出國報告（出國類別：國際會議）

參加第 27 屆肌萎縮性側索硬化症暨運動神經元疾病國際研討會

(27nd International symposium on ALS/MND) 心得報告

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摘要

感謝科技部的補助及台北榮總的支持，讓我有機會參加 2016 年 12 月 7 日至 12 月 9 日在愛爾蘭都柏林由 Motor Neuron Disease Association 及 International Alliance of ALS/MND 所舉辦的第二十七屆肌萎縮性側索硬化症及運動神經元疾病國際學術研討會(27th International Symposium on ALS/MND)。 此會議秉持了過去高品質的傳統，除了開幕及閉幕的 joint sessions 外，在 3 天內同步分別進行 7 個 Scientific Sessions 及 11 個 Clinical Sessions。 我所參加的 Scientific Sessions 內容豐富包含了 ALS/MND 主題各領域中近一年的新發現，其主題包括了 RNA Processing and Dysregulation、RNA and Neurodegeneration、Protein Misfolding and Aggregation、Therapeutic Strategies、Cell biology and Pathology、Epigenetics and Genomics、以及 Clinical Genetics。 而我同時也以學術壁報的形式發表了我們族群 ALS 病患的臨床突變基因的研究結果, 主題為 Mutational analysis of *TBK1* in ALS patients in Taiwan, 由於 *TBK1* 基因是於去年才被發現會造成 ALS，我們的研究在此相關主題研究中算是較早完成的，因而引起部分與會者的興趣。 整體而言，本次會議內容新穎豐富，有許多地方值得國內神經學界參考。

關鍵字: 肌萎縮性側索硬化症、運動神經元疾病

目次

一、 目的

肌萎縮性側索硬化症(amyotrophic lateral sclerosis; ALS)是因腦部及脊髓中運動神經元細胞漸進性退化而發生的疾病，進而導致病患表現出全身肌肉漸進性萎縮無力。 這樣的病症可以從肢體開始，也可以從口咽肌肉開始，但病患最後都會因肌肉無力而導致吞嚥咳痰及呼吸困難，而無法維持生存。 除了少部分病患會併發額顳型失智症(frontotemporal dementia; FTD)外，九成病患的智能及感覺能力都是完好的；諷刺的是，這些功能的完好常造成病患更大的痛苦。 ALS 疾病病程相當快，發病後平均餘命常為兩至三年，約五分之一的病患可以生存過 5 年，而僅有約十分之一的病患可活超過 10 年，目前無有效藥物。 因為此病的特別悲劇性，且曾發生在一些名人身上，如著名美國大聯盟棒球選手 Lou Gehrig 及史蒂芬霍金，歐美及日本對此疾病的研究如火如荼，遠遠超過一般與其發生率(約每十萬人中 1-3 人) 相當的罕見疾病。

我大概從六年前起開始對 ALS/MND (MND：運動神經元疾病) 的基因研究開始感到興趣。 我個人本身最早的研究興趣是遺傳性運動感覺神經病變(Charcot-Marie-Tooth disease; CMT)的基因研究，而 CMT 中有一類罕見的亞型為遺傳性運動神經病變(Hereditary Motor Neuropathy; HMN)。 這類疾病的病患只有運動神經病變而感覺神經是正常的，臨床表現也僅有肌肉萎縮無力的症狀，與 MND 相似，但症狀較輕。 因此關聯，我同時開始對 ALS/MND 的基因研究感到興趣，對此主題相關訊息也開始密切注意。

ALS 的研究在近年來有連續重大突破，每年都有重要的致病基因被發現，如 *TARDBP*（2008）、*FUS* (2009)、*C9ORF72* (2011 年底)、和 *TBK1*(2015)等基

因，進而幫助深入瞭解 ALS 的細胞分子致病機制，同時也提供家族性 ALS 病患切確的分子診斷。 我們在近幾年內，也跟隨國際的腳步對我們族群內 200 餘位不具有親屬關聯的 ALS 病友進行 *SOD1, TARDBP, FUS, OPTN, VCP, UBQLN2, SQSTM1, PFN1, HNRNPA1, HNRNPA2B1, MATR3*, 和 *TBK1* 等基因的突變分析；其中，30 餘個家族性 ALS 家系中百分之七十七的家系及 176 位散發性 ALS 病人中百分之十的病人的致病突變都能被明確地找出來。 由於我們這一系列的研究在世界各族群同領域的研究中算是較早期完成的，因而能在 2011-2016 連續發表五篇研究論文於神經醫學及老年醫學領域中名聲頗佳的 Neurobiology Aging 雜誌上。

