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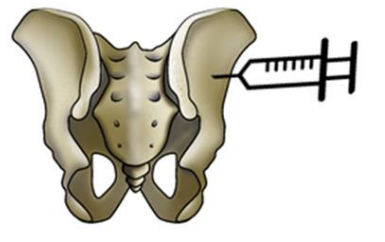
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# RNA-based immunoglobulin repertoire sequencing is a new tool for the management of monoclonal gammopathy of renal (kidney) significance.

Bone marrow aspirate and total RNA extraction

Light chain high-throughput sequencing

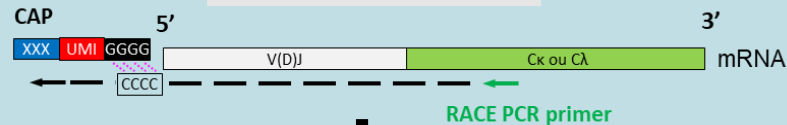
Light chain repertoire analysis and sequence alignment



RNA extraction



RACE-PCR

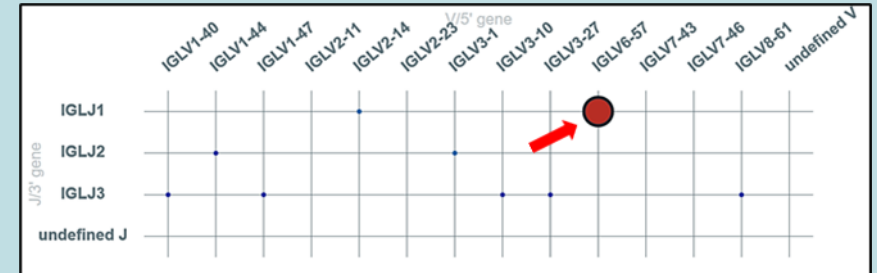


Amplification



Illumina sequencing

Rapid clone identification



Full-length VJ region of the monoclonal light chain

V

J

CONCLUSION:

RACE-RepSeq appears highly sensitive to detect subtle B/plasma cell clones in patients with monoclonal gammopathy of renal significance

## **RNA-based immunoglobulin repertoire sequencing is a new tool for the management of monoclonal gammopathy of renal (kidney) significance.**

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### **Keywords**

Next generation sequencing, immunoglobulin, AL-amyloidosis, monoclonal gammopathy of renal significance

## Abstract

The diagnostic approach of monoclonal gammopathy of renal significance is based on the detection of a monoclonal immunoglobulin in the blood and urine, and the identification of the underlying clone through bone marrow and/or peripheral blood cytologic and flow cytometry analysis. However, the monoclonal component and its corresponding clone may be undetectable using these routine techniques. Since clone identification is the cornerstone for guiding therapy and assessing disease response, more sensitive methods are required. We recently developed a high-throughput sequencing assay from bone marrow mRNA encoding immunoglobulins (RACE-RepSeq). This technique provides both full-length V(D)J region (variable, diversity and joining genes that generate unique receptors as antigen receptors) of the monoclonal immunoglobulin and the dominant immunoglobulin repertoire. This allows analysis of mutational patterns, immunoglobulin variable gene frequencies and diversity due to somatic hypermutation. Here, we evaluated the diagnostic performance of RACE-RepSeq in 16 patients with monoclonal-associated kidney lesions, and low serum monoclonal immunoglobulin and free light chain levels at diagnosis. Bone marrow immunohistochemical analysis was negative in all 11 patients so tested and 7 of 12 patients had no detectable clone matching the kidney deposits using flow cytometry analysis. By contrast, RACE-RepSeq detected a dominant clonal light chain sequence of matched isotype with respect to kidney deposits in all patients. Thus, high throughput mRNA sequencing appears highly sensitive to detect subtle clonal disorders in monoclonal gammopathy of renal significance and suggest this novel approach could help improve the management of this kidney disease.

## Translational Statement

High-throughput sequencing assay from bone marrow mRNA encoding immunoglobulins (RACE-RepSeq) is a very sensitive approach to detect small B/plasma cell clones in patients with monoclonal gammopathy of renal significance. This technique provides the full-length immunoglobulin variable domain sequences, allowing analysis of mutational patterns that may help to better understand mechanisms of immunoglobulin toxicity. Future studies are needed to evaluate the sensitivity of RACE-RepSeq analysis from bone marrow and blood samples for monitoring minimal residual disease, a new goal in the management of plasma cell disorders.

