

Signaling Networks in Gastric Cancer Cells Revealed by Phosphoproteomics

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Abstract

Signaling molecules in signaling pathways proceed to be phosphorylated in the signal transmission from membrane receptors to nucleus. Dysregulated phosphorylation has been implicated in a variety of human diseases including cancer. In this study, we combined SDS-PAGE prefractionation, TiO₂ phosphopeptide enrichment and LC-MS/MS technologies to identify the phosphoproteins and their regulatory sites in human gastric cancer cells. Totally 282 phosphorylation sites, corresponding to 245 unique peptides and 161 different proteins, were identified after the evaluation of ambiguous phosphosites. Among them, the phosphorylation of 109 (38.7%) sites and 36 (22.4%) proteins has not been previously reported. It was found that EGFR-ERK1/2 signaling network was mainly involved in the phosphorylation regulation of the identified proteins, suggesting that EGFR-ERK1/2 pathway plays a critical role in the network controlling gastric cancer cell process, and thus may be a drug target of anti-gastric cancer.

Keywords: Gastric cancer; Phosphorylation; Proteomics; Signaling networks; Drug targets

Abbreviations: MS: Mass Spectrometry; SCX: Strong-Cation exchange; IMAC: Immobilized Metal Affinity Chromatography; TiO₂: Titanium Oxide; DHB: 2, 5-Dihydroxybenzoic Acid; FPR: False-Positive Rate; PTM: Post-Translational Modification; GO: Gene Ontology; BP: Biology Process; MF: Molecular Function

Introduction

Reversible protein phosphorylation acts as a molecular switch controlling the regulation of signaling pathways that are involved in various biological responses, such as cell growth, differentiation, invasion and metastasis, and apoptosis (Cans et al., 2000). Dysregulated phosphorylation has been implicated in a variety of human diseases (Blume-Jensen et al., 2001). Protein kinases are the major players in protein phosphorylation. In cancer cells, mutation, rearrangement, translocation, over-expression cause constitutive activation of many protein kinases, and the hyper-activation of protein kinases has been implicated in cellular carcinogenesis (Hunter, 2000).

Human kinome contains 518 protein kinases. Among them, more than 150 protein kinases have been reported to be disease associated and thus have become major targets for anti-cancer drug development (Manning et al., 2002). Up today, 10 protein kinase inhibitors and over 100 kinase-targeted agents have been approved by the Food and Drug Administration of USA for clinical evaluation (Arslan et al., 2006; Roberts et al., 2007). The most successful examples of the kinase-inhibitory drugs are Gleevec (imatinib) targeting on BCR-ABL in leukemia and Herceptin targeting on HER2/ErbB2 in breast cancer (Arslan et al., 2006; Druker et al., 1996; Geyer et al., 2006).

In this connection, identification of phosphoproteins and their regulatory sites in cancer cells is the first step to discover the novel drug targets (Kim et al., 2005; Olsen et al., 2006; Rikova et al., 2007; Wu et al., 2007). Mass spectrometry (MS)-based phosphorylation identification has become a valuable tool in the target discovery. However, most phosphorylated proteins, especially phosphorylated protein kinases, are usually present in low abundance in cells. Protein (or peptide) fractionation

and phosphopeptide enrichment become necessary before MS analysis to identify phosphorylation proteins and sites. Commonly used fractionation techniques include SDS-PAGE separation and strong-cation exchange (SCX) chromatography (Kim et al., 2005; Wilson-Grady et al., 2008). Phosphopeptide enrichment mainly engaged affinity-based methods, using anti-phosphotyrosine antibodies, immobilized metal affinity chromatography (IMAC) and titanium oxide (TiO₂) (Beausoleil et al., 2004; Brill et al., 2004; Rush et al., 2005). With IMAC approach, the negatively charged phosphopeptides are purified by their affinity to metal ions such as Fe³⁺ or Ga³⁺. However, non-phosphorylated peptides containing multiple acidic residues are also frequently bound to metal ions (Ficarro et al., 2002). TiO₂ chromatography is an efficient alternative for phosphopeptide enrichment from peptide mixtures (Larsen et al., 2005; Thingholm et al., 2006).

Gastric cancer is the most frequent malignancy occurring in Southeast Asia and the second leading cause of cancer mortality in the world (Varon et al., 2009). Comprehensive analysis of protein phosphorylation in gastric cancer can provide important information for potential target discovery related to cellular signaling network. In the report, we applied SDS-PAGE protein prefractionation prior to TiO₂ phosphopeptide enrichment coupled with nano-LC-MS/MS to analyze the phosphorylation in SGC-7901 gastric cancer cells under serum-free culture conditions. The signaling network was mapped based upon the identified phosphoproteins. The results confirmed that the dysactivated phosphorylation events in gastric cancer were

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mainly regulated by EGFR-MAPK1/3 pathway, and suggested that dysactivated EGFR-MAPK1/3 pathway may be a drug target of anti-gastric cancer.

