Research paper

Spleen tyrosine kinase activity regulates epidermal growth factor receptor signaling pathway in ovarian cancer

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A B S T R A C T

Background: Spleen tyrosine kinase (SYK) is frequently upregulated in recurrent ovarian carcinomas, for which effective therapy is urgently needed. SYK phosphorylates several substrates, but their translational implications remain unclear. Here, we show that SYK interacts with EGFR and ERBB2, and directly enhances their phosphorylation.

Methods: We used immunohistochemistry and immunoblotting to assess SYK and EGFR phosphorylation in ovarian cancer tissues. We applied RNA-seq and phosphoproteomic mass spectrometry to investigate the SYK-regulated EGF-induced transcriptome and downstream substrates.

Findings: Induced expression of constitutively active SYK\textsuperscript{130E} reduced cellular response to EGFR/ERBB2 inhibitor, lapatinib. Expression of EGFR\textsuperscript{Y1187}, but not SYK non-phosphorylatable EGFR\textsuperscript{3F} mutant, resulted in paclitaxel resistance, a phenotype characteristic to SYK active ovarian cancers. In tumor xenografts, SYK inhibitor reduces phosphorylation of EGFR substrates. Compared to SYK\textsuperscript{KO} cells, SYK\textsuperscript{WT} cells have an attenuated EGFR/ERBB2-transcriptional activity and responsiveness to EGF-induced transcription. In ovarian cancer tissues, pSYK (Y525/526) levels showed a positive correlation with pEGFR (Y1187). Intense immunoreactivity of pSYK (Y525/526) correlated with poor overall survival in ovarian cancer patients.

Interpretation: These findings indicate that SYK activity positively modulates the EGFR pathway, providing a biological foundation for co-targeting SYK and EGFR.

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1. Introduction

Ovarian high-grade serous carcinoma (HGSC) is among the most aggressive female cancers [1]. While residual tumors after tumor cytoreductive surgery are sensitive to conventional chemotherapy, most patients eventually experience recurrence, leading to high cancer-related mortality. Extensive molecular analysis has shown that nearly all HGSC tumors have somatic p53 mutations and approximately 25% have a BRCA1/2 mutation [2]. Epidermal Growth Factor Receptor (EGFR) total protein expression and amplification studies have shown significant upregulation in HGSC [3,4]. The overexpression ranges from 30 to 98% of epithelial ovarian cancer cases, while the epithelial lining of the ovary has weak EGFR expression [3]. However, EGFR did not consistently correlate with disease aggressiveness [5].

To elucidate the mechanisms underpinning how ovarian HGSCs become resistant to chemotherapy, we previously compared the
proteomes between paired primary and recurrent post-chemotherapy HGSCs from the same women [6] to compile a list of proteins that were more frequently upregulated in recurrent tumors than in the matched primary counterparts. Among the frequently upregulated proteins, Spleen Tyrosine Kinase (SYK) was of particular interest because of its potential for therapeutic targeting with small molecular inhibitors [6]. Among SYK inhibitors, fostamatinib (prodrug of R406) has been recently approved by the US Food and Drug Administration to treat idiopathic thrombocytopenia purpura, in addition to other SYK inhibitors recently tested in phase 2 clinical trials for the treatment of hematopoietic malignancies [7–9]. Being the first oral SYK inhibitor, the selectivity of Fostamatinib is inferior to the newer class of SYK inhibitor, such as Entospletinib [10]. Compared to Fostamatinib, Entospletinib has increased selectivity for Janus kinase 2 (JAK-2), c-KIT, FMS-like tyrosine kinase 3 (FLT 3), RET, and VEGFR2 [11].

SYK is a non-receptor tyrosine kinase which initiates signaling via a number of cell surface receptors including integrins, Fc receptors, and complement receptors [12]. Several studies have suggested that SYK also participates in NF-κB-mediated transcriptional regulation and in PI3K-Akt-mTOR signaling [13–17]. Biologically, SYK activity is involved in tissue inflammation via the SYK-PI3K pathway which has been recently reported to be the critical determinant driving proinflammatory differentiation in γδT inflammatory cytokine-producing T lymphocytes [18]. Despite the fact that SYK signaling contributes to leukemogenesis, especially in the development of acute myeloid leukemia [9], its role in solid tumors is elusive and likely dependent on the tumor types and biological contexts [19–21]. Our previous study indicated that SYK activation promotes paclitaxel resistance in ovarian cancer cells, and SYK inhibition sensitizes paclitaxel-resistant tumor cells to treatment by enhancing microtubule stability [22]. Based on these results, targeting the SYK pathway is a promising strategy for enhancing tumor responsiveness to paclitaxel. We also reported that SYK directly phosphorylates cortactin and coflin, which are critically involved in the assembly and dynamics of actin filaments through phosphorylation signaling. In that report, we found that suppression of SYK activity inhibited ovarian tumor cell invasiveness by modulating actin dynamics [23].

