Research on the reactivation of Syk expression caused by the inhibition of DNA promoter methylation in the lung cancer

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Received August 18, 2010

The aim of this study was to study the expression of Syk gene and methylation in its promoter region in the lung cancer and to investigate the relationship between silencing of the Syk gene and DNA methylation of the Syk promoter region in lung cancer cell lines.

Real-time PCR and immunohistochemistry were used to examine the Syk expression in specimens from 3 lung cancer cell lines and 16 lung cancer patients (tumor tissues and adjacent normal tissues). MSP was used to analyze the methylation status of the Syk promoter region. We also investigated the role of restoring Syk expression by using a DNA methyltransferase inhibitor, 5-aza-CdR, in suppressing invasion of lung cancer cell lines.

No expression of the Syk gene was detected in the 3 lung cancer cell lines. In the 16 lung cancer patient samples, Syk expression was significantly lower in the tumor tissues than that in their adjacent normal tissues (P<0.05). Consistently, immunohistochemistry analysis of Syk protein expression showed that in the lung cancer tissues Syk protein expression was also significantly lower than that in their adjacent normal tissues. In the two lung cancer cell lines (NL9980, YTMLC-9) that lack the endogenous Syk expression, 4uM demethylation agent 5-aza-CdR treatment was able to reactivate the Syk gene expression, but not NCI-H446.

In conclusion, hypermethylation leads to silencing of the Syk gene in human lung carcinoma cell lines. Methylation of the Syk promoter and loss of Syk expression in lung cancer cell lines are independent biomarkers. Syk may be a potential tumor suppressor in human lung cancer.

Key words: 5-aza-2’-deoxycytidine, lung cancer, MSP, promoter methylation, Syk, tumor suppressor gene

Lung cancer is one of the most common malignancies in the world and a leading disease in industrialized countries, including China. Lung cancer development and progression occur through a complex, multistep process, including oncogene activation, mutation and loss of tumor suppressor genes.

Syk is a potential tumor suppressor gene, whose expression is frequently altered in tumors [1-2]. It is one of the two members of the Syk family (Syk and ZAP-70). Syk was thought to be a hematopoietic cell-specific signaling molecule and plays an essential role in maturation of lymphocytes and activation of immune cells [3-4]. Recent evidences show that its expression occurs also in many non-hematopoietic cell types. Down-regulation of Syk expression was first observed during breast cancer progression [1]. Its abnormal expression nowadays has also been detected in a number of other types of cancer, such as carcinoma ventriculi [5] and liver cancer [6]. Syk was found to be able to suppress tumorigenesis and metastasis, but the molecular mechanism of the Syk function still remains largely unknown.

DNA methylation has an essential regulatory function in mammalian development, suppressing gene activity by changing chromatin structure[7-8]. It has become apparent that aberrant DNA methylation of promoter region Cpg islands may serve as an alternate mechanism to genetic defects in the inactivation of tumor suppressor genes in human
malignancies [9-10]. DNA promoter hypermethylation is one of the mechanisms that lead to silencing of cancer suppressor genes. Increasing clinical evidence reveals a positive correlation of reduced Syk expression with increased risk for metastasis, indicating that Syk may be a potential new tumor suppressor[5, 11-13].

Although there is evidence of Syk as a putative tumor suppressor in several cancer types, the role of Syk in lung cancer has not been reported. In the present study, we examined Syk mRNA and protein expression levels in human lung cancer cell lines and primary tissue samples. We also analyzed methylation status of the Syk promoter and its relationship with the Syk mRNA expression in human lung cancer cell lines.

Materials and methods

**Cell lines and tissue samples.** Lung cancer cell lines NL9870, YTMLC-9, NCI-H446, and positive, negative controls breast cancer cell lines MCF-7 and MDA-MB-435S were provided by Tianjin Lung Cancer Institute. All cell lines were cultured in recommended media RPMI1640 (GIBCO, HEPES 4.76g/NaCO2 2.0g/RPMI-1640 10.4g/ddH2O 1000mL) supplemented with 10% newborn bovine serum (GIBCO), 1X-glutamine and 1Xpenicillin-streptomycin at 37°C in a humidified incubator with 5% CO2.

