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計畫名稱：以蛋白質體學技術來探討介貝類過敏可能之機制

計畫主持人：呂雅蕙 助理教授

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中文摘要

人類嗜鹼性白血球細胞對過敏和免疫調節作用在近年來逐漸受到重視。本研究利用免疫促進劑 (PMA 和 A23187) 去刺激人類嗜鹼性白血球細胞，觀察活化前後其細胞內所有蛋白質之變化，並採用二維電泳，配合介質輔助雷射脫附離子化四極棒式飛行時間分析儀 (MALDI-Q-TOF) 和蛋白質比對軟體，來進行差異性蛋白質體分析。實驗結果發現經 PMA 和 A23187 處理後，嗜鹼性白血球細胞會大量產生第二型 (Th2) 細胞激素如介白素 4, 5, 13 (IL-4, IL-5, Il-13)，使反應趨向於過敏方向，並同時鑑定到 2 個正向調節和 15 個負向調節蛋白質。進一步以蛋白質轉漬法分析異質核醣核蛋白 K (HNRPK) 和烯醇酶 (ENO1) 的產量，發現與二維電泳上蛋白質的表現量趨勢相吻合。這些蛋白質主要存在於細胞質、細胞核和細胞質膜。經生物資訊軟體分析比對後，這群蛋白質大部分和免疫、過敏失調和癌症形成有關。 **關鍵詞：** 人類嗜鹼性白血球細胞，蛋白質體學，免疫促進劑，第二型細胞激素

英文摘要

Basophil activation has been implicated in allergy reaction and immune modulation. Signaling transduction, mediator release and morphology change of basophils have been largely deciphered. This study examined the feasibility of using proteomic strategy to investigate the whole cell proteomic changes in KU812 basophils following activation with phorbol myristate acetate (PMA) plus ionophore (A23187). The two-dimensional electrophoresis (2D) coupled with matrix-assisted laser desorption/ionization-quadrupole/time-of-flight (MALDI-Q-TOF) was selected for studying comparative proteome differences; and cytokine mRNA expressions were monitored. The differentially displayed proteins were further validated by 1D- and 2D-Western blotting for protein expressions. IL-4, IL-5, and IL-13 mRNAs in KU812 were significantly induced; a phenomenon similar to the allergy reaction. The protein expressions of heterogeneous nuclear ribonucleoprotein K (HNRPK) and a-enolase (ENO1) were shown a good correlation according to the results from the 2D, and 2D-Western blots. Since the majority of the identified proteins were associated with immunological, inflammatory disorders and tumor genesis, this study in defining the proteomic changes in basophilic cells that occur in response to treatment with PMA plus A23187 can serve as biomarkers for characterization of *in vitro* immuno-stimulation.

Key words: basophil, proteomics, immuno-stimulants, Th2 cytokine

前言

Due to growing prevalence of allergic diseases worldwide, the exact molecular mechanisms of allergy reaction for prevention and/or treatment of allergy diseases are becoming increasingly important. Basophils and mast cells are major effector cells in allergic progression through their highly expressed FceRI on the cell surface[(1)]. These events trigger both preformed and newly synthetic chemical mediator secretions. Among them, IL-4, IL-5 and IL-13 are major cytokines for IgE production, facilitating Th2 differentiation and allergic responses[(2)]. Basophils could rapidly secrete large amount of IL-4 and IL-13, and constitutively express CD40L and CCR3 on the surface. Basophils have been detected in particular organs affected by allergic reactions, such as bronchial and airway biopsies from asthma patients, nasal lavage fluids from allergic rhinitis patients, as well as skin biopsies from atopic and contact dermatitis. Therefore, human basophils play a crucial role as a high priority source of IL-4, IL-13, histamine, and leukotriene-C_4 (LTC_4) that polarize the early type 2 immune response leading to allergy and asthma[(3-6)].

With the advent of proteomics and allergen sequence, biomic study of immunological molecular mechanisms enables more rapid advances in elucidating allergic inflammatory pathways through proteomic methodologies coupled with bioinformatic techniques. Proteomics characterizes cellular proteins, its abundance, modification state, protein complexes and interactions. The global changes in cellular protein expression can be visualized by two-dimensional gel electrophoresis (2D) and identified by mass spectrometry analysis.

Since IL-4 and IL-13 are central in allergic inflammation and share several biological properties, it is crucial to elucidate how human basophils synthesize and release such cytokines upon stimulation. The phorbol 12-myristate 13-acetate (PMA) induction of human basophils and KU812 cells brings about IL-13, not IL-4 secretion[(4,7)]. The ionomycin only initiates IL-4 not IL-13 production in KU812 cells, but it induces both IL-4 and IL-13 secretions in human basophils[(4,8)]. Regarding the release of histamine and LTC_4, PMA results in histamine slowly released at the maximum level within 1 hr, but no LTC_4 production[(9)]. The calcium ionophore, A23187, induces LTC_4 generation, an event which precedes histamine release[(10)]. Recently, combinational use of PMA and A23187/ionomycin for human immune cells such as mast cell, basophil, T cell, B cell and neutrophil seems to be a representative immuno-stimulant in allergic molecular mechanism, immune cell differentiation, signaling pathway, transcriptional factor activation, and protective lead compound screening[(11-13)].

The 2D couples with tandem mass spectrometry and validation assays were utilized to evaluate changes in comparative proteomes of human basophilic cells following treatment with PMA and A23187. The combination of PMA and A23187 induced IL-4, IL-5 and IL-13 expressions in human basophilic KU812 cells. The significantly regulated proteins are further classified based on metabolism, macromolecule binding, catalytic activity, association with inflammatory immunological diseases, tumorgenesis, and cell movement. Meanwhile, the

selected regulatory proteins as HNRPK and ENO1 were verified by 1D- and 2D-Western blotting**.** This study is the first of its kind to combine the use of immuno-stimulating treatment with proteomic platform and bioactivity validation assays to elucidate KU812 basophil activation induced by PMA plus A23187.

材料與方法

I. *Cell Culture*

Human basophilic leukemia KU812 cells were seeded at an initial concentration of 5×10^5 cells/mL in RPMI 1640 supplemented with 10% fetal bovine serum and maintained at 37°C in an incubator with controlled humidified atmosphere containing 5% CO_2.