# Introduction

In monoclonal gammopathy of renal significance (MGRS), renal lesions are independent of the tumor mass, but result from structural peculiarities in the variable (V) domain of the nephrotoxic immunoglobulin (Ig).<sup>1,2</sup> The serum level of the pathogenic Ig is usually low, complicating the diagnosis and assessment of response to chemotherapy. The initial hematological workup in MGRS patients is based on serum and urine protein electrophoresis/immunofixation, measurement of serum free light chains (FLC), and characterization of the underlying clone in the bone marrow (BM) and/or peripheral blood. However, depending on the type of MGRS-renal disorder, these techniques may fail to detect the nephrotoxic Ig and to identify the pathogenic clone. A previous study found that 1.9% of patients with AL-amyloidosis had no detectable abnormality using all available assays.<sup>3</sup> Moreover, 20% of patients with AL-amyloidosis display FLC levels below the threshold required for evaluation of hematological response, i.e. difference between involved and uninvolved FLC (dFLC) <50 mg/L, making therapeutic management a challenge. Recent studies suggest that such cases could be evaluated with the so called low-dFLC partial response criteria defined as achieving dFLC <10 mg/L in patients with a baseline dFLC between 20 and 50 mg/L.<sup>4,5</sup> However, in our experience, around 2% of patients with AL-amyloidosis are still not evaluable. As compared to AL amyloidosis, less patients with type I cryoglobulinemic glomerulonephritis (~90%) or immunotactoid glomerulonephritis (~70%) have a detectable monoclonal component. The detection rate is noticeably low in patients with proliferative glomerulonephritis with monoclonal Ig deposits (PGNMID), 80% of whom, have a negative hematologic evaluation.<sup>6-8</sup> Thus, more sensitive diagnostic techniques are required to guide therapeutic strategy in MGRS.

Next generation sequencing (NGS) has improved Ig sequencing and detection of minimal residual disease in B-cell malignancies.<sup>9</sup> The sensitivity of Ig sequencing on DNA is

limited by the number of cells and thus may be insufficient to detect subtle clones in MGRS patients.<sup>10</sup> We recently developed a high-throughput sequencing assay from BM mRNA encoding Igs (RACE-RepSeq). This RNA sequencing method has the ability to catch any Ig transcript, even after heavy somatic hypermutation, and to give access to the full-length V(D)J sequence.<sup>11</sup> Additionally, it has a strong sensitivity for the detection of small plasma cell clones, since every single secreting cell produces hundreds of copies of the Ig transcripts.<sup>11,12</sup>

The aim of this study was to assess the clone detection rate of RACE-RepSeq in patients with MGRS-associated renal lesions with low monoclonal Ig and FLC levels, in order to evaluate its value in the diagnostic workup of MGRS.

## Methods

Written informed consent was obtained from all study participants, recruited between 2015 and 2020. Kidney biopsy and bone marrow analysis were performed in all cases (Supplementary Methods).

A detailed protocol of RACE-RepSeq analysis is available in supplementary data. Briefly, total mRNA was extracted from BM samples (1 mL) using Tempus<sup>TM</sup> spin RNA isolation kit (Thermofisher), retrotranscribed to cDNA and then amplified using the 5'RACE PCR technique with unique molecular identifiers (UMI) to reduce amplification errors and quantitative bias. Two specific primers located in the 3' part of the constant domain of  $\lambda$ - and  $\kappa$ -LC genes and a generic adapter in 5' allowed the amplification of the entire corresponding repertoire of the patient (Figure 1 and Supplementary Table S1). An asymmetric paired-end sequencing strategy is employed to obtain full length V(D)J sequences on an Illumina MiSeq sequencer using a MiSeq Reagent Kit v2 (500 cycles). On average, the capacity of this kit allows exploring the simultaneous  $\kappa$ - and  $\lambda$ -LC repertoires of 30 patients with ~300,000 raw

reads per sample. Ig repertoire analysis was done using IMGT/HighV-QUEST (<http://imgt.org/>) and Vidjil software.<sup>13,14</sup>

