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LETTER TO THE EDITOR

A New Next-Generation Sequencing-Based Assay for Concurrent Preimplantation Genetic Diagnosis of Charcot-Marie-Tooth Disease Type 1A and Aneuploidy Screening

Charcot-Marie-Tooth (CMT) disease is the most common hereditary neuropathy, with a population prevalence of 1 in 2500. CMT disease type 1A (CMT1A), accounting for ∼70% of CMT1 cases and ∼50% of all CMT cases, is transmitted in an autosomal dominant manner. CMT1A maps to chromosome 17p11.2 and is caused, in the majority of cases, by a 1.4-Mb tandem duplication that includes the peripheral myelin protein22 (*PMP22*) gene (Li et al., 2013). The disease usually presents in the first 20 years of age, causing difficulty in walking or running, distal symmetrical muscle weakness and wasting, and sensory loss (van Paassen et al., 2014).

Preimplantation genetic diagnosis (PGD) for CMT1A is routinely undertaken (Harper et al., 2012) and, to our knowledge, is mainly based on linkage analysis with short tandem repeat markers (STRs) localized within the duplication region (De Vos et al., 2003; Lee et al., 2013). However, using linkage analysis, quite a few cases of diagnosis failure and misdiagnosis caused due to allele drop-out (ADO) and undetected recombination events have been reported in PGD (Wilton et al., 2009; Altarescu et al., 2013). Although multiple polymorphic markers are generally used to decrease the chances of diagnosis failure and misdiagnosis, for some families, the identification of mutation-proximal markers might be challenging owing to the lack of variability in these genomic regions. Moreover, the approach based on linkage analysis usually needs to be tailored to the disorder and to the individual family under investigation; the strategies also need to be optimized for a single cell or for a small number of cells. Both of these procedures can be time-consuming and labor-intensive. Furthermore, aneuploidy screening is considered to be significant in PGD of monogenic diseases, since a high percentage of aneuploidy in early human embryos is well-documented (Ambartsumyan and Clark, 2008; Mantzouratou and Delhanty, 2011) and the pregnancy rate would increase if euploid embryos are prioritized for transfer (Harton et al., 2013). Therefore, an approach that does not require individual-specific validation of linkage markers and that is capable of detecting the CMT1A duplication, combined with screening of aneuploidy in a single test, would potentially find widespread clinical application in PGD of CMT1A and other disorders caused by similar microduplications and microdeletions.

The rapid development of next-generation sequencing (NGS) technologies, which may ultimately provide a number of advantages, including reduced costs and enhanced precision as well as parallel and customizable analysis of multiple samples in a single sequencing run, has generated an increasing interest in determining whether NGS could be reliably applied to chromosomal copy number variation (CNV) analysis. Our previous study showed that low-coverage shotgun sequencing of approximately five million mapped sequencing reads allocated to sequential 60-kb sequencing bins across each chromosome could detect approximately 0.1 Mb CNVs with a total of 10 ng of input genomic DNA (Liang et al., 2014). Another study also demonstrated the potential of bioinformatic pipelines to overcome bias from single-cell whole genome amplification (WGA) and to detect CNVs larger than 1 Mb through low-coverage massively parallel sequencing (Zhang et al., 2013). We hypothesize that the NGS-based CNV analysis could be used for PGD to detect microduplications and microdeletions after single-cell WGA. Although existing studies have developed and validated NGS-based protocols for preimplantation genetic screening (PGS) of aneuploidy (Fiorentino et al., 2014) and for PGD of chromosomal abnormalities (Wang et al., 2014; Li et al., 2015), the clinical feasibility of the NGS-based CNV analysis in PGD for microduplication or microdeletion (e.g., the 1.4-Mb CMT1A duplication) syndromes has not been validated.

We present here an NGS-based reliable approach to detect the CMT1A duplication and determine the comprehensive chromosomal composition simultaneously at the single-cell level after multiple annealing and looping-based amplification cycles (MALBAC)-based WGA. The approach was applied to embryos from a female CMT1A patient; the results of NGS-based analysis were then compared to those of PGD based on linkage analysis (STRs within the 1.4-Mb region and

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single nucleotide polymorphism markers (SNPs) flanking the region).

The patient inherited the mutation from her affected mother, and a natural pregnancy was terminated after prenatal diagnosis of CMT1A by PCR combined with restriction enzyme digestion and linkage analysis in another unit. Multiplex ligation-dependent probe amplification (MLPA) using the SALSA P405-A1 CMT kit (MRC-Holland, Netherlands) was performed to further validate the presence of the mutation in our unit, which showed duplication in the female partner (proband, II1) and her affected mother (I1), and absence of duplication in her unaffected father (I2) and the male partner (II2) (Fig. S1). Subsequently, candidate STRs and SNPs within and flanking the CMT1A duplication region were amplified (using the conditions showed in Tables S1−S4) and tested for informativity in the family through segment length analysis and Sanger sequencing on the ABI 3130 genetic analysis system (Applied Biosystems, USA). Four informative STRs and nine informative SNPs were identified. A haplotype map was constructed for the family based on informative STRs (Fig. S2). The informative SNPs are listed in Table S5.

