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Research review paper

Technology platforms for molecular diagnosis of cystic fibrosis

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Abstract

Cystic fibrosis (CF) is one of the most common recessive genetic diseases in North America. So far, 1200 mutations causing CF have been identified. Several techniques such as allele specific oligonucleotide (ASO) dot-blot, reverse dot-blot, amplification refractory mutation (ARMS), and an oligo-ligation assay, are available to detect the most common mutations. However, detecting compound heterozygotes between ΔF508, the most common disease causing mutation, and other mutations which are rare is difficult as some mutations are common only to particular ethnic groups. Therefore, new diagnostic tests such as restriction enzyme assays and single stranded conformational polymorphism (SSCP) have been designed to recognize rare and population-specific mutations. This review will describe the most commonly used CF mutation detecting diagnostic techniques, as well as novel assays and techniques currently in development that might be employed in future.
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Keywords: Cystic fibrosis; ASO dot-blot; Reverse dot-blot; Microarray; Proteomics; Oligo-ligation assay

Contents

1. Introduction . . . 0
2. The most common diagnostic tests for cystic fibrosis . . . 0
   2.1. Allele specific oligonucleotide (ASO) dot-blot . . . 0
   2.2. Reverse dot-blot . . . 0
   2.3. Amplification refractory mutation system (ARMS) . . . 0
   2.4. Oligo-ligation assay (OLA) . . . 0
3. Novel diagnostic techniques in cystic fibrosis . . . 0
   3.1. Microarray . . . 0
   3.2. Randomly ordered fiber-optic gene arrays . . . 0
   3.3. Biospecific interaction analysis (BIA) . . . 0
   3.4. Multiplex primer-extension assay using ABI prism^R snapshot™ ddNTP primer-extension assay . . . 0
   3.5. Proteomics . . . 0
4. Conclusion . . . 0
Acknowledgements . . . 0
References . . . 0

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1. Introduction

Cystic fibrosis is one of the most common recessive genetic diseases in North America. This condition results from mutations in the cystic fibrosis transmembrane regulator (CFTR) gene, which was first identified in 1989 (Rommens et al., 1989). These mutations result in defects in chloride ion transport because CFTR functions as a chloride channel (Frizzell and Cliff, 1991). The primary symptoms of this disease are chronic bacterial infection, lung inflammation and elevated electrolyte levels in sweat (Sudbery, 2002). So far, approximately 1390 mutations of the CFTR gene have been identified (Hospital for sick Children, 2005). The eight most frequent mutations are ΔF508, G542X, W1282X, N1303K, 1717-1G→A, R553X, 2183AA→G, and I148T (Castaldo et al., 1999). The definitions of the abbreviations for cystic fibrosis mutations used in this paper are listed in Table 1. An estimate of the population frequency of mutations carried by CF-affected individuals that is based on data for European countries but, given the sample size, should be roughly similar in North America and Australia is shown in Table 2. Research suggests that over 90% of CF patients carry at least one ΔF508 allele and that almost 50% of CF cases are in individuals homozygous for ΔF508 (Sudbery, 2002). These data indicate that an initial test for the ΔF508 mutation is capable of identifying almost all individuals who are either affected by CF or who are carriers. For example testing for ΔF508 along with 20 common mutations identifies 70% of CF cases in Europeans (Sudbery, 2002). Several techniques such as allele specific oligonucleotide (ASO) dot-blot, reverse dot-blot, amplification refractory mutation (ARMS), and an oligo-ligation assay are available to detect the most common mutations (Schwarz, 1998). However, detecting compound heterozygotes between ΔF508 and rare mutations that are common only in particular ethnic groups is difficult. Therefore new diagnostic tests such as restriction enzyme assays and single stranded conformational polymorphism (SSCP) have been designed to recognize rare and population-specific mutations (Sudbery, 2002). This review will describe the most commonly used current CF diagnostic techniques, as well as novel assays and techniques that are might be employed to enhance our detection capabilities in the future.

