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Ras-MAPK pathway in patients with lupus nephritis

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ABSTRACT

Background Pathogenic mutations in genes encoding components of the Ras/mitogen-activated protein kinase (Ras-MAPK) pathway cause RASopathy. Here, we describe five unrelated patients with SLE carrying mutations associated with RASopathy and investigate the activity of the Ras-MAPK pathway.

Methods Pathogenic variants were identified by whole-exome/whole-genome sequencing. The activity of the Ras-MAPK pathway in peripheral blood mononuclear cells (PBMC) and kidneys was evaluated using RNA sequencing and datasets from the nephroseq database, respectively.

Results Five (likely) pathogenic variants in four Ras-MAPK genes were identified, including NRAS: c.G38A: p.G13D; ARAF: c.C1435T: p.R479C; KRAS: c.T341C: p.V114A; PTPN11: c.G455A: p.R152H and NRAS: c.G34A: p.G12S. Kidney injury is the main feature, presenting with nephrotic syndrome (2/5), proteinuria and haematuria (2/5). Acute kidney injury and rapidly progressive nephritic syndrome were noted in one patient each. Other clinical features included mucocutaneous lesions (5/5), cardiac involvement (4/5) and arthralgia (3/5). Laboratory abnormalities included hypocomplementaemia (5/5), presence of antiphospholipid antibodies (4/5), decreased regulatory T cells (3/3), pancytopenia (3/5) and persistent monocytosis (2/5). Kidney biopsy revealed lupus nephritis. Most patients responded well to standard therapy, with the exception of the patient with the NRAS p.G13D mutation who died. The Ras-MAPK pathway was activated in both PBMC and kidney of patients with LN as indicated by increased expression of NRAS, KRAS, RIT1, MRAS, PPP1CB, SHOC2, SOS2 and MAP2K1, as well as decreased expression of negative regulators of the Ras-MAPK pathway, CBL, LZTR1 and NF1.

Conclusion Kidney involvement may be the main feature of the clinical spectrum of RASopathy. Genetic screening should be considered for patients with early onset lupus.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Genetic variant studies provide new insights into immune mechanisms in SLE pathogenesis and offer new potential therapeutic targets.
⇒ Pathogenic mutations in genes encoding components of the Ras/mitogen-activated protein kinase (Ras-MAPK) pathway show a close association with autoimmune diseases.

WHAT THIS STUDY ADDS

⇒ Pathogenic mutations in genes encoding components of the Ras-MAPK pathway are important causes of lupus.
⇒ The Ras-MAPK pathway in both peripheral blood mononuclear cell and kidney is significantly activated in patients with lupus nephritis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study suggested a critical role of the Ras-MAPK pathway in the pathogenesis of SLE and provided new potential targets for precise therapy of both monogenic and more common, multifactorial disorders with lupus; however, the molecular mechanisms of the Ras-MAPK pathway in the development of SLE need to be studied in the future.

INTRODUCTION

SLE is caused by the inability of the immune system to distinguish self from non-self arising from loss of immune tolerance, which leads to aberrant autoimmunity and inflammation. In 35%–55% of patients, abnormal inflammation in the glomeruli can occur, which correlates with the development of lupus nephritis (LN). As with other diseases, causes of LN are multifactorial, with numerous environmental and genetic factors involved.[1] Most SLE cases are polygenic in nature. Over the past few decades, genome-wide association studies (GWAS) have identified hundreds of risk variants, and post-GWAS analyses, such as statistical fine mapping, expression quantitative trait loci and combined analyses with three-dimensional genome data, have helped explain the function of several risk variants.[2 3] These studies significantly improve our understanding of SLE and LN pathogenesis.

By contrast, the rise of high-throughput technologies such as whole-genome sequencing (WGS) and whole-exome sequencing (WES) has allowed the discovery of novel and rare or ultra-rare variants with strong effects in SLE/LN, which are identified in ~3%–10% of patients with early onset

SLE/LN.[4–6] Broadly, these alterations affect the complement system, extracellular and intracellular nucleic acid sensing and processing, type I interferon signalling and metabolic pathways.

Weiner *et al* found that common and rare variants implicate similar gene/pathway enrichment and genetic correlations for the same traits by systematically comparing the architecture of common and rare variants across 22 common traits and diseases in 394 783 UK Biobank exomes.[7] This means that the pathogenic gene/pathway identified in monogenic lupus may play critical roles in polygenic lupus and vice versa. In fact, previous studies have suggested the existence of this phenomenon. For example, TNFAIP3 has been identified as a risk gene for SLE by GWAS since 2008.[8 9] Recently, rare variants in TNFAIP3 were identified in both early onset and adult-onset SLE and LN.[10]

The Ras/mitogen-activated protein kinase (Ras-MAPK) pathway is a signal transduction cascade that is central to normal cellular processes such as proliferation, survival, differentiation and metabolism. Mutations in genes encoding components of the Ras-MAPK pathway are responsible for a class of disorders known as RASopathies, including Noonan syndrome (NS, OMIM: 163950), Costello syndrome (OMIM: 218040), cardiofaciocutaneous syndrome (OMIM: 115150), NS with multiple lentigines (OMIM: 151100) and NS with loose anagen hair (OMIM: 607721). Signalling upregulation occurring in RASopathies results from enhanced activity of Ras proteins (ie, KRAS, NRAS, MRAS, RRAS, RRAS2 and RIT1), upstream positive signal transducers and regulators (ie, SHP2, SOS1 and SOS2), proteins favouring transmission of Ras signalling to downstream transducers (ie, SHOC2 and PPP1CB) and tiers of the MAPK cascade (ie, BRAF, RAF1, MAP2K1 and MAPK1). Signalling upregulation also results from inefficient signalling switch-off operated by multiple feedback mechanisms (ie, defective function of CBL, NF1, LZTR1 and SPRED1).

Interestingly, an association of RASopathies and autoimmune diseases, such as thyroiditis, vasculitis and SLE has been reported, although it is rare. For example, the PTPN11 gene encodes the protein tyrosine phosphatase SHP2, which positively regulates the Ras-MAPK signal transduction pathway. Mutations in PTPN11 at the 12q24 locus cause NS, which is also concordant with linkage study reports of SLE in Hispanic and European American families.[11–13] Additionally, the p.F285S mutation in PTPN11 has been reported in a patient with both NS and SLE.[14] Moreover, we recently performed WES in patients with LN and identified several pathogenic genes encoding key proteins of the Ras-MAPK pathway, such as NRAS, KRAS, ARAF and PTPN11.[4] These studies suggest a close relationship between the Ras-MAPK pathway and the pathogenesis of LN. However, to the best of our knowledge, the systematic analysis of gene expression in the Ras-MAPK pathway in patients with LN has not been performed.

