Research report

A diagnostic gene chip for hereditary spastic paraplegias

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A B S T R A C T

Hereditary spastic paraplegias (HSPs) are a group of clinically and genetically heterogeneous monogenic neurodegenerative disorders. The gene screen of hereditary spastic paraplegias patients remains time consuming and costly because of their highly heterogeneous. As we know, there are some hot spots of mutation in many genes causing HSPs. Our aim was to develop a quick method for gene screen of HSP patients. The online mutation data banks of HSPs were searched and Chinese data for point mutations were mainly considered. Then mutations were comprehensively analyzed and ninety-six more common point mutations of HSPs disease genes were chose for the 96-plex GoldenGate assay diagnostic gene chip for HSPs. After that, we used this diagnostic gene chip to detect ninety-six clinically diagnosed HSP patients. For validation purpose, six previously Sanger sequenced cases with known point mutations were redetected on this array. The scores of all the ninety-six point mutations were between 0.601 and 0.993, and the call rate of the whole gene chip was 97.7% and its consistency was 99.0%. A patient suspected with a c.316G>C substitution in SPG6 was detected by the chip, which was further confirmed by polymerase chain reaction and sequencing. The high successful performance of this GoldenGate assay makes it a useful technique for preliminary genetic screening for HSP patients and it may be used in clinic in the future.

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1. Introduction

Hereditary spastic paraplegias (HSPs) are a group of clinically and genetically heterogeneous neurodegenerative disorders, which are of monogenic inheritance and primarily impair the pyramidal tracts. Traditionally, it has been divided into pure HSP and complicated HSP, depending on the presence of other neurological features in addition to spastic paraparesis (Harding, 1981, 1983). The genetics of HSP are complex and all modes of inheritance, including autosomal dominant (AD), autosomal recessive (AR), and X-linked recessive, have been described, besides several cases present as sporadic (Salinas et al., 2008). So far, at least a total of fifty-two chromosomal loci have been identified for HSPs, including seventeen of autosomal dominant, thirty of autosomal recessive and five of X-linked inheritance respectively (Boukhris et al., 2010; Slabicki et al., 2010; Montenegro et al., 2012; Gonzalez et al., 2013). Among them, twenty-eight genes responsible for the disease have been cloned (Boukhris et al., 2010; Slabicki et al., 2010; Bauer et al., 2012; Montenegro et al., 2012; Gonzalez et al., 2013).

The presence of clinical features may differ greatly among HSP patients caused by different gene mutations and even among patients in the same family (Boukhris et al., 2009). So far, a large number of novel mutations of disease genes have been identified by traditional method, such as polymerase chain reaction (PCR) and sequencing, plus Multiplex Ligation-dependent Probe Amplification (MLPA) (Depienne et al., 2007). All types of mutations have been reported, including site mutations, deletion, duplication, rearrangement and so on, while the majority are missense, nonsense and splice site mutations. However, the gene screen remains time-consuming and costly because of their highly heterogeneous clinical presence. Usually, during gene diagnosis, we always firstly screened the most common disease genes for the HSPs, such as SPG4 and SPG3A for autosomal dominant hereditary spastic paraplegia (AD-HSP), SPG11 and SPG15 for autosomal recessive hereditary spastic paraplegia (AR-HSP). But the exons of each gene are so many, for example seventeen exons for SPG4,
fourteen for SPG3, forty for SPG11 and forty-two for SPG15, and just the primer design, PCR and sequencing procedures make it time costly. However, both founder effects and mutational hot spots in some disease genes of HSPs were existed. For example, the c.715C>T substitution in SPG3A was a hot site and already reported in many countries such as China, Spain, America and Korea (Zhao et al., 2001; Li et al., 2007; Alvarez et al., 2010; Kwon et al., 2010).

The recently developed molecular diagnostic assays based on the hybridization of probe with target nuclear acids from clinical samples are allowing effective detection of various diseases with high speed, high sensitivity, and high specificity. The Illumina GoldenGate Universal-32 BeadChips can be deployed on the BeadArray platform for 96-plex, or 384- to 1536-plex assays (in multiples of ninety-six) in a single reaction over a three-day period (Fan et al., 2003). Also, GoldenGate gene chip allows customers to select the ideal solution for their loci multiplexing and sample throughput requirements and can be used to genotype rare variants (Tindall et al., 2010). The obvious advantage of this kind of gene-chip is its high performance and high throughput. It gives researchers the opportunity to take a snapshot of ninety-six single-nucleotide polymorphisms (SNPs) quickly and cost-effectively (Quackenbush, 2001). In order to explore a preferable screening method for genetically diagnosing HSP patients, we designed a 96-plex GoldenGate Universal-32 BeadChips using hot site mutations reported as common mutations for HSP patients, and practiced the chip in ninety-six clinically diagnosed HSP patients.

