The Role of Promoter Hypermethylation and Expression of Spleen Tyrosine Kinase in Mammary Carcinogenesis

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Background: *The spleen tyrosine kinase (SYK) has been considered as an important inhibitor of cancer cell growth and a potential tumor suppressor in human breast carcinomas. Loss of SYK expression occurs frequently in breast cancer cells together with the promoter hypermethylation suggests that methylation may play an important role in inactivating SYK. In contrast to full-length form of SYK (SYKL), a spliced variant or SYKS is frequently expressed in breast cancer cells.*
Objective: *To determine whether the expression of SYKL and its variant SYKS and the presence of DNA methylation in promoter of SYK might be associated with the risk and progression of mammary carcinoma.*
Material and Method: *Five breast cancer cell lines were examined for expression of SYKL and SYKS mRNA and SYK DNA methylation. One hundred and eight breast cancer tissues, 13 benign tumor tissues, and 35 adjacent non-cancerous tissues (ANCT) were extracted for examination of SYKL and SYKS mRNA. Genomic DNA of 83 breast cancer tissues and 13 benign tissues were isolated for examination of SYK DNA methylation. SYKL and SYKS were determined by quantitative reverse transcription-PCR. SYK DNA methylation was assayed by methylation-specific PCR.*
Results: *Two breast cancer cell lines with metastatic phenotype showed complete loss of SYKL mRNA together with SYK DNA methylation. The significant reduction of the SYKL mRNA expression in breast cancers and benign tumors in comparison to ANCT was observed (mean mRNA levels = 1.0297, 0.6294 and 0.3446 in ANCT, benign tumors and breast cancers, respectively). Complete loss of SYKL was found in 50% of breast cancer tissues but not in benign tumors and ANCT. SYK was methylated in 45% of breast cancer tissues compared with only 15% in benign tumor tissues. Furthermore, a significant correlation between SYK methylation and loss of its expression was observed. There was no significant association between SYKL expression or methylation and clinicopathological parameters. Two breast carcinoma cell lines showed SYKS expression. The expression of SYKS mRNA was found most frequently in breast cancer tissues (49%) and less in benign tumors (46%) and ANCT (25%). The SYKS expression showed a significant correlation with larger tumor size.*
Conclusion: *These findings suggest that SYK signaling pathway may play a crucial role in breast cancer development. The mechanisms responsible for SYKL inactivation may occur through DNA methylation or the presence of SYKS.*

Keywords: *Breast cancer, Methylation, Spleen tyrosine kinase, Tumor suppressor*

J Med Assoc Thai 2017; 100 (Suppl. 4): S13-S26
Full text. e-Journal: *http://www.jmatonline.com*

Breast cancer is the most commonly diagnosed neoplasm among women worldwide[1]. In Thailand, the incidence rate for breast cancer during 1993 to 1997 was 31.7 per 100,000 in Bangkok[2]. In addition, breast cancer retains the first place as a cause of cancer-related death in nonsmoking women. The failure in eradicating this disease is largely due to the lack of identification of a specific etiologic agent, the precise time of initiation, and the molecular mechanisms responsible for cancer initiation and progression.

Breast tumor development and progression are thought to occur through a complex, multistep process, including activation of oncogenes and mutation or loss of tumor suppressor genes. One group of proteins that play a critical role in breast cancer cell signaling pathway is tyrosine kinases. Overexpression of several receptor tyrosine kinases (RTKs) is observed in human breast cancers and is positively correlated with enhanced tumorigenesis. In contrast, non-receptor tyrosine kinases (NRTKs) such as Spleen Tyrosine Kinase (SYK)[3] and c-KIT[4], which were reported that they were reduced in breast cancer and have been

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proposed to play a role in carcinogenesis. Notably, SYK has been found as an important inhibitor of breast cancer cell growth and metastasis and SYK is proposed that as a potent modulator of epithelial cell growth and a potential tumor suppressor in human breast carcinomas[5]. However, there have been very few studies about its role in breast cancer development with conflicting results.

The clinical manifestation of breast cancer is the progression of disease to stages characterized by local invasion, followed by metastasis. The development of local invasion and distant metastasis are strongly associated with low therapeutic efficacy and poor survival. Indeed, improvements in local control have led to decrease in presentation of distant metastases. *SYK* is considered to be an anti-metastasis gene in human breast cancer[6]. Moreover, a loss of *SYK* expression is evident during progression in breast cancer from normal to invasive tumor. Therefore, *SYK* may be considered as a novel biomarker for tumor progression and useful for prognostic marker of mammary carcinoma.

The mechanism (s) responsible for the loss of *SYK* expression or SYK activity in breast cancer cells have remained unclear. So far two different mechanisms controlling *SYK* expression or SYK activity have been described, either alternative splicing[7] or hypermethy lation of its promoter[8]. The alternatively spliced variant of *SYK* or *SYK*S is identical to *SYKL*, except that it lacks a 69-bp sequence. *SYKS* creates an SYK protein isoform (SYKS) that lacks a 23-residue sequence (deletion) within the interdomain B (IDB) of SYK protein. The expression of SYKS in normal tissues is uncommon. The presence of alternatively spliced nonfunctioning SYK variant may explain the reduced of SYKL activity[7]. Therefore, the presence of specific splice variants may help to describe the loss of SYKL activity. Moreover, the hypermethylation of normally unmethylated CpG islands has been documented as a relatively frequent event in immortalized and transformed cells[9] and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancer[10,11]. There was a study demonstrated that *SYK* was frequently inactivated through an epigenetic pathway in breast cancer by methylation of *SYK* 5' CpG sites[8]. DNA methylation was frequently found in invasive tissues compared with adjacent normal tissue. Therefore, a study of *SYK* promoter hypermethylation and alternatively splice variance of *SYK* in tissue may have useful clinical application in the detection of breast neoplasia in patients with pre-invasive and early-stage breast cancer.

This study aimed to determine the relationship between methylation of *SYK* gene in promoter region, spliced variance of *SYK* and the level of *SYK* mRNA in various stages of human breast cancers. Moreover, the correlation with clinicopathological parameters and clinical outcomes in Thai population was determined.

Material and Method

Cell culture

The following human breast cancer cell lines were used in these studies: T47D, MCF-7, SKBr3, MDA-MB-231 and MDA-MB-435. They were kind gift from Dr. Suzanne Eccles (Institute of Cancer Research, UK). All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. All cell lines were maintained in DMEM containing 2 mM of L-glutamine, 100 units/ml of penicillin and 100 mg/ml of streptomycin supplemented with FCS. All cell culture reagents were purchased from GibcoBRL™ and all chemical reagents of the highest purity available were obtained from Sigma® (Sigma-Aldrich Company Ltd., Dorset, England) unless otherwise stated.

