty pairs of lung adenocarcinoma and corresponding nontumorous tissues at least 8 cm away from the tumor were obtained within 1 hour after the surgical lung tissue resection from patients who underwent surgery, and were immediately kept in liquid nitrogen until further analysis.

Sera were collected between January 2009 to December 2010 from 195 NSCLC patients at the time of diagnosis before tumor resection. The venous blood was collected by dry vacuum blood collection tube at room temperature, and allowed to stand at room temperature for 1 hour. After blood coagulation, it was centrifuged at 2000 *g* for 10 minutes in a centrifuge, and the serum was carefully aspirated and stored at −80°C.

2.2 | Ethics

The relevant experiments were in line with the requirements of the Declaration of Helsinki and had been reviewed and approved by the Institutional Review Board and Ethics Committee of SYUCC (GZR2017-186). For patients treated in our center, as a general standard procedure, we obtain their written informed consent for the use of clinical parameters and collected samples for further studies at the time of their admission. The records were anonymous and deidentified before use.

2.3 | Cell lines

The human NSCLC cell lines A549 and H226 and lung immortalized epithelial cell line 16HBE (Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS. The cells were incubated at 37°C in a humidified chamber containing 5% CO_2.

2.4 | Serum proteomic profiling C-reactive protein (CRP) complex purification from mixed serum

Serum samples from 10 LC patients were mixed, as were serum samples from 10 healthy controls. CRP complexes were purified from the mixed cancer serum or mixed control serum using the following method. In brief, after centrifugation at 10 000 *g* for 10 minutes at 4°C, 500 μL serum was diluted in 500 μL buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, 0.1% Nonidet P-40, pH 7.4), and CRP complexes

were precipitated by shaking overnight at 4°C using 500 μL anti-CRP carboxyl-coated polyethylene beads (Stagnant Water). The beads were washed 5 times with dilution buffer by centrifugation at 10 000 *g* for 10 minutes at 4°C, and the supernatant with unbound proteins was removed. The pellets were solubilized in 100 μL of triethyl-ammonium bicarbonate (TEAB) lysis buffer (20 mmol/L TEAB, 20 mmol/L DTT, 1% SDS) at room temperature for 10 minutes, heated to 95°C for 10 minutes, and then allowed to cool for a further 10 minutes at room temperature. Samples containing the affinity-purified proteins were subjected to an additional centrifugation step (10 000 *g* for 30 minutes at room temperature), and the supernatants were collected.

2.5 | Two-dimensional electrophoresis and MALDI time of flight/time of flight mass spectrometry analysis

Affinity-purified CRP complexes were subjected to 2-D separation, and 2-D electrophoresis (2-DE) runs were repeated 3 times. After electrophoresis, the gels were stained with blue silver, scanned in a densitometer (Molecular Imager FX; Bio-Rad) at a resolution of 600 dpi, and analyzed using PDQuest software (version 7.1.0; Bio-Rad). Protein spots showing 2-fold or higher change in density (Student's *t* test, $P < .05$) in a consistent direction were considered to be different and selected for further identification.

2.6 | Peptide extraction

Protein spots of interest were in-gel digested by trypsin. Gel pieces were first discolored in 50% acrylonitrile and 25 mmol/L ammonium bicarbonate and subsequently subjected to reduction in 10 mmol/L DTT and alkylation in 55 mmol/L iodoacetic acid. Following vacuum drying, the gel pieces were incubated with sequencing grade modified trypsin (Promega) at a final concentration of 0.01 mg/mL in 25 mmol/L ammonium bicarbonate for 16 hours at 37°C. Supernatants were collected, vacuum-dried, and redissolved in 50% acrylonitrile and 0.1% TFA for mass spectrometry (MS) analysis.

2.7 | Mass spectrometry

Tryptic peptides were finally dissolved in MALDI matrix (7 mg/mL α-cyano-4-hydroxycinnamic acid in 0.1% TFA and 50% acrylonitrile), spotted onto 192-well stainless steel MALDI target plates, and analyzed using an ABI 4800 Proteomics Analyzer MALDI time of flight/time of flight (TOF/TOF) mass spectrometer (Applied Biosystems). The MS together with the MS/MS spectra were searched against the IPI Human database version 3.24 using GPS Explorer version 3.0 software and MASCOT database search algorithms (version 2.0). The following search criteria were used: trypsin specificity, cysteine carbamidomethylation (C), and methionine oxidation (M) as variable modifications, 1 trypsin miscleavage allowed, 100 ppm MS tolerance, and 0.25 Da MS/MS tolerance. All identified proteins had protein scores greater than 59 ($P < .05$) and individual ion scores greater than 21 with expected values ($P < .05$). All MS/MS spectra were further validated manually.

2.8 | RNA extraction and quantitative PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription of total RNA (1 μg) was undertaken using SuperScript II reverse transcriptase. The quantification of IL-23R and the reference gene (*β-actin*) were carried out in triplicate on a LightCycler 480 II (Roche, Applied Science) using a SYBR green-based assay (Bio-Rad). The primers used in the quantitative (qPCR) reaction were as follows: IL-23R forward 5′- CAG GTC ACT ATT CAA TGG GAT GC-3′ and reverse 5′-GCA GTT CTT AAT TGC TGC TTG G-3′; and β-actin forward 5′-CGC GAG AAG ATG ACC CAG AT-3′ and reverse 5′-GGG CAT ACC CCT CGT AGA TG-3′.

