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RESEARCH ARTICLE

Global phosphoproteomic effects of natural tyrosine kinase inhibitor, genistein, on signaling pathways

Guang-Rong Yan[1], Chuan-Le Xiao[1]*, Gui-Wei He[1], Xing-Feng Yin[1], Nan-Peng Chen[1], Ya Cao[2]**, and Qing-Yu He[1]*

[1] Institute of Life and Health Engineering, and National Engineering and Research Center for Genetic Medicine, Jinan University, Guangzhou, P. R. China
[2] Cancer Research Institute, Xiangya School of Medicine, Central South University, Changsha, P. R. China

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Genistein is a natural protein tyrosine kinase inhibitor that exerts anti-cancer effect by inducing G2/M arrest and apoptosis. However, the phosphotyrosine signaling pathways mediated by genistein are largely unknown. In this study, we combined tyrosine phosphoprotein enrichment with MS-based quantitative proteomics technology to globally identify genistein-regulated tyrosine phosphoproteins aiming to depict genistein-inhibited phosphotyrosine cascades. Our experiments resulted in the identification of 213 phosphotyrosine sites on 181 genistein-regulated proteins. Many identified phosphoproteins, including nine protein kinases, eight receptors, five protein phosphatases, seven transcriptical regulators and four signal adaptors, were novel inhibitory effectors with no previously known function in the anti-cancer mechanism of genistein. Functional analysis suggested that genistein-regulated protein tyrosine phosphorylation mainly by inhibiting the activity of tyrosine kinase EGFR, PDGFR, insulin receptor, Abl, Fgr, Itk, Fyn and Src. Core signaling molecules inhibited by genistein can be functionally categorized into the canonial Receptor-MAPK or Receptor-PI3K/AKT cascades. The method used here may be suitable for the identification of inhibitory effectors and tyrosine kinases regulated by anti-cancer drugs.

Keywords:
Cell biology / Genistein / Phosphorylation / Signaling pathway / Stable isotope labeling by amino acids in cell culture / Tyrosine kinase

1 Introduction

Reversible protein phosphorylation plays a crucial role in the regulation of signaling pathways that control various biological responses, such as cell growth, differentiation, invasion and metastasis and apoptosis. Phosphorylation dysregulation has been implicated in various diseases including cancer. Protein phosphorylation is regulated by a balanced activity of protein kinases and protein phosphatases [1, 2]. The human kinome is composed of over 518 protein kinases (http://kinase.com), more than 150 of the protein kinases were reported to be disease associated [3, 4]. Most protein kinases phosphorylate serine and threonine residues, but a subset of protein kinases selectively phosphorylates tyrosine residues. These include 90 protein tyrosine kinases (PTKs) and 107 protein tyrosine phosphatases (PTPs). PTKs can be further divided into two main subgroups: receptor tyrosine kinases (RTKs) and non-RTKs. The RTK subgroup contains EGFR, PDGFR, FGFR and insulin receptor, whereas non-receptor PTKs (32 members)

Correspondence: Professor Qing-Yu He, Institute of Life and Health Engineering, and National Engineering and Research Center for Genetic Medicine, Jinan University, Guangzhou 510632, P. R. China
E-mail: tqyhe@jnu.edu.cn
Fax: +86-20-85227039

Abbreviations: ER, estrogen receptor; **PTK,** protein tyrosine kinase; **PTP,** protein tyrosine phosphatase; **pY,** anti-tyrosine phosphorylation; **RTKS,** receptor tyrosine kinases; **SILAC,** stable isotope labeling by amino acids in cell culture

*These authors contributed equally to this work.
**Additional correspondeing author: Professor Ya Cao, E-mail: ycao98@public.cs.hn.cn

comprise nine subfamilies, Src, Csk, Ack, Fak, Tec, Fes, Syk, Abl and Jak [2, 5, 6]. PTKs have become a major focus of anti-cancer drug development. The best known examples include tyrosine kinase inhibitor Gleevec (imatinib) targeting on BCR-ABL in leukemia and Herceptin targeting on HER2/ErbB2 in breast cancer [7–9].

Isoflavone genistein is a biologically active compound that exerts inhibitory effects on various cancer cells by inducing G2/M arrest and apoptosis [10, 11]. Because of its structural similarity to 17β-estradiol, genistein can compete with 17β-estradiol for binding estrogen receptor (ER). Genistein inhibited the activities of oestrogen agonist and antogonist through ERα- and ERβ-mediated pathways [12]. Recent studies have demonstrated that the anti-cancer effects of genistein could also be partly interpreted by its ability of inhibiting NFκB, ERK1/2 and Akt pathways, and regulating DNA topoisomerases, ribosomal S6 kinase, cell cycle proteins including cyclin B1, wee-1, CDK1 and apoptotic proteins, such as Bcl-2, Bcl-xL and BAX [13–15].

In addition, genistein is a known nature PTK inhibitor that can attenuate the growth of cancer cells by inhibiting PTKs-mediated signaling networks. *In vitro* experiments demonstrated that genistein repressed tyrosine-specific protein kinase activity of EGFR, $pp60^{v\text{-}src}$ and $pp110^{gag\text{-}fes}$ [16]. Genistein also inhibited the pro-oncogene HER2 protein tyrosine phosphorylation in breast cancer cells and delayed tumor onset in transgenic mice over-expressing HER2 gene [17]. Furthermore, genistein significantly downregulated the PTK-regulated proteins, EGFR and IGF1R, as well as the downstream substrates ERK1/2 [18]. Apart from these, most PTKs and tyrosine phosphorylation cascades mediated by genistein are largely unknown.

