의학박사 학위논문

A study on the relationship of genetic variation and drug metabolism of an enzyme involved in the metabolism of rifampicin

- Genotype and phenotype relationship of rifampicin metabolism -

리팜피신 대사에 관여하는 효소의 유전자 변이와 약물 대사와의 상관성에 관한 연구

- 리팜피신 대사의 유전형과 표현형의 관계 -

2012년 8월

서울대학교 대학원
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A thesis of the Degree of Doctor of Philosophy

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A study on the relationship of genetic variation and drug metabolism of an enzyme involved in the metabolism of rifampicin

- Genotype and phenotype relationship of rifampicin metabolism -

by

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A thesis submitted to the Department of Laboratory Medicine in fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine (Laboratory Medicine) at Seoul National University College of Medicine

August 2012

Approved by Thesis Committee

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논문 제목: 리팜피신 대사에 관여하는 효소의 유전자 변이와 약물 대사와의 상관성에 관한 연구

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Abstract

Rifampicin is a key bactericidal drug used for treatment of tuberculosis. Rifampicin has been known to be deacetylated in vivo resulting in its metabolite 25-desacetylrifampicin, but its metabolizing enzyme and its association with genetic variation is not elucidated yet. In this study, the presence of rifampicin metabolizing enzyme was verified and the genetic variation of surrogate enzyme and its association with the drug metabolism was investigated.

The activity of rifampicin metabolizing enzyme was measured in human liver microsome (HLM) by measuring the amount of 25-desacetylrifampicin after incubation of various amount of the HLM for various incubation time. After verifying the existence of the enzyme in HLM, carboxylesterase 2 was hypothesized to be the enzyme responsible for the deacetylation of rifampicin. Plasma concentrations of rifampicin and 25-desacetylrifampicin were measured for 35 tuberculosis patients who had been treated with first-line antituberculosis regimen.

Their DNA was extracted and sequencing of *CES2* gene covering whole 12 exons, 3' UTR, 5' UTR, and intronic regions previously reported to have variations was performed. Ten variations were detected and 2 were in candidate promoter region, 5 in intron, and 3 in 3' UTR. One of the variaion in 3' UTR was novel variation. There was a clear trend of increasing or decreasing concentration of

rifampicin as the number of variation increased in 6 variations. The frequency of variation was investigated by SNaPshot analysis of the 10 variation sites using DNA's from 100 healthy persons. The frequency was not different from that in NCBI or the previous reports except for the high frequency of the variation with deletion of 3 base pairs in 3' UTR. Patients with plasma rifampicin concentration ≧ 8 μg/mL had significant higher frequency of c.738A>G, c.4629A>G, c.10748G>A, and c.12027C>T variations. Reconstruced haplotype analysis showed that patients with one H9 haplotype had higher plasma rifampicin concentration. Promoter assay revealed decreased luciferase activity for c.738A>G.

The well known variation in *CYP3A5* gene, c.6986A>G was not associated with the change of plasma rifampicin concentration.

In conclusion, variations in *CES2* gene, espcecially c.738A>G may alter the metabolism of rifampicin.

Keywords: tuberculosis, rifampicin, metabolism, pharmacogenetics, variation, carboxylesterase

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List of Abbreviations

ANOVA	analysis of variance
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
HLM	human liver microsome
LC-MS/MS	liquid chromatography-tandem mass spectrometry
PCR	polymerase chain reaction
SAP	shrimp alkaline phosphatase
SNP	single nucleotide polymorphism
TBE	tris-borate-EDTA
UTR	untranslated region

Introduction

Tuberculosis is one of the oldest infectious diseases known to man. About one-third of the world's population has been infected by *Mycobacterium tuberculosis* and 1.7 million people died from the disease in 2009. The global incidence rate is still falling, but too slowly [1]. In Korea, the incidence of tuberculosis is very high which reaches up to fifty thousand people per year. The number of young patients are high, which underscores the importance of the early diagnosis and treatment [2].

Recently, the number of immunocompromised patients such as HIV infected or organ transplant patients are increasing and the latent tuberculosis are activated more frequently. The emergence and spread of multi-drug resistant (MDR) tuberculosis and extensive drug resistant (XDR) tuberculosis make the situation more complex and warrants the development of more effective treatment protocols [3].

Treatment of tuberculosis is based on the first-line anti-tuberculosis drugs including isoniazid, rifampicin, pyrazinamide, and ethambutol. Of them, isoniazid and rifampicin have the bacteriocidal effect and should be included in the treatment regimen [4]. The dosage and the duration of the drugs are well-established and widely used. However, in cases of delayed or minimal response, resistance to the drugs, risk of drug interaction, changes in the drug metabolism or in the drug level in patients with certain conditions such as diabetes, HIV infection, renal failure, or liver dysfunction, the

modification of the treatment protocol may be inevitable [5, 6]. In such cases, understanding of the drug metabolism and its effect on the pharmacological and side effect can enhance the treatment efficacy and contribute to lowering the incidence of tuberculosis and the emergence of drug resistant organisms.

For that end, pharmacogenetic approach is promising, as it assumes that the efficacy of drug metabolism is genetically determined which can be evaluated only once during the treatment period [7]. Isonizid is a well-known drug with its efficiency dependent on the genetic variation of an enzyme involved in its metabolism and correlated with drug level [8]. There are also many other drugs whose metabolism is correlated with genetic variation of some enzymes.

Rifiampicin is one of the most important drug for treatment of tuberculosis. It is also used for killing other mycobacterial species and has a role in treating methicillin-resistant *Staphylococcus aureus*, *Listeria* species, *Neisseria gonorrheae*, *Haemophilus influenzae*, *Legionella pneumophila* infections [9]. Rifampicin is known to metabolized via deacetylation and the serum half-life is 2-5 hours [10]. It is a well-known drug which induces some enzymes involved in the metabolism of many drugs [11]. Although rifampicin is widely used and affects the dosage of other drugs, the metabolism of it is not investigated clearly.

In this study, the genetic variation of the enzymes involved in the metabolism of rifampicin and its association with plasma rifampicin are investigated.

Materials and Methods

1. Study subjects

Thirty five patients with tuberculosis who were treated with first-line antituberculosis drugs and for whom the physician requested the monitoring of drug concentration were enrolled in this study. For analysis of frequency of genetic variation of rifampicin metabolizing enzymes, 100 healthy persons were enrolled. This study was approved by the institutional review board and written informed consents were obtained from all the tuberculosis patients and healthy persons.

