Sporadic mutations and expression analysis of Spleen Tyrosine Kinase gene in breast cancer: a preliminary report

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Spleen tyrosine kinase (Syk) is an intracellular receptor protein kinase involved in cell proliferation, differentiation and phagocytosis. Syk expression has been reported in cell lines of epithelial origin. The strong expression of Syk in mammary gland prompted research into its potential role in mammary carcinogenesis. Fresh Biopsy samples were collected from different hospitals of Pakistan. Single stranded conformational polymorphism and Semi quantitative reverse transcriptase polymerase chain reaction was used to investigate somatic mutations and expression alterations in twenty five breast cancer tumor tissues along with their adjacent normal control tissue. Statistical analysis was performed to explore Syk association with breast cancer risk. In the present study, DNA from tumor tissue was analyzed and mutations in the coding sequence and intronic sequence spanning the exonic region of the Syk gene were identified. Sequence analysis revealed two missense: g61096G>A, g65967G>A, one frame shift: g87413insA and one silent mutation g42841G>A in exonic region while nucleotide variations were also observed in intronic region including one splice site mutation. These mutations in Syk are first time being reported in breast cancer. In addition, this study also revealed that Syk mRNA expression was markedly reduced in tissues of breast cancer compared to their adjacent normal control tissue.

Key words: breast cancer, spleen tyrosine kinase, reverse transcriptase PCR, somatic mutations.

Introduction

Spleen tyrosine kinase (Syk) is a member of the non-receptor protein tyrosine kinase family Syk/ZAP-70, which is widely expressed in hematopoietic cells [1]. It is activated upon the binding of its tandem Src homology 2 (SH2) domains to immunoreceptor tyrosine-based activation motif (ITAM) within the receptor. This activation plays an essential role in lymphocyte activation and development of immune cells [2]. Initially, Syk was believed to be expressed only in hematopoietic cells, but now it has also been reported to express in non-hematopoietic cells such as epithelial cells, hepatocytes, fibroblasts, neuronal cells, and breast tissue [3]. It is involved in mediating diverse cellular responses such as proliferation, differentiation and phagocytosis [4].

Expression of Syk has been reported in cell lines of epithelial origin. Syk is considered to be a good tumor suppressor compared with other tyrosine kinases [5]. Studies have indicated that Syk expression varies in breast tissues. Most importantly its role in tumor suppression in breast cancer has been well reported. Syk is mostly expressed in normal breast tissue of humans, benign tumors and low grade cancer cels. Syk mRNA and protein expression have been reported to be downregulated or lost in invasive breast cancer [6].

The tumor suppressive activity of Syk is caused by simultaneous enhancement of cell-cell interactions and decreasing cellular motility [7]. In its aberrant form,

it is believed to result in abnormal mitotic progression and cell death [8-10].

The cause of loss of Syk expression in invasive breast cancer has remained unclear. Hypermethylation of CpG islands of the Syk gene promoter region can be one of the major causes of oncogenesis [11, 12]. In normal breast cells, full length Syk protein is expressed, which due to hypermethylation of the Syk promoter, loses normal expression and results in a shorter survival time. Overexpression of a kinase-deficient Syk mutant in a Syk-positive breast cancer cell line causes enhanced tumor growth [5], indicating the role of kinase domain in tumor suppression.

The present study has been planned to determine Syk mRNA expression in patients suffering from breast cancer. Both breast cancer and its adjacent normal control tissue (ANCT) were collected and semi quantitative reverse transcriptase-PCR was carried out. Expression was correlated with clinicopathological parameters of the sampled patients. Somatic mutations in these tissues were studied by PCR-SSCP method.

Material and methods

Tissue samples

Twenty five tumor samples with patient-matched normal epithelium were obtained at the time of surgical resection at the Pakistan Institute of Medical Sciences, Islamabad, Military Hospital Rawalpindi and Allied Hospital Faisalabad. Both tumor tissues and ANCT were verified histologically. These samples had been obtained after receiving informed consent from the patients according to a reviewed and approved protocol by the respective hospitals and CIIT departmental ethical committee. Resected tissues were collected in RNA later on and were stored at –80°C until further use. Clinicopathological data were collected from each patient's histopathology report.