MS-based quantitative proteomics has become a valuable tool for comprehensively characterizing protein expression and modification. However, a limited number of tyrosine-phosphorylated proteins were identified when using IMAC or TiO_2 phosphopeptide enrichment strategy because that tyrosine phosphorylation represents merely 0.05% of all the phosphorylation sites [19]. Analysis of tyrosine phosphorylation by MS has been greatly facilitated by the development of high specific anti-tyrosine phosphorylation (pY) antibodies for selectively enriching tyrosine phosphorylation proteins or peptides by immunoprecipitation. By coupling with stable isotope labeling by amino acids in cell culture (SILAC) or iTRAQ method, this immunoaffinity enrichment approach has been successfully used to analyze EGF, HER2, ephB2, insulin and Src-initiated tyrosine-phosphorylation signaling pathways [20–26].

To understand genistein-mediated protein tyrosine phosphorylation signaling pathways, we applied high-resolution MS in combination with tyrosine-phosphoprotein immunoaffinity enrichment and SILAC technologies to globally identify the tyrosine-phosphorylated proteins and sites regulated by genistein. We discovered 181 genistein-regulated tyrosine phosphorylation proteins containing 213 pY sites, many of which were not previously reported to be involved in the anti-cancer mechanism of genistein. Literature-based functional analysis was proceeded to categorize these inhibitory PTKs and effectors. This study provided us with comprehensive information of tyrosine phosphorylation for better understanding of the anti-cancer mechanism of genistein and the method used here may be suitable for the identification of inhibitory effectors of drugs.

2 Materials and methods

2.1 Flow cytometric analysis

Gastric cancer SGC-7901 cells were seeded at a density of $2–10^5$ *per* well in 6-well plates. After overnight incubation, the cells were exposed to genistein of 0, 20, 40 and 80 μM for 48 h, respectively; or were exposed to 40 μM genistein for 12, 24, 36 and 48 h, respectively. The cells were then collected and washed with PBS twice and fixed in ice-cold 70% ethanol at 4°C overnight. Subsequently, the cells were pelleted by centrifugation and re-suspended in staining solution (50 mg/mL propidium iodide and 5 kunit/mL of RNase A) for 30 min at 4°C in the dark. The DNA content of each cell was measured directly using a Becton Dickinson FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA). At least 10 000 events were counted. The proportion of DNA was analyzed using the Cell Quest and the Modfit LT version 3.0 Software.

2.2 Cell culture and SILAC labeling

Gastric cancer SGC-7901 cells were grown in RPMI 1640 containing ''light'' ($^{12}C_6$) or ''heavy''($^{13}C_6$) lysine supplemented with dialyzed FBS at 37°C in a humidified atmosphere with 5% CO_2 for 2 wk. At about 30% confluence, the ''heavy'' labeled SGC-7901 cells were treated with 40 μM genistein for 48 h, but the ''light'' labeled SGC-7901 cells were treated with only DMSO. The cells were harvested and suspended with radio immunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 0.25% deoxycholate, 1 mM EDTA, 1 mM DTT, 10 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail). The lysate was centrifuged at 13 200 rpm for 30 min. The supernatant fractions were collected and protein concentration was determined by bicinchoninic acid protein assay.

2.3 Immunoprecipitation

Cell extracts were prepared as described previously [27]. The ''light'' and ''heavy'' lysates were mixed at a ratio of 1:1 in protein weight, and the mixed proteins were precleared with

protein-G agarose at 4°C for 1 h. Then the supernatant was incubated with two anti-pY antibodies P-Tyr-100 (Cell Signaling) and 4G10 (Upstate) (mixed at a ratio of 1:1 by antibody weight) coupled to protein-G agarose beads at 4°C overnight. Precipitated immune complexes were then washed three times with PBS buffer, and the pellet was re-suspended in 5 × SDS sample buffer and boiled for 5 min. The eluted proteins were separated by 10% SDS-PAGE. The gel was stained with Coomassie Blue and the gel lanes were cut horizontally into 22 bands for in-gel trypsin digestion. For immunoblotting analysis, the immunoprecipitates were washed with radio immunoprecipitation assay buffer three times, and finally subjected to Western blot analysis by PI3K p110, intergrin β1, 14-3-3 ζ, chk2, Abl, Src, EGFR and PDGFR antibodies, respectively.

2.4 In-gel digestion

The protein bands were destained and digested with trypsin as described previously [28]. Briefly, the gel bands were further cut into small pieces and destained in 25 mM NH_4HCO_3/50% ACN, and then incubated with trypsin at 37°C overnight to allow digestion of proteins after reduction and alkylation. The tryptic peptides were extracted, and the peptide mixtures were concentrated by SpeedVac centrifuge to dryness and re-dissolved with 2% ACN in 0.1% formic acid before LC-MS/MS analysis.

2.5 Nano-LC-MS/MS analysis

The peptide mixtures were analyzed by reverse-phase liquid chromatography coupled with LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) as previously described with minor modification [23]. Briefly, the peptide mixtures were firstly loaded on a C_{18} reverse-phase column (100 μm id, 10 cm long, 5 μm resin from Michrom Bioresources, Auburn, CA, USA) using an autosampler. The peptide mixtures were eluted with 0–40% gradient buffer solution (Buffer A, 0.1% formic acid, and 5% ACN; Buffer B, 0.1% formic acid and 95% ACN) over 180 min. The eluate was then analyzed online in the LTQ-Orbitrap mass spectrometer operated in a data-dependent mode with capillary temperature of 200°C and spray voltage of 1.80 kV. A full MS scan with m/z 350–1800 was carried out in the Orbitrap at resolution $r = 100\,000$ at m/z 400, and followed by five MS2 scans in the LTQ with Dynamic Exclusion setting: repeat count of 2, repeat duration of 30 s and exclusion duration of 90 s. MS3 was further performed if an ion has a neutral loss of −98.00, −58.00, −49.00, −38.67, −32.67 or −24.50 Da in the MS2 and the ion is one of the top five most intense ions in the MS2. Conditions with 35% normalized collision energy, activation q of 0.25 and activation time of 30 ms were applied for MS2 and MS3 acquisitions.