II. *RT-PCR Analysis of Cytokine mRNA Expressions*

The KU812 cells (5×10^5 cells/mL) were treated by PMA (10 ng/mL) plus A23187 (1 μmol/L) or an equivalent amount of diluted dimethyl sulfoxide (DMSO) and incubated for 3 hours. After washing, total RNA was extracted, mixed with the reverse transcriptase (RT) mixture according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and incubated at 42°C for 50 minutes, followed by 95°C for 5 minutes. After each RT, PCR amplification was performed with the following primers. IL-4–sense, 5′ ATG-GGT-CTCACC-TCC-CAA-CTG-CT 3′; antisense, 5′ GTT-TTC-CAA-CGT-ACT-CTG-GTT-GGC3′. IL-5–sense, 5′GCT-TCT-GCA-TTT-GAG-TTT-GCT-AGC-T 3′; antisense 5′ TGG-CCG-TCA-ATG-TAT-TTC-TTT-ATT-AAG 3′. IL-6–sense, 5′ ATG-AAC-TCC-TTC-TCC-ACA-AGC-GC 3′; antisense, 5′ GAA-GAG-CCC-TCA-GGC-TGG-ACT-G 3′. IL-8–sense, 5′ATG-ACT-TCC-AAG-CTG-GCC-GTG-GCT 3′; antisense, 5′ TCT-CAG-CCC -TCT-TCA-AAA-ACT-TCT-C 3′. IL-13–sense, 5′ CCA-CGG-TCA-TTG-CTC-TCA-CTT-GCC 3′; antisense, 5′ CCT-TGT-GCG-GGC-AGA-ATC-CGC-TCA 3′. IL-1β–sense, 5′ ATG-GCA-GAA-GTA-CCT-AAG-CTC-GC 3′; antisense, 5′ ACA-CAA-ATT-GCA-TGG-TGA-AGT-CAG-TT 3′. IFN-g–sense, 5′ TGT-TAC-TGC-CAG-GAC-CCA-TAT-GTA-AAA 3′, antisense, 5′ CAT-CAC-TTG-GAT-GAG-TTC-ATG-TAT-TGC 3′, β-Actin–specific primers were as follows: sense, 5′ GTG-GGG-CGC-CCC-AGG-CAC-CA 3′; antisense, 5′ GTC-CTT-AAT-GTC-ACG-CAC-GAT-TTC 3′. The PCR process was performed with a Thermo Px2Thermal Cycler (San Jose, CA, USA). The products were electrophoresed in 2% agarose gel, stained with ethidium bromide, photographed, and quantitated by computer program.

III. *Two Dimensional (2D) Electrophoresis*

KU812 cells were resuspended in lysis buffer at 350 μL, as described previously[14]. The protein solutions (150 μg protein) were applied onto IPG strips (18 cm, pH 3-10NL)(GE Healthcare Life Sciences, Pittsburgh, PA, USA). After rehydration, isoelectric focusing (IEF) was conducted at 8000 V for 66000 Vh. The equilibrated strips were then transferred onto 12.5% homogenous polyacrylamide slab gel (18×18 cm), and separated proteins were then visualized with SYPRO® Ruby dye (Bio-Rad, Hercules, CA, USA). The 2D gel images were scanned using a Typhoon 9200 laser scanner (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and exported

to the image analysis software program using PDQuest software package version 7.1.1 (Bio-Rad, Hercules, CA, USA).

IV. *In-gel Digestion*

The in-gel digestion procedure was processed according to our previous study[14]. Briefly, the gel pieces were washed and rehydrated with trypsin (Promega, Madison, WI, USA) at 37°C for at least 16h. The extracted peptides were finally eluted with 5 μL of 75% acetonitrile/0.1% formic acid, and directly spotted on the sample plate of a MALDI-Q-TOF mass spectrometry.

V. *MALDI-TOF and MALDI-Q-TOF Mass Spectrometry.*

The trypsinized samples were premixed in a ratio of 1:1 (v/v) with the matrix solution (5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile, 0.1% v/v trifluoroacetic acid and 2% w/v ammonium citrate) and spotted onto the 96-well MALDI sample stage. The samples were analyzed by the Q-TOF Ultima™ MALDI instrument (MALDI™; Micromass, Manchester, UK), as described previously[9]. The MASCOT search engine (http://www.matrixscience.com) was used for MS/MS ions search using a MALDI-Q-TOF-MS hybrid quadrupole/orthogonal acceleration TOF spectrometer (MALDI™, Micromass, Manchester, UK). The product ion spectra generated by Q-TOF MS/MS were searched against NCBInr and Swiss-PROT databases for exact matches using the Mascot search program.

VI. *1D- and 2D-Western Blotting Verification of HNRPK and ENO1 Protein Level Expressions*

After 1D and 2D procedures, proteins were transferred onto PVDF membranes (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Primary antibodies including HNRPK and ENO1 were purchased from Abcam (Abcam, Cambridge, UK). After incubation with secondary antibodies, the immunoblots were visualized with the ECL detection kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA) then exposed to X-ray film, or Luminescent Image Analyzer LAS-4000 (Fujifilm, Tokyo, Japan).

VII. *Statistical Analysis*

Analysis of proteins regulation changed upon PMA plus A23187 treatment was done on three independent experiments. Each set of 2D gels was compared separately by an image comparison software. Different proteins that were found in all sets of experiment were selected for identification.

VIII. *Bioinformatics Tools for Protein Searches*

The protein search programs used the following sequential order of databases: NCBI (http://www.ncbi.nlm.nih.gov), Swiss-Prot/TrEMBL (http://www.expasy.ch/sprot) and Proteome (http://www.proteom.com/databases/HumanPD/ reports). In addition, the functional protein association networks or protein interactions were searched by STRING database (http://string.embl.de/). The combining pathway databases of BioCarta (http://www.biocarta.com), KEGG (http://www.genome.ad.jp/kegg/pathways.html) and the PubMed literature (http://www.ncbi.nlm.nih.gov/PubMed) were also used to search the correlated regulatory pathways of cellular response in KU812 and other immune cells. The two bioinformatic tools including our previous work as Bulk Gene Search System for Java (BGSSJ) [15]. The Ingenuity

Pathway Analysis (IPA) was applied in thoroughly compiling protein functional classification and interaction networks.

