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Global Analysis of Protein Expression in A549 Cells After Prolonged Nicotine Exposure by Using Label-free Quantification

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Abstract. *Background/Aim: Lung cancer is the leading cause of cancer death worldwide. Cigarette smoke is the most important risk factor for cancer development. Growing evidence indicates that prolonged nicotine exposure is a potential factor associated with tumorigenesis. Here, the effect of prolonged nicotine exposure on A549 cells was investigated, using label-free quantitative proteomics. Materials and Methods: Selection of an invasive subpopulation from the A549 cell line was performed to reveal the differential expression of proteins in relation to prolonged nicotine exposure, using Boyden chamber assays in combination with a proteomics approach. Results: One hundred proteins from the NicoA549-L5 subline showed significant change in expression compared to those from the A549-L5 subline and their A549 parental cell line. Heat shock protein, protein disulfide isomerase A3, profilin-1 and legumain were expressed at higher levels in A549 cells after prolonged nicotine exposure. Conclusion: These aberrant proteins might serve as novel cancer biomarkers for cigarette smokers.*

Lung cancer is the most common cause of cancer-related death worldwide (1, 2). In Thailand, lung cancer accounted for 14.1% of new cancer cases in 2018, which is the highest frequency among other cancer types (1). Lung cancer is broadly divided into two main types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC comprises approximately 10-15% of lung cancer cases whereas NSCLC comprises 85-90%. Although the diagnostic and treatment methods have been improved, the overall survival rate of lung cancer patient remains very poor with a 5-year survival rate <15% (3). Growing evidence indicates that cigarette smoke is by far the most important risk factor of lung cancer development (4-6). Approximately 80% of lung cancer deaths are associated with smoking. Lung cancer risk is therefore many times higher in smokers compared to non-smokers (7).

Cigarette smoke contains more than 5,000 chemical constituents, of which at least 60 are carcinogens (8). Nicotine, an alkaloid, is the main addictive component of cigarettes, and is generally considered to be a non-carcinogenic compound. However, an increasing number of studies have shown that nicotine can stimulate the progressive transformation of normal cells to the earliest stage in cancer development. This is because nicotine can promote proliferation, angiogenesis, invasion and migration through the nicotinic acetylcholine receptors (nAChRs) of various cancer types (9-13). For example, Dasgupta *et al.* (14) showed that nicotine increases proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) in A549 lung cancer cells. Additionally, *in vivo* studies showed that nicotine significantly promotes the outgrowth and number of metastatic nodules in mouse models of various cancer types (15-17).

Growing evidence indicates that prolonged nicotine exposure is a potential factor associated with lung tumorigenesis. Fararjeh *et al.* (18) investigated the effect of long-term exposure to low-dose of nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the epithelial HBL-100 cell line. HBL-10 cells were repeatedly

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exposed to nicotine for 23 cycles, and this long-term exposure to nicotine significantly increased proliferation, migration and invasion of HBL-10 cells. Additionally, malignant and metastatic phenotype tend to increase in long-term treatment of NCI-N417 SCLC cells with nicotine (19). Therefore, it is important to gain further insight into the molecular events during prolonged exposure to nicotine.

Toxicoproteomics is becoming a promising tool for discovering the molecular signature of prolonged toxicant exposure. It has been used to identify and quantify alterations in the levels of proteins following exposure to toxin, toxicant or other adverse chemicals in complex biological systems (20, 21). In this study, we aimed to investigate the effect of prolonged nicotine exposure on the A549 NSCLC cell line using label-free quantitative proteomics approach. In addition, we report here the first toxicoproteomic profiling of motile A549 cells in response to prolonged nicotine exposure during the process of invasion. The results demonstrated that prolonged exposure of nicotine promoted invasion in A549 cells. Legumain, heat shock protein HSP 90-alpha, heat shock-related 70 kDa protein 2, protein disulfide isomerase A3 and profilin-1 were significantly increased in A549 cells after prolonged exposure to nicotine. Our findings suggested that these aberrant proteins might serve as novel cancer biomarkers for cigarette smokers.

Materials and Methods

Cell culture. Human non-small cell lung cancer A549 cells were cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Merck Millipore, Burlington, MA, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained in a 37˚C incubator with 5% CO_2. Cell were passaged successively when achieving 80% confluency.

Cytotoxicity assay. A549 cells were used to determine the cytotoxic effect of nicotine using an MTT assay as described by Kuljittichanok *et al.* (22). Cells (5×10^3 cells/well) were seeded to 96-well plates and then treated with various concentrations of nicotine (1-40 mM; Sigma-Aldrich) for 24, 48 and 72 h. After incubation, the culture medium with nicotine was removed and replaced with fresh culture medium containing 0.5 mg/ml MTT (Sigma-Aldrich). The cells were then incubated for 2 h at 37˚C. Culture medium was removed, and formazan products were dissolved by adding 100 μl of dimethyl sulfoxide (DMSO) to each well. The absorbance was measured at 550 nm using a 96 well microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) and background subtraction was adjusted with the absorbance readings at 650 nm.

