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Differential Phosphoproteomics Analysis to Identify Proteins and Pathways of Human Lung Epithelial Cells During Exposure to *Burkholderia Pseudomallei*

การวิเคราะห์ความต่างของฟอสโฟโปรตีโอมิกส์เพื่อระบุโปรตีนและวิถีของเซลล์เยื่อบุผิวปอดของคนระหว่างการสัมผัสกับ *Burkholderia Pseudomallei*

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ABSTRACT

Burkholderia psudomalliei (Bp) is the causative agent of melioidosis, an infectious disease of humans and animals. At present there is no available human vaccine that protects against Bp, and with the current limitations of antibiotic treatment, the development of new preventative and therapeutic interventions is crucial. This study elucidated human epithelial host cell responses immediatly after exposure to Bp. Forty-five phosphoproteins of human lung epithelial cells were activated after exposure to Bp. Seven cellular pathway were found to be associated with 8 of those activated phosphoproteins. The results suggested the response of the host cell including cell proliferation, gene expression, differentiation, mitosis, cell survival, energy production and apoptosis. These results suggested the rapid response of the host cell and the possible importance of phosphoproteins in host responses to pathogen. Moreover, those phosphoproteins can be fulfill basic knowledge to insight host response to control Bp infection.

บทคัดย่อ

Burkholderia psudomalliei (Bp) เป็นสาเหตุของโรคเมลิออยโดสิสซึ่งเป็นโรคติดเชื้อของมนุษย์และสัตว์ ปัจจุบันยังไม่มีวัคซีนที่สามารถป้องกันโรคนี้และการรักษาด้วยยาปฏิชีวนะก็มีข้อจำกัด การพัฒนาการการป้องกันและการรักษาจึงเป็นเรื่องสำคัญและจำเป็น การศึกษานี้รายงานฟอสโฟโปรตีนที่สะท้อนการตอบสนองทันทีของเซลล์เยื่อบุผิวปอดของคนหลังจากสัมผัสกับเชื้อ Bp โดยพบว่ามีฟอสโฟโปรตีนจำนวน 45 ชนิดที่มีการแสดงออกหลังจากเซลล์สัมผัสกับเชื้อ Bp จากการวิเคราะห์พบว่าทมีฟอสโฟโปรตีน 8 ชนิดเกี่ยวข้องกับกระบวนการส่งต่อสัญญาณภายในเซลล์จำนวน 7 กระบวนการซึ่งเกี่ยวข้องกับการตอบสนองของเซลล์เจ้าบ้านหลายกระบวนการ ได้แก่ การเพิ่มจำนวนของเซลล์ การควบคุมการแสดงออกของยีน การแบ่งตัวเพิ่มจำนวนแบบ mitosis การดำรงชีพของเซลล์ การผลิตพลังงานและการตายของเซลล์ในแบบ apoptosis ผลการศึกษานี้ชี้ให้เห็นถึงการตอบสนองที่รวดเร็วของเซลล์เจ้าบ้านความสำคัญของฟอสโฟโปรตีนในการตอบสนองของเซลล์ต่อเชื้อโรค นอกจากนี้การศึกษาฟอสโฟโปรตีนเหล่านี้สามารถจะนำไปสู่ความรู้พื้นฐานเพื่อให้เข้าใจการตอบสนองของเซลล์มนุษย์เพื่อควบคุมการติดเชื้อ Bp

Keywords: *Burkholderia psudomalliei,* Bp1026b, A549, Phosphoprotein , Human lung epithelial

คำสำคัญ: *Burkholderia psudomalliei,* Bp1026b, A549, ฟอสโฟโปรตีน, เซลล์เยื่อบุปอดมนุษย์

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Introduction

Burkholderia psudomalliei (Bp) is the causative agent of melioidosis, an infectious disease of humans and animals. Bp is a gram-negative facultative intracellular environmental bacterium that can be found inhabiting the soil and water in the endemic areas especially in Southeast Asia and Northern Australia [1]. At present there is no available human vaccine that protects against Bp, and with the current limitations of antibiotic treatment, the development of new preventative and therapeutic interventions is crucial. Development of new diagnosis methods and vaccines against Bp will benefit from a thorough understanding of the pathogenesis of infection and the characteristics of naturally occurring immune responses which develop following environmental or experimental exposure to the organism.

As a facultative intracellular bacterium, Bp has ability to survive and multiply in both phagocytic and non-phagocytic cells of mammalian cells. Various in vitro models of infection have to been developed to study the interactions between the host cells and Bp. Macrophages play important role to eliminate Bp from host body. Human lung epithelial cells are particularly susceptible to Bp infection following exposure by inhalation and acted as an important physical barrier from infection by produce and distribute of surfactant [2]. However, the mechanisms of host against Bp still need to be more characterized.

