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An alternative to array-based diagnostics: Comparing arrayCGH to next-generation sequencing to evaluate fetal structural abnormalities.

Running title: Prenatal diagnosis using next generation sequencing.

Lesley Walker*[1], Christopher M. Watson[2,3], Sarah Hewitt, Laura A. Crinnion[2,3], David T. Bonthron[2,3], Kelly E. Cohen[1]

1: Department of Fetal Medicine, Leeds General Infirmary, Leeds, LS1 3EX, United Kingdom.

2: Yorkshire Regional Genetics Service, St. James's University Hospital, Leeds, LS9 7TF, United Kingdom

3: School of Medicine, University of Leeds, St. James's University Hospital, Leeds, LS9 7TF, United Kingdom

*Corresponding author

Address for correspondence:

Lesley Walker, MBChB MRCOG

Sub-specialty Trainee

Department of Fetal Medicine

Leeds General Infirmary

Leeds LS1 3EX

United Kingdom

Email: lesleywalker2@nhs.net

Telephone: +44 (0)113 392 7172

Abstract

Molecular diagnostic investigations, following the identification of fetal abnormalities, are routinely performed using array comparative genomic hybridisation (aCGH). Despite the utility of this technique, contemporary approaches for the detection of copy number variation are typically based on next generation sequencing (NGS). We sought to compare an in-house NGS-based workflow (CNVseq) with aCGH, for invasively obtained fetal samples from pregnancies complicated by fetal structural abnormality. DNA from 40 fetuses was screened using both 8x60K aCGH oligoarrays and low-coverage whole genome sequencing. Sequencer-compatible libraries were combined in a ten-sample multiplex and sequenced using an Illumina HiSeq2500. The mean resolution of CNVseq was 29 Kb, compared to 60 Kb for aCGH analyses. Four clinically significant, concordant, copy number imbalances were detected using both techniques, however genomic breakpoints were more precisely defined by CNVseq. These data indicate CNVseq is a robust and sensitive alternative to aCGH, for the prenatal investigation of fetuses with structural abnormalities.

Impact Statement

What is already known about this topic?

- Copy number variant analysis using next-generation sequencing has been successfully applied to investigations of tumour specimens and patients with developmental delay. The application of our approach, to a prospective prenatal diagnosis cohort, has not hitherto been assessed.

What does this study add?

- Next-generation sequencing has a comparable turnaround time and assay sensitivity to copy number variant analysis performed using array CGH.

- We demonstrate that having established a next-generation sequencing facility, high-throughput CNVseq sample processing and analysis can be undertaken within the framework of a regional diagnostic service.

Implications for clinical practice.

- Array CGH is a legacy technology which is likely to be superseded by low-coverage whole genome sequencing, for the detection of copy number variants, in the prenatal diagnosis of structural abnormalities

Keywords: Next-generation sequencing, copy number variation, fetal structural anomaly

Introduction

For several decades, invasive prenatal genetic testing has been offered to women at an increased risk of having a child with a chromosome abnormality. While G-banded karyotypes, which typically offer a resolution of between 5-10 Mb, were the original gold-standard assay, this technique has now been largely been superseded by array comparative genomic hybridisation (aCGH). By using aCGH genomic copy number variants can be detected at an increased resolution to karyotyping, allowing the identification of smaller deletions and duplications than was previously possible (Hillman et al. 2011; Bi et al. 2008; Breman et al. 2012; Wapner et al. 2012; Park et al. 2011). Consequently, a higher diagnostic detection rates have been reported for aCGH analysed cohorts of fetuses with structural abnormalities (Hillman et al. 2013; Saldarriaga et al 2015; ACOG 2013).

Recent advances in genomic analysis have been dominated by next-generation sequencing technologies, with successive models of Illumina instrumentation leading the field in terms of data volume and sequence quality. This has resulted in both diagnostic and research laboratories developing a range of novel sequence-based assays and informatics solutions. One such workflow, CNVseq, utilises low-coverage whole-genome sequencing for the detection of copy number variants (CNVs). Sequence reads are aligned to a reference genome and read counts are compared between a test sample and “normal” reference control (Chiang et al. 2009; Xie et al. 2009; Yoon et al. 2009; Zhao et al. 2013). Our laboratory has implemented this technique as a UK Genetic Testing Network approved assay that is currently being used for the molecular diagnosis of postnatally ascertained cases (Hayes et al. 2013).

As part of our ongoing research and development initiative we undertook a proof-of-concept study demonstrating how CNVseq can be usefully applied to fetal material from which poor quality DNA yields precluded an aCGH result (Cohen et al. 2015). The resolution of the abnormalities detectable from these data was limited by the per-run sequencing output, generated at that time, from an Illumina GAIIx. As NGS instrumentation has improved, the

per-run data yield has increased, a result of both extended read lengths and an increased number of clusters sequenced per run. In addition to increasing the resolution of detectable abnormalities, falling per-base sequencing costs are making sequence-based assays ever-more affordable.