Cell invasion assay. In vitro cell invasion assays were performed using a 6.5 mm Transwell chambers (8 μm pore size; Costar, Kennebunk, ME, USA) as described by Weeraphan *et al.* (23). Briefly, the upper surface of the membrane of the upper chamber was coated with 30 μg of Matrigel. Transwell chambers were then incubated overnight at 37˚C. Then, 100 μl of fresh serum-free media was added to the upper chamber, incubated at 37˚C for 1 h, after which all the media in upper chamber were removed. Five thousand of A549 cells were suspended with serum-free media containing nicotine at various concentration (0.1, 1.0, 5.0 and 10.0 nM) and then plated into the upper chamber. Cells incubated in serum-free media containing 0.5% (v/v) ethanol were used as a vehicle control. Then, 500 μl of complete medium were added to the lower chamber and incubated overnight at 37˚C in a CO_2 incubator. On the next day, cells that migrated across the filter membrane were fixed with 25% methanol for 15 min, and stained with 0.1% crystal violet for 15 min. Five random phase contrast images of migrated cells were taken and counted under 100× magnification. All experiments were performed in biological triplicates.

Prolonged nicotine exposure of A549 cells. A549 cells were cultured in RPMI 1640 (Gibco) containing 10% fetal bovine serum (Merck Millipore), 1% penicillin-streptomycin (Sigma-Aldrich) and 5 nM of nicotine. Briefly, 1×10^6 A549 cells were seeded and grown at 37˚C in an atmosphere of 5% CO_2. On the next day, cells were treated with 5 nM of nicotine and then passaged after an incubation period of 48 h. Cells cultured in media containing 0.5% (v/v) ethanol were included as a vehicle control. The prolonged nicotine exposure of A549 was designated as NicoA549-P5, and the prolonged exposure to vehicle as A549-P5. Cells were then harvested and kept at –80˚C for further analysis. All experiments were performed in biological triplicates.

Prolonged nicotine exposure of motile A549 cells. Subpopulations of A549 cells were selected according to their invasiveness capacity using 24 mm Transwell chambers. Briefly, the upper surface of the membrane of the upper chamber was coated with 400 μg of Matrigel and incubated as mentioned previously. One million A549 cells were suspended with serum-free media containing 5 nM of nicotine and then plated into the upper chamber. Cells in serum-free media containing 0.5% (v/v) ethanol were included as a vehicle control. Then, 2.6 ml of complete medium were added to the lower chamber and incubated for 48 h at 37˚C in a CO_2 incubator. The cells that migrated across the membranes were aseptically harvested and expanded for the next round of selection. The subline of the first-round selection was designated as A549-L1 and NicoA549-L1. Similarly, the sublines from 2, 3, 4, and 5 rounds of selection were designated as A549- and NicoA549-L2, -L3, -L4, -L5, respectively. A549-L5 and NicoA549-L5 cells that migrated across the membranes were harvested and kept at –80˚C for further analysis. All experiments were performed in biological triplicates.

Protein preparation for label free proteomic analysis. Ten micrograms of sample were resuspended in 50 mM ammonium bicarbonate (NH_4HCO_3) and protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Samples were reduced with 10 mM DTT at 95˚C for 5 min, alkylated with 1/10 volume of 200 mM iodoacetamide for 30 min at room temperature in the dark and then enzymatically digested with trypsin at a 1: 50 enzyme/protein ratio (Promega, Madison, WI, USA). Digestion was carried out overnight at 37˚C. The reaction was stopped by adding formic acid at a final concentration of 1% and the samples were completely dried by Speed Vacuum.

Label free LC-MS/MS analysis. Samples were prepared for label-free LC-MS/MS quantification by dissolving the digested samples