Therefore, in this study, we first report the characteristics of five patients with pathogenic mutations in genes encoding key components of the Ras-MAPK pathway. We further determine the expression pattern of genes in the Ras-MAPK pathway in both PBMCs and kidney tissues using RNA sequencing.

METHODS

Patients

All patients who met the diagnostic criteria for SLE and LN were evaluated at Jinling Hospital. A total of 2236 patients with unrelated LN and 287 family members (parents, siblings or offspring) underwent WES or WGS.

Whole-exome sequencing, whole-genome sequencing and sanger sequencing

One microgram of peripheral blood DNA was used for WES or WGS (BGI, Shenzhen, PR China). Data alignment was performed using BWA, and variants were annotated using Annovar. Variants were filtered using online databases, including gnomAD (https://gnomad.broadinstitute.org/), Kaviar (https://db.Systemsbiology.net/kaviar/) and dbSNP (https://www.ncbi.nlm.nih.gov/snp/), as well as an in-house database. Candidate pathogenic variants were further selected based on inheritance patterns. Finally, candidate variants were confirmed by Sanger sequencing.

RNA extraction and RNA sequencing

Total RNA was extracted from 2×10^6 PBMCs using the RNeasy Mini Kit (74104; Qiagen, Hilden, Germany). One microgram of RNA was used for library preparation. The libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) and sequenced on an Illumina NovaSeq (San Diego, California, USA). A set of 150 bp paired-end reads was generated and mapped to the human reference genome (GRCh38) using HISAT2 V.2.2.1. featureCounts was used to count the number of reads mapped to each gene. Differential expression analysis was performed using the edgeR package for R.

Statistical analyses

Data are expressed as mean±SD, and statistical evaluation was performed using a two-tailed t-test with GraphPad Prism V.8.0.2 (GraphPad Software, La Jolla, California, USA). A p value <0.05 was considered statistically significant.

RESULTS

Identification of pathogenic mutations in Ras-MAPK pathway genes in patients with lupus nephritis

We identified five pathogenic mutations in four Ras-MAPK genes in five patients, including NRAS: NM_002524: exon 2: c.G38A: p.G13D (P1); ARAF: NM_001654: exon 14: c.C1435T: p.R479C (P2); KRAS: NM_004985: exon 4: c.T341C: p.V114A (P3); PTPN11: NM_001330437: exon

4: c.G455A: p.R152H (P4); NRAS: NM_002524: exon 2: c.G34A: p.G12S (P5, figure 1A). Three mutations (NRAS p.G13D; NRAS p.G12S; PTPN11 p.R152H) have been identified as pathogenic variants in individuals with NS and/or lupus. The R479 site in ARAF and the V114 site in KRAS were highly conserved across species (figure 1B). The five mutations were absent or ultra-rare in the ChinaMap and gnomAD databases. They were predicted to be deleterious by multiple predictive models, with a combined annotation-dependent depletion score >20, a genomic evolutionary rate profiling score >4 and a pathogenicity score of Alphamissense ranging from 0.58 to 0.999 (figure 1C). These variants were classified as pathogenic or likely pathogenic according to the American College of Medical Genetics and Genomics criteria (figure 1C). Genetic screening of family members showed the healthy sister of P4 carried the same PTPN11 mutation, while her father and older sister did not. P5 carried a de novo NRAS mutation as it was not present in her parents.

Clinical features of the patients

Of the five patients, four were female. The ages of disease onset were 7, 15, 42, 39 and 14 years, respectively. All patients had kidney and mucocutaneous involvement (5/5, 100%). Kidney involvement manifested as nephrotic syndrome (P1, P2), moderate to nephrotic-range proteinuria accompanied by haematuria (P3, P4), or rapidly progressive nephritic syndrome and hypertension (P5). In addition, P1 and P4 developed acute kidney injury. Mucocutaneous involvement presented with erythema (P1, P2), vitiligo (P3), rash (P3, P4), light sensitivity (P3, P5), oral ulcers and alopecia (P3). Notably, cardiac manifestations were frequent (4/5, 80%), including mild mitral regurgitation (P1 and P2), decreased ejection fraction (P2), myocardial hypertrophy and pulmonary hypertension (P4) and enlargement of the left atrium and left ventricle (P5, figure 2A, a). Three patients (3/5, 60%) showed haematological manifestations, including pancytopenia (P3, P4, P5), neutropenia (P4) and Evans syndrome (P5). Neuropsychiatric symptoms were noted in two patients (2/5, 40%, P2, P3), such as seizures, mitral regurgitation, sleep disturbances and headaches. Splenomegaly and/or lymphadenopathy were observed in two patients (2/5, 40%, P3, P5) (figure 2A, b–c). Nausea, vomiting, abdominal bloating and distension were observed in one patient (P3). An overview of clinical symptoms occurring during the disease course is presented in tables 1 and 2.

Multiple autoantibodies of the patients

All patients had positive ANAs and specific SLE autoantibodies, including anti-double-stranded DNA in P1, P2 and P5, and anti-nRNP/Sm in P3 and P4. Antiphospholipid antibody was identified in four patients, including anticardiolipin in P1, P2 and P3, anti-β2 glycoprotein 1 antibodies in P3 and P5 and lupus anticoagulant in P2 and P3. However, no evidence of thrombotic or pathological pregnancy event was noted. In addition, organ-specific antibodies were observed. Two patients (P3, P4) showed positive antithyroid peroxidase, antithyroid-stimulating hormone receptor or antithyroglobulin antibodies, but they presented no evidence of hypothyroidism. One patient (P5) presented with a positive Coombs test, haemolytic anaemia, thrombocytopenia and splenomegaly, fulfilling the criteria for the diagnosis of Evans syndrome.

Immunological features of the patients

Complement analysis identified a significantly decreased level of complement C3 in all patients. Hyperimmunoglobulinaemia was identified in four patients (hyper IgG and IgA in P4; hyper IgM in P2; hyper IgE in P3, P5). Rheumatoid factor (RF) and Anti-Streptolysin O (ASO) levels were normal. Lymphocyte subset analysis showed significantly decreased CD4+ T cells and regulatory T (Treg) cells in all available patients. Increased B cells and persistent monocytosis were identified in the two patients with NRAS mutations (P1 and P5, figure 2B).