Protein phosphorylation mediate most of the signal transduction in eukaryotic cells by control many other cellular processes, modification the activity of substrates, including transcription, metabolism, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis, and differentiation. There is no previous report of host phosphoproteomics profile after exposure to Bp.

We aim to study of human host cells responses after exposure to Bp at early infection by identified human phosphoproteomics profile. The result from this study will gain new information of differential host responses to pathogen. This study will be the first to investigate the human phosphoproteomics after exposure to Bp.

Materials and Methods

Epithelial cell line preparation

A549 cell line will be used as epithelial cell. A549 cells will be cultured in RPMI-1640 supplemented with 10% FBS and incubated at 37^0C, 5% CO2 until 90% efficiency of cell growth. A549 cell will be collected by centrifugation at 1000 rpm for 5 minutes and then the supernatant will be removed. The cells will be re-suspended with new fresh cRPMI-1640 then counted by using hema-cytometer(by diluted 2 µl of cell suspension with 10 µl trypan blue) after that cell suspension will be diluted to 1.0 x 106 cells/ml with fresh cRPMI-1640. 10 ml of cell suspension will be transferred to cell culture dish and incubated at 37^0C, 5% CO2 for 24 hours to allowed cell adhesion to become monolayer epithelial cell line in the dish.

Bp1026b culture

Single colony of Bp1026b will be grown overnight in LB broth 3 ml at 37^0C with shaker incubator at 200 rpm. The bacterial suspension will be transferred to new LB broth 20 ml with 2% inoculums and then the tube will

be incubated at 37^0C with shaker incubator at 200 rpm to grown Bp to log phase. Bacterial cells will be collected by centrifugation 2,500 rpm for 10 minutes, then the supernatant will be removed and the bacterial cells will be re-suspended with new fresh cRPMI-1640. Then the Bp suspension will be adjusted with 0.5 Mcfarland standards and diluted into 1.0 x 107 cells/ml for 10 ml.

Bp1026b infection assay

Old media of A549 cell monolayer cell line (epithelial model) in cell culture dish will be removed the washed 2 times with RPMI-1640. The cell line will be infected with Bp1026b at MOI=10. The co-culture dish will be incubated at 37^0C, 5% CO2. The cells will be lysed and total protein will be collected at 0 s. Uninfect A549 cell will be used as control.

Phosphoprotein collection: Cell Lysis

The methods following Pierce® Phosphoprotein Enrichment Kit. Briefly, the uninfected and infected cells will be wash cells with 50mM HEPES, pH 7.0 after that HEPES wash buffer will be removed. The uninfected and infected cells will be lysed by adding 3 mL of Lysis/Binding/Wash Buffer with CHAPS, 1X Halt Protease Inhibitor EDTA-free and 1X Halt Phosphatase Inhibitor Cocktail to each cell culture dish. The lysed cells will be scraped and transferred into collection tube and placed cells on ice for 45 minutes before vortexing periodically. Then centrifuge lysed cells at 10,000 × g for 20 minutes at 4°C to pellet cellular debris and the supernatant will be collected into the new tube.

Phosphopratein enrichment

The methods following Pierce® Phosphoprotein Enrichment Kit. Briefly, the protein concentration of the supernatant by performing Lowry protein assay. The proteins will be adjust the concentration to 0.5mg/mL diluted with Lysis/Binding/Wash Buffer then invert column to suspend the resin. The column will be placed into a new tube. To remove the storage solution, the column will be centrifuged at 1000 × g for 1 minute at 4°C and the flow-through will be discarded. To equilibrate resin, Lysis/Binding/Wash Buffer with CHAPS will be added into the column. The column will be placed into new tube after that centrifuged at 1000 × g for 1 minute at 4°C and discarded the flow-through. Bottom of column will be plugged and added diluted lysate. The cap will be screwed to the top of the column and then inverted several times to mix. The column will be placed on a shaker for 30 minutes at 4°C. The bottom plug will be removed and then placed the column to new tube after that the column will be centrifuged at 1000 × g for 1 minute at 4°C to wash unbinding protein. For the wash step the resin will be washed 5 times by adding Lysis/Binding/Wash Buffer with CHAPS. To elute the phosphoproteins from the resin, after wash steps the column will be placed into new tube and centrifuged at 1000 × g for 1 minute at 4°C. The bottom of column will be plugged and transferred to a new tube. The Elution Buffer (without optional CHAPS) will be added and incubated at room temperature with occasional agitation for 3 minutes. The bottom of column will be removed and centrifuged at 1000 × g for 1 minute at 4°C. Repeat elution steps four times for a total of five elution fractions.

