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A New Targeted *CFTR* Mutation Panel Based on Next-Generation Sequencing Technology

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Searching for mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*) is a key step in the diagnosis of and neonatal and carrier screening for cystic fibrosis (CF), and it has implications for prognosis and personalized therapy. The large number of mutations and genetic and phenotypic variability make this search a complex task. Herein, we developed, validated, and tested a laboratory assay for an extended search for mutations in *CFTR* using a next-generation sequencing—based method, with a panel of 188 *CFTR* mutations customized for the Italian population. Overall, 1426 dried blood spots from neonatal screening, 402 genomic DNA samples from various origins, and 1138 genomic DNA samples from patients with CF were analyzed. The assay showed excellent analytical and diagnostic operative characteristics. We identified and experimentally validated 159 (of 188) *CFTR* mutations. The assay achieved detection rates of 95.0% and 95.6% in two large-scale case series of CF patients from central and northern Italy, respectively. These detection rates are among the highest reported so far with a genetic test for CF based on a mutation panel. This assay appears to be well suited for diagnostics, neonatal and carrier screening, and assisted reproduction, and it represents a considerable advantage in CF genetic counseling. *(J Mol Diagn 2017, 19: 788—800; http://dx.doi.org/10.1016/j.jmoldx.2017.06.002)*

Cystic fibrosis (CF) is a chronic, life-threatening genetic disease caused by loss-of-function mutations in the CF transmembrane conductance regulator gene (*CFTR*).[1,2]

Notwithstanding the considerable ethnic and geographic variability in the frequency of CF, the mean incidence of 1 in 2500 live births makes CF the most frequent severe autosomal recessive disease in the white population.[3] CF is characterized by wide genetic and clinical heterogeneity, which complicates diagnosis, prognosis, and therapy. From birth to adulthood, there is considerable variability in the severity and rate of disease progression in CF, with varying clinical presentations and different organs involved at different ages.[4] Often, great phenotypic variability arises from even a single *CFTR* genotype.[5,6]

More than 2000 different *CFTR* sequence variations, including CF-causing mutations and polymorphisms, have been reported (CFTR1 database, *http://www.genet.sickkids.on.ca*, last accessed May 15, 2017). Also in limited geographic areas, *CFTR* shows a complete catalog of mutation types in exons, introns, the 5′-flanking region, and the 3′-untranslated region, that is, point mutations, small insertions and deletions, complex alleles, and large genomic rearrangements.[5,7—12] This genetic heterogeneity greatly affects the allele *detection rate* (DR) of genetic tests, which is defined as the overall frequency of mutant alleles (of all of the mutant alleles present), as evidenced by a genetic test. For example, the DRs of the 23-mutation panel established by the American Congress of Obstetrician and Gynecologist and the

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American College of Medical Genetics differ substantially between various geographic regions, ranging from 49% to 94%.[13,14] This genetic heterogeneity is further enhanced if various well-recognized clinical forms of CF, such as classic CF, CFTR-related disorders, and congenital bilateral absence of vas deferens, are taken into consideration.[15,16] The same genetic test may have very different DRs, depending on its application in these different clinical forms.[17] In this article, CF refers to the classic form of the disease and excludes CFTR-related disorders and congenital bilateral absence of vas deferens, which will be specifically mentioned when appropriate.

Limiting the scope to CF, but considering geographic areas with high genetic heterogeneity, an extended Sanger sequencing protocol of the 5′-flanking region, 27 exons and proximal intronic flanking regions, plus selected deep intronic zones for specific intron mutations in *CFTR*, have shown DRs of up to 97%.[5,17] An additional search for large *CFTR* rearrangements showed a DR increment of approximately 2%.[5,9,17] The high DRs of approaches with extended genetic characterization seem to be confirmed by next-generation sequencing (NGS) technology, which has been recently used for sequence and copy number variation (CNV) analyses of *CFTR*.[18–27] NGS has been used mainly in validation studies and has been performed on a limited number of samples in comparisons of the performance of NGS to those of classic Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA). Although the NGS approach provides an obvious reduction in cost per base, and an actual possibility of multiple-gene analysis for multigenic disorders, the suitability of this approach in monogenic disorders is still debated. In particular, the classic sequencing approach and MLPA have shown excellent laboratory operative characteristics for *CFTR*.[5,17,28,29]