2. Measurement of enzyme activity

Enzyme activity was measured in human liver microsome (HLM). The buffer solution was prepared as 2.25% BSA in 5 mM $MgCl_2$, 100 mM $NaPO_4$, 1 mM EDTA, pH 7.4. One hudred μL of the buffer solution was mixed with 10 uL of HLM and 10 μL of 1 mg/mL rifampicin in methanol. The mixture was incubated for 30 minutes, one hour, two hours, three hours, and during overnight in a shaking incubator in 37℃. For stopping the enzyme reaction, 100 uL of methanol with 250 μg/mL of rifabutin was added. The rifabutin was used for internal standard of rifampicin and its metabolite 25-desacetylrifampicin. After centrifugation for 15 minutes at 1,900 g, the supernatant was separated and loaded onto the autosampler of the

liquid chromatography.

Rifampicin and 25-desacetylrifampicin was measured using LC-MS/MS. The mobile phase was a linear gradient of a mixture of 0.3% formic acid in 100% methanol (solvent A) and 0.3% formic acid in water (solvent B). The A and B solvents were mixed in 60:40 at a flow rate of 300 µL/min for 4 minutes. Ten µL of each sample was injected onto the C18 reversed-phase column and chromatographic separation was performed at 50℃ for 4 minutes.

A Xevo-TQMS was operated using the following settings; capillary voltage, 3.5 kV; cone voltage, 16-29 V; collision energy, 14-17 eV, depending on the analytes (Table 1). Quantification was achieved by multiple reaction monitoring (MRM) in positive ion mode. Integration of peak area and data analysis was performed using QuanLynx 4.1 software.

Table 1. MS/MS conditions and MRM transitions of the analytes

	Cone voltage (V)	Collision energy (ev)	MRM transition (*m/z*)
Rifampicin	29	17	823.5 > 791.5
25-Desacetyl rifampicin	16	14	781.5 > 749.5
Rifabutin	29	17	847.5 > 815.5

3. Analysis of the ratio of rifampicin and 25-desacetylrifampicin in plasma

Peripheral blood was collected in EDTA vacutainers from tuberculosis patients two hours after oral administration of rifampicin. After centrifugation, plasma was separated and frozen in -80℃ until analysis. Calibration curve was constructed with six concentrations of standard (0, 5, 10, 20, 40, and 80 mg/mL). Two quality control samples with low (5 mg/mL) and high concentration (20 mg/mL) were prepared by spiking stock solutions to blank serum. Stock solutions of rifabutin were combined and diluted in 50% methanol to produce the working internal standard solution (25 mg/mL).

4. PCR of *CES2* gene

For amplification and sequencing of *CES2* gene, GenBank accession number AY851164 was used as a reference sequence. Human genomic DNA was extracted from EDTA plasma using QIAamp Blood Mini kit according to the manufacturer's instructions and used as template PCR. Primers for PCR were designed to include the entire 12 exons, 5'-UTR, 3'-UTR, and promoter regions. The primer sequences and their predicted PCR product size for PCR are listed in Table 2.

PCR reactions were performed with 25 μL of a reaction mixture containing 2.5 μL (20-50 ng/μL) of the prepared template, 2.5 uL of 10X bufer, 2 μL of 2.5 mM dNTP, 0.625 U of Taq polymerase, and 0.4

μM of primer mixture, and 14 μL of distilled water. PCR conditions were as follows: an initial denaturation step at 94 °C for 10 min and 35 cycles of amplification consisting of denaturation at 95°C for 5 min, annealing at 58°C for 30 s, and extension at 72 °C for 1 min followed by a final extension step at 72 °C for 7 min. A PTC-200 (MJ Research Inc, Waltham, USA) was used for PCR reaction. The amplified PCR products were resolved by electrophoresis in a 2% agarose gel in 0.5X TBE buffer at 100 V for 40 min.

5. Sequencing of *CES2* gene

For sequencing reaction of the PCR product, additional primers were designed to cover as long sequences of the product as possible. The primer sequences for sequencing reaction only are listed in Table 3.

The PCR products were purified by adding 2 μL of ExoSAP-IT reagent (USB Corporation, Cleveland, Ohio, USA) to 5 μL of the PCR products and incubating for 15 min at 37℃ and for 15 min at 80℃. The DNA concentrations were measured using ND-1000 spectrophotometer (NanoDrop Tech, Rockland, USA) and diluted to 100 ng. After adding 0.1 μM of the sequencing primers, the mixtures were reacted with BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The reaction products were purified again using BigDye XTerminator Purification kit (Applied Biosystems) and analyzed with ABI Prism 3130xl genetic analyzer (Applied Biosystems). The sequence data were analyzed using

SeqScape Analysis 2.5 (Applied Biosystems) software.

6. SNaPshot analysis of the *CES2* gene

The SNaPshot analysis was performed for 9 SNP's from 5 regions with genomic DNA from 100 normal controls. DNA extraction and PCR reaction were performed as described in page 6. The 8 SNP's were analyzed by two sets of SNaPshot reaction and a small deletion (c.11884-11886del) by sequencing. The target SNP's, PCR primers, and SNaPshot primers, and the size of SNaPshot primers are listed in Table 4.

After purification of the PCR products, SNaPshot reactions were perfomed by incubating them with SNaPshot Multiplex kit (Applied Biosystems). One hundred ng of the purified PCR products and 0.4 μM of SNaPshot primers were mixed to two sets of reaction mixtures. The reaction products were purified again by adding 1 μL of SAP and the sequence was analyzed using the sequence analyzer. The data were analyzed using GeneMapper 4.0 (Applied Biosystems) software.

7. Analysis of c.6986A>G of *CYP3A5* gene

For analysis of c.6986A>G of *CYP3A5* gene, real-time PCR and melting curve analysis were performed. Human genomic DNA extracted from EDTA plasma was used as template. Primers and probes sequences are as follows. CYP3A5_F:

5'-TGCTCTACTGTCATTTCTAACCATAA-3'; CYP3A5_R: 5'-TGTACGACACACAGCAAC-3'; CYP3A5_D: 5'-LC Red 640-TCCAAACAGGGAAGAGATACTGAAAGACAA-Phosphate-3'; CYP3A5_A: 5'-ACCCAAGGCTTCATATGATGAAGGGTAATGT-Fluorescein-3.

Real-time PCR reactions were performed with 20 μL of a reaction mixture containing 2 μL of the prepared template, 2 μL of 2X QuantiTect Probe PCR Kit, 2.8 μL of DMSO, 0.4 μL of primer set (10 pmol/μL), 0.4 μL of probe set (10 pmol/μL), and 11.6 μL of distilled water. PCR conditions were as follows: an initial denaturation step at 95 °C for 10 min and 40 cycles of amplification consisting of denaturation at 95°C for 10 s, annealing at 58°C for 20 s, and extension at 72°C for 20 s. Melting curves were acquired by measuring the fluorescence during a temperature transition from 40°C to 80°C, with the rate of 0.2°C per second after 30 s of the first step. Fluorescence data were converted into melting peaks by plotting the negative derivatives of fluorescence with respect to temperature (-dF/dT) as a function of temperature.