2.9 | Immunohistochemistry

The paraffin-embedded lung adenocarcinoma tissues were sectioned into 4-μm-thick sections. The sections were dewaxed, rehydrated, and rinsed. The antigens were retrieved by heating the tissue sections at 100°C for 20 minutes in citrate (10 mmol/L, pH 6.0) solution. The sections were then immersed in a 3% hydrogen peroxide solution for 10 minutes to block endogenous peroxidase activity and incubated with the primary Ab human IL-23R Ab MAB14001 (1:100; R&D Systems) at 4°C overnight. Subsequently, the sections were incubated with a HRP-labeled secondary Ab (Zymed) at room temperature for 120 minutes. Finally, after washing, slides were incubated with DAB (Sigma) and immediately washed under tap water after color development, then all of the slides were counterstained with hematoxylin.

2.10 | Enzyme-linked immunosorbent assays of sIL-23R, IL-23, and IL-17

Serum sIL-23R levels were determined using a home-made ELISA kit developed by 2 Abs (R&D Systems): 100 μL human IL-23R Ab (5 μg/mL MAB14001) was added to each well of a 96-well enhanced-binding ELISA plate as coating Ab and incubated at 4°C overnight. Then each well was washed 3 times with the washing buffer. The blocking buffer (300 μL of 1× PBS and 3% BSA) was added to block the nonspecific binding sites; plates were incubated again at room temperature for 2 hours, which was followed by 3 washes with the washing buffer. Recombinant Human IL-23R Fc chimera protein (1400-IR-050; R&D Systems) was used as the standard. Blanks consisted of the assay buffer (1× PBS and 3% BSA). An aliquot (100 μL) for each of 7 standard solutions, blanks and test samples of serum was applied to wells of each plate (in

duplicate). Plates were incubated at 4°C overnight, which was followed by 3 washes with washing buffer. Then 100 μL human IL-23R biotinylated Ab BAF1400 (1.6 μg/mL) diluted in assay buffer was added, and plates were incubated at room temperature for another 2 hours. Plates were again washed 3 times with the washing buffer. Plates were developed by the addition of a 3,3',5,5'-tetramethylbenzidine substrate. Plates were incubated for 30 minutes at room temperature, and the reaction was then stopped by the addition of a stopping buffer (4M acetic acid and 0.5 M sulfuric acid). Measurements for the plates were obtained at 450 nm by using an automated plate reader. A commercial software package was used to calculate an 8-variable logistic curve.

The commercial ELISA kits of IL-23 and IL-17 (R&D Systems) was carried out according to the manufacturer's instructions.

2.11 | Serum soluble IL-23R bound with IL-23 in serum of NSCLC by ELISA assay

Interleukin-23 recombinant protein (100 μL) (800 pg 1887-ML-010; R&D Systems) was coated in the ELISA plate at 4°C overnight, with assay buffer (1× PBS and 3% BSA) as negative control. After washing with washing buffer (0.05% Tween-20) 3 times, 100 μL serum samples with high (50-100 pg/mL) or low (20-50 pg/mL) levels of sIL-23R validated by ELISA from 20 NSCLC patients were incubated for 2 hours at room temperature. After washing, binding sIL-23R was detected by human IL-23R Ab (1.6 μg/mL BAF1400; R&D Systems) similar to indirect ELISA, and plates were incubated at room temperature for another 2 hours. Plates were again washed 3 times with washing buffer. Plates were developed by the addition of a 3,3',5,5'-tetramethylbenzidine substrate. Plates were incubated for 10 minutes at room temperature, and the reaction was then stopped by the addition of a stopping buffer (4 M acetic acid and 0.5 M sulfuric acid). Measurements for plates were obtained at 450 nm by use of an automated plate reader.

2.12 | Statistics analysis

Statistical analysis was undertaken using SPSS 17.0 statistical software package (SPSS). The receiver operating characteristic curve was used to evaluate the overall prediction accuracy of each marker and determine the critical point. The difference between groups was determined by nonparametric tests. Multivariate prognostic analysis was carried out using a Cox regression model. Survival analysis was undertaken by the Kaplan-Meier method. The end-point was overall survival (OS), calculated from the start of diagnosis to the time of death or the last follow-up time. $P < .05$ was considered to be statistically significant in all cases. The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (www.researchdata.org.cn), with the approval RDD number RDDB2019000538.

3 | RESULTS

3.1 | Identification of sIL-23R in serum of NSCLC patients

We previously purified the CRP-bound components in serum samples from healthy controls or NSCLC patients using anti-CRP carboxyl-coated polyethylene beads and used 2-DE and subsequent MS to explore the serum soluble tumor markers (Figure 1A). Interleukin-23R fragments were only identified in the NSCLC serum frequently, and covered 12.2% of the amino acid sequence of IL-23R (IPI00915346) (Figure 1B,1). We hypothesized that the fragments of sIL-23R could be a new tumor serum marker of NSCLC.