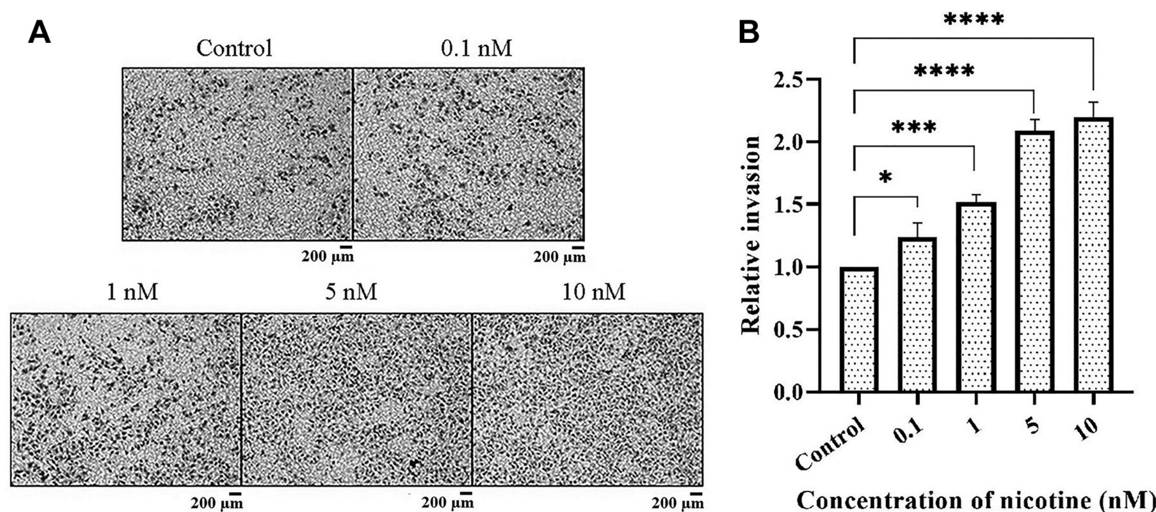

Figure 1. *Nicotine induces invasion of A549 lung cancer cells. (A) Representative images of the invasion assay using A549 cells treated with 0.1, 1.0, 5, and 10 nM of nicotine for 24 h. Scale bar, 200 µm. (B) Bar graph represents the relative invasion of A549 cells after treatment with various concentrations of nicotine. Data points represent the mean±SD. (*p<0.05, ***p<0.001, ****p<0.0001).*

in 0.1% formic acid in H_2O and separated on a Nanoflow liquid chromatography system (Thermo Fisher Scientific, Waltham, MA, USA). All samples were run in triplicate. Samples were injected into a C18 Acclaim PepMap RSLC (75 µm i.d. × 150 mm) column (Thermo Fisher Scientific) at a flow rate of 300 nl/min and the temperature of the column was maintained at 40˚C. The LC gradient was performed using 0.1% formic acid in 100% water (solution A) and 0.1% formic acid in 100% ACN (solution B) as follows: 1-50% B for 70 min, 50-90% B for 5 min, followed by 15 min with 90% B. One microliter of sample (100 ng/µl) was injected into the nano-LC system and then the separation was performed. Protein identifications were performed by Mascot software version 2.4.0. The search parameters were set up as follows: 1) database, Swiss-ProtFasta (released in October, 2018); 2) species, Homo sapiens; 3) digestion, trypsin; 4) instrument, ESI-TRAP; 5) fragment mass tolerance, 0.6 Da; 6) peptide mass tolerance, 1.2 Da; 7) maximum missed cleavages, 1. A false discovery rate (FDR) threshold of 1% was applied.

Western blot analysis. The proteins were extracted from the cells by homogenization in RIPA lysis buffer. The amount of protein in samples was quantified by the Bradford assay (Bio-Rad). Ten micrograms of protein samples were separated by 10% SDS-PAGE and proteins were then transferred to PVDF membranes (Pall Corp., Washington, NY, USA), which were blocked with 3% Bovine Serum Albumin (BSA) (Sigma-Aldrich) in Tris buffered saline with tween-20 (TBST) for 1 h at room temperature with agitation. Subsequently, the membranes were incubated with primary antibodies, including legumain (1: 10,000 dilution; cat.no. ab183028; Abcam, Cambridge, UK), HSP 90 (1:1,000 dilution; cat.no. 4874; Cell Signaling Technology, Danvers, MA, USA), PDI (1: 3,000 dilution; cat.no. 3501; Cell Signaling Technology), HSP 70 (1:1,000 dilution; cat.no. 4872; Cell Signaling Technology), Profilin (1: 5,000 dilution; cat.no. ab124904; Abcam), Histone H3 (1: 10,000 dilution; cat.no. 9715; Cell Signaling Technology) and β-actin (1: 10,000 dilution; cat.no. 3700; Cell Signaling Technology) in TBST buffer containing 3% BSA at 4˚C with agitation overnight. After washing 3 times with TBST buffer, the membranes were incubated with peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG as secondary antibodies (1:5,000 dilution; Dako, Santa Clara, CA, USA) in 3% non-fat dry milk for 1 h at room temperature with agitation. Membranes were then washed 3 times in TBST buffer. The immunoreactive bands were visualized using ECL Prime (GE Healthcare, Chicago, IL, USA) and detected by GE ImageQuant Las 4000 mini (GE Healthcare). All Western blot analyses were performed in biological triplicates.

