Chapter 1
Spinal Muscular Atrophy: Evaluation of New Emerging Methods for Carrier Screening and Diagnosis

Sim Joylynn, Jinghan Xie, Tan Grace Li Xuan, Liu Chun Ping, and Lai Poh San

Abstract Spinal muscular atrophy (SMA) is the most common genetic cause of infant mortality. Homozygous deletion of *Survival Motor Neuron (SMN)* 1 causes the disease in 95% of cases, but severity varies with the copy number of *SMN2*. The nearly identical sequences of the *SMN* genes make differentiation between them difficult, and current methodologies such as multiplex ligation-dependent probe amplification (MLPA) and real-time PCR (RT-PCR) present limitations for diagnosis. In this report, we analyse SMA samples of known copy number to compare new emerging technologies (Linked-Reads, Cytoscan array and next generation sequencing) against MLPA. MLPA analysis was the only method able to report the known copy numbers of the samples, making it still the most reliable means of SMA carrier screening and diagnosis. However, the three new methodologies are still emerging technologies for routine diagnostics compared to MLPA, and with further optimisation, they could greatly improve SMA carrier screening and diagnosis.

Keywords Spinal muscular atrophy (SMA) · SMN1 · SMN2 · Linked-Reads · Cytoscan (CYT) array · Whole genome sequencing (WGS) · Multiplex ligation-dependent probe amplification (MLPA)

1.1 Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive motor neuron disease with an annual incidence of about 1 in 6000 to 1 in 10,000 live births, and the carrier frequency is as high as 1 in 40 [1]. 95% of SMA patients are homozygous for

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SMN1 deletion. *SMN2* partially compensates for *SMN1* loss, lowering the severity of SMA. However, when two *SMN1* genes are carried in cis, this results in a silent carrier (i.e. a carrier that does not express the S*f*MA phenotype) [2, 3]. Further complicating diagnosis, *SMN1* and *SMN2* are nearly identical inversions [4], differing by only five base pairs: c.835−45G>A, c.840C>T, c.*3+100A>G, c.*3+214A>G and c.*248A>G [5]. Therefore, there is a need to (1) differentiate between *SMN1* and *SMN2*, (2) call copy number (CN) of *SMN1* and *SMN2* and (3) determine the phase of *SMN1* and *SMN2* for SMA clinical classification, prognosis, carrier identification and diagnosis [6]. Current methodologies such as multiplex ligation-dependent probe amplification (MLPA) and real-time PCR (RT-PCR) present some limitations. MLPA is unable to determine the phase of *SMN1* and *SMN2*, resulting in the inability to identify silent carriers with 2 *SMN1* on one chromosome but none on the other, and hence, false negative results in silent carriers. *SMN* genes are unevenly amplified in RT-PCR, which leads to inaccurate results. Therefore, in order to overcome these limitations, we evaluated new emerging methods—Linked-Reads, Cytoscan (CYT) array and whole genome sequencing (WGS)—against MLPA, the most commonly used method for carrier screening and diagnosis for SMA, by their ability to: 1. differentiate between *SMN1* and *SMN2*, 2. determine the CN of *SMN1* and *SMN2*, 3. locate structural variants in *SMN1* and *SMN2* and 4. phase alleles.

1.1.1 Hypothesis

We hypothesise that Linked-Reads, CYT array and WGS can overcome the limitations of current methods in determining patient or carrier status by differentiating between the two almost identical *SMN* genes, as well as calling CN in trans.

1.2 Methods

1.2.1 Sample Information

A total of six anonymised data sets from SMA patients with known *SMN1* and *SMN2* CN were provided by the National University of Singapore, Department of Paediatrics.

1.2.2 Technologies

1. Linked-Reads

DNA was sheared and put through size selection. The ChromiumTM system was then used for automated barcoded library construction. The barcoded libraries were then sequenced using Illumina Whole Exome Sequencing (WES). The data obtained was visualised on Loupe, a genome browser by 10× Chromium Platform designed for visualisation of Linked-Reads data [7]. The BAM file obtained was also visualised on Integrative Genome Browser (IGV).