## Results

We retrospectively analyzed 16 patients with biopsy-proven MGRS-associated renal disease (Table 1 and 2), and 8 control patients with immune thrombocytopenia and no evidence of monoclonal gammopathy by serum immunofixation (Supplementary Table S2). Eleven patients had dFLC <20 mg/L and 5 had negative serum/urine immunofixation at diagnosis. Search for a plasma/B-cell clone was negative in 11/11 patients using immunohistochemistry analysis of BM biopsies. Flow cytometry analysis was negative on peripheral lymphocytes in 8/9 tested patients and on BM in 5/12 patients. Finally, 2 patients had neither monoclonal gammopathy nor detectable clonal disorder.

Amongst 10 patients with  $\lambda$  AL-amyloidosis, BM flow cytometry analysis (n=7), allowed detection of a plasma cell clone in 2 patients (cases AL $\lambda$ 2 and AL $\lambda$ 10) and of a small lymphoplasmacytic clone in 2 patients (cases AL $\lambda$ 5 and AL $\lambda$ 7). However, the clone found in patient AL $\lambda$ 2 was of  $\kappa$  isotype, thus not related to glomerular  $\lambda$  LC deposits. Flow cytometry analysis detected an abnormal plasma cell population in patient AL $\lambda$ 4, but failed to confirm monoclonality. Among 3 patients with  $\kappa$  AL-amyloidosis, BM flow cytometry disclosed a lymphoplasmacytic clone in 1 of 2 tested patients (case AL $\kappa$ 3). No clone matching the renal deposits was found in the 2 patients with type 1 cryoglobulinemic glomerulonephritis, and the patient with immunotactoid glomerulopathy showed a small detectable  $\kappa$ -CLL clone using blood and BM flow cytometry (Table 1 and 2).

Using RACE-Seq, an IGLV or IGKV abundant clonotype was readily identified in all patients, ranging from 14 to 86% of total clonotypes. By contrast, the most abundant clonotype in the control group was between 0.2 to 3.4% of total clonotypes (Figure 2). Besides

the high detection rate of clonal LC sequences, RACE-RepSeq also provides the full-length VJ region of the LCs. Figure 3 depicts clonal sequences obtained in patients with MGRS in comparison with the corresponding germline sequences.

## Discussion

The present study indicates that RACE-RepSeq is highly sensitive to detect subtle B/plasma-cell clones in patients with MGRS. This novel approach appears promising for optimizing management and refining assessment of the hematologic response in MGRS subtypes associated with low clonal identification rate.

Besides its sensitivity to detect clones, RACE-RepSeq offers other interesting perspectives. It requires less than 1 ml of total BM aspirate to depict the dominant B-cell repertoire with characterization of full-length V(D)J region of produced Ig. Since sequence peculiarities in the V domain of monoclonal Igs govern their toxicity, RACE-RepSeq may help identify specific molecular profiles in various MGRS-related disorders. However, we cannot formally conclude that the most abundant LC sequence is the pathogenic one, especially when two clones of the same isotype were found (patients AL $\kappa$ 1 and AL $\kappa$ 3). Mass spectrometry (MS) analysis of laser-capture dissected deposits remains the only tool to gain information on the real deposited LC, but usually results in low V domain coverage or fails to identify any V gene due to the high mutation rate.<sup>15</sup> Coupling RACE-RepSeq for full-length sequence information and MS analysis would be a powerful tool to definitely identify the pathogenic Ig. RACE-RepSeq could also be useful to assess the monoclonal origin of certain renal diseases in which immunofluorescence studies lack enough sensitivity to differentiate between monoclonal and oligoclonal Ig deposits, such as monotypic fibrillary glomerulonephritis, IgA nephropathy with  $\lambda$ -LC restriction or PGNMID.<sup>16-19</sup>