3.2 | Interleukin-23R overexpressed in NSCLC cell lines and tissues

To investigate the source of sIL-23R, we detected the expression of IL-23R in lung cancer cell lines and tissue pairs. The relative expression of IL-23R mRNA was expressed differently in 2 NSCLC cell lines by qPCR: strongly in lung adenocarcinoma cell line A549, but low in lung squamous carcinoma cell line H226 and lung immortalized cell line 16HBE (Figure 2A). Similarly, 20 pairs of lung adenocarcinoma tissues expressed higher IL-23R mRNA than their adjacent normal tissues (Figure 2B) ($P < .001$). As shown in Figure 2C, IL-23R protein was highly expressed in lung adenocarcinoma tissues and sublocalized in cell cytoplasm of tumor cells, but hardly observed in NSCLC stromal or adjacent noncancer tissues by immunohistochemistry (IHC). Western blot analysis showed the bands of IL-23R had significant difference between cancer and normal tissues, and they were coincident with the relative expression of the IL-23R mRNA (Figure 2D).

3.3 | Serum soluble IL-23R binds to IL-23 in vitro

Given soluble cytokine receptor can work as an agonist or antagonist to its WT membrane receptor and regulate inflammation and immune function by binding the ligand, an ELISA experiment confirmed that sIL-23R indeed bound to IL-23 in the serum of NSCLC. Higher optical density values showed more sIL-23R binding with the coating IL-23 than the BSA, and it was sIL-23R concentration-dependent (Figure 2E).

3.4 | Association between serum IL-23R level and clinicopathologic characteristics of NSCLC patients

The levels of serum sIL-23R, IL-23, and IL-17 were detected by ELISA in 195 NSCLC patients. The association analysis between median sIL-23R levels and clinicopathologic parameters showed that elevated sIL-23R

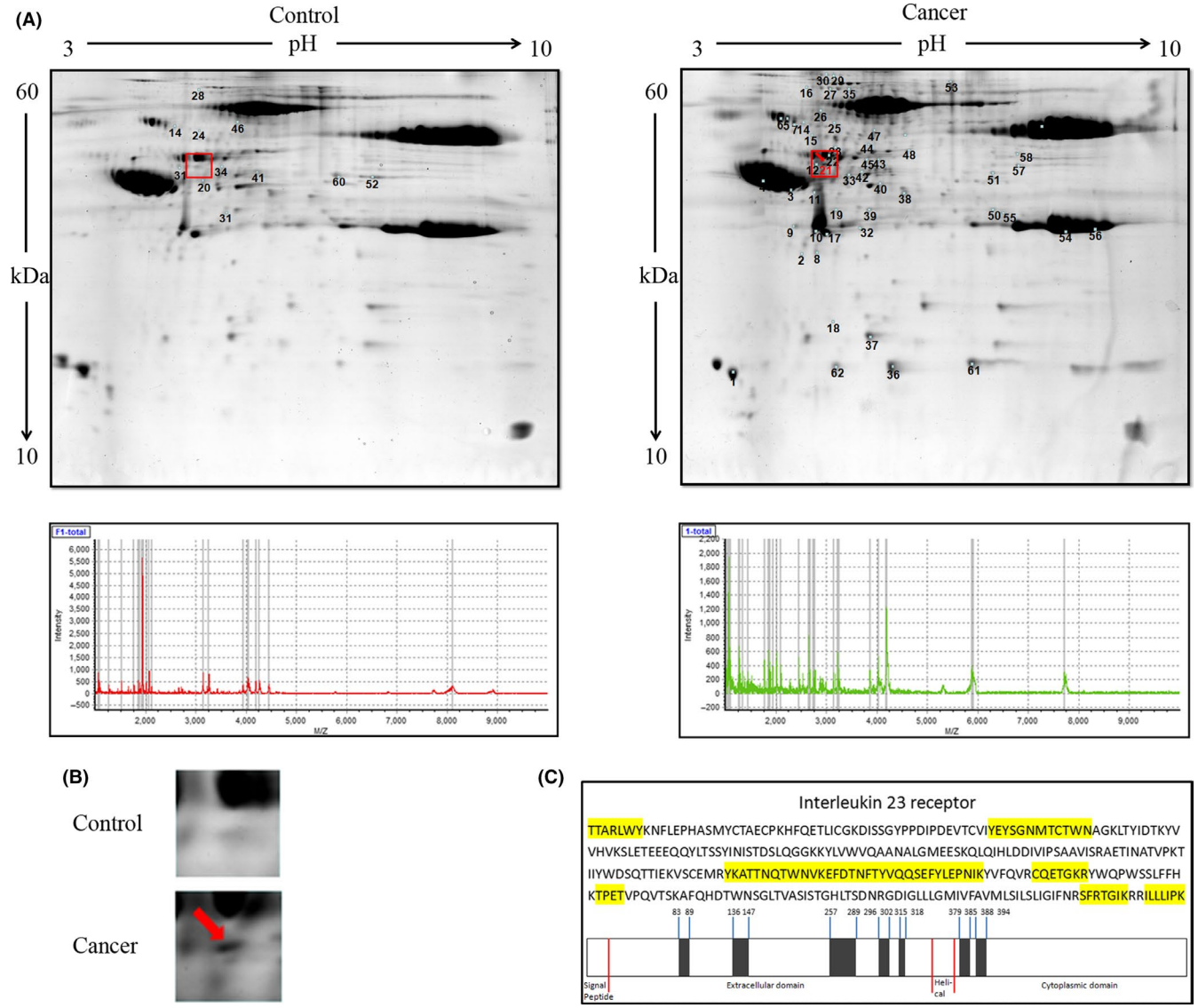

FIGURE 1 Identification of soluble interleukin-23 receptor (sIL-23R) in non-small cell lung cancer (NSCLC) and healthy control serum samples. A, Gel was visualized by silver staining. The mass spectroscopy (MS) results were analyzed after removing chemical and electrical signal noises, and through a series of processing such as data correction and normalization. Left panels, normal control; right panels, NSCLC. Red arrow in the box indicates the discrepant masses corresponding to sIL-23R. B, The protein spot with discrepancies between healthy controls and NSCLC patients was analyzed using MALDI time of flight (TOF)/TOF MS. C, sIL-23R fragments showed 12.2% amino acid sequence coverage in the MS analysis

levels were significant for patients with more advanced disease, including lymph node metastasis (P = .039) and clinical stage (P = .007) but not age, gender, smoking history, pathological grade, T stage, or M stage (Table 1). Moreover, the high levels of IL-23 were only associated with advanced T stage (P = .001) and clinical stage (P = .039) (Table S1).

