

NEXT GENERATION SEQUENCING: A TOOL FOR THIS GENERATION OF NEPHROLOGISTS

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ABSTRACT

The emergence of next generation sequencing (NGS) techniques has made the sequencing of whole genomes, transcriptomes, and epigenomes faster and more readily available than previous methods such as Sanger sequencing, which was developed in the 1970s. It is now 10 years since NGS began to revolutionise biological and medical research. Sequencing of RNA provides insights into up or downregulated gene expression patterns and therefore into molecular disease mechanisms. This can lead to the detection of new biomarkers that can be used as diagnostic tools in risk stratification, or even as new therapeutic targets. In nephrology, NGS plays a role in both basic and experimental research, but also in the clinical setting, whereby the diagnosis of innate genetic diseases such as ciliopathies or genetically moderated acquired diseases such as glomerulopathies has improved. NGS enables precise diagnosis and classification of common diseases of the kidneys and urinary tract, aids in both prognostic and predictive decision-making, and in the avoidance of unnecessary therapies. It also plays a role in the risk stratification of disease recurrence after transplantation. NGS is a robust method; however, the performance of NGS is dependent on the method of tissue storage, the extraction of DNA or RNA, and on the sequencing platform itself, as well as on the bioinformatic analyses performed, integration of clinical data, and comprehensive interpretation of the results. The aim of this article is to review and emphasise the importance of NGS as a tool for this generation of nephrologists.

Keywords: Review, nephrology, next generation sequencing (NGS), high-throughput nucleotide sequencing, DNA, transcriptome, epigenome.

INTRODUCTION

The invention of Sanger sequencing in the late 1970s represented a paradigm shift in the era of modern medicine.¹ The commercialisation of next generation sequencing (NGS), approximately 10 years ago, led to major improvements in both research and the diagnosis of renal diseases. With Sanger's chain termination DNA sequencing method, it is possible to sequence one gene at a time. On the other hand, NGS makes it possible to sequence the whole genome, exome, or predetermined panel of a patient's genes in a single sequencing reaction and in a much more time efficient manner. NGS also has the capacity to characterise all steps of transcription, translation, and methylation of DNA.²

The Human Genome Project, which aimed to sequence the whole human genome using Sanger sequencing, took a total of 14 years and was published in 2004.^{3,4} The National Human Genome Research Institute subsequently initiated and funded a sequencing technology development programme with the aim of reducing the duration and cost of genome sequencing. This led to a wave of new projects, and finally to the introduction of the contemporary commercial sequencing platforms. The first of these to be released was a method developed by Life Sciences (now Roche) in 2005.⁴ It was quickly followed by others, namely a sequencing platform developed by Solexa (now Illumina), in 2006, and the SOLiD platform developed by Applied Biosystems (now Life Technologies) in 2007.⁴

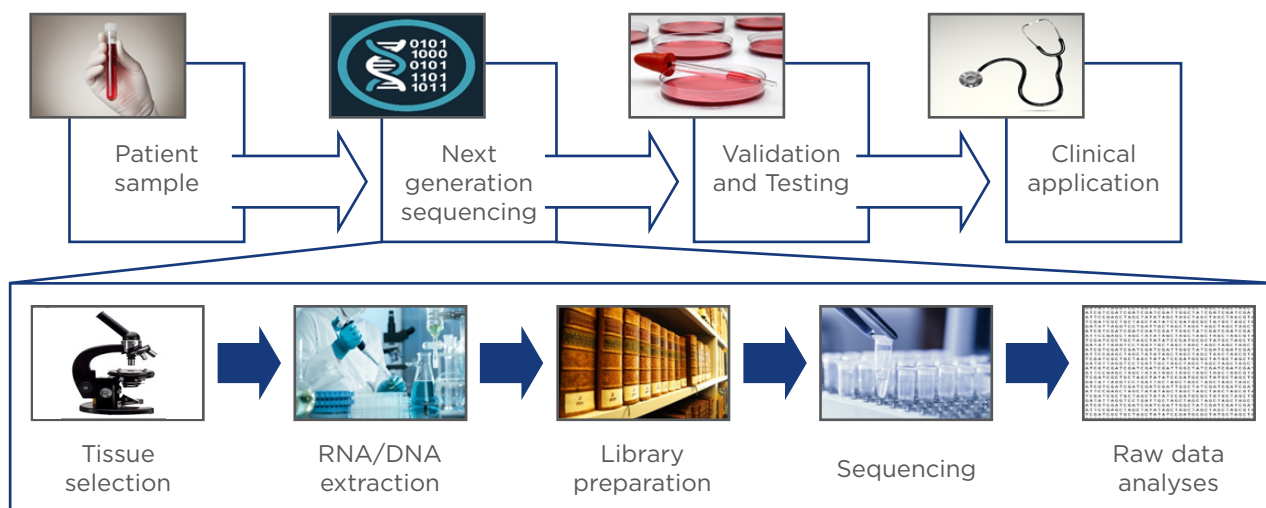


Figure 1: The upper section displays the application of next generation sequencing (NGS). The lower section illustrates the general workflow of NGS.

