A novel site of insertion of IS6110 in the *moaB3* gene of a clinical isolate of *Mycobacterium tuberculosis*

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Abstract

In *Mycobacterium tuberculosis*, genomic variation is generated mainly by insertions and deletions rather than by point mutations. RvD5 is one such deletion in *M. tuberculosis* H37Rv. Previous studies from our laboratory have shown the presence of *moaA3* gene in the RvD5 region in a large number of clinical isolates, that is absent in *M. tuberculosis* H37Rv and H37Ra. The present study was aimed at investigating the RvD5 locus of the clinical isolates by a detailed PCR analysis. Here we report a new point of insertion of the mobile genetic element, IS6110 in the genome of one clinical isolate of *M. tuberculosis*. The insertion has disrupted the *moaB3* gene, one of the ORFs in the RvD5 region, which is involved in the molybdopterins biosynthetic pathway. This insertion of IS6110 in the *moaB3* of the clinical isolate is different when compared to the insertion in the *moaB3* gene of *M. tuberculosis* H37Rv where 4kb RvD5 region has been lost by homologous recombination and only a truncated form of the gene is present. This finding is of relevance since IS6110 is a major element determining the genome plasticity of *M. tuberculosis* and its numerical and positional polymorphism has always been of special interest.

Introduction

It is well known that bacterial strains within a single species exhibit variations in their genetic profile. Deletions and rearrangements have been found in different members of the *M. tuberculosis* complex and have been correlated with attenuation and loss of virulence.¹⁻³ Regions of Difference (RD1-RD16) that range in size between 2 and 13 kb were identified in the members of the *M. tuberculosis* complex by various comparative genome analyses.^{4,5}

Six regions i.e., H37Rv-related deletions (RvD1 to RvD5 and *M. tuberculosis* specific deletion 1- TbD1), are absent in the *M. tuberculosis* H37Rv genome relative to other members of the *M. tuberculosis* complex. A major source of genetic polymorphism in *M. tuberculosis* is the insertion and deletion of these RDs. 4,6 Most RDs or DNA large sequence polymorphisms (LSP) are considered unique event polymorphisms and have been used to define the six major lineages and several sub-lineages of *M. tuberculosis*.⁷

IS6110 is found to mediate a number of genomic rearrangements in RD and RvD regions. Examples include deletion of the 7 kb locus RvD2 in *M. tuberculosis* H37Rv, which is present in the closely related avirulent derivative H37Ra,⁶ loss of RD2 region encoding the protein antigen MPB64 from some strains of *M. bovis* BCG² etc. The RvD2 region undergoes great variability in clinical isolates of *M. tuberculosis* and seems to represent a hot-spot for IS6110 transposition events.⁸ Our laboratory has been interested in studying the regions that are deleted in the laboratory strain *M. tuberculosis* H37Rv, but are present in clinical isolates of *M. tuberculosis*. RvD5 region which comprises several *moa* genes is one such deletion in the H37Rv strain. Primers designed to amplify an internal region of RvD5 were expected to give an amplicon of 1254 bp. Most of the clinical isolates screened using these primers gave the expected amplicon, except RGTB39. PCR amplification of this isolate gave an amplicon of 2.6 kb. Efforts to characterize this fragment led to the finding of an IS6110 insertion disrupting the *moaB3* gene in this region in the clinical isolate RGTB39.

Materials and Methods

M. tuberculosis strains and DNA isolation

A total of 105 clinical isolates of *M. tuberculosis* from different parts of Kerala in South India were used for the study. The standard strains included in the study are *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* and *M. bovis* BCG (Pasteur). The clinical isolates are the processed sputum samples of tuberculosis patients grown on LJ medium. These were later subcultured in Middlebrook 7H9 Broth (Difco Laboratories) supplemented with OADC enrichment (Difco Laboratories) and 0.5% glycerol (USB Corporation) at 37°C for four weeks. Biochemical characterization and DNA isolation were carried out as described earlier.¹