由於深刻感受到近年來國際間 ALS 相關研究的迅速進展，及我對 ALS 現今的各項基礎研究瞭解不足，並計畫未來投注更多時間在此一研究領域，因此參加此國際研討會。 我非常慶幸有參加此次會議，聽到許多令人非常欽佩的研究論文由其作者報告他們的研究細節內容，真是感到非常享受。

二、 過程

感謝科技部的補助及台北榮總的支持，讓我有機會參加 2016 年 12 月 7 日至 12 月 9 日在愛爾蘭都柏林由 Motor Neuron Disease Association 及 International Alliance of ALS/MND 所舉辦的第二十七屆肌萎縮性側索硬化症及運動神經元疾病國際學術研討會(27th International Symposium on ALS/MND)。此次會議是由愛爾蘭 ALS 病友會及國際 ALS 病友會聯盟所主辦，在研討會的前兩三天是國際 ALS 病友大會，緊接的才是與臨床及基礎研究相關的 ALS/MND 國際研討會。歐美的 ALS 病友會都非常活躍，且有不錯募款能力，不僅能主辦大型會議還常能設立獎項並提供研究經費，鼓勵各項 ALS 研究。 此會議秉持了過去高品質的傳統，除了開幕及閉幕的 joint sessions 外，在 3 天內同步分別進行 7 個 Scientific Sessions 及 11 個 Clinical Sessions。 我所參加的 Scientific Sessions 內容豐富包含了 ALS/MND 主題各領域中近一年的新發現，其主題包括了 RNA Processing and Dysregulation、RNA and Neurodegeneration、Protein Misfolding and Aggregation、Therapeutic Strategies、Cell biology and Pathology、Epigenetics and Genomics、以及 Clinical Genetics。其中，我個人認為特別精彩的演講如下：

1. Insights into the ALS/MND exposome 由荷蘭 Utrecht University 的 Dr. Roel Vermeulen 主講。
2. Untangling the ALS-X-Files 由美國 Duke ALS Clinic 的 Dr. Richard Bedlack 主講。
3. A precise medicine approach to ALS: What will it take? 由義大利 University of Torino 的 Dr. Adriano Chio 主講。

而我同時也以學術壁報的形式發表了我們族群 ALS 病患的臨床突變基因的研究

結果, 主題為 Mutational analysis of *TBK1* in ALS patients in Taiwan, 由於 *TBK1* 基因是於去年才被發現會造成 ALS，我們的研究在此相關主題研究中算是較早完成的，因而引起部分與會者的興趣。 這篇論文我們在投稿給此 ALS/MND 學術研討會的同時，就已經被 Neurobiology Aging 雜誌所接受刊登。 整體而言，本次會議內容新穎豐富，有許多地方值得國內神經學界參考。

三、 心得

在這次的會議中及我的反思之下，我獲得了兩個關於ALS前所未有的新觀念。 第一個是環境致病因素的重新強調。 從2008年迄今ALS臨床基因研究的蓬勃發展，發現了超過30餘種ALS的致病基因，但實際在臨床上所遇到的家族性遺傳的病例僅約十分之一，而帶有相同基因突變的病人也常有不同的發病年齡與疾病進展速度。 顯而易見地，非遺傳因素必定在此疾病中扮演相當重要角色。 而在ALS疾病中，先前關於環境因素的研究往往是僅針對於單一或數個環境因子，而未見到對ALS發生或病程有大影響力的環境因子，這有可能是ALS的發病是與多個環境因子的協同作用相關，或是根本尚未找到相關的重要環境因子。 而在實際的執行層面上而言，環境因子的深入研究是非常的困難。 傳統上常使用的就是問卷與訪談，所能得知的訊息有相當的侷限性。 伴隨著科技的發展，有兩個新的相關研究面向可以著力： 一是內在環境因子指標，可以藉者偵測體內所有代謝物質的變化 (Metabolomics)、所有基因受到甲基化等修飾情形 (Epigenomics)、及體內如重金屬等特殊物質的含量作為環境曝露後的結果代表。另一是大數據與穿戴式裝置的應用，藉由這兩個領域的進步可以收集分析龐大的個體行為與生理數據作為後續研究之用。 當環境因素被仔細地檢視之下，可以想見的其內容必為龐大而複雜，因此如同基因體(genome)的觀念，對於所有環境曝露的總體訊息則稱為exposome，而可以想像的，在不久的未來將有各種疾病的exposome wide association study (EWAS)，甚至同時考量全基因與環境曝露的Genome-Exposome wide association study。