8. *CES2* promoter assay

The expression construct of wild-type *CES2* containing an N-terminal Myc epitope was synthesized by reverse transcription polymerase chain reaction (RT-PCR) amplification of RNA isolated from human blood followed by cloning into the eukaryotic expression

vector pcDNA3 (Invitrogen). Thereafter, *CES2* expression constructs encoding sequence variations revealed in this study were recreated using QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). The variations tested were as follows: CA, CG, TA, and TG (the first nucleotide at c.453 and the second nucleotide at c.738). 293 cells were transfected with these mutants using the Lipofectamin 2000 system (Invitrogen), according to the manufacturer's recommendations.

9. Statistical analysis

The mean difference of drug concentration between different variations of the nucleotides were analyzed with analysis of variance. T-tests were performed to compare the mean drug concentration between different haplotypes of *CES2* gene. Hardy-Weinberg equilibrium test was performed for each locus of variation. IBM SPSS Statistics 19 (IBM Corparation, Armonk, NY, USA) was used for the statistical analysis. $P<0.05$ was considered as statistically significant. For haplotype reconstruction from genotype data of *CES2* gene, PHASE 2.1 was used [12, 13].

Table 2. Primer sequences for PCR amplification of *CES2* gene

Primer	Sequences (5' -> 3')	Product size (bp)
CES2r-1F	TTACCCATCTCCAGTCTTGCCCAT	1,240
CES2r-1R	TGCTCTATGCTCACGAACACCGAA	
CES2r-2F	AACCTGGTTGTGGAGGAGAGATGT	1,204
CES2r-2R	TTCCTCGGTGGCTTTCAGTTTCCT	
CES2r-3F	ACTGAGTCCTGAAAGCAAGGAGGA	766
CES2r-3R	TCACGAGTTCCACGCTGTCATCT	
CES2r-4F	AGTTAGAGACCAGCTTGGGCAACA	384
CES2r-4R	ACTGCGGTGTCAACAAGCAGTTTC	
CES2r-5F	AAGGGAGTGTTACAGGCAGGAAG	506
CES2r-5R	AATGCACGTGGGAATCCACGAAAC	
CES2r-6F	GGTGAATGTCAACACATGCAGCCA	795
CES2r-6R	TGTGCCCATTCTATAGGCCCAACA	
CES2r-7F	ATCTTGGGTGCCTAACCATTTGCC	841
CES2r-7R	AACTTTGCACAGGAGCCACAGAAC	
CES2r-8F	TGGAACAGTGTCAGAACTGCAGGA	1,375
CES2r-8R	CGGAGCCCAATAAACATGAGTGCT	
CES2r-9F	AGATTCCAGCCAGCTTTGGACCTA	1,023
CES2r-9R	AGGTAGACTGGAGAGATAGAGCGT	
CES2r-10F	AGCCAGAGGGAGCTTATTTCCTGT	1,086
CES2r-10R	TTGGAAACGGAGTCTCGCTCTTGT	
CES2r-11F	CAGAGGTTGCAGTGAGCTGAGATT	470
CES2r-11R	TGGCCACTCACAGATGTAAGAGCA	

Table 3. Primer sequences for sequencing reaction of *CES2* gene

Primer	Sequences (5' -> 3')
CES2r-1-1F	AAAGTGCTGGGATTACAGGCGTGA
CES2r-1-1R	TGGGAGGAAGCTTTCCAAGTCGAT
CES2r-2-1F	ATATCGAAGATCTCGCGTGCGTCGA
CES2r-2-1R	TCTGCTCCAGCGACCATCATGT
CES2r-3-1F	CCTGTGGACAAGGACAGGTTTGAA
CES2r-3-1R	TGTCCAGCAGTGGATCAGTGCCTT
CES2r-6-1F	ACCGCAGTGGAGTCAGAGTTTCTT
CES2r-6-1R	AGGGAAGGTCATGTTGAACTGGCT
CES2r-7-1F	ACTTCTCAGCACTGGAGACAAGCA
CES2r-7-1R	ACACAACAAGCGAAGACACACTCG
CES2r-8-1F	GGTGCATGCTGAGCCAAACAGTAA
CES2r-8-2F	CTCCTGTCCTGAGACAGAGGAAAT
CES2r-8-1R	ATGGGATGAGCCTGGAGCTATGAA
CES2r-9-1F	ACTCTTCAGACTGTGAGCTGTGCT
CES2r-9-1R	CAAACTTTCCCTGTAGACCAGGCT
CES2r-10-1F	ATGGCCCATACTTGTAATCCCAGC
CES2r-10-1R	AAGCCATCCTCCCATCTCATCCTT

Table 4. Primers used for SNaPshot reaction

Set	Target	PCR primer	PCR product (bp)	SNaPshot primer	SNaPshot primer size
Set 1	nt453 C>T	1F / 1-1R	801	CES Ty453F 5'-ttccctgcagccccagacc-3'	20 bp
	nt738 A>G	1F / 1-1R	801	CES Ty738F 5′-T(10)gtatttttagtagagatgag-3'	30 bp
	nt4629 A>G	4F / 4R	384	CES Ty4629F 5'-T(23)agctgtactccaagctactc-3'	43 bp
	nt6622 T>C	6F / 6R	795	CES Ty6622F 5'-T(33)cttccccagagcctgtggcc-3'	53 bp
	nt7035 G>A	6F / 6R	795	CES Ty7035F 5'-T(40)atgctgagaagcttctcact-3'	60 bp
Set 2	nt10748 G>A	9-1F / 9R	614	CES Ty10748F 5'-agggcagtggaagaaaaagc-3'	20 bp
	nt10769 G>A	9-1F / 9R	614	CES Ty10769R 5'-T(10)gtccttggcccccgagacct-3'	30 bp
	nt11651 A>G	10F / 10R	1086	CES Ty11651F 5′-T(23)agcctgctgtgcccacacac-3'	43 bp
	nt12027 C>T	10F / 10R	1086	CES Ty12027R 5′-T(33)ggcatgagccacagtgcctg-3'	53 bp

Results

1. Activity of rifampicin-metabolizing enzyme in HLM

To determine the existence of the rifampicin-metabolizing enzyme in HLM, various conditions of incubation time and enzyme concentrations were used. The formation of the presumed metabolite, 25-desacetylrifampicin, linearly increased as the incubation time was increased to 10 hours and it kept a steady state after 10 hours (Fig. 1A). The amount of 25-desacetylrifampicin also increased as the amount of HLM which was supposed to be proportional to the concentration of rifampicin metabolizing enzyme was increased (Fig. 1B). The formation of 25-desacetylrifampicin was minimal in human S9 or intestine microsome fractions.