Sequencing techniques within the various platforms are diverse, but all are described as NGS, massively parallel sequencing, or high-throughput sequencing.^{2,4-6} The three major advantages of NGS compared with Sanger sequencing are the cell-free system for library preparation, the simultaneous sequencing of potentially millions of reactions in parallel, and the independence of electrophoresis.⁴ Compared with other approaches (e.g. microarray), NGS offers the possibility of detecting novel findings that are not based on *a priori* assumptions. Sequencing of DNA enables the simultaneous analysis of the entire genome, which benefits comprehensive genetic diagnostics. Sequencing of RNA gives insight into up or downregulated expression patterns and therefore clarifies molecular disease mechanisms and altered pathways. This can lead to the detection of biomarkers that can be used as diagnostic or stratification tools, or as targets for therapies.^{2,7} NGS is a technology that is under continuous development. The vast volume of new data provides great opportunities, but also long lists of gene variations of uncertain significance. Efforts to standardise the analysis of sequencing results are ongoing. This includes the sharing of data and integration of clinical parameters to bring NGS closer to clinical requirements. This requires worldwide collaboration between genetic researchers, bioinformaticians, and nephrologists.⁷ **Figure 1** illustrates the general application of NGS.

Through improved diagnostics with greater effect on decision-making within both inherited and acquired kidney diseases, NGS helps to improve

patient management. In this review, we elaborate on the role of NGS for diagnostic, prognostic, and predictive measures in inherited and acquired kidney diseases, transplantations, and epigenetics. We emphasise the importance of NGS for today's generation of nephrologists.

TECHNICAL ASPECTS OF DNA AND RNA SEQUENCING

Tissue Storage

The robust performance of NGS depends both on the technical aspects of tissue storage and nucleotide extraction methods, as well as on the NGS technique itself.² Gold standard tissue storage for subsequent sequencing of extracted DNA or RNA is fresh frozen samples. As an alternative, an RNA stabilising solution can be used as a more practical alternative for the storage of fresh frozen tissues. The challenge is that most tissue specimens in pathological archives are formalin fixed and paraffin embedded (FFPE). FFPE tissue is inferior when it comes to molecular analysis as compared with fresh frozen specimens, especially because of the reduced yield and quality of isolated nucleotides. This is due to the process of fixation, embedding, and molecular modification, especially cross-linkage of nucleotides with formalin.⁸ However, we have shown that the results of messenger RNA sequencing of pairwise FFPE samples, stored with high quality stabilising solution, yields similar results, thus demonstrating the feasibility of NGS from FFPE tissues.⁹

Table 1: Sequencing platforms and characteristics.

Sequencing platform	Characteristics
Illumina technology	On a flow cell, fragments of double stranded DNA denaturated into single stranded DNA are amplified into clusters. During the reading process, the respective nucleotides are added, making this a 'sequence by synthesis' method. The nucleotides are reversible terminators and fluorescent.
Ion torrent technology	The reading of the sequence depends not on the emission of fluorescence, but on the emission of protons, which are detected by an ion sensor. Within this method, no amplification of fragments occurs. ²
Pacific bioscience technology	This technique consists of a single molecule real-time sequencing technology. Thus the sensors are able to detect one single molecule. The respective terminated nucleotides are added and recorded in real time. ²
SOLiD technology	Within this technique, a primer is linked to an adaptor. Labelled octamers are added to the primers and sample, and compete for ligation to the primer, whose fluorescence is then read. Thereafter, the octamer is cleaved until the next cycle. ⁴

These findings have been confirmed in a recent publication by a group from the USA.¹⁰

DNA and RNA Extraction

There are currently commercially available kits that enable the extraction of DNA and RNA from fresh frozen tissues and from FFPE samples. Using these, it is possible to extract nucleotides from leukocytes isolated from whole blood and from kidney biopsy sections. Laser capture microdissected (LCM) tissues are particularly interesting for RNA extraction, as they allow the selective analysis of gene expression patterns, for example in the analysis of glomeruli in particular glomerulopathies. We have also shown that it is possible to extract RNA of sufficient quality and quantity from a single kidney biopsy and from around 100 LCM FFPE glomerular cross-sections to enable NGS.¹¹

