科技部補助專題研究計畫成果報告
期末報告

粒線體調控物角質素8及18基因變異以及粒線體ND6基因之核醣核酸表現對於抗結核藥物性肝炎之性別特異性風險之影響

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中 文 摘 要 ： 自從isoniazid (INH)及rifampicin (RIF)在1950及1970年代開發成功後，結核病已有很有效的藥物可治療。然而結核病在台灣之發生率仍居高不下，其原因固然很多，但抗結核藥物引起肝炎，使得結核病患者中斷治療，無疑是治療失敗的重要原因。主持人曾作前瞻性研究，發現我國結核病病人發生抗結核藥物引起的藥物性肝炎之百分比為16.4%，發生藥物性肝炎之危險因子為： 1)女性；2) N-acetyl transferase 2 (NAT2)基因之slow acetylator; 3) 有B型肝炎且病毒量高者；4) 末期腎衰竭且未作透析。

為了瞭解抗結核藥物性肝炎最終之傷害，是否與製造ATP之粒線體複合體I (即NADH去氫酶)基因有關，主持人於2015年開始研究NADH去氫酶基因之SNP與抗結核藥物性肝炎之關係。研究結果顯示，NADH去氫酶基因中之ND4L subunit之SNP，僅發生於有抗結核藥物性肝炎之病人(男、女均有)，而ND5 subunit之SNP僅發生於有抗結核藥物性肝炎之女性。因此抗結核藥物性肝炎最終之傷害，可能與抑制粒線體複合體I (即NADH去氫酶)產生ATP有關。且此機轉可能有性別之差異性。

近年來科學家發現，Keratin(角質素)基因K8/K18之多型性與藥物性肝炎，尤其是致命之藥物性肝炎(包括抗結核藥物INH引起者)有關，而且角質素可改變粒線體的形狀與功能。同時亦有研究發現，血液中粒線體ND6 基因之mRNA表現量低之大鼠，較易發生CCl4引起之肝炎。但並無人報告上述各種現象是否與性別有關。因此主持人假設，女性發生抗結核藥物性肝炎之風險較高，可能與角質素基因K8/K18之多型性有關(因角質素可影響粒線體之功能)，且可能與血液中粒線體ND6基因之mRNA表現量有關。且這些K8/K18 SNP之genotype之分布，或ND6之mRNA表現量較低之百分比，在男性與女性不同，使女性發生抗結核藥物性肝炎之風險較高。

本計畫為期一年，研究方法為，分析300位肺結核病人(其中約有16%可能發生抗結核藥物性肝炎)，其: (A)白血球K8/K18基因SNP之genotype與haplotype，(B)血液中粒線體ND6基因之mRNA表現量，在有發生抗結核藥物性肝炎與未發生者之間有無不同，且在男性與女性之間有無不同，分析(A)與(B)之結果，與女性發生抗結核藥物性肝炎之風險較高有無相關性。

中 文 關 鍵 詞 ： 結核病、抗結核藥物性肝炎、女性、角質素、K8/K18基因、單核苷酸多型性、基因型、單套型、粒線體、NADH去氫酶、ND6、mRNA表現量

英 文 摘 要 ： Hepatitis due to anti-TB drugs is the most important adverse event of anti-TB chemotherapy. In 2007 to 2008 we conducted a study to investigate risk factors of hepatitis during anti-TB treatment. We found that anti-TB drugs-induced hepatitis occurred in 16.5% of TB patients. Risk factors included: 1) women; 2) N-acetyl transferase 2 slow acetylator; 3) High HBV viral load, and 4) end-stage renal disease.

To investigate whether final steps in anti-TB drugs-induced liver injury are related to mitochondrial ATP production failure, we studied mitochondrial complex I

(NADH dehydrogenase) gene variants in patients with liver injury due to anti-TB drugs and those without. We found that mitochondrial complex I ND5 SNPs occurred only in female patients with anti-TB drugs-induced liver injury. Thus final steps in anti-TB drugs-induced liver injury might be related to the ATP shortage from mitochondrial complex I dysfunction, and gender can influence the mechanism.

Recently keratin gene K8/K18 variants have been associated with drug-induced liver injury, including INH-induced, and keratins have been reported to modulate the shape and function of mitochondria. Rats with low mitochondrial ND6 mRNA expression in blood were found to be susceptible to CC14-induced liver injury. Therefore we hypothesized that K8/K18 SNP genotype (through affecting mitochondria function), and blood mitochondrial ND6 mRNA expression may be associated with anti-TB drugs-induced liver injury. The distribution of K8/K18 SNP genotypes, and the percentage of low ND6 mRNA expression may be different between females and males, contributing to the higher risk of anti-TB drugs-induced liver injury in females. We compare K8/K18 SNP genotype distribution and ND6 mRNA expression between TB patients with drug-induced liver injury and those without, and analyze if K8/K18 SNPs and low ND6 mRNA expression contribute to the higher risk of anti-TB drugs-induced liver injury in females than males.