Bioinformatics. Various proteins from Progenesis QI software were further analyzed through bioinformatics platforms such as STRING, which is a database for predicted signaling networks and protein interactions. Ontological analysis of the dysregulated genes was analyzed using the UniProt database to classify functions.

Statistical analysis. Data are expressed as mean±SD (standard deviation) of three independent observations. Statistical significance was calculated using a two tailed unpaired Student's *t*-test. A *p*-value of 0.05 or less was considered significant in this study.

Results

Cytotoxic effect of nicotine on A549 lung cancer cells. To elucidate the cytotoxic effect of nicotine, A549 cells were treated with various concentrations of nicotine and cell viability was examined by the MTT assay as shown in Supplementary Figure 1 (https://bc.cri.or.th/anticanres/supplementary.pdf). According to the results of the MTT cell viability assay, nicotine reduced cell viability in a time- and concentration-

dependent manner. The IC_{50} concentration of nicotine was determined to be 16, 12 and 7 mM for incubation periods of 24, 48 and 72 h respectively. Non-cytotoxic concentrations were selected for further studies.

Nicotine induces invasion of A549 lung cancer cells. Non-cytotoxicity concentrations were used to determine the invasive effect of nicotine on A549 cells using Boyden chambers assays. The invasive capacity of nicotine-treated A549 cells was significantly increased in a dose-dependent manner compared to that of untreated A549 cells (Figure 1). Compared to untreated cells, the highest effect of nicotine was observed at 5 nM and 10 nM by 2-fold increase (Figure 1B). Five nM nicotine was chosen as a reference concentration to study the effect of prolonged nicotine exposure.

Prolonged nicotine exposure enhances the invasive capability of A549 lung cancer cells. An invasive subpopulation of the A549 lung cancer cell line was selected to investigate the effect of prolonged nicotine exposure as illustrated in Supplementary Figure 2 (https://bc.cri.or.th/anticanres/supplementary.pdf). Results showed that an invasive subpopulation of A549 cells could be selected using Boyden chambers assays (Supplementary Figure 3A and B). The invasive capacity of sublines was gradually increased during subpopulation selection as compared to A549 parental cells by 1.25-fold to 2.6-fold (Supplementary Figure 3C and D). The invasive capacity of the nicotine-treated subline (NicoA549-L5) was significantly increased by 2.6-fold as compared to A549 parental cells.

Label-free quantitative proteomic analysis of untreated and nicotine-treated A549 cells in monolayer culture system. To determine whether metastasis-related proteins were altered by nicotine treatment, the label free quantitative proteomics was employed. The differential effect of nicotine on global protein expression was performed by comparing the protein profiles between nicotine treated and untreated A549 cells. The study was performed by LC-MS/MS with three replicates to ensure reproducibility. Differential protein expression was determined by calculation using Progenesis QI software version 3.1. The samples from the parental A549 and NicoA549-P5 were analyzed. Our investigation showed that 55 proteins from the monolayer system passed the cut off criteria of ANOVA p-value ≤0.05 and fold change greater than 1.5. Out of the total identified proteins affected by nicotine treatment, 27 proteins were up-regulated and 28 proteins were down-regulated in nicotine-treated A549 P5 cells compared to untreated parental cells, as shown in Supplementary Tables I and II (https://bc.cri.or.th/anticanres/supplementary.pdf). The protein with the greatest increase of 3.2-fold was lamina-associated polypeptide 2, isoforms beta/gamma (TMPO) whereas the protein with the greatest decrease of 2.6-fold was protein arginine N-methyltransferase 2 (PRMT2). Heat shock-related 70 kDa protein 2 (HSPA2), cathepsin D (CTSD), heterogeneous nuclear ribonucleoproteins C1/C2 (HNRNPC), legumain (LGMN) and heat shock protein HSP 90-alpha (HSP90AA1) showed high increase of 2.02 to 2.99 fold, whereas 40S ribosomal protein S10 (RPS10), plasminogen activator inhibitor 1 RNA-binding protein (SERBP1), carbonic anhydrase 12 (CA12), putative high mobility group protein B1-like 1 (HMGB1P1), ras-related protein Rab-10 (RAB10) and serine/arginine-rich splicing factor 4 (SRSF4) showed a 2.02-2.47 fold decrease. Legumain, heat shock-related 70 kDa protein 2, heat shock protein HSP 90-alpha, protein disulfide-isomerase A3 and profilin-1 were selected to validate expression using western blot analysis.

