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IMMUNOPATHOLOGY

Validation of a phospholipase A2 receptor antibody ELISA in an Australian cohort with membranous glomerulonephritis

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Summary

A commercial PLA2R Ab ELISA was validated by examining its ability to distinguish primary from secondary membranous nephropathy, correlating results with clinical markers of disease activity, and comparing its performance with an indirect immunofluorescence test (IIFT). PLA2R Ab levels were measured in 77 patients with biopsy proven membranous nephropathy, divided into either idiopathic (n = 61) or secondary groups (n = 6). In the idiopathic group, measures of contemporaneous disease activity (proteinuria, serum creatinine) were compared between seropositive and seronegative subjects. ELISA values were then compared with semi-quantitative results from an IIFT using PLA2R transfected HEK293 cells as substrate. The PLA2R Ab ELISA was positive in only 15 of 61 (25%) patients with idiopathic membranous nephropathy (IMN), but there was a significant negative relationship with time since diagnosis. Thus, in a subgroup of patients diagnosed within 6 months of analysis, the sensitivity was 6/15 (55%), rising to 6/8 (75%) in those recently-diagnosed patients who had not been treated. In the entire cohort, there was a significant positive correlation between ELISA values and degree of proteinuria, but our analysis did not control for variation of both variables with time. The PLA2R Ab ELISA also showed very high agreement with IIFT (96%). Therefore, the PLA2R Ab ELISA is a highly specific test for distinguishing primary from secondary membranous nephropathy that is most sensitive in newly diagnosed patients who have not received immunosuppression. Antibody levels correlated with degree of proteinuria, but this relationship was not shown to be independent of time. Both IIFT and ELISA platforms performed comparably.

Key words: Phospholipase A2 receptor antibody; membranous nephropathy; enzyme linked immunosorbent assay; sensitivity; specificity; indirect immunofluorescence test.

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INTRODUCTION

Membranous nephropathy is the most common cause of nephrotic syndrome in adults. Spontaneous complete remission of proteinuria occurs in up to 30% of patients at 5 years.[1,2] However, one third of patients with this condition will progress to end stage renal failure at 10 years if left untreated.[3] Membranous nephropathy may be either idiopathic (or primary), or secondary to infections, malignancy or drugs (see Table 1). Whilst the diagnosis of idiopathic membranous nephropathy (IMN) has traditionally been made by exclusion of secondary causes, recently a large proportion of cases thought to be idiopathic have been found in association with antibodies to M type phospholipase A2 receptor,[4] suggesting an underlying autoimmune pathogenesis.

The discovery of this association prompted an analysis of the utility of PLA2R Ab testing in the diagnosis, monitoring and prognosis of IMN. Sensitivity of the PLA2RAb for the diagnosis of IMN has varied between studies,[5] possibly due to varying intervals between diagnosis and measurement of the PLA2R Ab, and complicated by the commencement of immunosuppressive agents.[6] Despite this, the specificity of the PLA2RAb for IMN appears to be very high, with meta analyses estimating values at 99%, irrespective of the assay used.[6,7] PLA2RAb levels have also been found to correlate with disease activity as measured by proteinuria,[8] likelihood of spontaneous remission,[9] treatment response,[9,10] disease recurrence following kidney transplant[11] and likelihood of progression to renal failure.[9]

Although PLA2R Abs were initially detected by western blot, other platforms have been developed, including addressable laser bead immunoassay[12] and high sensitivity western blot assays,[13] both of which are mostly restricted to research settings. However, two assays which have been standardised and are currently available for routine diagnostic testing are the indirect immunofluorescence test (IIFT) and enzyme linked immunosorbent assay (ELISA). In contrast to the standardised IIFT, there are few published studies[14–17] that have evaluated the performance of this newer commercial assay; in particular, we are only aware of three head to head studies comparing the IIFT with the new ELISA, and to our knowledge, there are no published studies on the utility of this ELISA for monitoring disease activity in IMN. However, the ELISA platform offers clear benefits, including quantitative results, objective interpretation and facility for automation. Finally, there are no data on the performance of the PLA2R Ab ELISA in an Australian cohort.

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Table 1 Screening criteria to exclude a secondary cause of membranous glomerulonepritis (adapted from Behnert *et al.*[15])

Screening criteria
• A history of non-steroidal anti-inflammatory use, gold, penicillamine and recent antibiotic use • Physical examination to exclude lymphadenopathy, hepatosplenomegaly and palpable masses • Antinuclear antibody (ANA) • Anti-dsDNA • Thyroid function tests • Hepatitis B serology • Hepatitis C serology • FBC, EUC, eGFR, LFT, LDH, iron studies • Chest X-ray (or equivalent) • PSA (men > 50 years) • Mammogram (women > 40 years) • Gastroscopy, colonoscopy and renal ultrasound if clinically applicable (optional)

eGFR, estimated glomerular filtration rate; EUC, electrolytes/urea/creatinine; FBC, full blood count; LDH, lactate dehydrogenase; LFT, liver function tests; PSA, prostate specific antigen.

