Chapter 6

Drug Resistance in *Mycobacterium tuberculosis*

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Additional information is available at the end of the chapter

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Abstract

Tuberculosis (TB) remains to be a serious health problem worldwide. There is an increased transmission of *Mycobacterium tuberculosis* strains with drug resistance, hence complicating TB control. The deciphering of the *M. tuberculosis* genome, together with the implementation of new molecular biology tools, has allowed the identification of changes in nucleic acid sequences with a functional impact. These mutations have become important in the design of early-diagnostic kits to identify the resistance profile of *M. tuberculosis*. Since the conventional methods to determine the identity of *M. tuberculosis* strains based in cultures are laborious, time-consuming and performed by specialized technicians, the result is generated until 4 months after receiving the samples. During this time, patients with TB are not adequately treated, and resistant strains may be transmitted to the rest of the population. In this chapter, we describe the most relevant mutations in genes associated with drug resistance in *M. tuberculosis*, the analysis of gene expression to identify new markers of drug resistance strains, and the development of new antituberculosis drugs against drug-resistant strains.

Keywords: *Mycobacterium tuberculosis*, drug resistance, mutations, gene expression, antituberculosis drugs

1. Introduction

Tuberculosis (TB) has remained a serious health problem since *Mycobacterium tuberculosis*, the main agent of this disease, infects about one third population. In 2015, 10.4 million cases of TB were estimated and, although only a small percentage (5–10%) develops the illness, its control has complicated due to the emergence of drug resistance strains [1]. Tuberculosis regiment treatment includes the first line drugs rifampicine, isoniazide, ethambutol and pyrazinamide and strains that develop resistance to the two more effective antituberculosis drugs, isoniazide and rifampicine, named

multidrug resistance strains [2, 3]. The resistance phenomenon in *M. tuberculosis* has been highly related to mutations in specific genes [4], and this association has been the base for the implementation of rapid diagnostics kits [5] but unfortunately mutations do not explain completely the resistance in all cases [6, 7], suggesting that other mechanisms could be involved. New approaches to search new markers and mechanisms of resistance have been performed. One of these is the evaluation of changes in gene expression [8]. Together with the diagnostics of TB, the implementation of new schemes of treatments is necessary to restrict the transmission. The development of new drugs against drug resistance *M. tuberculosis* has resulted promissory to control TB [9].

2. Mutations that confer resistance in *Mycobacterium tuberculosis*

Although the rate of resistance to first and second line drugs in *M. tuberculosis* varies among countries, the resistance phenomenon has complicated the tuberculosis control worldwide. There two types of observed resistance in *M. tuberculosis*, one is the genetic resistance where mutations in genomic regions, on target genes, confer the capacity to avoid the drug effect; the second is the phenotypic resistance, where epigenomic modifications, including alteration of protein structures, generate resistance to drugs without mutation on DNA. Although the current knowledge of the molecular genetic basis of resistance to antituberculosis drugs has advanced rapidly the last years [10], there are unknown mechanisms in how bacilli is able to resist to drugs. Identification of clinical isolates with resistance to antituberculosis drugs would facilitate the timely and accurate diagnosis to initiate an appropriate treatment.

Many works have revealed, using microbiological and clinical data, mutational events in clinical isolates from patients with tuberculosis. Multidrug resistance appears to result from the sequential acquisition of mutations. Possible reasons for the acquisition of mutations include inadequate prescription and delivery of chemotherapy, poor compliance, or an insufficient number of active drugs in the treatment regimen [11]. Mutations or combinations of mutations have been found in strains displaying single or multidrug resistance. Here, we summarized the most common mutation found in clinical isolates that confer resistance to the first and second line antituberculosis drugs (**Table 1**).

2.1. Isoniazid

Due to its properties as a bactericidal drug, isoniazid has been widely used as the first line drug in the treatment against *M. tuberculosis* complex members. Mutations on *katG* and *mabA-InhA* genes have repeatedly been associated with isoniazid resistance [10] (**Table 1**). In the case of *katG*, the most common of mutation is S315, which is present in 50–95% of isoniazid resistant clinical isolates [12]. The occurrence of mutations is also observed in the promoter region of *mabA*/*inhA*. Mutations in the *inhA* promoter can also confer cross-resistance to ethionamide [13]. There are other genes as *ahpC*, *kasA*, and *ndh*, encoding for alkyl hydroperoxidase reductase, β-ketoacyl-ACP synthase, and NADH dehydrogenase, respectively, which have also been associated with isoniazid resistance.