第二個我所獲得的新觀念是避免先入為主的思考模式。 我一直先入為主地認為所有的另類療法 (Alternative medicine)對ALS是沒有療效的，對於這一部分的

相關訊息完全不在我之前的思考對象之內。 Duke ALS Clinic的Dr.Richard Bedlack有完全不同的想法，他一開始認為ALS的另類療法的存在對ALS病友與家屬有深刻的影響。 無論這些療法的效果，它們也可能有副作用，價格不合理地昂貴，同時影響正統ALS臨床試驗的收案，但是往往病人沒有正確的管道獲得這些療法的科學性評估。 因此他組織了一近百人的研究團隊並成立了一個Twitter頁面調查ALS病友們最感興趣的ALS另類療法，而後以熱門度為優先順序逐一調查評估這些另類療法，所得到的評估報告以open access的方式發表於ALS的專門學術期刊。 這些評估報告很快地就成為此期刊中下載率最高的文章們。 有趣的是，在這過程中，他們發現了23個病例有明確的醫療紀錄證實時當初是ALS但在接受不一的另類療法後疾病停滯並且進步。 而在這經驗中，他們開始研究這些病人的特性，同時也開始對其中之一 Lunasin，一種黃豆胜肽開始進行臨床試驗。 雖然Dr. Bedlack的研究還在進行中，最後的成果如何還很難說，我還是很贊同他的思維邏輯。 對於已存在的現象應該用多種角度來思考它的影響及背後所代表的意涵，而應該避免先入為主的觀念。 由於醫學有其應用性，對於臨床醫學上的難題，也許不應該執著於要先完全瞭解機制再針對機制設計研發治療方法，實證上有效後再研究背後原理也是很好的方式。

四、 建議事項（包括改進作法）

在這次第 27 屆肌萎縮性側索硬化症暨運動神經元疾病國際研討會中，台灣的參與者只有北投健康管理醫院的蔡清標院長與我，而鄰近的中國有超過二十位以上的與會者，這對我們來說是一個警訊。 建議政府應擴大鼓勵獎助醫師出國參加學術會議，增進專業涵養。 雖然政府及民間媒體常直強調台灣的醫療水準是非常傑出優越，身為臨床醫療專業的我們應該深知臨床醫學也如逆水行舟，不隨時努力進步就必定無法繼續保持卓越。

附錄（所發表的學術海報）

Mutational analysis of *TBK1* in ALS patients in Taiwan

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Background

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder that primarily affects upper and lower motor neurons. Approximately 10% of ALS cases are familial and more than 40 ALS disease genes have been identified, but only a few of them, including *SOD1*, *FUS*, *TARDBP* and *C9ORF72*, account for a significant number of ALS cases (Peters et al., 2015). The contribution of mutations in some newly identified genes, such as *TBK1* (Cirulli et al., 2015; Freischmidt et al., 2015), to ALS remains unclear because studies about them in various populations are still sparse.

The *TBK1* gene encodes the TANK-binding kinase 1 (TBK1) protein, which is able to phosphorylate multiple substrates, including optineurin (OPTN) (Wild et al., 2010), P62 (SQSTM1) (Pilli et al., 2012) and interferon regulatory factor 3 (IRF3) (Fitzgerald et al., 2003), and plays important roles in regulation of autophagy and inflammation (Lee et al., 2009; Xie et al., 2012). Recently, loss-of-function mutations in *TBK1* were identified in sporadic ALS (SALS) and familial ALS (FALS) in two large ALS genetic studies (Cirulli et al., 2015; Freischmidt et al., 2015). The defects in adaptor protein binding and kinase activity of the TBK1 proteins with loss-of-function mutations were also demonstrated by *in vitro* study (Freischmidt et al., 2015). Since TBK1 binds and phosphorylates OPTN and P62, which can in turn enhance autophagy and inflammation, the identification of *TBK1* as an ALS gene also highlights the role of OPTN, P62, autophagy and neuroinflammation in ALS pathogenesis.

To further understand the role of *TBK1* mutations in ALS in Han Chinese populations, we screened a cohort of 207 patients with ALS in Taiwan. Functional effects of the mutant gene products were also evaluated by *in vitro* studies.