Materials and Methods

Cell culture and lysate preparation

Gastric cancer cells SGC-7901 were cultured in RPMI1640 medium supplemented with 10% new born calf serum at 37°C in 5% CO₂. To maintain real protein phosphorylation level, the cells were serum-deprived for 12 h, followed by treatment with 1 mM sodium orthovanadate (Na₃VO₄) and 5 mM sodium fluoride (NaF) at 37°C for 30 min. Cells were washed with ice-cold PBS three times and then lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% SDS, 1 mM DTT, 10 mM PMSF, 1 mM NaF, 1 mM Na₃VO₄, and protease inhibitor cocktail). The cellular lysate was centrifuged at 13 200 rpm for 30 min and the supernatant was collected and stored in -80°C. Protein concentration was determined by BCA assay (Pierce).

SDS-PAGE separation and in-gel trypsin digestion

Cellular lysate was separated by 10% SDS-PAGE; the gel was stained with Coomassie blue R-250 and then excised into 10 band regions. Each band was further cut into approximately ~1 mm pieces. In-gel trypsin digestion was carried out as previously described (Yan et al., 2006). Briefly, the gel pieces were destained using 25 mM NH₄HCO₃/50% ACN, and then reduced, alkylated and digested with trypsin at 37°C overnight. Digests were extracted twice using a solution of 67% ACN/2.5% TFA. The extracted peptide solutions were dried in a SpeedVac centrifuge for phosphopeptide enrichment.

TiO₂ phosphopeptide enrichment

Phosphopeptides were enriched by following the protocol provided in the ProteoExtract phosphopeptide enrichment TiO₂ kit user manual (Calbiochem). Briefly, the peptide mixtures were diluted at least 1:4 with TiO₂ Phosphobind Buffer containing 2,5-dihydroxybenzoic acid (DHB) to achieve a final volume of 200 µl. The diluted peptide mixtures were mixed with TiO₂ Phosphobind Resin, and then incubated for 10 min at room temperature. The TiO₂ Phosphobind Resin was washed two times with Wash Buffer 1 and Wash Buffer 2 in kit, respectively. Finally, the phosphopeptides were eluted from the TiO₂ Phosphobind Resin by elution buffer, and the process was repeated one more time. The collected phosphopeptide solutions were evaporated to dry in a SpeedVac centrifuge for LC-MS/MS analysis.

Nano-LC-MS/MS analysis

A linear ion trap/Orbitrap (LTQ-Orbitrap) hybrid mass spectrometer (Thermo Electron, Bremen, Germany) coupled with nano-reverse-phase liquid chromatography was used to analyze the enriched phosphopeptides by following previously described procedures with minor modifications (Pan et al., 2008). Briefly, the purified phosphopeptides were loaded on a C₁₈ reverse phase column (100 µm i.d., 10 cm long, 5 µm resin from Michrom Bioresources, Auburn, CA) using an autosampler. The peptide mixtures were eluted with a 0-40% gradient solution (Buffer A, 0.1% formic acid, and 5% ACN; Buffer B, 0.1% formic acid and 95% ACN) over 180 min and were then online

detected in LTQ-Orbitrap mass spectrometer. The analysis was performed in a data-dependent mode in which acquisitions were automatically switched between MS and MS/MS. In each cycle, a full MS scan was carried out in the Orbitrap, followed by 5 MS2 scans for 5 most intense ions in the LTQ. When an ion has a neutral loss peak at -98.00, -58.00, -49.00, -38.67, -32.67, -24.50 Da in the MS2 scan and it is one of the 5 most intense ions in the MS2 spectrum, the ion was further selected for MS3 scan.

Phosphopeptide identification and phosphosite validation

Peak lists were produced by DTASuperCharge V 1.31 (SourceForge), and the derived peak lists were searched against a real and false IPI human database (V3.56) (containing 153,078 human protein entries) using the Mascot 2.2.04 search engine (Matrix Science, London, UK). The search conditions were set as follows: Precursor ion mass tolerance was 10 ppm, and fragment ion mass tolerance was 0.5 Da, tryptic specificity was applied with a maximum of two missed cleavages, Carbamidomethylation was as fixed modification, Oxidation (M), Phospho (ST), and Phospho (Y) were as variable modifications.