Drugs	MIC (μg/mL)	Drug mode of action	Gene	Target enzyme	Frequency of mutations (%) associated with resistance
Isoniazide	0.02–0.2	Inhibits mycolic acid synthesis and other multiple effects	*katG*	Catalase peroxidase	30–60
			InhA	Fatty acid enoyl acyl carrier protein reductase A	70–80
			ahpC	Alkyl hydroperoxidase reductase	Not known
			kasA	β-ketoacyl-ACP synthase	Not known
			ndh	NADH dehydrogenase	9.5
Rifampicin	0.05–1	Inhibits RNA synthesis	*rpoB*	β subunit of RNA polymerase	95
Streptomycin	2–8	Inhibits protein synthesis	*rpsL*	Ribosomal protein S12	65–67
			rrs	16S rRNA	
			gidB	7-Methylguanosine methyltransferase	33
Ethambutol	1–5	Inhibits arabinogalactan synthesis	*embCAB*	Arabinosyl transferase	70–90
Pyrazinamide	16–100	Disrupts plasmamembrane and energy metabolism (inhibits pantothenate and CoA synthesis)	*pncA*	Pyrazinamidase	>70
			IS6110 insertion		Not known
Fluoroquinolones	0.5–2.5	Introduces negative supercoils in DNA molecules	*gyrA*	DNA gyrase	42-85
			gyrB		
Kanamycin/ Amikacin	2–4	Inhibits protein synthesis	*rrs*	16S rRNA	>60
Capreomycin/ Viomycin	2–4	Inhibits protein synthesis	*rrs*	16S rRNA	40-100
			tlyA	rRNA methyltransferase	80
Ethionamide	2.5–10	Disrupts cell wall biosynthesis by inhibition of mycolic acid synthesis	*InhA*	Fatty acid enoyl acyl carrier protein reductase A	>60
			ethA	Flavin monooxygenase	>60
			ethR	Transcriptional repressor	Not known

Data taken and modified from [16] and [17].

Table 1. Genes associated with resistance to various anti-TB drugs.

2.2. Rifampicin

Rifampicin is highly bactericidal for *M.tuberculosis*, where this drug is able to bind to the β subunit of RNA polymerase, and this event induces hydroxyl radical formation in susceptible mycobacteria [14]. Resistance to rifampicin is acquired by mutations in a region of the 81-bp region of the *rpoB* gene, encoding the β subunit of RNA polymerase, and these mutations have been found in ~96% of rifampicin-resistant clinical isolates. The most frequent mutations are located in positions 516, 526, and 531. Also, there is evidence that mutations in the *rpoB* gene generate cross-resistance to rifamycins [15]. It is important to mention that not all mutations in *rpoB* are associated with rifampicin resistance [16].

2.3. Streptomycin

It has been considered as a second line antituberculosis drug, which binds to the 30S subunit of the ribosome and blocks protein synthesis. The resistance is provoked by mutations in the *rpsL* gene, which encoded the S12 protein, and the *rrs* gene, which encoded the 16S rRNA. The mutations in both genes are the main mechanism of streptomycin resistance, and it has been found in 65–67% of resistant clinical isolates [16]. There is another gene, *gidB*, involved in streptomycin resistance. This gene encoded a 7-methylguanosine (m(7)G) methyltransferase, and mutations have been found in 33% of clinical isolates resistant to streptomycin.

2.4. Ethambutol

Ethambutol has a target, the inhibition of the enzyme arabinosyl transferase, which is involved in the biosynthesis of cell wall arabinogalactan. The enzyme is encoded by the embB gene, harboured in the embCAB operon, and mutation in this gene is related to ethambutol resistance. The most frequent mutation found in the *embB* gene is located in codon 306. More than 68% of resistant clinical isolates have mutations in the *embB* gene [16].