2. Variations of *CES2* gene

As the deacetylating activity of rifampicin was observed in HLM, carboxylesterase 2 was hypothesized to be the rifampicin metabolizing enzyme. The *CES2* gene consisted of 11 exons and the whole exons and 5' and 3' UTR were included in the sequencing region for finding the variation of the gene. There were 10 variations in the *CES2* gene regions sequenced. Of them, two were in the promoter region, five in the intron, and 3 in the 3′ UTR. None were found in the exon. The 10 variations found and their frequencies are listed in Table 5. The

frequencies of the variation were not significantly different from those from NCBI's dbSNP and other previous reports except for the variation no. 9, which was 0.67 for GAA in c.11884-11886 while NCBI reported its frequency as 0.06. The variation c.12027C>T (variation no. 10) was not reported previously with a low frequency of variation.

3. Variations of *CES2* gene and their association with plasma rifamipicin concentration and the ratio to 25-desacetylrifampicin

When the plasma concentrations of the 35 patients with tuberculosis were analyzed, some associations could be predicted. Variations in c.738, c.4629, c.6622, c.7035, c.10748, and c.11884-11886 showed trends of a single direction of the change of the mean plasma rifampicin concentration as the number of variations increased (Table 6). The mean plasma rifampicin concentration increased in the variations in c.453, c.738, c.4629, c.7035, and c.10748, while that in the variation in c.11884-11886 decreased as the number of variations increased. When ANOVA was performed, variations in c.738, c.4629, c.10748, and c.11884 showed significant differences.

When the allele frequencies between patients based on the plasma rifampicin concentration of 8 μg/mL were compared, which is the lower limit of therapeutic range, four positions (c.738A>G, c.4629A>G, c.10748G>A, and c.12027C>T) showed significant difference in the allele frequency (Table 7). Of them, c.738A>G, c.4629A>G and c.10748G>A showed the exactly the same allele frequencies, which

were 0.15 in the patients with plasma rifampicin concentration < 8 μg/mL and 0.40 in the patients with plasma rifampicin concentration ≧ 8 μg/mL. All the patients showed the same genotype in those three positions. The change of the drug concentration of the two loci depending on the genotypes are presented in Fig. 2.

The 25-desacetylrifampicin to rifampicin ratio was significantly higher in the heterozygote than in the wild type of c.12027 (0.379±0.439 vs. 0.037±0.215, P<0.001). It was not significantly different in other 9 loci.

4. Frequencies of the variations of *CES2* gene in general population

The variations of *CES2* gene in 10 loci detected in the tuberculosis patients were analyzed in the 100 healthy persons (Table 8). The frequencies were very close to those of tuberculosis patients except for c.11884-11886del. It was 0.35 in healthy persons while 0.67 in tuberculosis patients. The variation in the position 12027 was not found in the healthy persons. When Hardy-Weinberg equilibrium test was performed, the frequency of c.11884-11886del deviated significantly from the Hardy-Weinberg equilibrium.

5. Haplotype analysis of CES2 gene

The haplotypes of the patients were estimated from the genotype

data by PHASE 2.1 software which uses Bayesian statistical method. There were 9 haplotypes (Table 9). Of them, H1 was the most frequent haplotype with estimated frequency of 0.434. H9, H4, and H6 were next frequently estimated haplotypes. When analyzing the relationship between specific haplotype and plasma rifampicin and 25-desacetylrifampicin concentrations, only H9 showed significant difference in plasma rifmapicin concentration. The patients with one H9 haplotype had higher plasma rifampicin concentration than those with no H9 haplotypes (13.7 vs. 9.1 μg/mL, P=0.023). Other parameters, such as age, height, weight, dose of rifampicin were not statistically different between patients with specific haplotypes.

6. Variation of *CYP3A5* gene

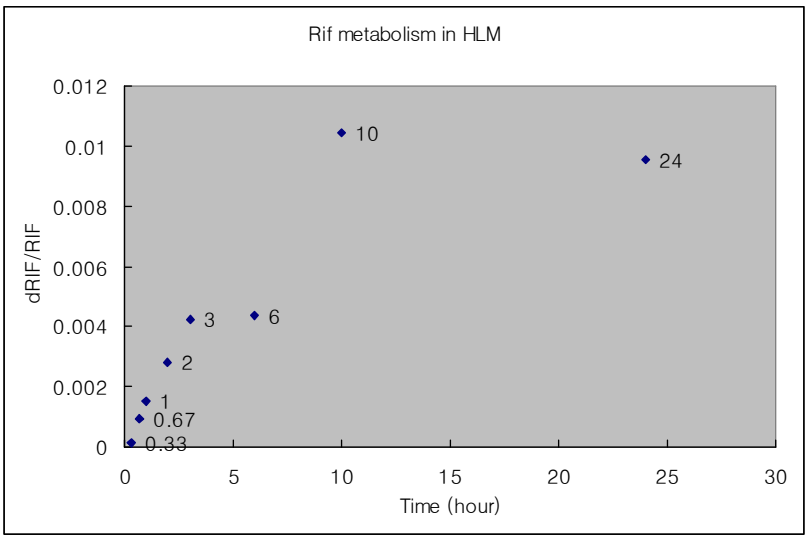
When analyzing the most frequent variation of *CYP3A5* gene, c.6986A>G, the frequency of the A>G variation was 0.80. Statistically significant differences were not found for plasma rifampicin and 25-desacetylrifmapicin concentrations between the two genotypes.

7. Promoter assay of *CES2* gene

As the variations in promoter regions resulted in significant difference in the plasma rifampicin concentration in tuberculosis patients, different combinations of promoter in *CES2* gene were constructed and their effect on luciferase activity was evaluated. Of

the CA, CG, TA, and TG constructs of c.453C>T and c.738A>G variations, the variation of c.738A>G decreased the luciferase activity by about 10% when the nucleotide of the c.458 was T, while the change was not constant when it was C. In terms of c.458C>T variation, the change was not consistent when the c.738 was A or G. The overall results of the promoter assay are presented in Fig. 3.

