行政院國家科學委員會補助專題研究計畫成果報告

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※　**細胞骨骼蛋白與擴張性心肌病變 (Cytoskeleton and Heart Failure)**　※

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計畫類別：個別型計畫

計畫編號：NSC 89－2314－B－002－190－

執行期間： 88 年 08 月 1 日 至 89 年 07 月 31 日

計畫主持人：朱樹勳

執行單位：台灣大學醫學院附設醫院心臟外科

中 華 民 國 89 年 10 月 31 日

行政院國家科學委員會專題研究計畫成果報告

計畫編號：NSC 89-2314-B-002-190

細胞骨骼蛋白與擴張性心肌病變 (Cytoskeleton and Heart Failure)

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壹、中文摘要

心臟血管疾病的病因皆相當複雜，用傳統的生化與遺傳的方法研究，通常是事倍功半。我們嘗試用蛋白基因體分析的方式來研究擴張性心肌病變(dilated cardiomyopathy; DCM) 作研究，以鑑別出與 DCM 相關的蛋白質與蛋白質修飾基。我們使用差別性萃取法(Differential extraction)純化心肌纖維蛋白後，使用二維膠質電泳(Two-dimensional gel electrophoresis)分離這些蛋白質，再用質譜儀作心肌纖維蛋白質的鑑定與修飾基的研究。舉 mlc-2 蛋白為例，我們發現一病人之 Ser-14 有 3.7% 被磷酸化，另外兩病人則都有近 17%的磷酸化。此項差異在臨床上之重要性，則尚待進一步的探討，尤其樣本數必須增加，以利統計學之比較分析。

關鍵詞：擴張性心肌病變、蛋白質基因組分析、質譜儀、二維膠質電泳

ABSTRACT

Cardiovascular diseases, like dilated cardiomyopathy (DCM), are usually very complex and the elucidation of pathogenic mechanisms is very difficult. Dilated cardiomyopathy (DCM) is a severe heart disease leading to heart insufficiency and most heart transplantations are indicated by DCM. We employed proteomics methods to analyze myocardial proteins derived from DCM myocardium, particularly myofibril proteins. We successfully isolated myofibril proteins using differential extraction method and then resolved them on a 2D gel electrophoresis system. The identities of proteins are verified using liquid chromatography-tandem mass spectrometry and specific modifications on these proteins can also be identified. Using myosin regulatory light chain 2 (mlc-2) as an example, we demonstrated that how modification is mapped and quantitatively determined using selected ion tracing approach. One patient in our reseach has 3.7% phosphorylation at Ser-14, while the other two both have a phosphorylation percentage of ~16%. The significance of this difference will be validated as more samples are analyzed.

Key word: dilated cardiomyopathy, proteomics, tandem mass spectrometry, two-dimensional gel electrophoresis

貳、源由與目的

Cardiovascular diseases, like dilated cardiomyopathy (DCM), are usually very complex and the elucidation of pathogenic mechanisms is very difficult. Dilated cardiomyopathy (DCM) is a severe heart disease leading to heart insufficiency and most heart transplantations are indicated by DCM. We proposed to employ proteomics methods to analyze cytoskeletal proteins derived from DCM myocardium, particularly the myofibril proteins. We aimed to identify the proteins that are differentially expressed in and to identify the protein modifications that are increased or decreased in DCM myocardium.

As the major acting macromolecules in cells, the proteome will be eventually altered by almost all, if not all, of the pathological processes. Elucidation of these alterations will greatly enhance our ability to understand how cellular functions are impaired and how these impairments can be fixed to restore cellular function. The study of the proteomic change can also help us to recognize what process causes the cellular changes and how a pathologic process is evolved.

The major advantage of proteomics over genomics is in the area of post-translational modifications. It is now clear that the activity of a protein often depends on its modification state. While the expression of a gene may be the same at different situations, if its modification status is different, the activity of a protein can be thus tuned on or off. Therefore, the study of protein modifications through a proteomics approach is a crucial and integral part to progress our understanding of living organisms.

參、結果與討論

Myofibril proteins are the major components of myocardial cytoskeleton. In order to explore their change in dilated cardiomyopathy, we employed a proteomics approach to monitor their expression as well as post-translational modifications.