In view of the diverse functions of SYK, we hypothesized that SYK activity may contribute to chemoresistance and recurrence in ovarian cancer through mechanisms in addition to the regulation of cytoskeletal dynamics. One of the important clues was based on evidence from our prior proteomic study suggesting that activated SYK phosphorylates several proteins with well-established roles in cancer pathogenesis [22]. Here, we show that both EGFR and ERBB2 are SYK phosphorylation substrates, a finding that has not been reported previously. It is well established that when phosphorylated, EGFR and ERBB2 translocate to nucleus, where they activate downstream genes that promote tumor progression in various types of epithelial cancers including ovarian carcinomas [24]. Thus, our findings reported here further our understanding of the regulation of the EGFR/ERBB2 signaling pathway. The identification of signaling cross-talk between the SYK and EGFR/ERBB2 pathways provides new insights into the pathobiology of ovarian cancer, which will impact future studies focusing on SYK-targeted therapy.
percentage of positive tumor cells and the intensity of staining. A composite score was calculated by multiplying the extent and intensity scores.

2.4. Cell lines and viability studies

Several ovarian cancer cell lines all with detectable SYK protein expression were included in this study: SKOV3, MPPC1, EFO21, Kuramochi, OVCAR3, KK, KOC7C, OVISE, OVA429, and OVCAR4. SKOV3 and OVCAR3 were obtained from the American Tissue Culture Center (Rockville, MD). MPPC1 cell line was previously established [28]. OVISE was obtained from the Japanese Health Science Research Resources Bank (Osaka, Japan). OVA429, EFO21, Kuramochi, KK, KOC7C and OVCAR4 were kind gifts from Dr. B. Karlan, Cedars Sinai Medical Center, Los Angeles, California, Dr. K. Nakayama, Shimane University, Japan and Dr. S. Baylin, Johns Hopkins Medicine, Baltimore, MD. All cell lines were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin, except for EFO21, which was maintained in the same medium with 20% (v/v) fetal bovine serum. The MPPC1 RT paxilax resistant cell line was maintained at maximum 25 nM paclitaxel as appropriate. Prior to experiments, the paclitaxel resistant cells were washed with PBS and replenished by regular medium for a minimum of 48 h before harvesting or treating with other conditions. SKOV3 and HeLa cells expressing SYK (WT, active mutant 130E) under the Tetracycline-off (Tet-off) inducible system were generated as previously described [22]. The inducible cells were maintained under 1–2 μg/ml doxycycline to suppress SYK expression. SYK expression was induced by removing doxycycline or treating with other conditions. SKOV3 and HeLa cells expressing SYK (WT, active mutant 130E) under the Tetracycline-off (Tet-off) inducible system were generated as previously described [22]. The inducible cells were maintained under 1–2 μg/ml doxycycline to suppress SYK expression. SYK expression was induced by removing doxycycline from the cells for at least 48 h. EGFR wild-type (EGFR WT) and mutant (EGFR MT) were constructed into the pcDNA6/V5-His A vector. The DNA fragment containing the coding region of mutated EGFR gene (EGFR MT) was synthesized by Express Mutagenesis (GenScript, NJ). The EGFR WT and EGFR MT were then transfected into SKOV3130E cells. For the 3D cell viability assays, 2000 cells/well were seeded in an ultra-low attachment plate (Corning) and spun at 300 × g for 3 min. The cell lines were then incubated in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin to promote spheroid formation. After 3 days, spheroids were treated with lapatinib (SelleckChem) at a concentration range of 1–2 μM for 1 week, after which viability was determined by the CellTiter-Glo® 3D Cell Viability Assay (Promega).

2.5. Generation of SYK knockout OVISE cells by CRISPR/Cas9 system

The pSpCas9n(BB)-2A-Puro (PX462) CRISPR/Cas9 vector from Addgene (plasmid no. 48141) was used in this study. Cloning was performed using a pair of CRISPR single-guide RNA (sgRNA) specifically targeting exon 2 of SYK: top strand-nicking sgRNA (CCGACAGGGG CAAACACCC) and bottom strand-nicking sgRNA (CAGCACCATACCC ATCGAG), which were designed and cloned as previously described [29]. OVISE cells were transfected using Lipofectamine® 3000 (Life Technologies), and positive cells were selected in the presence of 2 μg/ ml puroycin. Two to three weeks after transfection, single cell clones were isolated and screened for Cas9 expression. The knockout was verified by immunoblotting. Early passage (passage 1 or 2) knockout cells were used in experiments.