Table 1
The abbreviations of cystic fibrosis mutations used in this article

Mutations	Abbreviations
ΔF508	Phenylalanine at position 508 is deleted
G542X	Glycine at position 542 is replaced by stop codon
W1282X	Tryptophan at position 1282 is replaced by stop codon
N1303K	Asparagine at position 1303 is replaced by lysine
1717-1G>A	Glycine is replaced by alanine at the last nucleotide in the intron preceding nucleotide 1717 in the cDNA
R553X	Arginine at position 553 is replaced by stop codon
I148T	Isoleucine at position 148 is replaced by threonine
3120+1G>A	Glycine is replaced by alanine at the first nucleotide in the intron following nucleotide 3120 in the cDNA
2183AA>G	Alanine is replaced by glycine at 2183 and deletion of alanine at 2184
4016insT	Threonine is inserted after nucleotide 4016

2. The most common diagnostic tests for cystic fibrosis

2.1. Allele specific oligonucleotide (ASO) dot-blot

ASO dot-blot is one of the most widely used techniques to diagnose CF. Genomic DNA or cDNA from the patient is amplified by PCR and transferred onto nylon membranes as a "dot-blot". Membranes are hybridized with either a radiolabelled wild type allele specific oligonucleotide (ASO) or mutated ASO under stringent conditions. Following autoradiographic exposure, the combination of oligonucleotide hybridization is observed (Saiki et al., 1986). Using this combination an ASO dot-blot can clearly distinguish homozygous, heterozygous and wild type subjects (see Fig. 1). This assay is simple to perform, easy to interpret, but it requires the handling of radioisotopes. Moreover, each probe has to be separately hybridized to the nylon membrane containing the amplified DNA, and this makes the process complex where various mutations and polymorphisms are present (Saiki et al., 1989) and extremely impractical for high-throughput testing.

2.2. Reverse dot-blot

The reverse dot-blot technique was developed in order to overcome some of the problems with the ASO assay (Saiki et al., 1989). In this assay, homopolymer tails with terminal deoxyribonucleotidyltransferase are added to ASOs, which are attached onto nylon membrane by uv crosslinking. The target DNA is amplified with biotinylated primers and hybridized to immobilized oligonucleotides on the membrane. Hybridization is detected by adding streptavidin-horseradish peroxidase to the membrane where it will bind to any biotinylated DNA that has hybridized to the oligonucleotides. A positive signal can then be obtained by a

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Table 2
The distribution of CF mutations carried by affected individuals based on data from European countries (adapted from Sudbery, 2002)

Percentage of affected population	CF mutations
49%	ΔF508/ΔF508
21%	ΔF508/common mutation not ΔF508
21%	ΔF508/rare mutation
4.5%	Common mutation not ΔF508/rare mutation
2.25%	Common mutation not ΔF508/common mutation not ΔF508
2.25%	Rare mutation/rare mutation

simple non-radioactive colorimetric reaction (Saiki et al., 1989). This technique allows a number of amplified sequences to be analyzed simultaneously in one hybridization reaction (Saiki et al., 1989). Innogenetics has marketed INNO-LIPA kit which uses this technique to detect twelve frequent CF mutations and seventeen rare mutations sequentially. This technique can be automated at the post-PCR level, thus working time and errors can be reduced. The main drawback of this procedure is the cost (Tomaiuolo et al., 2003).

2.3. Amplification refractory mutation system (ARMS)

The multiplex ARMS kit, marketed by Cellmark Diagnostics, can be used to recognize the 20 most common CF mutations (Sudbery, 2002). This technique is also known as allele specific PCR (ASPCR) or PCR amplification of specific alleles (PASA). In this technique, three multiplex PCR reactions are performed in parallel for each individual. Each tube contains number of separate primers capable of detecting different mutations. In each tube, sets of primer pairs are designed to anneal a particular allele to produce distinct band, well separated from the products produced from other alleles amplified in the same tube. A band is only produced in presence of mutant allele and the absence of the band indicates that the allele is absent; therefore positive signals are easily differentiated in order to identify the exact mutations present. Since it is possible for one of the tubes to produce no bands, there are always positive controls to show that the PCR reaction is functioning properly in each tubes (Fig. 2, Sudbery, 2002). The ARMS assay is accurate, rapid and easy to perform (Houdayer et al., 1998), but it does not distinguish between homozygotes and heterozygotes except for the ΔF508 mutation (Tomaiuolo et al., 2003).

