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SHORT REPORT

High p16^{INK4a}, a marker of cellular senescence, is associated with renal injury, impairment and outcome in lupus nephritis

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ABSTRACT

Objectives Because a significant fraction of patients with lupus nephritis (LN) develops renal impairment, there is a need to better understand the mechanisms underlying disease progression. Here, we assessed for cellular senescence in the LN kidney, and its association with disease severity and outcome.

Methods We enumerated the number of cells positive for p16^{INK4a} protein, a marker of cellular senescence, by immunohistochemistry followed by digital quantification, on renal biopsies from 40 patients with active LN. We tested for an association of p16^{INK4a} with renal fibrosis, CD8$^+$ T cell infiltration, systemic disease and renal function at baseline and at 5 years.

Results The presence of p16^{INK4a}-positive cells was significantly associated with lower estimated glomerular filtration rate at baseline and 5 years post-treatment, independently of patient demographics and systemic disease parameters. It was also associated with higher baseline renal fibrosis and CD8$^+$ T cell infiltration. Interestingly, we observed marked spatial co-distribution of glomerular p16^{INK4a}-positive cells with CD8$^+$ T cells.

Conclusion We demonstrate, for the first time, that LN biopsies characterised by renal impairment display increased p16^{INK4a}-positive cells, associated with higher fibrosis and CD8$^+$ T cell infiltration. Cellular senescence may represent a kidney-intrinsic disease mechanism and potentially, a novel therapeutic target in LN.

Key messages

What is already known about this subject?

- Cellular senescence has been observed in renal ageing, certain renal diseases and in a mouse model of lupus nephritis, and was associated with renal damage.

What does this study add?

- This is the first demonstration of the occurrence of cellular senescence, based on the presence of p16^{INK4a}-positive cells, in a large series of lupus nephritis kidney biopsies, taken at baseline.
- The presence of p16^{INK4a}-positive cells is associated with renal fibrosis, CD8$^+$ T cell infiltration and functional impairment, but not with patient demographics or systemic disease parameters.

How might this impact on clinical practice?

- The striking codistribution of p16^{INK4a}-positive cells and CD8$^+$ T cells suggests a functional, interactive role in the pathogenesis of lupus nephritis, which may be targeted by senolytic therapies.
- The association with poor renal function 5 years post-treatment suggests the cellular senescence marker p16^{INK4a} in baseline renal biopsies may warrant exploration as a prognostic marker for poor renal outcome.

INTRODUCTION

Lupus nephritis (LN) is a severe complication of systemic lupus erythematosus (SLE), initiated by deposition of immune complexes or autoantibodies in glomerular basal membrane, followed by recruitment of inflammatory cells.[1] Renal injury leads to irreversible fibrosis, resulting in loss of kidney function. LN is treated with high-dose corticosteroids and other immunosuppressive agents.[2] One-third of patients nevertheless show a decline in renal function, with 5%–10% developing end-stage renal disease within 10 years.[3] Prognostic markers that would allow for timely treatment escalation or modification are hence eagerly sought, as are novel therapeutic targets.

Cellular senescence, triggered by stimuli such as telomere erosion, oxidative stress and chronic inflammation, ultimately leads to irreversible growth arrest through the accumulation of cyclin dependent kinase (CDK) inhibitors including p16^{INK4a} protein (*CDKN2A*): a major hallmark of cellular senescence.[4] Senescent cells remain metabolically active and undergo a number of morphological and physiological changes including the

upregulation of β-galactosidase activity[5] and acquisition of a proinflammatory, profibrotic senescence-associated secretory phenotype (SASP).[6] While essential in tissue repair and remodelling (e.g., during embryogenesis), cellular senescence can exert adverse effects in aging-related and chronic disease as well as cancer.[4] $p16^{INK4a}$ or β-galactosidase positive cells have been observed in renal ageing and certain kidney diseases, and were associated with histological lesions and renal impairment.[7] The presence of β-galactosidase positive cells correlated with proteinuria in MRL/*lpr* lupus-prone mice[8]; however, cellular senescence is yet to be clearly demonstrated in LN. Here, we report the occurrence of $p16^{INK4a}$-positive cells in kidney biopsies from (n=40) patients with active LN, and its association with renal injury and functional impairment.

