Efflux Pumps and Mutation Sequences Related to Drug-resistance of *Mycobacterium tuberculosis*: Potential Targets for Epitope Based Immunotherapy?

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Abstract

With the rapid development of methodologies used in the process of scrutinizing the detailed mechanisms involved in the *Mycobacterium tuberculosis* (*M. tuberculosis*, Mtb) infection, the role of efflux pumps has been drawing an increasing attention. These efflux pumps have a high relevance to the resistance of the anti-TB drugs, and they are mainly classified into several major families, such as, the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the resistance-nodulation-division (RND) superfamily. As to the substrates of these efflux pumps, some antibiotics and other compounds can be extruded in the case of multidrug resistance. Mutation sequences related to the drug-resistance of *M. tuberculosis* could also be used as potential targets for epitope identification, such as rpoB, katG, inhA, and embB. The vaccines based on epitopes from these targets can be used to enhance the effects of the chemotherapy and traditional vaccine therapy effects, especially to overcome the drug-resistance.

*Keywords: Drug-resistance; Mycobacterium tuberculosis; Efflux pump; Mutation; Epitope; Immunotherapy*

1. Introduction

During the past few decades, occurrence of drug-resistance in tuberculosis (TB) therapy has been highly concerned. Besides developing novel chemotherapeutic agents and strategies, the immunotherapy vaccine based on target antigens related to drug-resistance could be a potential strategy to overcome these problems. Since most of the papers related to TB epitopes and vaccines are focused on secreted antigens, the potential targets of efflux pumps and mutation sequences related to drug-resistance will be discussed here.

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2. Efflux pumps related to anti-TB drug-resistance

2.1. ABC Family

2.1.1. Rv0933
Rv0933, as the binding subunit of the PstSCAB operon, is always referred as a member of ABC family (Schmees et al., 1999). It provides energy for transport through ATP hydrolysis which promotes the functionality of the transporter (Wanner, 1996). And the over-expression of Rv0933 was correlated to the fluoroquinolone-resistance of Mycobacterium smegmatis (M. smegmatis) colony (Bhatt et al., 2000; Banerjee et al., 1998).

2.1.2. Rv0194
Over-expression of Rv0194 could significantly reduce the accumulation of ethidium bromide and could promote the resistance level of M. smegmatis. It was described that the over-expression of Rv0194 led to the increased minimum inhibition concentrations (MICs) of ampicillin, vancomycin, novobiocin, and erythromycin for M. smegmatis. Moreover, when reserpine, a widely used inhibitor, was added to the Rv0194-expressing strain, accumulation of ethidium recovered almost the same to its counterpart in wt M. smegmatis (Danilchanka et al., 2008).

2.1.3. Rv1218c
It was demonstrated that Rv1218c, a major ABC transporter of M. tuberculosis, caused the efflux of multiple substrates belonging to the chemical classes of novobiocins, pyrazolones, biaryl piperazines, bisanilinopyrimidines, pyrroles, and pyridines. In comparison to the wt strains, the mutants whose Rv1218c expression was inactivated showed 2– to 8-fold decrease in chemical resistance. But Rv1218c knock-out (KO) strain had no significant decrease in the MICs of many reference drugs (Balganesh et al., 2010).

2.1.4. Rv1819c (bacA)
Rv1819c is an inner membrane protein associated with maintenance of chronic infections in several diverse host-pathogen interactions. It was demonstrated that the maintenance of extended chronic infection and ultimate efficacy rather than the establishment of infection in mice was evidently influenced by the Rv1819c mutation within the strains. Deletion of bacA in M. tuberculosis led to increased bleomycin resistance (16– to 32-fold increase). And this phenotype was partially reversed in the complemented strain (Domenech et al., 2009).

2.1.5. Rv2209
Rv2209 exhibited dominant existence within the 30–day of Mtb infected lung samples. Interestingly, Fas protein is also exclusively shown within 30–day infection, which might promote the phthiocerol dimycocerosate (PDIM) and sulfolipids biosynthesis and this is required for the virulence and in vivo survival of Mtb. Therefore Rv2209 might be a conserved integral membrane protein involved in cell wall synthesis and cell process (Kruh et al., 2010).