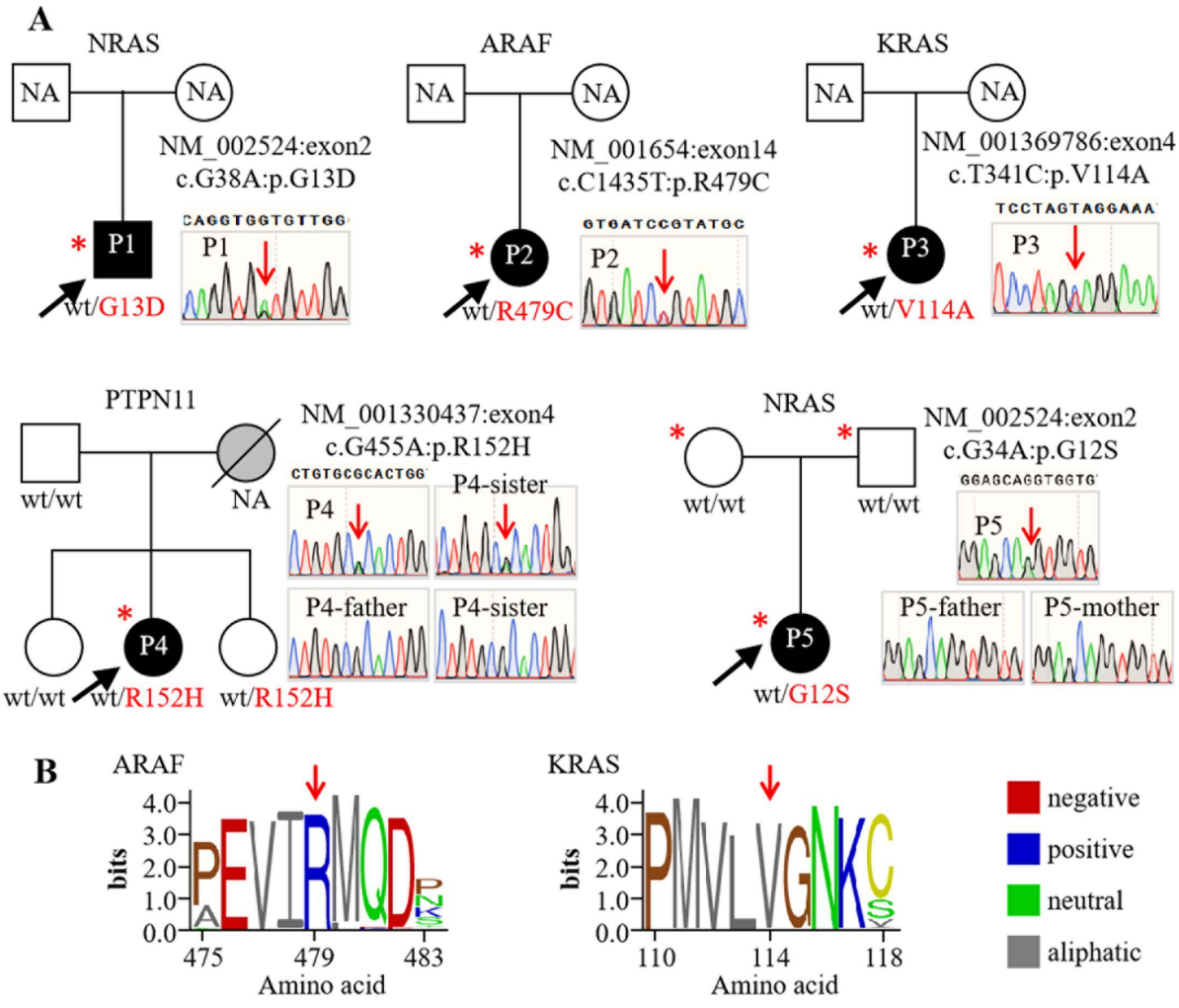
Kidney histopathological findings of the patients

Kidney biopsy was performed in four patients. According to the International Society of Nephrology/Renal Pathology Society classification of LN, the histopathological findings were categorised as LN-V in P1 and P3, LN-IV+V in P2 and LN-III+V in P4 (figure 2C and table 3).

P1 and P3 showed sclerotic glomeruli with massive epithelial erythrophilic deposits. No endocapillary hypercellularity, adhesion or fibrinoid necrosis was seen. Immunofluorescence (IF) staining revealed diffuse and granular deposition of IgG++, IgA+, C3++ and C1q+−++ along the capillary walls. Electron microscopy (EM) showed subepithelial electron-dense deposits and diffuse podocyte foot process effacement (figure 2C, a, e, i, c, g and k). In addition, interstitial inflammation, IgM deposits and a large number of lysosomes were observed in P1.

P2 showed globally sclerotic glomeruli and diffuse endocapillary and mesangial hypercellularity. ‘Platinum fungus’ was observed. Periodic acid-silver methenamine (PASM)-Masson staining showed mesangial, subendothelial and subepithelial erythrophilic deposits. IF staining revealed diffuse and granular deposition of IgG++, IgA++, IgM++, C3++ and C1q++ along the capillary walls, without extraglomerular deposits. Mesangial, subendothelial and subepithelial electron-dense deposits and focal podocyte foot process effacement were seen by EM (figure 2C, b, f and j).

P4 showed globally sclerotic glomeruli, endocapillary and mesangial hypercellularity, fibrocellular crescents and massive mesangial and subepithelial erythrophilic deposits. Karyorrhexis, interstitial inflammation, tubular atrophy and interstitial fibrosis were observed. IF staining revealed diffuse and granular deposition of IgG++, IgA+, IgM+, C3++ and C1q++ in the mesangium and along the capillary walls. Mesangial and subepithelial electron-dense



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Protein change	CADD score	Alphamissense score and class	GERP score	ACMG criteria and pathogenicity interpretation
NRAS p.G13D	25.1	0.999 likely_pathogenic	5.58	PS3 PM1 PM2 PP2 PP5 Pathogenic
ARAF p.R479C	35	0.866 likely_pathogenic	4.38	PM1 PM2 PP2 PP3 Likely pathogenic
KRAS p.V114A	26.8	0.855 likely_pathogenic	5.52	PM1 PM2 PP2 PP3 Likely pathogenic
PTPN11 p.R152H	24.3	0.58 likely_pathogenic	5.55	PM1 PM2 PP3 PP5 Likely pathogenic
NRAS p.G12S	24.9	0.992 likely_pathogenic	5.58	PS2 PM1 PM2 PP3 PP5 Pathogenic

Figure 1 Identification of five mutations in Ras-MAPK pathway genes in patients with lupus nephritis (LN). (A) Pedigrees of the five unrelated families and confirmation of the five mutations in Ras-MAPK pathway genes by Sanger sequencing. The proband is indicated by an arrow. Black shading indicates patients with LN. (B) Evolutionary conservation of the R479 site in ARAF and the V114 site in KRAS across various species. (C) The identified five mutations were predicted to be deleterious by multiple predictive models including CADD score, GERP score and Alphamissense score. They were interpreted as pathogenic or likely pathogenic according to the ACMG criteria. ACMG, The American College of Medical Genetics and Genomics; CADD, combined annotation-dependent depletion; GERP, genomic evolutionary rate profiling; NA, not available. *individuals who have undergone whole-exome or whole-genome sequencing.