2.4. Oligo-ligation assay (OLA)

OLA is another common technique used to detect CF mutations. In the OLA reaction, 2 oligonucleotides or probes are hybridized to a DNA sample in such a way that the 3′ end of the first or upstream probe is located next to the 5′ end of the second or downstream probe (Fig. 3, Glick and Pasternak, 2003). The region where the probes meet is right over a nucleotide position that will be altered in a mutant allele. After hybridization is complete, DNA ligase is added to the reaction. If the 3′ end of the first probe matches perfectly with the target DNA, it will be brought into close enough proximity to the second oligonucleotide that both probes can be ligated together. No ligation occurs when there is a mismatch between the 3′ end of first upstream probe and the target DNA. OLA is very accurate and it has the highest detection rate (Schwarz, 1998).This technique can be automated following ligation but requires a high level of operator proficiency (Tomaiuolo et al., 2003).

3. Novel diagnostic techniques in cystic fibrosis

Most of the CF diagnostic tests used to date have been highly informative and have been successfully applied in various research and diagnostic settings. Some of them, however, are tedious, technically complex to perform, and difficult to employ on a regular basis, especially in high-throughput settings (Feriotto et al., 2001). Therefore novel techniques are being designed to deliver a high level of accuracy, high-throughput screening, easily interpretable results, re-

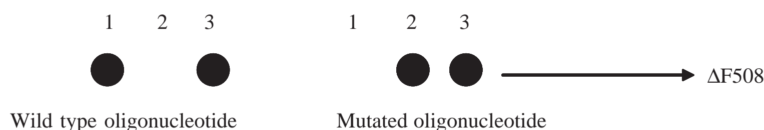

Fig. 1. Schematic representation of the allele specific oligonucleotide (ASO) dot-blot used to identify CF mutations. Subject 1 is healthy individual as no dot was detected with the mutated oligonucleotide. Subject 2 is homozygous for the mutation as a dot was detected only with the mutated oligonucleotide and subject 3 is heterozygous for the mutation as dots were detected with both the wild type and mutated oligonucleotides (modified from Castaldo et al., 1999).

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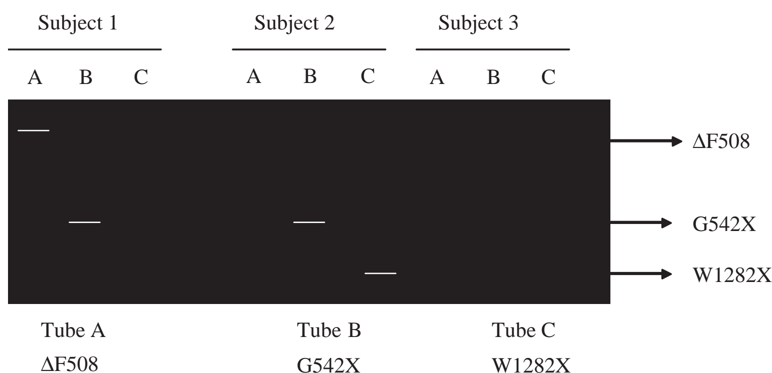

Fig. 2. Schematic representation of the multiplex PCR ARMs test used to detect CF mutations. The diagram shows tests on three different subjects. Subject 1 produces bands for both ΔF508 and G542X mutations in tube A and B . Therefore, subject 1 is a heterozygote containing both mutations. Subject 2 produces bands in tube B and C for G542X and W1282X mutations, respectively. This subject is a heterozygote containing both of these mutations. Subject 3 does not produce bands in any tubes. Therefore, this subject is a healthy individual. (modified from Sudbery, 2002).

duced costs, and detection of rare mutations. Some of these novel assays are described below.