Therefore, we sought to validate the commercial PLA2R Ab ELISA in an Australian cohort with biopsy proven membranous glomerulonephritis, to compare the performance of this ELISA against the IIFT, and finally, to verify previous findings of a relationship between PLA2R Ab and disease activity.

METHODS

Study recruitment

The study was conducted between April 2013 and November 2014. Subjects were enrolled by immunology and renal medicine departments in Australia, who were invited to send serum from patients with biopsy-proven membranous nephropathy. Where possible, values for creatinine and spot urinary protein were obtained concurrently. Patients were independently diagnosed to have either idiopathic or secondary membranous nephropathy by their enrolling clinician. Enrolling clinicians were also asked to provide demographic information such as sex, date of birth and disease specific information including use of immunosuppressive medication and where applicable, the underlying secondary disease. Patients with IMN and serum PLA2R Ab within six months of diagnosis were included in the 'prospective' subgroup, similar to the approach of Hofstra *et al.*[9] Medical records were obtained to determine clinical activity and immunosuppressive treatment. The study received ethics approval from the Westmead Hospital Human Research Ethics Committee.

Detection of PLA2R Ab by ELISA

All samples were tested by ELISA (EUROIMMUN, Germany) according to the manufacturer's instructions. Briefly, serum diluted at 1:100 was added to microtitre wells coated with recombinant PLA2R1 antigen. Following incubation and washing, an enzyme labelled anti-human IgG was added followed by a further incubation and washing step. A chromogenic substrate (3,3′,5,5′tetramethylbenzidine/H_2O_2) was added, followed by the addition of stop solution (0.5 M H_2SO_4).The optical density was measured at 450 nm. As per manufacturer guidelines, values of $\geq$20 RU/mL were considered positive, values between 14 and 20 RU/mL (deemed borderline by the manufacturer) and <14, were considered negative.

Detection of PLA2R Ab by IIFT

In a subset of 74 patients with adequate serum following ELISA testing, an IIFT (IIFT Mosaic; EUROIMMUN) was carried out using HEK 293 cells transfected with PLA2R1 as substrate, along with non-transfected cells as controls[10] using a screening dilution of 1:10. Slides were read by two independent readers (RS, MWL), blinded to PLA2R Ab ELISA results, whilst a third reader (SC) resolved any discrepancies.

Proteinuria index

Study centres varied in their measurement of proteinuria, with some measuring urinary albumin and others measuring urinary protein. In order to compare these disparate measures between patients and serially from a particular patient, the proteinuria index (PI) was calculated by dividing the urinary albumin to creatinine ratio (ACR) or urinary protein to creatinine ratio (PCR) by the upper limit of normal for each respective test.

Statistical analysis

In the prospective cohort, idiopathic membranous nephropathy was diagnosed using stringent criteria for exclusion of secondary disease (Table 1). However, in patients diagnosed greater than six months prior to enrolment in the study, in whom it was not feasible to reinvestigate for secondary causes, the gold standard for calculation of sensitivity and specificity was the referring clinician's assessment. Ninety-five percent confidence intervals were calculated for estimates of sensitivity and specificity. Because several variables were not normally distributed, Mann–Whitney U tests were conducted to detect differences in age, PI, serum creatinine and duration from diagnosis to PLA2R Ab testing between those with IMN who were antibody positive versus those who were negative. Between the same groups, the Z test for proportions was used to detect differences in the proportion of male patients and the proportion of those on immunosuppression. Spearman rank correlation coefficients were calculated in order to determine the relationship between PLA2R Ab and measures of disease activity, i.e., PI and serum creatinine. McNemar's test was used to test for systematic differences between IIFT and ELISA.

RESULTS

Study population

Seventy-seven patients were enrolled, the majority having IMN (61/77, 79%), and two-thirds of these being male, consistent with the epidemiology of this disease (see Table 2). Of the 16 patients with secondary disease, the majority (63%) were female, and the predominant secondary cause was systemic lupus erythematosus (9 cases). The other causes included prostate cancer (2 cases), acute myeloid leukaemia, rheumatoid arthritis, hepatitis B infection, hepatitis C infection and bovine serum albumin nephritis (one case each). There was no significant difference in interval between diagnosis and PLA2R Ab in those with idiopathic or secondary disease ($U = 371.5$, $p > 0.05$).

Diagnostic performance

Considering all patients with IMN, the PLA2R Ab ELISA was positive in 15 of 61 [sensitivity 25%, 95% confidence interval (CI) 16–37%], with a specificity of 100% (95% CI 81–100%) (Fig. 1). In the prospective cohort, in whom the PLA2R Ab test had been performed within 6 months of the renal biopsy, the sensitivity was higher (6/11, 55%; 95% CI