Patient materials

One hundred and eight newly diagnosed, previously untreated patients with breast cancer and thirty five of breast benign tissues at the Division of Head-Neck and Breast Surgery, Department of Surgery, Siriraj Hospital, Thailand were recruited between June 2004 to May 2005. All breast tissues were conserved in 0.5 ml of RNA stabilization reagent (RNA*later*™) in cryotube and were immediately stored at -70°C. This study was approved by the Siriraj Institutional Review Board. 'Node-positive cases' in this study were identified based on a histological diagnosis after a breast dissection. Patients who experienced no metastasis for at least 12 months post-operatively are scored as 'node-negative cases'. Tumors were staged according to the AJCCUICC TNM classification 7th edition.

DNA preparation

Genomic DNA was isolated from frozen tissue by digestion with 100 µg/ml proteinase K followed standard phenol-chloroform (1: 1) extraction and ethanol precipitation. The DNA sample was then resuspended in appropriate amount (50 to 500 mL) of TE buffer and stored at -20°C. DNA concentration was measured by absorbance at 260 nm. Spectrophotometric absorbance ratios (260/280 nm) should be consistently around 1.8 to 2.0.

Bisulfite modification

Genomic DNA was treated with sodium bisulfite[12]. Briefly, 2 mg/50 ml DNA was denatured by 5.5 ml NaOH (final concentration 3 M) for 6 min at 95°C. Thirty ml of 10 mM hydroquinone (Sigma Chemical Co.) and 520 ml of 3 M sodium bisulfite (pH 5.0), both was freshly prepared, was added and mixed followed by incubation under mineral oil at 55°C for 2 to 6 h. The modified DNA was purified using Wizard DNA purification columns (Promega, Madison, WI) and eluted into 50 ml of water (more detail; see Appendix). Modification was completed by 5.5 ml NaOH treatment (final concentration, 3 M) for 10 min at room temperature, followed by added 1 ml of 10 mg/ml glycogen, 17 ml of ammonium acetate and 200 ml of absolute ice-cold ethanol, then precipitated at -20°C overnight. Treatment of genomic DNA with sodium bisulfite converts unmethylated but not methylated cytosines to uracil, which are then converted to thymine during the subsequent PCR step, producing sequence differences between methylated and unmethylated DNA. The pellet was precipitated at high speed centrifuge (10,000 x g) for 25 min, and washed twice with 70% ethanol at 10,000 x g for 5 min. Excess ethanol was removed and the sample was allowed to air dry for 10 min. DNA was resuspended in 30 µl of TE buffer [20 mM Tris (pH 7.4)/5 mM EDTA] or SDW and subjected to PCR amplification using a primer set (forward 5'-GATTAAGATATATTTAGGGAATATG-3'; reverse 5'-CACCTATATTTTATTCACATAATTTC-3') that spanned the *SYK* CpG island. A 25-l reaction that contained 30 ng of bisulfite-treated DNA and PCR mixture (10x PCR buffer, 50 mM $MgCl_2$, 10 mM dNTPs, *Hot Start Taq* polymerase, 10 mM of each forward and reverse *SYK* CpG island primers) were processed in 35 thermal cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 1 min. Each PCR product (10 ml) was loaded onto 2% agarose gel and it was run at 100 Volts for 40 minutes. Stained gel with ethidium bromide and directly visualized under UV illumination. The product was expected to generate 664 bp.

Methylation-Specific Polymerase chain reaction (MSP)

Bisulfite-treated DNA was PCR-amplified as described above. One aliquot (2 µl) of diluted PCR product (40-fold) was subjected to nested duplex PCR amplification in a 25-µl volume. Methylation-specific primers were chosen to cover 9 CpG dinucleotides numbered 17 to 21 (forward) and 47 to 50 (reverse), both of which were consistently found heavily methylated. Similarly, unmethylation-specific primers covered 8 CpG dinucleotides numbered 18 to 22 (forward) and 35 to 37 (reverse). Primers specific for methylated DNA (forward 5'-CGATTTCGCGGGTTT CGTTC-3'; reverse 5'-AAAACGAACGCAACGCGAA AC-3') and unmethylated DNA (forward 5'-ATTTTGT GGGTTTTGTTTGGTG-3'; reverse 5'-ACTTCCTTA ACACACCCAAAC-3') was added to the same reaction and expected to generate 243-bp and 140-bp products, respectively (criteria for primer selection; see Appendix). A control without DNA was performed for each set of PCRs. Each PCR (10 ml) was directly loaded onto 2.0% agarose gel. Run reactions from each sample together to allow for direct comparison between unmethylated and methylated alleles. Include positive and negative controls. Horizontal gel can be run at 100 Volts for 40 minutes. Stained the gel with ethidium bromide, and directly visualized under UV illumination.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed for examining PCR products, and to estimate the amount of DNA. Five µl of the PCR products was mixed with 1 to 2 ml of dye and loaded into each well of 2% agarose gel. Electrophoresis was achieved at constant 100 Volts for approximately 40 min in 1X TAE buffer by using a horizontal electrophoresis apparatus (Mupid-2, Tokyo, Japan). Thereafter, the agarose gel was stained with ethidium bromide, visualized by a UV trans-illuminator, and photographed with a polaroid camera (Fotodyne, Hartland, USA). The DNA molecular weight marker used in the electrophoresis was Hae III-digested fX174 RF DNA fragments (New England BioLabs, Inc., Beverly, MA, USA.

RNA treatment techniques

Total RNA was isolated from each specimen by Trizol reagent. According, homogenize small section tissues using 1 ml of Trizol reagent in 2 ml screw-capped tube. The homogenized samples was transferred into a 1.5 ml of new microcentrifuge tube and incubated at room temperature for 5 min. Subsequently, they were then added 0.2 ml of chloroform, vortex sample for 15 sec and incubated at room temperature for 3 minutes. After incubation, the samples were centrifuged at 14,000 x g for 15 min at 4°C. The aqueous phase was transferred to a new 1.5 ml tube, RNA was then precipitated in the presence of 0.5 ml of isopropyl alcohol, mixed, and incubated at -20°C for at least 1 h. The samples were centrifuged at 14,000 x g for 10 min at 4°C. RNA pellet was washed twice with 1 ml of 75% ice-cold ethanol

and once with 95% ice-cold ethanol at 10,000 x g for 5 min.