3.1. Microarray

Microarray platforms are being developed that will facilitate detection of an increased number of CF mutations on a single chip in a cost effective way. Typically, the microarray technique involves the spotting of probes on the array (See Fig. 4). The mRNA from both test (sample from CF patient) and control samples are isolated where they are labelled with green and red fluorescent dyes, respectively. Then, both samples are mixed and hybridization to the probes attached to the microarray. The intensity of hybridization of any probe is proportional to the abundance of complementary mRNA bound to that probe. The fluorescence intensity can be quantified by a microarray scanner, and the level of gene expression can be compared between test and control samples by examining the ratio of red to green dye hybridized to a single spot (Galvin et al., 2004). Recently, microarrays have been successfully employed in preimplantation genetic diagnosis (PGD) of CF mutations, using F508 as a model (Salvado et al., 2004). A single microarray chip was constructed using oligonucleotides representing both the normal and F508 disease alleles. Target DNA was amplified by PCR, labelled with the fluorescent dye Cy3 and hybridized to the array. The performance of the array was evaluated by its capability to identify F508 mutations in the target DNA. Salvado et al. observed strong binding of the target DNA to the probes which allowed them to assign the expected F508 genotypes. They assessed the reliability and accuracy of the microarray diagnosis for F508 mutation in a blind test on 10 samples with either a normal, carrier or disease genotype. All samples were correctly genotyped using this technique (Salvado et al., 2004). Since this microarray was successful to detect F508 mutation, it may be possible to extend its capabilities to detect many mutations simultaneously within short period of time, especially in PGD where quick diagnosis is a major concern.

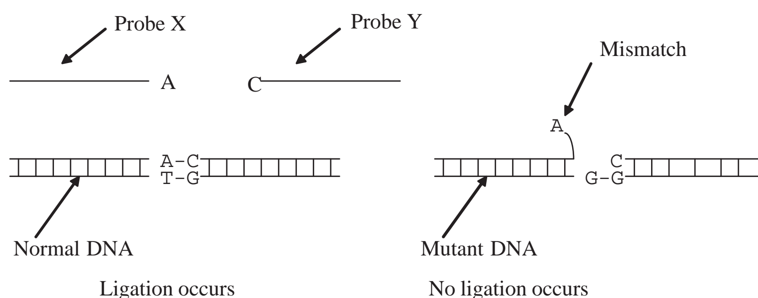

Fig. 3. The principle of oligonucleotide ligation assays (OLA). (modified from Glick and Pasternak, 2003).

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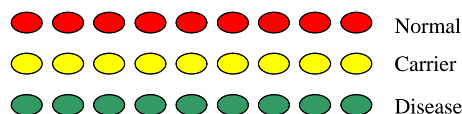

Fig. 4. Microarray data showing the hybridization of probes to indicate the presence or absence and quantity of mutant alleles present. Red, green and yellow represent normal, disease and carrier status, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Randomly ordered fiber-optic gene arrays

Microarray techniques entail target DNA labelling which is time consuming, and can also change the amount of target DNA present in the original sample (Steemers et al., 2000), therefore Steemers et al. developed randomly ordered fiber-optic gene arrays to screen unlabeled target DNA for CF mutations. Fig. 5A shows how randomly ordered microspheres, which are attached to Molecular Beacons (MB), are attached by biotin to the end of an optical fiber. Molecular beacons are DNA probes containing a hairpin loop that brings complementary 5′ (fluorophore) and 3′ (quencher) ends into close proximity, where they will bind (Steemers et al., 2000). The quencher can absorb energy emitted by fluorophore before any fluorescence can be detected, thus with no sample DNA bound, no signal is produced (Glick and Pasternak, 2003). Fig. 5B shows the organization of molecular beacons in greater detail. The sequence in the loop structures can be complementary to any wild type or mutated CF gene. Upon addition of unlabeled target DNA, the loop part of the MB base pairs with the appropriate complementary target sequence (Steemers et al., 2000). This hybridization separates the fluorophore and quencher moieties from each other, preventing the quenching of fluorophore by the 3′ end of the MB (Glick and Pasternak, 2003). Fig. 5C demonstrates how, when the complementary MB and target sequences hybridize, fluorescence hybridization patterns are imaged and analyzed (Steemers et al., 2000). This technique has been used to detect the most common CF mutation, ΔF508, successfully. This system provides high-throughput capabilities and fast hybridization times as multiple probes can be used simultaneously. It can also decrease the chance of both false positives and false negatives by including several copies of each probe in a random order at the end of each fiber (Steemers et al., 2000). Like microarrays, this technology has potential to detect many CF mutations simultaneously.