To establish a cutoff score threshold for a false-positive rate (FPR) of less than 1% (p<0.01), all spectra and all sequence assignments made by Mascot 2.2.04 were further processed and validated with the MSQuant 1.5 software for post-translational modification (PTM) score analysis. Filtering criteria applied to all phosphopeptides were used as previously described

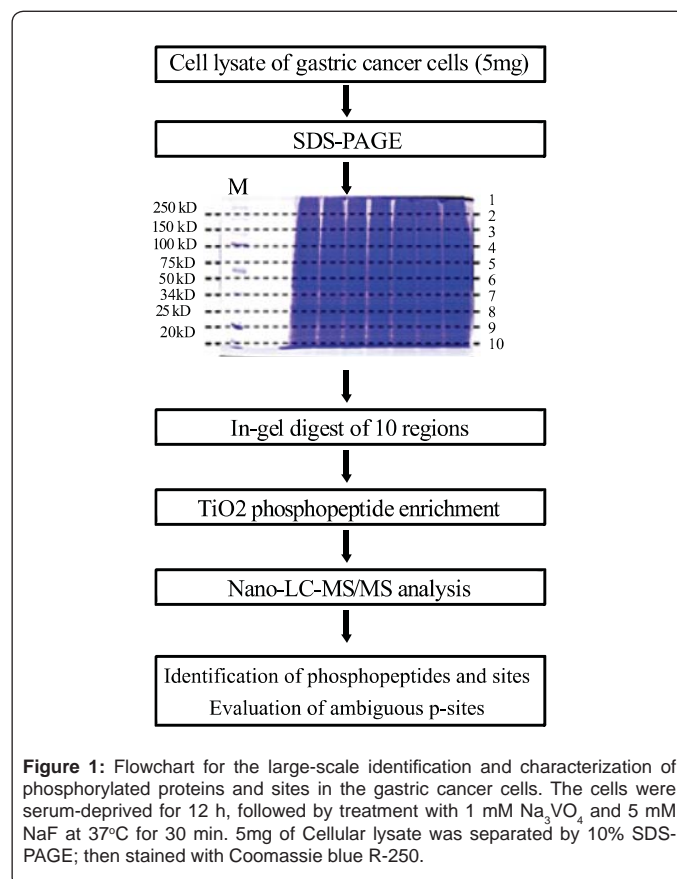


Figure 1: Flowchart for the large-scale identification and characterization of phosphorylated proteins and sites in the gastric cancer cells. The cells were serum-deprived for 12 h, followed by treatment with 1 mM Na₃VO₄ and 5 mM NaF at 37°C for 30 min. 5mg of Cellular lysate was separated by 10% SDS-PAGE; then stained with Coomassie blue R-250.