Next Generation Sequencing Techniques

Despite the extensive improvement over Sanger sequencing, the basis of NGS platforms remains a polymerase.¹² Common commercial sequencing platforms are given in Table 1.^{2,4} Before NGS is performed, the extracted nucleotides have to be processed to produce a copy DNA library through a library preparation method specific to the particular platform and sequencing approach.²

ROLE OF NEXT GENERATION SEQUENCING IN INHERITED KIDNEY DISEASES

Detection of Novel Gene Mutations

With whole exome sequencing, it is possible to sequence the entire protein-coding region

of the DNA. This method has the capacity to detect novel disease causing genes. In a recent publication, researchers sequenced the whole exome of a group of children with increased renal echogenicity. Causative mutations, in this case for nephronophthisis (NPHP), Alport syndrome, or renal tubulopathy, were identified in two-thirds of the affected individuals.¹³

Congenital Anomalies of the Kidney and Urinary Tract

The most common causes of chronic kidney disease (CKD) in children are congenital anomalies of kidney and urinary tract (CAKUT), followed by inherited kidney diseases such as polycystic kidney disease.¹⁴ Both are very heterogeneous disorders with changes to a single gene, as well as complex, multigenic alterations.¹⁴ NGS approaches allow the identification of disease-causing gene mutations in one single assay and therefore provide improved diagnostic possibilities.⁷ To date, more than 20 monogenic CAKUT-causing genes have been identified. Whole genome linkage analysis can identify genomic regions of interest by comparing shared genes between affected family members. This is how single, rare, deleterious variants leading to CAKUT have previously been detected.^{15,16}

Autosomal Dominant Polycystic Kidney Diseases

A study by Trujillano et al.¹⁷ demonstrated that using NGS to verify *PKD1* and *PKD2* mutations is superior, faster, and cheaper than the methods used routinely in the diagnosis of autosomal dominant polycystic kidney disease (ADPKD), such as Sanger sequencing.¹⁸ ADPKD can also

manifest in early childhood; which is phenotypically similar to other ciliopathies, such as NPHP. In these situations, NGS helps to set the precise diagnosis, thereby improving prenatal counselling and patient management. Patients with ADPKD have an increased prevalence of intracerebral aneurysms, especially when additional risk factors are present. Therefore, they require follow-up.¹⁹

Other Inherited Kidney Diseases

NPHP, chronic tubulointerstitial nephritis, is an autosomal recessive ciliopathy and the most common inherited cause of CKD in children.⁷ The classic NPHP mutation is only detected in 30–40% of cases. NGS has led to the identification of other causal gene mutations such as the *SDCCAG8* mutation, which was identified by sequencing of 800 candidate genes in 10 families.²⁰ Accurate diagnosis of NPHP is important due to its initial unspecific clinical manifestation in children, and because of extrarenal manifestations such as neurological anomalies, hepatobiliary disease, or retinal degeneration.¹⁹

The power of NGS has also been demonstrated in a study that showed that patients with Alport syndrome who exhibit mutations of *COL4A3* and *COL4A4* genes, develop nephrotic-range proteinuria with histologic findings of focal segmental glomerulosclerosis (FSGS). Importantly, these patients did not suffer from graft loss after transplantation as could have been expected from patients with FSGS.²¹ The diagnosis of Alport syndrome, as well as rarer diseases such as Sensebrenner's or Joubert's syndrome, has been tremendously accelerated through the widespread use of NGS. Diagnosis is now available in weeks in comparison to the months required by Sanger sequencing.^{22,23}

THE ROLE OF NEXT GENERATION SEQUENCING IN GLOMERULOPATHIES

NGS has led to the detection of genetic causes in diseases that had previously been regarded as essentially non-genetic. FSGS and IgA nephropathies are diseases in which mutational analysis has crucially influenced the understanding of the pathological mechanisms and their implications for clinical management.