Serum soluble IL-23R and IL-23 bind with each other, and sIL-23R was associated with N stage. To detect the influence of binding on sIL-23R lymph node metastasis promotion, we further analyzed the N stage among groups according to the ratio of serum sIL-23R/IL-23 (0-0.5 vs 0.5-1 vs >1). The statistical analysis revealed that the rank mean of N stage was the highest in the ratio of the >1 group ($P_{0\text{-}0.5 \text{ vs } >1}$ = .033, $P_{0.5\text{-}1 \text{ vs } >1}$ = .031). There was no significant difference between the groups 0-0.5 and 0.5-1 (Figure 3A). High level of sIL-23R was associated with advanced N stage of patients, but take the binding of sIL-23R with IL-23 in consideration, the group with a sIL-23R/IL-23 ratio of >1 had more advanced N stage than the 0-0.5 or 0.5-1 groups. For the association between T stage and IL-23/sIL-23R ratio, we found patients with more advanced T classification were in the group with an IL-23/sIL-23R ratio of 0.5-1 rather than in the 0-0.5 (P = .041) or >1 (P = .009) groups (Figure 3B). Our results were similar to Li et al's study, which proved IL-23 regulates proliferation of lung cancer cells in a concentration-dependent way.[13] In conclusion, we hypothesized that the stimulation of sIL-23R on lymph node metastasis could be neutralized by binding with IL-23. Vice versa, the binding of sIL-23R and IL-23 can also influence IL-23 function on T stage.