Methods

Mutational Analyses

Mutational analyses of the *TBK1* gene were carried out by direct nucleotide sequencing in a cohort of 207 unrelated ALS patients, of whom 32 patients had a positive family history and 168 had unknown genetic cause after mutations in *SOD1*, *C9ORF72*, *TARDBP*, *FUS*, *ATXN2*, *OPTN*, *VCP*, *UBQLN2*, *SQSTM1*, *PFN1*, *HNRNPA1*, *HNRNPA2B1*, *MATR3*, *CHCHD10* and *TUBA4A* had been excluded.

***In vitro* functional study**

The coding region of *TBK1* was cloned into pFLAG-CMV-5a (Sigma-Aldrich) and the *TBK1* missense variants, c.127A>G (p.I43V) and c.881G>A (p.G294D), were introduced into the wild-type (WT) expression plasmids using QuikChange Site-Directed Mutagenesis method (Stratagene). The expression vector for the FLAG-TBK1 R444X mutant was generated by amplifying and cloning the first 1329 bps of the WT *TBK1* coding sequence into the pFLAG-CMV-5a vector. The human IRF3 and OPTN expression constructs (pCMV-SPORT6-IRF3; pCMV-SPORT6-OPTN) were purchased from transOMIC.

HEK293T cells were used in the functional study and transient transfection was performed using Lipofectamine 2000 (Invitrogen). The relative abundance of the WT and mutant TBK1 proteins was determined by Western blotting. HEK293T cells were also co-transfected with both TBK1 and IRF3 expression constructs for evaluating IRF3 phosphorylation. HEK293T cells were co-transfected with both TBK1 and OPTN expression plasmids to do the coimmunoprecipitation (CoIP) analysis.

Results

Identification of the novel TBK1 variants

Mutational analyses of *TBK1* in the 207 patients with ALS revealed two missense variants and one nonsense variant, including p.I43V (c.127A>G), p.G294D (c.881G>A) and p.R444X (c.1330C>T) (Fig. 1). All three variants were identified in apparently sporadic cases and were not found in the 500 ethnically matched healthy controls. Despite of its rarity, *TBK1* p.I43V was likely a benign polymorphism because of its conservative amino acid exchange of isoleucine for valine, its occurrence at an evolutionary non-conserved residue and being predicted benign by Polyphen-2 and SNAP2. The other two variants, p.G294D and p.R444X, putatively result in a non-conservative amino acid substitution and truncation, respectively, of the TBK1 protein. These two variants are predicted to be disease-causing mutations by the CADD and Mutation Taster programs. Polyphen-2 and SNAP2 also predict *TBK1* p.G294D to be pathogenic and the 294th amino acid residue of the human TBK1 protein is evolutionarily conserved.

In vitro functional studies

To verify pathogenicity of the three novel *TBK1* variants, *in vitro* cell-based functional studies were performed. We first examined whether these variants would affect TBK1 expression by cell transfection studies and western blotting, which revealed that the R444X mutant TBK1 had a significantly smaller protein size and a reduced steady-state protein level, whereas both G294D TBK1 and I43V TBK1 had a similar size and levels compared with those of wild-type TBK1 (Fig. 2A). Human IRF3 is a TBK1 substrate. We analyzed the kinase activity of the TBK1 variants by assessing IRF3 phosphorylation and found that HEK293T cells expressing wild-type TBK1, G294D TBK1, or I43V TBK1 had similar amounts of phosphorylated IRF3, whereas cells expressing R444X TBK1 had no phosphorylated IRF3 (Fig. 2B). These findings suggested an impaired kinase function of R444X TBK1 and preserved kinase function of G294D TBK1 and I43V TBK1.

OPTN binds the C-terminus of TBK1 as its adaptor protein (Freischmidt et al., 2015). We next investigated this interaction between the TBK1 variants and OPTN by performing co-immunoprecipitation (Co-IP) of Flag–tagged wild-type TBK1 or the TBK1 variants with OPTN in HEK293T cells. We found an absent interaction by the R444X mutation but not by the G294D or I43V variants (Fig. 2C).

Clinical information of the TBK1 p.R444X mutation

By genetic analyses and *in vitro* functional verification, only *TBK1* p.R444X could be confirmed as a disease-causing mutation. The *TBK1* p.R444X mutation was identified in one single patient, so the frequency of *TBK1* mutation in our ALS population is approximately 0.5% (1/207). Patient H171, harboring the *TBK1* p.R444X mutation, initially presented with deteriorating working abilities, rough attitude, aggressive behaviors and intrinsic hand muscle atrophy and weakness at age 55. Then, she developed progressive proximal muscle weakness and atrophy, prominent disinhibition and more aggressive behavior. The diagnosis of ALS-frontotemporal dementia (FTD) was made at age 57. The Tc-99m ECD brain perfusion SPECT at age 57 showed a prominent frontotemporal lobe hypoperfusion. She became wheelchair-bound with dysarthria and dysphagia at age 58 and died of an out-of-hospital cardiac arrest at age 59.