The NGS-based approach to detect the CMT1A duplication was developed and validated using single lymphocytes. The lymphocytes were isolated from peripheral blood of II1, II2, and three random normal controls (C_1, C_2, and C_3) from unrelated families by using density gradient separation (Sigma-Aldrich, USA). Ten lymphocyte cells from each peripheral blood sample were retrieved individually to validate the reproducibility of the assay. The MALBAC single-cell WGA method was used for amplifying genomic DNA from a single lymphocyte (the electrophoresis results and the concentration of WGA products are showed in Fig. S3 and Table S6, respectively), according to the standard kit protocol (Yikon Genomics, China). Using an Illumina HiSeq 2500 platform, the amplified genome of each sample was sequenced for 10 million reads (the sequencing data are accessed in BioProject of NCBI: PRJNA298442, and the information of the data is summarized in Table S7). CNV analysis was performed as previously described (Zong et al., 2012). Briefly, read numbers were counted and displayed with a bin size of 1 Mb along the whole genome. For a genome region, the gain of copy number from two to three results in a 50% increase in read counts, and the loss of copy number from two to one results in a 50% decrease in read counts. We then focused on the 1.4-Mb CMT1A duplication (chr17:14,200,000−15,600,000) and 2 Mb of its flanking regions (chr17:12,200,000−14,200,000 and chr17:15,600,000−17,600,000) for CNV analysis. Read numbers were counted and displayed with a bin size of 100 kb along this 5.4-Mb region. Sliding windows of ten bins were used and displayed along the region. Relative read numbers were extracted and averaged, and then compared with those of the three random normal references. The 1.4-Mb duplication was directly detected by NGS in each of the 10 tested lymphocytes of II1, in comparison with the three random normal references, while the copy number of this region in lymphocytes of II2 was normal, as expected (Fig. 1A and B).

The assay was subsequently applied to embryos from the female CMT1A patient. She underwent a routine ovarian stimulation and intracytoplasmic sperm injection (ICSI) procedure. Among the 21 oocytes retrieved and subjected to ICSI, 19 were fertilized. Fourteen good-quality two-pronuclear embryos with more than six cells and less than 15% fragments on day 3 were biopsied for testing. A single blastomere was aspirated from each embryo and was amplified using the MALBAC method. The linkage analysis based on informative STRs and SNPs was performed. The spare WGA products were subjected to NGS for CNV analysis. The NGS results were analyzed blindly to the results of linkage analysis. Nine of the fourteen embryos were absent of CMT1A duplication based on linkage analysis (Fig. S2 and Table S8), which was concordant with NGS-based CNV analysis. Five of the nine CMT1A duplication-free embryos were non-transferable owing to aneuploidy (Embryos_3, 4, 8, 10, and 12). Of all the 14 embryos, nine (64.29%) showed chromosomal or segmental aneuploidy. Typical comprehensive results of embryos classified as normal (CMT1A-free and euploidy) or abnormal (CMT1A-affected and/or aneuploidy) are showed in Fig. 1C−H. The results of NGS-based CNV analysis for detection of the CMT1A duplication and aneuploidy screening, and the results of STRs/SNPs-based linkage analysis are summarized in Table 1.

To our knowledge, this is the first report of an NGS-based PGD procedure for CMT1A. The NGS-based approach enabled effective detection of the 1.4-Mb duplication directly at the single-cell level. Further, we note that the linkage status of Embryo_9, inferred from upstream (CMT1A-affected) and downstream (CMT1A-unaffected) SNPs, is contradictory (Table S8). One probable explanation is that an odd number of crossover(s) has occurred in the CMT1A recombination hotspot region. The occurrence of ADO in SNP 8 and SNP 9 resulting in a false-negative genotype may provide another explanation for the contradictory results. Thus, whether the affected chromosome has been transmitted from the proband to the embryo is equivocal and the gain or loss of copy number of the CMT1A region owing to possible recombination is indeterminate, both of which indicate that this embryo would not be appropriate for transfer. However, NGS-based CNV analysis definitely indicates a normal copy number of the CMT1A region (Fig. 1C and D), and thus avoids the discard of the unaffected embryo.