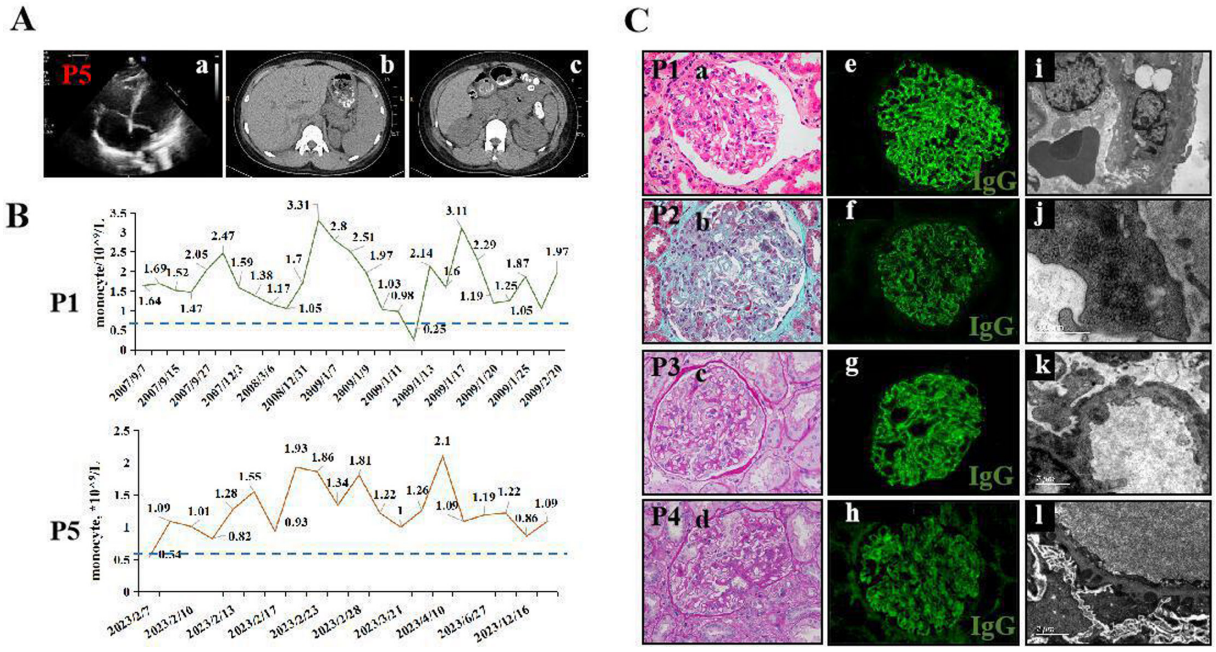

Figure 2 Clinical and pathological features of the five patients. (A) Clinical features of P5 with NRAS G12S mutation. (a) Enlarged left atrium and left ventricular diameter. (b) Splenomegaly. (c) Multiple small lymph nodes around the abdominal aorta. (B) Persistent monocytosis of the two patients with NRAS mutations (P1 and P5). Blue dotted lines indicate the upper range of monocyte counts. (C) Kidney histopathological lesions of patients P1–P4. Light microscopy: (a) swelling and proliferation of podocytes and infiltration of inflammatory cells in the glomerulus (H&E, ×400); (b) the enlarged glomerulus with mesangial and endocapillary hypercellularity and erythrophilic deposits in the subepithelial, subendothelial and mesangial regions (Masson, ×400). (c) Stiff capillary loops, paired endothelial cells and segmentally widened mesangium in the glomerulus (PAS, ×400). (d) Mesangial and endocapillary hypercellularity, widened mesangium and neutrophils in the glomerulus. It also demonstrates tubular atrophy and thickened tubular basal membrane. (PAS, ×400). Immunofluorescence: (e and g) the granular IgG deposits along the capillary wall (IF, ×400). (f and h) The granular IgG deposits in the mesangium and along the capillary wall (IF, ×400). Electron microscopy: (i, k and l) abundant electron-dense deposits in the subepithelial area and diffuse podocyte foot process effacement. (j) Tubuloreticular inclusions in the glomerular endothelial cells. PAS, periodic acid-Schiff.

deposits and diffuse podocyte foot process effacement were observed by EM (figure 2C, d, h and l).

Treatment and outcomes

All patients received methylprednisolone pulse therapy and prednisone in combination with various immunosuppressive therapies. P1 received a combined therapy with prednisone and cyclophosphamide (CTX) or tripterygium wilfordii (TW) prior to his visit to our centre. After kidney biopsy at our centre, methylprednisolone pulse therapy and prednisone with mycophenolate mofetil (MMF) and tacrolimus were successively prescribed. His proteinuria did not improve, and he developed acute kidney injury and recurrent gastrointestinal infections and died during follow-up.

P2 gradually improved after methylprednisolone pulse therapy followed by prednisone in combination with CTX, azathioprine, TW and hydroxychloroquine (HCQ), followed sequentially by MMF and HCQ. Proteinuria reduced to normal; however, complement levels did not recover during follow-up.

P3 received methylprednisolone pulse therapy followed by prednisone combined with tacrolimus and her proteinuria achieved partial remission. She was then treated with prednisone combined with CTX or MMF. Her proteinuria and complement levels remained normal for 8.5 years. Prednisone was used to maintain remission.

P4 received three rounds of methylprednisolone pulse therapy, followed by prednisone combined with tacrolimus or MMF. Her proteinuria and serum creatinine returned to normal. Complement C3 levels remained low during follow-up.

P5 received methylprednisolone pulse therapy followed by rituximab infusion. In addition, prednisone was used in combination with MMF and HCQ. Proteinuria, serum creatinine, haemoglobin and thrombocyte levels gradually returned to normal. Complement levels remained low during the 1-year follow-up.