First, we isolated the myofibril proteins by first extracting the non-myofibril proteins using high salt solutions. The insoluble portion is then solubilized and homogenized in the lysis buffer containing 0.1% of sodium dodecyl sulfate (SDS). Myofibril proteins in this

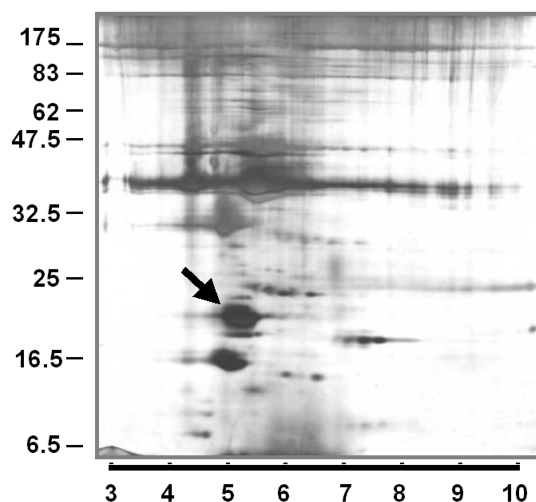


Figure 1. Two-dimensional electrophoresis of myofibril proteins. Two hundred fifty micrograms of purified myofibril proteins were loaded to IPG IEF 3-10 gel, and were resolved overnight for 19,000 V·hr. The gel was placed at the tope of a 11% polyacrylamide gel, which was run at 150 V until the dye front reached the bottom of the gel. The gel was developed using silver stain method. The arrow indicates the position of regulatory myosin light chain 2.

fraction were extracted using a methanol-chloroform method such that the detergents and salts were mostly removed. These myofibril proteins were then analyzed using two-dimensional electrophoresis, consisting of isoelectric focusing and SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Figure 1 shows a typical pattern of myofibril proteins in a 2D gel electrophoresis system. The identities of these proteins were verified using liquid chromatography-tandem mass spectrometry. The myofibril protein pattern was compared between donor and recipient hearts, but showing no distinct difference in terms of protein species and their relative abundance. In order to access whether the post-translational modifications were altered in DCM hearts, we employed a newly developed method to gauge the modification states.

APKKAKKRAG GANSNVFSMF EQTQIQEFKE AFTIMDQNRD
GFIDKNDLRD TFAALGRVNV KNEEIDEMIK EAPGPINFTV
FLTMFGEKLK GADPEETILN AFKVFDPEGK GVLKADYVRE
MLTTQAERFS KEEVDQMFAA FPPDVTGNLD YKNLVHIITH
GEEKD

Figure 2. The amino acid sequence of myosin regulatory light chain 2. The recovered peptide sequences were underlined with horizontal bars. The position of Ser-14 was highlighted. The coverage of the entire polypeptide is about 94% (155 out of 165 amino acids).

It has been reported that phosphorylation was incorporated at Ser-14 of cardiac myosin regulatory light chain 2 (mlc-2), therefore we tested our method on this particular protein. On the 2D gel, this protein has an isoelectric point of ~5.2 and an apparent molecular weight of 20 k. These are comparable to the corresponding parameters deduced from its amino acid sequence, which are 4.92 for the isoelectric point and 18,640 for the molecular mass. The gel spots containing the mlc-2 polypeptides were excised and subjected to in-gel trypsin digestion. The digestion product was then analyzed via liquid chromatography-tandem mass spectrometry.