2. CYT Array

Gene probes were deposited on a chip. cDNA, labelled with either green or red fluorescence, was generated from mRNA extracted. cDNA complementary base pairs with probes on the chip were analysed by fluorescence emission. The data obtained was visualised on Chromosome Analysis Suite (ChAS). Manual guides provided by Thermo Fisher Scientific Inc. were used in aiding the usage of software [8].

3. WGS

Patient DNA was sequenced through whole genome sequencing (WGS) at 40× read depth. The BAM data obtained was visualised on IGV. User guides provided by the Broad Institute (2018) were used in aiding the usage of software. Genome Reference Consortium Human Build 37 (hg19) was used as reference for WGS samples.

4. MLPA

DNA strands were denatured to separate the strands and hybridised with probes. The right probe oligo contains a stuffer sequence which is used to identify DNA pieces. The DNA was then amplified through PCR. MLPA amplicons are separated by length using capillary electrophoresis [9]. The measured fluorescence was visualised as a peak pattern and used to quantify each probe. CN was determined using probe ratio.

5. CN calling using SMN/mean read depth ratio

The c.840C>T site on exon 7 is the critical difference between *SMN1* and *SMN2*. Additionally, we were provided with the read depths of *SMN1* and *SMN2* exons 7 and 8 for each of our samples. Thus, using protocols modified from [10, 11], we determined the CN for our Linked-Reads and WGS samples by comparing the read depth of *SMN1* and *SMN2* exons 7 and 8 against each sample's overall mean read depth using the following formula.

$$\text{Copy number} = \frac{\text{Read depth of exon}}{\text{Mean read depth of sample}} \times 2$$

1.3 Results

1.3.1 *Linked-Reads*

Linked-Reads sequencing generates reads with an integrated barcode which traces the reads back to the original DNA molecule [12]. This allowed the reads to be mapped to the *SMN1* and *SMN2* genes, as shown from the read coverage in Fig. 1.1. The coverage of the c.840C site on *SMN1* and c.840*T* site on *SMN2* was also verified on IGV, confirming that Linked-Reads was able to differentiate between the SMN genes.

Structural variants (SVs) can be detected through calls and candidates recognised by Linked-Reads, where calls meet the higher-quality call threshold than candidates and occur in unambiguous regions of the reference genome. However, no SVs were called by Linked-Reads in our samples. A deletion of exon 7 in *SMN1* was observed in Sample *300,099* when viewing the reads in IGV, corresponding to the known *SMN1* CN of 0.

CN was also calculated by comparing the read depths of exons 7 and 8 in *SMN1* and *SMN2* against each sample's mean read depth. As seen in Table 1.1, there are discrepancies between the calculated and known CN, indicating that observed read depths underestimate the actual CN. This discrepancy could be due to difficulties in sequencing for the following reasons: 1. *SMN1* and *SMN2* genes are part of a 500 kb highly repetitive inverted duplication on chromosome 5, making it difficult to determine the organisation of this genomic region [13]; 2. high GC level of 54% in *SMN1* and *SMN2* [14], leading to a poor coverage of reads and less complete

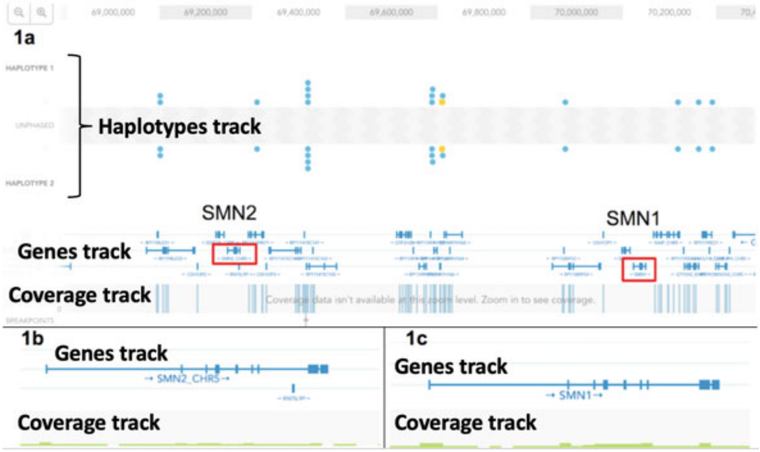


Fig. 1.1 **a** *SMN1* and *SMN2* on sample 300,097. **b**, **c** *SMN2* (left), *SMN1* (right). Green bar in the coverage track indicates read depth for the region. Genes and their exons are identified and labelled in the genes track