英文關鍵詞：tuberculosis, drug-induced liver injury, keratins, K8/K18, single nucleotide polymorphism (SNP), genotype, haplotype, mitochondria, ND6, mRNA expression, female

粒線體調控物角質素 8 及 18 基因變異以及粒線體 *ND6* 基因之核醣核酸表現對於抗結核藥物性肝炎之性別特異性風險之影響

Influence of genetic variants of mitochondria modulator keratin 8/18 and mitochondria *ND6* mRNA expression on gender-specific risk of liver injury due to anti-tuberculosis drugs（期末報告）

目錄

[BACKGROUND] 研究計畫之背景及目的:

[The importance of hepatitis during anti-tuberculosis treatment]

Tuberculosis (TB) remains a major and sometimes lethal infectious cause of death worldwide even in the 21st century, more than 40 years after the birth of the effective anti-TB medicine rifampicin. In our country, one of the main causes of a persistently high TB incidence (49 per 100,000 in 2013) is low treatment success rate (70% in 2011, far below the WHO's goal: 85%).[1] The most important cause of low treatment success rate is the development of hepatitis during anti-TB treatment (HATT).[2] In our previous studies, it resulted in modification or discontinuation of anti-TB treatment in 17-19 % of TB patients in our country,[3,4] and is difficult to manage.

Despite of investigations for decades, exact causes of HATT remain elusive. The best documented risk factor for HATT is slow acetylator phenotype/genotype of the *N-acetyltransferase 2* (*NAT2*) gene, which is associated with isoniazid (INH)-induced hepatotoxicity.[5,6] Other factors such as cytochrome P450 2E1 (*CYP2E1*),[7] glutathione S-transferase,[8] old age,[5,9-11] low body-mass index (BMI),[12] alcoholism,[13] concomitant hepatitis B virus (HBV),[14,15] hepatitis C viru s (HCV),[16,17] and HIV infection[17,18] have only been inconsistently associated with HATT.

[Hepatitis during anti-TB therapy: our first previous study]

In 2007 to 2008 we conducted a prospective study at National Taiwan University Hospital to evaluate the risk factors of HATT in TB patients, and observe the influence of HBV/HCV viral load in those who had concomitant HBV/HCV infection. In that study we also performed *NAT2* and *CYP2E1* genotyping to observe the simultaneous effects of genetic susceptibility and viral hepatitis.

We found that among the 360 TB patients we studied, HATT developed in 68 patients (**18.9%**), of which 59 (16.4%) was drug-induced hepatitis, and 9 (2.5%; all male) was acute flare-up of B or C viral hepatitis. Kaplan-Meier analysis and log-rank test revealed that: 1) women were twice more likely to have drug-induced HATT than men. 2) The risk of both drug- and virus-induced HATT were higher in patients with high initial viral load than those with low initial viral load or those without viral hepatitis (Table 1)[4].

Table 1. Risk factors of hepatitis during anti-TB treatment (HATT) in different subgroups of TB patients by univariate analysis[4]

Factor		Drug-induced HATT			Virus-induced HATT		
		No. at risk	No. (%) with HATT	*p*	No. at risk	No. (%) with HATT	*p*
Age	>65 y	151	25 (16.6)	0.881	31	4 (13)	0.869
	≦65 y	209	34 (16.3)		35	5 (14)	
Sex	Female	128	31 (24.2)	**0.003**	18	0	**0.045**
	Male		28 (12.1)		48	9 (19)	
HBV or HCV infected	High initial HBV VL*	23	9 (39.1)	**< 0.001**	23	5 (22)	**0.064**
	Low initial HBV VL	19	2 (10.5)		19	0	
	High initial HCV VL	12	4 (33.3)		12	3 (25)	
	Low initial HCV VL	12	2 (16.7)		12	1 (8)	
End-stage renal disease	Yes, under dialysis	9	2 (22.2)	0.079	2	0	**0.004**
	Yes, no dialysis	11	3 (27.3)		2	1 (50)	
NAT2 genotype	Slow acetylator	82	22 (26.8)	**0.002**	17	3 (18)	0.521
	Rapid acetylator	278	37 (13.3)		49	6 (12)	
CYP2E1 genotype	c1c1	215	36 (16.7)	0.801	45	6 (13)	0.942
	c1c2 or c2c2	145	23 (15.9)		21	3 (14)	

(*VL: viral load)

[Drug-induced hepatitis during anti-TB therapy: why women are twice risky than men?]

After completing our previous study, we want to answer why women are twice risky to develop drug-induced HATT than men (24% vs. 12%, Table 1). Actually for a long time women have been reported to have a higher risk of HATT than men, the hazarrd ratio was 1.5 to 3.3 [4,10,12,19]. One possible reason is the activity of cytochrome P450 3A4 (CYP3A4), the most abundant enzyme in the hepatic cytochrome P450 family that catalyzes phase I reaction of many drugs and produces toxic intermediates, is higher in women than men [20].

Yet the exact mechanism that leads to higher CYP3A4 activity in women than men is unknown. Single nucleotide polymorphisms (SNPs) in the coding region of the *CYP3A4* gene occur only rarely and cannot explain the difference in CYP3A4 activity between men and women [21]. The pregnane X receptor (PXR), a member of the nuclear receptor superfamily, is a known regulator of the *CYP3A4* gene. Because SNPs in the transcription factor binding sites of the *PXR* regulatory region (the

promoter and intron1) have been associated with altered *PXR* and *CYP3A4* expressions [22], it is possible that gene variants in the *PXR* regulatory region may contribute to differences in risk of drug-induced HATT between males and females. Therefore we conducted the following study to investigate the contribution of *PXR* SNPs to the increased risk of drug-induced hepatitis during anti-TB treatment in women.