METHODS

Patients and kidney biopsies

Patients were recruited at the Department of Rheumatology, Cliniques universitaires Saint-Luc (Brussels, Belgium). All met the 1982 revised ACR classification criteria for SLE and had biopsy-proven LN. Formalin-fixed paraffin-embedded (FFPE) renal biopsies were residual corporal material collected for diagnostic purposes between January 1996 and November 2019. Estimated glomerular filtration rate (eGFR) values were calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula. Patient consent was not required for the use of residual corporal material, in agreement with Belgian regulations on human studies.

Histology and immunohistochemistry

ISN/RPS 2003 classification of SLE renal biopsies, semi-quantitative sclerosis scores, and National Institutes of Health (NIH) activity index (AI) and chronicity index (CI) were retrieved from medical records. Immunostaining with anti-CD8 (C8/144B, Dako) and anti-$p16^{INK4a}$ (E6H4, Roche Ventana CINtec Histology) and Picrosirius Red (PSR) staining were performed on 5 µm FFPE serial sections. Slides were digitalised on an SCN400 scanner (Leica Biosystems, Germany) or a Pannoramic Confocal slide scanner (3DHistech, Hungary) at ×20 magnification. Computer-assisted quantification of the entire surface of sections was performed using Author™ V.2017.2 (Visiopharm, Denmark). Results shown are the number of $p16^{INK4a}$- or CD8-positive cells per μm^2 of tissue. Semi-quantitative PSR scores (scale: 2–6) are median (glomerular +interstitial fibrosis) scores from three (blinded) scorers. Further details are provided in online supplemental methods 1, online supplemental figure 1.

Statistical analyses

Statistical analyses were performed on GraphPad Prism V.9.1.0: Mann-Whitney or Wilcoxon matched-pair signed rank tests for two-group comparisons, Kruskal-Wallis for multigroup comparisons and Spearman's rank-order correlation coefficient.

RESULTS

$p16^{INK4a}$- positive cells in kidney biopsies from patients with active LN

We evaluated $p16^{INK4a}$ protein by immunohistochemistry on renal biopsies taken at diagnosis from 40 patients with active LN, including incident nephritis (n=31) and relapse (n=9). Demographic, biological and clinical data are summarised in online supplemental table 1. $p16^{INK4a}$-positive cells were detected in all, but with considerable variability between samples: from virtually none, to occasional scattered cells, to strongly positive areas (figure 1A, online supplemental figure 2A–D). Quantification of $p16^{INK4a}$ staining confirmed the heterogeneity of LN biopsies (range: 1.76×10^{-6} – 260×10^{-6}, median: 14.7×10^{-6} cells/μm^2) (figure 1B,C). Stained cells included mesangial cells, endothelial cells or podocytes in glomeruli, parietal epithelial cells in Bowman's capsules, proximal or distal tubular cells, and interstitial cells (figure 1D). Although the density of $p16^{INK4a}$-positive cells (per μm^2) was significantly higher in glomeruli than in interstitia (figure 1C), these values (per sample) showed a significant positive correlation (r=0.7591, p<0.0001) (online supplemental figure 3). Importantly, $p16^{INK4a}$ accumulation was not associated with patient age, gender or ethnicity (online supplemental figure 4A–C).

$p16^{INK4a}$ is associated with renal impairment at baseline and 5 years post-treatment

$p16^{INK4a}$ positivity was significantly associated with impaired renal function: biopsies from patients with eGFR <60 at the time of sampling had significantly higher $p16^{INK4a}$-positive cells/μm^2, and conversely, samples with high (>75th percentile) $p16^{INK4a}$ were associated with significantly lower baseline eGFR (figure 2A,B). The association with eGFR was true for both glomerular and interstitial $p16^{INK4a}$ (online supplemental figure 5A,B). Interestingly, whereas high glomerular $p16^{INK4a}$ was also associated with significantly higher proteinuria (urinary protein/creatinine ratio), which mainly reflects glomerular injury, interstitial $p16^{INK4a}$ was not (online supplemental figure 5C,D). In contrast to its association with poor renal function, $p16^{INK4a}$ was not associated with parameters of systemic disease such as serum anti double-stranded DNA antibody or C3 levels, or with ISN/RPS classification (online supplemental figure 6A–D). Slightly (non-significantly) higher $p16^{INK4a}$ was observed in biopsies from patients with longer duration between SLE and LN diagnosis, and in relapse compared with incident nephritis (online supplemental figure 7A,B). Importantly, analysis of only the incident LN cases upheld the association between $p16^{INK4a}$ and eGFR (online supplemental figure 7C), suggesting it is not solely dependent on kidney disease duration. Finally, high $p16^{INK4a}$ in baseline biopsies was also associated with lower eGFR at 5 years post-treatment (but not at 1 year), suggesting it may be predictive of poor long-term renal evolution (figure 2C,D).

