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Original article

Prognostic significance of glomerular and tubulointerstitial morphometry in idiopathic membranous nephropathy

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ABSTRACT

The purpose of our study was to investigate the prognostic value of clinical and pathological, in particular glomerular and tubulointerstitial morphometric variables in idiopathic membranous nephropathy (IMN). We prospectively followed 60 Caucasian patients diagnosed with idiopathic membranous nephropathy for at least 2 years or until primary outcome (≥50% permanent decrease in estimated glomerular filtration rate or death). Glomerular and tubulointerstitial morphometric variables at the time of renal biopsy were analyzed with respect to this outcome. Univariate analysis revealed that significant negative prognostic factors for this outcome were higher cholesterol and smaller albumin concentrations, higher creatinine and maximal 24-h proteinuria, higher grade of nephroangiosclerosis, higher glomerular basement membrane thickness and glomerulopathy index, higher interstitial fibrosis and tubular atrophy percentage and higher injury score. In multivariate analysis, only the maximal 24-h proteinuria and interstitial fibrosis and tubular atrophy percentage were independent predictors of this outcome. The results suggest that morphometric analysis, mainly quantitative measurement of interstitial fibrosis and tubular atrophy percentage, injury score, glomerular basement membrane thickness and glomerulopathy index could be used as an additional method for risk stratification of patients with idiopathic membranous nephropathy.

Introduction

Idiopathic membranous nephropathy (IMN) is one of the most common primary glomerulonephritides, accounting for 9.7–29.4% [3,9,17]. It is considered the most common cause of nephrotic syndrome in adults [22,25]. It has a very variable clinical course with all possible outcomes, ranging from spontaneous remission, with a reported incidence between 32 and 67%, to progressive deterioration and development of end-stage renal disease (ESRD), with a reported incidence between 12 and 44% [7,18,21,26]. Considering a variable clinical course, identification of specific and sensitive prognostic factors is of great importance for the selection of patients undergoing immunosuppressive treatment. Numerous prognostic factors have been validated in IMN, and for most of them, low specificity and/or sensitivity was found [4,7,12,16,20,22,25,27]. Glomerular and tubulointerstitial morphometric analysis is being used as a complementary method of routine analysis of renal biopsy in various diseases [23]. Regarding IMN, only a few studies used morphometric analysis [1,19,24]. The aim of this study was to validate glomerular and tubulointerstitial morphometric prognostic factors, as well as clinical factors, in our cohort of patients with IMN.

Material and methods

We included patients having undergone kidney biopsy in two Nephrology Departments in Zagreb, Croatia, between 1996 and 2009, and diagnosed with IMN. Patients with secondary forms of membranous nephropathy were excluded from the study. Age, gender, arterial blood pressure, serum creatinine, estimated glomerular filtration rate (EGFR), calculated according to the CKD-EPI formula [14], serum cholesterol, albumin and maximal 24-h proteinuria (until the biopsy) were recorded at the time of biopsy. In all patients, kidney biopsy was performed, and all specimens were processed for light, immunofluorescence and electron microscopy using standardized techniques. The histopathological parameters analyzed were as follows: Ehrenreich and Churg disease stage I–IV [6], semiquantitatively defined nephroangiosclerosis grade (0 – none, 1 – mild, 2 – moderate, 3 – severe), immunoglobulin