2.6. RNA isolation and reverse transcriptase-polymerase chain reaction

RNA was isolated and purified using the RNeasy Plus Mini Kit (Qiagen). The relative expression of SYK long and short isoforms in ascites tumors and cell lines was examined by reverse transcriptase followed by PCR. CDNA was synthesized using the iScript CDNA synthesis kit (BioRad), and PCR reactions were carried out using a primer set (forward, 5-AATCGGCACACAGGGGAAATG-3; reverse, 5 AGCTTTCGGTCAAGGAAAAC-3) to confirm the presence of the two transcripts. PCR products corresponding to long and short SYK isoforms were semi-quantitatively analyzed after separation by 3% gel electrophoresis, followed by densitometric analyses of band intensity using Image Lab software (Bio-Rad Laboratories).

2.7. In vitro kinase assay and ADP-Glo kinase assay

For the in vitro kinase assay, recombinant proteins active SYK (Sigma #56448), EGFR (Thermo #PV3872), ERBB2 (Thermo #PV3366), and CTNNB1 (Sigma #SRP5172) were added to a protein kinase buffer (50 nM Tris-HCl, 10 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, 0.01% Brij 35, pH 7.5) containing 100 μM ATP. The reactions were incubated at 37 °C for 1 h, and the products were separated on SDS-PAGE gels. The ADP-Glo kinase assay (Promega) was used to estimate the amount of ADP produced in the kinase reactions. Background luminescence signals arising from auto-phosphorylation or resulting from proteins alone were eliminated by subtracting signals obtained in reactions performed in the absence of SYK.

2.8. RNA isolation and RNA-seq

Isogenic OVISE ovarian cancer cell lines with SYK knockout (SYK KO) were treated for 24 h with 50 ng/ml EGFR (BD). RNA was isolated and purified using the RNeasy Plus Mini Kit (Qiagen). The quality of the total RNA was assessed using the Agilent 2100 Bioanalyzer RNA Nano Chip; RIN values ranged between 9.4 and 10.0. Paired-end index libraries were constructed using a standard protocol provided by Illumina. Sequencing was performed on the Illumina HiSeq2500 platform, in a 2 × 100 bp paired-end (PE) high output V4 chemistry configuration at GenWiz, Inc. Bioinformatics analysis was performed using Galaxy, an open access web-based program that contains a variety of next-generation sequencing analysis tools. Reads were processed and aligned to Homo sapiens reference genome build hg19 using Tophat gapped-read mapper (ver. 2.1.0). The aligned reads were processed with Cufflinks transcript assembly (ver. 2.2.1.0), and the resulting CTF files were amalgamated to UCSC hg19 RefSeq genes annotation file using Cuffmerge (ver. 2.2.1.0). Differential expression analysis was performed using Cuffdiff (ver. 2.2.1.3) to curate a list of genes whose levels of expression changes were significantly different (q-val < 0.05) between the tested genotypes.

2.9. Animal tumor xenograft studies and phosphoproteomic mass spectrometry

SKOV3IP cells were used to establish the subcutaneous tumor xenograft model. Briefly, tumor cells were mixed with an equal volume of Matrigel (BD Biosciences) and injected subcutaneously (5 × 106 cells/injection) into 4–6 week old female nu/nu mice. Tumor growth was monitored by measuring tumor diameters and calculating tumor volume. Treatment with drugs started when tumors reached an average volume of approximately 500 mm3. Three mice per group received intraperitoneal administration of vehicle control or R406 (10 mg/kg) four times in two days. DMSO vehicle control and R406 solution for injection were prepared in 10% 1-methyl-2-pyrrolidone, 40% PEG400, and 0.9% saline. All of the animal procedures were approved by the Johns Hopkins University Animal Care Committee. Upon completion of treatment, tumor xenografts were harvested, and snap frozen after washing with PBS containing phosSTOP.

Snap frozen tumor tissues were lysed using cold lysis buffer containing 6 M guanidine HCl, 10 mM Tris (2-carboxethyl) phosphine HCl, 40 mM 2-chloroacetamide, and phosphatase inhibitor cocktails 2 and 3 (Sigma) in 50 mM Tris–HCl, pH 8.5. Homogenized samples were heated to 95 °C, sonicated using 3 cycles of 30 s ON/30 s OFF sonication followed by 35, pH 7.5) containing 100 μM ATP. The reactions were incubated at 37 °C for 1 h, and the products were separated on SDS-PAGE gels. The ADP-Glo kinase assay (Promega) was used to estimate the amount of ADP produced in the kinase reactions. Background luminescence signals arising from auto-phosphorylation or resulting from proteins alone were eliminated by subtracting signals obtained in reactions performed in the absence of SYK.

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digestion strategy [30]. Immunoaffinity purification of phosphopeptides was carried out using anti-pTyr antibody, clone PT-66 (Sigma-Aldrich, MO). Phosphopeptide samples were subjected to a secondary digest with 1 μg of trypsin for 12 h at 37 °C. Samples were desalted using StageTips, dried via Speed Vac, and further enriched for phosphorylation using a PolyMAC-Ti kit according to the manufacturer’s instructions (Tymora Analytical, West Lafayette, IN).