[The association between PXR SNPs and higher risk of drug-induced HATT in women: our previous study (NSC 102-2629-B-002 -001 -)]

In this NSC study we hypothesized that certain genotypes and haplotypes of SNPs in *PXR* regulatory region may be risk factors for HATT, and the distribution of these genotypes and haplotypes may be different between male and female TB patients, contributing to the increased risk of HATT in females.

We selected 6 SNPs in the regulatory region of *PXR* gene based on known association with diseases [22,23]: rs3814055 (located in the 5' untranslated region), rs1248820, rs2461823, rs7643645 (all located in intron 1), rs6785049 (located in intron 5), and rs3814057 (located in the 3' untranslated region).

We enrolled TB patients, extracted genomic DNA from peripheral blood, sequenced these SNP sites and correlated with drug-induced hepatitis during anti-TB treatment. We found that among 355 TB patients (male, 65.6%), 70 (19.7%) developed HATT. Genotypes at **rs2461823** and **rs7643645** were significantly associated with risk of HATT **only in females**. AA at rs2461823 was a risk genotype, while AA at rs7643645 was a protective genotype (Table 2) [24].

Table 2. Frequency of drug-induced hepatitis during anti-tuberculous treatment (HATT) in males and females with different genotypes at six single nucleotide polymorphism (SNP) sites of the *PXR* gene

SNP	Loca-tion	Geno-type	Study population			Male			Female		
			No.	% with HATT	*p*	No.	% with HATT	*p*	No.	% with HATT	*p*
rs3814055	5'UTR	CC	238	19	0.742	156	16	0.916	82	24	0.761
		CT	99	22		60	18		39	28	
		TT	18	17		17	18		1	0	
rs12488820	Intron 1	CC	337	20	0.747	216	17	>0.999	121	26	>0.999
		TT	16	13		15	13		1	0	
rs2461823	Intron 1b	GG	128	18	0.052	89	19	0.729	39	15	**0.007**
		AG	172	17		114	15		58	22	

		AA	53	32		29	17		24	50	
rs7643645	Intron 1b	AA	95	13	0.142	65	17	0.368	30	3	**0.004**
		AG	171	22		117	19		54	30	
		GG	88	22		50	10		38	37	
rs6785049	Intron 5	GG	119	18	0.284	72	15	0.883	47	21	0.091
		AG	185	23		129	18		56	34	
		AA	51	14		32	16		19	11	
rs3814057	3' UTR	AA	73	15	0.431	50	18	0.636	23	9	0.117
		AC	205	22		139	18		66	30	
		CC	75	19		42	12		33	27	

The association with female hepatitis remained significant even by multivariate logistic regression analysis: genotype AA at rs2461823 (OR: 6.87 [2.55–18.52]) was associated with a > 6 times risk, and genotype AA at rs7643645 (OR: 0.14 [0.02–1.02]) was associated with a one-seventh risk of HATT **only in females**. Haplotype analysis showed that h001101 (OR: 2.30 [1.22–4.32]) and h000110 (OR: 2.25 [1.08–4.69]) haplotype were associated with increased risk of HATT **only in females [24]**.

[The association between PXR SNPs and risk of INH-, RIF- or PZA-induced HATT in females] [24]:

We further separated HATT into those due to isoniazid (INH), rifampicin (RIF) or pyrazinamide (PZA). We found that for INH-induced hepatitis, *NAT2* slow acetylator genotype and malnutrition were independent risk factors for INH-induced hepatitis in both males and females, but genotype AA at rs2461823 (OR: 10.5 [1.91–58.1]) and number of A allele at rs6785049 site (OR: 11.7 [1.06–129]) were independent risk factors **only in females** **(Table 3)**.

Table 3. Factors associated with isoniazid-induced hepatitis, by multivariate logistic regression analysis

Variables	***p***	**OR (95% CI)**
NAT2 slow acetylator	0.012	9.53 (1.65 – 55.0)
Malnutrition	0.034	5.87 (1.14 – 30.1)
AA in rs2461823 of *PXR* gene in women	<0.001	10.5 (1.91 – 58.1)
No. of A allele at rs6785049 in women	0.045	11.7 (1.06 – 129)

For RMP-induced hepatitis, we found that end-stage renal disease, number of A allele at rs6785049, and h000010 haplotype were independent risk factors in both male and female patients, but genotype AG at rs6785049 (OR: 3.09 [1.09–8.81]), and h001101 haplotype (OR: 5.51 [1.68–18.1]) were independent risk factors **only in females** (**Table 4**).

Table 4. Factors associated with rifampin-induced hepatitis, by multivariate logistic regression analysis

Variables	*p*	OR (95% CI)
End-stage renal disease	0.012	4.83 (1.42 – 16.4)
AG at rs6785049 in females	0.035	3.09 (1.09 – 8.81)
No. of A allele at rs6785049	0.038	3.09 (1.06 – 8.95)
h000010*	0.001	8.01 (2.30 – 27.9)
h001101* in females	0.005	5.51 (1.68 – 18.1)

* 0: common allele and 1: minor allele, by the order of rs3814055: C→T; rs12488820: C→T; rs2461823: G→A; rs7643645: A→G; rs6785049: G→A; rs3824057: A→C.