Activation of Ras-MAPK pathway in peripheral blood mononuclear cell from P5 with NRAS p.G12S mutation

We next evaluated the activity of the Ras-MAPK pathway using PBMC from P5. The results revealed notable alterations in gene expression patterns within this pathway. Specifically, we observed a significant upregulation in the expression levels of NRAS, RIT1, PPP1CB, BRAF and SHOC2 in P5, when compared with those from healthy controls (figure 3A). Notably, the expression levels of the five genes exceeded those observed in each individual healthy control. Furthermore, the fold-change

Table 1 Clinical characteristics of the five patients

Patients	P1	P2	P3	P4	P5
Gender	Male	Female	Female	Female	Female
Age of disease onset	7	15	42	39	14
SLE family history	N	N	N	N	N
Fever	Y	Y	Y	N	N
Haematological	N	N	Leucopenia, thrombocytopenia, anaemia	Anaemia, leucopenia, thrombocytopenia, neutropenia	AIAH, immune thrombocytopenia, Evans syndrome, leucopenia
Neuropsychiatric	N	Seizure, mitral regurgitation, sleep disturbances	Headache, cranial MRI showing small ischaemic foci in both frontal lobes and left ventricular anterior horn	N	N
Mucocutaneous	Facial erythema	Erythema on the extremities and face, map tongue	Light sensitivity, vitiligo, rash on the trunk, oral ulcers, alopecia	Papular rash on the hands	Light sensitivity, dry mouth, dry eyes
Serosal	Pericardial effusion	N	Bilateral pleural effusion, pericardial effusion	Pericardial effusion	Pericardial effusion
Musculoskeletal	Arthralgia	N	Arthralgia, bilateral shoulder and elbow pain	N	Arthralgia
Cardiovascular	Mild mitral regurgitation	Mild mitral regurgitation, decreased left ventricular ejection fraction	N	Pulmonary hypertension, myocardial hypertrophy	Enlargement of the left atrium and ventricle.
Gastrointestinal	N	N	Nausea, vomiting, abdominal bloating and distension	N	N
Hepatosplenomegaly, lymphadenopathy	N	N	Multiple lymphadenopathy in bilateral axillae and mediastinum	N	Splenomegaly, multiple small lymph nodes around the abdominal aorta
Renal manifestation	Nephrotic syndrome	Nephrotic syndrome	Nephrotic-range proteinuria, haematuria	Moderate proteinuria, haematuria, acute kidney injury	Rapidly progressive nephritic syndrome, elevated serum creatinine, increased blood pressure

AIAH, autoimmune hemolytic anaemia; N, no; Y, yes.

in expression of these genes ranged from 2.5 to 6.0, highlighting a considerable increase in their transcript abundance in P5. Conversely, the expression of CBL and LZTR1, two negative regulators of the Ras-MAPK pathway, was found to be decreased in P5 (figure 3B). The expression level of LZTR1 was lower than those observed in each individual healthy control. These findings collectively indicate the activation of the Ras-MAPK pathway in the patient harbouring the NRAS p.G12S mutation, thereby providing insights into the potential molecular mechanisms underlying the observed phenotypic characteristics in this patient.

Activation of Ras-MAPK pathway in PBMC from patients with LN

Since common and rare variants display similar gene/pathway enrichment, we next evaluated the expression of NRAS, KRAS, ARAF and PTPN11 in PBMC from patients with LN (n=22). We found that the expression of NRAS and KRAS was significantly elevated in patients with LN

Table 2 Overview of clinical and laboratory characteristics in the patients

Features	Patients, n (%)
Recurrent fever	3 (60%)
Haematological	3 (60%)
Anaemia	3 (60%)
Thrombocytopenia	3 (60%)
Leucopenia	3 (60%)
Neutropenia	1 (20%)
Neuropsychiatric	2 (40%)
Mucocutaneous	5 (100%)
Serosal	4 (80%)
Pericardial effusion	4 (80%)
Pleural effusion	1 (20%)
Musculoskeletal	3 (60%)
Cardiovascular	4 (80%)
Gastrointestinal	1 (20%)
Hepatosplenomegaly, lymphadenopathy	2 (40%)
Kidney manifestation	5 (100%)
Acute kidney injury	1 (20%)
Rapidly progressive glomerulonephritis	1 (20%)
Nephrotic syndrome	2 (40%)
Autoantibodies positive	5 (100%)
Hypocomplementaemia	5 (100%)
Antiphospholipid antibody positive	4 (80%)
Decreased CD4+ T cells	5 (100%)
Decreased regulatory T cells	3/3
Monocytosis	2 (40%)

compared with healthy controls (p=0.0388 and 0.0266, respectively), while the expression of ARAF and PTPN11 was comparable with that observed in healthy controls (figure 3C). We further examined the expression of additional key genes in the Ras-MAPK pathway. Notably, we found that the expression of PPP1CB, RIT1, MRAS, SHOC2 and SOS2 was also increased in patients with LN. The fold-change in the expression of these genes ranged from 1.3 to 2, suggesting a moderate but consistent upregulation. Additionally, we observed a significant decrease in the expression of CBL and LZTR1 in patients with LN compared with healthy controls (figure 3D). Collectively, these findings indicated the activation of the Ras-MAPK pathway in PBMCs of patients with LN, highlighting potential alterations in signalling pathways that may contribute to the pathogenesis of this condition.

Activation of Ras-MAPK pathway in kidney of patients with LN

We finally investigated the expression patterns of key genes in the Ras-MAPK pathway in kidney tissues by using gene expression datasets obtained from the nephroseq database. We found that the expression of NRAS, KRAS, MRAS, MAP2K1 and RIT1 was significantly upregulated in kidney tissues derived from patients with LN compared with those from healthy living donors across two distinct datasets (figure 4A-D), while the expression levels of ARAF and PTPN11 were comparable between the two groups (figure 4A-B). Furthermore, we observed a significant downregulation of NF1 and LZTR1, two negative regulators of the Ras-MAPK pathway, in kidney tissues of patients with LN compared with healthy controls (p=0.00002 and 0.013, respectively; figure 4C-D).

To gain additional insights into the clinical relevance of these alterations, we performed correlation analysis, which showed that the expression of NRAS, KRAS and PTPN11 in kidney tissues of patients with LN was negatively correlated with estimated glomerular filtration rate levels (figure 4E). These results indicated the activation of the Ras-MAPK pathway in kidney tissue of patients with LN.

DISCUSSION

In the current study, we found that mucocutaneous, cardiac manifestations and reduction of Treg cells are dominant features of patients harbouring mutations in genes encoding components of the Ras-MAPK pathway. Furthermore, we identified that the Ras-MAPK pathway is activated in both PBMC and kidney tissues from patients with LN. The findings illustrate a close association of Ras-MAPK pathway activity with SLE pathology.