PCR amplification of RvD5 region

Primer pairs used in this study are *moaFP1*

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5'-CCCATCGTGGTCGTTTACC-3' and *moaRP1* 5'-CGATGGCAGCGTTTACAG-3' for the flanking sequences, *moaFP2* 5'-TGTCATAGGAGCTTCCGACC-3' and *moaRP2* 5'-AACATTCGGCATCATTGCTT-3', and *moaFP3* 5'-AGTTTGGTCATCAGCCGTTT-3' and *moaRP3* 5'-AACCAAGTCCAGGAGGGAAC-3' for the internal sequences. PCR was performed in a final volume of 30 µL using 1.25 units of Taq DNA Polymerase (Promega Corporation) for each reaction. After initial denaturation, amplification was done in a PCR thermal cycler (BioRad) for 35 cycles of 94°C/ 1 min, 64°C/ 1 min, 72°C/ 1 min followed by a final extension of 72°C/ 7 min.

Cloning and sequencing

pGEMT Easy vector (Promega) was used to clone the 2.6 kb PCR product. Using the six *moa* PCR primers, template DNA was sequenced using the fluorescent big dye terminator cycle sequencing ready reaction kit (PE Biosystems) in an automated sequencer (ABI Prism 310). The sequencing reaction

mixtures, each containing 300 ng of template DNA, 3.2 pmol of primer, 2.0 μ L of Prism Ready Mix and 1.13 μ L of 25mM $MgCl_2$, were subjected to 25 cycles of denaturation (at 96°C for 30 s), annealing (at 55°C for 15 s), and extension (at 60°C for 4 min). Six different sequencing reactions were carried out i.e., the forward sequences with the three forward primers - moaFP1, moaFP2 and moaFP3 and the reverse sequences with the three reverse primers - moaRP1, moaRP2 and moaRP3.

Aligning sequences and homology search

DNA sequences identified in this study were compared to sequences in the GenBank and EMBL databases. They were also subjected to BLAST analysis in the *M. tuberculosis* DNA sequence database (<http://genolist.pasteur.fr/TubercuList/>) and the *M. bovis* database (<http://genolist.pasteur.fr/BovList/>). For DNA sequence alignment, the program BL2SEQ in the SDSC Biology Workbench was utilized.

IS6110 Fingerprinting

Genomic DNA of the standard strain *M. tuberculosis* H37Rv and three local isolates was digested with *Pvu* II (New England Biolabs), separated on 0.8 % agarose gel and blotted onto a nylon membrane (Hybond). After prehybridization at 65°C for 3 hours, a 245bp PCR product of IS6110 from *M. tuberculosis* H37Rv labelled with α - ^{32}P dCTP probe was denatured, quick chilled and allowed to hybridize the membrane. After overnight hybridization at 65°C, the blot was washed with increasing stringency of SSC-SDS and exposed to an activated Phosphor screen (Kodak). The screen was then scanned using Personal Molecular Imager FX (BioRad) and the picture was visualized using the software Quantity One (BioRad) to obtain the IS6110 RFLP pattern.

Results

PCR

To investigate the RvD5 region and to find out the presence of moaA3 and its adjacent regions in the clinical isolates of *M. tuberculosis* from Kerala, primers were designed to amplify the flanking sequences. One such primer pair (moaFP1 moaRP1) was expected to amplify a region encompassing the whole of *moaB3* gene and a part (768bp) of *moaA3* gene. The expected size of the PCR product was 1254 bp. In our laboratory we have a repository of clinical isolates of *M. tuberculosis* collected from tuberculosis patients in

Kerala. PCR screening of these isolates using the primers moaFP1 and moaRP1 gave the expected product of 1254 bp in all isolates tested. But one of the isolate RGTB39 generated a PCR product of size 2.6 kb (Figure 1).