2.6 Phosphopeptide identification and quantatition and phosphosites validation

Peak lists for the database search were produced in the MASCOT generic format using DTASuperCharge V 1.31 (SourceForge), and the derived peak lists were searched using the MASCOT 2.2.04 search engine (Matrix Science, London, UK) against a real and false IPI human database (V3.56), respectively. The following search criteria were employed: full tryptic specificity was required; two missed cleavages were allowed; Carbamidomethylation was set as fixed modification, whereas Oxidation (M), Phospho (ST) and Phospho (Y) were considered as variable modifications. Precursor ion mass tolerances were 10 ppm for all MS acquired in the Orbitrap mass analyzer, fragment ion mass tolerance was 0.5 Da for all MS2 spectra acquired in the LTQ.

Mass spectra of identified phosphopeptides with peptide score >10 were further processed and validated with the MSQuant 1.5 software for PTM score analysis as well as quantitation [29], and all spectra of these potential and decoy phosphopeptides were confirmed by manual interpretation of MS/MS ion spectra using the criteria as described previously [30, 31]. Briefly, three filters for peptide identification were applied: (i) peptide score threshold was 10; (ii) PTM score threshold was 14 and (iii) at least three consecutive b- and/or y-ion series were required [32]. The estimated false-positive rate based on the decoy database search was $<2\%$.

For phosphopeptides with multiple potential tyrosine phosphorylation sites, the probabilities for phosphorylation at each site were calculated from the PTM scores as described previously [29]. Phosphorylation sites that were occupied with probability >0.75 were reported as class I phosphorylation sites. For class II sites, localization probability was between 0.75 and 0.25. Phosphorylation sites with localization probability <0.25 were discarded.

2.7 Western blot analysis

Protein extracts were electrophoresed on 10%-denaturing gels and then electroblotted onto PVDF membranes. The membranes were incubated with pY p-Tyr-100 and 4G10 antibodies at 4°C overnight, followed by incubation with corresponding secondary antibodies at room temperature for 2 h. The antibody-bound proteins were detected using the SuperSignal chemiluminescence system (ECL, Pierce, USA) followed by exposure to autoradiographic film.

3 Results

3.1 Effect of genistein on SGC-7901 cells

To analyze the effect of genistein treatment on cells, SGC-7901 cells were treated with different concentrations of

genistein for 48 h or with 40 μM genistein for different time, and then the DNA content was measured by flow cytometric analysis. As shown in Fig. 1, SGC-7901 cells at G2/M phases significantly increased in dose- and time-dependent manners, suggesting that genistein inhibited cell growth by inducing a G2/M arrest. Under the same conditions of genistein treatment, we did not detect significant effects of genistein on apoptosis (data not shown). This observation is consistent with previous studies showing that the induction of apoptosis needed longer time genistein treatment [33]. These results confirmed that genistein inhibited cell growth mainly due to G2/M cell cycle arrest.

3.2 Protein tyrosine phosphorylation inhibited by genistein

Genistein has been previously proven to be a natural PTK inhibitor that inhibits tyrosine phosphorylation cascades, such as EGFR, IGF1R and HER2 [16–18]. To detect other PTKs inhibited by genistein, we analyzed the effect of genistein on tyrosine phosphorylation by Western blotting using pY antibodies p-Tyr-100 and 4G10. Figure 2 shows that genistein changed the tyrosine phosphorylation patterns of the proteins in gastric cancer cell, indicating that tyrosine-phosphorylated proteins were regulated by genistein. As shown in Fig. 2, that the two different pY antibodies p-Tyr-100 and 4G10 specifically detected different tyrosine-phosphorylated proteins. This is consistent with the fact that a particular antibody is often made toward its specific cellular targets [20]. In this regards, we used both the two pY antibodies to extensively immunoprecipitate and collect the tyrosine-phosphorylated proteins.

3.3 Identification of genistein-regulated tyrosine phosphorylation

The "heavy" ($^{13}C_6$)-lysine-labeled cells were treated with 40 μM genistein for 48 h, whereas the "light" ($^{12}C_6$)-lysine-labeled cells were treated with only DMSO. Their lysates were mixed at a ratio of 1:1 by protein weight; tyrosine-phosphorylated proteins in the mixed lysates were immunoprecipitated by using two different anti-pY antibodies (p-Tyr-100:4G10, 1:1 in weight) and then separated by SDS-PAGE gels. Each gel was divided into 22 bands and subjected to in-gel digestion and analysis by LC-MS/MS. Totally 224 tyrosine-phosphorylated proteins containing 261 tyrosine phosphorylation sites were identified. To precisely assign the tyrosine phosphorylation sites within a peptide, we used PTM score to calculate the probabilities of tyrosine phosphorylation at each site [29]. More than 90% (237) of the tyrosine phosphosites were found to be class I phosphorylation site with high confidence ($p > 0.75$). Figure 3A shows a representative MS/MS spectrum for a phosphosite-containing peptide in the detection, and all other MS/MS spectra are available *via* the hyperlinks in the Supporting Information Table S1.