For PZA-induced hepatitis, multivariate logistic regression analysis revealed that genotype AG at rs7643645 (OR: 2.85 [1.33–6.11]) was an independent risk factor for both male and female patients, but genotype AA at rs2461823 (OR: 7.29 [2.54–20.9]), number of G allele at rs7643645 (OR: 1.84 [1.19–2.85]), and h000110 haplotype (OR: 5.10 [1.92–13.5]) were independent risk factors **only in females** (**Table 5**).

Table 5. Factors associated with pyrazinamide-induced hepatitis, by multivariate logistic regression analysis

Variables	*p*	OR (95% CI)
AA at rs2461823 in females	<0.001	7.29 (2.54 – 20.9)
AG at rs7643645	0.007	2.85 (1.33 – 6.11)
No. of G allele at rs7643645 in females	0.006	1.84 (1.19 – 2.85)
h000110* in females	0.001	5.10 (1.92 – 13.5)

* 0: common allele and 1: minor allele, by the order of rs3814055: C→T; rs12488820: C→T; rs2461823: G→A; rs7643645: A→G; rs6785049: G→A; rs3824057: A→C.

Thus our hypothesis is proved that SNPs in *PXR* regulatory region are associated with increased risk of drug-induced HATT among women.

However, this gender-dimorphic association between *PXR* and drug-induced HATT may not be the sole factors that lead to increased risk of drug-induced HATT in women. Other factors or genes may also contribute to woman's higher risks of drug-induced HATT. Besides, despite of our observations, we still do not know what

substances are directly responsible for the final damage to liver cells. Therefore in 2015 we conducted a study to investigate if females are more vulnerable during the final stage of hepatic damage (i.e. the stage of mitochondrial energy supply) of HATT than males.

[*The association between gender-specific risk of isoniazid hepatitis and mitochondrial NADH dehydrogenase gene polymorphism* Isoniazid 藥物性肝炎之性別特異性風險與粒線體 NADH 去氫酶基因多型性之關聯性] (科技部 104-2629-B-002-001-)

In this study we investigated whether INH-induced (or other anti-TB drug-induced) hepatotoxicity was associated with underlying mitochondrial complex I (also known as NADH dehydrogenase) genetic mutations or not.

Previous studies performed in western countries have shown that, up to 20% of INH-treated patients developed increased ALT activity, and 1% of recipients developed severe hepatotoxicity including liver failure and mortality [25]. Our own study shows that INH-induced hepatitis developed in 2.3% of INH recipients [4], and risk factors for INH-induced hepatitis included: *N-acetyltransferase* gene (*NAT2*) slow acetylator and malnutrition (for men and women alike), AA genotype in rs2461823 and number of A allele at rs6785049 of *PXR* gene (**only in women**) [24]. However, we know very little about the molecular mechanisms that cause INH-induced liver injury, and whether or not the molecular mechanisms exert sex-dimorphic influences in men and women. **Previous experiments showed that INH [26] or its major metabolite hydrazine [27] may interfere with mitochondrial function.** However, these mitochondrial changes (mitochondrial oxidant stress) were not sufficient to cause overt hepatocyte injury, because hepatic mitochondria have a large reserve capacity. Lee KK et al showed that INH alone did not induce cell injury in cultured mouse hepatocytes. However, co-exposure of hepatocytes to INH and nontoxic concentrations of the complex I inhibitors rotenone or piericidine resulted in massive ATP depletion and cell death [26]. They found that **the toxic metabolite hydrazine is a solubilized mitochondria complex II inhibitor,** and concluded that underlying **pharmacological or genetic** inhibition of mitochondria complex I (which alone is not acutely toxic), can trigger INH-induced hepatocellular injury.

[Genetic mutations associated with mitochondrial complex I deficiency]

Mitochondria have an inner and an outer membrane. The respiratory chain, located in the inner membrane, is controlled by 2 separate genetic systems in the

genomic and mitochondrial DNA (mtDNA). mtDNA is a small, closed circular and double-stranded molecule containing 37 genes, of which 24 are needed for translation and 13 encode subunits of the respiratory chain [28]. **Complex I, also named nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase or NADH dehydrogenase,** is composed of at least 46 subunits and its defect are the most frequent deficiencies of the respiratory chain [29].

Polymorphisms or mutations in mtDNA coding NADH dehydrogenase have been associated with neurological diseases [29], breast cancer [30], and asthma in girls but not in boys [31]. Because the reported association with breast cancer and asthma in girls shows **gender dimorphism**, we decide to investigate if single nucleotide polymorphisms (SNPs) in mitochondrial NADH dehydrogenase gene are associated with INH-induced hepatitis and the increased risk of INH-induced HATT in females. **Our hypothesis was that mitochondrial *NADH dehydrogenase* gene SNPs, which can be potential causes of mitochondrial complex I deficiency, are associated with INH-induced hepatocellular damage (INH metabolite hydrazine being a known mitochondrial complex II inhibitor), and that genotypes/haplotypes at SNPs of NADH dehydrogenase gene can be different between females and males, contributing to the increased risk of INH hepatitis in females.**

[Our preliminary results]:

From August 2015 up to now, we have performed mitochondrial DNA sequencing on peripheral blood leukocytes from 10 TB patients with (hepatitis group) and 10 TB patients without (control group) anti-TB drug-induced hepatitis. The preliminary results show that both hepatitis and control group patients had SNPs in mitochondrial NADH dehydrogenase (complex 1) genes. However, the polymorphisms in *ND4L* gene (which codes for NADH dehydrogenase subunit 4L) occur only in patients (both male and female) with anti-TB-drug-induced hepatitis, not in control group. Moreover, SNPs in the ND5 gene (which codes for NADH dehydrogenase subunit 5) occur only in female patients with hepatitis, not in male patients with hepatitis, and not in control group (**Table 6**).