Cloning and sequencing of 2.6 kb PCR product

The 2.6 kb band was gel eluted and cloned in pGEMT Easy vector. The ends of the insert were sequenced using moaFP1 and moaRP1 primers. The forward primer gave a sequence which had homology only with 211bp of *moaB3*. The latter part of the sequence shared homology with a portion of IS6110 transposases seen in the genomes of both *M. tuberculosis* H37Rv and in *M. tuberculosis* CDC1551. As expected, the sequence obtained using reverse primer had homology with moaA3. To

get the internal sequences of the 2.6 kb fragment, two sets of internal primers - a) moaFP2 and moaRP2 and b) moaFP3 and moaRP3 - were used. moaFP2 and moaFP3 gave the rest (remaining part) of the IS6110. moaRP2 and moaRP3 primers gave the rest of the *moaA3* and the *moaB3* genes. Details of the primers and overlapping sequences obtained are shown in Figure 2.

Sequence analysis

Analysis of the sequence data revealed that in RGTB39, the *moaB3* gene was disrupted by an IS6110 insertion breaking the gene into two fragments. A comparison of *moaB3* and its neighbouring genes in *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551 and RGTB39 is given in Figure 3. The entire 375 bp long *moaB3* gene is undisturbed in both *M. tuberculosis* CDC1551 and in *M. bovis*. But in *M. tuberculo-*

Figure 1. PCR of 10 representative clinical isolates of *M. tuberculosis* from Kerala using primers moaFP1 and moaRP1. Lane 1: Marker DNA (DNA double digest (*Hind* III / *Eco* R I)); Lanes 2-11: clinical isolates RGTB29, 39, 70, 86, 110, 123, 142, 144, 154 and 177 respectively. RGTB39 shows a 2.6 kb band and the rest show the 1254 bp band.

Figure 2. Schematic representation of the genome position of IS6110 insertion in the *moaB3* gene of RGTB39. The different sequences obtained to get the final assembly of the 2.6 kb PCR product are shown as striped arrows. The genome coordinates (*M. bovis*) of the primer positions and the genes are also shown.

sis H37Rv *moaB3* is truncated, with only the last 137 bp of the gene being present. The first 238 bp of the *moaB3* gene along with the other ORFs comprising the RvD5 region is deleted. Interestingly in RGTB39, none of these scenarios are observed. The IS6110 insertion is after 164 nucleotides and the rest of *moaB3* gene is present downstream of IS6110 sequence. Also, the insertion of IS6110 in *moaB3* is in the opposite orientation when compared to that in *M. tuberculosis* H37Rv.

IS6110 RFLP

IS6110 RFLP of RGTB39, RGTB123 and H37Rv were carried out to find out the number of copies of IS6110 in RGTB39. H37Rv served as the positive control (multicopy IS6110) where as RGTB123 served as the negative control which had no copy of IS6110. We have previously reported a large number of single and no copy strains of *M. tuberculosis* from our region⁹ but RGTB39 turned out to be a strain with more than ten copies of IS6110 (Data not shown).

GenBank Submission

The 2612bp sequence amplified from RGTB39 using primers *moaFP1* and *moaRP1* was completely sequenced, analysed and submitted to Genbank (GenBank:GU994139).

Discussion

Earlier experiments conducted in our laboratory to screen the different RDs and RvDs showed the absence of *moaA3*, a gene in the RvD5 region, in some of the clinical isolates from Kerala.¹ Screening larger number of isolates using different primers from RvD5 region showed an IS6110 insertion in the RvD5 region of one clinical isolate, RGTB39. Though initially it seemed to be similar to *M. tuberculosis* H37Rv, it later turned out to be different for three reasons - a) Firstly, the orientation of IS6110 is reverse in RGTB39 when compared to that in *M. tuberculosis* H37Rv. b) In *M. tuberculosis* H37Rv, the *moaB3* gene is truncated (Rv3324A) with only the last 137 nucleotides being present. The rest of the gene along with three other ORFs is lost in the RvD5 deletion. However in RGTB39 the whole of the *moaB3* gene is present flanking the IS6110 insertion. c) The point of insertion of IS6110 in both the strains is different, since in *M. tuberculosis* H37Rv only the last 137 nucleotides remain, whereas in RGTB39 the last 211 nucleotides of the gene are present (Figure 3). Thus, this locus can be considered as a new site of insertion of IS6110. It is interesting to note here that the IS6110 insertion in RGTB39 seems to be an independent event.