Since the tryptic digestion was performed after immunoprecipitation pull-down with the anti-phosphotyrosine antibodies, the ratios of all the labeled peptides can be used to quantify the tyrosine phosphorylation levels. Among the

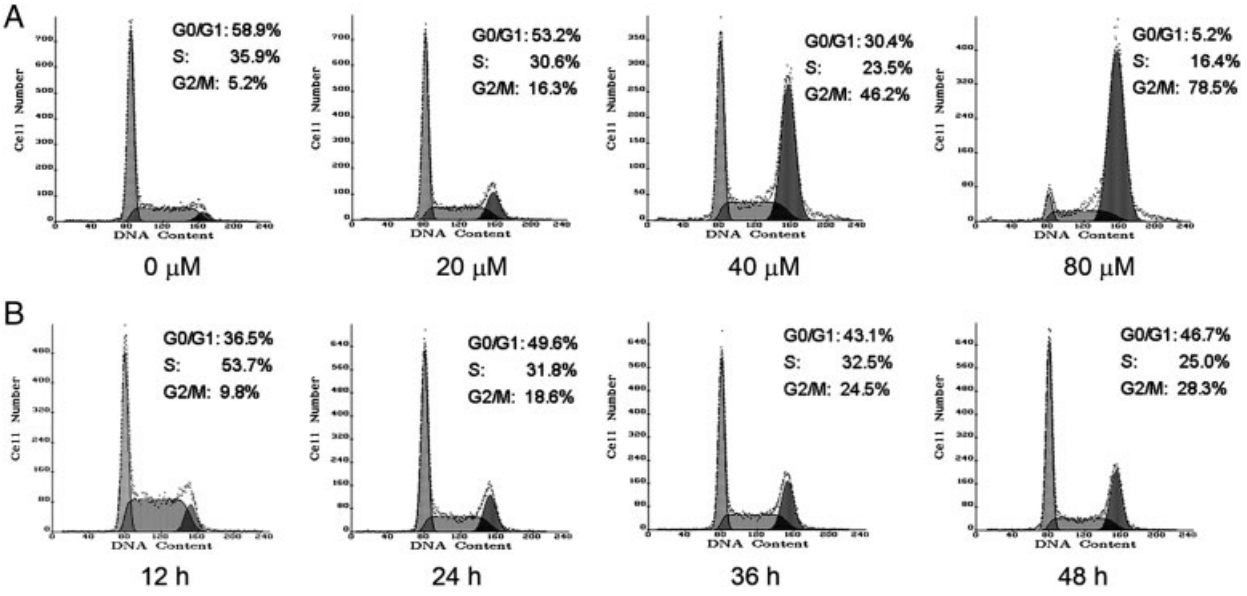

Figure 1. Genistein induced G2/M arrest of SGC-7901 cells in a dose- and time-dependent manner. (A) The gastric cancer SGC-7901 cells were exposed to genistein of 0, 20, 40 and 80 μM for 48 h, respectively; (B) The cells were exposed to 40 μM genistein for 12, 24, 36 and 48 h, respectively; and the cells were then stained by propidium iodide. The DNA content of each cell was measured using a Becton Dickinson FACSort flow cytometer.

224 tyrosine-phosphorylated proteins, 181 proteins containing 213 phosphorylation sites had 1.5-fold or higher changes in the peak intensities in response to genistein treatment. Figure 3B shows an MS example of the quantitative analyses by SILAC. Supporting Information Table S1 lists these altered tyrosine phosphopeptides with change ratios, their phosphosites and corresponding proteins; and Supporting Information Table S2 lists the mean ratio and their SD. The tyrosine phosphorylation of 174 proteins among 181 identified proteins was inhibited by genistein. This result can be understood given the fact that genistein is a natural PTK inhibitor that usually downregulates the tyrosine phosphorylation of the downstream substrates by inhibiting the activity of PTKs [16].

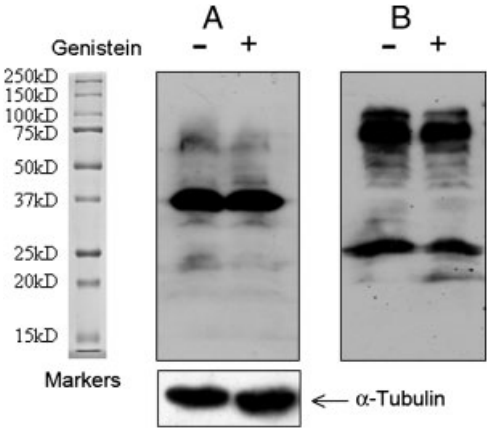

Figure 2. Protein phosphotyrosines were regulated by genistein, and anti-pY antibodies p-Tyr-100 and 4G10 specially detected by different tyrosine-phosphorylated proteins. SGC-7901 cells were treated with 40 μM genistein or DMSO (control) for 48 h, protein phosphotyrosine level was detected by Western blotting using anti-pY antibodies p-Tyr-100 and 4G10, respectively.

Interestingly, the tyrosine phosphorylation level of seven proteins was found to be stimulated by genistein. It is well known that protein tyrosine phosphorylation is regulated by both PTKs and PTPs. The tyrosine phosphorylation of PTPs increased the activation of PTPs [34]. For example, SHP2 is a ubiquitously expressed SH2 domain containing PTP. Phosphorylated Tyr-542 and Tyr-580 could be engaged to the N-SH2 and C-SH2 domains, respectively, in an intramolecular manner to stimulate SHP2 activity [35]. In this study, we found that the tyrosine phosphorylation of five protein phosphatases were inhibited by genistein, suggesting that genistein may decrease the activation of protein phosphatases by downregulating their tyrosine phosphorylation levels so that the tyrosine phosphorylation of their downstream substrates was upregulated. Furthermore, genistein could upregulate the phosphorylation of some effectors