Gene mutations involved in the Ras-MAPK pathway cause a disorder named RASopathy, which shares common traits, such as craniofacial dysmorphisms, short stature, cardiac malformations, variable cognitive delay and an increased risk of cancer development. There were no obvious craniofacial abnormalities and short stature in our cohort, but the patients presented with varying degrees of cardiac involvement, including hypertrophic cardiomyopathy, regurgitation, reduced ejection fraction and cardiac dilatation. Patients with KRAS and NRAS mutations had manifestations of lymphoproliferative diseases, which is consistent with previous studies.[15 16] Notably, it was reported that 52% of patients with RASopathy presented with at least one autoantibody; 14% of the patients fulfilled the criteria of autoimmune disorders, such as autoimmune thyroiditis, SLE, coeliac disease, antiphospholipid syndrome, vitiligo and autoimmune hepatitis.[14] In our cohort, there were prominent autoimmune manifestations, such as the presence of SLE, antiphospholipid antibodies, autoimmune thyroid disease-associated antibodies, Evans syndrome and vitiligo. These autoimmune manifestations in our cohort have been previously described in RASopathy, except for Evans syndrome. Evans syndrome has both immune thrombocytopenia and autoimmune haemolytic anaemia. Sixty-five per cent of paediatric Evans syndrome were found to have an underlying immune-related monogenic disorder.[17] This study provides the first association of Evans syndrome with NRAS gene mutation, which is likely very rare. These findings warranted the importance of genetic testing in

Table 3 Kidney biopsy findings of the patients

	P1	P2	P3	P4
Light microscopy				
No. glomeruli	31	30	28	40
Sclerotic glomeruli (score)	1	1	1	1
Cellular/Fibrocellular crescents (score)	0	0	0	2
Endocapillary hypercellularity (score)	0	3	0	1
Platinum fungus (score)	0	2	0	0
Neutrophils/Karyorrhexis (score)	0	0	0	1
Fibrinoid necrosis (score)	0	0	0	0
Interstitial inflammation (score)	1	0	0	2
Tubular atrophy (score)	0	0	0	2
Interstitial fibrosis (score)	0	0	0	2
Thrombotic microangiopathy	0	0	0	0
Collapsing glomerulopathy	0	0	0	0
Immunofluorescence				
IgG	2+	2+	2+	2+
IgA	1+	2+	1+	1+
IgM	1+	2+	–	1+
C3	2+	2+	2+	2+
C1q	1+	2+	2+	2+
Electron microscopy				
Electron-dense deposits	Subepithelial	Mesangial, subendothelial, subepithelial	Subepithelial	Mesangial, subepithelial
Podocyte foot process effacement	Diffuse	Focal	Diffuse	Diffuse
Tubuloreticular inclusions	–	+	+	+
Renal biopsy diagnosis	LN-V	LN-IV+V	LN-V	LN-III+V
AI	1	5	0	6
CI	1	1	1	5

AI, activity index; CI, chronicity index; LN, lupus nephritis.

patients with LN with cardiac involvement, lymphoproliferative disorders and Evans syndrome.

Heterozygous variants in genes within the Ras-MAPK pathway are the common ways of pathogenesis, such as NRAS, ARAF, KRAS, PTPN11, SOS1, SHOC2, NF1 and RIT1. In addition, somatic mutations in NRAS, KRAS and NF1 rarely occur in malignancies. NRAS p.G13D heterozygous variant has been reported in patients with juvenile myelomonocytic leukaemia (JMML), autoimmune lymphoproliferative syndrome (ALPS) and SLE. NRAS p.G12S heterozygous variant has been identified in individuals with JMML, myelodysplastic syndrome, multiple myeloma and NS. Functional studies have shown that variants at the p.G12 and p.G13 amino acid residues in NRAS cause constitutive activation of NRAS signalling.[15 18 19] PTPN11 p.R152H heterozygous variant has been reported in an individual with NS.[20] Advanced modelling of protein sequence and biophysical properties support that this missense variant has a deleterious effect on PTPN11 protein structure/function. However, no functional studies were performed. KRAS p.V114A and ARAF p.R479C variants are novel and have not been reported previously. All five variants identified in our patients are heterozygous, consistent with previous reports. This is the first report establishing a link between these variants and SLE as well as LN, thereby expanding both the genotypic and phenotypic spectrum of Ras-MAPK pathway-associated diseases. Genetic screening of family members found that one sister of P4 carried the PTPN11 mutation but without any symptoms. This may be explained by intrafamilial variability with milder phenotypes.