2.1.6. DrrABC
It has been reported that DrrABC operon was involved in active transport of antibiotic and phthiocerol dimycocerosate (PDIM) across the membrane (export). Luis R. Camacho et al reported that a mutant strain (Δ drrC) was reversed to restore and translocate the PDIM production after
being introduced with the plasmid conferring the drrC gene. The results indicated the necessity of drrABC operon to utilize the PDIM. Besides, the attenuated mutants of *M. tuberculosis* devoid of PDIM and strains without these molecules in the cell wall displayed drastically increased initial rates of PDIM up-take than did Mt103 (*M. tuberculosis* clinical isolate). And the PDIM-less strain was proved much more sensitive to the antimicrobial compounds than that of the wild type strain under the 0.1% SDS. This effect suggested that DrrABC could help maintain the cell wall impermeability by promoting the translocation of PDIM within *M. tuberculosis* (Camacho et al., 2001).

2.2. MFS Family

2.2.1. Rv1258c

Rv1258c is a tetracycline/amino-glycoside resistance (Tap)-like efflux pump. The cytosolic accumulation of drugs was prevented by the transcription of this Tap-like pump, which was believed to be a crucial factor contributing to the drug resistance of *M. tuberculosis*. An over-expression of Rv1258c was confirmed when coexistent with rifampicin, which could explain the formation of the efflux-mediated rifampicin resistance in some clinical cases (Ainsa et al., 2008; Siddiqi et al., 2004; Jiang et al., 2008). In addition, the mutation frequency of Mtb strains was significantly decreased due to the combination of piperine and rifampicin (RIF) when compared to the rifampicin single treatment group (Sharma et al., 2010). It was reported that streptomycin (STR) resistance could be due to over-expression of Tap (1258c). And this over-expression was believed to be caused by mutations upstream of whiB7 (Reeves et al., 2013). In addition, the MIC of multiple drugs (2’-N-ethylnetilmicin, 6’-N-ethylnetilmycin, gentamicin, p-aminosaliclylate, spectinomycin, tetracycline, triclosan, and vancomycin) to the TAP KO strain were 2– to 4–fold higher than that of the wild type strains (Ramon-Garcia et al., 2011). It was also described that under the induction of isoniazid (INH), Rv1258c was over-expressed and this was also highly related to the ethidium bromide (EtBr) efflux activity of the INH induced strains. Additionally, the over-expression of other efflux pumps like efpA, mmpL7, mmr, and P55 were also detected. All the results illustrated the significance of efflux activity necessary to the in vivo survival of *M. tuberculosis* under anti-TB drug pressure (Rodrigues et al., 2012). Therefore, it could be a promising target for us to find effective protective CTL epitopes to the treatment of drug-resistance *M. tuberculosis*.

2.2.2. Rv0849

In Rv0849 KO strains, the MIC of amikacin (but not many other reference drugs) decreased by 2–fold, and this decrease could be restored to 8–fold higher after being complemented reaching the same level within the over-expression strains (Balganesh et al., 2012).

2.2.3. Rv1410c (P55)

Under the induction of INH, Rv1410c (P55) could be over-expressed, which caused the efflux of INH and EtBr. However, this efflux activity could be reversed by verapamil (Rodrigues et al., 2012). In addition, isolates harboring P55 plasmid displayed increase in the MICs of 8–fold to tetracycline and streptomycin, 4–fold to gentamicin, and 16–fold to 2’- and 6’-N-ethylnetilmicin, respectively (Silva et al., 2001).
2.2.4. Rv2846c (efpA)
It was reported that the strains with deletion of efpA exhibited a higher susceptibility to several anti-TB drugs (2-fold decrease in MICs of ethidium bromide, acriflavine, and fluoroquinolone) compared to the wild type. However, they showed a 2– to 8-fold increase in MICs of some other antimicrobial compounds such as rifamycins (rifampin, rifamycin SV, and rifabutin), isoniazid, chloramphenicol (CAP), and erythromycin (Li et al., 2004). Also, exposure to INH obviously induced the over-expression of efpA. And this change seemed positively mediating the efflux of EtBr and INH, which could be effectively reversed by verapamil (Rodrigues et al., 2012).