Functional and interaction analysis. The up-regulated and down-regulated proteins from Supplementary Tables I and II were analyzed by UniProt database to classify functions as shown in Supplementary Figure 4 (https://bc.cri.or.th/anticanres/supplementary.pdf). Proteins were classified as follows: transcription, metabolic process, response to stimulus, translation, mRNA processing, protein transport, nucleosome assembly, ion transport, proteolysis, apoptotic process, protein targeting, and others, with the major group being involved in transcription at 18.18%. The protein-protein interactions of 55 proteins with 1.5 up-and downregulation fold change were analyzed by the STRING database and were used to predict functions using the GO pathway. Forty four out of 55 proteins showed good interaction as shown in Figure 2. Three interesting clusters were involved in regulation of metabolic process (24 proteins), regulation of gene expression (20 proteins), protein transport (22) and binding proteins (36 proteins). Some proteins were involved in many functions such as LGMN, HSPA2, HSP90AA1, profilin-1 (PFN1), HNRNPC, 40S ribosomal protein S15a (RPS15A) and peroxiredoxin-5, mitochondrial (PRDX5).

Validation of differential protein expression in untreated and nicotine treated A549 cells using western blot analysis. To confirm the expression of proteins from LC-MS/MS, LGMN, HSPA2, HSP90AA1, protein disulfide-isomerase A3 (PDIA3) and PFN1 were selected for western blot analysis. The results, shown in Figure 3A, indicate that the expression levels of all selected proteins were higher in nicotine treated A549 cells, when compared to untreated control cells, especially regarding legumain and profilin-1.

Label free quantitative proteomic analysis of untreated and nicotine treated A549 cells in the invasion system. Since label free quantitative mass spectrometry does not require large amounts of protein, we used the invaded cells from the Boyden transwell chambers to compare differential expression between