Table 6 Genetic variants observed in NADH dehydrogenase gene of mitochondrial DNA in TB patients with or without hepatitis due to anti-TB drugs

	SNPs observed in:

Gene	Male patients with drug hepatitis	Female patients with drug hepatitis	Male patients without drug hepatitis	Female patients without drug hepatitis
ND1	+	+	+	+
ND2	+	+	+	+
ND3	+	+	+	+
ND4L	**+**	**+**	**-**	**-**
ND4	+	+	+	+
ND5	**-**	**+**	**-**	**-**
ND6	Not completed yet	Not completed yet	Not completed yet	Not completed yet

Currently the study is still going on.

[Keratin 8 and 18 genetic variants in drug-induced liver injury]:

Yet mitochondrial complex I polymorphisms or mutations are probably only one of the many mechanisms that can contribute to mitochondrial ATP exhaustion and hepatocyte necrosis. Recently It has been reported that keratin 8 and 18 genetic variants that occurred in a highly conserved amino acid residues were found in patients with fatal drug-induced (including INH-induced) liver injury.[32] In that study, however, the influence of gender was not analyzed, because of the small size of samples. Keratins are a subgroup of intermediate filaments, found mainly in epithelial tissues, and can be divided into the acidic type I (including K9 - K28 and K31 - K40) and basic type II (including K1 - K8 and K71 - K86) intermediate filaments.[33] Human adult hepatocytes contain only K8/K18, while most other cell types contain more complex combination of keratins.[34] Keratins are cytoprotective proteins and keratin gene mutations predispose the host to > 60 diseases which can be reproduced in animal models. [35] In mouse liver, mutations of K8/K18 do not cause severe disease in basal conditions, but predispose the animal to severe liver injury under stresses including apoptotic, oxidative and drug-induced.[36] Human association studies have found that keratin variants were over-expressed in patients with end-stage liver diseases, acute hepatic failure, chronic hepatitis C virus infection, primary biliary cirrhosis and acetaminophen-induced liver injury.[37,38] Yet in these studies the influence of gender was not mentioned.

[Keratins modulate the shape and function of mitochondria]:

As for possible mechanisms that underlie the cytoprotective function of keratins, a recent investigation has found that mouse liver with disrupted keratins have smaller mitochondria, K8-null mouse livers have decreased ATP content, K8-null mitochondria

have decreased cytochrome c and increased sensitivity to calcium ion induced permeability transition.[39] Thus keratins influence the shape and function of mitochondria, and keratin gene mutations may contribute to human diseases through their effects on mitochondria and ATP production.

[Blood mitochondrial gene expression profile to identify individuals susceptible to anti-TB drug induced liver injury]

From the literatures described above, mitochondrial toxicity is an important mechanism for drug-induced liver injury. Thus identifying variants of mitochondrial genes or mitochondria-related genes (like *K8/K18*) may help us discover individuals who are susceptible to drug-induced liver injury. Yet genetic methods may not be the only technique that can help us identifying this susceptible population. Yun JW and colleagues have reported that mouse with low level of blood *ND6* RNA expression showed significantly higher susceptibility to carbon tetrachloride- (CCl4-) induced liver injury.[40] However, in this animal study, all the rats were male, and gender effect was not addressed. *ND6* is the mitochondrial gene coding for NADH dehydrogenase subunit 6.[28] As stated above, NADH dehydrogenase (or complex I) catalyzes the transfer of electrons from NADH to ubiquinone and builds up electrochemical potential for the generation of ATP. Thus complex I dysfunction can result in ATP shortage and hepatocyte necrosis.[28] In mouse experiment *NAD6* mutation has been associated with ATP production defect, over-production of reactive oxygen species and tumor metastasis,[41] although the effect of gender was not investigated in that study, either, and there has been no human study on the possible role of *ND6* mutation or expression in drug-induced liver injury.

[Our hypothesis on the association of K8/K18 genetic variants and blood ND6 expression with susceptibility to anti-TB drugs-induced liver injury]:

With animal and human studies linking *K8/K18* genetic variants to hepatic failure and drug-induced liver injury, and animal experiment showing low blood *ND6* expression in CCL4-induced liver injury, we hypothesized that:

(1) *K8/K18* genetic variants may be associated with anti-TB drugs-induced liver injury (possibly through modulating mitochondria function), and that genotypes/haplotypes at SNP of *K8/K18* may be different between females and males, contributing to the increased risk of anti-TB drugs-induced liver injury in females.

(2) Blood mitochondrial *ND6* gene expression may be lower in patients with anti-TB

drugs-induced liver injury, and may serve as a biomarker for the identification of patients susceptible to anti-TB drugs-induced liver injury, especially females.