結果

I. *Cytokine Expressions of KU812 after PMA plus A23187 Treatment*

We treated human basophil cells KU812 with PMA (10 ng/mL) and A23187 (1 μmol/L). The PMA structure was analogous to diacylglycerol that activates protein kinase C. The A23187 was a calcimycin that greatly increases the ability of Ca^{2+} crossing biological membranes. In RT-PCR, mRNA expressions of the cytokine genes were normalized to each sample by using b-actin gene as internal controls. The up-regulation of IL-4, IL-5, IL-6, IL-8, IL-13, and IFN-g (no IL-1b), was detected after PMA plus A23187 stimulation at 3 hours. The typical Th2 cytokines consisting of IL-4, IL-5 and IL-13 were apparently enhanced more than 2 fold changes (Figure 1). The IL-5 elevated to the highest level at 5 fold changes among all test cytokines. The combination treatment with PMA and A23187 stimulated KU812 cells elevation of Th2 type cytokine mRNA expression levels.

II. *Expression Changes of 17 Identified Proteins in KU812 after PMA plus A23187 Treatment*

The time-course experiments were conducted at least three times and showed good reproducibility among different cell batches and 2D gels (Supplementary Figure 1). The 2D patterns revealed that the largest number of significantly-regulated proteins occurred at 6h treatments using the PDQuest analysis. We identified 2 up-regulated proteins (heterogenous nuclear ribonucleoprotein K (HNRPK) and eukaryotic initiation factor 4A-1 (EIF4A1), and 15 down-regulated protein expressions including protein set (SET), 40S ribosomal protein SA (RPSA), tubulin b-5 chain (TUBB), heat shock protein 60 (HSPD1), actin b-chain (ACTB), actin a-chain (ACTA), tryptophanyl-tRNA synthetase, cytoplasmic (WARS), a-enolase (ENO1), tyrosyl-tRNA synthetase, cytoplasmic (YARS), fructose-bisphosphate aldolase C (ALDOC), furmarate hydratase (FH), 26S protease regulatory subunit (PSMC6), fructose-bisphosphate aldolase A (ALDOA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and profilin-1 (PFN1) at 6h treatment (Table 1). Statistically significant variations were defined as a minimum 2-fold changes and $p<0.05$ when comparing to protein expression levels among control and treatment groups.

The 2 up-regulated protein spots were shown in a representative 6h-treatment gel, and 15 down-regulated protein spots were revealed in control group (Figure 2). The computerized close-up vision for each spot was collected (Figure 3), and optical density of identified spots from three independent experiments were analyzed and piled up into histograms (Table 1). To identify protein spot individually, the 3D diagram utility provided by PDQuest was subjected to careful examination of each spot. The HNRPK and EIF4A1 levels had 2-fold elevation with time increases at 6h. In contrast, the SET, RPSA, ALDOA levels were markedly reduced by 4-fold at 6h time point but gradually increased at 12h. The remaining down-regulated proteins reached a

peak at 3h and declined at 6h down to 30-50% levels comparable to untreated cells at 3h. Furthermore, we identified specific isoforms and several spots for one protein identity for HNRPK (up-regulated) and ENO1 (down-regulated). The five protein spots of HNRPK revealed 3 up-regulated and 2 down-regulated isoforms (data not shown); however, only one up-regulated spot was shown due to statistical significance. On the other hand, the three ENO1 spots showed significant reductions at 6h compared to control group. These spots with varied mobility indicated possible alternative splicing, post-transcriptional or post-translational modifications for ENO1 and HNRPK.

III. *Verification of Significantly-Regulated Proteins by 1D- & 2D-Western Blotting*

From the time-course 1D-western blotting analyses (Figure 4A), the 6h treated cells seemed to have a slightly higher HNRPK expression than the untreated KU812 cells. In contrast, ENO1 exhibited a slightly elevated level in the untreated KU812 cells. We further conducted 2D-western blotting analysis to better understand the expressions of isoforms for HNRPK and ENO1 (Figure 4B). The acidic isoform (indicated by a) of HNRPK in 6h PMA plus A23187-stimulated KU812 cells was significantly higher than that in the untreated KU812 cells. This spot might result from inducible phosphorylation of HNRPK by PMA plus A23187 treatment. Other HNRPK isoforms did not show significant variances. On the contrary, almost all ENO1 isoforms (indicated by a to e) exhibited down-regulation after 6h PMA plus A23187 treatment. These 2D and 2D-western blotting results showed a good correlation.

IV. *Subcellular and Functional Classification*

We used 2 bioinformatic tools including our previous work as Bulk Gene Search System for Java (BGSSJ) shown in Figure 6, and Ingenuity Pathway Analysis (IPA) shown in Table 2 to thoroughly compile protein functional classification and interrelationships. The PMA plus A23187-stimulated proteins correlate primarily to immunological disorder (ACTA, ACTB, ALDOA, ENO1, GAPDH, RPSA, TUBB), and tumorgenesis (ACTB, ALDOA, ALDOC, ENO1, FH, PFN1, TUBB) (Table 2). From subcellular localization analysis, the identified proteins were mainly distributed in the cytoplasm, followed by nucleus and plasma membrane (Figure 5A). The proteins in the nucleus consist of HNRPK, SET, RPSA, PSMC6, and PFN1 according to BGSSJ and IPA databanks. Primary metabolism on carbohydrate, protein, and nucleic acid plays an important role in the physiological process (Figure 5B). From molecular function classification, the identified proteins abundantly bind to protein, nucleotides, nucleic acid, and metal ion (Figure 6C).

討論

Basophils comprise less than 1% of peripheral blood leukocytes and serve as primary Th2 type effector cells during allergic inflammation and parasite infection[5, 16]. Activated basophils produce rapid cytokine release due to sufficiently preformed IL-4 and IL-13 mRNA transcripts in basophil ontology[17]. In addition to IL-4 and IL-13, IL-5 and IFN-g were greatly elevated in PMA plus A23187-stimulated KU812 cells within 3h in this study (Figure 1). The phenomenon is similar in chronic urticaria patients that PMA plus ionomycin heavily enhanced both IL-4 and IFN-g productions in CD4+ lymphocyte[18]. Human basophils are a more effective source of IL-4 than mast cells or eosinophils. Human basophils release equivalent IL-4 amount as FceRI-activated Th2 cells. The activated basophils provided early IL-4 source which might drive naïve CD4 T cells to IL-4-producing Th2 phenotypes *in vitro* and *in vivo*[19-20]. Even IL-4 deficient basophils still could inhibit naïve CD4 T cells differentiate into Th1 cells, although they did not help in Th2 differentiation[21]. Basophils might serve as a potential connection between innate and adaptive immunity. Basophils serve as an initiator and a good therapeutic target in IgE-mediated chronic allergic inflammation from basophil-depleting mAb studies[22].

