

Spleen Tyrosine Kinase Inhibition Modulates p53 Activity

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ABSTRACT: Spleen tyrosine kinase (SYK) is a cytoplasmic enzyme that promotes survival and proliferation of B cells. SYK inhibition has shown promising results in the treatment of arthritis and chronic lymphocytic leukemia (CLL). However, in other context, it has been shown that SYK overexpression in epithelial cancer cells induced senescence in p53-dependent mechanism, which underscored its antineoplastic activity in vitro. Here, we show that SYK was induced in response of DNA damage in parallel with p53 levels. In addition, using chemical inhibitors of SYK reduced p53 levels in HCT116 and HT1080 cell lines, which underlines the role of SYK inhibition on p53 activity. Furthermore, SYK inhibition modulated the cell growth, which resulted in a decreasing in cell death. Interestingly, SYK expression showed a positive prognosis in patients with solid tumors in correlations with their survival rates, as expected negative correlation was seen between SYK expression and survival rate of patients with CLL. In conclusion, these findings demonstrate that SYK inhibition modulates p53 expression and activity in HCT116 and HT1080 cells. Reconsidering using of SYK inhibitors in clinical setting in the future should be evaluated carefully in accordance with these findings to prevent the formation of secondary malignancies.

KEYWORDS: SYK, p53, apoptosis, HCT116, HT1080

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The tumor suppression activity of p53 mediates response of damaged cells.¹ It is a barrier against cellular transformation through its regulation of proteins and genes at transcriptional and posttranscriptional levels, which results in apoptosis and/or cell cycle arrest.¹ Many factors participate in determining the cell fate decision after p53 induction. Apoptosis is an important cell response that is initiated by p53 through which cell enters program of cell death after stress.² This can be achieved through regulation of proapoptotic genes such as BCL2 family members that stabilizes p53 in damaged cells.³ Permanent cell cycle arrest or senescence is another cellular response to stress, which is initiated by p53 activation to prevent cellular transformation. During senescence, cells remain metabolically active and acquire distinguished morphological features. Premalignant tumor is characterized by the presence of senescent cells at high levels, which are disappeared after entering to malignant stage of tumor. Analysis of human tumors demonstrates an essential role of p53 in tumor suppression. For example, more than 50% of human tumors of different types harbor TP53 mutations.⁴ p53 prevents malignant transformation, it acts as a sensor of stress, such as DNA damage, oncogenic upregulation, and ribosomal dysfunction.⁵ In cells which undergo high level of stress, p53 pushes program of cell death of apoptosis or irreversible program of cell cycle arrest of senescence to prevent transformation of cells.⁵ However, in cells which are exposed to low level of stress, p53 initiates protective survival responses, such as temporary cell cycle response and DNA damage repair.² These cellular responses depend on ability of p53 to work as an activator of genes involved in apoptosis and or senescence.⁶ Therefore,

there is a demand to find out a new modulator of p53 activity to understand the p53 pathway more widely.

Spleen tyrosine kinase is an enzyme that is highly expressed in all hematopoietic tissues. It involves in development and proliferation of B cells.⁷ Normally, B-cell receptor activation stimulates downstream cascade that involves SYK activation and subsequently B-cell activation, which leads to its development or proliferation. SYK activation has an implication on allergy and autoimmunity,^{8,9} B-cell malignancies,¹⁰ and viral oncogene^{11,12}; thus, abnormal SYK signaling due to, for example, mutations can lead to the previous disorders. Therefore, kinase inhibitors have been developed to target and inhibit its abnormal stimulation. R112, is a SYK selective inhibitor, it ameliorates patients with allergic rhinitis,¹³ fostamatinib is another example of SYK inhibitor that has been tested and has showed promising results in reducing severity of arthritis in vivo.^{14,15} SYK inhibition also has been suggested to be a therapeutic target for lymphoma and leukemia. GS-9973 (Entospletinib), for example, is a selective inhibitor of SYK that has shown clinical benefit for relapsed CLL patients.¹⁶

A previous work by Bailet et al¹⁷ has shown a new role of SYK in senescence initiation in p53-dependent mechanism. This unpredicted observation has not been followed. Here, we show that SYK inhibition by entospletinib and fostamatinib downregulated p53 and its apoptotic activity after DNA damage.