A



B

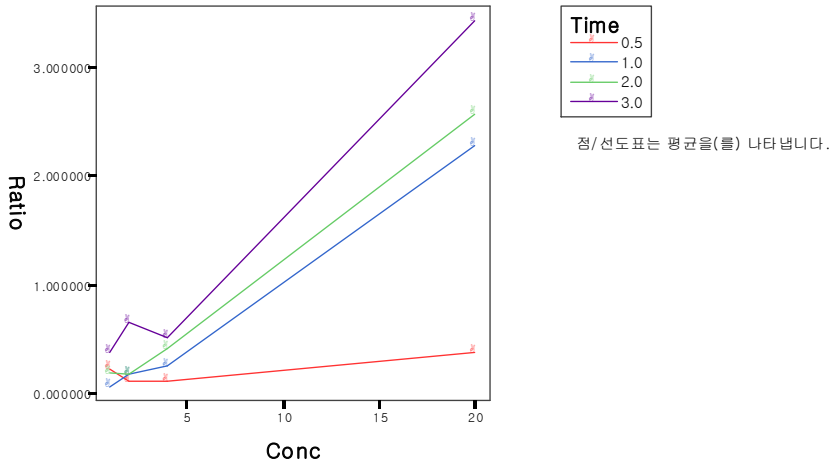


Fig. 1. Formation of 25-desacetylrifampicin with varying length of incubation time (A) and amount of HLM (B). Abbreviations. dRIF, 25-desacetylrifampicin; RIF, rifampicin

Table 5. List of variations of *CES2* gene and frequencies

SNP no.	Location	Position	Variation	Frequency		
				Pts	NCBI*	Kim
1	promoter	453	C/T	0.22	0.12	
2	promoter	738	A/G	0.33	0.37	
3	intron 1	4629	A/G	0.33	0.33	
4	intron 2	6622	T/C	0.20	0.11	
5	intron 3	7035	G/A	0.34	0.45	
6	intron 10	10748	G/A	0.33	0.37	
7	intron 10	10769	G/A	0.03	0.06	0.03
8	3′ UTR	11651	A/G	0.31	0.16	
9	3′ UTR	11884	GAA/del	0.67	0.06	
10	3′ UTR	12027	C/T	0.03		

* Frequencies were cited from NCBI's dbSNP. Pts, patients.

Table 6. Frequencies of *CES2* gene variations and corresponding plasma rifampicin concentration

SNP no.	Position	Variation	Rifampicin concentration (μg/mL)*			P value
			wild	hetero	homo	
1	453	C/T	9.2±2.9	13.0±6.7	9.4±1.5	0.072
2	738	A/G	8.9±2.9	10.5±3.1	13.9±7.4	0.034
3	4629	A/G	8.9±2.9	10.5±3.1	13.9±7.4	0.034
4	6622	T/C	9.9±3.2	10.3±3.6	7.8±2.1	0.615
5	7035	G/A	8.9±2.9	10.9±3.2	13.2±7.2	0.066
6	10748	G/A	8.9±2.9	10.5±3.1	13.9±7.4	0.034
7	10769	G/A	10.1±4.6	13.7±1.5		0.285
8	11651	A/G	9.4±2.9	9.6±3.3	14.1±8.1	0.068
9	11884	GAA/del	14.7±7.8	10.5±3.1	8.9±2.8	0.015
10	12027	C/T	10.6±4.4	5.1±0.1		0.093

*Plasma rifampicin concentration was measured from the samples taken two hours after oral administration of rifampicin in tuberculosis patients. Data are presented as mean±SD.

Table 7. Allele frequencies of the *CES2* gene of tuberculosis patients based on plasma rifampicin concentration of 8 μg/mL

SNP no.	Position	Variation	Frequency in < 8 μg/mL*	Frequency in ≧ 8 μg/mL	P value
1	453	C/T	0.15	0.26	0.322
2	738	A/G	0.15	0.40	0.044
3	4629	A/G	0.15	0.40	0.044
4	6622	T/C	0.22	0.19	0.752
5	7035	G/A	0.20	0.40	0.111
6	10748	G/A	0.15	0.40	0.044
7	10769	G/A	0.00	0.04	0.364
8	11651	A/G	0.20	0.36	0.193
9	11884	GAA/del	0.85	0.64	0.083
10	12027	C/T	0.10	0.00	0.023

*Plasma rifampicin concentration was measured from the samples taken two hours after oral administration of rifampicin in tuberculosis patients.

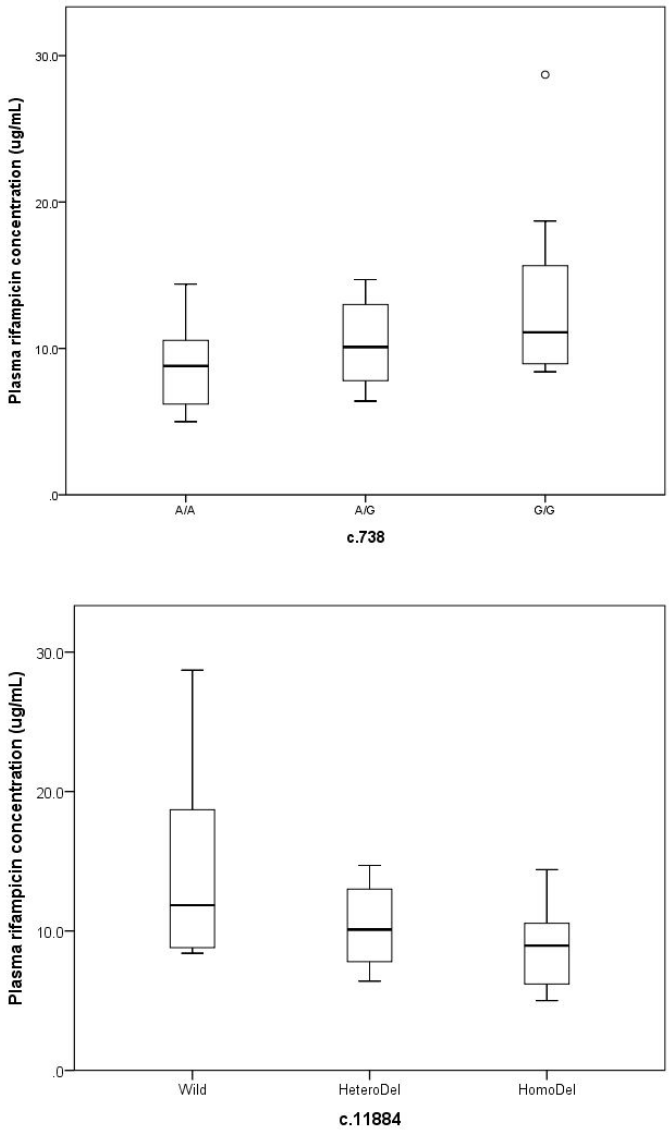


Fig. 2. Change of plasma rifampicin concentration from different genotypes of the two representative loci. Plasma rifampicin concentration increases (upper panel) or decreases (lower panel) as the genotype change from wild type to homozygous variation.