Table 1.1. CN call for *SMN1* and *SMN2* exons 7 and 8 of samples 300,097–99

	Calculated CN				Known CN			
Sample	SMN1		SMN2		SMN1		SMN2	
	exon 7	exon 8	exon 7	exon 8	exon 7	exon 8	exon 7	exon 8
300,097	1	1	0	0	2	2	1	1
300,098	1	2	0	1	2	2	2	2
300,099	0	1	1	1	0	0	3	2

assembly; 3. low input DNA mass of 0.4–0.5 ng, which was below the recommended range of 1–3 ng, affecting the performance of sequencing.

Linked-Reads is able to phase alleles by assembling long reads from short reads, creating a phase block by utilising continuous reliable heterozygous variants (phasing quality > 23) to connect the reads [15]. However, in this analysis, reads in *SMN1*, *SMN2* and their flanking regions in samples 300,097–30,099 were not assigned to either haplotype as there were insufficient single-nucleotide variants (SNVs) [15] present in our samples that were informative for Long Ranger to determine phase blocks.

1.3.2 CYT Array

Both *SMN* genes are labelled as *SMN1* and *SMN2* simultaneously by ChAS (Fig. 1.2), showing that ChAS is unable to differentiate between *SMN1* and *SMN2*.

Mean weighted Log2 ratio and smooth signal values were calculated by ChAS and used to determine the CN. Log2 ratio indicates gain or loss in genetic material, with a ratio of 0 indicating a CN of 2. The Log2 ratios of *SMN1* and *SMN2* in CYT34 and CYT221 are close to 0, indicating that CN for both genes in both samples is 2. Smooth signal is a smoothed calibrated estimate which can represent non-integer CN. It uses the Gaussian function to reduce noise within the array, thus allowing for a more accurate CN to be determined. The smooth signal values of *SMN1* and *SMN2* in CYT34 and CYT221 are also close to 2, corroborating the Log2 ratio calculated CN of both samples. However, there is a discrepancy between the calculated and the known CN (Table 1.2), as Cytoscan is unable to differentiate between highly

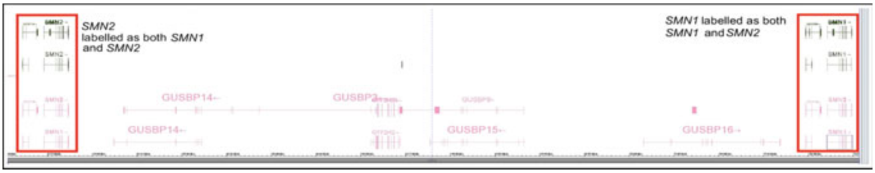

SMN2
SMN2 labelled as both SMN1 and SMN2
SMN2
SMN2
SMN1
GUSBP14
GUSBP3
GUSBP14
GUSBP15
SMN1 labelled as both SMN1 and SMN2
SMN1
SMN1
GUSBP16
SMN2
SMN1

Fig. 1.2 *SMN1* and *SMN2* annotation in ChAS

Table 1.2. Mean weighted Log2 ratio, smooth signal values, calculated and known CN of samples CYT34 and CYT221

Sample/gene	Data type	Mean	Calculated CN	Known CN
CYT34/*SMN1*	Weighted Log2 ratio	– 0.03	2	2
	Smooth signal	1.969	2[a]	
CYT34/*SMN2*	Weighted Log2 ratio	– 0.051	2	1
	Smooth signal	1.942	2[a]	
CYT221/*SMN1*	Weighted Log2 ratio	0.159	2	1
	Smooth signal	2.398	2[a]	
CYT221/*SMN2*	Weighted Log2 ratio	0.191	2	3
	Smooth signal	2.402	2[a]	