Methods

Cell culture

HT1080 and HCT116 were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine



serum (Gibco) and penicillin-streptomycin (50 units/mL). About 1 μ M entospletinib (GS-9973) and 0.5 μ M fostamatinib (R788) were used to inhibit SYK in this study, and 1.5 μ M doxorubicin (Sigma-Aldrich) was added to induce DNA damage.

Immunoblot analysis

For lysate extraction, medium was removed and plates were washed once with 1 \times phosphate-buffered saline (PBS) and trypsinized, collected, and kept on ice. About 100 μ L of radioimmunoprecipitation assay buffer pH 7.4 was added and the cell pellet was suspended and incubated for 20 minutes on ice. Cells were ruptured by passing through a syringe 5 times or with sonication and centrifuged for 15 minutes at 12000 rpm, and the supernatant was transferred into tubes where 35 μ L of 4 \times loading buffer was added. Concentrations were determined using Bradford protein assay (Fermentas). About 10 to 20 μ g of total protein per sample was subjected to 10% or 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to Immobilon-P membrane (Millipore). Primary antibodies used were, anti-p53 (DO-1; Santa Cruz: sc-129), anti-SYK (N-19): sc-1077, anti- β -actin (ab8227; Abcam). A C-DiGit Scanner (Li-COR) was used for protein visualization.

Flow cytometry

To assess the percentage of cell death after SYK inhibitor addition, a propidium iodide (PI) staining followed by a fluorescence-activated cell sorting analysis was performed. Cells were split into 6-well plates and left to grow for 24 hours before treatment. Media from each sample was aspirated and collected in labeled 15-mL Falcon tubes. To wash the cells, 2 mL of 1 \times PBS was added to the plates and kept in the same tube of the media. About 1 mL of trypsin was added to the plates and cells were collected in the same tubes of the media and 1 \times PBS. The tubes were then centrifuged at 200g for 5 minutes and the pellet was then washed twice with 1 \times PBS. For fixation, 1 mL of 70% ethanol was mixed with the pellet and placed at -20°C for at least 30 minutes. Cells were taken out of the freezer and were centrifuged at 200g for 5 minutes. The cells were then washed once with 1 mL of 1 \times PBS. The pellet was resuspended in 300 μ L of PI buffer: 50 μ g/mL of PI, 10 μ g/mL RNase A, 1 \times PBS and transferred to polystyrene round-bottom tubes. The tubes were incubated for 30 minutes at 37°C in the dark. In total, 10000 events were recorded for each sample using the Becton Dickinson FACSCanto II and FACSDiva 6.0 software (Becton Dickinson) for acquisition and analysis.

Bioinformatics analysis

PPISURV¹⁸ was used to correlate survival rates in patients with cancer to the expression level of SYK. In each data set, samples were grouped with respect to expression rank of the

gene, which reflects relative messenger RNA expression level and introduces no normalization bias. The “low-expression” and “high-expression” groups are those where expression rank of the gene is less or more than average expression rank across the data set. This separation of patients into “low” and “high” groups in the data set along with survival information is next used to find statistical differences in survival outcome.

Statistical analysis

The statistical analysis of mean values and standard deviations was performed using Prism 7 (GraphPad) for Mac version. All error bars represents the standard deviation. *P* value was calculated using 2-tailed unpaired *t* test.