2.5. Pyrazinamide

This pro-drug is converted to its active form, pyrazinoic acid, and it only kills non-growing persistent bacteria. The mutations of the *pncA* gene are scattered along this genomic region, and it is the main mechanism of pyrazinamide resistance. The majority of pyrazinamide-resistant clinical isolates (72–99%) have showed mutations on the *pncA* gene sequence. Due to a high correlation between mutations and pyrazinamide resistance, it has been suggested that the use of mutations to predict the resistance profile to pyrazinamide; however, there are silent mutations that do not confer resistance [16].

2.6. Fluoroquinolones

Fluoroquinolones are able to inhibit the activity of DNA gyrase. When the activity of DNA gyrase is affected, the chromosomal DNA acquires a supercoiled conformation [17]. Then, mutations on *gyrA*, encoding DNA gyrase, are strongly associated with fluoroquinolone

resistance. There are many reports where mutations located in the *gyrA* region are present in 42–85% of clinical isolates resistant to fluoroquinolones (Louw et al., 2009). Also, mutations on the *gyrB* gene have also been associated with fluoroquinolone resistance, where 3% of clinical isolates harbor the mutation in this gene. The most common mutations of the *gyrA* gene are located in codons 90, 91 and 94 [16].

2.7. Amikacin/kanamycin

Amikacin and kanamycin are considered as second-line antituberculosis drugs. It has been identified that the *rrs* gene as the target of action of these drugs; however, the molecular mechanisms that confer resistance are focused to inhibition of protein synthesis [18]. About 60% of the clinical isolates resistant to amikacin/kanamycin have mutations on the *rrs* gene [17]. The most common mutations are located at the position 1400 of the *rrs* gene, which causes high-level resistance these drugs.

2.8. Ethionamide

This prodrug requires to be activated by the mono-oxygenase EtaA/EthA. It has been described as the only bactericidal agent against *M. tuberculosis*. Ethionamide inhibits mycolic acid synthesis. Mutations in *inhA* also confer resistance to ethionamide. Frequency of mutations on *etaA*/*ethA*, *ethR,* and *inhA* genes in clinical isolates resistant to ethionamide reaches 60% [16].

Mutations described in *M. tuberculosis* have led to the implementation of rapid molecular diagnostic kits with the aim to diagnose TB and detect drug resistance in a shorter period compared to drug susceptibility testing based on the culture of the microorganism [19].

Within the rapid methods approved by the WHO, there are real-time PCR-based assays, as Xpert MTB/RIF, the line probe assays Genotype MTBDR*plus* and Genotype MTBDR*sl.* The XpertMTB/RIF tests allow *M. tuberculosis* detection as well as resistance to rifampicine. A multicenter study in which 6648 patients were evaluated, the Xpert MTB/RIF test allowed the detection of 90.3% of the TB confirmed cases based on culture, as well as 67.1% of the TB cases diagnosed by microscopy. For detection of rifampicine resistance, a sensitivity of 94.4% and specificity of 98.3% were reported, and an indeterminate rate of 2.4%, which was lower than that of culture diagnose with 4.6% [20]. On the other hand, the Genotype MTBDR*plus* allows detection of resistance for the first line drugs, while Genotype MTBDR*sl* detects resistance to the second line drugs. A meta-analysis of Genotype MTBDR*plus* reported a pooled sensitivity of 0.91, 0.96, and 0.91 and a pooled specificity of 0.99, 0.98 and 0.99 for the detection of isoniazide-, rifampicine-, and multidrug-resistance, respectively. Both, sensitivity and specificity settings have 95% confidence intervals [21]. Finally, in a multicenter study realized in 2012, the accuracy of the Genotype MTBDR*sl* was evaluated in 200 *M. Tuberculosis* isolates; in this study, the sensitivity reported was between 77.3 and 92.3% for the detection of resistance to fluoroquinolones, ethambutol, amikacin, and capreomycin while for kanamycin was 42.7 and 22.6% for XDR detection; the specificity was 82% for all drugs [22].