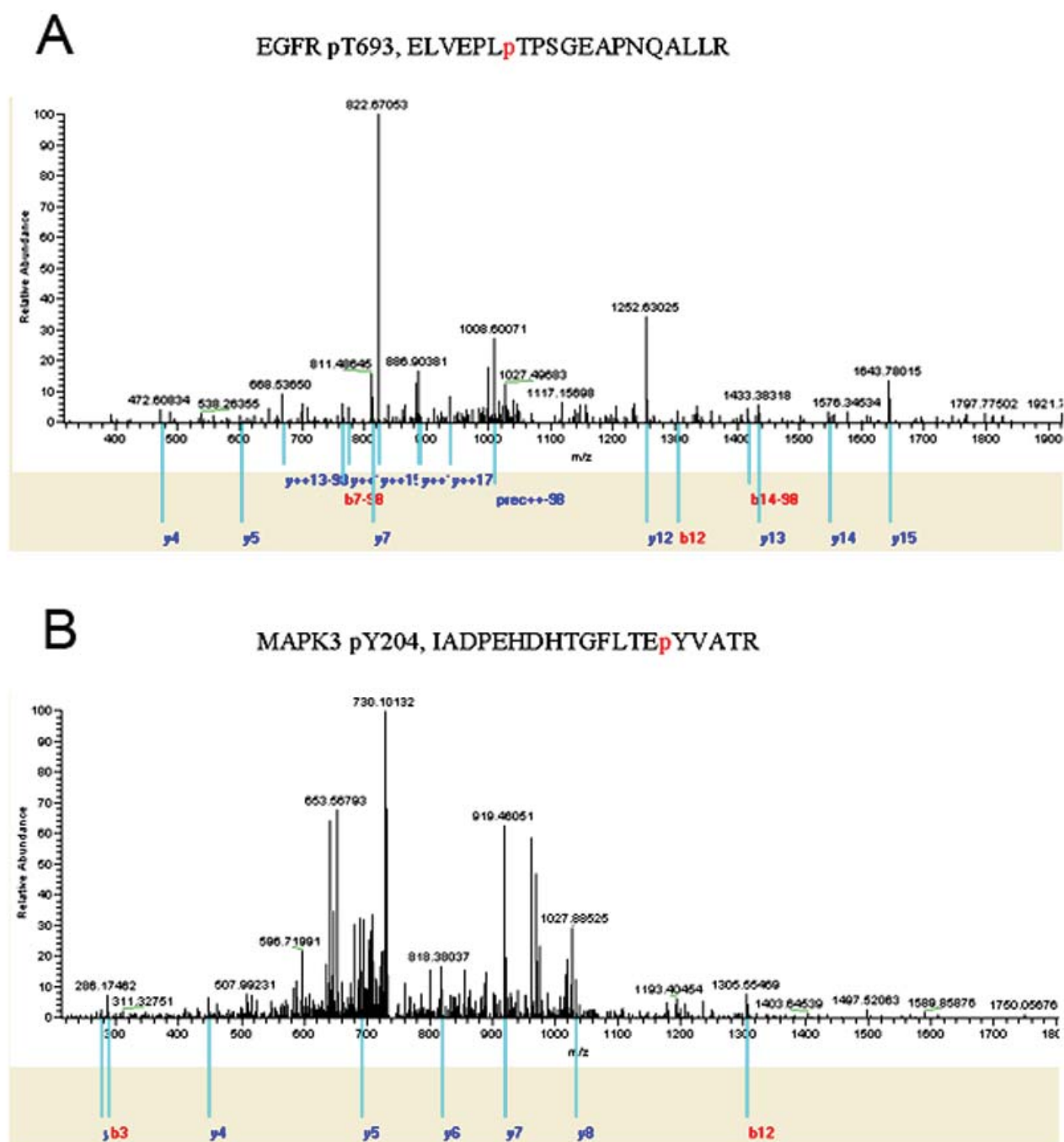


Figure 2: Representative spectra of Ser/Thr phosphopeptide ELVEPLpTPSGEAPNQALLR in EGFR protein (A) and Tyr phosphopeptide IADPEHDHTGFLTEpYVATR in MAPK3 protein (B). In the MS/MS analysis, b and y ions of various peptides were detected and the localization of the phosphorylation sites were determined by PTM scores in MSQuant software analysis as described previously (Olsen et al., 2006). The matched b and y ions for a given phosphopeptide sequence are colored red and blue, respectively.

(Olsen et al., 2006): (1) Peptide score threshold was 13, (2) PTM score threshold was 14, (3) the sum of peptide score and PTM score was higher than 38. The estimated FPR based on the decoy database search was less than 1%. And all spectra of these phosphopeptides were further confirmed by manual interpretation of MS/MS ion spectra using the criteria as

previously described (Macek et al., 2007; Nichols et al., 2009).

For ambiguous phosphosites, the probabilities for phosphorylation at each site were calculated based on PTM scores (Olsen et al., 2006). Phosphorylation sites with localization probability >0.75 were reported as class I phosphosites, the probability between 0.75 and 0.25 as class II

sites. Phosphorylation sites with localization probability <0.25 were discarded.

Results and Discussion

Identification of phosphorylation proteins and sites

To minimize the phosphorylation events induced by growth factors, cytokines and hormone in serum, SGC-7901 cells were cultured in serum-free condition. Figure 1 shows the work flow for the phosphorylation analysis, in which cellular proteins were separated by SDS-PAGE and digested with trypsin, and the resulting peptide mixtures were then subjected to TiO₂ phosphopeptide enrichment, followed by nano-LC-MS/MS detection. The MS/MS spectra were searched against human IPI protein database (V3.56) containing sequences in the forward direction and in the reverse direction by using Mascot search engine for phosphorylation assignment.

We identified 282 phosphorylation sites, mapping to 245 unique peptides and 161 different proteins at a FPR of less than 1%. The entire dataset containing the phosphorylation proteins and sites identified in the study is summarized in supplementary Table S1 (http://life-health.jnu.edu.cn/phospho/Supplementary_data.xls). Figure 2 shows the representative MS/MS spectra for phosphosites-containing peptides in the detection; all other MS/MS spectra are available via the hyperlinks provided in Table S1. To precisely assign the ambiguous phosphosites within a peptide, the probabilities of phosphorylation at each site were calculated based on PTM score as previously described. Among 282 phosphorylation sites, we could localize 188 (66.7%) phosphosites with high confidence as class I phosphorylation site. Around 65.3% of the phosphoproteins identified here were found to be singly phosphorylated, and other fractions were either doubly (25.7%), triply (7.8%), or more highly (1.2%) phosphorylated. It must be pointed out that, more than 600 phosphosites were firstly assigned before ambiguous phosphosite checking, indicating that the evaluation of ambiguous sites was necessary for the accuracy of the phosphoproteomic analysis.

Characterization of phosphoproteins

To find out novel phosphorylated proteins and sites, peptide sequences for phosphorylation sites were adjusted to ±6 aa from the central position and then were matched to the Phosphosite database, the most comprehensive database of phosphorylation data. The matching results are available in the supplementary Table S2 (http://life-health.jnu.edu.cn/phospho/Supplementary_data.xls). Some 61.4% of phosphorylated sites identified here have been found in other studies, suggesting that our phosphorylation site information was credible. The remaining 109 phosphorylation sites (38.6%) and 36 phosphorylation proteins (22.4%) detected in this work have not been described in the Phosphosite database. These newly discovered phosphorylation sites and proteins may implicate new functions for these molecules, and their biological roles in gastric carcinogenesis warrant further investigation.