DNase treatment of RNA

In order to eliminate DNA contamination in RNA samples, DNAase treatment was routinely employed. In brief, 10 to 100 mg of total RNA (in 300 ml DEPC-water) was mixed with 33 ml of 10 x PCR buffer, 1 ml of 10 units of human placental ribonuclease inhibitor and 1 ml 10 units of DNase I. After incubation for 45 min at 37°C, the DNase I was inactivated by adding 335 ml of phenol: chloroform (3: 1). Samples were vortexed 5 sec, left on ice for 10 min, and centrifuged at 13,000 x g for 15 min at 4°C. The supernatant (aqueous phase) was carefully transferred into a new microfuge tube containing 900 ml of ice-cold isopropanol and 120 ml of 3 M Sodium Acetate (pH 5.2) and incubated at -20°C overnight. The RNA pellet was collected by cold centrifugation at 13,000 x g for 15 min, rinsed once with 500 ml of ice-cold 70% ethanol and 95% ethanol, centrifuged at 10,000 x g for 5 min. The RNA pellet was air-dried for 10 min and dissolved in DEPC-water with the appropriate amount.

Estimations of RNA concentration and quality were performed on 1 ml of the RNA sample by measuring the OD_{260} and OD_{280} in a GeneQuant (Pharmacia). Spectrophotometric absorbance ratios (260/280 nm) were consistent from 1.8 to 2.0. In some cases, the integrity of RNA was checked by ethidium bromide (1 mg/ml) staining of 0.8% agarose/TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0) minigel electrophoresis. The RNA samples were stored at -70°C until used.

Reverse transcription of RNA (cDNA synthesis)

In order to synthesize cDNA, RNA was converse to DNA by reverse transcription reaction. In brief, the template RNA solution (10 ng to 2 µg) was thawed on ice. The primer solution, 10x Buffer RT, dNTP aliquots, and RNase-free water was thawed at room temperature. After thawing all solutions were stored on ice immediately. Each solution was mixed by vortexing, and centrifuged briefly to collect residual liquid from the sides of the tubes. Following, prepare the dNTP mix C on ice according to Table 5, mixed thoroughly by vortexing, and centrifuged briefly to collect residual liquid from the sides of the tubes. RNase inhibitor was diluted to a final concentration of 10 units/µl in ice-cold 1x Buffer RT (dilute an aliquot of the 10x Buffer RT accordingly using the RNase-free water supplied) and mixed carefully by vortexing for no more than 5 sec, and centrifuged briefly to collect residual liquid from the sides of the tubes and then a fresh dilution of RNase inhibitor was prepared. The master mix contains all components required for the labeling reaction except the template RNA. The template RNA was added to the individual tubes containing the master mix, mixed thoroughly and carefully by vortexing for no more than 5 sec, centrifuged briefly to collect residual liquid from the walls of the tube and incubate for 2 h at 37°C. Following, 2 ml RNase A (10 mg/ml) was added and vortexed for no more than 5 sec and then incubated the reaction mix for 10 min at 65°C, for 5 min at 93°C and then cooled immediately on ice.

This reaction mixture was incubated at 42°C for 60 min, denatured at 95°C for 5 min, quickly chilled on ice and then used immediately for PCR or stored at -20°C. The conditions for RT were the same for each of the comparison samples and b-actin control including all the components, volume, temperature, and reaction time.

Reverse transcriptase Polymerase chain reaction (RT-PCR)

RT-PCR was carried out in the 0.2-ml microcentrifuge tube containing the following solution into the PCR mixture (10x PCR buffer, 50 mM $MgCl_2$, 10 mM dNTPs, *Taq* polymerase, 10 mM of each forward and reverse *SYK*L and *SYK*S primers and cDNA template). The PCR primers of *SYK*L are as follows: Syk-S1 forward, 5'-CATGTCAAGGATAAGAACA TCATAGA-3' and Syk-AS1 reverse, 5'-AGTTCACC ACGTCATAGTAGTAATT-3'. A 541-bp product was expected for *SYKL*. The PCR primers of *SYKS* are as follows: forward, 5'-TTTTGGAGGCCGTCCACAAC-3'; reverse, 5'-ATGGGTAGGGCTTCTCTCTG-3' that spans the 69-bp deletion region was used to amplify *SYK* cDNA. A 152-bp product was expected for *SYK*S transcripts. RT-PCR was performed in a GeneAmp PCR 9700 system (Applied Biosystem, USA). The amplified product was analyzed by electrophoresis on a 2.0% agarose gel (Seakem LE agarose, USA) and stained with ethidium bromide (BioRad, USA). These were visualized under ultraviolet light using UV transilluminator (SynGene, USA).

β-actin was used to check RNA integrity and as an internal control. Sequences of PCR primer sets of β-actin (in 5'-3' direction) were as follow: forward -TCGACAACGGCTCCGGCAT, backward - AAGGTGG TGCCAGATTTTC, Products size: 239 bps. Typical PCR reaction mixtures contained 2.5 ml of 10x PCR buffer, 10 mM of dNTPs mix, 2.0 units of *Taq* DNA polymerase,

10 mM of each upstream and downstream primer, and SDW up to an end-volume of 25 ml. Finally 2 ml of cDNA were added and the reaction mix was overlaid with mineral oil. The PCR reaction was carried out in a TRIO-Thermoblock thermal cycler. Amplification cycles consisted of denaturing the cDNA for 1.3 min at 94°C, primer annealing for 2 min at 50°C and primer extension during incubation for 2 min at 72°C with the last extension step for 10 min. The optimal condition for each primer pair was achieved by adjusting the annealing/extension temperature and time. A negative control reaction was always prepared which contained water or non-RT RNA in place of the DNA template.

In order to quantify the level of mRNA expression between different samples, preliminary experiments were performed which established the range of PCR cycles in which exponential amplification occurred for each primer pair studied. A PCR cycle was chosen in the middle of the linear range of amplification. Ten-ml aliquots of the RT-PCR products were electrophoresed in 2% agarose gels containing 1 x TAE (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.0) and 1 mg/ml of ethidium bromide. Molecular weight markers (1 Kb Ladder) were loaded into the end well of each gel for estimation of the size of PCR products. Electrophoresis was carried out at a constant voltage of 80 (small gel) or 120 V (large gel) for 20 min. Bands were visualized by examining the gel under UV light and captured using a Mitsubishi UVP video copy processor connecting to the ImagePro Software. The intensity of bands with background subtraction was measured using Quantiscan Image Analysis software (Cambridge, UK). Pilot studies had shown the similar results compared with the conventional radio-isotopic (^{32}P) technique. In addition, a series of PCR templates (0.5 mg, 1 mg, 2 mg and 4 mg of reverse transcribed-tRNA) were prepared and underwent the PCR reaction with appropriate number of cycles to confirm the linear correlation between the amount of starting mRNA and the signal intensity of PCR products. Relative density was calculated by dividing specific gene signals by the β-actin signal. In order to control gel-to-gel variability, each PCR products from the breast cancer cell line, MDA-MB-231 was also electrophoresed as a control. The level of mRNA was calculated as the ratio of tissue sample to MDA-MB-231 on the same photograph and was then corrected as a ratio to the corresponding β-actin level.