Table 8. Frequencies of the variations of *CES2* gene in general population

SNP no.	Location	Position	Variation	Frequency	Hardy-Weinberg chi-square
1	promoter	453	C/T	0.20	0.0158
2	promoter	738	A/G	0.31	0.0332
3	intron 1	4629	A/G	0.30	0.2268
4	intron 2	6622	T/C	0.19	0.8901
5	intron 3	7035	G/A	0.30	0.2268
6	intron 10	10748	G/A	0.31	0.6413
7	intron 10	10769	G/A	0.05	3.4437
8	3′ UTR	11651	A/G	0.26	0.4154
9	3′ UTR	11884	GAA/del	0.35	8.0354
10	3′ UTR	12027	C/T	0.00	0.0000

Table 9. Haplotype frequency of *CES2* gene of the tuberculosis patients

Haplotype	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Estimated frequency
H1	C	A	A	T	G	G	G	A	del	C	0.434
H2	C	A	A	T	G	G	G	G	del	C	0.026
H3	C	A	A	T	A	G	G	A	del	C	0.014
H4	C	A	A	C	G	G	G	A	del	C	0.168
H5	C	A	A	C	G	G	G	A	del	T	0.023
H6	C	G	G	T	A	A	G	G	wild	C	0.072
H7	C	G	G	T	A	A	A	A	wild	C	0.028
H8	T	G	G	T	A	A	G	A	wild	C	0.014
H9	T	G	G	T	A	A	G	G	wild	C	0.214

P1 – P10 denotes the nucleotide position of the *CES2* gene; P1 453, P2 738, P3 4629, P4 6622, P5 7035, P6 10748, P7 10769, P8 11651, P9 11884, and P10 12027, respectively. As some haplotypes which were not estimated for any patient were not included, the sum of the esitmated frequency is less than one.

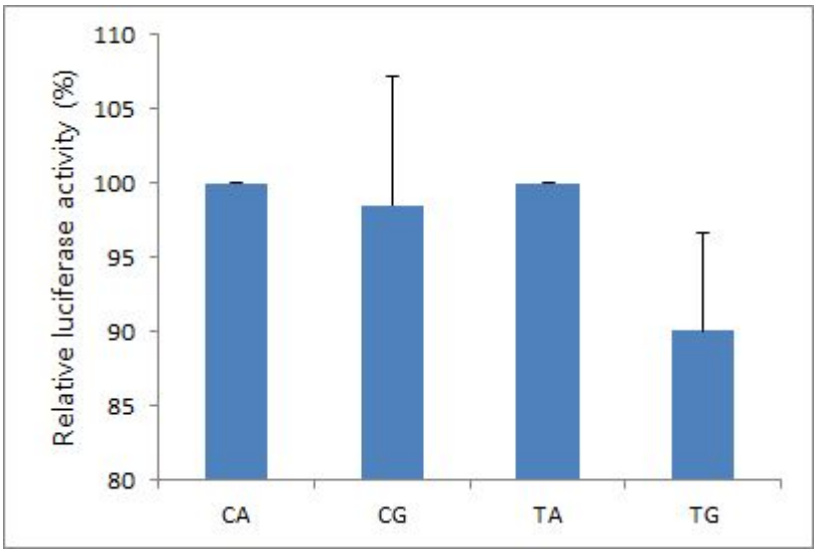


Fig. 3. Promoter analysis of the two promoter regions of *CES2* gene. When c.453 was T and c.738 was G (TG), the luciferase activity was decreased by about 10% when compared to TA. When the combination was CG, the change was not consistent. The luciferase activity was normalized to 100% for CA and TA combinations.

Discussion

Rifampicin is one of the most widely used antibiotics to treat tuberculosis and other infectious diseases. It also modifies the metabolism of other drugs by inducing liver microsomal enzymes. Despite its wide use and importance in combined treatment, the metabolism of rifampicin has not been elucidated. The metabolite of rifampicin in vivo has been known as 25-desacetylrifampicin from decades ago [14] and many investigators studied about pharmacokinetics of rifampicin on the assumption [15, 16]. The deacetylation of rifampicin makes the more polar metabolite and its penetrance to the cell wall is decreased [14], which leads to the decreased bactericidal activity of rifampicin. In the bile, all the antibiotic activity is known to be due to the deacetylated metabolite [17].

The prevalence and incidence of tuberculosis is not well controlled, especially in Korea, although the socioeconomic status has improved. The long treatment period and the emergence of the drug resistant bacteria make many clinicians skeptical about the current treatment protocol. Targeted therapy by adjusting the dose and period on the basis of the patients' metabolic characteristics of the drug is a promising alternative to the current protocol [18]. The development of a new drug that can replace the bactericidal drugs is in progress [19], but the practical use of it will take much time. For the targeted therapy with rifampicin to be successful, the metabolic enzyme and its variations, and their associations with drug metabolism should be

determined.

In this study, the enzyme responsible for the metabolizing the rifampicin was found to be present in liver microsome. It was minimally present in S9 or intestinal microsome which is widely used fraction for investigation of drug metabolism. The result confirms the previous report that the activity of the enzyme is within liver and it is a kind of esterase [20]. However, the enzyme could not be identified because the enzyme activity was two low to be detected by current technique due to the dilution of the HLM by fractionation. This may be the reason why most investigators could not find the enzyme for decades. Instead of finding the enzyme by laborious and ineffective fractionation and enzyme assay, I hypothesized the carboxylesterase 2 may be the responsible enzyme for the metabolism of rifampicin as this enzyme converts many prodrugs by esterification. Of the many enzymes involved in hydrolysis of ester bond, the enzyme that is responsible for the metabolism of rifampicin was known as B esterase [19], which is inhibited by organophosphates [21]. Carboxylesterase, acetylcholinesterase, and butyrylcholinesterase belong to the B esterase. Among them, carboxylesterase is the most important enzyme and has the most broad substrate specificity. Furthermore, the structure of the rifampicin is similar to CPT-11 which is the representative prodrug that is metabolized by carboxylesterase [21].

There is a recent study that arylacetamide deacetylase is the rifampicin metabolizing enzyme [22]. This study proved that recombinant arylacetamide deacetylase showed a similar deacetylating

activity to HLM. However, the authors did not fractionate HLM and purify the enzyme, and thus it seems to be the indirect evidence of the confirmation.