In-gel digestion

The SDS-PAGE gel will be washed with DW (10 minutes x 2 times). Then the gel will be cut into 1 mm2 cubes and transfer to 96 well-plate. Gel-pieces will be washed with 200 µL of DW and shaken for 10 minutes and

then all liquid will be removed. Coomassie blue dye will be removed by adding 200 L of 25 mM NH_4HCO_3 and shaking for 10 minutes and then all liquids will be removed (perform in triplicate). The chemicals will be removed from gel-pieces by adding 200 uL of DW shake for 10 minutes and all liquid will be removed (perform in triplicate). Then, 200 L of ACN will be added and shaken for 10 minutes and all liquid will be removed (for dehydration) (perform in duplicate). The gel pieces will be dried for 10 minutes at room temperature inside the cabinet. Then, 50 uL of 10 mM DTT in 10 mM NH_4HCO_3 will be added and incubated at 56°C for 1 hour. Then, the 50 uL of 100 mM IAA in 10 mM NH_4HCO_3 will be added and all liquid will be removed after incubated for 1 hour at room temperature. Then, 200 JL of ACN will be added and all liquid will be triplication removed after shaking for 5 minutes. Tryptic digestion will be performed by adding of 10 JL of trypsin (10 ng/ul trypsin in 10mm NH_4HCO_3) and incubated at 37°C for 3 hours. Then, the peptides will be extracted by adding 50 pl of 50% ACN in 0.1% formic acid and shaking at room temperature for 10 minutes (perform in triplicate). Then, protein solution will be transferred into new low binding 96 well-plates and incubated at 40°C until the protein solution is dried. Then, the extracted protein will be storaged at -20 °C until the protein analysis will be performed.

LC-MS/MS

The adjustment of background intensity will be performed by 12 µl/well of 0.1% formic acid diluted by LC-Ms grade water. The protein samples will be re-suspended with 0.1% formic acid in LC-Ms grade water and mixed by auto-pipette for 100 times, then the samples will be transferred into the low binding tubes. The protein samples will be centrifuged at 10,000 rpm for 10 minutes and transferred into the LC-Ms/Ms injection vial. Then, the 4.5 µl of protein suspension will be injected to LC-MS/MS (SYNAPT G1 HDMS mass spectrometer, WATERS).

Nano scale LC separation of tryptic peptides will be performed with a Nano Acquity system equipped with a Symmetry C18 5 µm, 180-µm x 20-mm Trap column and a BEH130 C18 1.7 µm, 100-µm x 100-mm analytical reversed phase column. The samples will be initially transferred with an aqueous 0.1% formic acid solution to the trap column with a flow rate of 15 µl/minute for 1 minute. Mobile phase A will be added by water with 0.1% formic acid, and mobile phase B will be 0.1% formic acid in acetonitrile. The peptides will be separated with a gradient of 15–50% mobile phase B over 15 minutes at a flow rate of 600 nl/minute followed by a 3 minutes rinse with 80% of mobile phase B. The column temperature will be maintained at 35°C. The lock mass will be delivered from the auxiliary pump of the Nano Acquity pump with a constant flow rate of 500 nl/minute at a concentration of 200 fmol/ul of fibrinopeptide B to the reference sprayer of the Nano Lock Spray source of the mass spectrometer. All samples will be analyzed at once and the tryptic peptides analysis will be performed using a SYNAPTTM HDMS mass spectrometer (Waters Corp., Manchester, UK). For all measurements, the mass spectrometer will be operated in the V-mode of analysis with a resolution of at least 10,000 full-width half-maximum. All analyses will be performed using positive nanoelectrospray ion mode. The time-of-flight analyzer of the mass spectrometer will be externally calibrated with fibrinopeptide B from 50 to 1600 m/z with acquisition lock mass corrected using the monoisotopic mass of the doubly charged precursor of [Glul] fibrinopeptide B. The reference sprayer will be sampled with a frequency of 20 seconds. Accurate mass LCMS data will be acquired with data direct acquisition mode. The energy of trap will be setting at collision energy of 6 V. In transfer collision energy control,

low energy will be set at 4 volts. The quadrupole mass analyzer will be adjusted such that ions from m/z 300 to 1800 were efficiently transmitted. The MSMS survey was over the range 50 to 1990 Da and scan time was 0.5 second.