Here, we report our experience using a CNVseq informatics workflow, in combination with an updated sequencing platform, to assess genomic copy number variants in a cohort of patients prospectively recruited with fetal structural abnormalities. As routine molecular diagnostic investigations were concurrently undertaken on the patients in this cohort, we are able to report the sensitivity of this technique compared to that of aCGH.

Materials and Methods

We present a cohort of 53 prospectively recruited cases from our centre, a tertiary referral fetal medicine unit, which covers a population of approximately 5 million people across the Yorkshire and Humber region of Northern England. Our study inclusion criteria included either; (i) one or more structural anomalies identified on an ultrasound scan, (ii) an isolated nuchal translucency of ≥3.5 mm, (iii) two or more ultrasound variants. Patients underwent invasive testing comprising either amniocentesis or chorionic villus sampling (CVS). Each participant was counselled in person and provided with a detailed patient information leaflet. Phenotypic descriptions were collected on anonymised data collection forms with a unique patient identifier. Women under the age of 16 were not eligible for recruitment. Ethical approval for this study was granted by the Bradford Leeds Research Ethics Committee (reference: 15/YH/0508).

DNA was extracted from prenatal samples using QIAamp DNA micro (QIAGEN Ltd., Manchester, UK) and iGENatal (IGEN Biotech, Madrid, Spain) extraction kits. Tissue cultures were established for samples with low concentration DNA extractions, as determined using a Quibit® fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Diagnostic quantitative fluorescence (QF)-PCR was performed to exclude aneuploidy of chromosomes 13, 18 and 21, triploidy and monosomy X. Array-CGH was subsequently processed on a BlueGnome ISCA 8x60k oligoarray following manufacturer's protocols (Illumina Inc, San Diego, CA, USA). Data were analysed using BlueFuse Multi software v.4.1. Identified variants were reported according to criteria defined by the Association for Clinical Genetic Science, Royal College of Obstetricians and Gynaecologists, American College of Medical Genetics standards and guidelines and local policy (Association for Clinical Cytogenetics. 2009; The Royal College of Pathologists. 2015; Kearney et al. 2011). Expected values for the quality control metrics calculated by the BlueFuse software are detailed in Supplementary Table 1. All samples not meeting these criteria were classified as "suboptimal" (Table 1) (Illumina. 2014).

In addition to aCGH, each sample was processed using a previously validated CNVseq workflow (Watson et al. 2014). Briefly, 200 ng (batches 1, 2 and 4) or 500 ng (batch 3) of genomic DNA, quantified using a Qubit® dsDNA Broad Range Assay (Thermo Fisher Scientific, Waltham, MA, USA), was sheared using a Covaris S2 (Covaris Inc., Woburn, MA, USA). Fragment size was assessed using an Agilent Bioanalyzer high sensitivity chip (Agilent Technologies Ltd., Stockport, UK). Illumina compatible whole genome sequencing libraries were prepared using NEBNext® Ultra™ reagents (New England Biolabs, Ipswich, MA, USA). AMPure bead size selection producing a library insert size of approximately 200bp was performed. End-repair and adaptor ligation were undertaken as outlined in the manufacturer's protocol. The quality and concentration of each final library was determined using an Agilent Bioanalyzer and a Quant-iT™ Picogreen® assay (Thermo Fisher Scientific, Waltham, MA, USA). Equimolar concentrations of 10 libraries (11 for batch 4), were pooled for sequencing. This was performed using single-end 51bp reads across two lanes of a HiSeq2500 Rapid flowcell (Illumina Inc., San Diego, CA, USA). Raw sequence data was converted to FASTQ.gz format using CASAVA v.1.8.3. Sequence reads from each sample were aligned to an indexed

human reference genome (hg19) using bwa aln v.0.6.2 (http://bio-bwa.sourceforge.net) (Li and Durbin. 2009). Duplicate reads were marked and removed from coordinate sorted BAM files using Picard v.1.85 (http://broadinstitute.github.io/picard/). Genomic coordinates of uniquely mapped test and reference reads (those with a MAQ value ≥37) were extracted using samtools v.0.1.18 (https://sourceforge.net/projects/samtools) (Li et al. 2009). Read counts were adjusted to account for variations in local GC% and the resulting output was loaded into the R module DNA copy v.1.32.0 which segments the data into regions of equal copy number (Venkatraman and Olshen. 2007). Quality control criteria for CNVseq analyses were based on empirically determined metrics established while validating our post-natal CNVseq workflow.