Lung cancer tissues and their matched adjacent non-cancerous tissues were obtained from 16 patients with lung cancer undergoing surgical resection in our department. All specimens were placed in liquid nitrogen immediately after resection and stored at -80°C until RNA or genomic DNA extraction. No patient had received chemotherapy or radiation therapy prior to surgery. All patients were confirmed to have lung carcinoma by pathologic test.

The pathological sections of human lung cancer tissues were taken from Tianjin Medical University General Hospital pathology department.

**Syk gene expression assay.** RNA isolation and reverse transcription reaction. Total cellular and tissues RNA was isolated by using Trizol (Invitrogen) reagent according to the manufacturer’s recommendations. Cellular RNA was isolated from 5×10⁶ to 1×10⁸ cells by 1ml Trizol decomposition and tissues samples were grounded into a fine powder using a mortar and pestle, incubated in Trizol solution (100g/L) for 15min. Then 1/5 volume of chloroform was added. After vigorous agitation standing for 5min, the inorganic phase was separated by centrifugation at 12000g for 15min at 4°C, RNA was then precipitated in the presence of equimolar of isopropanol and centrifuged at 12000g for 10min at 4°C. RNA pellets were washed with 75% iced ethanol and centrifuged at 8000g for 5min at 4°C then dissolved in diethylypyrocarbonate (DEPC)-treated H2O. Total RNA quantified and concentration determination using UV spectrophotometer (Beckman Coulter) by absorbency at 260/280 nm and 1.2% denaturing agarose gel. For Real-time PCR analysis, 2ug RNA was reverse transcribed using Reverse Transcriptase M-MLV (Takara), Ribonuclease inhibitor (Takara) and dNTP mixture (Takara), according to the manufacturer’s protocol, the cDNA templates were subjected to PCR amplification.

**Real-time PCR.** 2.5ul of the resultant cDNA of 3 lung cancer cell lines and 16 pairs of lung cancer and matched adjacent non-cancerous tissues were mixed with 2XSYBR Premix Ex Taq™ (Takara). The primer (5uM) sets used were: Syk, forward 5’-ACTTGGTACCGGGTGAAT-3’, reverse 5’-GGGTGCAAGTTCTGGCTCAT-3’; GAPDH, forward 5’-AGAAGGCTGGGGCTCATTTGCAGGG-3’, reverse 5’-GTCACTGGCGCTTCTCACACATG-3’. The amplifications were performed in ABI-7500 Real-time PCR System according to the manufacturer’s protocol. Each sample ran in triplicate for each gene. An initial denaturation step was at 95°C for 10s followed by 40 cycles of denaturation at 95°C for 5s, annealing at 60°C for 30s, and extension at 72°C for 30s.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded sections of lung malignant tumors and adjacent nonneoplastic tissues were subjected to immunostaining with a rabbit polyclonal antibody against Syk c-20 (Santa Cruz). Briefly, 5-uM-thick tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval by boiling in sodium citrate buffer (10mM, pH 6.0). The sections were incubated at 4°C overnight with Syk primary antibody (1:200 dilution, Cell Signaling, Beverly, MA) and then stained with 3,3’-diaminobenzidine. After visualization of immunoreactivity, the sections were counterstained with hematoxylin and mounted. The immunostained sections were evaluated without any knowledge of the patients’ clinical information and status of MSP and Real-time PCR of Syk. Normal lung tissues were taken as the internal positive controls. The stains were graded as follows: (a) positive when immunoreactivity is equivalent to that seen in normal lung cells or is moderately decreased; and (b) negative when immunoreactivity is weak or zero.

**DNA Methylation analysis.** DNA extraction, purification, bisulfite modification and MSP (Methylation-specific PCR). Genomic DNA from cell lines or frozen lung cancer tissues was extracted by using a Dneasy kit (Takara). Genomic DNA was treated with methylSEQr™ Bisulfite Conversion Kit (Applied Biosystems). Briefly, 300ng gDNA was treated with methylSEQr™ Denaturation Buffer, incubated at 37°C for at least 15min. Then the methylSEQr Conversion Reagent agent was added to each sample, followed by incubation at 50°C for 16h. The modified DNA was purified using methylSEQr™ Purification Columns. It was treated again with NaOH (0.1M) and precipitated DNA was resuspended in 50uL of TE buffer (3mM Tris (PH8.0)/0.2mM EDTA) and subjected to MSP amplification. Methylation-specific primers were designed to cover 9 CpG dinucleotides numbered 17-21 (forward) and 47-50 (reverse). Similarly, unmethylation-specific primers were designed to cover 8 CpG dinucleotides numbered 18-22.
Results