Each phosphoprotein was searched against the Uniprot database (release in March 3, 2009) and analyzed with Gene Ontology (GO). The subcellular location data of 92 (57.1%) phosphoproteins were annotated in Uniprot database or 107

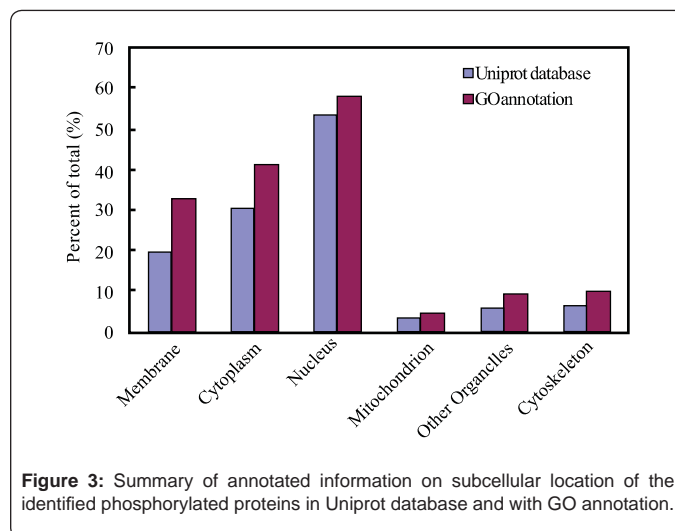


Figure 3: Summary of annotated information on subcellular location of the identified phosphorylated proteins in Uniprot database and with GO annotation.

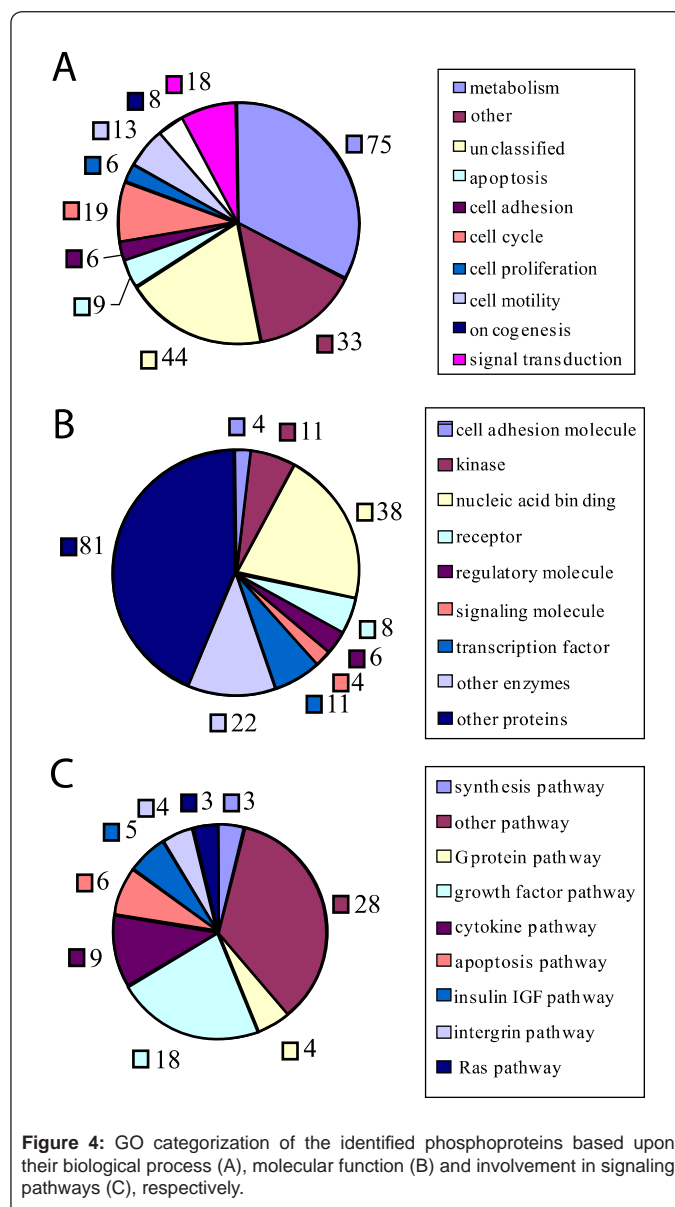


Figure 4: GO categorization of the identified phosphoproteins based upon their biological process (A), molecular function (B) and involvement in signaling pathways (C), respectively.