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G deposition grade (0 – lack of deposition, 1 – mild, 2 – moderate, 3 – severe), complement C3 deposition grade (0 – lack of deposition, 1 – mild, 2 – moderate, 3 – severe), presence of secondary focal segmental glomerulosclerosis (FSGS) and heterogenicity of immune deposits (synchronous electron dense deposits with a single stage in all analyzed glomeruli were arbitrarily classified as homogenous type and others having various stages as heterogeneous type, according to Yoshimoto et al. [30]). Morphometric analysis was carried out by a semiautomatic image analysis procedure, using the optical microscope Olympus BX41 with camera Olympus DP71 connected with PC and with ImageJ image analysis software (http://rsb.info.nih.gov/ij/). Glomerular morphometry was carried out by analysis of light microscopy (PAS-stained images with a magnification of ×400). In each case, 5–10 glomeruli, cut through hilum or having complete outline of Bowman's capsule, were selected for glomerular morphometry. Biopsies with less than 5 glomeruli were not included in the study. After opening the image, ImageJ measurement tools were calibrated by micrometer specific to the magnification into standard units (mm, μm and nm). Glomerular morphometric parameters measured were: glomerular diameter (GD), tuft diameter (TD), glomerular area (GA), tuft area (TA), mesangial matrix and membranes area (MA), urinary space area (UA = GA − TA), capillary space area (CA = TA − MA), tuft volume fraction (TVF = (TA/GA) × 100), urinary space volume fraction (UVF = (UA/GA) × 100), membranes and mesangial matrix volume fraction (MVF = (MA/GA) × 100) and capillary space volume fraction (CVF = (CA/GA) × 100), as described earlier by Rayath et al. [24]. After obtaining color image by Image/Color/Split channels tool, red, green and blue channels of the image were separated, and for further analysis, the green channel was kept, because it gives the sharpest glomerular image. Using a free-hand tool from the menu bar and tracing the outline of the glomerulus and then the tuft, an area was selected as region of interest (ROI), and then, using Analyze/Measure tool, GA, TA and MA measured. MA was measured by Image/Type/8-bit tool to convert the green channel of the original image to grayscale, and then the threshold for staining detection was set by selecting Image/Adjust/Threshold tool. The final grayscale image was created in which black areas approximately represent mesangial matrix and membrane areas (MA), as reported earlier by Rayath et al. [24]. For every glomerular morphometric parameter measured, the mean of all values measured in a single biopsy was used as reference value for the individual patient. Morphometric measurement of interstitial fibrosis and tubular atrophy (IFTA) was carried out by analyzing Masson-trichrome stained images with a magnification of ×400 (areas of fibrosis are stained blue). After separating the glomerules and medulla from the cortex, blue color was defined as ROI by selecting one blue area with a free-hand tool and then by clicking the Image/Adjust/Color threshold tool and Sample button, which removed pixels not falling into the selected color range. After that, the image is converted into binary (8-bit), and the whole biopsy cylinder is marked as ROI. Then, using Analyze Particles tool, the Area fraction was determined which represents IFTA (in percentage) (described earlier in detail by Rangan and Tesch [23]). Injury score (IS) is a marker of chronic damage and has recently been shown to have prognostic value in focal segmental glomerulosclerosis [29]. It is calculated as IS = (number of segmental sclerotized glomeruli + number of globally sclerotized glomeruli)/total number of glomeruli + IFTA (expressed as an absolute number). Electron microscopy was carried out using JEOL JEM-1400 electron microscope. Glomerular basement membrane thickness (GBMT) was ascertained on images at a magnification ×8000. GBMT was determined as a harmonic mean of 100 orthogonal intercepts across the glomerular basement membrane (GBM) measured from at least 5 glomerular capillary loops by line tool of ImageJ software on the acquired images after calibration for magnification. Harmonic mean was multiplied by $8/3\pi$ to correct the measuring error due to oblique sectioning of capillary walls [13,24]. In each measurement, GBMT was defined as a distance between endothelial cell and podocyte membrane, and included intramembranous immune deposits. Glomerulopathy index (GPI) was calculated by the formula GPI = 1/10 × GBMT + MVF (according to Rayat et al. [24]).

Follow-up started at the time of biopsy. It was minimally 2 years and continued until February 2011 or until the primary outcome. Serum creatinine and 24-h proteinuria were measured every 3 months during follow-up, and EGFR was calculated. Combined primary outcome was renal failure (RF, defined as ≥50% permanent decrease in EGFR from baseline values) or death.

Statistical analysis was performed using the SPSS version 17.0 for Windows and MedCalc version 12.2. Normally distributed variables were expressed as mean ± standard deviation and compared using Student's *t*-test. Nonparametric continuous variables were expressed as median and interquartile range and compared using Mann–Whitney *U-test*. Categorical variables were expressed in percentage and compared using χ^2-test or Fisher's exact test. Univariate comparisons for outcomes were performed by Kaplan–Meier curves and log-rank test. Receiver operating characteristics (ROC) curves analysis was made to determine area under curve (AUC) and to calculate the sensitivity and specificity of various clinical and morphometric baseline variables in the prediction of primary outcome, using the most discriminative thresholds (cut-off values). A multivariate Cox proportional hazard model was constructed to determine independent variables associated with primary outcome. Only variables associated by univariate analysis were included in a multivariate model. For all analyses, $p < 0.05$ was considered significant.

Results

Sixty Caucasian patients were included in this study. Nephrotic syndrome was present in 93.3% of the patients. Tables 1, 2 and 3 show the baseline clinical, histological and morphometric parameters with respect to primary outcome. The patients were treated nonrandomly, following guidelines [2,5,8,22]; 85% of the patients with immunosuppressives (56.7% with glucocorticoid + alkylating agent, 18.3% with glucocorticoid + cyclosporin and 10% with glucocorticoid alone), and 90% of the patients received renin-angiotensin inhibiting drugs. Patients were followed for a median of 48 months (range 6–132 months). During follow-up, 12 patients reached primary outcome (20%), two patients died (one of thromboembolic incident; for the other one, the cause of death was unknown). The estimated probability of survival without primary outcome was 79.0 ± 6.8% at 60 months and 62.7 ± 10.0% at 84 months (Kaplan–Meier survival analysis). In univariate analysis, higher serum creatinine (lower EGFR), higher serum cholesterol and lower albumin concentration were associated with primary outcome. Pathohistological and morphometric variables associated with primary outcome were higher nephroangiosclerosis grade, higher GBMT and GPI, higher IFTA percentage and higher IS. Other morphometric and pathohistological variables tested were not associated with primary outcome. The ROC analysis showed that the most discriminative variables in the prediction of primary outcome were IFTA and IS (Table 4). The optimal cut-off value of IFTA was 18%, and that of IS 0.322 (Figs. 1 and 2). Kaplan–Meier survival analysis showed that renal and patient survival (primary outcome) was significantly higher in patients with IFTA ≤ 18% (Fig. 3) and IS ≤ 0.322 (Fig. 4). Cox proportional hazards model included variables selected by univariate analysis. The results are shown in Table 5. The only independent predictors of primary outcome were maximal 24-h proteinuria (hazard ratio, HR = 1.127) and IFTA (HR = 1.029). We also created a similar Cox proportional hazards