PMA plus A23187 induces histamine[12], IL-4, IL-5, IL-6, IL-8 and IL-13 protein secretions in activated basophil cells[11]. They also enhance CD40 ligand[13], and phosphorylation of cytoplasmic p38 MAPK, p44/42 MAPK, p54/46 SAPK/JNK, as well as nuclear c-Jun phosphorylation both on ser63 and ser73[23]. PMA and A23187 increased DNA-binding activity of phosphorylated c-Jun, JunB, JunD, c-Fos, and FosB nuclear extracts of KU812 cells[23].

Heterogeneous nuclear ribonucleoproteins (hnRNP) are predominantly nuclear RNA-binding proteins that involve in a range of RNA life including splicing, processing, export of the mature RNAs and translation, and signal transduction that influence gene expression [24]. The hnRNP family was recently regarded as an important target of the autoimmune response in rheumatic diseases. The hnRNP-K (HNRPK) was associated to rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and mixed connective tissue disease[25]. The HNRPK was significantly up-regulated by 6h PMA plus A23187 stimulated KU812 cells. From 2D-western blot of HNRPK, a spot (indicated by a) appeared at the more acidic side. HNRPK were phosphorylated at multiple sites, especially on tyrosine residues, in response to various signals. HNRPK was assumed to be a target of SH2-containing protein tyrosine kinases such as Src, Lck, Lyn and Fyn[24]. B cell receptor (BCR) activation did not change HNRPK concentration in 1D immunoprecipitated protein, but greatly enhanced 1D and 2D immunoblotting with anti-phosphotyrosine antibodies, which cause inducible phosphorylation of HNRPK[26]. Our immunoblotting results agreed with the phenomena of BCR activation, indicating that PMA plus A23187 might induce phosphorylation of HNRPK in KU812 cells (Figure 4B). Regarding cancer biology, HNRPK increased transcriptional activation of the c-*myc* gene and oncogene c-Src related to cancer development, survival, and migration. The HNRPK was identified as the potential targeted protein of cancer metastasis from loss-of-function screening by randomized intracellular antibodies. Furthermore, the cytoplasmic accumulation of HNRPK played an

important role in metastasis[27] and colorectal cancer [28]. The HNRPK protein elevated at 6h PMA plus A23187 treatment might enhance DNA and RNA binding activity, transcriptional and translational modulating activity. The PTM study of HNRPK on KU812 cells upon treatment with PMA and A23187 is under way.

Enolase is a multifunctional protein abundantly expressed in the cytoplasm and serves as Myc-binding protein (MBP-1) in the nucleus. Enolase is a plasminogen receptor on the surface of the hematopoetic, epithelial and endothelial cells. Auto-antibodies to airway epithelial cells (A549) were examined in sera from patients with severe asthma[29]. Anti-enolase antibodies have been incriminated in various inflammatory and autoimmune diseases including SLE, RA, mixed cryoglobulnemia (MC), systemic sclerosis (SSc), Behcet's disease, multiple sclerosis, Hashimoto's encephalopathy (HE), and paraneoplastic retinopathy [30]. Anti-enolase antibodies could serve as prognostic indicators for autoimmune or neoplastic disorders but not diagnostic biomarkers due to broad distribution in numerous diseases. ENO1 was also a highly conserved fungal allergen and its IgE-binding epitopes were elucidated. PMA treated hematopoietic cells such as B cells, T cells, monocytes, neutrophils showed greater amounts of ENO1 on their surface. The translocation of ENO1 seemed to be associated with its hydrophobic domain, posttranslational acylation or phosphorylation. The isoform of ENO1 detected by our 2D-western blot at 6h PMA plus A23187 treatment were significantly decreased (Figure 4B). Such drastic change of ENO1 expressions in the treated-KU812 cells with PMA plus A23187 might be associated with the ENO1 location or its posttranslational modifications. This area needs to be further investigated.

The tubulin beta-5 chain (TUBB) is a major constitute of microtubules responsible for cell mobility. It belongs to MHC I binding protein and is associated to NK-mediated cytotoxicity. The TUBB was identified as autoantigen in allergic rhinitis, and 52% of allergic rhinitis patients possessed anti-TUBB autoantibodies[31]. Pharmacological and morphological data revealed that the cytoskeleton as tubulins and actin associated to histamine secretion in basophil and mast cells. Furthermore, IL-3 and anti-IgE alone did not promote tubulins synthesis, but combinational treatment can induce both histamine release and tubulins up-regulation[32].

The 60 kDa heat shock protein (HSPD1) resulted in mitochondrial protein transportation and macromolecule assembly. The HSPD1 were proven to possess dominant immuno-modulatory capability in innate, adaptive immune systems, autoimmune, and tumor immunology[33]. Although the dichotomous immunoregulatory ability is still not completely elucidated, the intracellular (self) HSPD1 is generally regarded as chaperonin and anti-inflammatory. On the other hand, extracellular (from pathogenic organisms) HSPD1 is associated to pro-inflammatory[34]. Due to admirable immunogenicity of HSP family, HSP have been designed for their adjunct use in vaccines[34]. HSPD1 down-regulation in KU812 cells upon PMA and A23187 stimulation (Table 1) might point toward immunological inflammatory pathway.

The actin family was mainly involved in cell motility and ubiquitously expressed in eukaryotic cells. In rat basophilic leukemia (RBL-2H3), both F-actin and myosin were remolded into membrane ruffles and aggregations, and followed by parallel stress fibers located on the

ventral membrane originally from plasma membrane during secretion of histamine after PMA or A23187 treatment[35].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ENO1 and ALDOA are key enzymes in glycolysis. Fumarate hydratase (FH), a major enzyme in citric acid cycle, function as interconversion of malate and fumarate. PMA plus A23187 synergistically activate H_2O_2 generation in cultured porcine thyroid cells through activating protein kinase C and increasing cytoplasmic free calcium, respectively[36]. Oxidizing agents as H_2O_2 were shown to carbonylate and induce polypeptide degradation in glycolytic enzymes including ENO1, pyruvate kinase, and GAPDH[37]. ENO1 was a primary target of H_2O_2- or O_2^--mediated carbonylation in *Escherichia coli* [38]. Posttranslational modification of glycolytic enzymes was recognized as an intracellular sensor of oxidative stress[39]. We detected down-regulation of glycolytic enzymes including ALDOA, ALDOC, FH, ENO1 and GAPDH in PMA plus A23187 treated KU812 basophils. Therefore, the carbohydrate catabolism was reduced possibly through the production of oxidizing agents induced by the PMA plus A23187 treatment.