[a] value rounded down to whole number

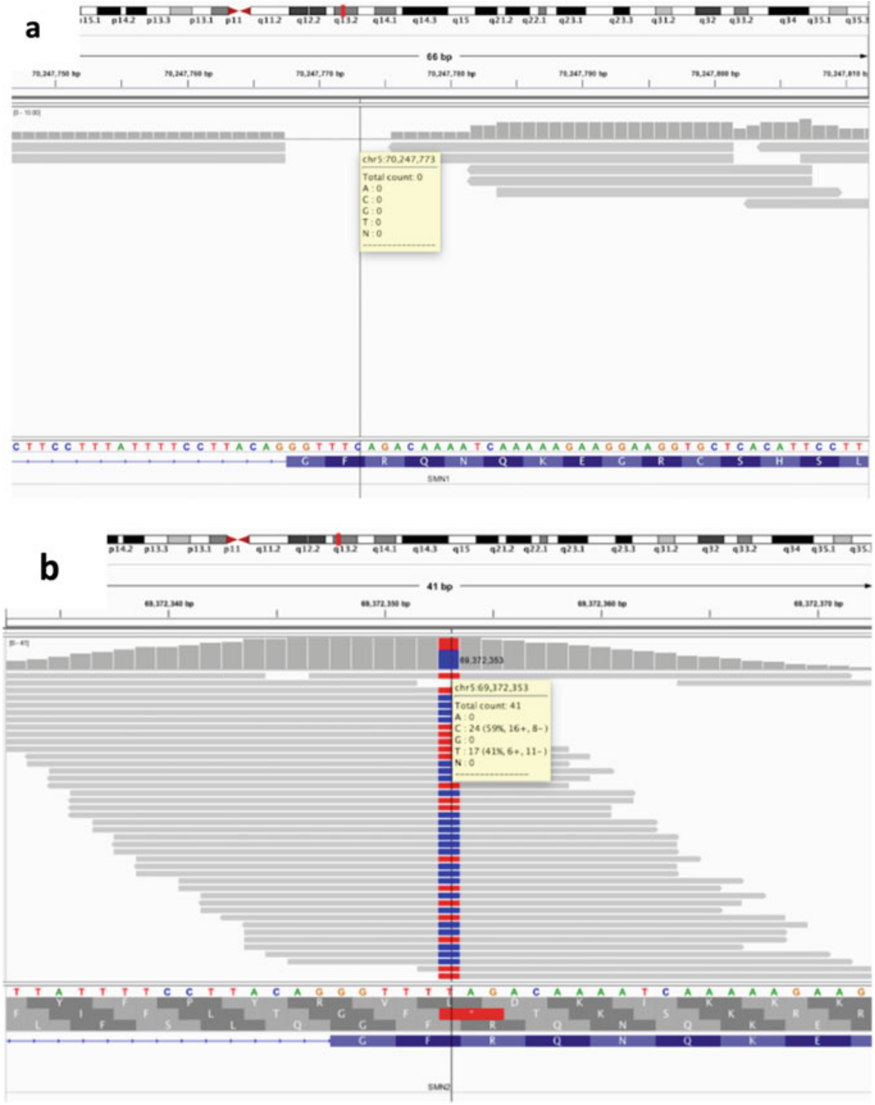
homologous regions such as *SMN1* and *SMN2* [15].

1.3.3 WGS

WGS was able to identify the *SMN1* and *SMN2* genes, as indicated by the presence of reads in these regions. Reads that align with the reference sequence are displayed in grey. However, WGS was not able to differentiate between the two genes well. Out of the five base pair differences in the *SMN2* genes, a misalignment of *SMN1* reads to *SMN2* was observed in four of these regions in sample NGS-1108 (Fig. 1.3). Critically, at the crucial c.840 site, no reads were observed in *SMN1*, but reads were mapped to C (24 reads) and T (17 reads), respectively, in *SMN2* (Fig. 1.3). This misalignment reflects WGS's inability to distinguish between homologous regions such as the *SMN* genes [16] (Table 1.3).