3.3. Biospecific interaction analysis (BIA)

Recent advances in surface plasmon resonance (SPR) based biosensors have enabled researchers to use biospecific interaction analysis (BIA) for monitoring various molecular reactions in real time. BIA is one of the most rapid (40 min), easy and automatable approaches for detecting point mutations of CF. This procedure monitors real-time hybridization between allele specific oligonucleotide probes and target DNA that is a single stranded PCR product obtained from normal subjects, a heterozygous, or a homozygous CF patient. This method has been used to detect the W1282X mutation, which is the most common in the Ashkenazi population (Feriotto et al., 2001). This optical-based technique uses sensor chips to which target

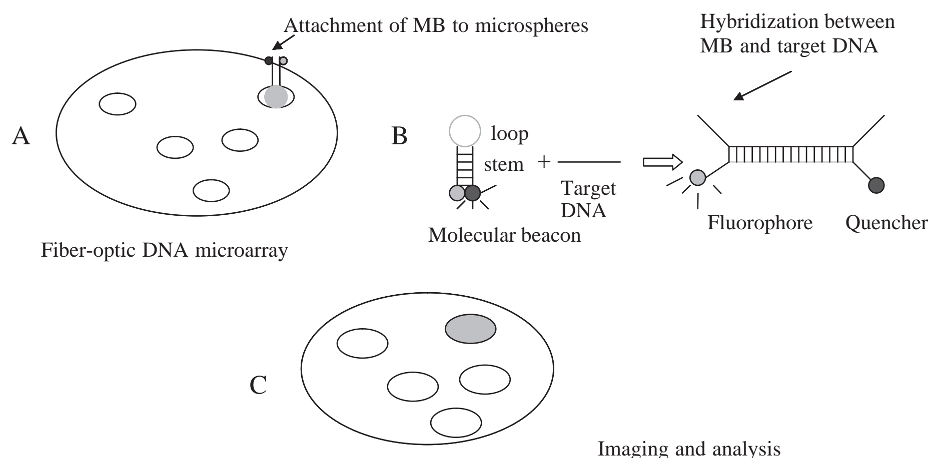

Fig. 5. Schematic representation showing the principles of fiber optic gene arrays (Modified from Steemers et al., 2000).

DNA has been immobilized and detects and quantifies changes in the refractive index of the chip following hybridization of allele specific DNA probes to the target DNA (Feriotto et al., 2001). Using this method, a one-step non-radioactive diagnosis can be performed (Feriotto et al., 2001). This technique could be beneficial for pre implantation diagnosis (PGD) where the speed of diagnosis is critical.

3.4. Multiplex primer-extension assay using ABI prism^R snapshot™ ddNTP primer-extension assay

There is currently no assay that can detect both 3120+1G→A and I148T mutations, which are the second most common mutations in African American and French Canadian populations, respectively. Therefore, a multiplex primer-extension assay has been developed to detect these two CF mutations rapidly and simultaneously using the ABI prism^R snapshot™ ddNTP primer-extension assay. The principle of this technique is described and illustrated in Fig. 6. The addition of fluorescently labelled ddNTP to the 3′ end of a sequencing primer can be detected by primer-extension reaction (PER) using an ABI genetic analyzer (see Fig. 7). In order to identify a specific polymorphism or mutation, the specific ddNTP matching the sequence of the mutant allele is labelled with a fluorescent dye, but can only be added if the target sequence contains a complementary nucleotide, indicating it is a mutant allele (Brown et al., 2001). The lasers in the analyzer detect oligonucleotides containing the fluorescent dyes. Researchers are speculating that further manipulation of the assay will allow more than two mutations to be analyzed, making it more cost effective. They also believe multiplex assays based on this technique will identify additional CF mutations (Brown et al., 2001). Unlike microarrays or DNA chip based techniques, this technique would not be able to detect hundreds of mutations simultaneously, however.