The prognosis of MGRS depends on the rapid achievement of deep hematologic response, whose accurate assessment often remains a challenge.<sup>1</sup> In multiple myeloma, negative



minimal residual disease measured by next generation flow cytometry (NGF) or NGS on DNA has been shown to correlate with improved progression-free and overall survival.<sup>9,20</sup> Our results suggest that RACE-RepSeq may represent a valuable additional tool to precisely assess hematologic response and thus guide duration and adaptation of chemotherapeutic regimens. A precise comparison of the sensitivity of RACE-RepSeq with those of NGF and NGS on DNA deserves to be carried out. Additionally, in unpublished preliminary data including 14 patients with MGRS, RACE-RepSeq shows a significant correlation between BM and blood for the detection of clonal plasma cells. Thus, this technique could represent an additional noninvasive tool for frequent monitoring of the hematologic response. In clinical practice, the value of RACE-RepSeq, appears promising although it remains to be evaluated in larger cohorts of MGRS patients.

**Disclosures:** All the authors declared no competing interests

## Supplementary Material

### Supplementary Methods

Supplementary Figure S1. Comparison of light chain sequences obtained by TOPO cloning and Sanger sequencing (TOPO) with sequences obtained by RACE-RepSeq after UMI correction (NGS). Depicted sequences are the consensus of <10 sequences in TOPO (6 to 9) and >500 sequences after UMI correction in NGS (572 to 1671). Lambda light-chain variable regions are highlighted in yellow and junction regions in green.

Supplementary Table S1. Primer sequences used for RNA-based Immunoglobulin Repertoire Sequencing (RACE-RepSeq)

Supplementary Table S2. Main characteristic of patients with immune thrombocytopenia (control group)

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## Figure legends

Figure 1. Schematic representation of the high-throughput sequencing assay from bone marrow mRNA encoding immunoglobulins performed in patient AL $\lambda$ 8.

Figure 2. (a) Most abundant clonotype found in each patient. (b) Comparison between MGRS and immune thrombocytopenia patients (\*\*\*) $p < 0.001$ , Mann-Whitney test).

Figure 3. Deduced amino acid sequences of the monoclonal  $\lambda$  (a) and  $\kappa$  (b) light chain VJ domains in MGRS patients compared to germline sequences according to IMGT numbering. Mutated amino acids are highlighted in green.

Table 1. Clinical, immuno-hematological and RACE-RepSeq analysis of lambda light chain-associated MGRS patients

	ALλ1	ALλ2	ALλ3	ALλ4	ALλ5	ALλ6	ALλ7	ALλ8	ALλ9	ALλ10	CryoIgGλ
Sex	Male	Male	Female	Female	Female	Male	Female	Female	Female	Female	Male
Age (years)	71	63	62	73	55	69	75	86	70	75	61
Hematological findings											
SPEP M-spike (g/dL)	0	0.2	0	0	1.0	0	1.0	0.4	0.4	0.5	0
UPEP M-spike (g/24h)	0	<0.1	0	0	<0.1	0	0	<0.1	<0.1	0	0
Serum immunofixation	Negative	IgGκ	Negative	Negative	IgMλ	IgMλ	IgMλ	IgGλ	IgGλ	IgMλ	Negative
Urine immunofixation	Negative	λ	Negative	Negative	IgMλ	λ	Negative	IgGλ	IgGλ	Negative	Negative
Kappa (mg/L)*	8	16	52	15	10	12	33	44	23	11	12
Lambda (mg/L)*	24	42	24	31	26	42	26	42	52	47	21
dFLC	16	26	-28	16	16	30	-7	-2	29	36	9
Kappa/Lambda ratio*	0.33	0.38	2.17	0.48	0.38	0.29	1.27	1.05	0.44	0.23	0.57
Bone marrow findings											
Plasma cell (%)	1	3	1	3	2	1	2	5	3	1	<1
IHC	No clone	ND	No clone	ND	No clone	ND	No clone	No clone	No clone	No clone	ND
Flow cytometry	No clone	No λ clone κ-PC clone (1% of cells)	ND	No clone**	LP clone (2% of cells)	No clone	LP clone (3% of cells)	ND	ND	λ-PC clone (0.2% of cells)	No λ clone κ-CLL clone
RACE-RepSeq analysis											
Total reads	263431	271927	349826	200368	253297	277565	246454	240136	232753	198025	167247
Number of clonotypes	396	64	757	308	527	764	400	353	330	895	567
Most abundant clonotype (% of total clonotypes)	IGLV1-44/J7 (57)	IGLV1-47/J3 (44.5)	IGLV1-47/J2 (16)	IGLV2-8/J2 (37)	IGLV2-14/J1 (75)	IGLV2-14/J1 (46)	IGLV2-14/J1 (26)	IGLV6-57/J1 (88)	IGLV6-57/J2 (62)	IGLV8-61/J3 (43)	IGLV3-21/J3 (32)
Other abundant clonotype (% of total clonotypes)	None	IGKV1-5/J5 (24)	None	None	None	None	None	None	None	None	None