2.3. RND Family

2.3.1. mmpL7
mmpL7 gene is necessary for the proper localization of PDIMs. The mutants unable to synthesize or translocate PDIMs are more sensitive to detergent than the wild type (wt) strains, which indicated that in addition to being important virulence factors, extractable lipids of M. tuberculosis play a role in the cell envelope architecture and permeability (Camacho et al., 2001). Over-expression of mmpL7 induced by INH in some strains was believed in contributing to the higher MICs for INH and EtBr. And this over-expression could be reversed by verapamil (Rodrigues et al., 2012).

2.4. SMR Family

2.4.1. Rv3065 (mmr)
Although the MICs of any known drugs tested were not affected by the KO of Rv3065, the over-expression and complementation strains of KO exhibited an 8-fold increase in the MICs of ethidium bromide (Balganesh et al., 2012; De Rossi et al., 1998). Moreover, Rv3065 was indicated to be an efflux pump causing the INH resistance that could be drastically reversed by verapamil (Rodrigues et al., 2012). The mmr gene was also conferring the resistance to TPP, ethidium bromide, erythromycin, acriflavine, safranin O, and pyronin Y, but not streptomycin, ciprofloxacin, doxorubicin, rhodamine 123, rifampin, chloramphenicol, tetracycline, proflavine, sulfadiazine, and cetyltrimethylammonium bromide (De Rossi et al., 1998).

3. Mutation targets related to anti-TB drug-resistance

Artificial site-directed mutation has been always used as an important technique to confirm the binding sites between the pathogen proteins and some toxic compounds. However, active mutations might occur within M. tuberculosis to decrease the burden of pressure brought by toxic compounds. In this part, important mutations related to drug resistance will be discussed to reveal the potential value of these sequences to be used as target antigens. Currently, as the occurrence of MDR and even XDR strains, most widely used anti-TB drugs appear no longer potent as they were before. Hence, some researches were performed to explore the correlation between the drug resistant phenotypes and the mutations of related targets.

3.1. rpoB
S531F, and S531L could lead to the MIC increase of rifampin at least 32–fold and rifabutin at 8– to 32–fold, respectively. As shown above, most frequent mutations occurred at positions 516, 526, or 531 (Nakata et al., 2012). Besides, mutations were identified in 32 rifampicin-resistant strains at positions 509, 511, 516, 522, 526, 531, 533, 550 and 572. And the mutation S531L was believed the most frequent (42.1%). Also, mutations occurred at positions 526 (18.4%), 516 (10.5%) and 511 (7.9%) are regardless of the geographical difference (Chen et al., 2010). Moreover, on the basis of 267 clinical isolates a multiplex allele-specific PCR was developed. The hot rpoB mutation sites were located at 531, 526 and 516 (58%, 25.2% and 9.1%, respectively). Specifically, 57.3% multi-drug-resistant strains were tested with the mutation S531L, while 12.6% were tested with the mutation H526Y and 7.7% appeared with mutation D516V. Interestingly, among all the above multi-drug-resistant isolates Beijing strains occupied the biggest proportion (Prammananan et al., 2008). According to some previous studies, strains with the same rpoB genotype but different geographic backgrounds had similar drug resistance profiles (Williams et al., 1998; Moghazeh et al., 1996). The resistance patterns mutation frequencies within rpoB gene, which varied in different geographic areas, indicated that the resistance structures of isolates evolved under the selective pressure of anti-TB therapies and the spread of various genetic clones (Lin et al., 2013).

3.2. KatG
Mutations involved in inhA and KatG were believed mainly correlated to INH resistance. From the study of Belay Tessema et al, 35 of 260 clinical isolates were confirmed isoniazid resistant. Furthermore, 33 of the 35 isolates were proved mutants with S315T while the other two had a nucleotide mutation within the promoter region. However, the mutation KatG (S315T) was associated with high level of drug resistance, whereas strains harboring an inhA promoter mutation only had lower level of resistance. In addition, rather than the common S315T mutation, a novel mutation type S315G was also identified. Although this mutant only causes a moderate deficiency in INH activation in vitro, it is sufficient to lead to the insufficient INH-NAD production for bactericidal effects under normal treatment regimen (Tessema et al., 2012; Suarez et al., 2009). In addition, under the condition of hydrogen peroxide, katG became less capable to catalyze the efficient inhibition of InhA caused by the IN-NAD adduct. Furthermore, the adduct formation rate mediated by wild-type KatG was about 20–fold greater than by the isoniazid resistant KatG [S315T] mutant under optimal conditions. However, the poor efficiency of the KatG [S315T] mutant can be restored with an increased concentration of INH, which appeared consistent with reduced affinity of KatG for INH binding to the resting enzyme (Zhao et al., 2006). Moreover, according to the X-ray crystal information of the mutant, the most important change is the evidently decreased dimension of the access channel within the KatG, which made it rather difficult to contact and react with katG (Plinke et al., 2011).