2. Materials and methods

2.1. Patients

Ninety-six clinically diagnosed HSP patients from unrelated Chinese Han families were collected, which comprised of sixty-four males and thirty-two females with mean age at onset of 11.7 ± 8.9. To further test the sensitivity of the HSP gene chip, six samples containing known mutations in SPG3A, SPG4 and SPG11 were included as positive controls, while sixty-six patients were previously genetically screened by sequencing and the rest twenty-four were newly clinically diagnosed as HSP. The sixty-six genetically screened cases included twenty-one AD-HSP patients precluded from SPG4, SPG6A, SPG6, SPG7, SPG12, SPG7c and SPG10 mutations by direct sequencing and MLPA. Fifteen AR-HSP patients precluded from SPG11, SPG15, SPG5 and SPG7 mutations, and thirty sporadic HSP patients precluded from SPG4, SPG6A, SPG6, SPG7, SPG12 and SPG42 mutations. The twenty-four newly clinically diagnosed HSP cases included eight AD-HSP, four AR-HSP and twelve sporadic cases. All the patients were clinically diagnosed according to Harding criteria (Harding, 1981, 1983). DNA samples were extracted from peripheral venous blood by common method and diluted to a concentration of 50 ng/μl by 1 × TE. All participants or their legal guardian signed informed consent for participating in both the clinical and genetic studies. This study also got approval by the Ethics Committee of Xiangya Hospital of Central South University.

2.2. Designation of the HSP gene chip

In order to find out all the hot spots of HSPs, we searched both PubMed [http://www.ncbi.nlm.nih.gov/pubmed] and Chinese database (Wan Fang Database) [http://www.wanfangdata.com.cn/] using the terms ‘HSP OR SPG OR hereditary spastic paraplegia for mutation reports. And we also reviewed the authors’ reference database for further mutation reports. Then all of the site mutations were collected and the sequence were obtained using UCSC Genome Bioinformatics database for corresponding 60 bp upstream and downstream of the mutation sites. A designability rank score was calculated for each SNP via Illumina’s Assay Design Tool, where a score <0.4 predicted a low success rate, between 0.4 and 0.6 a moderate success rate, and >0.6 a high success rate for the conversion of a SNP into a successful GoldenGate assay. After that we selected the most commonly reported ninety-six SNPs which had a final score above 0.5, and avoid any other mutation in the 60 bp segment flanking the mutation analyzed. We prepared the GoldenGate Universal-32 BeadChips via the Illumina, Inc.

2.3. Usage of the HSP gene-chip

The newly designed HSP 96-plex GoldenGate Universal-32 BeadChips was used to diagnose the ninety-six HSP patients according to the Illumina’s user manual (GoldenGate Genotyping Assay, Manual). As it was the first time we confronted this gene chip, and ninety-six samples for one gene chip could be detected as multiples of thirty-two once, we performed the genetic diagnosis of ninety-six samples by the gene chip for two steps. Firstly, we detected thirty-two patients in order to avoid unnecessary practice errors as a whole. After that we conducted the genetic screen for the rest sixty-four patients. The GoldenGate assay was based on the use of two Allele-Specific (ASO) and one Locus-Specific Oligo (LSO) per SNP locus. To maximize the function of the gene chip, 250 ng of genomic DNA was used for each genotype. During the primer hybridization process, the assay oligonucleotides hybridize to the genomic DNA sample bound to paramagnetic particles. Following hybridization, several wash steps were performed, reducing noise by removing excess and mis-hybridized oligonucleotides. Extension of the appropriate ASO and ligation of the extended product to the LSO joins information about the genotype present at the SNP site to the address sequence on the LSO. These joined, full-length products provide a template for PCR using universal PCR primers P1, F2, and P3. Universal PCR primers P1 and P2 were Cy3- and Cy5-labeling. After downstream-processing, the single-stranded dye-labeled DNAs were hybridized to their complement bead type through their unique address sequences. Hybridization of the GoldenGate Assay products onto the BeadChips allows for the separation of the assay products in solution, onto a solid surface for individual SNP genotype datum. After hybridization, the BeadScan system was used to analyze fluorescence signal on the BeadChip, which was in turn analyzed using software for automated genotype clustering and calling. For each SNP, the amplification product for homozgyous genotypes displayed normally a signal in either the Cy3 or Cy5 channels, whereas the heterozygous genotype at this locus should display a signal in both channels. The automatic allele calling was done using the Illumina GenomoStudio software with default value and three clusters on a graph based on the fluorescence obtained.