Results

SYK inhibition has shown an effective action against some B-cell malignancies and autoimmune diseases.^{13–16} However, it has been reported that SYK overexpression induced senescence in p53-dependent mechanism in melanoma cells.¹⁷ The tumor suppressive action of SYK has not been followed adequately; here, we show that inducing DNA damage of HCT116 and HT1080 cells that have wild-type p53 upregulated SYK expression in parallel with p53 expression. In addition, SYK inhibition with entospletinib and fostamatinib decreased p53 activation after DNA damage in both cell lines, as shown in Figure 1. This underlines the role of SYK activation in p53 activity.

After demonstrating the ability of SYK inhibition in reducing p53 levels, we studied the role of SYK inhibition in cell death. As shown in Figure 2, PI staining of HCT116 cells after adding doxorubicin, entospletinib reduced percentage of cell death as represented by sub-G₁ in the left side of the histogram comparing with the doxorubicin alone. This suggests that the activity of SYK could play a role in cell death through p53 activity.

It has been believed that SYK is an oncogene that mediates prosurvival effects of B cells.¹⁰ However, previous work by Bailet et al¹⁷ has shown a tumor suppression effect in other context. To understand the heterogeneity of SYK in cancers, we used PPISURV¹⁸ to analyze correlations of SYK expressions with survival rates of patients with different cancers. We found that as expected, high expression of SYK in patients with CLL correlated with poor prognosis (Figure 3). However, interestingly, high expression of SYK in solid tumors such as colon, breast, ovarian, cervical, and lung correlated with good prognosis. These data propose that SYK could have a dual role in cancer between proliferative and suppressive action.

Discussion

It has been shown that SYK upregulation induced senescence, and the induction depends on a functional p53.¹⁷ We have found that DNA damage of cancer cells upregulated SYK expression in parallel with p53 expression. Using SYK chemical inhibitors attenuated p53 expression after DNA damage.