Despite the excellent operative characteristics of experimental approaches based on both extended sequencing and large rearrangement search, which are welcomed for diagnostic purposes, these approaches may not be suitable for other health programs, such as neonatal and carrier screening. In fact, in addition to the obvious problems of cost and time, the functional characterization of all of the possibly identified sequence variations and the subsequent assignment of their clinical significance[30] may be impossible tasks. Consequently, several efforts have been made to develop experimental approaches based on selected panels of known CF-causing mutations.[31,32] In this case, the DR of the selected panel of mutations in populations of various ethnographic origins and/or with various CF clinical forms invariably arose as a crucial variable.[17] NGS platforms seem to be particularly suited to the development of mutation panels with high a mutation number and, consequently, a high DR. However, at the moment, only a small number of articles have addressed specific *CFTR* mutation panels based on NGS approaches.[33]

In this work, we developed, validated, and tested a high-throughput, NGS-based approach using a customized mutation panel containing 188 CF-causing mutations in *CFTR*. We analyzed 1828 subjects referred for diagnosis and a case series of 1138 patients with CF (646 patients from northern Italy and 492 patients from central Italy). We called this assay *188-CF-NGS*. This approach revealed DRs of 95.0% and 95.6% in CF patients from central and northern Italy, respectively. Also in geographic regions with high genetic heterogeneity (such as Italy), a suitably customized and reasonably large panel of *CFTR* mutations, together with an NGS-based approach, allows the attainment of a high DR that is definitely suited for diagnostic purposes, at least in CF, and for neonatal and carrier screening.

Materials and Methods

Study Design and Case Series

We designed a specific, customized panel of 188 CF-causing mutations, described in Supplemental Table S1. The mutations were included based on their high frequency in the Italian population and their documented associations with CF. Frequency information was obtained using the data on the frequency of CF mutations in Italian patients in the literature,[5,34–39] from the 2010 Report of the Italian CF Register (*http://www.registroitalianofibrosicistica.it*, last accessed January 31, 2017), as well as from personal communication with some Italian CF centers and laboratories. The characterization of the mutations as CF causing was obtained from data on the functional effect from the North American CFTR2 (Clinical and Functional Translation of CFTR, *https://cftr2.org*, last accessed January 31, 2017) project[40] and from the literature.[5,34,35,41] With this panel of *CFTR* mutations, we optimized an NGS-based assay we called *188-CF-NGS*.

Our assay underwent six steps of validation (Figure 1). If allowed by the overall number of mutant alleles, each mutation was validated in at least three independent samples. Also, possible differences in quality, analytical sensitivity, and analytical specificity of the 188-CF-NGS assay between analysis of dried blood spots (DBSs) and analysis of genomic DNA were evaluated.

The first validation step was the analysis of 48 selected DBSs (referred to the Newborn Screening Laboratory, ASST Fatebenefratelli Sacco—PO Ospedale dei Bambini "V. Buzzi," Milan, Italy) that were previously investigated by mass spectrometry assay (*Mass Spectrometry Assay*). The DBSs were obtained from neonates screened as positive through the immunoreactive trypsinogen assay. The composition of this subset of DBS samples was as follows: eight homozygotes, 26 compound heterozygotes, four heterozygotes, and 10 with no *CFTR* mutation.

The second validation step was the analysis of 24 genomic DNA samples extracted from peripheral blood (referred to the Medical Genetics Laboratory, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy), which had been genotyped by confirmatory methods (*Confirmatory Methods*). The subset of 24 genomic DNA samples consisted of nine compound heterozygotes, eight heterozygotes, and seven with no *CFTR* mutation.

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