2.4. Statistical analysis

Genotype calls were made using the Genotyping module of the GenomeStudio software. Different indexes were calculated by the GenomeStudio software. Several indexes were used to check the automated genotype calling and the sample clustering. Sample-specific call rates were calculated as percent successfully genotyped SNPs among DNA samples. Similarly, per-SNP call rates were calculated as percent successfully genotyped samples. Summary statistics were calculated among all SNPs and samples except for the poorly called samples in order to preclude the influence of the unqualified sample on the efficiency of the gene chip. The homozgyous and heterozygous clusters were checked visually and revised, and only the most reliable calls were retained. A color mark was then given to each SNP as follows: Black failed; Red was homozgyous of AA; Purple was heterozygous of AB; Blue was homozgyous of BB. A and B were named according to Illumina SNP Genotyping technical note ‘TOP/BOT’ Strand and ‘A/B’ Allele.

2.5. Consistency

The consistency between the SNP genotyping obtained using the GoldenGate assay and the Sanger sequencing was figured out by the accordance of the successful calls and previously sequencing results. Plots that appeared to be “unusually” clustered (i.e. failed ones and suspected mutations) were further confirmed by PCR and sequencing.

2.6. Positive control DNA samples with known mutations

To further test the sensitivity of the HSP gene chip, six samples containing known mutation of SPG3A(c.715C>T), SPG4(c.477T>A), and SPG11(c.6856C>T, c.5977T>C, c.6681A>T, c.3004C>T) were included as positive controls.

3. Results

3.1. SNPs of the HSP gene-chip

By using the database search, 968 mutations reported for fourteen cloned disease genes of HSPs were found out, including 540 site mutations. The sequence of each site mutation was obtained using UCSC Genome Bioinformatics database for corresponding 60 bp upstream and downstream of the mutation sites. Out of the 474 analyzed SNPs, 403 SNPs had a score >0.6 (designability rank = 1), forty-nine SNPs ranked between 0.4 and 0.6 (designability rank = 0.5) and twenty-two SNPs ranked between 0 and 0.4 (designability rank = 0). Finally ninety-six most common site mutations and their corresponding sequences were chose, the final scores of which ranged from 0.601 to 0.993, and which matched the Illumina criterion of absence of other known SNPs in their vicinity and with sufficient sequence information upstream and downstream of the SNPs. The ninety-six SNPs included seven SPG3A mutations, twenty SPG4 mutations, one SPG6 mutations, one SPG8 mutations, five SPG10 mutations, four SPG31 mutations, one SPG42 mutations, five SPG5 mutations, six SPG7 mutations, twenty-one SPG71 mutations.
Genotyping c.1417 mutations, four SPG15 mutations, five SPG17 mutations, twelve SPG1 mutations, and four SPG2 mutations respectively. The detailed information of the ninety-six SNPs was available in Supplementary material Table 1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.brainresbull.2013.07.002.

3.2. Calls rate of the HSP gene-chip

The vast majority of SNPs (90 out of 96) gave a clear genotyping which were successful for nearly all accessions (93.7% of the collection). Most of the SNPs yielded one clear clusters representing the wild-type homozygous genotypes. Only six SNPs were classified as a genotype failure because of its bad performance in the first step (Fig. 1a–f), with the call rate below 95.0%. In the pictures discussed, the presence of many outlier samples in the original analysis resulted in failure calls or being incorrectly genotyped as mutations which was precluded by Sanger sequencing. These outliers reduced the SNP call rate to a value below 95.0%. Except the failed six SNPs the per-SNP call rates of the other ninety SNPs ranged from 95.8% to 100.0%. One sample was missed during the operation, without which the call rate of the whole gene chip was 97.7% (with the failed six SNPs included).

3.3. Consistency

According to our previous sequencing data, 2210 calls had been sequenced. Among the 2210 calls, we defined fourteen plots as failure and eight plots as false positives during gene chip test, with eleven failed plots and six false positive plots in the first step and three failed plots and two false positive plots in the second step respectively. Therefore, the consistency of this chip was 99.0%.