Expression of Syk in lung cancer cell lines and tissues
(Fig. 1, Tab. 1, 2)

Expression of Syk mRNA in 3 lung cancer cell lines by Real-time PCR showed that, all the cell lines had low-level Syk mRNA expression compared to the positive control breast adenocarcinoma cell line MCF-7 or the negative control breast duct cancer cell line MDA-MB-435S. The mRNA expression of Syk gene in all the lung cancer cell lines that we examined were lost (P<0.05).

Immunohistochemistry (Fig. 2A-F)

Immunohistochemistry results show us: In SP×100, the difference between neoplasm and adjacent tissues is exist in every histopathological types. The specific rabbit polyclonal antibody against Syk c-20 is adhere to the cytoplasmic and stained claybank, which is mainly confined to the connective tissue adjacent to cancer, but in the lung cancer part which is absented. In SP×400, we hardly find the staining sites in all histopathological types.

Table 1. Syk expression in the lung cancer cell lines (x ± S)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>x ± S</th>
<th>MCF7</th>
<th>MDA-MB-435S</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>3479646.090±224965.900</td>
<td>–</td>
<td>0.005</td>
</tr>
<tr>
<td>MDA-MB-435S</td>
<td>524.707±46.744</td>
<td>0.005</td>
<td>–</td>
</tr>
<tr>
<td>NL9980</td>
<td>1493.522±4.392</td>
<td>0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>YTMLC-9</td>
<td>94.521±12.564</td>
<td>0.005</td>
<td>0.002</td>
</tr>
<tr>
<td>NCI-H446</td>
<td>24.714±5.025</td>
<td>0.005</td>
<td>0.001</td>
</tr>
</tbody>
</table>

As shown in Table 1, compared with the positive control MCF-7 and negative control MDA-MB-435S, the mRNA expression of Syk gene in all the lung cancer cell lines that we examined were lost (P<0.05).

Table 2. Syk expression in lung cancer tissues (2^ΔΔCt)

<table>
<thead>
<tr>
<th>No. specimens</th>
<th>Cancer tissues</th>
<th>Normal tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.865</td>
<td>1.093</td>
</tr>
<tr>
<td>2</td>
<td>1.860</td>
<td>4.836</td>
</tr>
<tr>
<td>3</td>
<td>0.237</td>
<td>3.335</td>
</tr>
<tr>
<td>4</td>
<td>0.456</td>
<td>0.419</td>
</tr>
<tr>
<td>5</td>
<td>0.377</td>
<td>1.205</td>
</tr>
<tr>
<td>6</td>
<td>0.613</td>
<td>0.573</td>
</tr>
<tr>
<td>7</td>
<td>0.597</td>
<td>1.429</td>
</tr>
<tr>
<td>8</td>
<td>0.309</td>
<td>0.901</td>
</tr>
<tr>
<td>9</td>
<td>0.464</td>
<td>0.631</td>
</tr>
<tr>
<td>10</td>
<td>1.077</td>
<td>0.702</td>
</tr>
<tr>
<td>11</td>
<td>1.432</td>
<td>1.780</td>
</tr>
<tr>
<td>12</td>
<td>0.693</td>
<td>1.727</td>
</tr>
<tr>
<td>13</td>
<td>0.655</td>
<td>0.728</td>
</tr>
<tr>
<td>14</td>
<td>0.541</td>
<td>0.906</td>
</tr>
<tr>
<td>15</td>
<td>0.477</td>
<td>0.726</td>
</tr>
<tr>
<td>16</td>
<td>0.515</td>
<td>0.238</td>
</tr>
</tbody>
</table>

As shown in Table 2, in the 16 lung cancer patients, the Syk expression was lower in the tumor tissues than that in their matched adjacent normal tissues (P<0.05).
Syk protein expression in lung cancer and adjacent tissues of human large cell lung cancer (Fig.3 SP×100, Fig.4 SP×400), human lung squamous carcinoma (Fig.5 SP×100, Fig.6 SP×400) and human lung small cell carcinoma (Fig.7 SP×100, Fig.8 SP×400). Blue arrows show the cancer tissues and cells, and red arrows show the positive region that tumor adjacent tissues dyed by rabbit polyclonal antibody against Syk c-20. In SP×100, the difference between neoplasm and adjacent normal tissues is found in every histopathological types. The specific rabbit polyclonal antibody used was anti-Syk c-20.