Human carboxylesterase 2 is a member of serine esterases and metabolizes ester and some other functional groups. The gene encoding carboxylesterase 2 is located on chromosome 16q22.1 and consists of 12 exons. It is a well known enzyme that converts irinotecan into its active metabolite SN-38 [23] and specific for heroin, cocaine, 6-acetylmorphin, procaine, and oxybutyinin [24]. There are some reports on the variations in *CES2* gene. Saito et al. found 9 variations and one of them was nonsynonymous variation [25]. Kim et al. found 12 novel variations in *CES2* gene. One of them was nonsynonymous, one altered the 3' splice acceptor site, and two were synonymous [26]. Kubo et al. demonstrated the functional deficiency of the protein and found a novel nonsynonymous SNP [27]. In this study, however, the nonsynonymous or splice site variations were not detected at all. This is quite unexpected as above representative three studies were from Japanese people who are ethnically similar to Koreans. One variation at 3' UTR found from a tuberculosis patient was not reported previously, but it was not found in general population. Therefore, its frequency may be extremely low and seems to have little functional significance. The allele frequency was similar to that in NCBI and previous reports except for the deletion of 3 base pairs. The deletion significantly deviated from the Hardy-Weinberg equilibrium which showed higher frequency of deletion than expected.

There was a clear trend of increasing or decreasing concentration of plasma rifampicin depending on the degree of variation. Of 10 variations found in this study, 6 showed such phenomenon. It suggested that the variation may be associated each other and might affect the metabolism of the drug in any way. When the frequency of the variations were compared based on the plasma rifampicin concentration of 8 μg/mL, which is the lower limit of therapeutic concentration, patients with subtherapeutic rifampicin concentration had significantly lower frequency of the variation allele at c.738A>G, c.4629A>G, and c.10748G>A. This suggests that patients with wild type allele have increased rifmapicin metabolizing activity and thus have lower plasma rifampicin concentration than patients with variant allele. The opposite relation was observed at c.12027C>T. However, patients with the homozygote of the variant allele were not found. Although the estimated haplotype reconstructed from the 10 loci of variations was not associated with the change of the plasma rifampicin concentration, haplotypes with at least above 3 loci may be associated with the drug metabolism. The ratio of 25-desacetylrifampicin to rifampicin was not significantly different except for one locus, c.12027. However, there were only 2 patients with heterozygous variation and one of them showed extremely high 25-desacetylrifampicin concentration, which may be an outlier. Therefore, the association of the genetic variations with the metabolic ratio is not clear. The reason may be that the blood sampling time was at peak concentration, not at the fully distributed time.

The results of promoter analysis suggests that of the two loci of promoter with variation, c.738A>G affects the transcription of the enzyme. The change from A to G resulted in decreased luciferase activity, which may result in the decreased metabolism of the drug and increased plasma concentration. The fact that the variation in the promoter region of an enzyme can affect the enzyme activity has been proved in another study [28].

Rifampicin is known as the potent inducer of CYP enzymes like *CYP3A4* and *CYP3A5* [11]. Rifampicin blood levels and half-life are decreased during prolonged administration possibly due to auto-induction of its metabolizing enzyme [29]. In this study, a well known variation in *CYP3A5* gene and its association with plasma rifampicin concentration was studied. However, there was no association between c.6986A>G genotypes and plasma rifampicin concentration. The result suggest that *CYP3A5* does not affect metabolism of rifampicin or variation in another position may change the metabolism of rifampicin.

In summary, the variations in *CES2* gene was associated with the change of plasma rifampicin concentration and the variation in the promoter region, c.738A>G may decrease the metabolism of rifampicin resulting in increased plasma rifampicn concentration.

References

1. World Health Organization. 2010/2011 Tuberculosis Global Facts. Available at http://www.who.int/entity/tb/publications/2010/factsheet_tb_2010.pdf (Accessed November 2011).

2. Current situation of tuberculosis and its control in Korea. Kim HJ. J Korean Med Assoc 2006;49(9):762-772.

3. Gandhi NR, Nunn P, Dheda K, Schaaf HS, Zignol M, van Soolingen D, Jensen P, Bayona J. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. Lancet 2010;375(9728):1830-43.

4. Blumberg HM, Burman WJ, Chaisson RE, Daley CL, Etkind SC, Friedman LN, Fujiwara P, Grzemska M, Hopewell PC, Iseman MD, Jasmer RM, Koppaka V, Menzies RI, O'Brien RJ, Reves RR, Reichman LB, Simone PM, Starke JR, Vernon AA; American Thoracic Society, Centers for Disease Control and Prevention and the Infectious Diseases Society. American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America: treatment of tuberculosis. Am J Respir Crit Care Med 2003;167(4):603-62.

5. Kimerling ME, Phillips P, Patterson P, Hall M, Robinson CA, Dunlap NE. Low serum antimycobacterial drug levels in non-HIV-infected tuberculosis patients. Chest 1998;113(5):1178-83.

6. Mehta JB, Shantaveerapa H, Byrd RP Jr, Morton SE, Fountain F, Roy TM. Utility of rifampin blood levels in the treatment and follow-up of active pulmonary tuberculosis in patients who were slow to respond to routine directly observed therapy. Chest

2001;120(5):1520-4.
7. Ferraldeschi R, Newman WG. Pharmacogenetics and pharmacogenomics: a clinical reality. Ann Clin Biochem 2011;48(Pt 5):410-7.
8. Donald PR, Sirgel FA, Venter A, Parkin DP, Seifart HI, van de Wal BW, Werely C, van Helden PD, Maritz JS. The influence of human N-acetyltransferase genotype on the early bactericidal activity of isoniazid. Clin Infect Dis 2004;39(10):1425-30.
9. Mandell GL, Bennett JE, Dolin R. Principles and practice of infectious diseases. 7th ed. Churchill Livingstone Elsevier, 2011.
10. Burman WJ, Gallicano K, Peloquin C. Comparative pharmacokinetics and pharmacodynamics of the rifamycin antibacterials. Clin Pharmacokinet 2001;40(5):327-41.
11. Niemi M, Backman JT, Fromm MF, Neuvonen PJ, Kivistö KT. Pharmacokinetic interactions with rifampicin : clinical relevance. Clin Pharmacokinet 2003;42(9):819-50.
12. Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 2001;68(4):978-89.
13. Stephens M, Donnelly P. A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet 2003;73(5):1162-9.
14. Rifampicin desacetylation in the human organism. Maggi N, Furesz S, Pallanza R, Pelizza G. Arzneimittelforschung 1969;19(4):651-4.
15. Acocella G, Mattiussi R, Segre G. Multicompartmental analysis of serum, urine and bile concentrations of rifampicin and desacetyl-rifampicin in subjects treated for one week. Pharmacol Res Commun 1978;10(3):271-88.