Steroid Resistant Nephrotic Syndrome and Focal Segmental Glomerulosclerosis

Nephrotic syndrome in children is classified into steroid resistant nephrotic syndrome (SRNS) and steroid sensitive nephrotic syndrome (SSNS). There is no causal treatment for SRNS, which inevitably leads to end-stage renal disease (ESRD).²⁴ SRNS is thus one of the main causes of ESRD in children and adolescents.²⁵ FSGS is a syndrome defined by specific glomerular damage and scarring. SRNS is a cornerstone of FSGS and often the underlying histopathological syndrome of SRNS in children.^{26,27}

Exome sequencing of around 1,700 international families with SRNS revealed a single-gene cause in one-third of the patients. The earlier the patients developed nephrotic syndrome, the more often single gene causes were identified. In 1% of the patients, a mutation within the genes influencing the coenzyme Q10 pathway was detected. This might be a new biomarker for classification, but also a new target for treatment of SRNS.²⁵ Amongst children with sporadic SRNS, heterogeneous genetic alterations were as frequent as 58%, but were not identified in children with steroid sensitive disease.²⁸ Recently, a more time and cost efficient NGS technique was developed.²⁹ The technique allows for mutation analysis of 21 genes associated with SRNS. Eighty-one adult subjects with primary FSGS/SRNS were also investigated by Gast et al.³⁰ with a targeted NGS panel. They found that mutations within various collagen IV genes were the most frequent mutations detected. SSNS on the other hand, can also be genetically influenced, illustrated by *EMP2* mutations associated with familial SSNS.²⁴

Genetic testing is also important when estimating the risk of recurrence of FSGS after transplantation. For example, none of the patients with mutations of *NPHS2* had a recurrence of FSGS after transplantation.³¹ In this context, living donors can also be tested for silent mutations, which would increase the risk of recurrence.²⁷

IgA Nephropathy

The most common glomerulonephritis is IgA nephropathy, which leads to ESRD in 15–40% of cases. The epidemiology of IgA nephropathy is very heterogeneous. It is frequent within the Asian population, but rare in Africa. This suggests a genetic cause or predisposition.³² A study examined the expression profiles of IgA nephropathy, via

transcriptome sequencing of microRNA (miRNA) with NGS techniques. Results showed that 85 miRNAs were differentially expressed and either up or downregulated in IgA nephropathy.³³ Mitochondrial DNA (mtDNA) was sequenced from the blood of patients with IgA nephropathy prior to kidney transplantation. It was found that patients with ESRD due to IgA nephropathy had more variations in mtDNA than healthy patients.³⁴

Membranous Nephropathy

miRNA was isolated and sequenced from peripheral blood lymphocytes of patients with membranous nephropathy (MN).³⁵ It was shown that miRNA profiles from patients with MN differ from healthy patients; more dysregulated miRNA could be found in patients with MN. Interestingly, more downregulated than upregulated miRNA were apparent in patients with MN, compared with the controls. miRNA could therefore play a role in the pathogenesis of MN, and could be used both as a diagnostic tool and as a potential therapeutic target.³⁴

Diabetic Nephropathy

Diabetic nephropathy (DN) is a main cause of ESRD in adults and is on the rise due to the increasing prevalence of diabetes worldwide,^{36,37} although only 40% of all patients with Type 2 diabetes develop diabetic nephropathy. There is a known genetic and hereditary disposition for increased susceptibility to developing diabetic nephropathy, which has been investigated by NGS.³⁶ NGS might also yield novel therapeutic approaches to DN: transforming growth factor beta-1 (TGF- β 1) influences the development and progression of diabetic nephropathy through glomerulosclerosis and tubular interstitial fibrosis.³⁷ A study sequenced the transcriptome of tubular cells stimulated with TGF β 1, which lead to insights into activated pathways such as NF κ B. The following sequencing of kidney biopsies with DN revealed that patients shared 179 regulated genes with the *in vitro* tubular cell line. TGF β 1 might therefore be a candidate gene to target.³⁷

THE ROLE OF NEXT GENERATION SEQUENCING IN KIDNEY TRANSPLANTATION

NGS plays a role within the assessment before, and the monitoring and diagnosis of complications after kidney transplantation.

Human Leukocyte Antigen Typing

In recent years, an abundance of new human leukocyte antigen (HLA) variants have been published. This has rendered HLA typing increasingly challenging, costly, and time consuming. NGS has increased sensitivity and facilitated the assessment of HLA status. HLA typing with NGS was compared with Sanger sequencing on a large scale in 2014. NGS was superior in time and cost efficiency. Results were concordant between both techniques.^{38,39}