(66.5%) in GO. The classification diagram displayed in Figure 3 showed that the most abundant groups were cytoplasmic and nuclear proteins. Interestingly, quite a lot of membrane phosphoproteins were listed in Uniprot database (18, 19.6%) and in GO annotation (35, 32.7%). The likely reason is that we used high-stringent lysis buffer containing 2% SDS detergent to easily lyse cellular membrane, instead of low-stringent detergent NP-40, Triton X-100, urea or sodium to extract proteins from cells. In addition, 3 identified phosphoproteins were annotated as secretive proteins in Uniprot database, suggesting that the change of phosphorylation level of proteins in cellular secretion or body fluid may be used as cancer biomarkers. Many identified phosphoproteins can be categorized into different subcellular locations, and for this reason, the sum of the proteins listed in the different compartments is more than 92 in Uniprot database and 107 in GO annotation, respectively.

A GO analysis was used to classify the functions of these identified phosphoproteins in cell process by Panther classification system (<http://pantherdb.org>). Totally 142 (88.2%) identified phosphoproteins were annotated in the classification system. Among them, 107 (75.4%) phosphoproteins were annotated for their biology process (BP), 108 (76.1%) for their molecular function (MF), and 26 (18.3%) for their involvement in pathways. Figure 4 demonstrates the category diagrams. Among the pathway annotation, our identified phosphoproteins MAPK1 and MAPK3 were found to implicate in 19 different signaling pathways, including apoptosis, Ras pathway, EGF pathway, FGF pathway, insulin IGF pathway, integrin and interleukin pathways, PDGF pathways, TGF-beta, Toll receptor and VEGF pathways. Furthermore, the MAPK 1/3 upstream kinase MAP3K2 and their terminal effector kinase RPS6KA3 identified here were also found to be involved in these signaling