Discussion

We screened a Taiwanese cohort of 207 unrelated patients with ALS for mutations in the *TBK1* gene and identified a novel nonsense mutation, p.R444X, in one patient with apparently sporadic ALS-FTD. Although no segregation analysis was possible because the p.R444X mutation was found in only one single case, its pathogenicity is supported by the following points. First, the mutation is absent in 1,000 ethnically matched control chromosomes and present with an extremely rare allele frequency (1/ 120,418) in the ExAC database. Second, the p.R444X mutation was predicted to be pathogenic by the CADD and Mutation Taster programs. Third, *in vitro* functional study demonstrated that the p.R444X mutation resulted in a truncated TBK1 protein product, low protein expression, and loss of the kinase function and binding ability to OPTN. TBK1 functions as a homodimer, and the TBK1 R444X mutant protein loses approximately half of its amino acid residues, including its C-terminal adaptor-binding motif and most of its scaffold dimerization domain. Therefore, the p.R444X mutation may impair formation of TBK1 homodimers and lead to its functional loss.

Mutations in *TBK1* may be associated with a wide clinical spectrum of diseases. In the study by Freischmidt et al., patients with *TBK1* mutations may present with typical ALS, lower motor neuron syndrome, progressive bulbar palsy, ALS-FTD, or FTD, and 50% of the ALS patients with *TBK1* mutations presented cognitive impairment, usually progressing to fulminant FTD in the later disease stage. Our patient with the *TBK1* p.R444X mutation also manifested both ALS and FTD. These findings support the role of *TBK1* mutations in ALS-FTD spectrum disorders and add *TBK1*, in addition to *C9ORF72*, *TARDBP*, *FUS*, and *VCP*, to the list of genes involved in both ALS and FTD.

In conclusion, this study identified a novel *TBK1* mutation, p.R444X, in one out of 207 unrelated Han Chinese patients with ALS in Taiwan and stresses on the importance to consider *TBK1* mutation as a possible cause of ALS in patients with unknown genetic causes.

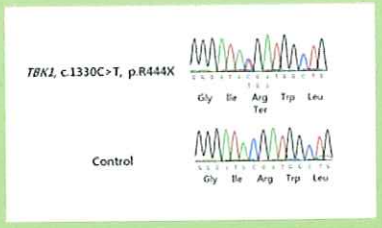

Fig. 1. The novel *TBK1* mutation, p.R444X (c.1330C>T), identified in this study.

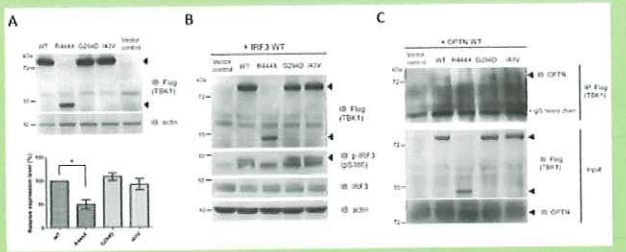

Fig. 2. *In vitro* functional analysis of the TBK1 variants. (A) Representative immunoblots (IB) and associated densitometric analyses for TBK1 levels in HEK293T cells expressing WT, R444X, G294D, I43V TBK1, or empty vector (vector control) were shown. The asterisk indicates $p < 0.05$ ($n = 5$). (B) Cell lysates from HEK293T cells co-transfected with IRF3 expressing plasmids and the indicated Flag-tagged TBK1 constructs were immunoblotted to examine the kinase activities by observing phosphorylated IRF3 with a phosphor-specific pSer386 antibody. Arrowhead in the second panel indicates the phosphorylated IRF3. (C) Interaction between TBK1 and its adaptor protein optineurin (OPTN). HEK293T cells were co-transfected with OPTN expressing plasmids and indicated Flag-tagged TBK1 constructs. Cell lysates were co-immunoprecipitated using anti-Flag antibody. Arrowheads point to the signals corresponding to OPTN in the 1st and 3rd panel or Flag-tagged TBK1 in the 2nd panel.

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Conflict of interest statement

The authors have no conflicts of interest to disclose.

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