3. Searching for new markers to identify drug resistance of *Mycobacterium tuberculosis*

In the understanding and linking-up of genetic associations with the drug resistance phenotype in *M. tuberculosis*, mutations in specific genes have been the most common association as previously described; nevertheless, not all resistant *M. tuberculosis* strains have the related mutations previously reported suggesting that other mechanisms could be involved in this phenomenon [6, 23, 24]. For this purpose, the expression level of some genes has been studied. One of them is efflux pump genes, these are important elements that play a role in the extrusion of drugs out of the cells conferring *M. tuberculosis* resistance to drugs [25]. The efflux pumps have been classified in super families: ATP-binding cassette (ABC), major facilitator super-family (MFS), resistance nodulation division (RND), small multidrug resistance (SMR), and multidrug and toxic-compound extrusion (MATE) [23]. In *M. tuberculosis*, the efflux pumps consist of (a) 12 mycobacterial large membrane proteins (MmpL) belonging to RND-type transporters [26], (b) 37 ABC transporters (26 complete and 11 incomplete) from which 21 are putative exporters which include antibiotic transporters that represent the 2.5% of the genome [27, 28] and (c) 16 putative MFS drug efflux pumps [29]. Some findings have reported efflux pump genes to be overexpressed in drug resistance *M. tuberculosis* strains (**Table 2**) [25, 30-32]. The importance of efflux pumps involved in drug resistance has led to suggest the analysis of the implementations of a combined therapy of antituberculosis drugs together with efflux pump inhibitors [23].

Locus	Symbol[a]	Gene name[a]	Drug-resistant phenotype	References
Rv2688c		Antibiotic-transport ATP-binding protein ABC transporter	XDR	[32]
Rv1634		Drug efflux membrane protein	XDR	[32]
Rv2936	*drrA*	Daunorubicin-dim-transport ATP-binding protein ABC transporter drrA	XDR	[32] [30]
Rv2937	*drrB*	Daunorubicin-dim-transport membrane protein ABC transporter drrB	XDR	[32] [30]
Rv0820	*phoT*	Phosphate-transport ATP-binding protein ABC transporter phoT	XDR	[31]
Rv2136c	*uppP*	Conserved membrane protein	MDR	[25]
Rv2846c	*efpA*	Membrane efflux protein efpA	MDR	[30]
Rv0849		Conserved membrane transport protein	MDR	[30]
Rv1250			MDR	[30]
Rv1410		Aminoglycosides/tetracycline-transport membrane protein	MDR	[30]
Rv1634		Drug efflux membrane protein	MDR	[30]
Rv2994		Conserved membrane protein	MDR	[30]

Locus	Symbol[a]	Gene name[a]	Drug-resistant phenotype	References
Rv2333c	*stp*	Involved in transport of drug across the membrane (export)	MDR	[30]
Rv2459		Conserved membrane transport protein	MDR	[30]

[a]Data obtained from TB database and/or TubercuList, XDR: extensively drug-resistant.

Table 2. Overexpressed efflux pump genes in drug-resistance *Mycobacterium tuberculosis* strains.

Furthermore, studies in drug resistance strains have reported other genes as differentially expressed between sensitive and drug-resistant strains. Functional categories of these genes are among others, stress response and translation (**Table 3**). On the other hand, expression of intergenic regions (IGs) has also been associated with a drug resistance phenomenon in *M. tuberculosis* [8, 25, 31], suggesting that an additional analysis is necessary to evaluate and confirm the contribution of these regions in drug resistance.

With the aim to demonstrate the resistance association between the level of expression of some genes and drug resistance, assays using recombinant strains of *M. tuberculosis* as well as other *Mycobacterium* strains treated with different drugs and/or overexpressing genes of interest have been analyzed [25, 33-35]. The new findings related to differences of gene basal expression between susceptible and resistant *M. tuberculosis* strains can contribute to identify newly genetic drug-resistant markers that could contribute in the early diagnosis of drug-resistant tuberculosis, which could be applied in the establishment of a more efficient drug therapy [8, 30].

Locus	Symbol[a]	Gene name[a]	Expression level modification	Drug-resistant phenotype	References
Rv1181	*pks4*	Polyketide beta-ketoacyl synthase pks4	I	XDR	[31]
Rv1182	*ppA3*	Polyketide synthase associated protein papA3	I	XDR	[31]
Rv1184c		Hypothetical exported protein	I	XDR	[31]
Rv0826		Conserved hypothetical protein	I	XDR	[31]
Rv1483	*fabG1*	3-oxoacyl-[acyl-carrier protein] reductase fabG1	I	XDR	[31]
Rv1592c		Conserved hypothetical protein	I	XDR	[31]
Rv1623c	*cydA*	Membrane cytochrome D ubiquinol oxidase subunit I cydA	I	XDR	[31]
Rv2585c		Conserved lipoprotein	I	XDR	[31]
Rv2621		Transcriptional regulator	I	XDR	[31]
Rv3269		Conserved hypothetical protein	I	XDR	[31]
Rv0287	*esxG*	Esat-6 like protein esxG	I	MDR	[8]
Rv0288	*esxH*	Low molecular weight protein antigen 7 esxH	I	MDR	[8]