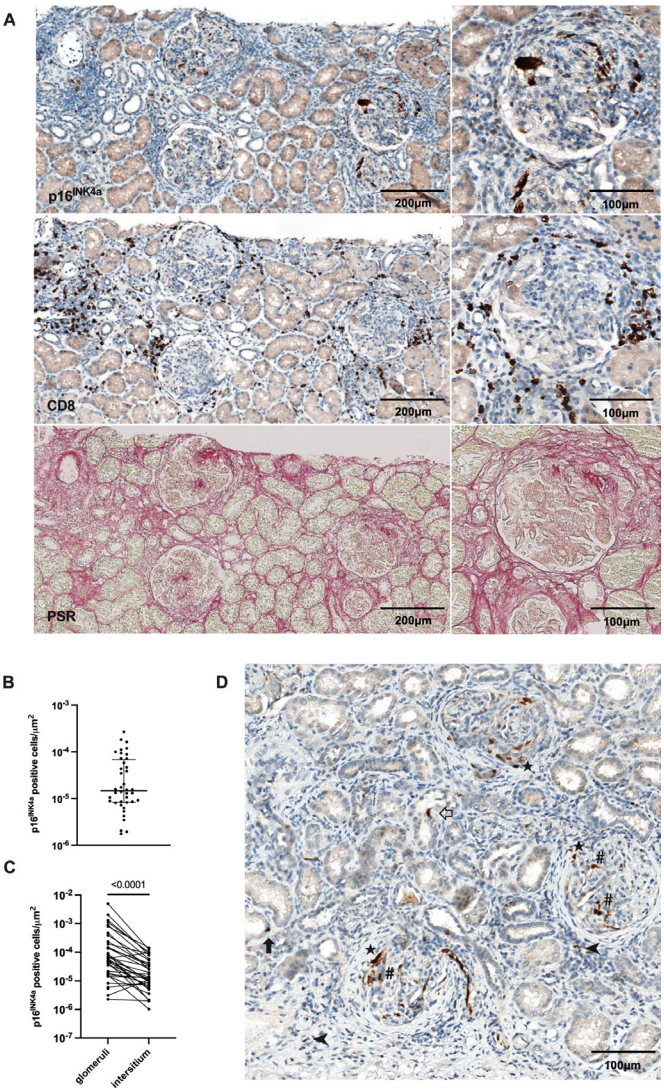

Figure 1 p16^{INK4a}-positive cells in kidney biopsies from patients with active LN. (A) p16^{INK4a}, CD8 and PSR staining of FFPE serial sections from an LN kidney baseline biopsy. Overview (left panels), close-up (right) showing p16^{INK4a}-positive cells within a glomerulus, periglomerular CD8-positive cells and collagen deposits in and around Bowman's capsule of the same glomerulus. (B) Quantification of p16^{INK4a} (positive cells/µm^2) showing degree of heterogeneity among samples. (C) Quantification of p16^{INK4a} (positive cells/µm^2) in glomerular vs interstitial areas of the same samples. *p value*: Wilcoxon matched-pair signed rank test. (D) Representative image from a sample showing p16^{INK4a}-positive cells within glomeruli (#), in Bowman's capsules (filled star), proximal tubules (filled arrow), distal tubules (arrow) and dispersed in interstitium (arrowhead). FFPE, formalin-fixed paraffin-embedded; LN, lupus nephritis; PSR, picrosirius red.