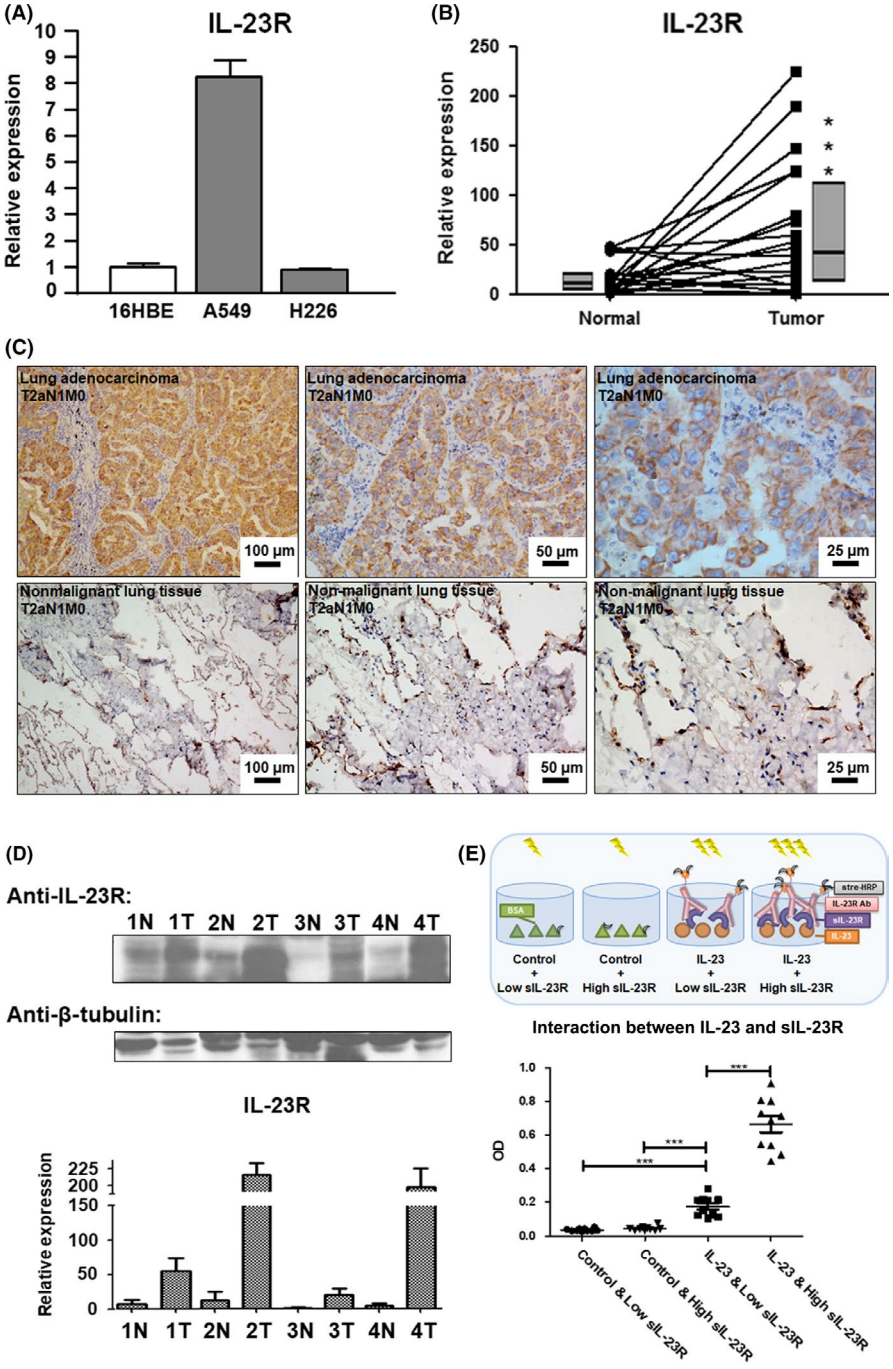

FIGURE 2 A, Relative expression of interleukin-23 receptor (IL-23R) in lung adenocarcinoma cell line A549, lung squamous carcinoma cell line H226, and lung immortalized cell line 16HBE. Original mean value of relative expression of 16HBE was 3.84×10^{-6}. For simplification, we set the value of 16HBE as 1, and the other values of cell lines and tissues were corrected by the baseline. B, Each pair of data points connected by a line represent the relative expression of IL-23R from tumor and adjacent noncancerous tissues of a single donor. Black line with gray block next to each group indicates median and 25%-75% percentile. ***$P < .001$. C, Immunohistochemistry detected IL-23R overexpression and sublocalization in lung cancer (LC) cell cytoplasm but not on membrane, stromal, or adjacent noncancer normal tissues. Tissue sections were derived from 2 patients with lung adenocarcinoma (T2aN1M1). The 10×, 20×, and 40 × microscopic field images were taken separately. D, Western blot analysis detected IL-23R in lung adenocarcinoma tissues and paired noncancer tissues. Graph below shows relative expression of IL-23R mRNA in the same patch of LC tissue pairs by quantitative PCR (qPCR). Values were corrected by the baseline of the relative expression level of 16HBE to 1. As internal standards, western blot analysis used β-tubulin and qPCR used β-actin. E, Interaction between IL-23 and sIL-23R in NSCLC patients' serum. Optical density (OD) values of IL-23 coated wells were significantly higher than control. ***$P < .001$

Interleukin-17 was the downstream cytokine of the IL-23/IL-23R/STAT3 pathway stimulated by IL-23 in the Th17 cells.[15] To identify the regulatory signal delivered by the sIL-23R-IL-23 complex on IL-17, we analyzed the correlations between sIL-23R/IL-23 and serum levels of IL-17. It is generally considered as significant when one value shows 2-fold or higher change than the other. Therefore, we chose 0.5 and 2 as the 2 cut-off values of the sIL-23R/IL-23 ratio. The subsequent analysis showed the intermediate group with ratio of 0.5-2 had the lowest rank mean of IL-17 ($P_{<0.5 \text{ vs } 0.5\text{-}2} = .033$, $P_{0.5\text{-}2 \text{ vs } >2} < .001$) (Figure 3C), but there was no significant difference between the <0.5 and >2 groups. The level of sIL-23R was similar to IL-23 in the group with the sIL-23R/IL-23 ratio of 0.5-2, in which the level of IL-17 was lower than that of the <0.5 group (the level of IL-23 was significantly higher than sIL-23R). We hypothesized that the stimulation of IL-23 on IL-17 was blocked by the binding of sIL-23R. The level of IL-17 in the >2 (the level of sIL-23R was higher than IL-23) group was also significantly higher than that of 0.5-2. In addition, an extra pairwise correlations analysis revealed that the Spearman's rank correlation coefficients between sIL-23R and IL-17 was 0.384 ($P < .001$). These results implied that sIL-23R might inhibit IL-23 in the traditional stimulation of IL-17, but might have a weak

TABLE 1 Serum levels of soluble interleukin-23 receptor (sIL-23R) and clinical characteristics of patients with non-small cell lung cancer

Characteristics	All (n = 195)	Serum sIL-23R level		*P**
		Low	High	
Age, years				
≤60	92	28	64	.513
>60	103	27	76	
Gender				
Female	60	13	47	.176
Male	135	42	93	
Smoking status				
Smoker	112	30	82	.703
Non-smoker	82	24	58	
Grade				
Well differentiated	8	1	7	.181
Moderately differentiated	84	12	72	
Poorly differentiated	74	4	70	
T status				
T1 and 2	148	47	101	.190
T3 and 4	43	8	36	
N status				
N0	73	27	46	**.039**
N1/2/3	117	27	90	
M status				
M0	152	47	105	.151
M1	41	8	33	
Stage				
IA, IB, IIA	70	27	43	**.007**
IIB, IIIA	65	18	47	
IIIB, IV	59	10	49	

Note: Bold type indicates statistical significance.

**P* values assessed using Mann-Whitney *U* test and Kruskal-Wallis test.

promotion function on IL-17. We hypothesized the balance of sIL-23R and IL-23 could influence the maturation of Th17 cells by downstream IL-17.

3.5 | Prognostic value of serum sIL-23R/IL-23 ratio and related cytokines in patients with NSCLC

The OS of patients with NSCLC (n = 195) was plotted using the Kaplan-Meier method and a log rank test. The groups with low sIL-23R level showed a significantly better 5-year survival rate than the elevated sIL-23R groups (Figure 4A). These results indicated that higher sIL-23R was associated with poor prognosis in patients with NSCLC. In addition, high levels of IL-23 and IL-17 were associated with lower survival rate in patients with NSCLC (Figure S1).