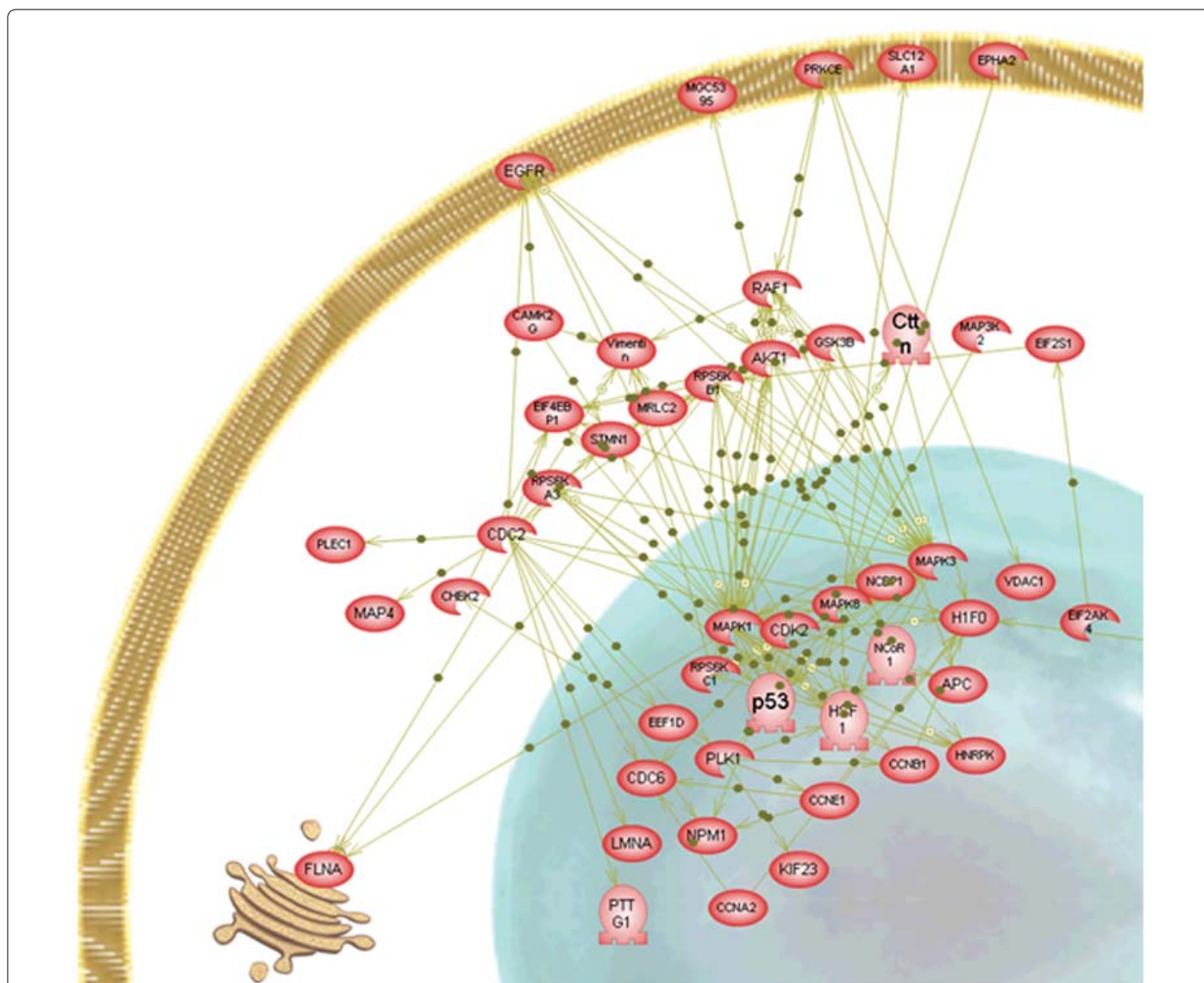


Figure 5: The phosphorylation regulation networks of the identified phosphoproteins mapped by Pathway Studio program. The relationship between these identified phosphoproteins and various signal molecules were mapped only based upon their phosphorylation regulation. These phosphorylation regulations have been reported by other literatures.

pathways. The data suggest that MAPK1/3 (ERK1/2) signaling pathway may play a critical role in gastric cancer cellular process concerning cell proliferation and anti-apoptosis.

Discovery of protein kinase motifs

Phosphorylation of protein kinases usually represents their activation state, and is directly associated with the switch of signal transmission in the cell. Phosphorylation specificity of protein kinases on downstream substrates typically depends on the amino acid sequence surrounding the target phosphorylation site. To discover protein kinases that were involved in the phosphorylation of the identified phosphosites, the Uniprot IDs of our identified phosphoproteins were simultaneously submitted to the Scansite program to search for specific kinases involved in the specific phosphosites identified (Obenauer et al., 2003). Totally, 32 phosphosites (11.4%) were

found to be phosphorylated by 12 different protein kinases at highest stringency level. Furthermore, most of the 32 sites were phosphorylated by protein kinases Akt, GSK3, ERK1 (MAPK3), and CK2, suggesting that these protein kinases were dysactivated in gastric cancer based on the fact that gastric cancer cells were cultured in serum-free medium.

Mapping of signaling networks

Reversible phosphorylation plays a switch role in the regulation of signal transmission from cellular membrane receptor to nucleus. We used Pathway Studio software to map the signaling networks involved in our identified phosphoproteins. Pathway Studio software can dynamically create and draw protein interaction networks and pathways based upon information from the reported literature. All interactions mapped by Pathway Studio are linked to the original references.