The inconsistency between 1D- and 2D-western blots might be caused by the following reasons: (1) the alternative splicing transcript variants of HNRPK and ENO1 could not be recognized by 1D-western blots; (2) phosphorylated HNRPK was not revealed by 1D-western blots, but the more acidic isoform of HNRPK up-regulated in the PMA plus A23187 treated KU812 cells (shown in the 2D-western blots). This finding agreed with the inducible phosphorylation of HNRPK in the BCR activation[26], and in response to the oxidative stress that was possibly induced by PMA plus A23187 treatment[36]; (3) the isoforms of ENO1 largely shifted in the pI and Mw range of the 2D gel[40] so that the selected 2D region for western blots could include the major (not all) isoforms of ENO1.

In summary, numerous differentially expressed proteins were identified in PMA plus A23187-activated KU812 cells. Most of these proteins were the key factors involved in metabolism, macromolecule binding, and catalytic activity, and were associated with inflammatory immunological diseases, tumorgenesis, and cell movement. HNRPK and ENO1 variations were consistently validated by protein level exanimations. However, further functional study of these proteins may lead to better understanding of the activation mechanisms for PMA plus A23187 treatment.

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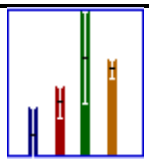
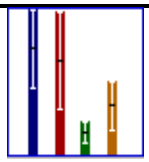
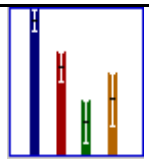
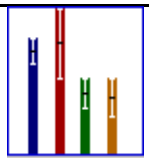
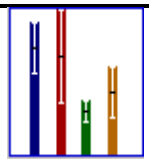
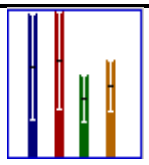
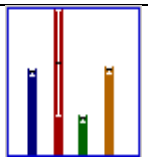
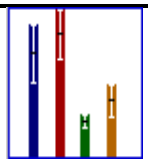
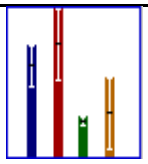
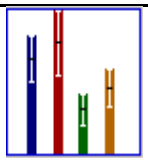
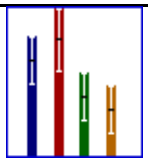
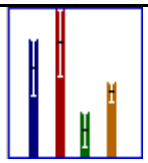
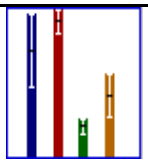
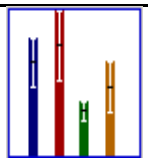
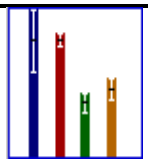
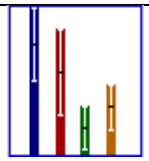
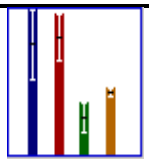
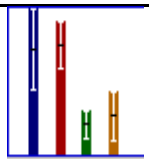
Table 1. Summary of significantly regulated proteins in total cellular lysates of KU812 cells in a time course treatment study with PMA plus A23187 using the MALDI-Q-TOF analysis

Spot	Change	Accession	Protein name	Score	Theoretic		SC[b]	Matched sequences
	Con 3h 6h 12h	Sprot[a]			Mr (kD)	p*I*		
Up-regulated								
1		P61978	Heterogeneous nuclear ribonucleoprotein K (HNRPK)	37	50.9	5.39	2	NLPLPPPPPPR
2		P60842	Eukaryotic initiation factor 4A-1 (EIF4A1)	126	46.1	5.32	8	GIYAYGFEKPSAIQQR AEVQKLQMEAPHIIVGTPGR
Down-regulated								
3		Q01105	Protein SET (SET)	55	33.5	4.23	8	LRQPFFQK SGYRIDFYFDENPYFENK
4		P46783	40S ribosomal protein SA (RPSA)	85	32.8	4.79	11	AIVAIENPADVSVISSR FTPGTFTNQIQAAFREPR
5		P07437	Tubulin β-5 chain (TUBB)	211	49.6	4.78	10	ISVYYNEATGGKYVPR LHFFMPGFAPLTSR MAVTFIGNSTAIQELFKR
6		P10809	Heat shock protein 60 (HSPD1)	214	61.0	5.70	6	TLNDELEIIEGMKFDR KISSIQSIVPALEIANAHR ISSIQSIVPALEIANAHR
7		P60709	Actin β-chain (ACTB)	275	41.7	5.29	23	AVFPSIVGRPR VAPEEHPVLLTEAPLNPK LCYVALDFEQEMATAASSSSLEK SYELPDGQVITIGNER DLYANTVLSGGTTMYPGIADR

8		P62736	Actin α-chain (ACTA)	57	42.0	5.23	4	SYELPDGQVITIGNER
9		P23381	Tryptophanyl-tRNA synthetase, cytoplasmic (WARS)	126	53.1	5.83	7	ISFPAIQAAPSFSNSFPQIFR ALIEVLQPLIAEHQAR
10		P06733	α-enolase (ENO1)	70	47.1	7.01	3	LAMQEFMILPVGAANFR
11		P54577	Tyrosyl-tRNA synthetase, cytoplasmic (YARS)	51	59.1	6.61	3	TVVSGLVQFVPKEELQDR
12		P06733	α-enolase (ENO1)	258	47.1	7.01	12	EIFDSRGNPTVEVDLFTSK LAMQEFMILPVGAANFR AGYTDKVVIGMDVAASEFFR
13		P06733	α-enolase (ENO1)	67	47.1	7.01	8	LAMQEFMILPVGAANFR AGYTDKVVIGMDVAASEFFR
14		Q6FH94	Fructose-bisphosphate aldolase C (ALDOC)	75	39.4	6.41	4	TPSALAILENANVLAR
15		P07954	Furmarate hydratase (FH)	33	54.6	8.85	4	THTQDAVPLTLGQEFSGYVQQVK
16		P62333	26S protease regulatory subunit (PSMC6)	63	44.1	7.10	3	EVIELPLTNPELFQR

17		P04075	Fructose-bisphosphate aldolase A (ALDOA)	51	39.4	8.30	5	IGEHTPSALAIMENANVLAR
18		P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	128	36.0	8.57	11	VIHDNFGIVEGLMTTVHAITATQK LISWYDNEFGYSNR
19		P07737	Profilin-1 (PFN1)	126	15.0	8.44	12	TFVNITPAEVGVLVGK TFVNITPAEVGVLVGKDR

[a]Accession number from UniProtKB/Swiss-Prot.

[b]SC stands for Sequence Coverage from Mascot search result.