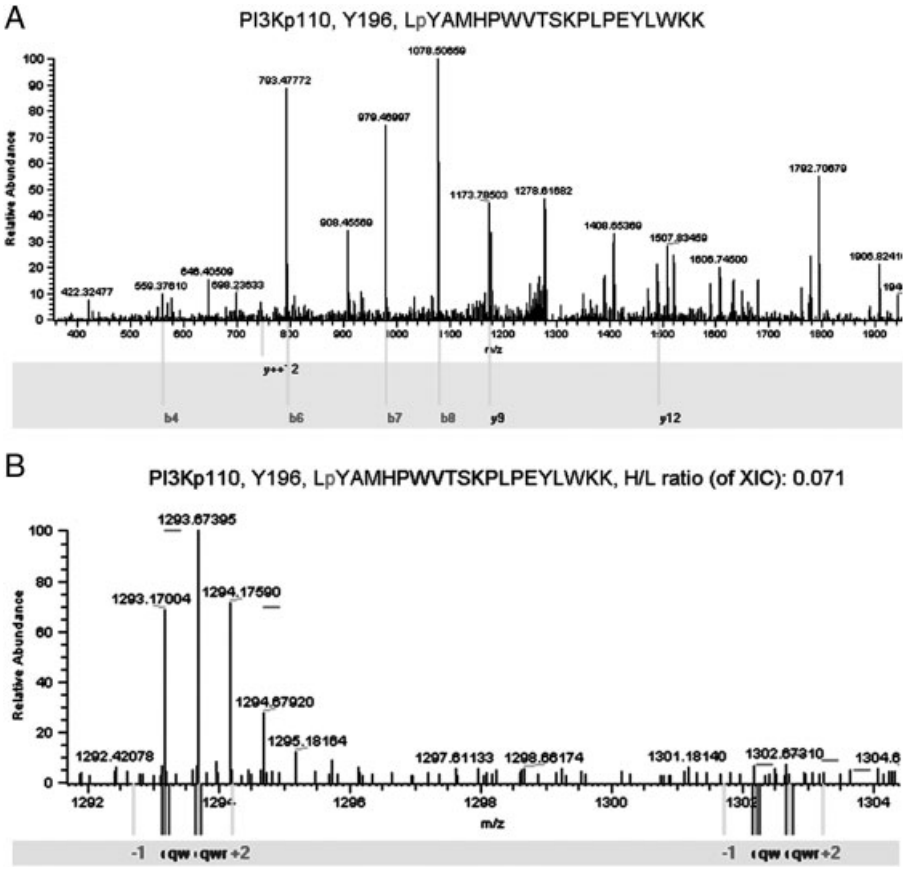

Figure 3. Representative MS/MS spectra and SILAC quantitative analysis. (A) MS/MS spectrum of the peptide LpYAMHPWVTSKPLPEYLWKK, which bears the phosphotyrosine at Y196 of PI3Kp110. In the MS/MS analysis, b- and y-ions of various peptides were detected and the localization of the phosphorylation sites was determined by PTM scores in MSQuant software analysis as described previously [28]. The matched b- and y-ions for the given phosphopeptide sequence are labeled under the x-axis. (B) A mass spectrum showing a peptide pair LpYAMHPWVTSKPLPEYLWKK from PI3Kp110 with three lysine and doubly charged ion differing by 9 Da and exhibiting an *H*/*L* (of XIC) ratio of 0.071.

possibly by indirect manners. Phosphorylation sometimes attenuates the activation of signaling molecules by negative feedback [36]. Mann and coworkers also indicated that the phosphorylation of signaling molecules not only activates but also deactivates their activation [29].

As summarized in Table 1 and Supporting Information Table S2, many signaling transducers were identified to be regulated by genistein, including nine protein kinases, eight receptors, five protein phosphatases, seven transcriptical regulators and four signal adaptors. Several of these molecules including PI3K, MAP3K12 and MAP4K3 have been shown to participate in genistein-regulated signaling pathways [13]. The majority of the identified signaling transducers have not been reported previously to be associated with genistein-regulated signaling pathways, although they may be involved in molecular functions such as G2/M progression, apoptosis, cell adhesion, DNA excision repair and cytoskeletal arrangement. These newly identified signaling transducers are potential genistein targets and effectors; defining the roles of these proteins would be particularly

Table 1. Genistein-regulated receptors, adaptors, kinases, phosphatases and transcriptical factors and their phosphorylation sites