Primary tumors were grouped into 4 levels according to amount of *SYKL* mRNA expression by semi-quantitative RT-PCR, including level 0, 1+, 2+ and 3+. Level zero is a clearly loss of *SYK* mRNA expression whereas other levels were categorized by the density ratio with β-actin. The density range from 0.10 to 0.99, 1.00 to 1.99, and above 2.00 were grouped into level 1+, 2+ and 3+, respectively.

Statistical analysis

The frequency of *SYKL*, *SYKS* mRNA expression and *SYK* DNA methylation among different types of breast tissues were tested using the Pearson's χ^2 test. The association between the expression of *SYKL*, *SYKS* and *SYK* DNA methylation and clinicopathological parameters was evaluated by Chi-square test with Fisher's exact test. The correlation between the loss of *SYK* and *SYK* DNA methylation was tested by the Phi and Cramer's V tests and all statistical analysis was performed using the SPSS statistical software package version 11.5. A *p*-value less than 0.05 was considered to be statistically significant.

All experiments were performed in triplicate and repeated on at least 2 separate occasions. For evaluation of mean of *SYKL* mRNA expression, the ANOVA was used to determine the difference between 3 groups by Stat View program. A p-value <0.05 was considered to be statistically significant.

Results

Expression of SYKL in breast cancer cell lines

Since *SYKL* is considered to be a potent modulator of epithelial cell growth and a potential tumor suppressor in human breast carcinomas, the expression of *SYKL* mRNA was examined in 5 breast cancer cell lines, namely T47D, MCF-7, SKBr3, MDA-MB-231, and MDA-MB-435. The results are shown in Fig. 1. Three breast cancer cell lines expressed *SYKL* at unequal levels according to type of cancer cell lines. In contrast, metastatic breast cell lines, MDA-MB-231 and MDA-MB-435 clearly lost *SYKL* expression.

Expression of SYKL in breast tissues

To examine the expression of full-length *SYK (SYKL)* in breast cancer patients, quantitative RT-PCR analysis of *SYK* mRNA derived from breast cancer tissues, benign tumors and adjacent non-cancerous tissues (ANCT) was performed. *SYKL* mRNA and marker were examined using agarose gel electrophoresis (Fig. 2). Relative mRNA expression levels of the *SYK* in breast tissues were normalized to the level of β-actin mRNA expression in the respective sample. The mean mRNA levels of *SYK* among breast cancer, benign tumor and ANCT showed the significant difference, which

were 0.6294, 0.3446 and 1.0297, respectively. The mean mRNA of ANCT was significantly increased in comparison to the benign ($p = 0.0114$) and breast cancer tissues ($p = 0.0137$) as shown in Fig. 3. Of the 104 cancer cases analyzed, 50% (52/104) showed no detectable level of *SYKL* mRNA. Fig. 4 showed the frequency of *SYKL* expression at various levels in breast cancer tissues. The relative mRNA expression levels of *SYKL* were categorized into 4 levels (0, 1+, 2+ and 3+) according to the criteria described in Chapter IV. *SYKL* expression was also detected in benign tumors and ANCT at low (level 1+) and moderate levels (level 2+). The frequency of *SYKL* expression in breast tissues was represented in Table 1 and graph showed in Fig. 5. In 35 paired samples of human breast cancer and ANCT, 20 cancer cases (65%) demonstrated the decreased levels of *SYK* mRNA compared to ANCT.

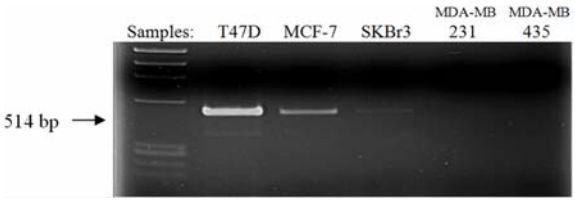

Fig. 1 Gel electrophoresis result of *SYKL* mRNA expression by RT-PCR in breast cancer cell lines. This gel photographs consists of the sample from the breast cancer cell lines. First lane as a marker and the samples name is labeled above. *SYKL* show at the 514-bp band.

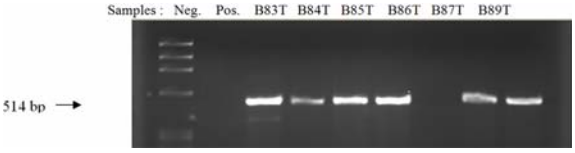

Fig. 2 Gel electrophoresis result of SYKL mRNA expression by RT-PCR in breast tissues. This gel photographs consists of 6 samples from the breast cancer tissues in which the sample name is labeled above and the first lane show a marker. *SYKL* is detected at the 514-bp band. There are 5 out of 6 breast cancer tissues express *SYKL* at unequal level.

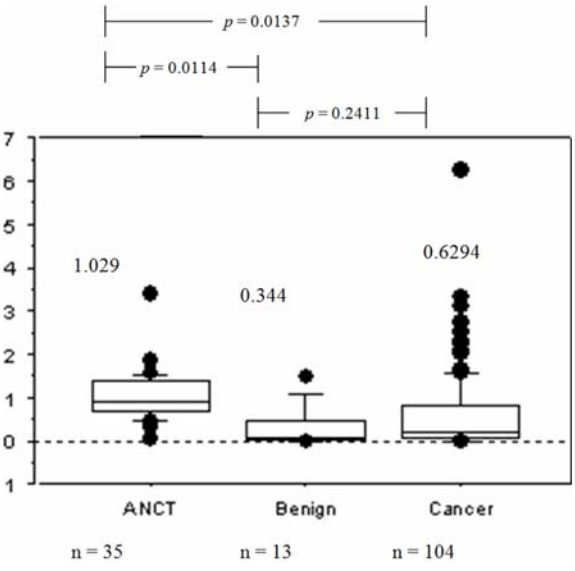

Fig. 3 The mean mRNA level of *SYKL* in breast tissues.

Correlation between SYKL expression and clinicopathological parameters in Thai patients

The relationship between the level of *SYKL* mRNA expression in the breast cancer tissues (n = 104) and clinicopathologic factors (age, tumor size, histopathological grading, lymph node metastasis, estrogen receptor, progesterone receptor and HER2 status) was examined by Pearson's Chi-square test and

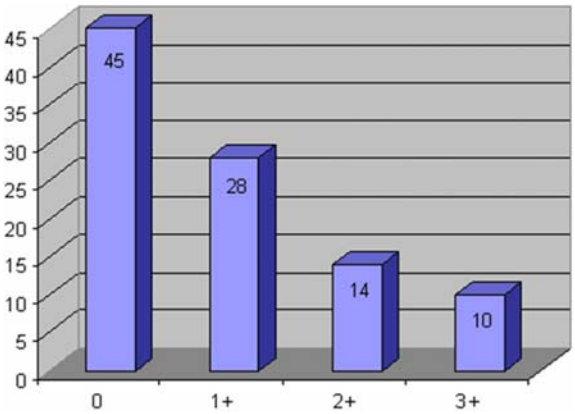

Fig. 4 The frequency of *SYKL* expression at various levels in breast cancer tissues.