Furthermore, we analyzed the prognostic value of the sIL-23R/IL-23 ratio. We selected 2 cut-off values of 0.43 and 2.56 to divide patients into 3 groups according to the largest Youden index, clinical significance, and cases of each group. The group with ratio of 0.43-2.56 (n = 61) had the best OS compared to the other 2 groups ($P_{<0.43 \text{ vs } 0.43\text{-}2.56}$ = .044, $P_{0.43\text{-}2.56 \text{ vs } >2.56}$ = .036). However, there was no significant difference between groups with ratio of less than 0.43 (n = 70) and greater than 2.56 (n = 64) (Figure 4B-D). Moreover, to detect the possible underlying reasons, the cut-off values of sIL-23R/IL-23 were also used in the comparison analysis of N and T stage and IL-17. Results were similar to those shown in Figure 3 for IL-17, but there was no significant difference in the N or T stage among the groups divided by the OS ratio (Figure S2).

Results of the univariate Kaplan-Meier analysis and the multivariate Cox regression model with respect to OS rate showed that age, grade, TNM stage, sIL-23R, IL-23, IL-17, and the sIL-23R/IL-23 ratio were significantly correlated with survival in the univariate Kaplan-Meier analysis. To determine whether the characteristics could be independent prognostic factors for outcomes, we carried out a multivariate analysis. In this analysis, age, grade, TNM stage, and the sIL-23R/IL-23 ratio were recognized as independent prognostic factors

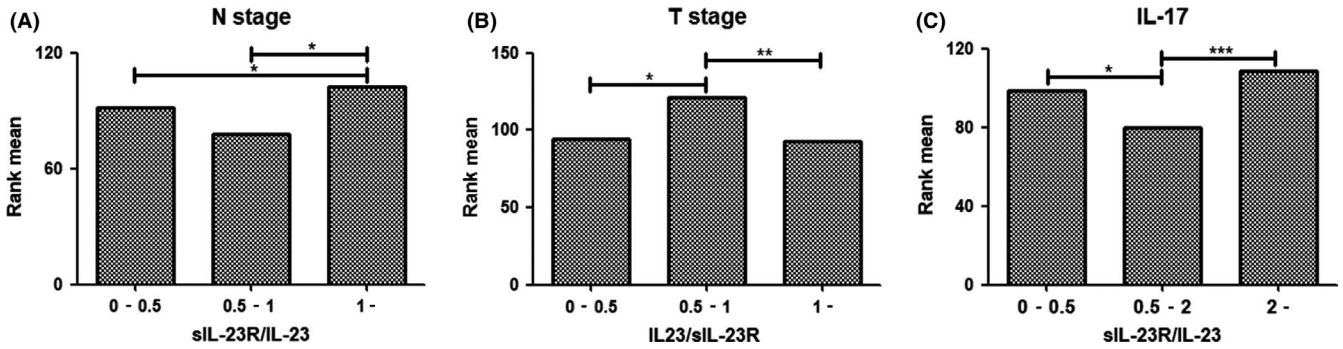

FIGURE 3 Rank sum test analysis of the relationship between the balance of 2 cytokines, soluble interleukin-23 receptor (sIL-23R) and IL-23, and N stage (A), T stage (B) and IL-17 (C) of non-small cell lung cancer patients. The ordinate was the rank mean. *P < .05, **P < .01, ***P < .001