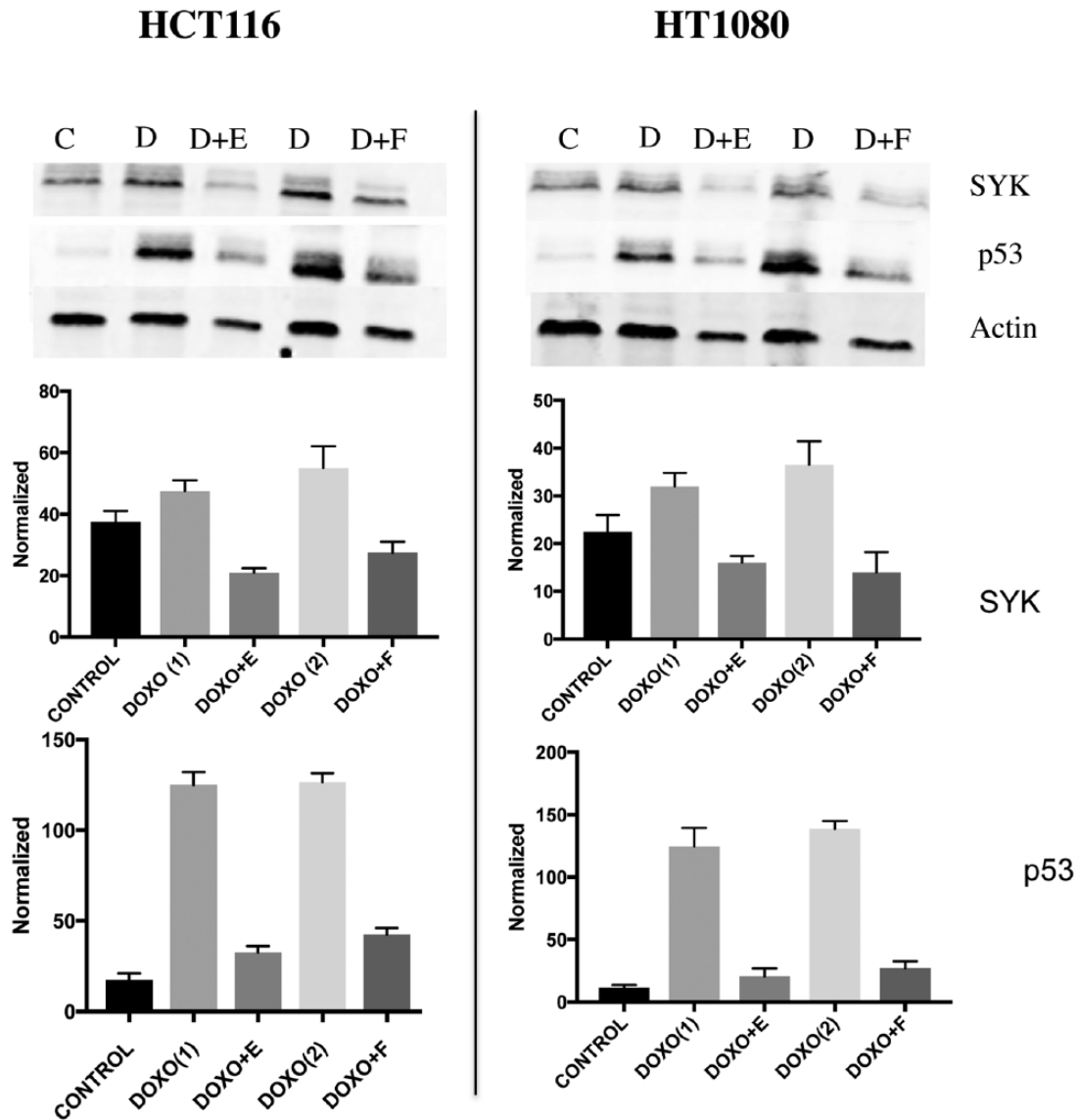


Figure 1. SYK inhibition by entospletinib (E) and fostamatinib (F) reduces p53 level (D). HCT116 and HT1080 cells were treated with 1.5 μ M doxorubicin or 1 μ M doxorubicin (D) and 1 μ M entospletinib or 0.5 μ M fostamatinib for 24 hours. Entospletinib and fostamatinib reduced p53 levels in both cell lines. Error bars showed p53 and SYK quantification from different experiments.

We have also shown a positive correlations between SYK expression and survival rates of patients with different solid tumors, and negative correlation was seen between SYK expression and survival rate of CLL patients. This could explain the tumor suppressive activity of SYK in solid tumors, which has been suggested before.¹⁷ SYK is a tyrosine kinase that shows a role in signal transduction of B cells. Normal function leads to B-cell maturation, and its inhibition by small molecules has shown a therapeutic potential in patients with allergic rhinitis¹³ and arthritis^{14,15} and also has been proposed to be a potential therapeutic target for leukemia and lymphoma.¹⁶

This study illustrates a correlation between SYK and p53 activity after DNA damage in cancer cells. However, the exact mechanism behind it is not clear; SYK could directly phosphorylate p53, that subsequently increases its stability, and SYK inhibition may affect p53 phosphorylation, results in

p53 degradation by MDM2. Another proposed mechanism is through BTK feedback loop. BTK has a direct influence on p53 activity.¹⁹ SYK is the upstream target of BTK, so SYK phosphorylation could phosphorylate BTK that results in p53 upregulation.²⁰ Another suggested mechanism could be through transcriptional regulation of p53 target genes through methylation of, for example, Set7/9²¹ and Smyd2²² or through SUMOylation of p53²³ that leads ultimately to p53 regulation. It has been reported that SYK expression induced senescence in melanoma cells.¹⁷ However, in our study we noticed cell death effects after DNA damage induction in HT1080 and HCT116 cells. This could be due to the strong effect of doxorubicin, which normally induces apoptosis rather than senescence due to high p53 expression, in addition, cell background also plays a role in cell fate decision between senescence and apoptosis.²⁴⁻²⁶