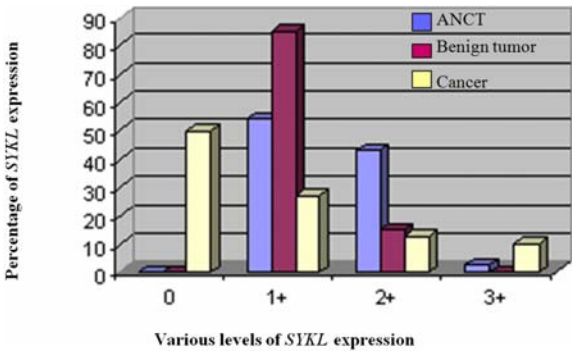

Fig. 5 The percentage of *SYKL* expression at various levels in breast tissues.

Fisher's exact test. There were no significant relationships between *SYKL* expression and clinicopathological finding (Table 2).

Expression of SYKS in breast cancer cell lines

In human, an alternatively spliced form of *SYK* or *SYKS* was identified and found that it lacks 69-bp sequence in interdomain B. It has been proposed to have a different function distinct from *SYKL*[7]. Therefore, RT-PCR was performed to examine the expression of *SYKS* in breast cancer cell lines. The expression of *SYKS* was found in two out of 5 breast

Table 1. Number (percentage) of subjects with *SYKL* expression in breast tissues

	SYKL Expression Levels				*p*-value
	0 n (%)	1+ n (%)	2+ n (%)	3+ n (%)	
ANCT	0 (0)	19 (54)	16 (46)	0 (0)	
Benign tumor	0 (0)	11 (85)	2 (15)	0 (0)	0.151
Cancer	52 (50)	28 (27)	14 (13)	10 (10)	<0.001

Table 2. Clinicopathological features of breast cancer according to the expression of *SYKL*

Characteristic	*SYKL* expression		*p*-value[a]
	Positive[b] (n = 24)	Negative[b] (n = 80)	
Age (year)	50.1±11.2	52.6±12.5	NS
Histopathological Grade			0.295
Well & Moderate-differentiated	9	37	
Poorly-differentiated	9	24	
Tumor size			0.355
T1	5	21	
≥T2	18	52	
Lymph node metastasis			0.321
Negative	11	41	
Positive	11	29	
Perineural invasion			0.643
No	5	19	
Yes	1	3	
Perivascular invasion			0.370
No	11	41	
Yes	11	31	
Pathological staging			0.567
I-II	16	53	
III-IV	6	19	
Hormone Receptor			0.183
Positive	16	43	
Negative	5	26	
HER2			0.504
Positive	3	16	
Negative	7	28	

[a] Pearson chi-square test and Fisher's exact test.
[b] Positive = level 2+ and 3+, Negative = level 0 and 1+

cancer cell lines. Both MCF-7 and SKBr3 expressed *SYKS* at 152-bp band along with *SYKL* at 221-bp band whereas T47D expressed only *SYKL* at 221-bp band. On the other hand, MDA-MB-231 and MDA-MB-435 did not express both isoforms of *SYK* (Fig. 6).

Expression of SYKS in breast tissues

To examine the expression of an alternatively spliced *SYK* variant (*SYKS*) in breast cancer tissues, benign tumors and ANCT, the expression of *SYKS* mRNA in primary breast tissues was determined by RT-PCR (Fig. 7). A shortened form of *SYK* transcript at 152-bp band was present along with the 221-bp band (Fig. 7). The 152-bp products matched the expected size of PCR amplification from *SYKS* transcript[7] and the sequence of the longer PCR product (221 bp) was identical to that of *SYKL* cDNA. Table 3 and Fig. 8 showed the expression of *SYKS* in breast cancer, benign and ANCT specimens. *SYKS* expressed in 49% (53/108), 46% (6/13), and 25% (9/36) of breast cancer tissues, benign tumors and ANCT, respectively.

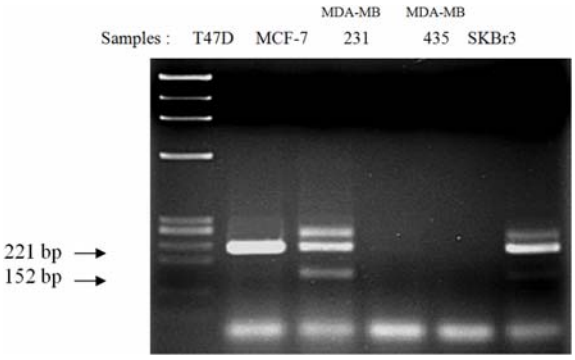

Fig. 6 Gel electrophoresis result of *SYKS* mRNA expression by RT-PCR in breast cancer cell lines. This gel photograph consists of the sample from the breast cancer cell lines in which the cell lines name is labeled above and the first lane show a marker. *SYKS* show at the 152-bp band and *SYKS* always express along with *SYKL* at 221-bp band.

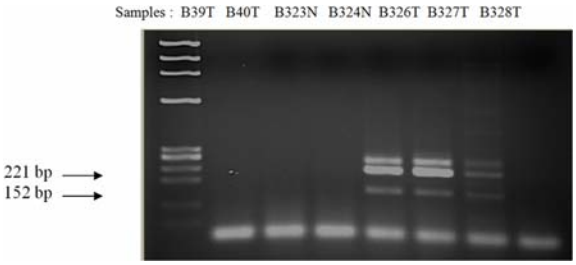

Fig. 7 Gel electrophoresis result of *SYKS* mRNA expression by RT-PCR in breast tissues. This gel photographs consists of 7 samples from the breast cancer tissues in which the sample name is labeled above. *SYKS* show at the 152-bp band. 3 of 7 breast cancer tissues express *SYKS* along with *SYKL* at 221-bp band.

Correlation between SYKS expression and clinicopathological parameters in Thai patients

Breast cancer patients (n = 108) were stratified by clinicopathological parameters including age, tumor size, histopathological grading, lymph node metastasis, estrogen receptor, progesterone receptor and HER2 status, and their relations with the *SYKS* expression were evaluated by Pearson's Chi-square test and Fisher's exact test (Table 4). A significant correlation between *SYKS* expression and tumor size >2 cm was found ($p = 0.008$) and *SYKS* expression had a potential to correlate with advanced pathological stages but it not reach significant ($p = 0.074$). Their relationship was shown in Fig. 9.