Mutational analysis

To detect the mutations in the Syk gene, fifteen sets of primers were used to amplify the entire coding region (exons 1–16) of the Syk gene as described previously [5]. Genomic DNA was extracted from tumor as well as ANCT tissue using phenol chloroform method [13, 14].

Each PCR was performed in 10 µl reaction mixture containing approximately 20 ng DNA (2 µl), 10 mM of the specific primer (1.5 µl) and 5 µl of PCR master mix (Fermentas). Amplification was carried out with initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing temperature for 45 s and 72°C for 45 s with final extension at 72°C for 7 min.

PCR products were resolved on 2% agarose gel and analyzed. PCR-single stranded conformation polymorphism (SSCP) followed by DNA sequence analysis was performed to detect any sequence variations.

Sequencing results were analyzed by BioEdit software version 8.

mRNA expression analysis

The expression levels of Syk mRNA were examined in twenty five breast tumor tissues along with their adjacent normal control tissues, and RNA was isolated from human breast tissue as described previously [15].

Extracted RNA was reverse transcribed to synthesize cDNA using SuperScript III First Strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. Synthesized cDNA was amplified for Syk gene using primers designed with primer blast software. The sequence of primer used for analysis of Syk mRNA expression was (forward) CCACTGTGGCCAGCACGAGG and (reverse) GATGCCACCAGGGCAGCCTG. The primers were checked to avoid amplification of non-specific products. PCR reactions were carried out in the final volume of 10 µl of reaction mixture consisting of 2 µl cDNA, 5 µl PCR master mix (fermentas), 1 µl primer forward and 1 µl primer reverse. The reaction mixture was submitted to initial denaturation at 94°C for 2 min, followed by 30 rounds of amplification at 94°C (15 s) for denaturation, 65°C (30 s) for annealing, and 72°C (1 min) for extension, with final extension at 72°C for 2 min. Housekeeping gene GAPDH was used as an internal control. Amplified products were analyzed on 2% agarose gel.

Statistical analysis

Expression of the Syk gene was correlated with histopathological results by Fisher's Exact test using statistical analysis software Graph pad Prism.

Results

Mutational analysis

To investigate the possible role of mutations in the Syk gene for downregulation of its expression in breast tumor samples, genomic DNA was analyzed by PCR-SSCP analysis. Sequencing results confirmed sequence variations in exon 5, 7, 10, and 15 (Fig. 1) and predicted change and type of genetic variations are mentioned in Table I. Intronic variations were also detected in intron 1-2, 9-10, 14-14'15'-16&16 (Fig. 2 and Table II).

mRNA expression analysis

Uniform expression of GAPDH was observed in all control and breast tissue samples (Fig. 3A). Uniform expression of Syk was observed in all control breast tissues. However, the Syk expression was related to the stage