Table 2. Functional classification of significantly regulated proteins by Ingenuity Pathway Analysis (IPA) Software

Function	Protein symbol
Immunological Disease	
acute allergic pulmonary eosinophilia	ACTB[a], ALDOA, ENO1, GAPDH, TUBB
immunological disorder	ACTA, ACTB, ALDOA, ENO1, GAPDH, RPSA, TUBB
rheumatoid arthritis and autoimmune disease	ACTA, ALDOA, ENO1, RPSA
Cancer	
tumorgenesis and neoplasia	ACTB, ALDOA, ALDOC, ENO1, FH, PFN1, TUBB
carcinoma, primary and malignant tumor	ALDOA, ALDOC, ENO1, PFN1, TUBB
lung cancer, lung tumor, lung adenocarcinoma, lung carcinoma	ALDOA, ALDOC, ENO1
growth and development of breast cancer cell lines	HNRPK, PFN1
attachment and invasion of melanoma cell lines	RPSA
migration of fibrosarcoma cell lines	HNRPK
apoptosis of tumor cells	GAPDH, ENO1
Cellular Assembly and Organization	
formation of filaments	ACTA, PFN1, TUBB
formation of microspikes and actin	PFN1
formation of mitotic spindle and spindle fibers	TUBB
formation of myofilaments	ACTA
formation of nucleosomes	SET
binding of microsomal membrane	GAPDH
binding of ribosome	EIF4A1
Inflammatory Disease	
inflammatory disorder	ACTA, ALDOA, ENO1, GAPDH, HSPD1, RPSA
rheumatic arthritis disease	ACTA, ALDOA, ENO1, RPSA
iridocyclitis, and uveitis	HSPD1
acute respiratory distress syndrome	GAPDH

Cellular growth and proliferation	
growth of cells	ACTB, EIF4A1, ENO1, HNRPK, HSPD1, PFN1, RPSA, SET
growth of breast cancer cell lines	HNRPK, PFN1
Cellular Movement	
cell movement	ACTB, HNRPK, HSPD1, TUBB, WARS, YARS
cell movement of fibrosarcoma cell lines	HNRPK
chemotaxis and homing of mononuclear leukocytes, blood cells and lymphatic system cells	HSPD1, YARS
migration of eukaryotic cells	HNRPK, HSPD1, WARS, YARS
invasion of melanoma cell lines	RPSA
Carbohydrate metabolism	
glycolysis	ALDOA, ENO1
metabolism of fumaric acid	FH
quantity of UDP-N-acetylglucosamine	GAPDH
binding of lipopolysaccharide	RPSA
Small Molecular Biochemistry	
aminoacylation	WARS, YARS
recovery of ATP	HSPD1
production of niric oxide	HSPD1, PFN1
Gene expression	
activation of synthetic promoter	ACTB, GAPDH, HNRPK, SET
recruitment and binding of mRNA	EIF4A1
transactivation of protein binding site	ENO1
transactivation of synthetic promoter	GAPDH, HNRPK

[a] Refer to Table 1 for protein abbreviations.

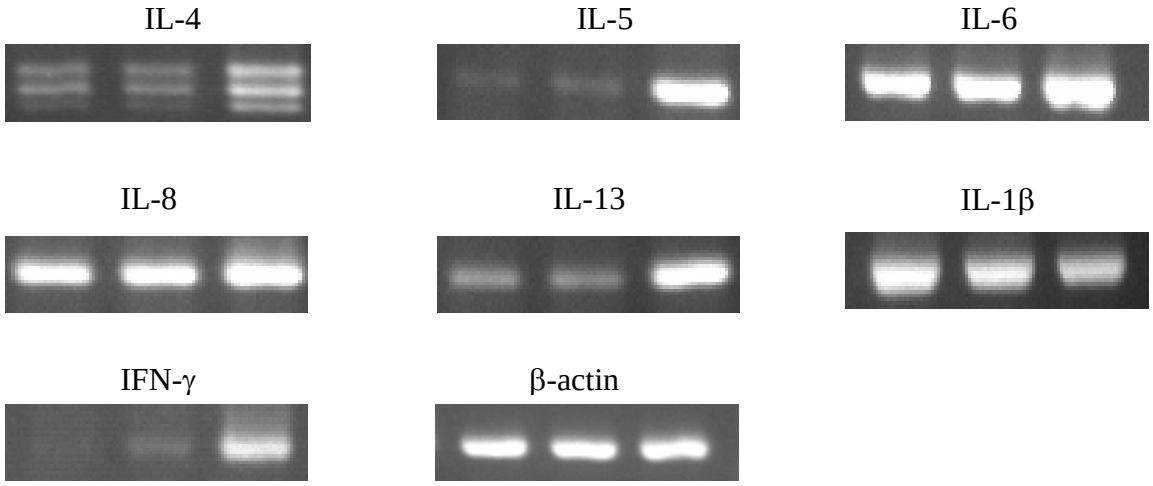


Figure 1. RT-PCR patterns for cytokines of human basophil cell line KU812.
Blank (left): no treatment; Control (middle): treated only with an equivalent amount of diluted DMSO; Treated (right): stimulated with PMA plus A23187 for 3h.