LC-MS/MS data analysis

The data from LC MS/MS analysis wall be analyzed by Decyder MS 20 differential analysis software (Amerssham biose ES, UK) and searched by mascot database (www.ncbi.nlm.nih.gov, National Center for Biotechnokenty Information, U.S. National Library of Medicine, USA). The parameters for analysis will be 0.1 unit mlz shift tolerance and 5.0% mlz shape tolerance. Group-to-group comparisons will be performed by Jvenn (http://jvenn.toulouse.inra.fr/app/ index.html) comparisons will be performed by the highest intensity of each phosphoproteins. The values will be normalized with BSA external intensity control. The p-values < 0.05 will be considered statistically significant. Pathway analysis was performed by using tools and database in http://www.pantherdb.org/

Results and Discussion

A total of 497 and 514 phosphoproteins were identified from unexposed A549 epithelial cell and the cell after immediately exposure to Bp, respectively. There were 45 unique phosphoproteins found only in the exposure to Bp condition. Whereas, 28 phosphoproteins were uniquely found only in the unexposed cell. There were 469 phaophoptoteins found in both conditions.

Among 45 unique phosphoptoteins, pathway analysis found 8 proteins associated to 7 pathways showed in table 1 including: 1) Integrin signalling pathway (P00034); it involves actin reorganization and activation of mitogen-activated protein kinase (MAPK) and other signalling cascades [3]. The MAPK regulates cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis. 2) Huntington disease (P00029); this pathway involves in vesicle endocytosis, regulation of calcium channels via the PSD-95 complex, and regulation of apoptosis via BDNF [4]. 3) p53 pathway feedback loops 2 (P04398); the p53 transcription factor is the key gatekeeper in the cellular response to stress signals that lead to one of three different types of cellular response: cell cycle arrest, cellular senescence or apoptosis [5]. 4) Co-factor and vitamin metabolism; the retinal dehydrogenase 1 binds free retinal and cellular retinol-binding protein- bound retinal. It can convert/oxidize retinaldehyde to retinoic acid. It belongs to the aldehyde dehydrogenase family. This protein is a part of vitamin biosynthesis. One of the importance role of the vitamin is determining apoptosis [6-7]. This leads to propose that the cell after exposure to BP might determine its apoptosis by this pathway too. 5) Glycolysis (P00024): A well-documented metabolic pathway, which involves a series of enzymatic reactions to convert one molecule of glucose to two molecules of pyruvate. The cell might start to collect energy molecules, ATP for further dealing with the pathogen [8]. 6) PDGF signaling pathway (P00047): Basically, platelet derived growth factor (PDGF) plays a critical role in cellular proliferation and development. The PDGF receptors belong to the family of receptor protein tyrosine kinases (RPTK) [9-10]. Upon ligand binding, the receptors chains trans-phosphorylate each other, resulting in

recruitment of adaptor molecules via SH2 domains and leading to phosphorylation of other cellular proteins. 7) Blood coagulation (P00011), the blood coagulation cascade and fibrinolytic pathway [11].

This is the first report of phosphoproteins that reflect the response of pulmonary epitheial cells after immediately exposure to Bp. The human cell response in at least 7 pathways which involved several activities including cell proliferation, gene expression, differentiation, mitosis, cell survival, energy production and apoptosis. These associated pathways involved by only 8 unique phoshoproteins. But totally 45 unique proteins were found but almost of them have not been studied the function. There should be several activities that the cells response to the immediately exposure to the pathogen.

These results suggested the rapid response of the host cell and the possible importance of phosphoproteins in host responses to pathogen. Moreover, those phosphoproteins can be fulfill basic knowledge to insight host response to control Bp infection.

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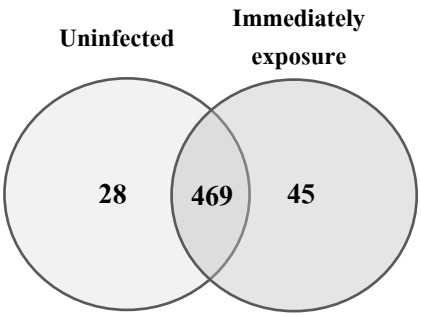

Figure 1 Venn diagram shows number of phosphoproteins found in uninfected control A549 epithelial cell and in the cells immediately exposure to *Burkholderia psudomalliei* .

Table 1 Associated cellular pathway of phosphoproteins uniquely found in A549 epithelial cell after immediately exposed to *Burkholderia psudomalliei*.

	Pathway	Proteins
1	Integrin signaling pathway (P00034)	Filamin
2	Huntington disease (P00029)	Synaptojanin
3	p53 pathway feedback loops 2 (P04398)	Rb
4	Co-factor and vitamin metabolism	Retinal Dehydrogenase 1
5	Glycolysis (P00024)	Triosephosphate isomerase
6	PDGF signaling pathway (P00047)	1. Ets 2. GTPase-activating protein Rho (Rho)
7	Blood coagulation (P00011)	Kininogen