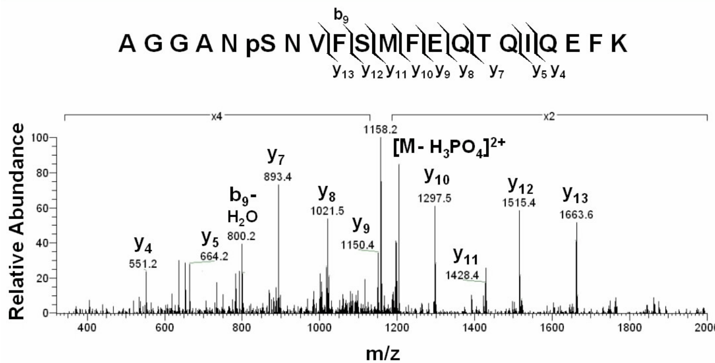
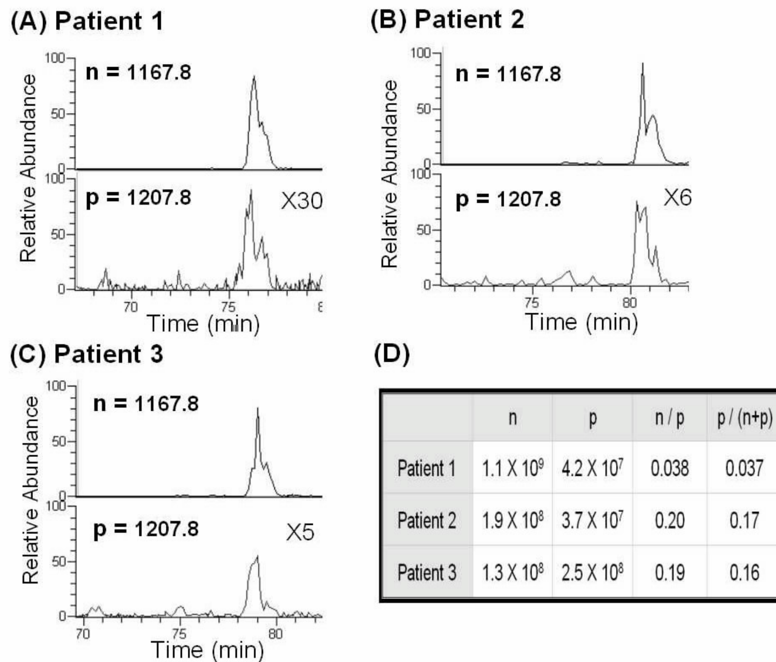


Figure 3. Tandem mass spectrum of Ser-14 phosphopeptide. The Ser-14 phosphopeptide was subjected to collision-induced dissociation experiment and the resulted fragment ions were resolved by a LCQ ion trap mass spectrometer.

Figure 2 shows the identification of peptides derived from mlc-2 proteins. More than 90% of the amino acid sequence can be recovered, showing the good throughput of our method. Mostly importantly, all the phosphorylatable amino acids can be verified. Selected ion tracing method was used to identify potential phosphopeptides, which revealed only a phosphorylated peptide. Tandem mass spectrometry was then used to map the phosphorylation site.

In the tandem mass spectrum of this phosphopeptide, a predominant fragment ion of *m/z* 1158.2 was seen, which represented the parent ion with a loss of a charge-less phosphoric acid molecule. This signature feature indicates that this peptide is indeed a phosphopeptide. As y_4 to y_{13} ions have no mass shift in the spectrum, this excludes the possibility that the phosphate group is on either Ser-18 or Thr-23. Since Ser-14 is the only phosphorylatable residues besides these two residues, this result strongly indicates that Ser-14 is the phosphorylation site.



	n	p	n / p	p / (n+p)
Patient 1	1.1×10^9	4.2×10^7	0.038	0.037
Patient 2	1.9×10^8	3.7×10^7	0.20	0.17
Patient 3	1.3×10^8	2.5×10^8	0.19	0.16

Figure 4. Determination of phosphorylation states at Ser-14. The mlc-2 protein collected from the recipient hearts was analyzed for the phosphorylation state. In panels A, B and C, the upper tracing indicates the elution of unmodified species and the lower chromatograph reveals the presence of the singly phosphorylated peptide. The area under each peak was integrated and tabulated in panel D. Both n/p and p/(n+p) ratios are calculated and presented.

At last, we determined the Ser-14 phosphorylation states on mlc-2 protein derived from different patients. As shown in Figure 4, mlc-2 proteins carried a 3.7% Ser-14 phosphorylation for patient 1, while 16~17% of Ser-15 was phosphorylated in myocardium of patients 2 and 3. These results indicate that it is feasible to monitor phosphorylation state using our method

肆、計畫成果自評

In this report, we showed that myofibril proteins could be isolated and resolved using a 2D gel system. Liquid chromatography-tandem mass spectrometry is an appropriate method to identify the proteins in the gel. If we are interested in the modification state of a particular DCM-associated protein, it can be determined using this newly developed LC/MS method. Currently, we have no definite conclusion on what modification is related to DCM. This will await the collect samples from more patients.

Nevertheless, we will use this approach to do more detailed analysis on modification states of cardiac myofibrils at different disease conditions. Most of cardiovascular diseases are very complex and this might be one of the better strategies to solve this kind of biomedical problems. However, we also understand that it is more suitable for experiments using a larger sample number. Currently, several possibilities are being evaluated.