DNA methylation of SYK in breast cancer cell lines

It has become clear that epigenetic alterations play a critical role in the initiation and progression of cancer. It has been demonstrated that aberrant methylation is a widespread epigenetic phenomenon

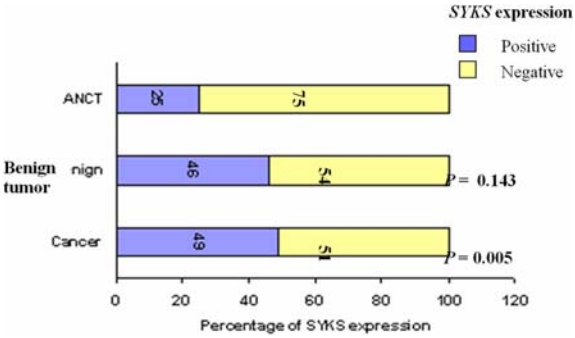

Fig. 8 The frequency of *SYKS* expression in breast tissue.

Table 3. Number (percentage) of subjects with *SYKS* expression in breast tissues

	SYKS expression		*p*-value
	Positive n (%)	Negative n (%)	
ANCT	9 (25)	27 (75)	
Benign	6 (46)	7 (54)	0.143
Cancer	53 (49)	55 (51)	0.005

Table 4. Clinicopathological features of breast cancer according to the expression of *SYKS*

Characteristic	*SYKS* expression		*p*-value[a]
	Positive (n = 53)	Negative (n = 55)	
Age (year)	51.9±12.1	52.0±12.2	NS
Histopathological			0.074
Grade			
Well & Moderate-differentiated	22	27	
Poorly-differentiated	21	12	
Tumor size			0.008
T1	8	19	
≥T2	43	30	
Lymph node metastasis			0.104
Negative	24	30	
Positive	25	17	
Perineural invasion			0.356
No	12	12	
Yes	1	3	
Perivascular invasion			0.111
No	24	31	
Yes	25	18	
Pathological staging			0.246
I-II	34	38	
III-IV	15	11	
Hormone Receptor			0.471
Positive	30	32	
Negative	14	17	
HER2			0.220
Positive	11	8	
Negative	15	20	

[a] Pearson chi-square test and Fisher's exact test

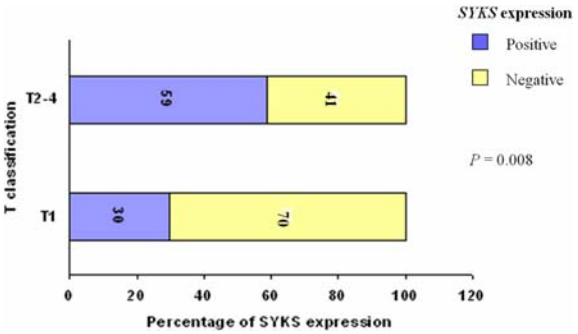

Fig. 9 The relationship between *SYKS* expression and tumor size in breast cancer tissues.

in cancer and may be among the earliest changes to occur during oncogenesis[13]. Aberrant methylation may lead to deregulation of gene expression. In human cancer, most notably, tumor suppressor genes (p16/CDK2, Rb, VHL, and BRCA1), mismatch repair genes (hMLH1), and others such as estrogen receptor a, E-cadherin, 14-3-3s, death-associated protein kinase, and Thrombospodin-1, are repressed by CpG island hypermethylation in cancer tissues[14,15]. There are strongly biological evidence showed no mutations, translocation, or homozygous deletions involving the *SYK* gene in naturally occurring neoplasm[8]. Coopman et al[3] suggested that the loss of *SYK* expression occurs at the transcriptional level. A few mechanisms could explain the loss of expression when the chromosomal rearrangement of exonic mutation appears to be minimal, such as aberrant methylation of the 5'-regulatory sequences, promoter mutations, loss of transcriptional activators, or binding of suppressor proteins to the promoter. Hypermethylation of *SYK* was reported to be associated with loss or reduction of *SYK* gene expression in breast cancer cell lines[8,16].

Yuan et al[8] explored the potential role of methylation in silencing *SYK* expression, they found the CpG island exhibited extensive methylation in three *SYK*-negative lines. In contrast, no methylation was observed in DNA from two *SYK*-positive cell lines. In the present study, DNA methylation of *SYK* in breast cancer cell lines which are T47D, MCF7, SKBr3, MDA-MB-231 and MDA-MB-435 were examined by methylation-specific PCR (MSP) (Fig. 10). The results showed that T47D, MCF7, MDA-MB-231 and MDA-MB-435 were methylated but SKBr3 was not. DNA methylation might cause the loss of *SYK* in breast cancer cell lines; especially in metastatic cell lines (MDA-MB-231 and MDA-MB-435) that completely loss of *SYK* mRNA and it seem that methylation is the main cause of this result.

DNA methylation of SYK in breast tissues

The expression of DNA methylation of *SYK* in breast cancer tissues of patients was examined using methylation-specific PCR (MSP) (Fig. 11). *SYK* was hypermethylated in 45% (37/83) of breast cancers and 15% (2/13) of benign lesions (Table 5 and Fig. 12). There were no significant relationships between the level of *SYK* DNA methylation in breast cancers and clinicopathologic factors (Table 6).

Comparison of SYK methylation with SYK mRNA expression

To explore the potential role of methylation in silencing *SYK* expression, methylation status of *SYK* DNA and mRNA expression in primary breast tissues were compared (Table 7 and Fig. 13) There was a significant correlation between SYK methylation and loss of SYK expression by 76% (29/38) of *SYK* methylation exhibited loss of *SYK* mRNA expression (p = 0.049). However, 56% (31/55) of breast tissues that lacked *SYK* expression did not have *SYK* methylation.

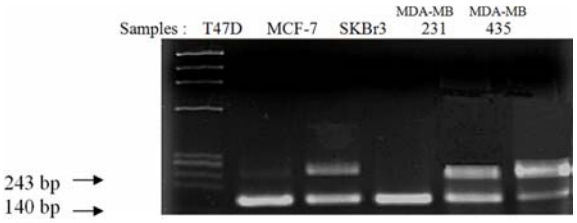

Fig. 10 Gel electrophoresis result of *SYK* DNA methylation by MSP in breast cancer cell lines. This gel photographs consists of the sample from the breast cancer cell line in which the sample name is labeled above. Methylated *SYK* DNA show at 243-bp bands (upper lane) and unmethylated *SYK* DNA show at 140-bp bands (lower lane). Of 5 breast cancer cell lines, *SYK* DNA of MDA-MB-231 and MDA-MB-435, MCF-7 and T47D are methylated whereas SKBr3 are not methyalted.