3.4. Successful diagnosis performance of the HSP gene-chip

All the six genetically diagnosed positive cases were successfully detected by this chip (Fig. 2a–f). One suspected mutation in the genotype clusters of rs104894490 (c.316G in SPG6) was found in a proband from twenty-four newly clinically diagnosed HSP cases with autosomal dominant family history (Fig. 3). The complete pedigree consisted of five generations and HSP was diagnosed in
twelve family members with four living patients (Fig. 4a). The family members were further confirmed by PCR and sequencing (Fig. 4b and c). We Sanger sequenced the corresponding locus in family members including II1, II4, III1, III5, III6, III7, III8, III11, III14, IV3, IV7, IV9, IV11, IV12, IV14, IV16, IV18, V1-2 and V5-7. Patients III6, IV7, IV12 and IV16 carried the c.316G>C mutation while other family members did not. So, the c.316G>C (p.Gly106Arg) sequence change completely cosegregated with the disease phenotype. The mutation p.Gly106Arg in SPG6 was reported by our group in another family (Du et al., 2011) and also researches in other countries (Reed et al., 2005; Munhoz et al., 2006), which confirmed it was a mutation for hereditary spastic paraplegia rather than a neutral polymorphism. At twenty-two years of age, the proband had noticed symmetrical wasting and weakness of both lower limbs, which slowly progressed. When she first came to our hospital at age forty-eight, the physical examination revealed a marked spastic paraplegia with pes cavus and severe wasting of the lower limbs. The other three patients developed similar problems. As the disease progressed, all the patients displayed a markedly spastic gait and required a cane, walker, or wheelchair to move. Urinary urgency was a common symptom but no cognitive impairment. Brain-stem auditory evoked potential (BAEP) and visual evoked potential (VEP) were normal. Spinal evoked potential (SEP) showed decreased velocity of Posterior Tibial Nerve and the latency periods of bilateral cortical potentials were elongated.

4. Discussion

Hereditary spastic paraplegias are a group of clinically and genetically heterogeneous neurodegenerative disorders, sometimes cannot be differentiate from hereditary spinocerebellar ataxias, Parkinson’s disease or other neurodegenerative disorders via mere clinical presentation (Wang et al., 2009; Guidubaldi et al., 2011). In these cases, the gene diagnosis becomes the golden standard. However the high genetic and clinical heterogeneity of HSPs make gene diagnosis very difficult. Luckily there are some
suspected hot spots of mutation in many genes leading to HSPs (Kaneko et al., 2006; Du et al., 2011; Gonzalez et al., 2013).

Recent advances in molecular-level interaction and detection technology are upgrading the clinical diagnostics by providing new strategy of diagnosis. Particularly, DNA microarrays can be efficiently used in clinical diagnosis which involve discovery of disease-relevant genes or agents causing diseases, mutation analysis. The performance of DNA-microarray-based diagnosis is continuously improved by the integration of other tools. Patents on the applications of DNA microarrays in disease diagnosis involved complex diseases such as chronic inflammatory diseases and type 1 diabetes, monogenic autosomal recessive disorders such as ataxia telangiectasia, spinal muscular atrophy and Friedreich's ataxia (Yoo et al., 2009). Most recently, genome-wide association (GWAS) studies, whole exome sequencing and whole-genome sequencing are becoming useful ways for detecting disease related and disease causative genes (Chung and Chanock, 2011; Pareek et al., 2011; Singleton, 2011). But they are costly and time-consuming for following analysis with above 5000 dollars for whole exome sequencing and about 16,300 dollars for whole-genome sequencing in China, only a limited number of patients can afford it.

Illumina, Inc. produces microarrays with high performance and multifunction (Steemers and Guderson, 2005). The Illumina GoldenGate Universal-32 BeadChips are capable of multiplexing 96, 384 or 1536 SNPs (in multiples of 96) in a single reaction over a three-day period. Also, GoldenGate gene chip allows customers to select the ideal solution for their loci multiplexing and sample throughput requirements. In order to explore a preferable screen method for gene diagnosis of HSP patients, we designed a 96-plex GoldenGate Universal-32 BeadChips, and practiced the chip by detecting ninety-six clinically diagnosed HSP patients. By employing this method, we have developed the diagnostic gene chip for hereditary spastic paraplegias successfully. In this study we present the successful application of SNP-array based molecular gene diagnosis of HSPs. This HSP diagnostic gene chip is featured for its high consistency and performance, which could be used for research and clinical application to screen HSP patients. We emphasize this form of indirect DNA analysis as the first step in the approach to genetically diagnose clinically diagnosed HSP patients, because it is fast and inexpensive with only three days and not more than 50 dollars cost.