Figure 2. Syk protein expression in lung cancer and adjacent tissues of human large cell lung cancer (A SP×100, B SP×400), human lung squamous carcinoma (C SP×100, D SP×400) and human lung small cell carcinoma (E SP×100, F SP×400).
Methylation of the Syk Promoter in lung cancer cell lines
(Tab. 3, Fig. 3,4)
MSP analyses of the Syk promoter methylation status using genomic DNA extracted from 3 lung cancer cell lines, products of 243 and 140bp were expected for methylated (M) and unmethylated (U) DNA, on the side, MDA-MB-435S as methylation positive control and MCF-7 as methylation negative control.

Syk negative lung cancer cell lines (NL9980, YTMLC-9 and NCI-H446) were treated with or without 4uM 5-aza-CdR for 3 days. The Syk mRNA expression was examined by Real-time PCR in treated cell lines and compared with that in untreated cell lines. In cell lines NL9980, YTMLC-9 the Syk mRNA expression was up-regulated after the de-methylation drug treatment (P<0.05), but the Syk mRNA expression in NCI-H446 was approximately the same as that in the control group (P>0.05).

Discussion
Syk is a new putative tumor suppressor gene, which constitutes an autonomous family of nonreceptor tyrosine kinases together with zeta-activated protein of 70 kDa (ZAP-70). The locus mapped to chromosome 9 at band q22, contain two adjacent Src homology 2 (SH2) domains and C-terminal kinase domains with two interdomain regions, unlike Src-family kinases which lack an SH3 domain[14-16], and which was first recognized as a 40 kDa proteolytic fragment derived from a p72 tyrosine kinase present in porcine spleen[17]. Syk is ubiquitously expressed in hematopoietic cells and has been extensively studied as an effector of B cell receptor (BCR) signaling. It is involved in coupling activated immunoreceptors to downstream signalling events that mediate diverse cellular responses including proliferation, differentiation and phagocytosis. BCR engagement induces signaling cascades mediated by three families of nonreceptor tyrosine kinases: the ZAP-70 family (ZAP-70, Syk), the Src family (Lyn, Fyn, Btk), and the Tec family (Btk, Itk, Etk). After BCR activation, Syk-dependent signaling pathways regulate the clonal expansion, differentiation, or apoptosis of B cells. Phospholipase C (PLC)-r2 and phosphatidylinositol 3-kinase (PI3-K) are key targets of Syk tyrosine phosphorylation after BCR cross-linking. In B cells, Syk phosphorylation of PLC-r2 results in downstream activation of the ERK and kinases, whereas PI3-K phosphorylation by Syk mediates Akt activity Syk also preferentially phosphorylates the α-tubulin subunits of microtubules, which has been proposed to regulate the ability of the microtubule cytoskeleton to function as a scaffold for the assembly of signaling complexes.[18-22]