[*Goals of our current proposal*]

In the current 2-year proposal, we will test our hypothesis by:

1) Investigate SNPs in *K8/K18* gene in TB patients who develop anti-TB drugs-induced liver injury and in TB patients without drugs-induced liver injury.
2) Compare the distribution of *K8/K18* SNP genotypes/haplotypes between the two groups and check if certain SNP genotypes/haplotypes are associated with anti-TB drugs-induced liver injury.
3) Check if certain *K8/K18* SNP genotypes/haplotypes are associated with anti-TB drugs-induced liver injury **only in female patients**.
4) Investigate if *K8/K18* SNPs are associated with severity of anti-TB drugs-induced liver injury, and if the association shows gender dimorphism.
5) Compare the blood mitochondria *ND6* RNA expression between patients with anti-TB drugs-induced liver injury and those without, and investigate if the potential difference shows gender dimorphism.

[METHODS] 研究方法

[*Ethics approval*]

Request for ethics approval for the study has been submitted to the Ethics Committee of National Taiwan University Hospital.

[*Study population*]

The inclusion criteria are (All participants must be □ 20 years old):

1. Patients with culture- or histology proved tuberculosis (TB)

The exclusion criteria are:

1. Patients who are treated with corticosteroids or other potentially hepatotoxic drugs.

[*Study protocol*]

1. For TB patients who agree to participate in the study, liver function test (AST, ALT, bilirubin, gamma-GT, alkaline phosphatase, albumin), renal function test (creatinine), fasting blood sugar, uric acid, hemogram, HBsAg and anti-HCV will be checked. (These are the routine blood tests before treatment initiation, not research blood tests.)
2. For TB patients, blood will be drawn at time zero (before initiation of anti-TB therapy) for DNA and RNA extraction (for K8/K18 genetic variants and mitochondria *ND6* mRNA expression). (These are for the purpose of research.)
3. For TB patients, blood will also be drawn 2, 4, 6, 8, 10 and 12 weeks after the initiation of anti-TB therapy to check for hepatitis during anti-TB therapy, and HBV/HCV viral load if needed. (These are routine tests, not for research purpose.)
4. For healthy controls blood will be drawn when they join the study for DNA and RNA extraction (for K8/K18 genetic variants and mitochondria *ND6* mRNA expression).

[*Definition of liver injury during anti-TB treatment*]

Liver injury during anti-TB treatment was defined as an increase in serum AST and/or ALT to > 3 times the upper limit of normal (ULN) if symptomatic, or > 5 times the ULN if asymptomatic.

[*Anti-TB therapy*]

All TB patients receive standard anti-TB treatment including daily INH (H), RIF (R), ethambutol (E), plus pyrazinamide (PZA, Z) in the first 2 months, and daily HR for the following 4 months. The regimen would be modified if necessary by the primary care physician in case of concomitant hepatic/renal diseases, adverse events or susceptibility test.

[*Diagnosis of anti-TB drugs-induced liver injury*]

The diagnosis of INH- or RIF-induced liver injury requires a positive rechallenge test (at least doubling of serum AST or ALT level after rechallenge), whereas PZA-induced liver injury is diagnosed either by rechallenge or by exclusion.

[*SNP study of keratin 8/keratin 18 gene*]:[32]

Genomic DNA will be obtained from EDTA-anticoagulated peripheral blood with DNA extraction kit (Qiagen, Valencia, CA, USA). The mutational K8/K18 hotspots including K8 exons 1, 6, 8 (corresponding to amino acids 1-108, 328-400, 421-483), K18 exon 1 (amino acids 1-139) are amplified using the following primers (Table 7):

Table 7 K8/K18 Primers and length of amplified products

Exon	Primer	Size (bp)
K8		
1F:	TGCCTCTACCATGTCCATCA	392
1R:	CGGGACTACCAGGAGAAAGG	
2F:	TAGACCTTTTTGCTCTCTCCT	282
2R:	GAGCAGGTGACTTCAGTTGGG	
3F:	CTCATATCCTCATCTCTGTGA	279
3R:	ACTTAGGAATATTTAGGGACA	
4F:	TGGCAACTAGAAAGTCCTGTG	279
4R:	AGCCTCTGGTTGAGTCTCAGG	
5F:	CACTTGCCCTCTTCCCCACAG	333
5R:	CACCCCCAACCCGGCCCATAC	
6F:	CATACCCAACCTGACCTACTTACC	369
6R:	AGAACAACAGGACCCCAAGTC	
7F:	TGACCGGACCTGCTTCCCTAT	293
7R:	AGGTCACTGTGAGCGACTGAG	
8F:	TACCTCTGTCCCTCACCAGG	300
8R:	CTCCTGTTCCCAGTGCTACC	
K18		
1F:	CAAAGCCTGAGTCCTGTCCTTT	481
1R:	AGTTGAGGTCCCTCCTACCCCTTAC	
2F:	CTGGCTTTCTATTCATGGAAC	201
2R:	AACTACCCAGCCTGGGGAGCA	
3F:	CCTCTGATCACCTCCACTCCT	202
3R:	GGCCAGTGGCCCCTGCTTGC	
4F:	CACTTTTGCCCCTGTCACCTTTAG	237