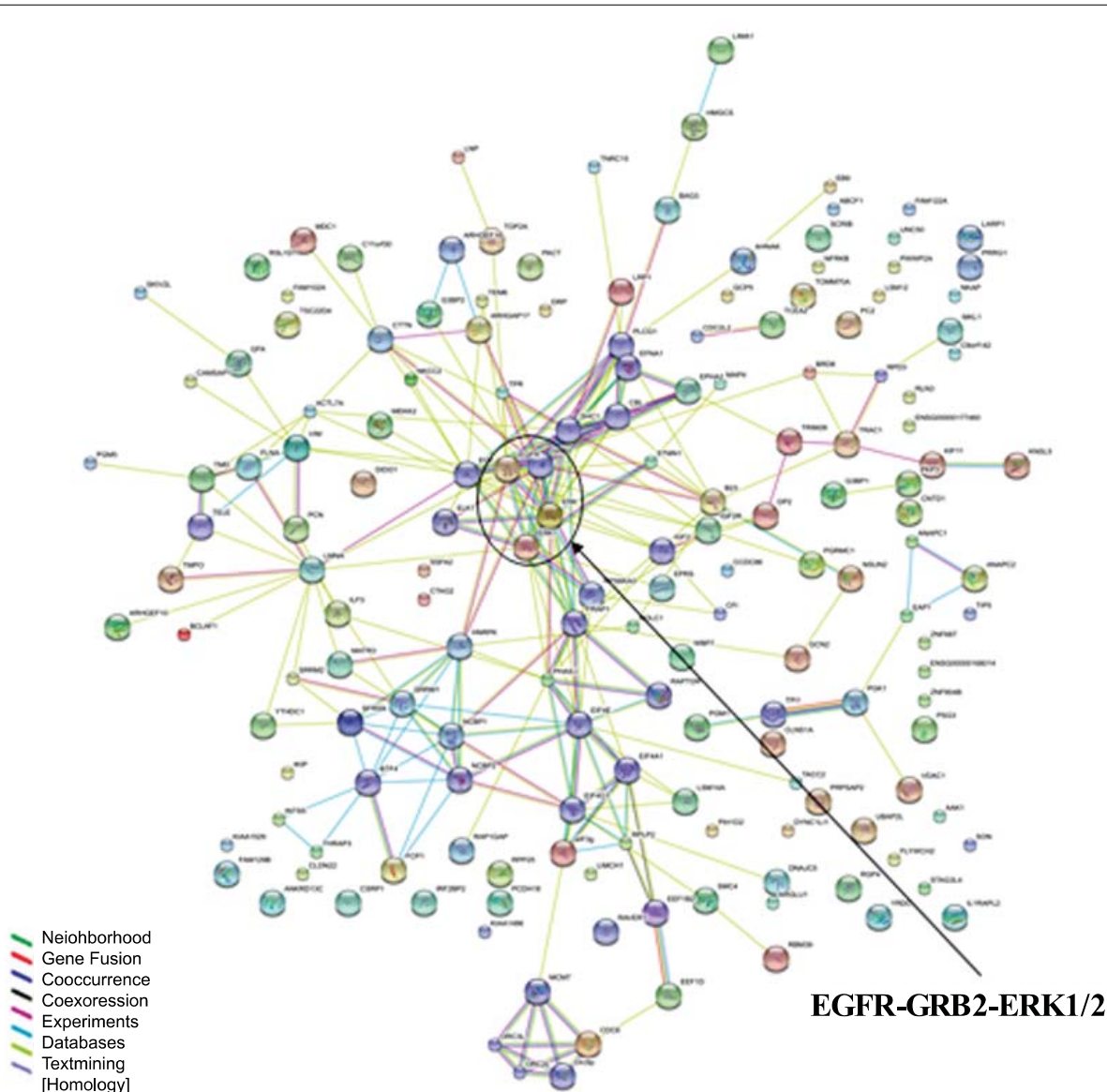


Figure 6: The protein-protein interaction networks of the identified phosphoproteins predicted by STRING program. The following sets of STRING were employed: organism, required confidence (score), interactions shown were "homo sapiens", "medium confidence (0.400)", "no more than 20 interactions", respectively, and the other parameters were default settings.

The promodification (mainly phosphorylation) regulation of protein kinases and phosphatases on our identified phosphoproteins was analyzed by Pathway Studio. Figure 5 shows that the identified phosphoproteins mainly involved in the EGFR, MAPK1/3 (ERK1/2), Akt signaling pathways. The phosphorylation regulation networks can be viewed in our webpage <http://life-health.jnu.edu.cn/phospho/Gastric-phospho-pathway/Phospho-regulation-gastric.html>. The signaling pathways have been reported to be associated with the carcinogenesis of many cancers including gastric, liver, lung, breast, nasopharyngeal and prostate carcinomas (Patel et al., 2009; Sundaram, 2006).

We then analyzed the relationship between these identified phosphoproteins and various signal molecules based upon phosphorylation regulation, protein-protein interaction, and direct regulation. The results showed that most of our identified phosphoproteins involved in EGFR, MAPK1/3, p53 signaling pathway that is known to regulate cell proliferation and anti-apoptosis in various cancer cells (Patel et al., 2009; Sundaram, 2006). This interaction network can be viewed in our webpage <http://life-health.jnu.edu.cn/phospho/Gastric-phospho-pathway/gastric-pathway.html>, and all the original references about the regulation and interaction are available in the webpage. These data further supported the notion that MAPK1/3 is a critical signal molecule serving as a signal node in gastric cancer cells.

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) is another system for mapping protein-protein interaction networks (Jensen et al., 2009). We used the system to further analyze the protein-protein interaction networks of our identified phosphoproteins. As shown in Figure 6, most of the 161 identified phosphoproteins involved in the protein-protein interaction networks via direct interaction, and the interaction networks can be viewed in our webpage http://life-health.jnu.edu.cn/phospho/Figure_6.tif. Notably, EGFR, MAPK1/3, and their signal adaptors GRB2, SHK1 were found to be signal nodes in the networks, again suggesting that EGFR-MAPK1/3 pathway plays a crucial role in the biological process of gastric cancer cells.

Taken together, both Pathway Studio and STRING analysis showed that our identified phosphoproteins can be functionally categorized into EGFR-MAPK1/3 cascades. *Helicobacter pylori* infection has been implicated in gastric pathogenesis via the mechanism of dys-activation of the EGFR and MAPK pathways (Ding et al., 2008; Yan et al., 2009). And MAPK 1/3 (ERK 1/2) was also constitutively phosphorylated in gastric cancer cells (Wu et al., 2008). In this study, we identified that MAPK1 at Tyr-187, MAPK3 at Tyr-204, MAP3K2 at Ser-163, Ser-164, and EGFR at Thr-693 were phosphorylated in the gastric cancer cells. Previous studies have shown that the phosphorylation at these sites represents the activation of these protein kinases (Li et al., 2008; Meloche et al., 2007), indicating that EGFR-MAPK1/3 signaling pathway was hyper-activated in gastric cancer. Collectively, our data suggest that the EGFR-MAPK1/3 pathway plays a critical role in gastric pathogenesis, and that EGFR and MAPK1/3 may be the therapeutic targets of anti-gastric cancer.

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