Genetic screening method for analyzing survival motor neuron copy number in spinal muscular atrophy by multiplex ligation-dependent probe amplification and droplet digital polymerase chain reaction

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To the Editor: Spinal muscular atrophy (SMA) is an autosomal recessive disease caused by a deficiency of the survival motor neuron 1 (SMN1) protein, which causes the loss of motor neurons in the anterior horn of the spinal cord.[1] A genetically similar gene, *SMN2*, has a translationally silent C-to-T transition at Position 6 in its 7th exon that causes only 10% correctly spliced full-length and functional SMN protein via alternative splicing. But in SMA-affected individuals, *SMN2* is the sole source of SMN protein and defined to be a disease-modifying gene because of the relationship between its copy number (1–8) and disease severity.[2] Numerous assays have been revealed to quantify copy numbers of *SMN1* and *SMN2* in DNA samples. Currently, multiplex ligation-dependent probe amplification (MLPA)[3] and an emerging method of droplet digital polymerase chain reaction (ddPCR)[4] are two widely used genetic screening methods. Therefore, the reproducibility of the two different technologies was compared for assessing copy numbers of *SMN1* and *SMN2*.

Twenty-four patients were collected from a clinical trial (No. NCT04010604). Human induced pluripotent stem cells (iPSCs) of SMA type I with 2 or 3 *SMN2* copies, and SMA type II with 3 *SMN2* copies were obtained from Coriell Cell Repositories (i-1, i-2, and i-3, respectively). The informed consents were obtained from all families. The study has been approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University.

As the Supplementary Table 1, http://links.lww.com/CM9/A332 shown, copy numbers of *SMN1* ranged from 0 to 3 copy number variations (CNVs) and copy numbers of *SMN2* ranged from 1 to more than 4 CNVs. Seven samples of MLPA results for copy numbers of *SMN1* and nineteen for copy numbers of *SMN2* could not be assigned to a specific copy number, because their ratios fell within an ambiguous range as defined by the kit instruction or were not consistent in two tests. MLPA test illustrated that the iteration rate of copy numbers of *SMN1* was 74.1% (20/27), and that of *SMN2* was 29.6% (8/27). It is too arduous to get a reliable result for more than 4 copies of *SMN* because MLPA just only assesses for less than or equal to 4 copies of *SMN* with a ratio below 2.15. Besides, the copy number results of *SMN2* of MLPA for i-1 were 2 in Test 1 and 3 in Test 2, i-2 were more than 4 in these two tests, and i-3 were also more than 4 in these two tests, which were not consistent with Coriell Cell Repositories as shown above. Collectively, these results indicated that it could only obtain the copy number values within the ratio range and could not detect samples with the copy numbers exceeding 4 for MLPA.

While analyzing the ddPCR results, it was found that the iteration rates of copy numbers of *SMN1* and *SMN2* were both 100% (27/27) for ddPCR [Supplementary Table 1, http://links.lww.com/CM9/A332]. On the one hand, the results of P-10, F2-II-2, F2-II-3, F2-III-2, F2-III-4, F3-I-1, and F3-I-2 for copy numbers of *SMN1* were consistent with ddPCR, whose MLPA results were uncertain. On the other hand, there were no consistent results for copy numbers of *SMN2* detected by MLPA except for the samples of P-4, P-6, P-10, F1-II-2, F1-II-2-f, i-2, i-3, and SD-019, while the results of ddPCR were reproducible and all were matched in these two tests. In addition, the copy numbers of *SMN1* and *SMN2* obtained from ddPCR is in concordant with the clinical phenotypes [Supplementary Table 1, http://links.lww.com/CM9/A332]. Furthermore, the copy number results of *SMN2* of ddPCR for 3 iPSCs were the same as that of Coriell Cell Repositories. Taken together, ddPCR provides a level of specificity unachievable by MLPA,

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Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000001102

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Chinese Medical Journal 2020;133(20)