Genomic databases including OMIM (https://omim.org), the Database of Genomic Variants (http://dgv.tcag.ca) (MacDonald et al. 2014), and Decipher (https://decipher.sanger.ac.uk) (Firth et al. 2009) were used to determine the clinical significance of variants identified by both the aCGH and CNVseq workflows.

Results

Fifty-three women consented to participate in the study between January and August 2016. An abnormal QF-PCR result was obtained for thirteen samples and these were excluded from further analysis (Supplementary Table 2).

The remaining 40 samples were analysed using both the aCGH and CNVseq workflows (sample CS08 was repeated as initial output data was uninterpretable). Twenty-three samples were obtained by amniocentesis and 17 by CVS. Thirty-two were used directly while 8 required culturing to increase the total cell count prior to DNA extraction (6 samples were obtained by amniocentesis and 2 were obtained by CVS). Of the 8 specimens that required culturing, 3

produced suboptimal microarray results yet robust CNVseq results were generated from all samples.

The DNA concentration of extracted samples ranged from 13-656 ng/µl. For eleven samples these were categorised as being low (<100 ng/µl). These samples provided an insufficient mass of DNA to meet the suggested input requirement for CNVseq library preparation. Despite this, all low concentration DNA samples provided robust CNVseq results and only 1 had a suboptimal array result.

Seven of the 40 microarray results were determined to be of “suboptimal” quality for reporting purposes following the application of manufacturer recommended quality control parameters and review of these data by experienced cytogeneticists. A mean of 4 (range 1-8) variant calls per case were generated by the BlueFuse Multi algorithm. These automatic calls included variants smaller than our reporting size threshold.

Per-batch sequencing metrics for all CNVseq data are summarised in Supplementary Table 3. Intra-batch per-sample read distributions were most dispersed for pool 1 (range 6.6-16.0%) and tightest for pool 3 (range 8.5-11.2%). CNVseq assay resolution is dependent on the number of uniquely mapped reads per-sample; the minimum number was 17.4 million reads (maximum 46.5 million; mean 28.2 million) providing a minimum average resolution of 17 kb (maximum 46 kb; mean 29 kb) (Supplementary Table 4). The mean number of calls generated per-sample was 38. Sample CS01 was an apparent outlier, having 191 calls, of which only 46 were >30 kb. The median number of calls per case for the CNVseq cohort was 33. This increased number of calls, identified in comparison to the aCGH dataset, is not surprising given the non-targeted nature of these data.

Excluding benign CNVs, and those CNVs that did not intersect disease causing genes, clinically significant sub chromosome-level imbalances were identified in 3 cases (Table 2).

The detection of identified CNVs were concordant between both aCGH and CNVseq datasets. A low-level trisomy 2 mosaicism was evident in fetus CS12, which presented with coarctation of the aorta. As the sample was obtained by CVS, and in light of the unlikely fetal phenotype, it was reported as a likely confined placental mosaicism (Figure 1).

Case CS19 presented with intrauterine growth restriction, posterior fossa abnormality and echogenic bowel. Both aCGH and CNVseq workflows identified a heterozygous terminal-arm deletion of 21 Mb between 10p12.31 and 10p15.3 (Figure 2). This region encompasses 88 genes of which 16 are listed as pathogenic in the OMIM database. This was the only terminal-arm deletion identified in the cohort.

The smallest clinically significant variant was identified in case CS25 which presented with echogenic kidneys and polyhydramnios. Although the aCGH quality control metrics classified the data from this array as being of suboptimal quality, the 1.4-1.9 Mb interstitial deletion, located at 17q12, was clearly distinguishable. Furthermore, the presence of this variant was corroborated by the CNVseq data (Figure 3). This phenotype has been previously described secondary to mutations or deletions of TCF2 (OMIM: 137920) (Gilboa et al. 2016).

A further copy number imbalance was detected in case CS27, a fetus that presented with bowel dilatation and polyhydramnios. The 22q11 deletion was approximately 2.6 Mb in size (Figure 4). This microdeletion syndrome, also referred to as Velocardiofacial or Di George Syndrome, encompasses more than 50 genes and is a known pathogenic variant likely to be responsible for the ultrasound features seen in this case.

Discussion

This study has demonstrated that NGS-based CNV-Seq technology can be used to investigate structurally abnormal fetuses with comparable genomic resolution, quality control metrics and turnaround times as array CGH in the prenatal setting. CNV-Seq successfully identified all of the genomic variants detected by array CGH, with similar resolution achieved despite the batch processing of ten samples in the CNV-Seq workflow. This potential for high-throughput analysis is an advantage of sequencing-based analysis over array CGH.