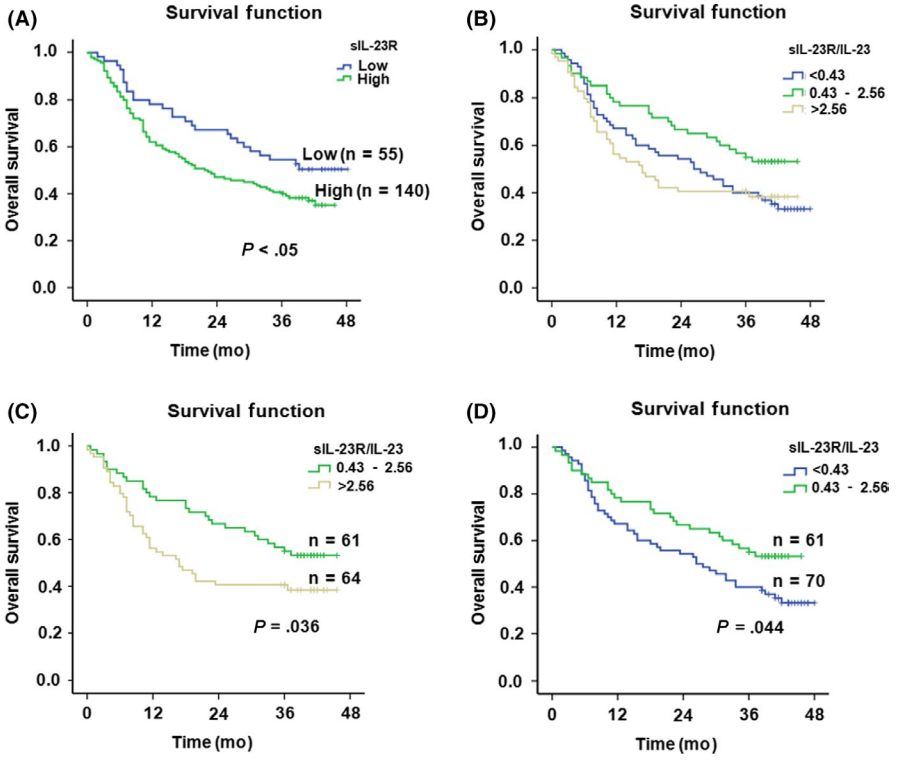

FIGURE 4 A, Kaplan-Meier analysis of serum soluble interleukin-23 receptor (sIL-23R) for non-small cell lung cancer (NSCLC) patient prognosis. B, Merged survival curves of NSCLC patient subgroups with high (>2.56), intermediate (0.43-2.56), and low (<0.43) sIL-23R/IL-23 ratios. C, Overall survival was significantly worse in the >2.56 sIL-23R/IL-23 ratio group than the 0.43-2.56 group ($P = .036$). D, NSCLC patients with intermediate (0.43-2.56) sIL-23R/IL-23 ratio showed better survival ($P = .036$) than those with low (<0.43) ratio

TABLE 2 Univariate and multivariable logistic regression analysis of variables associated with non-small cell lung cancer

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	*P* value	HR	95% CI	*P* value
Age (>60 years)	1.499	1.032-2.177	.034	2.008	1.282-3.146	**.002**
Gender (reference: male)	0.870	0.583-1.299	.496	–	–	–
History of tobacco use	1.122	0.774-1.626	.542	–	–	–
Grade (well vs moderately vs poorly differentiated tumor)	1.831	1.255-2.670	.002	1.863	1.291-2.689	**.001**
TNM stage (I/IIA vs IIB/IIIA vs IIIB/IV)	2.274	1.769-2.855	<.001	2.254	1.687-3.011	**<.001**
sIL-23R (>0.451 pg/mL)	1.551	1.007-2.389	.046	1.012	0.590-1.738	.965
IL-23 (> 3.020 pg/mL)	1.835	1.266-2.661	.001	1.202	0.730-1.979	.470
IL-17 (> 0.241 pg/mL)	1.783	1.232-2.580	.002	1.016	0.634-1.627	.947
sIL-23R/IL-23 (0.43-2.56)	1.628	1.068-2.482	.024	1.945	1.147-3.299	**.014**

Note: Bold type indicates statistical significance.

Abbreviations: –, gender and history of tobacco use are not included in the multivariate Cox regression analysis; CI, confidence interval; HR, hazard ratio; IL, interleukin; sIL-23R, soluble interleukin-23 receptor.

(Table 2). Thus, our findings indicate that the balance of IL-23 and sIL-23R is involved in the prognosis of NSCLC patients.

4 | DISCUSSION

The IL-23/IL-23R axis regulates immune responses against pathogens and plays a major role in the differentiation and maintenance of Th17 cells and the development of autoimmune diseases and cancers.[16] Interleukin-23R has been proved to have many mRNA splicing variants. A soluble form of IL-23R (sIL-23R), termed △9, can work as an antagonist and compete with its membrane-associated counterparts for the ligands to modulate the molecular pathway in Th17 cells.[11] Interleukin-23R has been found expressed in LC and is involved in LC proliferation.[13] As yet, there has been no study about the clinical significance of serum sIL-23R and its binding with IL-23 in NSCLC.

Previously, we have found several fragments of IL-23R in the CRP-binding complex from serum of NSCLC patients, but not in the normal controls, by proteomics. Quantitative PCR and western blot analyses revealed the overexpression of IL-23R in NSCLC cell strains and tumor tissues compared to lung immortalized cell line or normal paired tissues. In addition, IHC sublocalized IL-23R in the cell cytoplasm of tumor cells, but not on the membrane like its WT immune cells, which implied that sIL-23R was a truncated form with serum soluble properties. As a comparison, IL-23R can be hardly observed in tumor stromal or in pairwise nontumor tissue. We hypothesized that the elevated level of sIL-23R in serum originated from the overexpression of NSCLC cells.

A home-made double-Ab sandwich ELISA kit revealed the correlation between sIL-23R level and patients tumor lymph node metastasis from 195 NSCLC patients for the first time. An indirect ELISA validated the binding of the soluble cytokine receptor, sIL-23R, and its WT natural ligand, IL-23, in the serum of NSCLC in vitro. Moreover, the role of sIL-23R as a tumor lymph node metastasis promoter might be neutralized by binding IL-23. Vice versa, binding with sIL-23R produced IL-23 effects on NSCLC proliferation, consistent with Li et al's study,[13] namely, with the level of IL-23 increasing, the proliferation of LC cells was promoted, whereas too high concentration of IL-23 inhibited proliferation of LC cells instead. We hypothesized that cancer cells might overexpress IL-23R and secrete sIL-23R to promote tumor lymph node metastasis and neutralize extra IL-23 to keep it at low and proliferation-promoting levels.

The correlation between high levels of sIL-23R with poor prognosis of NSCLC patients indicated the potential of sIL-23R as a new serum tumor marker. Increasing levels of IL-23 and IL-17 were also associated with poor prognosis of NSCLC patients, which were similar to the results of other studies.[17,18] Moreover, we found that too low or too high sIL-23R/IL-23 ratios suggested a poor prognosis of NSCLC patients. The Cox regression analysis found that sIL-23R/IL-23, but not sIL-23R, IL-23, or IL-17, could be an independent prognostic factor. Taking together, we hypothesized that sIL-23R and IL-23 were both tumor markers with a role in promoting tumor lymph node metastasis and tumor proliferation, respectively; but the binding made the 2 antagonists neutralize the cancer-promoting effect.