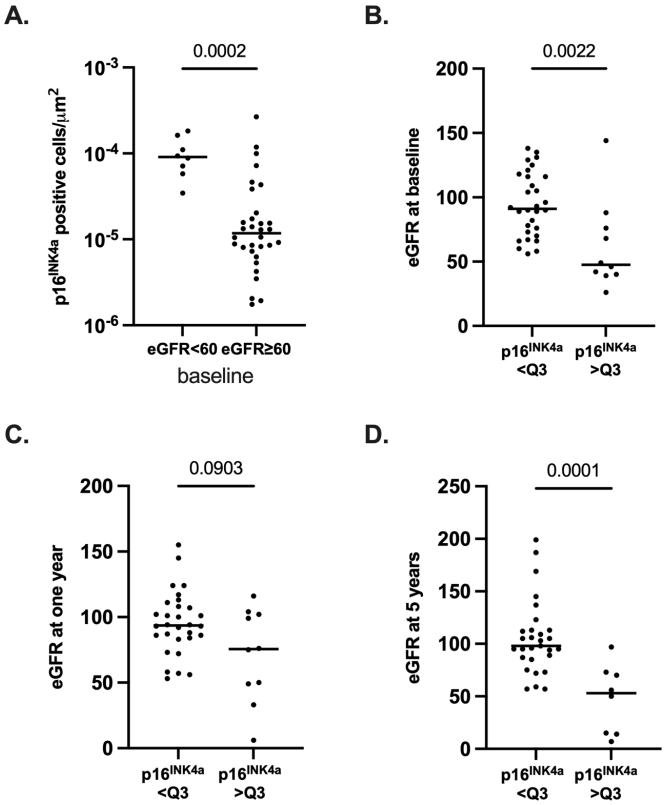

Figure 2 Association between p16^{INK4a}-positive cells and renal impairment in LN. (A) Quantification of p16^{INK4a} (positive cells/µm^2) in samples from patients with renal impairment (estimated glomerular filtration rate: eGFR <60 mL/min/1.73 m^2) at baseline. (B–D) eGFR in patients with p16^{INK4a}-high (>75th percentile, 'Q3') vs p16^{INK4a} low-to-moderate (<Q3) baseline kidney biopsies. Decreased eGFR at baseline (significant) (B) 1-year (non-significant) (C) and 5 years post-treatment (significant) (D) in p16^{INK4a}-high vs p16^{INK4a} low-to-moderate group. Horizontal bars: medians. *p values:* Mann-Whitney U test.

p16^{INK4a} is associated with renal CD8$^+$ T cell infiltration and fibrosis

It has been shown that CD8$^+$ T lymphocytes are the predominant immune cell type infiltrating the LN kidney,[9] and that their presence is associated with renal disease severity.[9 10] We found that p16^{INK4a}-high biopsies displayed significantly higher CD8$^+$ T cell infiltration (figure 3A). They were also significantly associated with increased fibrosis: collagen deposition as reflected by semi-quantitative scores of PSR staining, and sclerosis and NIH CI scores provided by a nephropathologist (figure 3B–D). NIH AI scores, in contrast, were not significantly different between the p16^{INK4a} groups (data not shown). Intriguingly, in examining tissue sections, we noticed what seemed to be a spatial co-distribution between strong p16^{INK4a} staining (that tended to be glomerular) and clustered CD8-positive cells (most often periglomerular) in certain LN biopsies (figure 1A, online supplemental figure 2). We, therefore, quantified the number of CD8$^+$ T cells located within a 30 µm radius around each glomerulus, the thickness of its Bowman's capsule, and the number of p16^{INK4a}-positive cells within it (online supplemental figure 1). This was done on all glomeruli visible on the three serial sections, across all samples. Glomeruli with high p16^{INK4a}-positive cell accumulation displayed significantly higher periglomerular CD8$^+$ T cells (figure 3E), as well as increased thickness of Bowman's capsules, reflecting glomerular fibrosis (figure 3F).