3.5. Proteomics

Recent advances in the field of proteomics have made it possible to identify novel disease markers that can be used as diagnostic tools. In the CFTR gene, various factors other than mutations are responsible for the phenotypic variability of CF. Comparing total protein expression profiles (proteomics) between CF patients and normal individuals with a two-dimensional electrophoresis (2-DE), can help to understand cellular alterations responsible for CF. Proteins that interact differently with the wild type and mutant CFTR can also be recognized by this approach. Therefore, protein profiling is promising since it can identify novel disease markers for diagnosis of CF mutations that are not based on CFTR gene analysis (Roxo-Rosa et al., 2004). The process of proteomics involves four major steps. First, separation of total protein is obtained by two-dimensional electrophoresis (2-DE). Second, detection of proteins is achieved by one of the different staining methods such as coomassie blue or silver. Third, bioinformatics analysis is carried out to produce 2-DE maps for database comparison. In this approach, individual protein spots are quantified and spot patterns

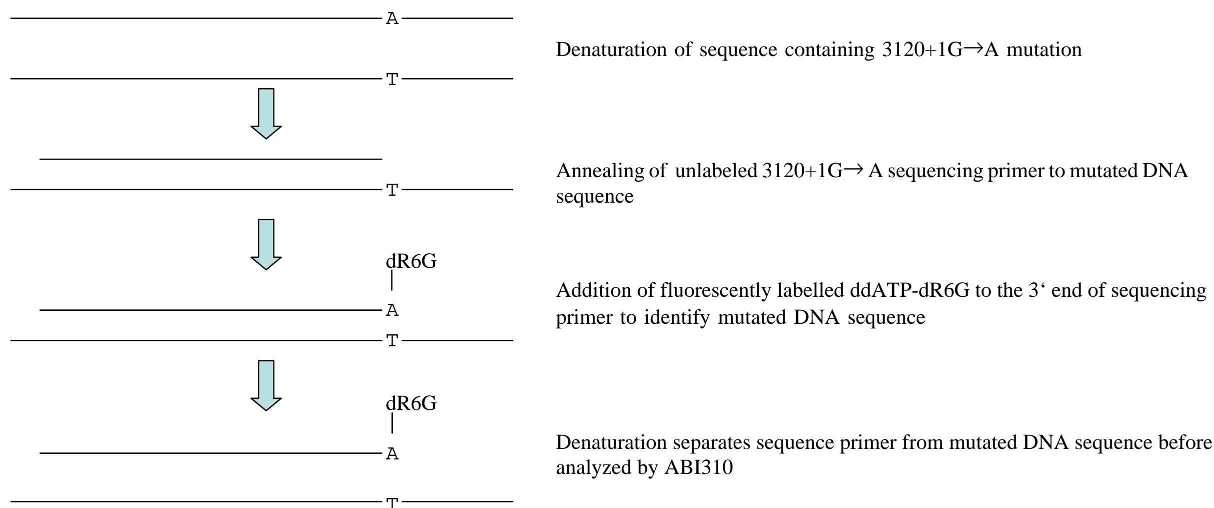

Fig. 6. Multiplex primer extension assay to detect the 3120+1G→A mutation. In order to detect I148T mutation, a different dideoxynucleotide (ddGTP-dR110) would be added instead of ddATP-dR6G, along with a different specific pimer (modified from Brown et al., 2001).