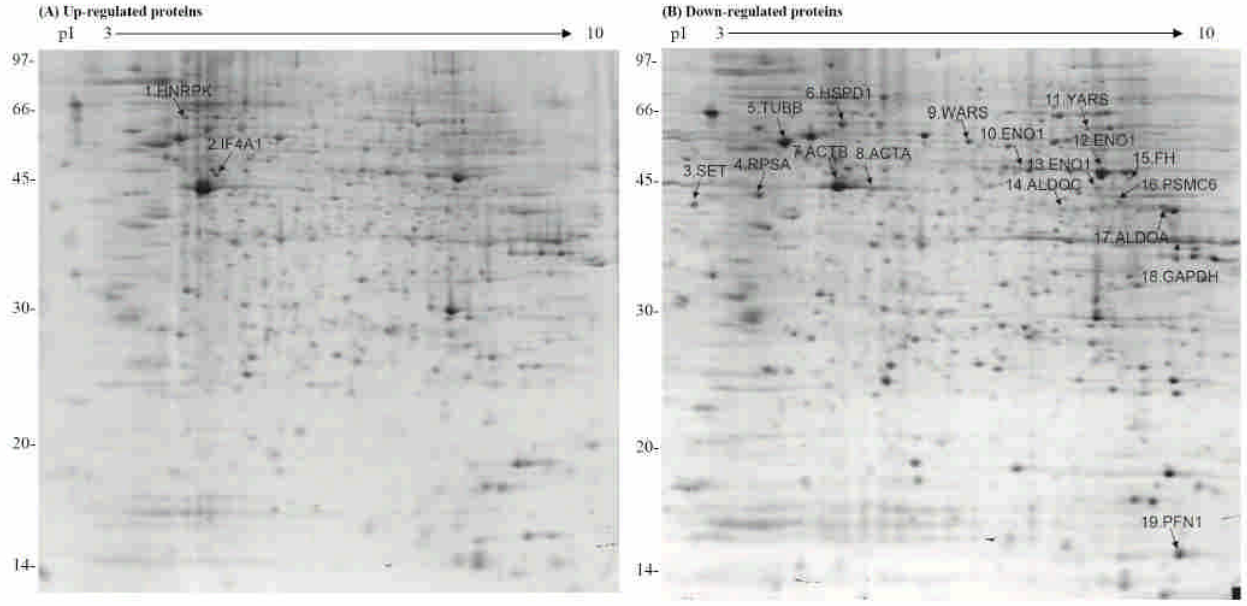


Figure 2. The representative 2DE gel for 6 hr PMA plus A23187 treatment.

Protein spots present in the pI range of 3-10 and molecular weight range of 14-97 kD. (A) The up-regulated proteins are shown in the gel of 6h treatment group. (B) The down-regulated proteins are shown in the gel of control group. The 2D experiments for each group were conducted at least three times with good reproducibility among different cell batches and 2D gels (Supplementary Figure 1). Statistically significant variations were defined as a minimum 2 fold changes in expression level and $P<0.05$ at 6 h compared to protein expression level in control group.

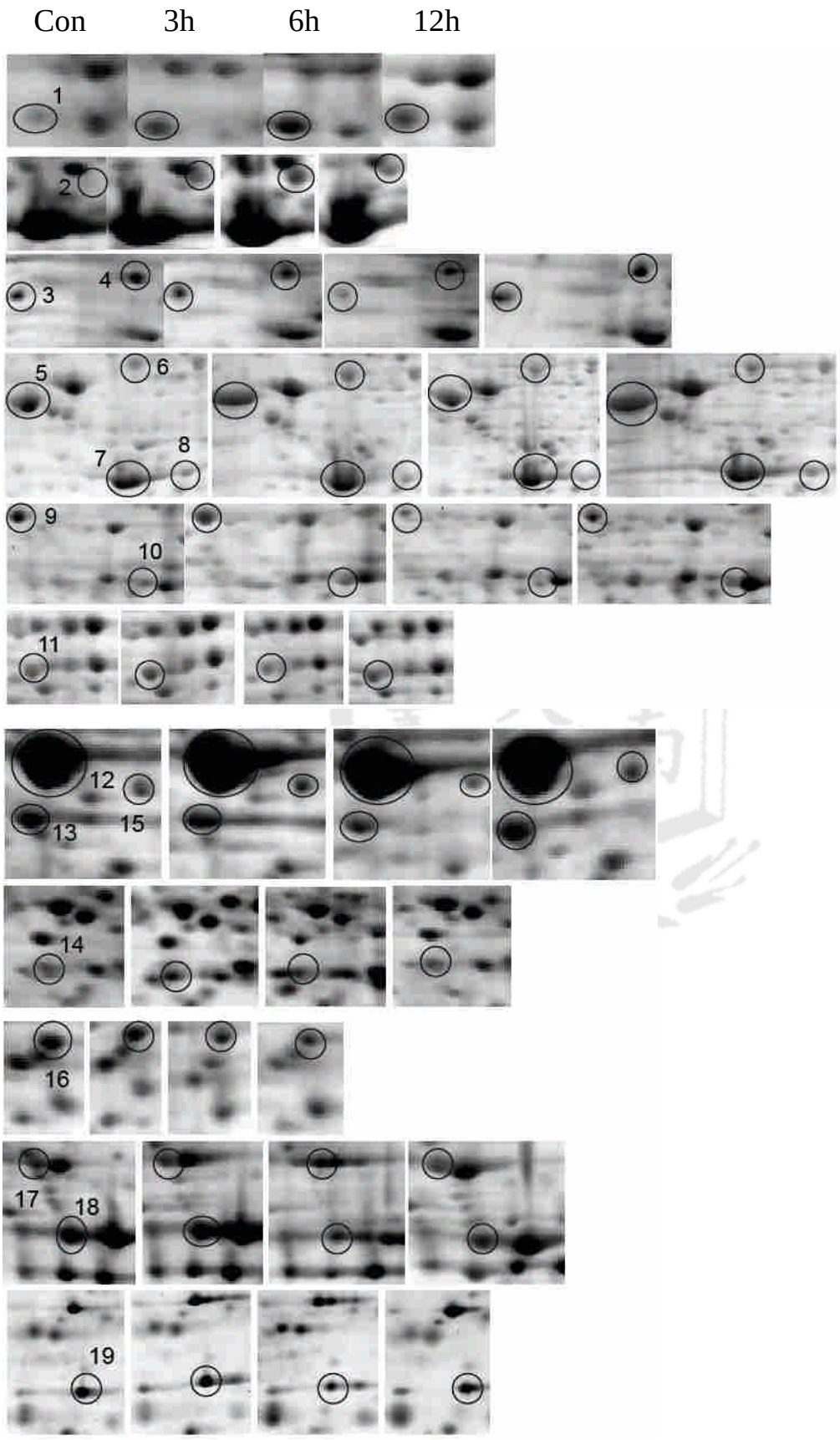


Figure 3. Close-up view of PMA plus A23187-dependent expression of 17 identified proteins. Gel sections (1-19) represent varied expression of the indicated protein spots at different time periods.