Other than the 1.4-Mb CMT1A duplication, CNVs are known to be associated with various severe diseases, such as the 22q11.2 deletion/duplication syndrome (Morrow et al., 1995) and Williams−Beuren syndrome (Osborne et al., 2001). Previous PGD application focused more on monogenetic diseases such as cystic fibrosis and chromosomal or segmental imbalances in embryos from translocation carriers. Few studies reported PGD for diseases caused by CNVs, especially small CNVs, and thus very little is known about the feasibility of PGD for these disorders. Some of this is attributed to the technical limitations on the detection of small CNVs at the single-cell level. The current study

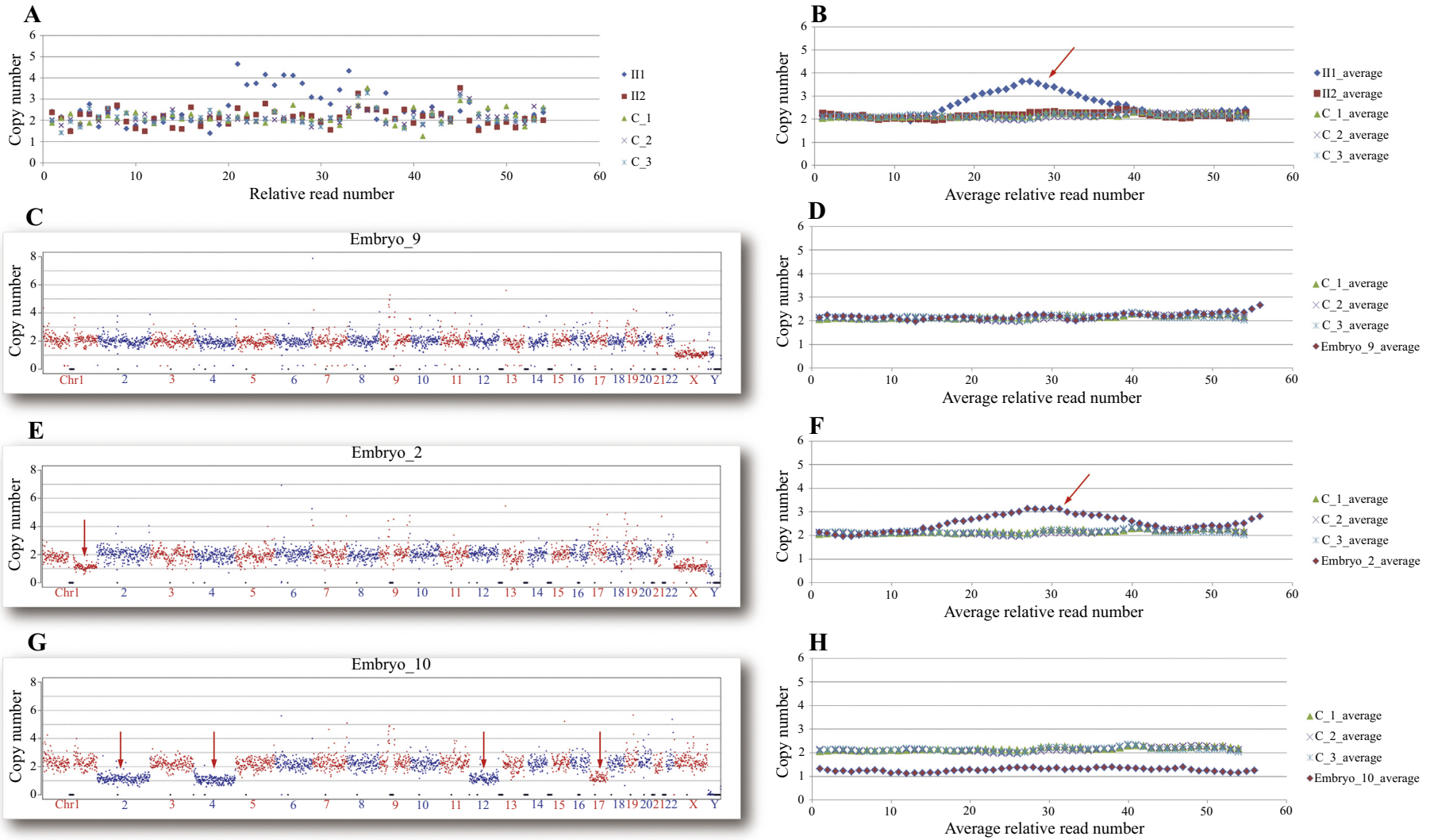

Fig. 1. The next-generation sequencing (NGS)-based copy number variation (CNV) analysis for detection of the Charcot-Marie-Tooth disease type 1A (CMT1A) duplication and aneuploidy screening in single lymphocytes and embryos.