Discussion

The present study examined whether the expression of *SYK* and mechanisms responsible for its inactivation as well as the expression of spliced variant of *SYK* could have an implication in progression of breast cancer. We demonstrated that low tumorigenic breast cancer cell lines including T47D, MCF-7 and SKBr3 expressed *SYKL* mRNA at different levels ranged

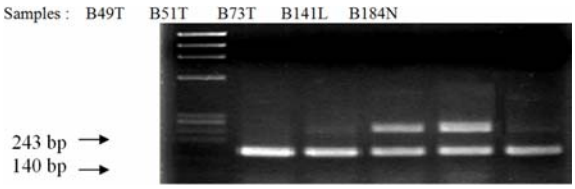

Fig. 11 Gel electrophoresis result of *SYK* DNA methylation by RT-PCR in breast tissues. This gel photographs consists of the sample from the breast cancer tissues in which the sample name is labeled above. This figure shows also unmethylated, slightly methylated, and strongly methylation *SYK* DNA.

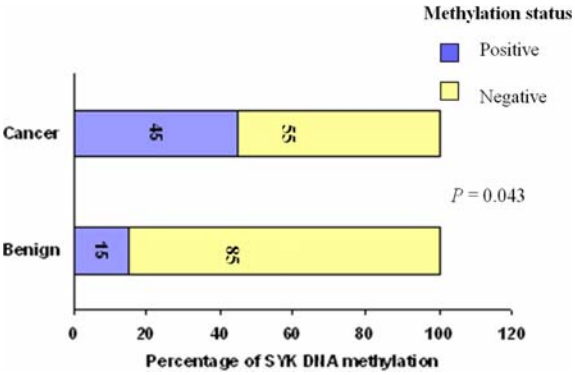

Fig. 12 The frequency of *SYK* DNA methylation in breast cancer tissues and benign tumor.

Table 5. Number (percentage) of subjects with *SYK* DNA methylation in breast cancer and benign

	SYK methylation		*p*-value
	Positive n (%)	Negative n (%)	
Benign	2 (15)	11 (85)	
Cancer	37 (45)	46 (55)	0.043

Table 6. Clinicopathological features of breast cancer according to the DNA methylation of *SYK*

Characteristic	*SYK* Methylation		*p*-value[a]
	Positive (n = 37)	Negative (n = 46)	
Age (year)	51.0±13.3	54.6±12.4	NS
Histopathological Grade			0.123
Well & Moderate-differentiated	21	17	
Poorly-differentiated	11	18	
Tumor size			0.155
T1	10	7	
≥T2	26	37	
Lymph node metastasis			0.548
Negative	19	24	
Positive	15	18	
Perineural invasion			0.346
No	8	14	
Yes	2	1	
Perivascular invasion			0.452
No	21	25	
Yes	12	17	
Pathological staging			0.456
I-II	24	31	
III-IV	9	14	
Hormone Receptor			0.449
Positive	21	26	
Negative	10	15	
HER2			0.428
Positive	6	9	
Negative	13	14	

[a] Pearson Chi-square test and Fisher's exact test

Table 7. Correlation between methylation of *SYK* and loss of *SYK* mRNA expression in breast tissues

SYK Methylation	*SYKL* Expression		*p*-value
	Positive[a] n (%)	Negative[a] n (%)	
Positive	9 (24)	29 (76)	
Negative	24 (44)	31 (56)	0.048

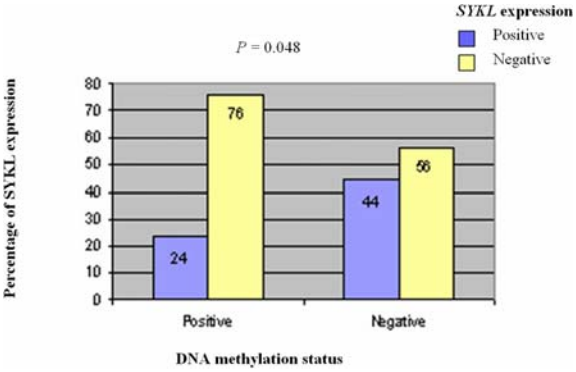

Fig. 13 Correlation between *SYKL* expression and DNA methylation.

from highest to lowest, respectively. Both MDA-MB-231 and MDA-MB-435 which are breast cancer cell lines with metastatic phenotype did not express *SYKL*. In addition, the expression of *SYKL* mRNA was decreased significantly from ANCT, benign tumors and breast cancers, respectively. These data confirmed the existence of SYK silencing in breast cancer cells of patients. No relationship between the expression of *SYKL* in breast cancer tissues and clinicopathological parameters was determined in consistent with previous

report[17]. Our results also show that *SYK* mRNA expression in breast tumors as determined by RT-PCR methodology is independent of tumor stage and therefore it is unlikely to play a prognostic role in patients with breast cancer. However, the overexpression of *SYK* in breast cancer may reflect that SYK might play another essential role in tumor progression which remains unknown at present. Nevertheless, an evidences support a role for SYK in gastric carcinogenesis. SYK protein expression was identified only in the nuclei of gastric cancer cells, because SYK in the nucleus is respected to the functioning SYK (SYKL)[18]. They found the loss of nuclei SYK expression was closely related to the malignant property of gastric cancer in the context of tumor depth and lymph node metastasis, especially in early gastric cancer.

With regard to the position of SYK, SYK protein is localized in the nucleus of breast cancer cells and only SYKL was detected in the nucleus while SYKS was detected in the cytoplasm[7]. Wang et al[7] found that the invasive ability of breast cancer cells was significantly suppressed by the expression of SYKL that presence in the nucleus whereas SYKS lacks this activity. Hence, the expression of *SYKL* mRNA which was detected by RT-PCR may not have a prognostic role in patients with breast cancer because of the presence of splice variants of *SYK* mRNA[19] represents another limitation to our RT-PCR methodology. The detection of SYKL protein in the nucleus of breast cancer cells or detection of specific *SYKS* mRNA may improve on this diagnostic requirement.

The expression of alternatively spliced variant of *SYK (SYKS)* was found in breast cancer tissues about 49% (53 of 108) which is more than in benign tissues (46%) and ANCT (25%), respectively. It seems the expression of *SYKS* is increases according to the number of abnormal cells in tissue, suggesting a contribution of *SYKS* to mammary tumor initiation and progression. A significant association between *SYKS* expression and increased tumor size was found. The trend towards associations with several unfavorable factors was also observed. These data support the role of *SYKS* in breast cancer progression and *SYKS* by itself may have oncogenic functions in breast cancer. These alternate mechanisms of *SYKS* need to be investigated.