4R:	GTCTGCCTCCCTCCCACACCTT	
5F:	CTGCCAAGGTGTGGGAGGGAG	211
5R:	AGGTGATGTGAAGGCACTCAC	
6F:	CAGAAGGCCAGCTTGGAGAAC	270
6R:	ATCTCCTGATCCCAGCACGTG	
7F:	GGGCTTGGTCTTCTGTTACAG	163
7R:	GGGTACCCTGCTTCTGCTGGC	

These primers are based on the sequence M34482.1 for K8 and AF179904.1 for K18. Amplified products are purified and genotyped using the Sequenom MassARRAY® system (iPLEX GOLD) according to the manufacturer's instructions. Briefly, PCR and single-base extension primers (SBE) are designed using the MassARRAY assay design 3.1.2.5 software (Sequenom MassARRAY system) that allows iPLEX reactions for SBE designs with the modified masses associated with the termination mix. The reaction products are dispensed onto a 384-element SpectroCHIP bioarray (Sequenom) using a MassARRAY nano-dispenser and assayed on the MassARRAY platform. Mass differences are detected with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The MassARRAY Workstation v.3.3 software is used to process and analyze the iPLEX SpectroCHIP bioarray while the Typer Analyzer v.3.4 software is used to analyze all genotypes obtained from the assays.

[RNA isolation]:

Total RNA will be isolated from peripheral blood drawn into EDTA-anticoagulated tubes using RNA extraction kit.

[Reverse transcription and quantitative real-time PCR] [40]

RT-PCR is performed using mitochondrial ND6-specific primers (Reference sequence ENSRNOT00000051268):

Sense: 5'-TGTCTAGGGTTGGCGTTGAA-3'

Anti-sense: 5'-AACCAACATCCCACCCAAAT-3'

The annealing temperature is 65°C. The cycle number is 35. The product size is 150 bp. For quantitative real-time PCR we will use primers and probes designed by Applied Biosystems and supplied as Assay-on-Demand Gene Expression Assay mix which contains a 20X mix of unlabelled PCR forward and reverse primers and TaqMan probe.

[*Sample size*]:

In 1 year (we were allowed one year for this project) we plan to enroll 100 TB patients. Of them about 60 would be male patients, and about 40 would be female.

We expect that about 12% (n=7) of male and 24% (n=10) of female TB patients would develop anti-TB drugs-induced liver injury. Totally about 17 patients would develop anti-TB drugs-induced liver injury (prevalence about 17%).

[Data and statistical analysis]:

All SNPs will be tested for Hardy-Weinberg equilibrium.[42] Linkage disequilibrium is analyzed using Haploview.[43] Haplotype frequencies are calculated from *K8/K18* genotype data and analyzed using EM algorithm by TagSNPs.[44] The distribution of genotypes and haplotypes at SNP sites is compared between TB patients with and those without anti-TB drugs-induced liver injury, and between female and male patients with anti-TB drugs-induced liver injury. Mitochondrial *ND6* mRNA expression levels of patients with and without drug-induced liver injury are compared. The association between K8/K18 SNPs, mitochondrial *ND6* expressions and clinical factors including drug-induced liver injury and gender is analyzed using chi-square method, uni- and multi-variate logistic regression model.

We will analyze if (1) K8/K18 SNPs (2) mitochondrial *ND6* mRNA expression is associated with increased risk of anti-TB drugs-induced liver injury in females.

[RESULTS]:

We enrolled 20 patients with pulmonary TB (male n=12, 60 %). Among them, 10 patients had developed drug-induced liver injury (DILI) during anti-TB therapy, and 10 patients had not. We performed *K8* and *K18* SNP study on their peripheral blood leukocytes using real-time qPCR methods. Primers for the SNP sites are shown in Table 7 (see Methods).

We found that in all 20 participants, all the SNP genotypes we investigated in *K8* and *K18* gene were wild type. Due to time limit (we were allowed one-year for this project), we were not able to study more cases. From the results, it is likely the DILI due to anti-TB drugs is not associated with *K8* and *K18* gene polymorphism. The results are compatible with previous reports, which shows that K8 and K18 polymorphisms are very rare among Asian people.[32]

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計畫成果自評:

From the results, it is likely the DILI due to anti-TB drugs is not associated with *K8* and *K18* gene polymorphism. The results are compatible with previous reports, which shows that K8 and K18 polymorphisms are very rare among Asian people.[32]

科技部補助專題研究計畫項下：

日期：2017 年 6 月 17 日

出國人員 姓名	李麗娜	服務機構 及職稱	台大醫學院附設 醫院檢驗醫學部
會議時間	2017 年 5 月 19 日至 2017 年 5 月 24 日	會議地點	美國華盛頓(哥倫比亞特區)
會議名稱	(中文)2017 年美國胸腔學會國際會議 (英文)2017 American Thoracic Society International Conference		
發表論文 題目	(中文) 粒線體 DNA 變異在抗結核藥物肝傷害的重要性 (英文) Mitochondrial DNA Variants in Drug-Induced Liver Injury by Anti-Tuberculosis Agents		