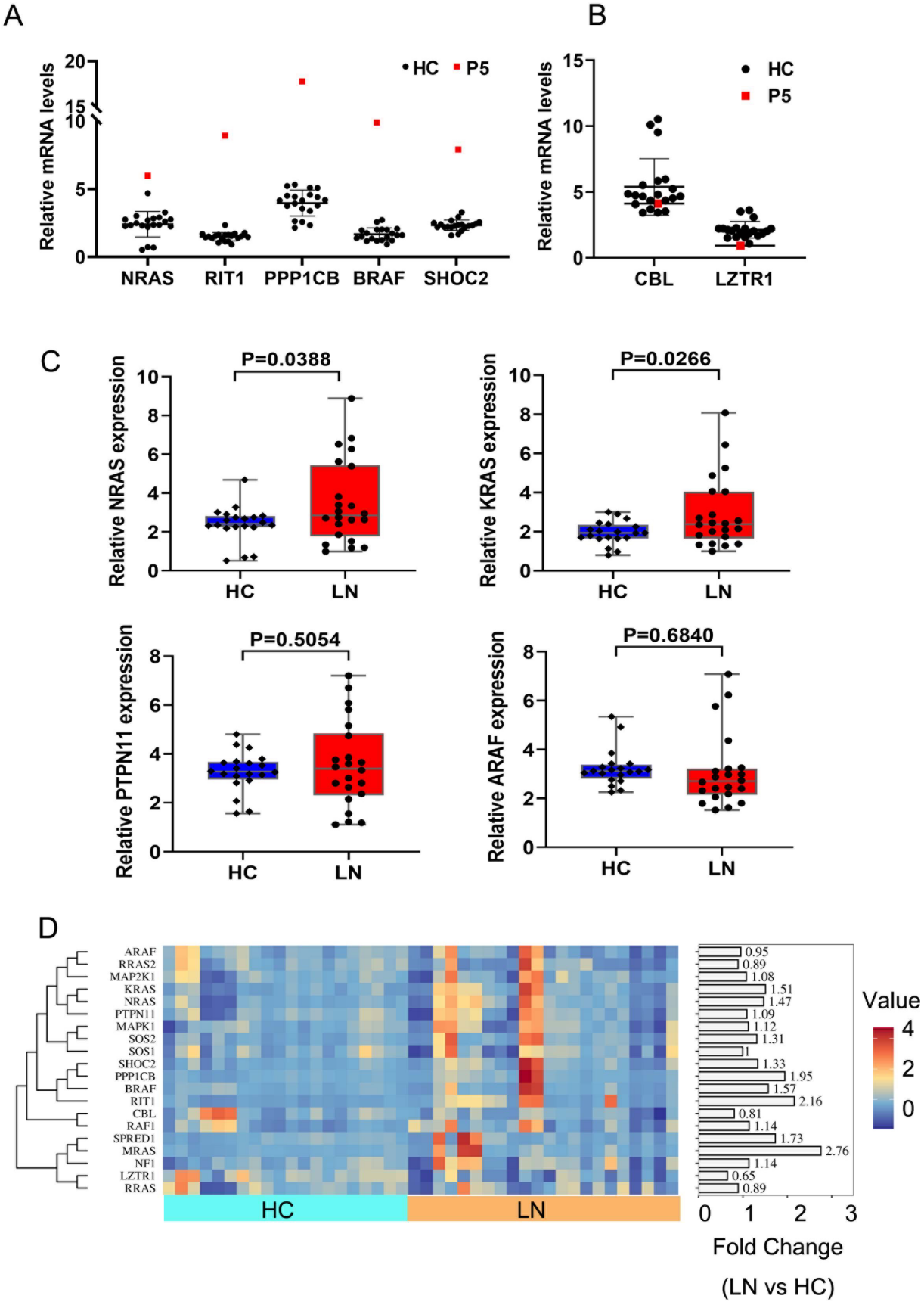

Figure 3 Activation of the Ras-MAPK pathway in PBMC from patients with LN. (A) Upregulation of NRAS, RIT1, PPP1CB, BRAF and SHOC2 in PBMC from P5. (B) Downregulation of CBL and LZTR1 in PBMC from P5. (C) Relative expression of NRAS, KRAS, PTPN11 and ARAF in PBMC from patients with LN. (D) Dysregulation of key genes in the Ras-MAPK pathway in PBMC from patients with LN. HC, healthy control; LN, lupus nephritis; PBMC, peripheral blood mononuclear cells.

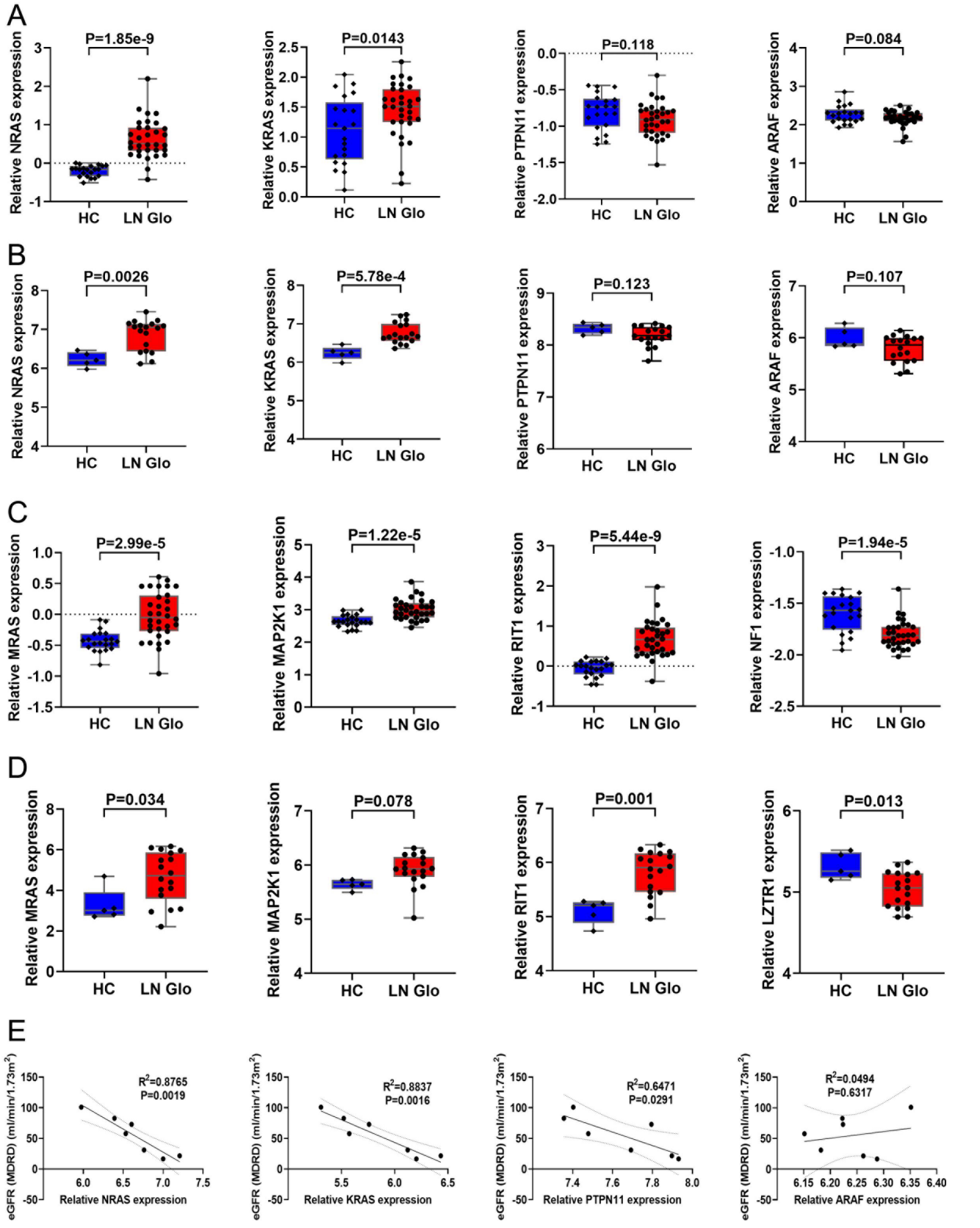

Figure 4 Activation of the Ras-MAPK pathway in kidneys of patients with LN. (A-B) Relative expression of NRAS, KRAS, ARAF and PTPN11 in the kidneys of patients with LN in datasets Ju CKD Glom (A) and ERCB Lupus Glom (B), respectively. (C-D) Differentially expressed key genes in the Ras-MAPK pathway in the kidneys of patients with LN in datasets Ju CKD Glom (C) and ERCB Lupus Glom (D), respectively. (E) Correlation analysis of relative NRAS, KRAS, PTPN11 and ARAF expression and eGFR levels using the dataset ERCB Lupus TubInt. Datasets were downloaded from the nephroseq database. eGFR, estimated glomerular filtration rate; HC, healthy control; LN, lupus nephritis.