Coopman et al[3] suggested that regulation of the *SYK* expression in breast cancer occurs at the transcriptional level. Hypremethylation of *SYK* at its CpG island has been proposed that it is a cause of SYK gene silencing. Yuan et al[8] found *SYK* 5' CpG hypermethylation in 30% (6/20) of breast cancer cell lines, and the aberrant methylation status was strongly associated with loss of *SYK* expression. *SYK* hypermethylation was found in majority (4/5) of breast cancer cell lines examined in this study. In addition, *SYK* was hypermethylated in 45% (37/83) of unselected breast cancer tissues and 15% of benign lesions. There was no significant correlation between the *SYK* methylation status and clinicopathological parameters with respect to age, tumor size, pathological grade, lymph node status, and expression statuses of hormone receptor (ERa and PgR), and HER2/neu.

To determine whether methylation was responsible for the loss of *SYK* expression, the correlation between the loss of *SYK* expression and its methylation status was determined. 76% with *SYK* methylation exhibited loss of *SYK* mRNA expression, which is evidence that methylation plays a major role in inactivating *SYK*. However, 56% of breast cancer lacked *SYK* expression that did not show *SYK* methylation, and thus it seems methylation might be not completely shut down *SYK* expression or other mechanisms besides methylation are responsible for silencing *SYK* expression.

In conclusion, *SYK* expression is reduced during human breast cancer progression and support a role for *SYK* function in breast cancer development. Methylation of *SYK* is one of the potential mechanisms responsible for silencing *SYK* expression. The excessively presence of the spliced variant of *SYK* or *SYKS* might influence in breast cancer initiation and progression. The loss of *SYKL* mRNA and the expression of *SYKS* and hypermethylated *SYK* may provide a valuable addition to other susceptible genes that are being assembled to facilitate prognosis of breast cancer progression.

What is already known on this topic?

Several RTKs play an important role in breast carcinogenesis. Members of NRTKs such as SYK have been proposed as a potential tumor suppressor and novel biomarker in human breast carcinomas although the underlying mechanisms have remained unclear.

What this study adds?

This study confirm the role of SYK in human breast cancer development and progression via two mechanisms both alternative splicing and hypermethylation of its promoter. This finding may have useful clinical application in management of breast cancer patients.

Acknowledgements

This study was supported by the educational research grant of the Faculty of Medicine Siriraj Hospital, Mahidol University.

Potential conflicts of interest

None.

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บทบาทของภาวะโปรโมเตอร์ไฮเปอร์เมทิเลชั่น และการแสดงออกของจีน spleen tyrosine kinase ในกลไกการเกิดมะเร็งเต้านม

พรชัย โอเจริญรัตน์, บุศรากร ทะไกรราช

ภูมิหลัง: Spleen tyrosine kinase (SYK) ได้รับการศึกษาว่าเป็นตัวยับยั้งที่สำคัญของการเจริญเติบโตของเซลล์มะเร็งและมีบทบาทเป็น tumor suppressor ในมะเร็งเต้านมของมนุษย์ การสูญเสียการแสดงออกของจีน SYK พบได้บ่อยในเซลล์มะเร็งเต้านมโดยเกิดร่วมกับ promoter hypermethylation บ่งบอกว่าการเกิดภาวะ methylation อาจมีบทบาทสำคัญในการยับยั้งจีน SYK ส่วน spliced variant ของจีน SYK หรือ SYKS มีการแสดงออกที่พบบ่อยในเซลล์มะเร็งเต้านมซึ่งตรงข้ามกับ full-length form ของจีน SYK หรือ SYKL

วัตถุประสงค์: เพื่อศึกษาการแสดงออกของจีน SYKL และ SYKS และการเกิด DNA methylation ในตำแหน่ง promoter ของจีน SYK และหาความสัมพันธ์กับความเสี่ยงและการลุกลามของมะเร็งเต้านม

วัสดุและวิธีการ: ศึกษาการแสดงออกของจีน SYKL และจีน SYKS และการเกิด SYK DNA methylation ในเซลล์มะเร็งเต้านม 5 สายพันธุ์ ศึกษาการแสดงออกของจีน SYKL และจีน SYKS โดยวิธีการ quantitative reverse transcription-PCR ในเนื้อเยื่อจากมะเร็งเต้านม 108 ราย เนื้องอกปกติ 13 ราย และเนื้อเยื่อเต้านมปรกติข้างเคียง 35 ราย ทำการสกัด DNA จากเนื้อเยื่อมะเร็งเต้านม 83 รายและเนื้องอกปรกติ 13 รายเพื่อหาการเกิด SYK DNA methylation โดยวิธี methylation-specific PCR

ผลการศึกษา: พบการสูญหายไปทั้งหมดร่วมกับการเกิด DNA methylation ของจีน SYKL ในเซลล์มะเร็งเต้านมสองสายพันธุ์ที่มีคุณสมบัติแพร่กระจายได้ พบมีการลดลงอย่างมีนัยสำคัญในการแสดงออกของจีน SYKL ในเนื้อเยื่อมะเร็งเต้านมและเนื้องอกเต้านมเมื่อเทียบกับเนื้อเยื่อเต้านมปกติ (ค่าเฉลี่ยของ mRNA = 1.0297, 0.6294 และ 0.3446 ในเนื้อเยื่อปรกติ เนื้องอกธรรมดา และเนื้อเยื่อมะเร็งเต้านมตามลำดับ) พบมีการสูญหายไปของจีน SYKL ใน 50% ของเนื้อเยื่อมะเร็งเต้านมแต่ไม่พบในเนื้องอกธรรมดาและเนื้อเยื่อเต้านมปกติ พบมี methylation ของจีน SYK ใน 45% ของเนื้อเยื่อมะเร็งเต้านมเมื่อเทียบกับ 15% ในเนื้องอกธรรมดา ยิ่งไปกว่านั้น ยังพบมีความสัมพันธ์อย่างมีนัยสำคัญระหว่างการเกิด methylation และการไม่แสดงออกของจีน SYK แต่ไม่มีความสัมพันธ์กับคุณลักษณะทางคลินิกและพยาธิวิทยา พบมีเซลล์มะเร็งเต้านมสองสายพันธุ์มีการแสดงออกของจีน SYKS การศึกษาในเนื้อเยื่อพบมีการแสดงออกของจีน SYKS ในเนื้อเยื่อมะเร็งเต้านมเกือบครึ่ง (49%) และมีการแสดงออกที่น้อยกว่าในเนื้องอกธรรมดา (46%) และเนื้อเยื่อปรกติ (25%) การแสดงออกของจีน SYKS มีความสัมพันธ์กับขนาดของก้อนมะเร็งที่ใหญ่ขึ้น

สรุป: การศึกษานี้แสดงให้เห็นว่ากลไกที่เกี่ยวข้องกับจีน SYK มีบทบาทที่สำคัญในพัฒนาการของมะเร็งเต้านม กลไกที่ทำให้ยับยั้งการทำงานของจีน SYKL อาจเกิดผ่านกระบวนการ DNA metylation หรือการเกิดจีน SYKS