Cell Mediated Rejection

T-cell mediated graft rejection (TCMR) is due to donor T cell responses towards major histocompatibility complexes. The mechanisms of TCMR were investigated through sequencing of the β locus of T cell receptors (TCR β) in patients who underwent a combined kidney and bone marrow transplant. These patients commonly display immune tolerance without immunosuppressive therapy. TCR β was sequenced using NGS prior to transplantation in order to monitor them during the adaptation towards tolerance. Results showed that donor reactive T cells were diminished in tolerant patients after transplantation. Characterisations of T cells with NGS prior to transplantation might therefore provide a new biomarker, improving the prediction of transplant outcome.⁴⁰ miRNA-10b is significantly downregulated in patients with rejected kidney allografts. A study showed that human renal glomerular endothelial cells transfected with miRNA-10b inhibitors showed increased apoptosis, proinflammatory cytokine release, and other cellular aspects also seen in graft rejection. These findings led the authors to suggest that miRNA-10b could be a novel therapeutic target in cell mediated graft rejection.⁴¹

Monitoring of Rejection

Snyder et al.⁴² described the potential use of NGS to non-invasively monitor the condition of the transplanted organ via cell-free DNA (cfDNA). Whole genome sequencing of the recipient was performed using NGS prior to the transplantation. By focussing on single nucleotide polymorphisms (SNPs), a unique fingerprint of the recipient's cfDNA was identified. After transplantation, donor cfDNA was sequenced and distinguished from host DNA through comparison in SNPs. Any increase in donor cfDNA was associated with 'injury'. In the case of heart transplant, an increase in donor

cfDNA was highly associated with acute rejection and even preceded the clinical event. This diagnostic and predictive approach could also have implications for kidney transplantation.⁴²

Diagnosis of Infections and Other Complications After Transplantation

Viral infections play an important part in the morbidity and mortality of patients after kidney transplantation.⁴³ Viral specific T cells can be detected through sequencing. NGS is of invaluable benefit in this case, because the T cell receptor has potentially indefinite variations according to the respective antigen immunisation.⁴⁴ A study showed that post-transplantational characterisation of T cells from urine, blood, and allograft with NGS helped to distinguish differential diagnosis such as infections as a reason for post-transplantation complications.⁴⁴ NGS can also be used to identify novel infectious pathogens. This was demonstrated by the discovery of a new arenavirus through the sequencing tissues originating from kidney transplant autopsies. This could not be achieved using routine diagnostics, such as polymerase chain reaction, microarray, or serological assays.⁴⁵

ROLE OF NEXT GENERATION SEQUENCING IN EPIGENETICS

Recent studies report that epigenetic variations contribute to individual patient susceptibility to develop progressive CKD.⁴⁶ Epigenetic research has advanced greatly with the emergence of NGS, which has allowed the detection of epigenetic modifications.⁴⁷ Epigenetics reflects functionally relevant changes to the genome without changing the underlying DNA sequence. Examples of such epigenetic changes are DNA methylation, histone modifications, but also long non-coding RNA fragments.⁴⁸ Epigenetic modifications can influence transcriptional regulation and thus affect gene expression, a phenomenon that is described as molecular memory. Each cell type has its specific epigenome modulated by environmental influences.^{46,48} This cell type specific epigenome is defined during development and differentiation and preserved during cell division.⁴⁹ Environmental

conditions, such as nutrition or smoking, can cause changes in the epigenome.⁴⁷

Exploration of epigenetic modification using NGS can lead to new insights into the pathological mechanisms of kidney diseases. Altered DNA methylation for example, often activates or silences genes, and therefore alters gene expression in the kidney and consequently the rate of renal disease progression. The combination of chromatin immunoprecipitation assays with NGS is a strong method for identifying genome-wide DNA binding sites for transcription factors and other proteins. This can be used in epigenetic studies, e.g. for altered binding of transcription factors and histone modifications.^{50,51}

Epigenetics in Kidney Diseases

The epigenome is a factor in the development of complex gene-environmental diseases, including CKD.⁴⁹ Epigenetic dysregulation is supposed to add to cardiovascular morbidity.⁵² As an example, miRNAs that can be identified via NGS represent epigenetic regulators influencing progression of cardiovascular disease.⁵² Genetic variants and epigenetic changes in chromatin affect gene transcription in response to environmental stimuli. This is important in the regulation of diabetes development, including vascular changes, and can be analysed by NGS.⁵³ Recently, there has been progress regarding the definition of the contribution of epigenetics to the progression of fibrosis. Fibrosis is the common pathway of progressive CKD, but displays a huge variance in individual progression.⁴⁵ Recent studies of a genome-wide methylation screen show that epigenetic modifications lead to perpetuated fibroblast activation and fibrogenesis in the kidney.⁵⁴

CONCLUSION

NGS of the whole genome, exome, or epigenome plays a crucial role for today's generation of nephrologists, both in basic and experimental research, but also in the clinical setting. Diagnosis and classification of kidney diseases have improved, but also therapeutic decision-making, which helps to avoid unnecessary therapies.

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