In the aspect of tumor-associated immunity, the binding of sIL-23R with IL-23 might also be immunosuppressive on maturation of Th17 cells, as the level of downstream IL-17, the specific cytokine of Th17 cells, was lower when the level of sIL-23R was similar to IL-23. In addition, sIL-23R could have a function of stimulating IL-17 independently, but needs further experimental verification. The correlations between sIL-23R and the IL-23/IL-17/Th17 axis could reveal sIL-23R involvement in tumor-associated immunity. Moreover, the prognostic role of the sIL-23R-IL-23 complex might rely on Th17 cells, but not on N or T stage promotion, as shown in Figure S2. T-helper 17 cells have complex biological functions and their contribution to NSCLC is controversial.[15] In our study, the Th17-related cytokines were all associated with poor prognosis of LC patients. Therefore, we hypothesized the promoting role of Th17 in NSCLC.

The main problem with this study is the lack of mechanisms for sIL-23R to improve lung cancer lymph node metastasis and tumorigenesis. We need further exploration of the sIL-23R/IL-23 complex. This study was also limited in its small sample size, single-center design, and retrospective nature.

In conclusion, we found sIL-23R promotes lymph node metastasis and affects prognosis in NSCLC patients. The sIL-23R binds its natural ligand IL-23 and the balance of sIL-23R and IL-23 in circulation can be a new prognostic marker for patients with NSCLC.

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DISCLOSURE

The authors declare no conflicts of interest.

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REFERENCES

1. Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015. *Cancer J Clin*. 2016;66(2):115-132.
2. Rao JS, Gondi C, Chetty C, Chittivelu S, Joseph PA, Lakka SS. Inhibition of invasion, angiogenesis, tumor growth, and metastasis by adenovirus-mediated transfer of antisense uPAR and MMP-9 in non-small cell lung cancer cells. *Mol Cancer Ther*. 2005;4(9):1399-1408.
3. O'Callaghan DS, O'Donnell D, O'Connell F, O'Byrne KJ. The role of inflammation in the pathogenesis of non-small cell lung cancer. *J Thorac Oncol*. 2010;5(12):2024-2036.
4. Howlader NNAKM. *SEER Cancer Statistics Review, 1975–2013, Based on November 2015 SEER Data Submission, Posted to the SEER Web Site, April 2016*. Bethesda, MD: National Cancer Institute; 2016.
5. Parham C, Chirica M, Timans J, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. *J Immunol*. 2002;168(11):5699-5708.
6. Langrish CL, McKenzie BS, Wilson NJ, de Waal Malefyt R, Kastelein RA, Cua DJ. IL-12 and IL- 23: master regulators of innate and adaptive immunity. *Immunol Rev*. 2004;202(1):96-105.
7. Zheng Y, Danilenko DM, Valdez P, et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature*. 2007;445(7128):648-651.
8. Lee E, Trepicchio WL, Oestreicher JL, et al. Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. *J Exp Med*. 2004;199(1):125-130.
9. Kan SH, Mancini G, Gallagher G. Identification and characterization of multiple splice forms of the human interleukin-23 receptor alpha chain in mitogen-activated leukocytes. *Genes Immun*. 2008;9(7):631-639.
10. Yu RY, Brazaitis J, Gallagher G. The human IL-23 receptor rs11209026 A allele promotes the expression of a soluble IL-23R-encoding mRNA species. *J Immunol*. 2015;194(3):1062-1068.
11. Yu RY, Gallagher G. A naturally occurring, soluble antagonist of human IL-23 inhibits the development and in vitro function of human Th17 cells. *J Immunol*. 2010;185(12):7302-7308.
12. Baird AM, Leonard J, Naicker KM, Kilmartin L, O'Byrne KJ, Gray SG. IL-23 is pro-proliferative, epigenetically regulated and

modulated by chemotherapy in non-small cell lung cancer. *Lung Cancer*. 2013;79(1):83-90.
13. Li J, Zhang L, Zhang J, et al. Interleukin 23 regulates proliferation of lung cancer cells in a concentration-dependent way in association with the interleukin-23 receptor. *Carcinogenesis*. 2013;34(3):658-666.
14. Zheng J, Jiang L, Zhang L, et al. Functional genetic variations in the IL-23 receptor gene are associated with risk of breast, lung and nasopharyngeal cancer in Chinese populations. *Carcinogenesis*. 2012;33(12):2409-2416.
15. Marshall EA, Ng KW, Kung SH, et al. Emerging roles of T helper 17 and regulatory T cells in lung cancer progression and metastasis. *Mol Cancer*. 2016;15(1):67.
16. Akitsu A, Iwakura Y. Interleukin-17-producing gammadelta T (gammadelta17) cells in inflammatory diseases. *Immunology*. 2018;155(4):418-426.
17. Song L, Ma S, Chen L, Miao L, Tao M, Liu H. Long-term prognostic significance of interleukin-17-producing T cells in patients with non-small cell lung cancer. *Cancer Sci*. 2019;110(7):2100-2109.
18. Cam C, Karagoz B, Muftuoglu T, et al. The inflammatory cytokine interleukin-23 is elevated in lung cancer, particularly small cell type. *Contemp Oncol (Pozn)*. 2016;20(3):215-219.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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