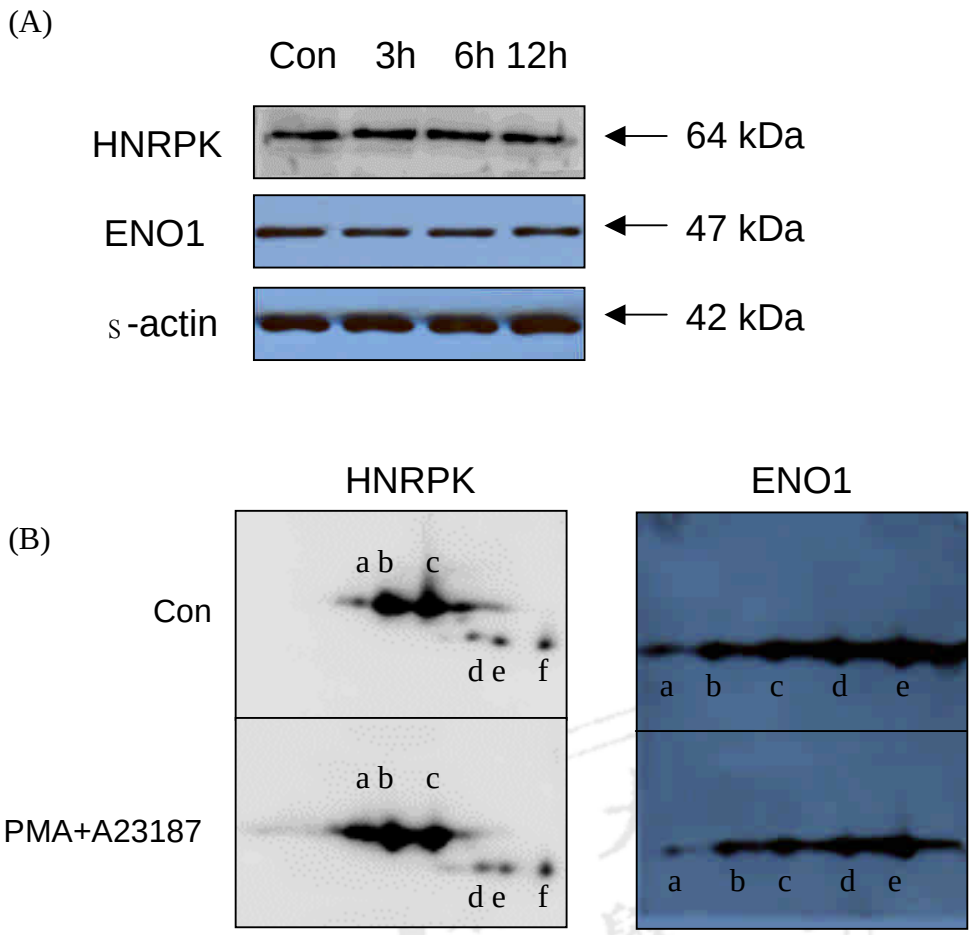


Figure 4. HNRPK and ENO1 validations were achieved by 1D- and 2D-Western blotting. (A). 1D-Western blotting of KU812 cells at differential time points. (B). 2D-Western blotting of KU812 cells at 6 h PMA plus A23187 treatment.

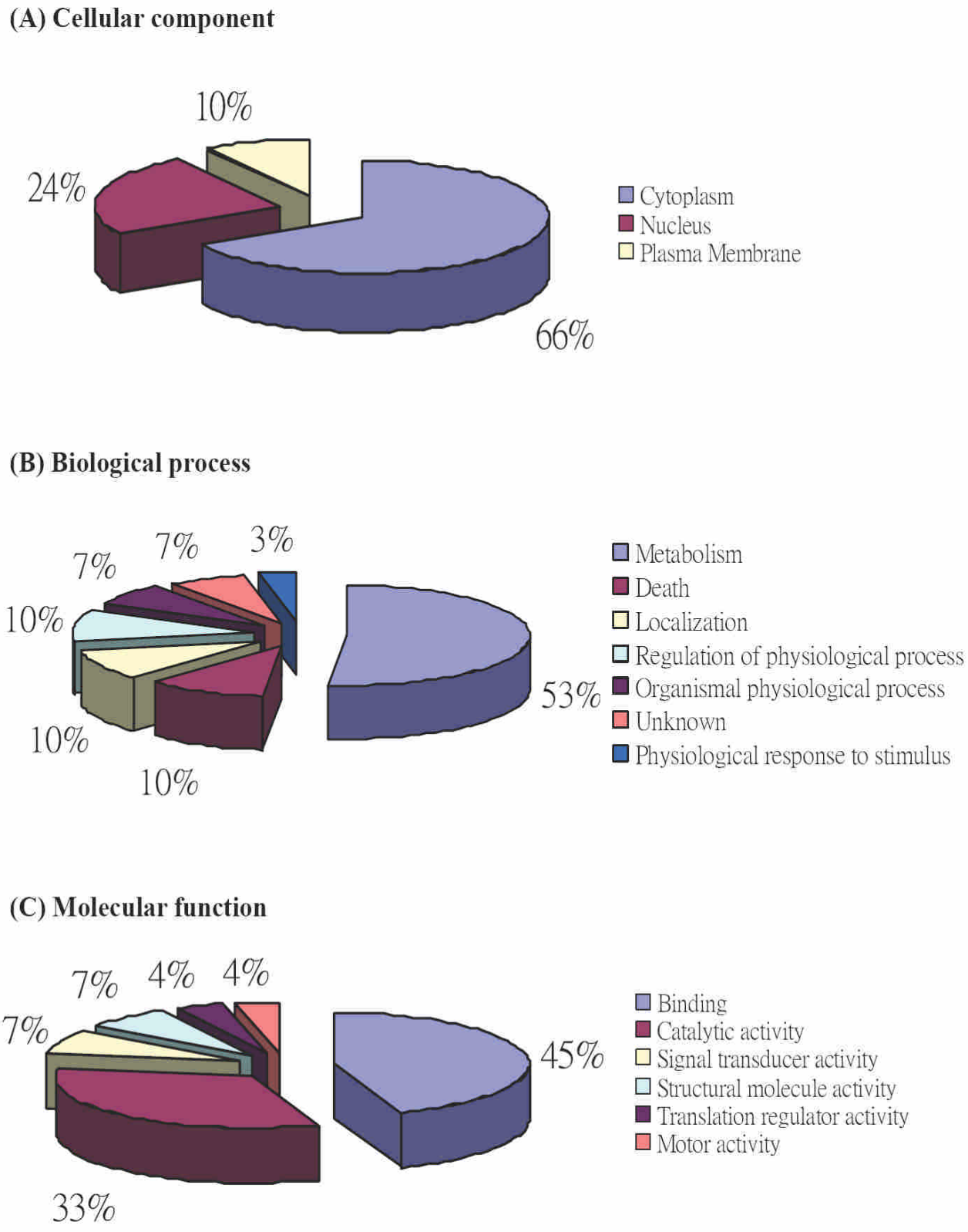


Figure 5. Functional classification assay conducted by the Bulk Gene Search System for Java (BGSSJ).

(A). Localization, (B). Biological process, and (C). Molecular function.