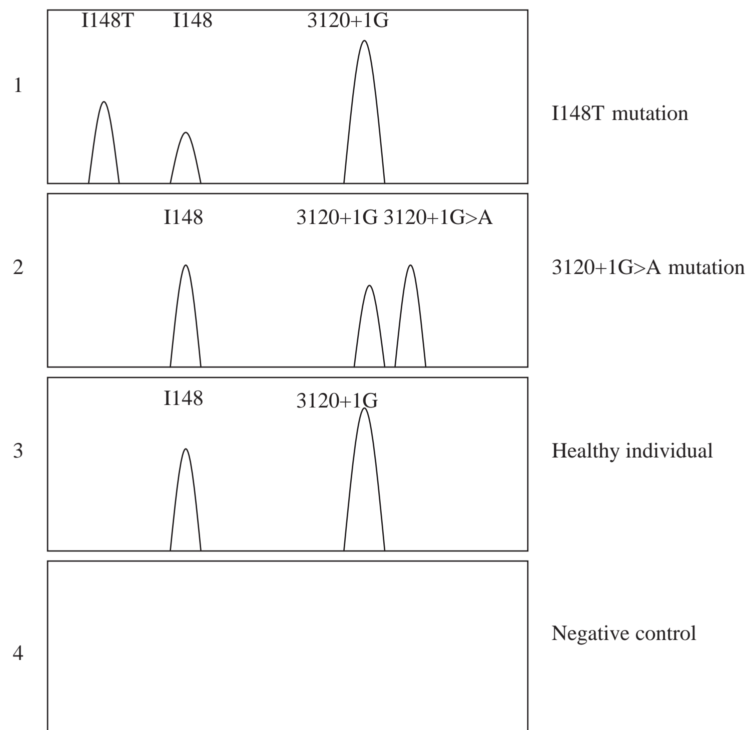

Fig. 7. The electropherograms of four primer extension reactions (PERs) analyzed on an ABI 310 genetic analyzer to detect I148T and 3120+1G>A CF mutations. 1) I148T heterozygote peak was detected only for the I148T mutation, not for 3120+1G>A. 2) 3120+1G>A heterozygote peak was detected only for 3120+1G>A, not for I148T. 3) A healthy individual was analysed so no peaks were detected for either the I148T or 3120+1G>A mutations 4) represents negative control (Modified from Brown et al., 2001).

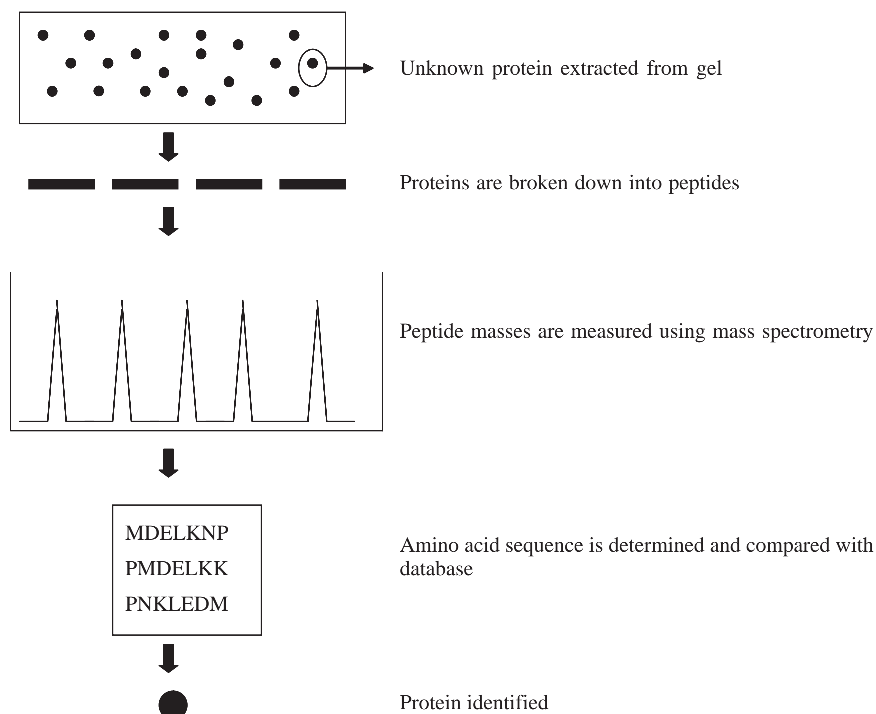

Fig. 8. The principle of proteomics.

are matched among multiple gels (disease versus control). Fourth, spots of interest are identified by mass spectrometry after excising them from 2-DE gels. Peptides are excised by trypsin digestion and their masses are measured by mass spectrometry. Peptide-sequence tags are produced by fragmentation of selected peptides, and they are used to search a protein sequence in a database so that protein of interest can be identified (Roxo-Rosa et al., 2004). At present however, this technique is neither fast nor cheap. This technique is, however, very promising for the development of non-gene based assays that can be utilized in the diagnosis of CF (Fig. 8).