A: The copy number (y-axis) for the 1.4-Mb CMT1A duplication region (chr17:14,200,000−15,600,000, corresponding to the x-axis value of 20,−34) and 2 Mb of its flanking regions (chr17:12,200,000−14,200,000, corresponding to the x-axis value of 0−20, and chr17:15,600,000−17,600,000, corresponding to the x-axis value of 34−54). Read numbers are counted with a bin size of 100 kb along this 5.4-Mb region and are displayed as the relative read numbers for each bin (each single dot). **B:** Relative read numbers are averaged with a statistical sliding window of 10 bins and are compared with those of the three random normal references (C_1, C_2, and C_3) from unrelated families, indicating a CMT1A duplication (slant red arrow) in II1 (blue dot) and normal copy number in II2 (red dot). **C−H**: The results of aneuploidy screening and CMT1A-duplication detection of Embryo_9 (**C** and **D**, respectively), Embryo_2 (**E** and **F**, respectively), and Embryo_10 (**G** and **H**, respectively) are showed, indicating that Embryo_9 is euploidy and is unaffected by CMT1A, that Embryo_2 is affected by CMT1A (slant red arrow) with a segmental monosomy of chromosome 1 (−1q) (vertical red arrow), and that Embryo_10 is unaffected by CMT1A with the monosomy of chromosomes 2, 4, 12, and 17 (vertical red arrow).

Table 1
The results of PGD and aneuploidy screening based on NGS and linkage analysis

CMT1A PGD and aneuploidy screening	Embryo ID													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
NGS-based CNV analysis	A[a]	A	UA[b]	UA	A	A	UA	UA	UA	UA	A	UA	UA	UA
SNPs linkage analysis	A	A	UA	UA	A	A	UA	UA	?[c]	?	A	UA	UA	UA
STRs linkage analysis	A	A	UA	UA	A	A	UA	UA	UA	UA	A	UA	UA	UA
Aneuploidy screening by NGS	XX, +1, +8, +9, +22	XY, −1q	XY, +21	XX, −1, −8, −10q, −12	XY, +6p, −13q	XX	XX	XY, −16	XY	XX, −2, −4, −12, −17	XX, +4, −8, +9, −10, +15, +17, +19, +20	XX, −17q, −21	XX	XX
Transferrable	N[d]	N	N	N	N	N	Y[e]	N	Y	N	N	N	Y	Y

[a] A, affected by CMT1A; [b] UA, unaffected by CMT1A; [c] ?, the linkage status is indeterminate because contradictory genotyping results are observed between upstream and downstream SNPs; [d] N, not transferrable; [e] Y, transferrable. The embryos which are affected by neither CMT1A nor aneuploidy are transferrable. CMT1A, Charcot-Marie-Tooth disease type 1A; CNV, copy number variation; SNPs, single nucleotide polymorphism markers; STRs, short tandem repeat markers.

indicates the potential applicability of NGS-based analysis in PGD for small CNVs associated with different syndromes. Although only a limited number of samples were evaluated in this study, we propose that this methodology is reliable for identifying CNVs with a segment size of at least ~1 Mb at the single-cell level by low-depth sequencing of ~10 million reads.

Although the patients in monogenic PGD cycles are often not at risk for fetal aneuploidy, it is still strongly recommended to perform aneuploidy screening due to the high percentage of aneuploidy in human preimplantation embryos. In this study, ~64% embryos showed chromosomal or segmental aneuploidy. Aneuploidy was also highly prevalent (~56%) in CMT1A-free embryos. Previous studies indicated that in double-factor PGD, 30%−40% embryos unaffected by monogenic abnormalities turned out to be aneuploid (Obradors et al., 2008; Brezina et al., 2011). Importantly, aneuploidy of chromosome 17, on which the CMT1A duplication region is located, was observed in Embryo_10 (monosomy) and Embryo_11 (trisomy) (Table 1). In fact, the linkage analysis results of Embryo_10 were difficult to interpret. The haplotype based on STRs was indeterminate because unexpected single peaks (single segment length) were observed in D17S2229, D17S220, and D17S2217 (the single peak in D17S227 was expected) (Fig. S2). A number of factors may cause this, for example, a single copy of the region covered by all the four STRs or ADO of STRs showing unexpected single segment length. Meanwhile, contradictory genotyping results were observed in upstream and downstream SNPs (Table S8), which may result from an odd number of crossover(s) in the CMT1A recombination hotspot region or the occurrence of ADO of SNP 6, SNP 8, and SNP 9. Regardless of the low probability of concurrent ADO in multiple linkage markers, it cannot be totally excluded. With regard to Embryo_11, although it could be excluded for transfer based on linkage analysis, the authentic copy number of the CMT1A duplication region was not considered. Aneuploidy of the chromosome(s), on which the implicated mutation(s) is located, would increase the incidence of diagnosis failure or misdiagnosis if informative linkage markers are insufficient or drop out, or only the mutations themselves can be detected owing to the low availability of linkage markers (i.e., PGD for *de novo* mutations). Therefore, in this context, aneuploidy screening may also facilitate the definite and accurate diagnosis in PGD for monogenetic diseases.

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SUPPLEMENTARY DATA

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