In the cases where a clinically-significant imbalance was detected, genomic breakpoints were more easily defined using CNV-Seq. The digital nature of sequencing-based technology allows exact genomic positions to be elucidated, and genomic breakpoints and variant sizes can be inferred from these positions. Deleted probes on the array platform may fall out with of the actual genomic breakpoints and impact on the $\log_2$ ratio for that region, reducing the ability to accurately pinpoint genomic size or positions. Such information may be of clinical value, especially where breakpoints are close to or intersect with clinically-important genes. It has been reported previously that low level mosaicism is easier to identify using a CNVseq, rather than aCGH workflow (Grotta, 2015). The specimen with confined placental mosaicism in this cohort was more easily visualised in the CNVseq karyogram compared to the array result. However, it is not possible to be certain of this with only a single mosaic sample analysed.

One of the chief advantages of is the ability to influence platform resolution by in silico manipulation of window size, which can be performed after processing. This is not possible using an aCGH platform where genomic resolution is fixed by the probe density defined by the reagents. CNV-Seq resolution is also adjustable by alterations in the number of samples processed within the batch; a smaller number of samples will share the same number of reads, increasing the read-count per patient and allowing smaller imbalances to be detected. Higher order multiplexing of tens of samples is possible, but at the cost of reduced platform resolution. Such compromises may be important if the desired analysis was limited to large scale genomic abnormalities such as trisomies or large deletions and duplications. Overall, our current

CNVseq workflow configuration produces a per-patient resolution that significantly exceeds that obtained from the aCGH assays.

For the purposes of this study, the genomic resolution was chosen to mimic that of the array CGH platform across the genome represented by probes within. In this study no additional clinically-significant variants were detected by the CNV-Seq platform, but a larger number of detected calls were made by the analysis pipeline when compared to array CGH. This is not surprising given the non-targeted nature of the platform. Importantly, the CNV-Seq platform did not generate any false positive calls which would have impacted on the clinical management of the fetal structural abnormality, and the majority of the calls were below the pre-defined thresholds for further investigation.

This increase in potential calls did have an effect on the post-processing time required to analyse and report a CNVseq sample although the processing time for the technology was similar for both platforms.

Strategies could be employed to further reduce analysis time, improving clinical utility of this platform. Filters can be introduced to remove frequently-occurring benign copy number variants, and reporting thresholds altered to limit the requirement for molecular confirmation studies. It is important to note that despite the increased genomic resolution of the CNVseq workflow, no additional variants of uncertain significance were detected. This finding will provide reassurance to clinicians who are naturally wary of the challenges posed by prenatal variants of uncertain significance.

Strengths of this study include the introduction of a novel technology into the prenatal setting, offering a credible alternative to array CGH analysis. Although beyond the scope of this work, analysis of postmortem samples or DNA from pregnancy loss tissue such as stillbirths may also benefit from this technology, especially when DNA is fragmented or in very small

quantities. Currently, failed array experiments lead to a ‘no result’ clinical report. The addition of NGS-based technology allows repeat analysis and potentially, increased information for parents.

The study is limited by the number of participants recruited, which resulted in a small number of clinically-significant variants to base analysis upon. The relative infrequency of genomic abnormalities in structurally abnormal fetuses is a barrier common to most studies of genomic analysis.

The initial capital costs of establishing a CNVseq workflow are undoubtedly more significant than those associated with purchasing a microarray scanner. Despite this, many regional genetics laboratories in the UK are now equipped with sequencing core facilities that provide an opportunity to perform high throughput NGS.

Conclusion

We demonstrate CNVseq to be a reliable and robust alternative to aCGH when used for the prenatal diagnosis of structural abnormalities. Sequencing has a similar turnaround time and comparable detection rate of copy number variants when compared to aCGH. The most significant initial difficulty to widespread implementation is the high capital cost of establishing the required next-generation sequencing infrastructure. As large, population-scale programmes such as the 100K Genome Project are conducted, it is likely that sequencing based-methods will become a first-line test for prenatal diagnostics in the near future.

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Declaration of interests

The authors report no declarations of interest.

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Figure Legends

Figure 1: Case CS12 showing **(A)** A BlueFuse Multi view datapoints across all chromosomes and **(B)** a comparable karyogram view from the CNVseq workflow. The low-level trisomy 2 is visible by the raised baseline segmentation for this chromosome (horizontal red line).

Figure 2: Case CS19 showing **(A)** a heterozygous terminal deletion of the chromosome 10 p-arm from the BlueFuse Multi software. **(B)** The corresponding deletion as detected by the CNVseq workflow.

Figure 3: Case CS25 showing **(A)** a heterozygous interstitial deletion on the chromosome 17 q-arm from the BlueFuse Multi software. **(B)** The corresponding deletion as detected by the CNVseq workflow.

Figure 4: Case CS27 showing **(A)** a heterozygous interstitial deletion on the chromosome 22 q-arm using the BlueFuse Multi software. **(B)** The corresponding deletion as detected by the CNVseq workflow.