For LC-MS analysis, phosphopeptide samples were injected into an Easy nLC 1000 chromatography system coupled online to a high resolution LTQ Orbitrap Velos Pro mass spectrometer (Thermo Scientific). The phosphopeptides were separated on a 45 cm analytical column maintained at 50 °C, which was prepared using 2.1 μm ProntoSIL C18 beads (Bischoff Chromatography, Leonberg, Germany). The mass spectrometer was operated in a full MS scan (m/z 300–1700), followed by CID fragmentation of the top 15 most abundant ions (NCE 30%). The dynamic exclusion window was set to 10 ppm, and the exclusion time was set to 60 s. The raw LC-MS data were analyzed using the MaxQuant software version 1.5.8.3 against the Uniprot Homo sapiens database (downloaded August 2017). A phosphorylation site localization score of 0.75 was used as the cut-off.

2.10. Statistical analysis

Results are shown as mean ± SEM. Statistical evaluation was performed using Graphpad Prism including two-way ANOVA and Pearson correlation analysis. p < .05 is considered significant.

3. Results

3.1. SYK activity affects EGFR and ERBB2 phosphorylation

Based on our phospho-proteomic analysis, we found that EGFR and ERBB2 phosphorylation status was related to SYK pathway activity. Thus, we determined whether SYK affected EGFR and ERBB2 phosphorylation using in vitro kinase reactions and ADP-Glo kinase assay to measure ATP consumption by ADP formation. We observed a dose-dependent increase in ADP with increasing amounts of ERBB2 or EGFR added to a reaction mixture containing a fixed amount of active recombinant SYK (Fig. 1A and B), suggesting that ERBB2 and EGFR were directly phosphorylated by SYK. The estimate-specific activities were 6.4 nmole/min/mg for ERBB2 (rate constant = 46.4 min−1, Mw 137,910 Da) and 48.5 nmole/min/mg for EGFR (rate constant = 361.2 min−1, Mw 134,277 Da). For comparison, in control experiments, we did not observe a significant increase in SYK-dependent CTNNB1 phosphorylation (Supplemental Fig. 1A). Indeed, the ERBB2 (pY877) and EGFR (pY1197 and p Y869) phosphopeptides in Figure 1A identified in our Stable Isotope Labeling by/with Amino acids in Cell culture (SILAC) data [22] contained acidic residues in the −1 or +1 position adjacent to the tyrosine residues, a motif which has been implicated as a target motif for direct SYK phosphorylation [31]. We did not perform an in vitro kinase reaction using STAT3 because it has been previously established as a direct SYK substrate [32].

Next, we determined whether SYK was able to regulate EGFR and ERBB2 phosphorylation by measuring EGFr-stimulated phosphorylation of EGFR and ERBB2 in MPS1TR cells treated with SYK inhibitors. We found that two different SYK inhibitors, R406 (700 nM) and entospletinib (700 nM), decreased the levels of EGFR-stimulated phosphorylation of EGFR (pY1197) and ERBB2 (pY877) (Fig. 1C). We also observed a corresponding reduction in the levels of p-EGFR (Y1197) and p-ERBB2 (Y877) in OVISE SYK0 cells as compared to OVISE SYKWT cells (Supplemental Fig. 1B). To corroborate these results, we also used the SYK Tetracycline-off (Tet-off) inducible system, in which SKOV3 was induced to express either wild-type SYK (SYKWT) or a constitutively active mutant; SYK130E, tagged with green fluorescent protein (GFP). The results showed that induced expression of SYKWT or SYK130E proteins increased the levels of pERBB2 (pY877) and pEGFR (pY1197) (Fig. 1D).

3.2. SYK interacts with EGFR/ERBB2, and its inhibition in vivo reduces phosphorylation of downstream EGFR substrates

To determine whether SYK interacts with EGFR/ERBB2 in a cellular system, we performed co-immunoprecipitation with pSYK (Y525/526) to detect the presence of pEGF and pERBB2. The cytoplasmic, membrane, and nuclear subcellular fractions of OVISE cells, which have high endogenous levels of SYK, were immunoprecipitated using a pSYK (Y525/526) antibody (Fig. 1E). pEGFR (Y1197) and pERBB2 (Y877) levels were high in the pSYK immunoprecipitated membrane and nuclear fractions. Similar results were observed in another SYK expressing ovarian cancer cell line KOC7C (Supplemental Fig. 1C). These data suggest that pSYK interacts with these receptor kinases predominantly at the cell membrane and nucleus.