IPI number	Protein name	Tyrosine phosphopeptide[a)]	pY site	Mean *H*/*L* ratio[b)]
Receptors				
IPI00016422	DCC	(p)YSLLGGSNLLISNVTDDDSGM(p)YTCVVT(p)YK	Y287, Y308, Y314	0.323
IPI00217561	Intergrin β	LSEGVTISpYKSpYCKNGVNGTGENGR	Y397, Y400	0.04775
IPI00235003	FAS	pYIT(p)TIAGVM(p)TLpSQVK	Y259	0
IPI00006217	GRIN2D	LVTIGSGKVFATTGpYGIALHK	Y789	0.544
IPI00013375	Orexin receptor 1	DpYLpYPK	Y39, Y41	0.048
IPI00375308	P2RX5	PYVIAKNK	Y21	0
IPI00384590	GPR97	PYWLNYEAHLMK	Y70	0
IPI00796324	GABRG3	AGpYPKEEMIYRWR	Y3	0
Adaptor proteins				
IPI00016373	Rab13	GAMGIILVpYDITDEK(p)SFENIQNWMK(p)SIK	Y88	0.233
IPI00031084	PSD2	FYAVLKGTILpYLQKDEYRPDK	Y552	0.0807
IPI00853219	RAPGEF2	PYIMIpSKD(p)T(p)TAKEVVIQAIR	Y621	0.007
IPI00152665	SH3TC1	IYpTRLATIpYHNFLLDREK	Y1287	0.075
Kinases				
IPI00000879	TXK	ALpYDFLPREPCNLALR	Y91	0.118
IPI00555727	MAP3K12	PYISREMC(p)TLV(p)SKVR	Y5	0
IPI00219510	MAP4K3	FDPPLRKEpTEPHHELDLQLEpYGQGHQGGpYFLGANK	Y358, Y366	0.202
IPI00607753	Chk2	ILKIpYSLSRFSK	Y204	0.1565
IPI00436355	PRKG1	pTpYNIILRGIDMIEFPKK	Y582	0
IPI00641400	PIK3C3	ENLDLKLTPpYKVLATSTK	Y17	0
IPI00292690	PIK3CG	LpYAMHPWVTSKPLPEYLWKK	Y196	0.071
IPI00008883	NUAK2	GTpYGKVKK	Y108	0
Phosphatases				
IPI00009066	PTPRQ	SFSILWDPP(p)TIVTGKF(p)SpYR	Y121	0.0017
IPI00295577	PTPRB	RpYLVSIK	Y1421	0.0795
IPI00019812	PPP5C	CApYQILVQVKEVLSKLSTLVETTLK	Y210	0.14425
IPI00026987	DUSP10	(p)SA(p)TIVIApYLMKHpTRM(p)TM(p)TDApYK	Y422, Y435	0
IPI00033054	CTDSPL2	KVpYADKLLNILDPK	Y355	0.059
Transcriptical regulators				
IPI00002849	ELK4	KNKPNMNpYDKLSR	Y56	0.011
IPI00020985	EP300	TAVpYHEILIGpYLE(p)YVKK	Y1414, Y1421,Y1424	0.00075
IPI00001520	SSX2	ASEKIF(p)YV(p)YMKR	Y48, Y50	0.132
IPI00007273	ZBTB1	DNHpYQINSIQKK	Y516	0.1
IPI00039808	DACH1	HLVGGLH(p)TvpY(p)TKLKR	Y232	0
IPI00181670	GRHL1	IEEPKRVLLpYVR	Y345	0.142
IPI00220289	CHD6	ALLVYCVKHpYK	Y1145	0.1967

a) Class I phosphosites are indicated by a lower case ''p'' in front of the amino acid in the peptide sequence, whereas class II phosphosites are indicated by a parenthesized ''(p)''.

b) Their SD were listed in the Supporting Information Table S2.

useful for understanding the anti-cancer mechanism of genistein.

Among the 181 genistein-regulated tyrosine phosphoproteins, 87 have not previously been identified as tyrosine-phosphorylated proteins in other context by matching to Phosphosite database, the most comprehensive database of phosphorylation data. The other 94 known tyrosine phosphoproteins contained 110 pY sites, 12 sites of which have been identified in other studies, but were not previously known to be regulated by genistein and the remaining 98 sites were firstly identified here. Supporting Information Table S3 lists the matching results.

3.4 Validation of differential tyrosine phosphoproteins

The alterations of tyrosine phosphorylation for representative phosphoproteins in response to genistein treatment were validated by immunoprecipitation with anti-phosphotyrosine antibodies (p-Tyr-100 and 4G10 were at a ratio of 1:1 by antibody weight) and Western blotting with protein-specific antibodies. As shown in Fig. 4, the tyrosine phosphorylation level of PI3 K p110, intergrinβ, 14-3-3ζ, Chk2 was downregulated by genistein, fully consistent with the results from the SILAC quantitative proteomic analysis.

3.5 Discovery of PTKs associated with genistein-regulated phosphoproteins

To find out the specific PTKs associated with the specific tyrosine phosphorylation sites on the phosphoproteins regulated by genistein, the UniProt IDs were simultaneously submitted to the algorithm Scansite to scan for related kinase motifs (http://scansite.mit.edu). The resulting kinase motifs associated with the identified phosphorylation sites are listed in Supporting Information Table S4. These data suggest that the tyrosine phosphorylation of the genistein-regulated phosphoproteins was mainly mediated by tyrosine kinases Abl, Fgr, Itk, Fyn, Src, EGFR, PDGFR and insulin receptor. Among these protein kinases, EGFR, Src and insulin receptor have been previously demonstrated to be inhibited by genistein [16–18], the other tyrosine kinases may be novel inhibitory targets of genistein. The downregulation of the tyrosine phosphorylation of Abl, Src, EGFR and PDGFR was further confirmed by Western blotting analysis (Fig. 5). In addition, 80 genistein-regulated phosphoproteins were found to contain SH2 domain and interact with signaling adaptors, Crk, Grb2, Nck, Shc, PLCγ, and PI3K p85. Signaling adaptors have been proven to link RTKs with Ras/MAPK pathway, suggesting that nearly half of our identified phosphoproteins may be involved in the RTKs-Ras/MAPK pathway by interacting with signaling adaptors.

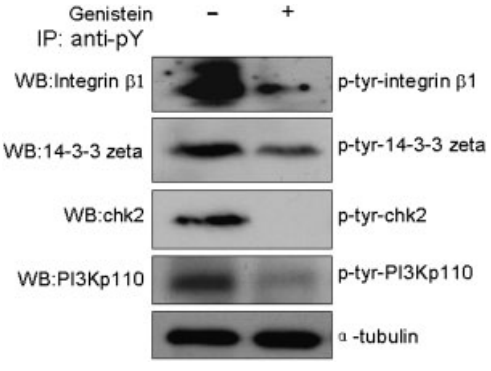

Figure 4. Validation of the SILAC-based quantitation by immunoprecipitation and Western blotting of representative tyrosine phosphoproteins. The tyrosine phosphoproteins were immunoprecipitated with anti-phosphotyrosine antibodies (p-Tyr-100 and 4G10 were mixed with 1:1 ratio by antibody weight), followed by Western blotting analysis with anti-integrin β1, 14-3-3 ζ, chk2 and PI3Kp110, respectively.