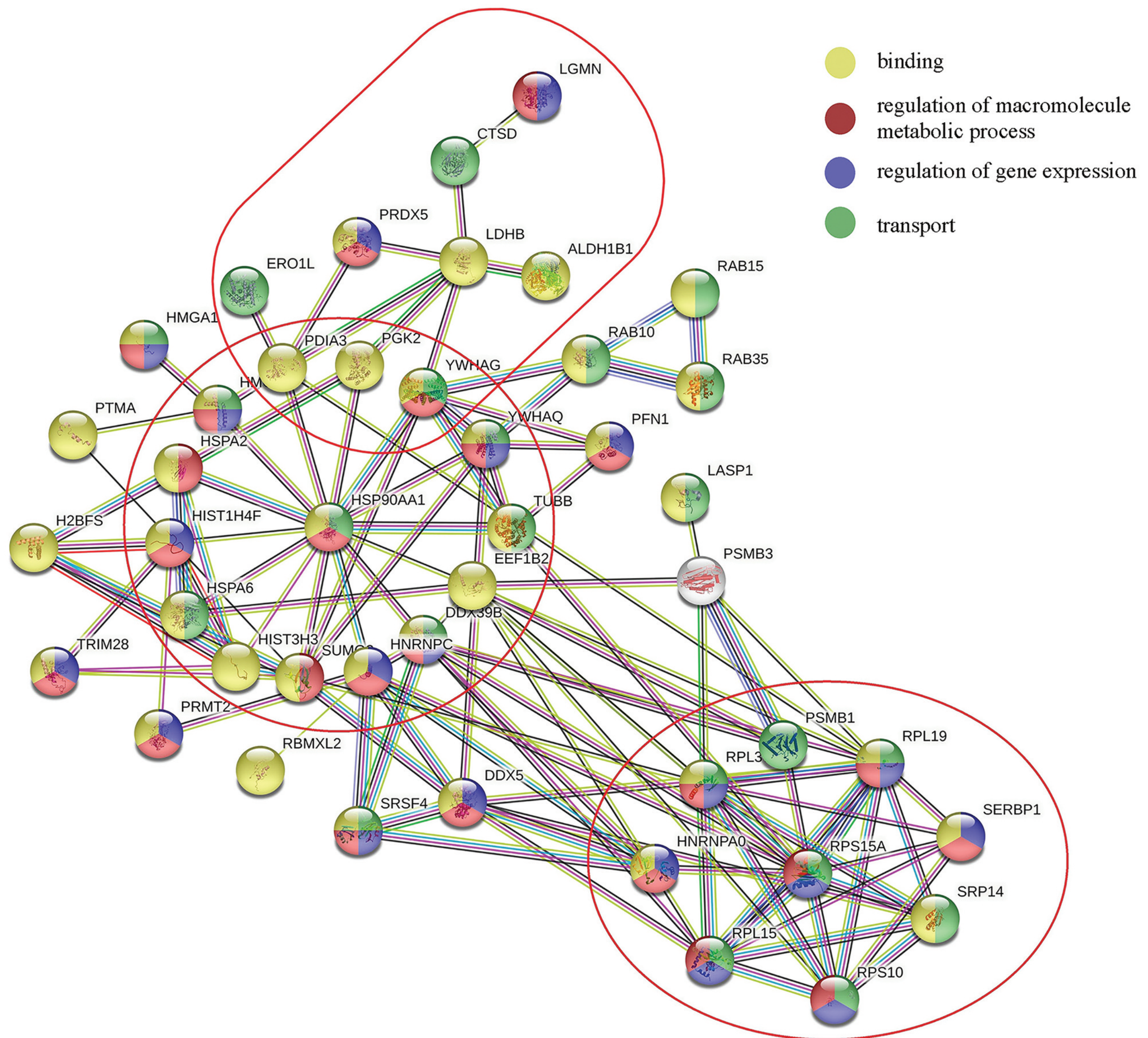

Figure 2. *Nicotine induces differential protein expression in A549 cells growing as a monolayer culture (flask). The differentially expressed proteins were used to search the STRING database to predict their protein-protein interactions.*

cells treated with nicotine and untreated cells. Progenesis QI software version 3.1 was then used to identify and analyze the differential protein expression between invaded cells with and without nicotine treatment. Our results showed that 100 proteins exhibited differential expression in the invasion system, using cut-off criteria: ANOVA p-value ≤0.05, and fold change greater than 1.25. From the 100 identified proteins affected by nicotine treatment, 24 proteins were up-regulated and 76 proteins were down-regulated in nicotine treated A549-L5 cells, compared to untreated A549-L5 cells, as shown in Supplementary Tables III and IV (https://bc.cri.or.th/anticanres/supplementary.pdf). The highest increase in expression levels of the up-regulated proteins was 2.03-fold for Obg-like ATPase 1 (OLA1), whereas the lowest expression levels in down-regulated proteins was 1.85-fold for sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (ATP2A2). The up-regulated and down-regulated proteins from Supplementary Tables III and IV were then analyzed using the UniProt database to classify functions as shown in Supplementary Figure 5 (https://bc.cri.or.th/anticanres/supplementary.pdf). Proteins were classified as follows: translation, metabolic process, mRNA processing, transcription, cell division, response to stimulus, cytoskeleton, cell migration,