Given that active SYK phosphorylated EGFR and ERBB2 by interacting with them, we speculated that a SYK inhibitor would suppress phosphorylation of EGFR, ERBB2, and their downstream phosphorylated substrates in vivo. Immunocompromised nude mice bearing SKOV3IP tumor xenografts were treated with R406, and tumors were collected for global phosphoproteomic analysis. R406 treatment alone did not significantly reduce tumor growth. In total, 588 unique phosphopeptides belonging to 394 unique proteins were identified (Table S1). The numbers of unique phosphopeptides in tumor with and without R406 are 510 and 567, respectively. Fewer unique phosphopeptides were identified in tumors from the R406 treated group, presumably a consequence of inhibition of SYK kinase. The downregulated phosphoproteins included SYK itself and, importantly, EGFR and ERBB2, providing evidence that the phosphorylation of these receptor tyrosine kinases was dependent on SYK activity. In addition, phosphorylation levels of several known EGFR downstream substrates decreased in tumors from animals treated with R406; these included the adapter molecule CRK [33], 1-phosphatidylinositol 3,4,5-trisphosphate phosphodiesterase gamma-1 [34], hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) [35], GRB2 associated binding protein 1 (GAB1) [35], and signal transducing adapter molecule 2 (STAM2) [36] (Table S1). These findings provide compelling evidence that SYK inhibition affects EGFR phosphorylation signaling.

3.3. SYK activity promotes tumor development through EGFR signaling

Since SYK kinase activity has been shown to promote paclitaxel resistance [22], we investigated whether SYK-induced EGFR phosphorylation might also be involved. To explore this possibility, we generated a non-phosphorylatable EGFR mutant (EGFRWT) containing phenylalanine substitutions at Y869, Y1172, and Y1197, which are the presumbly SYK-dependent phosphorylation sites based on our SILAC data [22] (Table 1). In the presence of EGFRWT, SYK130E-induced cells (-Tet, expressing the constitutively active SYK130E) exhibited increased resistance to paclitaxel (IC50 = 8.2 nM) compared to SYK0 non-induced cells (+Tet; IC50 = 2.7 nM). In contrast, expression of the non-phosphorylatable EGFRWT mutant in SYK130E-induced cells (-Tet) reduced paclitaxel resistance (IC50 = 5.4 nM) compared to cells expressing EGFRWT (Fig. 2A and B). These data suggest that EGFR phosphorylation is involved in SYK-induced paclitaxel resistance. Moreover, SKOV3 cells expressing SYK130E were more resistant to the EGFR/ERBB2 dual kinase inhibitor, laptinib (IC50, SYK130E = 150.8 μM as compared +Tet = 1.8 μM), further suggesting that SYK kinase activity enhanced EGFR signaling (Fig. 2C).

3.4. SYK activity modulates the EGFR-dependent transcription

The signaling cross-talk between SYK and EGFR raises the possibility that SYK may indirectly regulate EGFR-mediated transcription.
Therefore, we generated SYK knockout OVISE ovarian cancer cells to determine if SYK kinase activity had the capacity to modulate the EGFR-dependent transcriptome. Compared to OVISE SYKWT cells, OVISE SYKKO cells had a reduced growth rate (Fig. 2D), as observed using two independent SYKKO cells clone. We determined changes in transcript levels in well-established nuclear EGFR-regulated genes \[37–41\] in response to EGFR pathway activation by adding EGF to cultures of the isogenic pairs of SYKWT and SYKKO cells. While SYKWT cells exhibited increased expression levels of Aurora-A, cyclin D1, Myc, and COX-2 genes when stimulated by EGF, SYKKO cells exhibited no significant changes after EGF treatment, indicating a failure to activate the EGFR pathway in SYKKO cells (Fig. 2E). In contrast, overexpressing SYK130E increased the expression levels of Aurora-A, Myc and COX-2 genes.

To identify SYK-regulated genes and EGF responsive genes, we then performed RNA-seq using SYKWT and SYKKO cells with and without exogenous EGF stimulation (Fig. 3A). In the absence of EGF, we identified 1925 differentially expressed genes between SYKWT and SYKKO cells (termed “SYK-regulated genes”) (Fig. 3B). These included 1015 upregulated and 910 downregulated genes in SYKKO cells (FDR \(< 0.01\), Table S2). Expression levels of 74 genes known to belong to the EGF/EGFR- and ERBB2-signaling pathways were altered in SYKKO cells (Fig. 3C).

By comparing SYKWT cells with and without EGF stimulation, we identified 1208 genes that were altered (corresponded to “EGF-responsive genes”) (Fig. 3B). These included 1015 upregulated and 910 downregulated genes in SYKKO cells (FDR < 0.01, Table S2). Expression levels of 74 genes known to belong to the EGF/EGFR- and ERBB2-signaling pathways were altered in SYKKO cells (Fig. 3C).