3.6 Signaling pathways involved in tyrosine phosphorylation regulation

To map genistein-regulated phosphotyrosine signaling pathways, we used Pathway Studio program to analyze the phosphorylation regulation of tyrosine kinases on genistein-regulated phosphoproteins based on post-modification (mainly phosphorylation) (http://www.

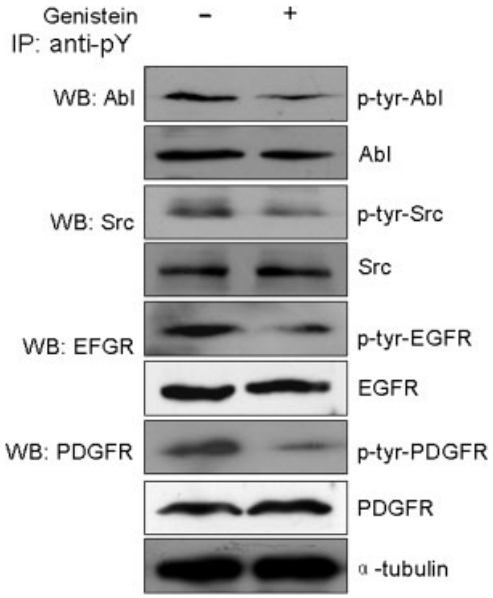

Figure 5. Genistein downregulated the tyrosine phosphorylation level of tyrosine kinases. The tyrosine phosphoproteins were immunoprecipitated with anti-phosphotyrosine antibodies (p-Tyr-100 and 4G10 were mixed at a ratio of 1:1 by antibody weight), followed by Western blotting analysis with anti-Abl, Src, EGFR and PDGFR, respectively.

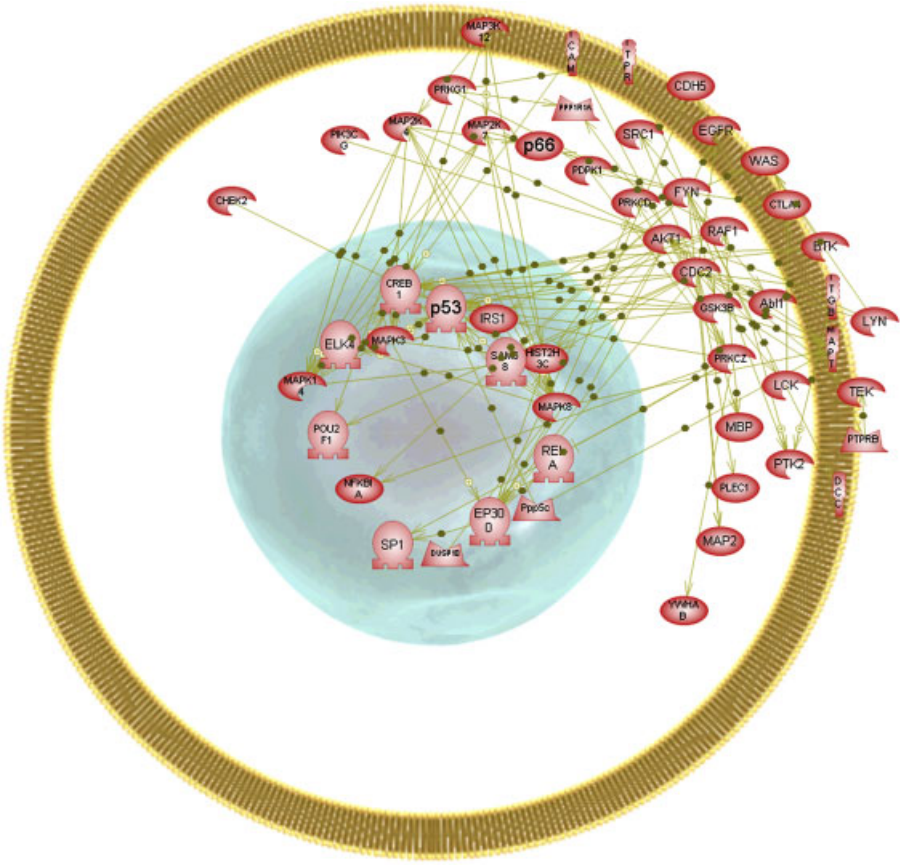

Figure 6. The phosphotyrosine signaling networks were regulated by genistein. The tyrosine phosphoproteins regulated by genistein were functionally categorized into signaling pathways.

ariadnegenomics.com/products/pathway-studio/). Pathway Studio software provides a desktop graphical user interface with capability to dynamically create and draw protein interaction networks and pathways based on information from the literature. All interactions shown by Pathway Studio were linked to the original citation where the fact was drawn. Pathway Studio analysis showed that most signaling molecules inhibited by genistein can be functionally categorized into the canonial RTK-Adaptor-Ras-Raf-MAPK-Transcriptical factor cascade, in which tyrosine kinases EGFR, Abl, Lck, Fyn and Src may be signal nodes in the phosphorylation regulation (Fig. 6), consistent with the predicted results from Scansite analysis. Among these tyrosine kinases, Src, Lck, Fyn and Fgr belong to Src family.