Figure 3. Schematic representation of the comparison of RvD5 and the flanking regions in H37Rv, CDC1551 and RGTB39. In CDC1551 the *moaB3* gene is intact. In RGTB39 it is disrupted after 211 bp. In H37Rv, the gene is truncated with only a 137 bp fragment in the genome. The region spanning the expected PCR product of 1254 bp in CDC1551 and the region spanning the 2.6 kb PCR product in RGTB39 are shown in dotted lines.

In clinical isolates, IS6110 preferentially inserts into certain regions of the genome of *M. tuberculosis*, such as the *ipl* or the IS preferential locus. Six alternative *ipl* sequence locations have been reported for the integration of IS6110.¹⁰ In addition, SiteMapping, a DNA microarray-based methodology has been used to identify eight previously unknown regions for insertion of IS6110.¹¹ Polymorphisms found in *plcD* gene in isolates of virulent *M. bovis* were attributed to IS6110 insertions.¹² A recent study has described a hotspot for the insertion of IS6110 in the area of the region of difference (RD) 724.¹³

IS6110 transposition events may disrupt open reading frames¹⁴ or regulatory domains. In addition, IS-mediated deletion events have been suggested to be an important mechanism driving mycobacterial genome variation.⁶ Homologous recombination between directly repeated IS6110 elements has been proposed as a likely mechanism for genomic deletions in clinical isolates.¹⁵ The integration of an insertion element can result in either up or down regulation of genes in which it resides. One such case where IS6110 acts as a promoter in *M. tuberculosis* infected macrophage has been reported.¹⁶ It is equally possible that IS6110 insertion may disrupt existing promoter regions leading to down regulation of gene expression. An IS6110 insertion site could possibly be responsible for a strain's increased capacity for transmission and replication. More than 60% of characterized IS6110 insertions have been found to disrupt coding regions. But multiple copies of a gene could

compensate for natural knockouts created by IS6110 insertion as in the case of PPE (Pro-Pro-Glu) genes of *M. tuberculosis*.¹⁴ The nature of any alteration will depend upon the function of the products of the genes involved. Gene knock out studies are needed for the confirmation of the role of such genes on the intracellular survival of the pathogen.

In the present study, the IS6110 insertion was found in *moaB3*, a gene belonging to the multi gene family of the molybdopterin biosynthetic pathway. Molybdopterin is a cofactor required for nitrate reductase and many other enzymes involved in anaerobic metabolism. Genes involved in the molybdopterin cofactor biosynthesis pathway are present in almost all organisms. A study using a promoter trap vector has identified two of the *M. tuberculosis* genes in this pathway - *moaX* and *moeB1* - as upregulated in the bacteria present in mouse lungs upon infection.¹⁷ Therefore, *moa* genes may be important in the intracellular survival of the pathogen. Since the new insertion disrupting *moaB3* was found in a clinical isolate obtained from a patient with pulmonary tuberculosis, it could be argued that *moaB3* gene may not be essential for pathogenesis, but may play a role in the variation of virulence of clinical isolates. It could also be that other members of the multigene family compensate for the loss of function. Since *M. tuberculosis* dedicates 21 genes to the biosynthesis of the molybdopterin cofactor¹⁸ this may be important in the life of the pathogen, but other genes may be expected to complement the loss of activity of *moaB3*.

Conclusions

Genome variation is the main underlying reason for phenotypic differences observed between organisms of the same species. Genome comparison by multiple sequence alignment of six known genomes of mycobacterial strains (CDC1551, F1, C, Haarlem, H37Rv and H37Ra) revealed that most of the variation results from deletion and transposition events preferentially associated with insertion sequences and genes of the PE/PPE (Pro-Glu/Pro-Pro-Glu) family, but not with genes implicated in virulence. The comparison of multiple genomes demonstrates that *M. tuberculosis* genome is currently undergoing an active process of gene decay, analogous to the adaptation process of obligate bacterial symbionts.¹⁹ So IS6110 mediated genomic rearrangements possibly have a great influence on the generation of genomic variation and ultimately the evolution of *M. tuberculosis*.

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