Abbreviations : AL, light-chain amyloidosis; CryoIgGλ, Type 1 cryoglobulinemic glomerulonephritis with IgGλ deposits; dFLC, difference between lambda and kappa light chain serum concentrations; IHC, immunohistochemistry analysis of bone marrow biopsy; LP clone, lymphoplasmacytic clone; MGRS, monoclonal gammopathy of renal significance; M-spike, monoclonal spike; ND, not done; PC clone, plasma cell clone; RACE-RepSeq analysis, high-throughput sequencing assay from bone marrow mRNA encoding immunoglobulins; SPEP, serum protein electrophoresis; UPEP, urine protein electrophoresis

\*Normal range (Freelite™) : kappa = 3.3-19.4 mg/L, lambda = 5.71-26.3 mg/L, kappa/lambda ratio = 0.26-1.65

\*\*Detection of less than 0.2% of abnormal plasma cells (CD19-, CD56-, CD27+, CD28+, CD81+, CD117-) without monotypic appearance

Table 2. Clinical, immuno-hematological and RACE-RepSeq analysis of kappa light chain-associated MGRS patients

	ALκ1	ALκ2	ALκ3	CryoIgGκ	ITG
Sex	Male	Male	Male	Male	Male
Age (years)	61	40	63	42	72
Hematological findings					
SPEP M-spike (g/dL)	0	0.4	0.2 and 0.1	<0.1	0
UPEP M-spike (g/24h)	0	0	<0.1	0	0
Serum immunofixation	IgGκ/IgMκ	IgGκ	2 IgMκ	IgGκ	Negative
Urine immunofixation	Negative	Negative	κ	Negative	Negative
Kappa (mg/L)*	21	14	16	53	18
Lambda (mg/L)*	13	12	7	14	11
dFLC	8	2	9	39	7
Kappa/Lambda ratio*	1.6	1.16	2.28	3.78	1.6
Peripheral blood flow cytometry	No clone	ND	No clone	No clone	κ-CLL clone
Bone marrow findings					
Plasma cell (%)	1	2	2	1	2
IHC	No clone	No clone	No clone	No clone	ND
Flow cytometry	No clone	ND	LP clone (0.1% of cells)	No clone**	κ-CLL clone
RACE-RepSeq analysis					
Number of sequences	316713	260701	263006	149169	592467
Number of clonotypes	1706	682	709	562	239
Most abundant clonotype (% of total clonotypes)	IGKV1-16/J4 (14)	IGKV3-11/J4 (78)	IGKV1D-39/J4 (50)	IGKV1-33/J2 (39)	IGKV3-20/J1 (87.5)
Other abundant clonotype (% of total clonotypes)	IGKV4-1/J1 (8.5)	None	IGKV2D-28/J4 (7)	None	None

Abbreviations : CryoIgGκ, Type 1 cryoglobulinemic glomerulonephritis with IgGκ deposits; dFLC, difference between kappa and lambda light chain serum concentrations; IHC, immunohistochemistry analysis of bone marrow biopsy; ITG, immunotactoid glomerulopathy with IgGκ deposits; LP clone, lymphoplasmacytic clone; M-spike, monoclonal spike; RACE RepSeq analysis, high-throughput sequencing assay from bone marrow mRNA encoding immunoglobulins; SPEP, serum protein electrophoresis; UPEP, urine protein electrophoresis

\*Normal range (Freelite™) : kappa = 3.3-19.4 mg/L, lambda = 5.71-26.3 mg/L, kappa/lambda ratio = 0.26-1.65

\*\*A small kappa light chain positive B-cell clone (0.7% of bone marrow cells) with an immunophenotype of chronic lymphocytic leukemia was detected 6 months later