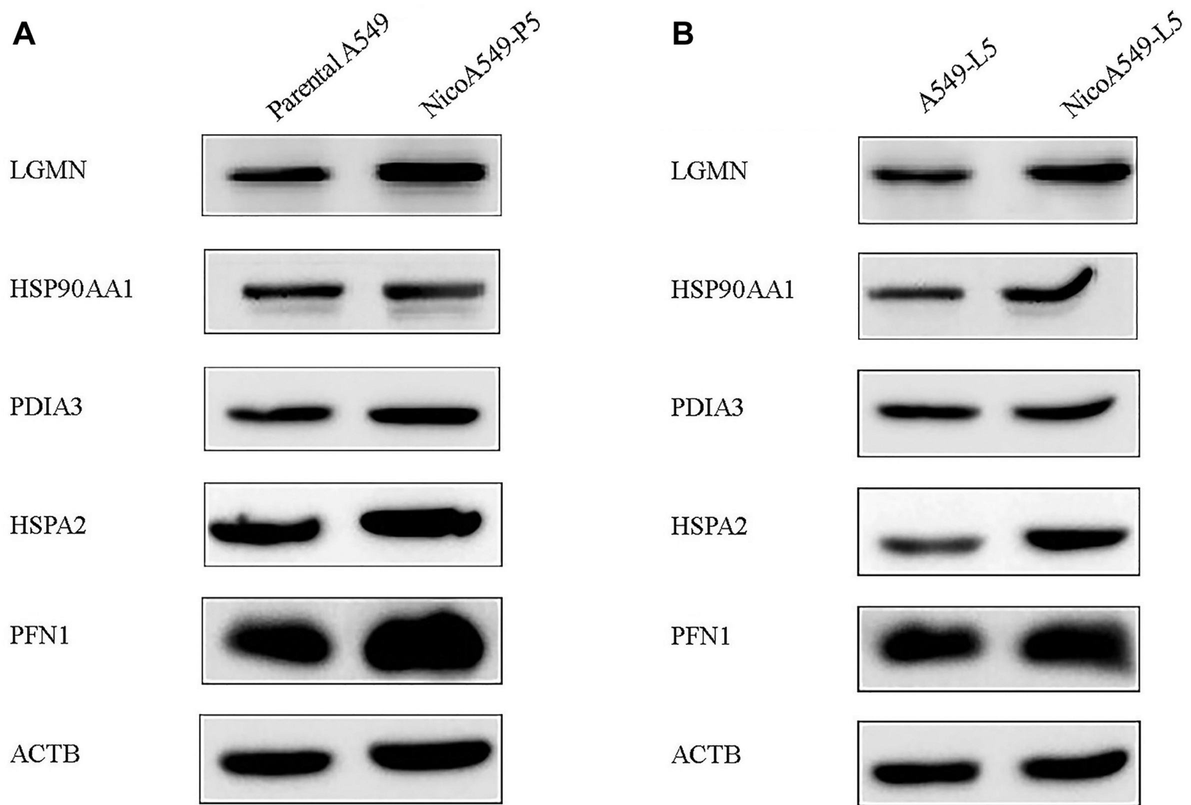

Figure 3. *The expression levels of the dysregulated proteins in cells growing in monolayers and invasion system were verified by western blot analysis. (A) Western blot showing the expression of selected proteins in untreated and nicotine treated A549 cells from monolayer system. (B) Western blot showing the expression of selected proteins in untreated A549-L5 and nicotine treated A549-L5 cells from invasion system.*

protein targeting, nucleosome assembly, apoptotic process, and others. The major proteins involved in translation were 20.00% of the proteins.

Validation of the identified proteins from LC-MS/MS from invasion system. To validate the expression levels of proteins from LC-MS/MS, LGMN, HSPA2, HSP90AA1, PDIA3, and profilin-1 (PFN1) were selected for western blot analysis. The results, shown in Figure 3B, indicate that the expression levels of all selected proteins were increased in nicotine treated A549 cells, when compared to untreated control cells.

Discussion

The aim of our studies was to investigate the effect of prolonged exposure to nicotine on non-small cell lung cancer using the A549 cell line as a model. Thus, the effect of nicotine on cytotoxicity and invasion was studied, and proteomic techniques were used to analyze differential protein expression in A549 cells treated or not treated with nicotine. The label-free quantitative proteomic technique was adapted to ensure that small amounts of protein, such as 100 ng, would be sufficient to examine changes in the levels of expression. First, the expressed proteins from nicotine-treated and untreated cells in the monolayer culture system were determined to reveal the effect of prolonged nicotine treatment on A549 cells. Then, results were confirmed by studying the proteins collected from the cells invading through the Boyden chamber. Since the A549 cells were highly invasive, sufficient proteins could be obtained from the invaded cells to show differences in expression with and without nicotine treatment. The advantage of using the Boyden chamber in our work was that there was no interference by cell proliferation, and any changes found should only involve cell motility.

Based on the results of MTT cell viability assay, the IC_{50} concentration of nicotine was determined to be 16, 12 and 7 mM for incubation periods of 24 h, 48 h and 72 h, respectively. In agreement with Tao Gao *et al.* (24), nicotine did not show any significant effect on the A549 cell line at the dose of 0.01 μM. The invasive property of A549 cells was studied using Boyden chamber assays at non-cytotoxic

concentrations. In agreement with the reports of Dasgupta *et al*. (14) and Sun *et al*. (25), our results indicated that nicotine induced A549 cell invasion in a dose-dependent manner. To gain insight into the molecular events resulting from prolonged nicotine exposure on cell invasion, an invasive subpopulation of A549 lung cancer cell line was selected. In comparison with A549 parental cells, the invasive capacity was gradually increased during subpopulation selection by 1.25-fold to 2.6-fold. Yi *et al*. (26) found that five sublines from the human lung cancer cell line CL1 showed 4-fold to 6-fold higher invasive capability through the basement membrane matrix, as compared to the parental cells. Our results agree with those of Amaro *et al*. (27), who isolated an invading subpopulation of breast cancer MDA-MB-231 cells by repeated selection, which showed greater invasive potential as compared to parental cells. Interestingly, our findings indicated that the invasive capacity of NicoA549-L5 was significantly increased by 2.6-fold, as compared to parental cells. In agreement with previous studies, prolonged exposure to nicotine enhanced the invasive and metastatic properties in numerous types of cancer cells, such as breast, lung, and oral cancers (19, 28).

The label-free quantitative mass spectrometry technique was used to compare the proteins from nicotine-treated and untreated A549 cells in the monolayer culture system. The results showed that 55 proteins were up-regulated and down-regulated. According to protein-protein interaction analysis, these proteins could be linked into 3 interesting clusters including protein transport, protein binding, regulation of metabolic process and gene expression. LGMN, HSP90AA1, PDIA3, HSPA2 and PFN1 were selected for validation by immunoblotting and all 5 proteins showed increased expression following treatment with nicotine, in agreement with the mass spectrometry results. The functions of these five proteins are proteolysis for LGMN, response to stimulus for HSP90AA1, PDIA3 and HSPA2 and metabolic process for PFN1. We further confirmed our results by label-free quantitative LC/MS/MS of nicotine-treated and untreated cells, by collecting proteins from the cells which invaded through the Boyden chamber. We identified 100 proteins from the invasion system and identified the same up-regulated proteins for validation.