In the genistein-regulated phosphotyrosine signaling networks, protein kinases EGFR, Src, MAPKs, PI3K, Akt, CDC2, and transcriptical factors p53, CREB and NFκB have been demonstrated to be regulated by genistein in other studies [13, 16–18, 37, 38], suggesting that our quantitative proteomic results were credible. The remaining signaling molecules were identified for the first time to be involved in genistein-regulated phosphotyrosine signaling pathways. The signaling networks of the regulation can be viewed in http://life-health.jnu.edu.cn/phospho/p-Tyr-genistein/Tyr-phosphoregulation.html. It must be pointed out that our identified phosphoproteins were only partially included into the shown signaling pathways because nearly 50% of them were novel phosphoproteins with no information in literature for Pathway Studio software to map into the signaling networks.

4 Discussion

In canonial RTKs and G-protein-coupled receptors signaling pathways, activated receptors recruit adaptor proteins that bring to the plasma membrane guanine exchange factors for small GTPases (*e.g.* Ras, Rac and Rho), which then transmit the signal to downstream effectors. For example, Ras signals to Raf and activates MAPK cascade to regulate the activity of tanscriptical factors and terminal effector kinases [20]. The current global characterization successfully identified various signaling molecules in the canonial signaling cascade from ''signal initiator'' receptors to ''signal transducer'' adaptor proteins and protein kinases and then to ''terminal effector'' transcriptical factors and effector kinases. Genistein directly or indirectly regulated these signaling molecules in the cascade to exert its inhibition to gastric cancer cells.

In molecular level, genistein has been shown to inhibit the activity of ERK1/2 (MAPK1/3) and PI3K/AKT [13, 16]. This study specified that genistein repressed the tyrosine

phosphorylation of two PI3K isoforms PIK3CG (p110 gamma) at Tyr-196 and PIK3C3 at Tyr-17. Although MAPK1/3 was not directly identified here, we found that the phosphorylation levels of MAPK upstream kinase MAP3K12 at Tyr-5 and MAP4K3 at Tyr-358 and Tyr-366 were downregulated by genistein. These results indicated that genistein inhibited the activity of MAPK by suppressing the activity of its upstream kinase, suggesting that MAPK is the effector but not the direct inhibitory target of genistein.

It has been established that MAPK and PI3K/AKT signaling pathways were always activated by cell surface receptors such as RTKs and G-protein-coupled receptors. In this connection, we revealed that the tyrosine phosphorylation of eight cell surface receptors was inhibited by genistein. Except for FAS, seven other receptors including GRIN2D, HCRTR1, P2RX5, GPR97, DCC, GABRG3 and intergrin-β1 have been proven to be involved in the activation of the MAPK and PI3K/AKT pathways [39–44]. The architecture of the MAPK pathway usually includes a set of adaptors (Shc, GRB2, Crk, *etc.*) linking the receptors to guanine nucleotide exchange factors (Sos, C3G, *etc.*) that transduce the signal to small GTP-binding proteins (Ras, Rab), which in turn activate the core unit of the cascade composed of MAPK. We here discovered that several signal adaptors, including Ras-related protein Rab-13, PSD2, Rap guanine nucleotide exchange factor 2 and SH3TC1, were phosphotyrosine inhibited by genistein, providing a reasonable basis for linking the receptors to downstream MAPK signaling pathway.

Interestingly, tyrosine-phosphorylated receptors also stimulated the MEK/ERK, PI3K/AKT pathways *via* non-RTKs Fak, Src, Fyn, Lck or Pyk [40, 42, 43, 45]. Our Scansite and Pathway Studio analysis (Fig. 6) showed that these tyrosine kinases Abl, Lck, Fyn, Src, Fgr and Itk may play important roles in mediating genistein-regulated signaling pathways from cell surface receptors to MAPK and PI3K/AKT. These results further suggested that genistein inhibited the activation of MAPK and PI3 K by acting on the starting signaling molecules in the canonial cascade, implicating that these cell surface receptors and non-RTKs may be the inhibitory targets of genistein.

The endpoints of the MAPK cascade include the MAPK-activated protein kinases (MAPKAPK) and transcriptical factors that regulate genes involved in apoptosis, inflammation, cell growth and differentiation [4]. Firstly, we found here that seven novel transcriptical regulators and their regulatory sites were mediated by genistein, several of which have been shown to involve in the cell cycle or apoptosis, including transcriptical regulator ELK4, EP300 [46, 47]. Pathway Studio analysis further demonstrated that the phosphorylation of these transcriptical regulators was mainly regulated by MAPK and PI3K/AKT cascades (Fig. 6). In addition to our identified transcriptical regulators, Pathway Studio analysis also demonstrated that the confirmed genistein-regulated phosphorylation of transcriptical factors CREB, NFκB, STAT3 and HIF-1α was mediated by MAPK and PI3K/AKT cascades [13]. Interestingly, a co-activator of these transcriptical factors, EP300, was found to be phosphotyrosine inhibited by genistein [48–49]. The phosphorylation of EP300 has been proven to be regulated by MAPK1 (ERK2) [49], suggesting that genistein downregulated the phosphorylation of EP300 by inhibiting the activity of MAPK1.

In conclusion, the current quantitative phosphoproteomics identified a substantial proportion of the tyrosine phosphoproteins and their regulatory sites to be regulated by genistein. Besides several of the phosphoproteins that have been previously implicated in genistein-mediated signaling pathways, many other proteins may be novel inhibitory effectors and targets with no previously known function in genistein-regulated signaling networks. Most of these phosphoproteins can be functionally categorized into the canonial Receptors-MAPK or Receptors-PI3K/AKT cascades. Newly identified PTKs involved in the phosphotyrosine regulation of these phosphoproteins may be potential genistein-inhibitory targets warranted to be further explored in the future. This is the first comprehensive analysis to characterize the effectors and tyrosine kinases in genistein-regulated phosphotyrosine signaling pathways. This tyrosine-phosphoproteomics method can be used for the global identification of novel inhibitory effectors and tyrosine kinases mediated by other anti-cancer drugs.

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