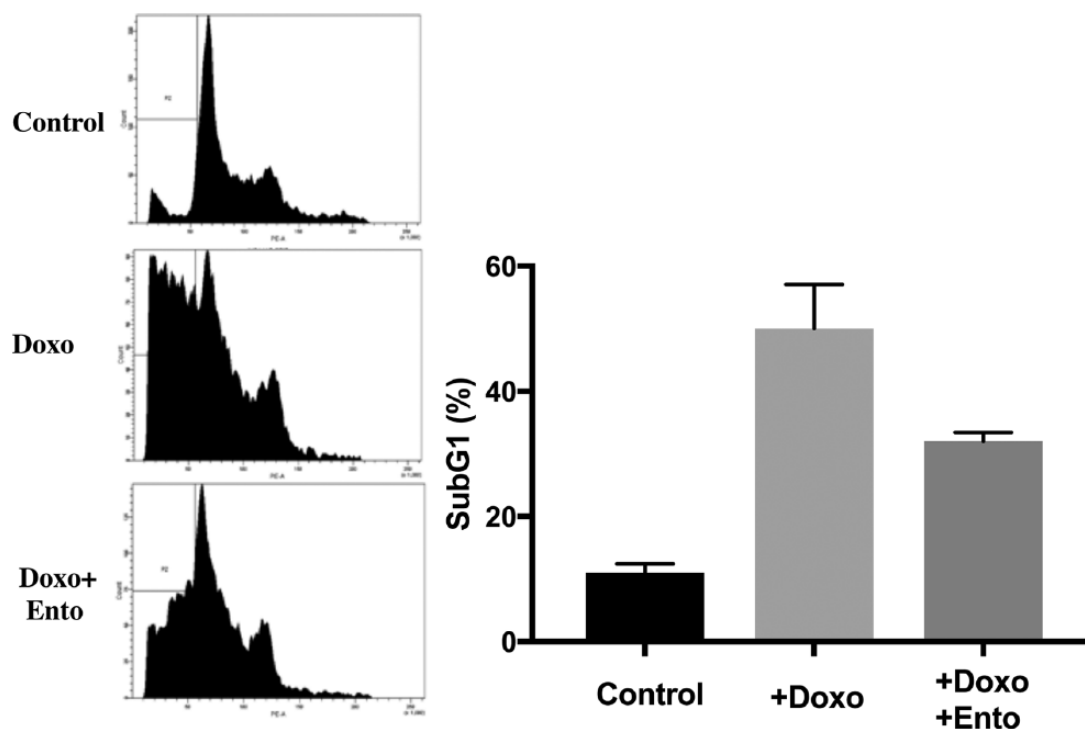


Figure 2. SYK inhibition rescues cell death after doxorubicin addition. Fluorescence-activated cell sorting analysis of propidium iodide–stained HCT116 treated with dimethyl sulfoxide (control), 1.5mmol/L doxorubicin or doxorubicin and 1 μ M entospletinib for 48 hours. Numbers indicate the percentage of events in the sub-G₁ phase of the cell cycle (dead cells).