3.3. InhA
Compared with wild-type InhA of H37Rv, purified enzyme protein with I194T showed 5–fold greater in Km without a significant increase in Vmax, which means the decreasing rate of reaction (Leung et al., 2006). The existence of S94A in INH resistant isolates was confirmed, and this mutation was proved helpful to establish the pH range over which the enzyme is active. I21V mutation in InhA was also believed related to the INH resistance. The MIC of INH to some mutant strains harboring the mutation of I21V was increased by 16–fold. Moreover, I47T and I21V mutation might produce negative impact for NADH binding to inhA, and thus result in the occurrence of INH resistance to certain degree (Basso et al., 1998). Diana Machado et al also found
I194T was helpful to cause the INH resistance of clinical MDR isolates (Machado et al., 2013; Silva et al., 2003; Segall et al., 2007). Plus someone revealed that transduction of the inhA (S94A) allele was sufficient to confer clinically relevant levels of resistance to isoniazid killing and inhibition of mycolic acid biosynthesis in *M. tuberculosis*. This resistance correlated with the decreased binding of the INH-NAD inhibitor to InhA. Also, a hydrogen-bonding network is disrupted by the loss of the hydroxyl group in the S94A substitution (Vilcheze et al., 2006). Similar report provided the evidence of a water-mediated H-bond between NADH and the protein frame. And the mutations like S94A, I21V could destroy the H-bond network, which lead to the less efficient interaction between antimicrobial compound complex and the enzyme inhA (Pantano et al., 2002). As for the mutations I21V, I47T, I194T details about their effects on the interaction between INH and the protein frames were also provided. The binding and inactivation studies clearly demonstrated that NADH became more extractable from a binary complex with an increased Kd value in the cases of clinical strains of *M. tuberculosis*. Furthermore, this reduced affinity would decrease the amount of enzyme present in an inactive form, thereby resulting in INH resistance (Zhao et al., 2006).

3.4. embB

So far even though a full respect to the ethambutol resistance has not been acquired, two frequent mutations M306I and M306V of embB were illustrated related to ethambutol resistance (Tessema et al., 2012). Claudia Plinke er al reported that these mutants could lead to the MICs of ethambutol increase 2- to 4-fold. Meanwhile, *In vivo* experiment showed that the selected embB306 GTG mutant required more ethambutol to control its growth in the lung compared to wild-type H37Rv (Basso et al., 1998). Also, a study developed among multi-drug-resistant *Mycobacterium tuberculosis* isolates in Henan, China revealed that embB306 was the most prevalent mutation both in ethambutol-susceptible MDR and ethambutol-resistant MDR cases, two less common mutations embB406 and embB497 were also found. Furthermore, M306I and M306V were believed to be the dominant types among the embB mutations during the ethambutol resistance. Other less frequent mutations like N399T, E405D and G459D were detected as well (Park et al., 2012). And this indicates that apart from the geographical difference similarity can be discovered in mutations relevant to ethambutol. Apart from this, Angela M. Starks et al asserted that Mutations at embB position 306 can serve as important molecular indicators of ethambutol resistance in *Mycobacterium tuberculosis*. Because the proportion of rpoB point mutation has reached 66% within the spontaneous mutants while 55% of these were detected mutations at embB 306. Additionally, the MIC of ethambutol for spontaneous mutants was increased 2– to 8-fold compared to the pan-susceptible *M. tuberculosis* strains from which the mutants were generated (Starks et al., 2009).

The epitopes from these potential targets discussed here could be used to develop vaccines against drug-resistance *M. tuberculosis*. We have identified the first T cell epitope from the efflux pump (Zhu et al., 2011). But up to now, most of the research work is focused on secreted antigens which are believed to contribute to the virulence of Mtb. Since the drug-resistance of Mtb is becoming very urgent and serious problems. We hope more research will be done in the future to use the drug-resistance related antigens as immunotherapy targets of *Mycobacterium tuberculosis*. 
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