Animal and human immunologic models have shown that the activation of the Ras-MAPK signalling pathway may lead to autoimmunity.[21 22] Autoreactive immature B cells usually reside in the bone marrow, but constitutively active mutation of RAS protein can change this pattern, alter the Ras-ERK pathway and result in breakdown of B cell self-tolerance and secretion of autoantibodies.[23] SHP-2, encoded by PTPN11, is important for the maintenance of resting lymphocytes and regulation of the transcription factor nuclear factor-kappa B, which plays a role in antibody production and activation of natural killer cells.[14] Moreover, Ras-MAPK signalling controls the expression of Foxp3 in T lymphocytes and plays a role in controlling the differentiation of Foxp3+ Treg cells and maintaining immune tolerance.[24] Defective immunosuppressive activity of Treg cells has been reported in patients with NRAS p.G13D mutation.[25] A reduction in Treg cells is an immunological hallmark of patients in our cohort, indicating that gene mutations in the Ras-MAPK pathway may cause autoimmune diseases by impairing Treg cells. These studies highlight the involvement of different genes of the Ras-MAPK pathway in autoimmunity. In our cohort, this involvement was corroborated by the presence of autoantibodies in patients harbouring mutations in NRAS, KRAS, PTPN11 and ARAF.

Previous studies indicated a role for Ras activation in kidney fibrosis. For instance, Ras activation has been described in cultured mesangial cells or kidney fibroblasts when stimulated with advanced glycation end-products or epidermal growth factor.[26 27] Ras-induced MAPK and ERK activation has been found in an experimental model of UUO-induced tubulointerstitial fibrosis.[28 29] In addition, transforming growth factor-β signalling depends on Ras-MAPK pathway inputs for the induction of kidney fibrosis.[29 30] Our study provides compelling evidence for the activation of the Ras-MAPK pathway in kidney tissues of patients with LN, implicating this pathway in the pathogenesis of kidney involvement in LN. This finding necessitates further exploration to elucidate the precise mechanisms involved.

The mechanism of Ras-MAPK pathway activation in LN is unclear. Rare mutations in genes within the Ras-MAPK pathway are an important cause, as we noted that the patient with the NRAS p.G12S mutation exhibited marked activation of the Ras-MAPK pathway. This finding underscores the importance of genetic analysis in identifying patients with Ras-MAPK pathway alterations and highlights the potential for targeted therapies in treating such conditions. Common variants may be another factor, as the single nucleotide polymorphisms associated with PTPN11 have been identified as risk variants for SLE.[31] Consistent with this, our study showed that the expression of key genes in the Ras-MAPK pathway was activated, such as RIT1 and MRAS, even in patients who did not carry a rare mutation in this pathway. Previous research has demonstrated that the overexpression of RIT1 is associated with the activation of the Ras-MAPK pathway, along with enhanced phosphorylation of p38 MAPK and AKT.[32] Additionally, YAP signalling-induced MRAS expression leads to the formation of a complex with SHOC2, which subsequently accelerates the activation of MAPK and AKT signalling in cancer cells. Conversely, MRAS knockout attenuates the phosphorylation of these kinases.[33] These observations imply that dysregulation of key genes within the Ras-MAPK pathway could represent a novel mechanism underlying the pathogenesis of LN, suggesting potential new therapeutic targets.

Our results also support a recent study that confirms both common and rare variants converge on similar genes and pathways associated with the same traits.[7] These findings suggest that both rare and common variants provide rich sources to improve our understanding of disease pathogenesis. Therapeutics targeting genes associated with rare variants have the potential to benefit a large patient population, extending beyond those who carry specific mutations.

In the era of precision medicine, the optimal therapeutic agent for these patients could potentially be a Ras-MAPK signalling inhibitor. Covalent KRAS p.G12C inhibitors, such as sotorasib (AMG510) and adagrasib (MRTX849), have been approved by the US Food and Drug Administration for patients with non-small-cell lung cancer harbouring the KRAS p.G12C-mutant.[34] Additionally, pan-RAS inhibitors, MEK inhibitors and MAPK inhibitors have been developed and show broad implications in antitumour therapeutics.[35–38] We anticipate that these therapies will be highly effective for patients with Ras-MAPK signal-mediated immune dysregulation. Moreover, regarding the impaired Treg-cell function and immune tolerance in such patients, therapeutic strategies aimed at restoring immune tolerance, such as Treg-cell infusion and Treg-promoting therapies (eg, rapamycin, interleukin (IL)-10, IL-2, tumour necrosis factor receptor 2 agonists), may prove clinically effective.[39] For example, low-dose IL-2 has been shown to be both safe and effective in SLE.[40] A phase I clinical trial (NCT02428309) using polyclonal Tregs in SLE found that the administered Tregs were able to traffic to the site of autoimmunity, leading to a dynamic shift from Th1 to Th17 responses.[41] In the future, careful consideration will be necessary to tailor an appropriate treatment regimen when implementing Treg therapy.

Our study has several limitations. First, genetic screening of some family members and functional studies of novel variants were not conducted. Second, the expression data of key genes in the Ras-MAPK pathway in kidney tissues were primarily obtained from the European-American population. Therefore, these results need to be further validated in the Chinese population in future studies.

In summary, we present the phenotypic features of five patients with lupus with pathogenic mutations in genes encoding components of the Ras-MAPK pathway. The widespread application of genetic testing technology provides new diagnostic and therapeutic options for autoimmune diseases. Monogenic diseases, such as RASopathies, may help advance our understanding of disease pathogenesis and contribute to the development

of targeted therapies for more common, multifactorial disorders with lupus.

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Contributors CZ and XJ performed the experiments, analysed the data and drafted the initial version of the manuscript. YZ, JZ and XG performed the bioinformatics analysis. CZ, XJ, YJ, JY, QZ, DL and HZ enrolled the patients and collected and interpreted clinical and pathological information. CZ and ZL designed the study. ZL reviewed and edited the manuscript. All authors approved the submitted version of the manuscript. ZL is the guarantor.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval The study was approved by the Institutional Review Boards of Jinling Hospital (2022DZKY-061-01). All of the patients and family members provided written informed consent.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information. All data associated with this study are present in the paper. Primary data of DNA-based and RNA-based assays can be accessed by contacting the corresponding authors (ZL).

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