For the past few years, the researchers discovered that Syk also is a general physiological function in a wide variety of nonhematopoietic cells, and promoter methylation of anti-on-
cogene is a mechanism for the gene silencing. Syk is expressed in mammary epithelial cells [1], airway epithelial cells [23], human nasal fibroblasts [24], vascular endothelial cells [25], neuron-like cells [26], hepatocytes [27] and melanocytes [28]. The recent identification of Syk as a potent modulator of tumor epithelial cell growth has generated a need for further exploration of the role of nonreceptor tyrosine kinases in wide cancer progression and metastasis. These initial results offer the promise that novel molecular targets may be identified both for the prevention of tumor development and for inhibition of metastatic tumor spread. Some studies reported that Syk is deleted in thlioma tissues. Cooperman et al. [22] tested the expression of Syk in a panel of well-characterized human breast cancer cell lines. They suggested that loss of Syk mRNA expression may be associated with the progression to a malignant phenotype in breast cancer. Toyama et al. [29] measured Syk mRNA expression in paired samples of primary breast cancer and matched, adjacent non-cancerous tissues by real-time quantitative RT-PCR. They found that patients with reduced Syk expression were at significantly increased risk for distant metastasis and showed a significantly poorer prognosis. Yuan et al. [30] is of the opinion that hypermethylation is one of the causes leading to silencing of the Syk gene in human breast cancer. They discovered that 30% (6/20) breast cancer cell lines had hypermethylation in CpG island of the Syk promoter region, which correlated with silence of the Syk gene. The Syk expression was reactivated after treatment with the de-methylation agent 5-aza-CdR. They also found the Syk promoter methylation in 32% (12/37) breast cancer tissues but not in adjacent normal tissues. They concluded that Syk as a potential tumor suppressor gene, was silenced in breast cancer and suppressed the tumor invasive level. Similar result was also reported in liver cancer [31]. Ding et al. [11] found that Syk expression was low and was associated with its promoter hypermethylation in breast cancer tissues. In addition, the methylation frequency is significantly higher in lymphatic metastasis group than that in lymphatic metastasis negative one. In the study of 61 carcinoma ventriculi patients, Wang S and their colleagues [5] found that Syk expression level is lower and methylation in its promoter is higher in tumor than that in adjacent normal mucosa. Liu et al. [12] also reported the same result. In pre-B cell acute lymphoblastic leukemia, Goodman et al. [13] found that the correlation between loss of Syk expression in mRNA or protein and its promoter hypermethylation was closely related to leukaemia occurrence and evolution.

However, reports on Syk in the field of lung cancer are rare. In our study, we explored the mRNA and protein expression of Syk in lung cancer cell lines, lung cancer and adjacent non-cancerous tissues. Our results showed that Syk expression was readily detectable in normal lung tissue and epithelium, but was reduced or absent in the invasive lung tumor cell lines. The level of Syk mRNA expression in lung cancer tissues is significantly lower than that of adjacent noncancerous tissues. Immunohistochemistry of normal and pathologic human breast tissue samples also verified the Syk expression in normal lung epithelium and Syk expression absence in invasive lung carcinomas. Our results indicate that loss of Syk mRNA and protein expression may be correlated to lung tumorigenesis. We also examined methylation status of the CpG islands in the Syk promoter in lung cancer cell lines, and found hypermethylation in cell lines NL9980 and YTMLC-9. Demethylation is expected to reactivate gene products that are silenced by promoter hypermethylation. When we used a DNMT inhibitor 5-aza-CdR, reactivation of Syk was detected in methylation positive cell lines.

To our surprise, in the experiments we have found some different results in the SCLC NCI-H446 from the other two NSCLC cell lines (NL9980, YTMLC-9). Through the PCR test, we know that the Syk also has the expression diminution in the NCI-H446. However, through the test to the Syk gene promoter methylation, there is no methylation found. This means that the expression diminution of NCI-H446 cell line is not triggered by promoter methylation. The gene mutation, the base deletion, and etc. might be the possible reasons, but the true mechanims needs a further research and discovery. This point of view is consistent with the classification of lung cancer during our clinical experience. In the classification of pathology, the lung cancer cells are often grouped to NSCLS and SCLS. Each of them has its own specific pathology mechanisms, characteristics, staging and treating fashion, one of which is completely different from the other. From the experiment conclusion, we can have the assumption that the gene promoter methylation is an important factor which induces the expression diminution of NSCLC.

Taken together, our findings in lung cancer are in agreement with reports for other types of carcinomas found in the literature [1, 5, 11-13, 29-31]. The association of the promoter hypermethylation with the loss or reduction of Syk expression may provide a new way to treat lung cancer [32-33].

In conclusion, hypermethylation leads to silencing of the Syk gene in human lung carcinoma. Methylation of the Syk promoter and loss of Syk expression in lung cancer are independent biomarkers. Syk may be a potential tumor suppressor in human lung cancer.

Acknowledgement. We thank Prof. Biao He and Dr. Si-cheng Zhao for critically reading the manuscript.

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