Comparison of the IPA canonical pathway between SYK-regulated and EGF-responsive genes indicated significant overlapping. We found 63.3% (19/30) of the SYK-regulated pathways were also identified as being EGF responsive pathways (Fig. 3D, Table S4 and S5).

To determine how the SYK pathway affected the response of cancer cells to EGF, we deduced a list of EGF responsive genes that become differentially expressed genes between SYKWT and SYKKO cells (Table 1). These included 1208 genes that were altered (corresponded to “EGF-responsive genes”) (Fig. 3B). These included 1015 upregulated and 910 downregulated genes in SYKKO cells (FDR < 0.01, Table S2). Expression levels of 74 genes known to belong to the EGF/EGFR- and ERBB2-signaling pathways were altered in SYKKO cells (Fig. 3C).

Comparison of the IPA canonical pathway between SYK-regulated and EGF-responsive genes indicated significant overlapping. We found 63.3% (19/30) of the SYK-regulated pathways were also identified as being EGF responsive pathways (Fig. 3D, Table S4 and S5).

To determine how the SYK pathway affected the response of cancer cells to EGF, we deduced a list of EGF responsive genes that become
dysregulated under SYKKO conditions by subtracting the EGF-responsive genes in SYKKO (Table S6) from the EGF-responsive genes in SYKWT cells. Among the 1208 EGF-responsive genes found to be altered in SYKWT cells, 496 were also altered in SYKKO cells, leaving 712 genes (58.9%) as being dysregulated by SYK knockout (Fig. 3B). Through IPA-based analysis of these 712 “SYK-dependent” genes, we identified their deduced molecular and cellular functions. Functions related to cellular movement, cell death and survival, cellular assembly and organization, cellular function and maintenance, and cell development comprised the top five (Fig. 3E). The associated canonical pathways of these “SYK-dependent” genes (Table S7) included STAT3 and PTEN pathways. These data provide further evidence of the involvement of SYK in EGFR signaling.

3.5. Correlation of phospho-SYK (Y525/526), phospho-EGFR (Y1197), and phospho-STAT3 (Y705) in human ovarian carcinoma tissues

To further determine the biological significance of SYK phosphorylation of EGFR and its downstream target, STAT3, we performed immunohistochemistry on ovarian HGSC tissues, and determined the expression patterns of pSTAT3, pEGFR, pERBB2, and pCTNNB1, using their phosphorylation site-specific antibodies. The specificity of the antibodies used was either previously reported or confirmed here by immunoblotting (Supplemental Fig. 2C and 3). We observed a positive correlation between nuclear pSYK (Y525/526) and nuclear p-STAT3 (Y705) (p < .0001 and r = 0.45) (Fig. 4A and B), and a positive correlation between nuclear expression of pSYK (Y525/526) and pEGFR (Y1197) (p < .0001; r = 0.42) (Fig. 4C and D). A weaker positive correlation was observed between pSYK (Y525/526) and p-ERBB2 (Y877) (p = .049; Pearson r = 0.206) (Fig. 4C and E).

We observed intense nuclear pSYK (Y525/526) immunoreactivity, and both intense cytoplasmic and nuclear total SYK immunoreactivity (Fig. 5A). To further corroborate the subcellular localization of pSYK (Y525/526) and total SYK, we isolated subcellular fractionations and found that pSYK (Y525/526) expression was abundant in the soluble nuclear fraction of all cell lines tested (OVISE, KOC7C, Kuramochi, and EFO21), but remained relatively low in the chromatin bound fraction (Fig. 5B). In addition, pSYK (Y525/526) expression was detected in the cytoskeletal fraction in the majority of the cell lines, confirming its role in cytoskeletal organization as previously reported [22]. Among all four cell lines, cytoplasmic expression of total SYK was significantly greater than nuclear expression (Fig. 5B).

3.6. SYK isoform in ovarian high grade serous carcinomas

There are two SYK isoforms, long SYK(L) and short SYK(S), distinguished by their subcellular localizations. While previous biochemical analyses showed that SYK(L) and SYK(S) had comparable intrinsic protein tyrosine kinase activity in hematopoietic cells [42], it was unclear whether the linker region (between SH2 and kinase region) present in SYK(L) but not in SYK(S) affected binding for other proteins and SYK function. For example, as compared to SYK(L), SYK(S) had reduced binding ability to phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) [43]. In the current study, RT-PCR results showed that all 10 ovarian cancer cell lines (SKOV3, MPSC1, EFO21, Kuramochi, OVCAR3, KK, KOC7C, OVISE, OVCAR4, and OVCAR4) exhibited higher expression levels of nuclear translocatable SYK(L) than cytoplasmic SYK(S) (Supplemental Fig. 5B). We extended this study to 82 ovarian cancer samples acquired from tumor ascites (Supplemental Fig. 5C and D), and observed that ovarian carcinoma cells also predominantly
expressed SYK(L) (Supplemental Fig. 5C). SYK(S)/SYK(L) ratios were mostly less than one, with the exception of a single sample (Supplemental Fig. 5D). We detected SYK(L) expression in all samples tested, while SYK(S) was detected in only 51 (62%) of the 82 samples. These findings indicated that SYK(L) was the predominant isoform in ovarian cancer specimens, a result supporting the nuclear localization of pSYK in HGSC tissues.