一、參加會議經過

美國胸腔協會每年五月舉辦之國際會議為全世界最大之胸腔醫學大會，每年都有來自全世界之胸腔內、外科醫師、科學家、流行病學家、衛生行政專家、呼吸治療師、護理人員等熱烈參與，參與人數超過一萬人，發表超過 6000 篇之新論文，是全世界胸腔醫學領域之專業人員最重視之會議。今年(2017)之年會在美國首都華盛頓市舉行，大會之研討主題包括: 肺癌之最新標靶治療、免疫療法、致癌基因及抗藥基因研究，肺癌之篩檢，肺結核菌之基因研究，結核病之分子免疫學研究及最新診斷方法，非結核性分枝桿菌感染，肺部之細菌、黴菌、病毒感染，流感、禽流感、胸腔重症、慢性阻塞性肺疾、氣喘病、職業性肺部疾病、急性呼吸窘迫症候群、呼吸衰竭及機械式呼吸、長期呼吸照護、睡眠呼吸終止症候群、肺泡蛋白沉著症、類肉瘤、肺動脈高壓、間質性肺炎、肺臟移植、環境因子造成的肺疾等。

職參加此次會議主要目的有三:

一. 發表論文；

二. 汲取胸腔醫學最新之觀念、最新的研究成果、最尖端的研究技術；
三. 與各國學者討論、切磋，汲取他人寶貴的經驗，作為改進我們研究方法、設計將來研究主題之參考。

職在本次大會中，發表壁報論文一篇，題目為: Mitochondrial DNA Variants in Drug-Induced Liver Injury by Anti-Tuberculosis Agents (粒線體 DNA 變異在抗結核藥物肝傷害的重要性)。

職在研討會中，得以接觸到與抗結核藥物之副作用，尤其是抗結核藥物引起之肝傷害有關之研究，這些知識對於職的研究方向、研究方法以及研究結果之判讀均有莫大的幫助。在本次研討會發表的抗結核藥物引起之肝傷害研究中，職的論文是唯一有關粒線體 DNA 變異的，因此職亦感到職本身在抗結核藥物引起之肝傷害之研究方面，也有其獨特之處，值得更深入研究下去。

除了發表論文外，職亦參與多場討論會，汲取多國學者在胸腔疾病及重症領域之最新研究成果，並交換意見。各國學者對於我們的研究，深感興趣，亦給予我們許多寶貴的意見。職參與此次年會在抗結核藥物引起之肝傷害領域之尖端知識方面，深感收獲甚豐，對日後職之研究方向與實驗設計有很大的幫助。

二、與會心得

此次參加 2017 年美國胸腔學會國際會議，不僅能將我們過去數年之研究成果發表，讓世界各國的學者都認識到，我們在抗結核藥物引起之肝傷害領域之基礎、臨床及流行病學方面之研究，也讓我們得

知目前各國正進行之最新有關胸腔疾病及重症之研究，研習有關這些疾病之新觀念、新知識、新發現、新的研究方法與技術，並與各國學者討論、切磋，向他們請教我們研究上遇到的困難與瓶頸，也向他們提供我們在研究結核病及其他胸腔疾病之經驗，對我們的研究工作，不論是在實驗室的技術、臨床上的處理、統計學上的方法、將來研究的方向等，均有極大的幫助。

三、考察參觀活動(無是項活動者略)

四、建議

感謝科技部提供經費，讓職出國發表論文，參加國際會議，增廣見聞及專業知識，拓展視野，研習最尖端的醫學科技，職受益良多。懇請科技部能繼續支持我們的研究，也希望我們明年也有新的研究成果在國際上發表。

五、攜回資料名稱及內容

ATS 2017 International Conference Final Program

六、其他

105年度專題研究計畫成果彙整表

計畫主持人：李麗娜	**計畫編號：**105-2629-B-002-003-
計畫名稱：粒線體調控物角質素8及18基因變異以及粒線體ND6基因之核醣核酸表現對於抗結核藥物性肝炎之性別特異性風險之影響	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		

		其他	0		
	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	1	人次	聘用大學生一名擔任part-time研究助理
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表　□未發表之文稿　■撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   近年來科學家發現，Keratin(角質素)基因K8/K18之多型性與藥物性肝炎，包括抗結核藥物INH引起者，有關，而且角質素可改變粒線體的形狀與功能。同時亦有研究發現，血液中粒線體ND6 基因之mRNA表現量低之大鼠，較易發生CCl4引起之肝炎。但並無人報告上述各種現象是否與性別有關。因此主持人假設，女性發生抗結核藥物性肝炎之風險較高，可能與角質素基因K8/K18之多型性有關(因角質素可影響粒線體之功能)，且可能與血液中粒線體ND6基因之mRNA表現量有關。且這些K8/K18 SNP之genotype之分布，或ND6之mRNA表現量較低之百分比，在男性與女性不同，使女性發生抗結核藥物性肝炎之風險較高。

   本計畫在一年期間，以real-time QPCR 分析20位結核病病人白血球K8/K18之多型性，發現無論是有或沒有發生抗結核藥物性肝炎，其白血球K8/K18之基因都是wild type，並沒有多型性。此與過去文獻上報告，亞洲人K8/K18基因多為wild type一致，表示我國結核病病人發生抗結核藥物性肝炎之風險可能與K8/K18之多型性關係不大。

4. 主要發現

本研究具有政策應用參考價值：□否 ■是，建議提供機關科技部,

（勾選「是」者，請列舉建議可提供施政參考之業務主管機關）

本研究具影響公共利益之重大發現：■否 □是

說明：（以150字為限）