LGMN or asparagine endopeptidase plays a role in cell invasion and migration (29). There have been several studies on legumain expression in cancer cells, but the expression of legumain in A549 NSCLC treated with nicotine has never been investigated. Our study is the first report showing higher expression of legumain in A549 cells when treated with nicotine, both in the monolayer system and the invasion system. Legumain is known to be expressed in various human tissues such as placenta, kidney, liver, spleen, and testis (30), and has been shown to degrade fibronectin, the key component of extracellular matrix protein (31). Legumain has also been found to cleave pro-gelatinase A into gelatinase A and plays a role in extracellular matrix remodeling and degradation to enable cell invasion and migration (29). Emerging evidence also indicates that the overexpression of legumain increases migratory activity *in vitro* and promotes invasive and metastatic phenotypes *in vivo*. Thus, legumain may be involved in tumor invasion and metastasis by degradation of extracellular matrix proteins (32).

Heat shock proteins (HSPs) are vital for the folding of newly formed proteins. HSPs are overexpressed and involved in the invasion and metastasis of various human cancers (33-35). This family of proteins are induced by many stresses including nicotine (36). Our results showed higher expression of HSPA2 and HSP90AA1 in A549 cells, when treated with nicotine in both the monolayer system and the invasion system. HSP90AA1 is a member of the heat shock protein 90 family which is present in most mammalian cells. It has been found to be related to tumor migration and invasion (37, 38). Wu *et al*. (39) reported that nicotine can increase expression of HSP90AA1 protein. Furthermore, HSP90AA1 has been shown to promote prolegumain intracellular stability and secretion. Disrupting the interaction between HSP90AA1 and prolegumain could reduce tumor metastasis (40). These results may explain our findings that both legumain and HSP90AA1 are overexpressed after the treatment of cells with nicotine. HSPA2, a member of heat shock protein 70 (HSP70) chaperon family, plays a central role in cellular motility, migration, and invasion (41, 42). Increased expression of HSP70 was found in A549 cells in response to hypoxic injury and exposure to zinc oxide nanoparticles (43, 44). The exposure to cigarette smoke has been reported as another factor inducing up-regulation of the expression of HSP70 in rat brain (45).

Protein disulfide isomerase (PDI) is a molecular chaperone which is involved in the maintenance of cellular homeostasis by supporting the folding of synthesized proteins. Increasing data indicates that PDI promotes the survival and progression of various cancers (46). The current study showed that treatment of A549 cells with nicotine led to increased expression of PDIA3 in both the monolayer and invasion systems. Our findings are consistent with several previous studies showing that PDIA3 could be involved in the induction of cancer cell migration and invasion (47, 48). PFN1 is a small actin-binding protein that regulates actin remodeling and supports cancer cell metastases (49). Our studies showed higher expression of profilin-1 in A549 cells when treated with nicotine in both the monolayer and the invasion system. Moreover, the expression of profilin-1 was significantly higher when cells growing in monolayer were treated with nicotine. Previous studies on the role of nicotine treatment on the expression of profilin-1 by Paulo *et al*. (50) showed significantly different relative abundance of profilin-1 in nicotine-treated and untreated pancreatic cells. Our

results, together with these previous reports, suggest that nicotine can induce the expression of profilin-1 and lead to metastasis in A549 cells.

In conclusion, our studies indicate that nicotine is correlated with the metastasis of lung cancer, which can be further studied by label-free quantitative proteomics to identify the precise targets of action. For the first time, our studies show high expression of legumain in A549 cells when treated with nicotine. Legumain expression appears to be associated with nicotine treatment and increased tumor invasion. Our findings suggest that legumain, heat shock protein HSP 90-alpha, heat shock-related 70 kDa protein 2, protein disulfide isomerase A3 and profilin-1 could be significant biomarkers for lung cancer therapy. Taken together, these results provide strong data to support the idea that prolonged exposure of nicotine can promote invasion in NSCLC, leading to metastasis. This study testifies to the strength of quantitative proteomics in biomarker identification, allowing the discovery of new therapeutic targets for lung cancer.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this work.

Authors' Contributions

SK conducted the study. DC and CS identified proteins using LC-MS/MS. PC provided technical assistance. CV analyzed data using Progenesis QI software. CS, CW and TR conceived and designed the study. SK, CW and CS analyzed and wrote the manuscript. PS helped with manuscript preparation. JS read and corrected the manuscript. All Authors read and approved the final manuscript.

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