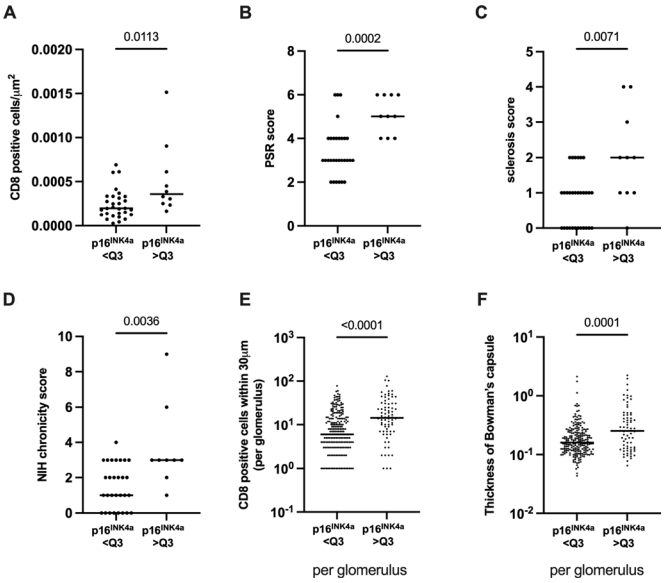

Figure 3 Association between p16^{INK4a}-positive cells, CD8$^+$ T cell infiltration and fibrosis in LN kidney biopsies. (A) Significantly higher CD8$^+$ T cells (positive cells/µm^2) in p16^{INK4a}-high (>75th percentile, "Q3") vs p16^{INK4a} low-to-moderate (<Q3) biopsies. (B–D) Significantly higher fibrosis in p16^{INK4a}-high vs p16^{INK4a} low-to-moderate biopsies: collagen deposits (semi-quantitative PSR staining scored on a scale from 2 to 6) (B), sclerosis (glomerulosclerosis and interstitial fibrosis on a scale from 0 to 6) (C), and NIH chronicity index (on a scale from 0 to 12) (D). (E, F) Glomeruli with high p16^{INK4a} (>Q3) show significantly higher numbers of periglomerular CD8$^+$ T cells (quantified in a 30 µm radius around the glomerulus) (E), and significantly thicker Bowman's capsule (fibrous tissue surrounding the capsule, normalised to area of the glomerulus) (F). Horizontal bars: medians. *p values:* Mann-Whitney U test. PSR, picrosirius red.

DISCUSSION

This is the first demonstration, in a large series of patients, that p16^{INK4a}, a major hallmark of cellular senescence, is associated with disease severity in LN. IHC for p16^{INK4a} protein is widely used to assess for senescence ex vivo, and has been associated with severity of other renal diseases.[7] Selective elimination of p16^{INK4a}-positive cells in mouse models of ageing and induced nephropathy has moreover been shown to relieve fibrotic lesions and improve renal function,[11 12] suggesting a key role in renal pathogenesis. Why cellular senescence may occur in LN is unknown. The inflammatory, oxidative environment of the LN kidney may be a source of cell stress. Several proinflammatory factors have been implicated in senescence induction,[13 14] including interferon-β[13] that has been linked to the senescent phenotype of bone marrow mesenchymal stem cells from patients with SLE.[15]

While our study highlights a significant association between the abundance of p16^{INK4a}-positive cells and LN severity, the use of additional markers of cellular senescence, in a larger cohort, will be essential to confirm its relevance in LN. If and how cellular senescence contributes to disease progression (or whether it simply reflects tissue injury) remains to be investigated. A detrimental effect may be exerted through the profibrotic, proinflammatory secretome (SASP) typical of senescent cells. In keeping with this hypothesis, we describe a tight spatial co-distribution between p16^{INK4a} positive cells, fibrosis and CD8$^+$ T cell infiltration in LN kidneys. Another pathogenic mechanism may involve the accumulation of functionally incompetent cells, for instance, renal progenitor cells (RPC, a subset of parietal epithelial cells located in the Bowman's capsule of glomeruli).[16] While healthy RPCs regenerate glomerular and tubular structures thanks to their capacity to proliferate and differentiate into renal cell subsets,[16] senescent RPCs could hamper tissue repair.[17] Finally, while the SASP is particularly suited to engaging the immune system (including CD8$^+$ T lymphocytes)[18] for the clearance of senescent cells, the latter can upend the process by inhibiting cytolytic cells.[18] It has been suggested that persistence of senescent cells (due to the overwhelming or inhibition of the immune response) tips the balance from a positive to a negative impact.[16] Further in vitro experiments will be required in order to dissect the role of p16^{INK4a}-positive cells in the pathogenesis of LN. This may have important implications for therapy, the first open-label pilot study using the senolytic drugs dasatinib plus quercetin (DQ) having shown promising results in idiopathic pulmonary fibrosis and diabetic kidney disease.[19 20]

Finally, the association of high baseline p16^{INK4a} with impaired renal function 5 years post-treatment initiation suggests it may be a promising predictor of disease severity. It would be of interest to assess for p16^{INK4a} and its associated markers (fibrosis and CD8$^+$ T cell infiltration) in 1-year follow-up biopsies as well as in a larger cohort, as the evolution of these markers from baseline to 1 year may better reflect treatment response than either time point alone.

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Competing interests Bernard Lauwerys is currently employed at UCB Pharma, Anderlecht, Belgium.

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Patient consent for publication Not applicable.

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