Received: 23-05-2020 **Edited by:** Ning-Ning Wang

because of the presence of an ambiguous result beyond the interpretation interval and the maximum ratio range for MLPA results when using a dosage quotient for interpretation.[3] Moreover, it requires normal control for MLPA system while ddPCR does not require reference DNA samples but only compare to its endogenous control. In addition, the five steps of MLPA are essential but take cumbersome process to obtain results.[3] On the contrary, ddPCR is a time-efficient way for its simple steps.[4]

Among the SMA patients in our cohort, an uncustomary pair of SMA siblings was found whose symptoms varied largely. To test whether the CNV of *SMN2* was the cause of inconsistent symptoms for the pair of siblings reported by us,[5] the MLPA was conducted to determine the copy numbers of *SMN2* first. However, the copy numbers of *SMN2* of the siblings were erratic in different tests, although the same healthy control was chosen [Supplementary Table 1, http://links.lww.com/CM9/A332]. The proband (F1-II-2), one of the siblings, carried 4 copies of *SMN2*, and his sister (F1-II-1) carried 2 copies of *SMN2*. However, when the second test was performed, the copy numbers of *SMN2* of the proband and his sister were 4. Considering the different results of MLPA, the ddPCR was performed then to verify the copy numbers of *SMN2* for this pair of siblings using DNA extracted from PBMC and fibroblasts. For both of the siblings, ddPCR assay showed 0 copy of *SMN1* and 4 copies of *SMN2* in three independent tests. Finally, it became clearly that there were other modifiers caused the inconsistent phenotype for the siblings. Thus it could be seen that when patients who have inconsistent phenotype were met, as the most basic and common factor to determine the severity clinical phenotype of SMA patients, it is better to select a reproducible and reliable genetic screening method for copy number quantification of *SMN2*, then to provide the basis for further discussing SMA-severity genetic modifiers.

The copy numbers of *SMN1* of one SMA family who carried a rare mutation of *SMN1*c. 844 C>T using T-A cloning was also verified. In this study, it was found that the copy numbers of *SMN1* of SMA Type I *SMN1* (c.844C>T+0) was 1 by MLPA but 0 by ddPCR, and *SMN1* (c.844C>T+1) carrier was 2 and 1, respectively [Supplementary Table 1, http://links.lww.com/CM9/A332]. In direct comparison, there was a great difference between MLPA and ddPCR for *SMN1* c.844C>T mutation detection and the MLPA results represent the true numbers were confirmed. It reminds us that it needs to combine ddPCR and MLPA results in copy number detection of *SMN1* for further screening of asymptomatic carriers. Furthermore, it would be possible that the faulty copy number generated from ddPCR is caused by the probe design, which may bind the point mutations exactly. Although the major of SMA cases are associated with homozygous deletion of *SMN1*, compound heterozygous mutations of *SMN1* are also non-negligible. It suggests that the copy number of *SMN1* and its complex compound heterozygous status can only be effectively determined by combining multiple methods.

Overall, by comparing methods of MLPA and ddPCR, the ddPCR was described as a robust platform for analysis of copy numbers of *SMN1* and *SMN*2, so as to further provide the explanation for the phenotype-genotype correlation as well as the treatment of this disease. In addition, it was recommended that *SMN1* compound heterozygous mutation needs joint analysis of multiple methods.

Funding

This work was supported by grants from the National Natural Science Foundation of China (Nos. 81771230, U1905210), the Joint Funds for the Innovation of Science and Technology of Fujian Province (Nos. 2017Y9094 and 2018Y9082), the National Key Clinical Specialty Discipline Construction Program, the Key Clinical Specialty Discipline Construction Program of Fujian, and the Startup Fund for Scientific Research of Fujian Medical University (Nos. 2018QH1050 and 2018QH2035).

Conflicts of interest

None.

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How to cite this article: Hong JM, Zhao M, He J, Huang XJ, Zhao ZY, Chen WJ, Wang N, Li JJ. Genetic screening method for analyzing survival motor neuron copy number in spinal muscular atrophy by multiplex ligation-dependent probe amplification and droplet digital polymerase chain reaction. Chin Med J 2020;133:2510–2511. doi: 10.1097/CM9.0000000000001102