Locus	Symbol[a]	Gene name[a]	Expression level modification	Drug-resistant phenotype	References
Rv1037c	*esxI*	Esat-6 like protein esxI	I	MDR	[8]
Rv1642	*rpmI*	50S ribosomal protein L35 rpmI	I	MDR	[8]
Rv1630	*rpsA*	30S ribosomal protein S1 rpsA	I	MDR	[8]
Rv3487c	*lipF*	Esterase/lipase lipF	R	MDR	[8]
Rv3418c	*groES*	10 kda chaperonin groES	R	MDR	[8]
Rv1161	*narG*	Respiratory nitrate reductase alpha chain narG	R	MDR	[8]
Rv1819c		Drugs-transport transmembrane ATP-binding protein ABC transporter	R	MDR	[25]

[a]Data obtained from TB database. XDR: extensively drug-resistant, MDR: multidrug-resistant, I: gene with induced expression in the resistant strain analyzed, R: gene with repressed expression in the resistant strain analyzed.

Table 3. Differential expressed genes in drug-resistance *Mycobacterium tuberculosis* strains.

4. Development of new drugs against *Mycobacterium tuberculosis* drug resistance strains

Even though tuberculosis antibiotic treatment therapy is described, drug resistance in *M. tuberculosis* complicates the TB control. In 2015, 480,000 MDR tuberculosis cases were estimated and, in addition, 100,000 more cases were added which had resistance to rifampicin [1], these cases are more likely to develop multi-drug resistance. Drug-resistant TB has led to the implementation of new therapeutic regimens involving second line drugs, once drug susceptibility testing results are available [36].

Drug therapy for a patient infected with a susceptible *M. tuberculosis* strain lasts 6 months with diverse combinations of the first-line drugs rifampicine, isoniazid, ethambuthol, and pyrazinamide, while treatment therapy for a patient with DR tuberculosis can last up to 20 months and include a fluoroquinolone, an injectable aminoglycoside plus an oral bacteriostatic second line drug and a first line drug (for details consult D'Ambrosio et al. [36]).

Because the problem of resistant tuberculosis is increasing, searching for new drugs continues with the aim of improving the therapeutic regimens currently used, shorten treatment duration in addition to find more effective drugs for latent TB and drug-resistant strains. The development of new antituberculosis drugs implicates the following stages: basic research, discovery of new antituberculosis compounds or drugs, preclinical and clinical studies conformed by phases I, II, and III to finally get to the technology transfer; all these processes entail long periods of time [37]. In this continuous search for better antituberculosis drugs, many natural, semi-synthetic, and synthetic compounds have been evaluated *in vitro* and *in vivo*. We will mention some new drugs that are based on the structure of first line drugs, among which some analogues have been described with activity against sensitive and drug resistant *M. tuberculosis* strains. Thereby based on ethambutol, some of the novel described

compounds comprise SQ109 and analogues based on carbamate prodrugs [38], S2824 and analogues with a homopiperazine ring [39], 1,2 diamines [40], ferrocenyl compounds [41] and dihydrosphingosine-ethambutol analogues [9]. Within pyrazinamide analogues, it has been described POEs (pyrazinoic acid esters) and 5-Cl-substituted pyrazinoic acid derivatives [42]. However, it is necessary to consider the adverse effects of these compounds. For isoniazide-based compounds, there has been reported aromatic and heterocyclic aldehydes containing electron-withdrawing or donating groups [43], and rifampicin has been described within the rifamycins as well as among others as rifabutin, rifapnetine, rifalazil, and rifametane [44].

As general conclusion, although mutations are commonly associated with drug resistance in *M. tuberculosis*, other studies are necessary to discover genetic markers that support the early diagnostic of drug resistance in strains that enable the establishment of optimized therapeutic schemes limiting their transmission.

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