4. Conclusion

Among newly developing techniques, re-sequencing microarrays, multiple genotyping arrays and comparative genome hybridization arrays hold the great promise to diagnose various genetic diseases including CF. In order to replace expensive DNA sequencing assays, re-sequencing microarrays are being developed (Kaufman and Strom, 2003). Furthermore, a low-density microarray is being fabricated for multiple SNP (single nucleotide polymorphism) analysis that will identify 25 CF mutations and 6 polymorphisms simultaneously. Comparative genome hybridization arrays may revolutionize prognostic testing since it has potential to scan entire genome for insertions and deletions (Kaufman and Strom, 2003). All techniques are being designed to provide quick, easy, accurate, and reliable diagnostic tests. Given that the mutations involved are different in nature (i.e. some are point mutations, some are deletions) it will be difficult to come up with one technique that will detect them all in one quick easy step.

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References

Brown NM, Bernacki S, Pratt VM, Stenzel TT. Fluorescent, multiplexed, automated, primer-extension assay for 3120+1G→A and I148T mutations in cystic fibrosis. Clin Chem 2001;47:2053–5.

Castaldo G, Fuccio A, Cazeneuve C, Picci L, Salvatore D, Raia V, et al. Detection of five rare cystic fibrosis mutations peculiar to southern Italy: implications in screening for the disease and phenotype characterization for patients with homozygote mutations. Clin Chem 1999;45:957–62.

Feriotto G, Ferlini A, Ravani A, Calzolari E, Mischiati C, Bianchi N, et al. Biosensor technology for real-time detection of the cystic fibrosis W1282X mutation in CFTR. Hum Mutat 2001;18:70–81.

Frizzell RA, Cliff WH. Back to the chloride channel. Nature 1991;350:277–8.

Galvin P, Clarke LA, Harvey S, Amarlal MD. Microarray analysis in cystic fibrosis. J Cyst Fibros 2004;3:29–33.

Glick BR, Pasternak JJ, 2003. Molecular biotechnology: principles and applications of recombinant DNA. 3rd ed. Washington (DC): ASM press; 2003. p. 240–9.

Hospital for sick children, Toronto. Cystic fibrosis mutation database. http://www.genet.sickkids.on.ca/cftr/; 2005.

Houdayer C, Cazeneuve C, Cougoureux E, Magnier C, Tredano M, Aymard P. Clinical evaluation of the CF(12)m cystic fibrosis DNA diagnostic kit. Clin Chem 1998;44:1346–8.

Kaufman HW, Strom, CM. From peapods to laboratory medicine: molecular diagnostics of inheritable diseases. MLO Med Lab Obs 2003;35:30-2, 36, 38, 40.

Rommens JM, Ianuzzi MC, Kerem BS, Drum ML, Melmer G, Dean M. Identification of the cystic fibrosis gene: chromosome walking and jumping. Science 1989;245:1059–65.

Roxo-Rosa M, Davezac N, Bensalem N, Majumder M, Heda GD, Simas A, et al. Proteomics techniques for cystic fibrosis research. J Cyst Fibros 2004;3:85–9.

Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA. Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. Nature 1986;324:163–6.

Saiki RK, Walsh PS, Levenson CH, Erlich HA. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc Natl Acad Sci 1989;86:6230–4.

Salvado CS, Trounson AO, Cram DS. Towards preimplantation diagnosis of cystic fibrosis using microarrays. Reprod Biomed Online 2004;8:107–14.

Schwarz MJ. DNA diagnosis of cystic fibrosis. Ann Clin Biochem 1998;35:584–610.

Steemers FJ, Ferguson JA, Walt DR. Screening unlabeled DNA targets with randomly ordered fiber-optic gene arrays. Nat Biotechnol 2000;18:91–4.

Sudbery P, 2002. Human molecular genetics. 2nd ed. Harlow (England): Pearson education limited; 2002. p. 147–52, 257–58.

Tomaiuolo R, Spina M, Castaldo G. Molecular diagnosis of cystic fibrosis: comparison of four analytical procedures. Clin Chem Lab Med 2003;41:26–32.