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Study of the Genes Responsible for the Drug Resistance of *Mycobacterium tuberculosis* and Their Application for Rapid Diagnosis

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Introduction

Control of drug-resistant tuberculosis has become one of the major problems all over the world. However, the detection of drug-resistant *Mycobacterium tuberculosis* by culture takes at least 3 weeks. Thus, treatment is prescribed empirically. Patients who fail to respond to drugs remain infectious and they may serve as a source of transmission of infections from patients to patients, patients to health care workers, and from patients to their relatives. Thus, the establishment of a rapid, simple and reliable method for detecting drug-resistant *M. tuberculosis* is one of the most urgent subjects in the treatment of tuberculosis patients.

Mode of action of anti-tubercle drugs

Five drugs (Rifampicin (RFP), Isoniazide (INH), Ethanbutol (EB), Streptomycin (SM), and Pyradinamide (PZA) are now used as first line drugs for treatment of tuberculosis. In addition to these, so called second line drugs such as kanamycin (KM), enviomycin (EVM) are used. The mode of action of these drugs were studied and reported. RFP act on the beta subunit of RNA polymerase and inhibit the RNA synthesis. INH inhibits the biosynthesis of mycolic acid on the lipid rich cell wall. EB inhibit the synthesis of arabino-galactan attached on the peptide glycan. Amino-glycoside antibiotics such as SM and KM bind to the ribosome and inhibit the protein synthesis. Pyrazinnoic acid, a metabolite of pyradinamide, inhibits the eukaryotic-like fatty acid synthetase I.

Molecular mechanism of acquiring drug resistance

To understand the molecular basis of resistance might become helps for the establishment of a novel method for detection of drug-resistant *M. tuberculosis* strains. This approach has been pursued, and the detection of drug-resistant *M. tuberculosis* by genetic methods has become possible.

Near 95% of rifampicin-resistant strains carry mutation(s) on a certain part of the RNA polymerase beta subunit gene (*rpoB*). About 80% of streptomycin-resistant strains carry mutation(s) on either ribosomal protein S12 gene (*rpsL*) or 16S ribosomal RNA gene (*rrs*). A region, which is different from that associated with the SM resistance, on the *rrs* is associated with the kanamycin resistance. It has also been known that the resistance to isoniazid (INH) is due to mutations in the catalase-peroxydase gene (*kat* G), the NADH-dependent 2-trans enoyl-acyl carrier protein reductase gene (*inhA*) operon or the alkyl hydroperoxide reductase gene (*ahpC*). The gene responsible for resistance to ethambutol has been reported to code

arabinosyltransferases in *M. avium* and *M. tuberculosis*. The *pncA* gene coding pyrazinamidase/ nicotinamidase turned out to be a gene responsible for pyrazinamide resistance. Caceres *et al.* reported that the overexpression of the D-alanine racemase gene confers resistance to D-cycloserine (CS) in *M. smegmatis*. In addition, the mutation(s) responsible for the fluoroquinolone (FQ)-resistant mutations were found on the *gyrA* gene as has been seen in other bacteria.

Applications for rapid diagnoais

As applications of these findings, many methods were used for detection of point mutations. Telenti *et al.* sequenced the *rpoB* gene by using an automatic sequencer and identified the mutation site associated with rifampicin resistance. However, direct sequencing method is not practical because it requires sequencing of a large number of genes and a costly apparatus. Thus, the PCR-SSCP (PCR-single strand conformational polymorphism) method, the heterodupulex method, the PCR- RFLP (PCR-restriction fragment length polymorphism) method, the molecular beacon method and so forth have been developed as a practical gene amplification method, each has its own shortcomings.

Recently, a line hybridization assay (INNO-LiPA Rif.TB kit: Innogenetics, Belgium) was developed for the purpose of detection of rifampicin resistance on the basis of defferential hybridization (26). Although this method has significantly improve conventional methods, there still remain a few problems to fully meet needs of accuracy and speed of diagnosis in clinical practice. Since this is a method where oligonucleotides are adsorbed onto a filter, the number of lines positioned in a unit area is limited. For example, if more mutant sequences associated with rifampicin resistance are to be covered, there will be nearly 20 kinds of sequences as for positive sequences alone. Consequently, many kinds of filters must be prepared. In the INNO-LiPA Rif.TB kit, in order to avoid this problem, 5 sequences isolated from different sites on the genome derived from wild-type tubercle bacillus are used and those that are not completely fused are determined as resistant for convenience. A DNA microarray comprises oligonucleotide probes arranged on a silicon chip at high density in an array. The technique was developed for the purpose of detection of rifampicin resistance and species determination of mycobacteria. There also remain technological and economical problems such as chip preparation cost, formation of a precise array on a silicon chip and high-resolution device for reading results.

Oligo array for detection of drug resistant *M. tuberculosis*

To overcome shortcomings described above, we have established a simple and cost saving method for detection of drug resistant *M. tuberculosis*. Wild and mutant type oligonucleotides having an amino group at 5' end was synthesized by using an oligonucleotide synthesizer in a conventional manner and spotted on a poly-carbodiimide coated slide glass. The oligonucleotides involved in this array consisted of 36 wild (susceptible) type, 67 mutant (resistant) type, and four for the discrimination of *M. tuberculosis* and *M. avium-intracellurare* complex (MAC). The *rpoB* gene fragment for RFP, *katG*, *inhA* gene fragment for INH, *embB* gene fragment for EB, *rrs* and *rpsL* gene fragment for SM, and *rrs* gene fragment for KM resistance discrimination were amplified by PCR using biotinylated antisense primer and hybridized to the oligonucleotide array on the slide glass. The biotin on the array was detected by horseradish peroxidase labeled avidin-biotin complex, followed by

color reaction using TMB peroxidase reagent. After the detection of chemical color development, images were stored as TIFF images in a computer by using a scanner for OA. Pigmentation observed in these images, which was formed by the chemical color development, indicates that the oligonucleotide immobilized on the substrate (capture oligo) and the nucleic probe in the test sample are perfectly complementary to each other, forming a duplex.

By this method about 90% of RFP resistant *M. tuberculosis* could be detected. This was very close to the ratio of the RFP resistant *M. tuberculosis* carrying mutation(s). In the case of KM resistant *M. tuberculosis*, all the strains carrying mutation(s) (about 70% of resistant strains) could be identified by our oligo array. In contrast to these, slightly lower number that theoretical one were detected. The Oligo array for detecting the EB resistant *M. tuberculosis* is now in progress.

Many mutations responsible for the drug resistance of *M. tuberculosis* were identified during this decade. By using this information, many trials have been performed to establish rapid, simple, and reliable method for the detection of drug resistant *M. tuberculosis*. Each method possessed own shortcomings such as time or cost consuming. The Oligo array system established in our laboratory will be one of the best methods for the detection of drug resistant *M. tuberculosis*. However, the present system should be developed by getting new information of the mutations responsible for the drug resistance and adding spots on the oligo array.