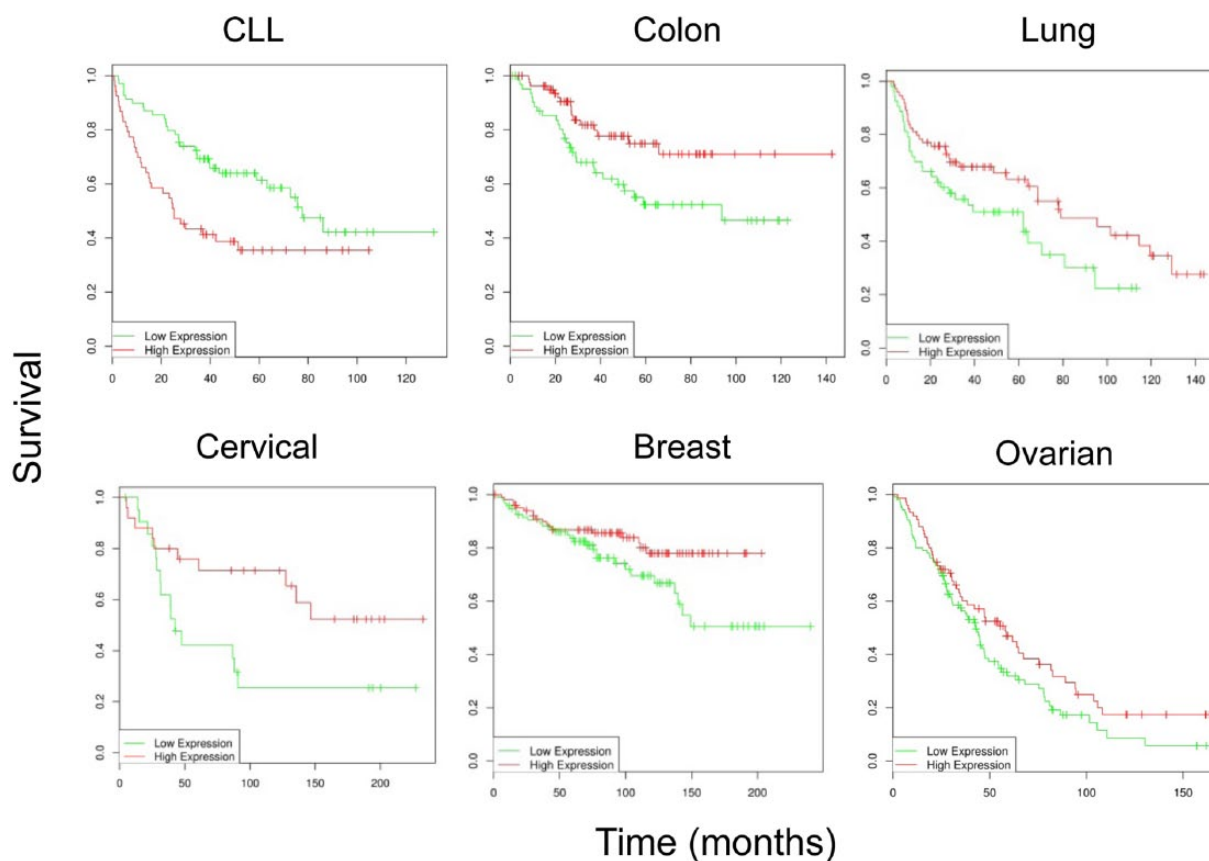


Figure 3. Correlations between SYK expressions and survival rates of patients with different cancer types. Kaplan-Meier survival curves of patients with chronic lymphocytic leukemia, breast, lung, cervical, ovarian, and colon cancers, segregated according to high (red) or low (green) expression of SYK, obtained from public databases through a bioinformatics analysis using PPISURV (www.bioprofiling.de). Each graph represents a different Gene Expression Omnibus data set. The comparisons between SYK expressions and survival rates for each data set are significant ($P < .01$).

Small molecules for SYK inhibition have been developed to treat allergic diseases and other blood malignancies.^{13–16} However, our data clearly has revealed a complete reduction in p53 after SYK inhibition, which could result in malignant transformation due to p53 reduction. Our study demonstrates that SYK inhibition modulates p53 activity in cancer cells. This is supported by the fact that SYK inhibition is able to inhibit cell death after inducing DNA damage. These observations are supported by our bioinformatics study that shows a positive prognostic value of SYK expression in patients with solid tumors in correlations with their survival rates, and negative correlation was seen between SYK expression and survival rate of patients with CLL. This clearly supports the cumulative data that demonstrate the antineoplastic properties of SYK in solid tumor. However, cell context and microenvironment could modulate this effect; therefore, more research should be conducted to evaluate the role of SYK modulation in solid tumor in vivo. In addition, clinical use of SYK inhibitors should be reevaluated carefully in accordance with these findings.

Author Contributions

MA conceived, designed the study, analyzed the data, and wrote the manuscript.

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