3.7. Association between levels of pSYK (Y525/526) and clinical outcome in ovarian cancer patients

We next assessed the clinical significance of nuclear pSYK levels in two independent patient cohorts, one consisting of 42 primary HGSC tissues (with residual tumor <1 cm) from the Johns Hopkins Hospital, and the second consisting of 112 HGSC ascites samples collected from the Norwegian Radium Hospital. In both cohorts, tumors were divided into two groups according to median staining levels: low (including zero and H-scores below median) and high (H-score above median). We found that within the same cohort, tumors with higher levels of nuclear pSYK (Y525/526) were associated with shorter overall survival than those with lower levels of nuclear pSYK (Y525/526) \(p = 0.0074\), log-rank test (Fig. 5C). Similarly, a positive association between high nuclear pSYK (Y525/526) and lower survival rates (Fig. 5E). In contrast, total nuclear SYK levels did not impact patient survival \(p > 0.05\) (Fig. 5F).

4. Discussion

EGFR signaling is one of the most studied tyrosine kinase pathways in human cancer [3,44,45], and targeting its members has become a mainstay in precision cancer medicine [46,47]. Both EGFR and ERBB2 belong to the EGF receptor tyrosine kinase family. The EGR pathway is activated upon binding of EGF to its cognate receptor localized on the cell membrane, initiating nuclear translocation and downstream transcription regulation [48]. Their activities can also be regulated by multiple mechanisms including transcriptional upregulation by MED12 and phosphorylation by upstream kinase effectors or by collateral kinases [49]. The complexity of EGFR pathway regulation is critical for cancer cells to adapt to an ever-changing tumor microenvironment, and can explain unresponsiveness or resistance to the anti-EGFR mono-therapy. As an example, EGFR and ERBB2 have been shown to be overexpressed in epithelial ovarian cancers through transcriptional upregulation and gene amplification [3,44], but clinical trials evaluating anti-EGFR or anti-ERBB2 therapies in ovarian cancer have shown minimal clinical benefit [50]. From these perspectives, the results reported here are significant, as we demonstrate that both EGFR and ERBB2 are substrates of SYK, and have shown that SYK-mediated phosphorylation enhances EGFR/ERBB2 signaling activity. These findings identify crosstalk between SYK and EGFR/ERBB2 pathways as a new translational research focus in ovarian cancer.

EGFR tyrosines can be phosphorylated by JAK2 and by ERBB2 at Y1069 [45,51] and by c-Src at Y869, Y915, Y944, Y1092, and Y1125 [52,53]. In this study, we reported novel SYK phosphorylation target sites (EGFR sites Y869, Y1069, Y1172, Y1197 and ERBB2 sites Y877, Y1005) and accordingly add additional data to map for the signaling interactome of EGFR in cancer cells. It is of interest to note that in addition...
to EGFR/ERBB2 and STAT3, SYK also acts as an upstream kinase that phosphorylates several downstream members. This includes PIK3R1, a component of the PI3K signaling pathway, which is involved in ovarian cancer invasion and metastasis [54,55]. Thus, SYK is a molecule of potential promise for targeted therapy. The levels of active SYK in nuclei are associated with disease aggressiveness, and inactivation of SYK using small compound inhibitors can simultaneously suppress multiple signaling pathways known to be associated with advanced malignant behaviors in human cancers (Supplemental Fig. 4). The orally efficacious SYK inhibitor, fostamatinib (prodrug of R406), has been available to treat immune-mediated thrombocytopenia purpura, and another SYK inhibitor, entospletinib (GS-9973), has been tested in clinical studies for use in treating rheumatoid arthritis and hematological malignancies [10,56–59]. Both drugs are relatively safe, with mild but reversible side effects in patients. Thus, clinical studies to test these currently available drugs to treat ovarian cancer patients warrants further consideration.