1.3.4 MLPA

SMN1 and *SMN2* genes were identified and differentiated by MLPA (Table 1.4) using probes specific to *SMN1* and *SMN2* exons 7 and 8. CNs were deduced from probe ratios provided by the manufacturer [9]. Sample O221 had a single copy of *SMN1* exon 7 and exon 8 and is, therefore, a carrier of SMA. Sample O34 was detected to have two copies of *SMN1*, which indicates that the patient is unaffected. However, this is only true when the *SMN1* genes occur in trans. As MLPA does not phase alleles, it cannot confirm that O34 is not a silent carrier. Sample O34 has two copies of *SMN2* exon 7, but only one copy of *SMN2* exon 8. This indicates a deletion of *SMN2* exon 8 in sample O34. Hence, the second copy of *SMN2* is not a full functional



```
chr5:70,247,773
Total count: 0
A : 0
C : 0
G : 0
T : 0
N : 0
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```
chr5:69,372,353
Total count: 41
A : 0
C : 24 (59%, 16+, 8-)
G : 0
T : 17 (41%, 6+, 11-)
N : 0
```

Fig. 1.3 **a** c.840C on *SMN1* of sample NGS-1108. No reads were observed. **b** c.840T on *SMN2* of sample NGS-1108. Bar is coloured in proportion to the read count of each base. Cytosine is in blue, and thymine is in red

Table 1.3. CN call for *SMN1* and *SMN2* exons 7 and 8 of sample NGS-1108

Calculated CN				Known CN			
SMN1		SMN2		SMN1		SMN2	
exon 7	exon 8	exon 7	exon 8	exon 7	exon 8	exon 7	exon 8
0	1	0	1	2	2	1	1

Table 1.4. Determined CN from probe ratio, in comparison with known CN for sample O34

	O34			O221		
Gene/exon number	Probe ratio	Calculated CN	Known CN	Probe ratio	Calculated CN	Known CN
SMN1 exon 7	0.75	2	2	0.66	1	1
SMN1 exon 8	0.89	2	2	0.61	1	1
SMN2 exon 7	0.76	2	2	2.49	5	5
SMN2 exon 8	0.44	1	1	1.97	4	4

gene. MLPA can be considered a reliable tool for determining CN, as the obtained CN was consistent with the known CN (Table 1.4).

1.3.5 Comparison of Linked-Reads, CYT Array and WGS with MLPA

These newly emerging methods—Linked-Reads, CYT array and WGS—were compared against MLPA. Linked-Reads is capable of differentiating between the highly homologous *SMN1* and *SMN2* genes, a critical factor which measures up to MLPA and sets it apart from CYT array and WGS. Linked-Reads identification of molecules is more reliable than CYT array as it uses different "identification codes" for each molecule [12], whereas CYT array uses microarray analysis, which utilises probes [17] that are similar for the highly homologous *SMN1* and *SMN2* genes. This is also a drawback of WGS as reads from *SMN1* may misalign to a highly homologous *SMN2* gene during sequence assembly due to short read length.

In this analysis, we used a simple method of estimating CN using Linked-Reads and WGS data by comparing the read depths of *SMN* exons 7 and 8 to each sample's mean read depth, due to the lack of access to sophisticated computational pipelines or software (such as those described by [18]) for determining CN. Our method gives an approximate estimation which would have to be confirmed either computationally or through wet experiments. Nevertheless, as we expect CN to be either in terms of deletion or between two to four, the exact value of the increased read depth can be rounded off. While Linked-Reads was able to detect a deletion of exon 7 in *SMN1* corresponding to sample *300,099*'s *SMN1* CN of 0, the CNs of *SMN1* and *SMN2* could not be accurately determined using Linked-Reads and WGS data, unlike in MLPA.

Due to the lack of heterozygous SNVs in close proximity within SMN regions, Linked-Reads was not able to determine the haplotype of *SMN1* and *SMN2* in our samples, which is important for identifying silent carriers of SMA. However, if there were adequate heterozygous SNVs in SMN regions, Linked-Reads would be able to determine phase blocks and resolve haplotypes [19], giving it a huge advantage over the other three methods.

1.4 Conclusion

Considering Linked-Reads' ability to differentiate between *SMN1* and *SMN2* genes, identify SNPs, and its potential ability to identify SVs and phase alleles to determine haplotypes, Linked-Reads can be viewed as a possible tool for carrier screening and diagnosis as it presents the ability to overcome limitations of MLPA and RT-PCR, which is the inability to phase *SMN1* and *SMN2* and uneven amplification of genes, respectively. Although Linked-Reads and WGS were not able to call *SMN* gene CNs accurately in this analysis, further work can be done to optimise the technology to be up to par with MLPA's ability to call CN. To overcome the limitation of low read depth, normalisation of read counts can be done to account for GC bias [19], and DNA input mass can be increased to 1 ng per library [20].

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