In this study, we have demonstrated the suitability of a 96-plex GoldenGate assay for detecting site mutations of HSPs. Ninety SNPs (93.75%) gave excellent genotyping results according to the criteria defined by Close et al. (2009). Sensitivity and specificity are often tested by means of a positively testing cohort with known mutations that have been identified with a well-accepted technology. The reliability of the technique was also evaluated by comparing the results of the GoldenGate SNP genotyping with Sanger sequencing data for certain genotypes. The results were consistent between the two techniques in almost all cases with 14 failed plots and 8 miscalled plots, together counting for 0.995%. We here suggest considerations regarding mutation selection before genotyping and screening before analysis. Because this gene-chip is used to detect point mutation, we recommend that the point mutation should

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**Fig. 3.** Genotyping of c.316G in SPC6 indicate a site mutation carrier. Note: Genotyping of all the 96 patients at the position of c.316 in SPC6. Each dot indicated the genotype of one patient. The blue dots indicated homozygous genotype of GG. The black dots indicated failure one, which was excluded mutation at this position by sequencing. The red dot indicated a homozygous mutation which was further confirmed by sequencing as genotype of GC.

**Fig. 4.** Analyses of the family with c.316G>C mutation in SPC6. Note: (a) The pedigree of the family. The small squares indicated male; circles, female; filled shape, affected; black arrow, proband; slashed, deceased. Arrows on the pedigrees indicated the proband. (b) The representative Sanger sequence results of the healthy members in this family. (c) The representative Sanger sequence results of the HSP patients in this family revealed c.316G>C substitution mutation in SPC6. Family members including II1, II4, IV3, III2, III3, III4, III6, III7, III8, III10, IV1, IV2, IV3, IV4, IV5, IV6, IV7, IV8, IV9, IV10, IV11, and V1-2 were sequenced for this mutation locus, but only patients III4, IV3, IV12 and IV15 carried the mutation. The sequence change completely cosegregated with the disease phenotype.
better be of high mutation frequency, the designability rank be higher than 0.5 and any other mutation in the 60 bp segment flanking the mutation analyzed should be avoided. For rare and single copy variants, genotyping technologies must accurately perform cluster calling. In this study successful cluster calling was observed for both rare autosomal and single copy X-linked variants. Because ninety-six samples for one gene chip could be detected as multiples of thirty-two, we performed the genetic diagnosis of ninety-six samples by the gene chip for two steps. Firstly, we detected thirty-two patients, and found six SNPs were failure, with eleven failed plots and six false positive plots. But, when we conducted the rest sixty-four patients secondly, all the SNPs were successfully called, with only three failed plots and two false positive plots. So, although we did not repeat the experiment with the same sample, it suggested good reproducibility when we used other DNA samples. And we also have produced similar diagnosis gene chip for Parkinson's disease with good reproducibility (unpublished data). Accordingly, although six SNPs were failure, it was not because of the gene-chip itself, it was our inexperience in the first step. With our experience improved, all the call rates were also increased above 95.0% (during the second step). By applying HSP 96-plex GoldenGate Universal-32 BeadChips to diagnose the newly clinically diagnosed HSP patients, we detected a suspected mutation in SPG6. From the genotyping map, we suspected the patient as homozygous CC at position c.316 in SPG6, the same mutation locus as we reported before (Du et al., 2011; Reed et al., 2005; Munhoff et al., 2006). However, we confirmed the patient as heterozygous GC by Sanger sequencing. We contributed this discordance to the instability of that SNP, because we can see four failed calls at this SNP and the corresponding call rate was 95.8%. To make sure whether this gene chip was capable of detecting HSP patients, we included six HSP gene mutation carriers among the ninety-six patients. The genotypes of the six genetically diagnosed HSP patients were in accordance with the previous sequencing results, which further confirmed the performance of this gene chip. But the obvious limitation of this diagnosis gene chip was that it could only detect the designed ninety-six SNPs. So, in order to improve the gene diagnosis, patients that are diagnosed by this gene chip should be further confirmed by sequencing. MLPA and other gene diagnosis techniques for other mutations. In another word, patients that are diagnosed negative by this gene chip screen cannot be excluded from HSPs.

During the past twenty years, DNA microarray-based techniques have been predominantly applied to detection of pathogens and disease-causative agents. But their widespread use has been hampered for the limited identification capability. To increase its practical usage in diagnosis, it should become more affordable, convenient to practice, accurate, and maybe portable. One example is on-site pathogen and SNPs detection which will enable sensitive on-site diagnosis of specific diseases in clinical. Indeed, much progress is being made on the use of the DNA microarray platform for disease diagnosis. With all these advances, it is expected that the DNA microarray or its sister technologies will play an increasingly important role in disease diagnosis in the near future.

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