In ovarian cancer cells treated with paclitaxel, our unpublished data showed that SYK mRNA is upregulated during the breakpoint after each cycle of the chemotherapy. While the expression of pSYK can be affected by other external cues mediated by phosphorylation signaling, our current data suggest that SYK overexpression during tumor recurrence is transcriptomically regulated. While SYK is conventionally considered to be a cytoplasmic non-receptor tyrosine kinase, our data also underscore the important role of nuclear SYK, whose expression levels are associated with the aggressiveness of HGSC. The long isoform of SYK transcript contains a nuclear localization signal [60], and is the predominant form associated with ovarian cancer (Supplemental Fig. 5A-D). Similar nuclear SYK immunoreactivity can be detected in gastric carcinoma [61], hepatocellular carcinoma [62], triple negative breast carcinoma [28], and in tissues and cells of lymphoid and epithelial origins [28,62–64]. These findings raise the question of why SYK is present in the nucleus. One possibility is that SYK functions in the phosphorylation of transcriptional regulators, and is thus a regulator of their transcriptional activities [65]. Supporting this view, SYK has been reported to regulate the activity of the transcriptional regulator Ikaros and its import into the nucleus [66]. Our data showed that SYK knockout resulted in changes of EGFR-regulated genes. Thus, SYK activity can also control the transcription of downstream targets of EGFR, and can impact EGF growth factor-mediated transcription in cells.

Fig. 4. Correlation of pSYK(Y525/526) with pSTAT3(Y705) and of pSYK(Y525/526) with pEGFR(Y1197) in high-grade serous ovarian carcinomas. (A) Immunohistochemical analysis of tissue microarrays containing high-grade serous carcinoma sections to detect expression of pSTAT3(Y705). The same tissues were also stained using pSYK(Y525/526) antibody (n = 95). Two representative tissues, one with low and one with high expression of pSYK(Y525/526) and of pSTAT3(Y705) are shown (scale bar 400 μm). (B) Association between nuclear expression levels of pSYK(Y525/526) and of pSTAT3(Y705) using Pearson correlation analyses in tumors. (C) Representative photomicrographs of a high-grade serous ovarian carcinoma stained with antibodies against pSYK(Y525/526), pEGFR(Y1197), or pERBB2(Y877) (scale bar 100 μm). (D) Pearson correlation between pSYK(Y525/526) and pEGFR(Y1197). (E) Pearson correlation between pSYK(Y525/526) and pERBB2(Y877).
The function of nuclear EGFR is more defined. It has been reported to act as a co-transcriptional activator for cyclin D1 [37], control proliferating cell nuclear antigen (PCNA) activity [67], and regulate DNA repair through DNA-PK activity [68]. In addition, several biological roles of the EGFR pathway in ovarian cancer have emerged. EGFR has been suggested to participate in promoting omental dissemination of ovarian cancer cells by inducing a paracrine loop between tumor and stromal compartments of the omental microenvironment [69]. It has been shown that inhibiting EGFR pathway activity in ovarian cancer cells is important to induce tumor dormancy [49] and targeted reduction of EGFR induces mitophagy and death of ovarian cancer cells through activation of MTOC2 and Akt [70]. The evidence shown in these studies provides a novel approach for pharmacological inhibition of EGFR activity as a potential treatment for EGFR-positive cancers. However, monotherapy targeting only the EGFR pathway may not provide an effective clinical benefit; rather, combination therapy that also includes targeting of the EGFR pathway could be promising [71]. Since SYK and EGFR may both be co-activated in HGSC tissues, and co-activation is associated with worse clinical outcomes, co-targeting these kinases may result in higher efficacy. In fact, dual inhibition of kinases has been proposed as an effective way to treat ovarian cancer in preclinical models [72]. From this perspective, the combination of EGFR inhibitor and SYK inhibitor may warrant further clinical investigation.

In summary, results presented in this report provide evidence that SYK phosphorylates the key members in the epidermal growth factor receptor (EGFR) signaling pathway, suggesting that SYK promotes tumor progression in ovarian cancer through multiple mechanisms in addition to modulating cytoskeletal proteins and their dynamics [22]. Our findings indicate that nuclear SYK activity contributes to disease aggressiveness in ovarian cancer by regulating the EGFR/ERBB2 signaling pathway. Efforts to unravel the intricate signaling interactions among SYK, EGFR/ERBB2, STAT3, and PI3K are needed to elucidate how SYK signaling orchestrates these pathways. It will be equally important to identify the nuclear proteins that are direct substrates of the SYK long isoform. Determining the effects of inhibiting SYK signaling in animal models and in future clinical trials will be crucial to determining the promise of targeting the SYK pathway using clinically available SYK inhibitors for effective cancer treatment.
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Authors’ contributions

Conception and design: Y. Yu, T-L. Wang, I-M. Shih; Development of methodology: Y. Yu, Y. Suryo Rahmanto; Acquisition of data: Y. Yu, Y. Suryo Rahmanto, Y-A. Shen, B. Davidson, A.N. Fader; Analysis and interpretation of data: Y. Yu, Y. Suryo Rahmanto, L. Ardighieri, A. Ayhan, B. Davidson, I-M. Shih; Writing, review, and/or revision of the manuscript: Y. Yu, S. Gaillard, T-L. Wang, I-M. Shih.

Declaration of Competing Interest

The authors report no potential conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.08.055.

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