16. Pullen J, Stolk LM, Degraeuwe PL, van Tiel FH, Neef C, Zimmermann LJ. Pharmacokinetics of intravenous rifampicin (rifampin) in neonates. Ther Drug Monit 2006;28(5):654-61.
17. Acocella G, Bonollo L, Garimoldi M, Mainardi M, Tenconi LT, Nicolis FB. Kinetics of rifampicin and isoniazid administered alone and in combination to normal subjects and patients with liver disease. Gut 1972;13(1):47-53.
18. Vuilleumier N, Rossier MF, Chiappe A, Degoumois F, Dayer P, Mermillod B, Nicod L, Desmeules J, Hochstrasser D. CYP2E1 genotype and isoniazid-induced hepatotoxicity in patients treated for latent tuberculosis. Eur J Clin Pharmacol 2006;62(6):423-9.
19. Ginsberg AM, Spigelman M. Challenges in tuberculosis drug research and development. Nat Med 2007;13(3):290-4.
20. Jamis-Dow CA, Katki AG, Collins JM, Klecker RW. Rifampin and rifabutin and their metabolism by human liver esterases. Xenobiotica 1997;27(10):1015-24.
21. Liederer BM, Borchardt RT. Enzymes involved in the bioconversion of ester-based prodrugs. J Pharm Sci 2006;95(6):1177-95.
22. Nakajima A, Fukami T, Kobayashi Y, Watanabe A, Nakajima M, Yokoi T. Human arylacetamide deacetylase is responsible for deacetylation of rifamycins: rifampicin, rifabutin, and rifapentine. Biochem Pharmacol 2011;82(11):1747-56.
23. Choi SA, Lee JY, Wang KC, Phi JH, Song SH, Song J, Kim SK. Human adipose tissue-derived mesenchymal stem cells: characteristics and therapeutic potential as cellular vehicles for prodrug gene therapy against brainstem gliomas. Eur J Cancer 2012;48(1):129-37.
24. Satoh T, Hosokawa M. The mammalian carboxylesterases: from molecules to functions. Annu Rev Pharmacol Toxicol 1998;38:257-88.

25. Saito S, Iida A, Sekine A, Kawauchi S, Higuchi S, Ogawa C, Nakamura Y. Catalog of 680 variations among eight cytochrome p450 (CYP) genes, nine esterase genes, and two other genes in the Japanese population. J Hum Genet 2003;48(5):249-70.
26. Kim SR, Nakamura T, Saito Y, Sai K, Nakajima T, Saito H, Shirao K, Minami H, Ohtsu A, Yoshida T, Saijo N, Ozawa S, Sawada J. Twelve novel single nucleotide polymorphisms in the CES2 gene encoding human carboxylesterase 2 (hCE-2). Drug Metab Pharmacokinet 2003;18(5):327-32.
27. Kubo T, Kim SR, Sai K, Saito Y, Nakajima T, Matsumoto K, Saito H, Shirao K, Yamamoto N, Minami H, Ohtsu A, Yoshida T, Saijo N, Ohno Y, Ozawa S, Sawada J. Functional characterization of three naturally occurring single nucleotide polymorphisms in the CES2 gene encoding carboxylesterase 2 (HCE-2). Drug Metab Dispos 2005 ;33(10):1482-7.
28. Park HD, Kim YK, Park KU, Kim JQ, Song YH, Song J. A novel c.-22T>C mutation in GALK1 promoter is associated with elevated galactokinase phenotype. BMC Med Genet 2009;10:29.
29. Nitti V, Delli-Veneri F, Ninni A, Meola G. Rifampicin blood serum levels and half-life during prolonged administration in tuberculous patients. Chemotherapy 1972;17(2):121-129.

국문 초록

리팜피신은 결핵 치료에서 살균 효과가 있는 중요한 약물이다. 리팜피신은 체내에서 탈아세틸화되어 대사물인 25-desacetylrifampicin을 생성하는 것으로 알려져 왔다. 그러나, 대사 효소와 유전적 변이와의 연관성은 아직까지 규명되지 않았다. 본 연구에서는 리팜피신을 대사하는 효소의 존재를 확인하고 후보 효소의 유전적 변이와 약물 대사와의 관계를 규명하였다.

리팜피신을 대사하는 효소의 활성도는 사람 간 마이크로좀에서 측정하였다. 다양한 양의 사람 간 마이크로좀을 다양한 시간 동안 배양한 후 25-desacetylrifampicin의 양을 측정하였다. 사람 간 마이크로좀에 해당하는 효소가 존재하는 것을 확인한 후에 카르복실에스테라제 2를 리팜피신의 탈아세틸화에 관여하는 효소로 가정하였다. 일차 항결핵약물 요법을 받는 35명의 결핵 환자의 혈장 리팜피신과 25-desacetylrifampicin 농도를 측정하였다.

DNA를 추출한 후 *CES2* 유전자의 12개의 엑손 전체, 3‘ UTR, 5. UTR, 이전 보고에서 변이가 있는 것으로 알려진 인트론 부위를 포함하여 염기서열분석을 시행했다. 10개의 변이가 발견되었는데 2개는 프로모터로 추정되는 부위, 5개는 인트론, 3개는 3 ’UTR에 있었다. 3‘ UTR에 있는 1개의 변이는 새롭게 발견된 변이였다. 변이의 수가 증가할수록 리팜피신의 혈장 농도가 변화하는 변이는 6개였다. 정상인 100명을 대상으로 하여 10개의 변이가 있는 위치에 SNaPshot 분석을 시행하여 변이의 빈도를 구하였다. 변이의 빈도는 NCBI 또는 이전에 보고된 것과 다르지 않았는데 3’ UTR에 3개의 염기쌍이 결실된 변이는 빈도가 높았다. 혈장 리팜피신 농도가 8 μg/mL 이상인 환자는 c.738A>G, c.4629A>G, c.10748G>A,

c.12027C>T 변이의 빈도가 유의하게 높았다. 일배체형을 재구성하여 분석한 결과 H9 일배체형이 하나 있는 환자에서 혈장 리팜피신 농도가 더 높았다. 프로모터 분석에서는 c.738A>G에서 루시퍼라제 활성도가 감소하였다.

CYP3A5 유전자의 잘 알려진 변이인 c.6986A>G는 혈장 리팜피신 농도와 관련이 없었다.

결론적으로 *CES2* 유전자의 변이, 특히 c.738A>G는 리팜피신의 대사를 변화시킬 수 있다.

주요어: 결핵, 리팜피신, 대사, 약물유전학, 변이, 카르복실에스테라제
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