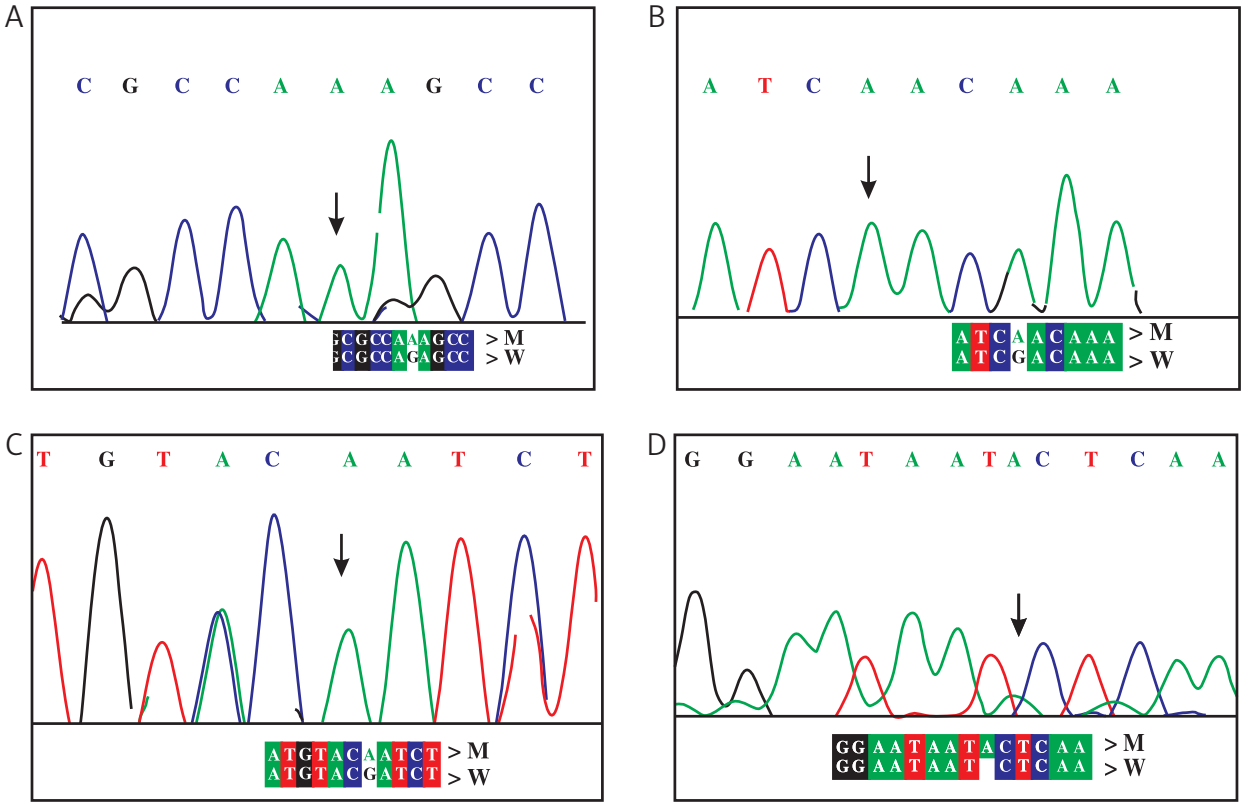

Fig. 1. Nucleotide sequences of a fragment of Syk of exon 5 (A), exon 7 (B), exon 10 (C) and exon 15'(D). The first three images show the transition from G>A resulting in amino acid change Gln>Gln (A) and Asp>Asn (B ,C) and frame shift mutation given in D

Table I. Sequencing results of exon variants of the Syk gene

Reported and novel exonic variants identification in Syk gene			Function
exon	change in nucleotide	codon	type of mutation
5	G>A (rs35758162)	CAG>CAA	Gln>GlnSilent
7	G>A	GAC>AAC	Asp>AsnMissense
10	(insertion of A)		Frameshift
15'	G>A	GAT>AAT	Asp>AsnMissense

of breast cancer as indicated in Fig. 3B. Syk and GAPDH expression was the same in control breast tissues.

Downregulation of Syk expression was not detected in stage I tumor samples as compared with controls, but samples of stage II and stage III showed downregulation of Syk in comparison with its expression in the adjacent normal control tissue.

Expression of the Syk gene with respect to clinicopathological parameters

Correlation of the *Syk* gene expression in clinicopathologically classified normal and tumor tissues was also determined using Fisher's Exact test (Table III). Patients with Grade III and stage III tumor have statistically significant p-value (0.013*) for the loss of expression as compared with normal expression of the *Syk* gene. Invasive ductal carcinoma; 15 (15) patients (p-value = 0.002**), with tumor size > 2 < 5; 9 (9) patients (p-value = 0.0294*); and > 5 cm; 11 (11) patients (p-value = 0.0001****), also showed statistically significant values for the loss of expression as compared with normal expression of the Syk gene.

Discussion

In order to assess the role of *Syk* in breast cancer patients among the Pakistani population, this preliminary research project studied mutational as well as expressional analysis of this gene in 25 breast cancer tissues.

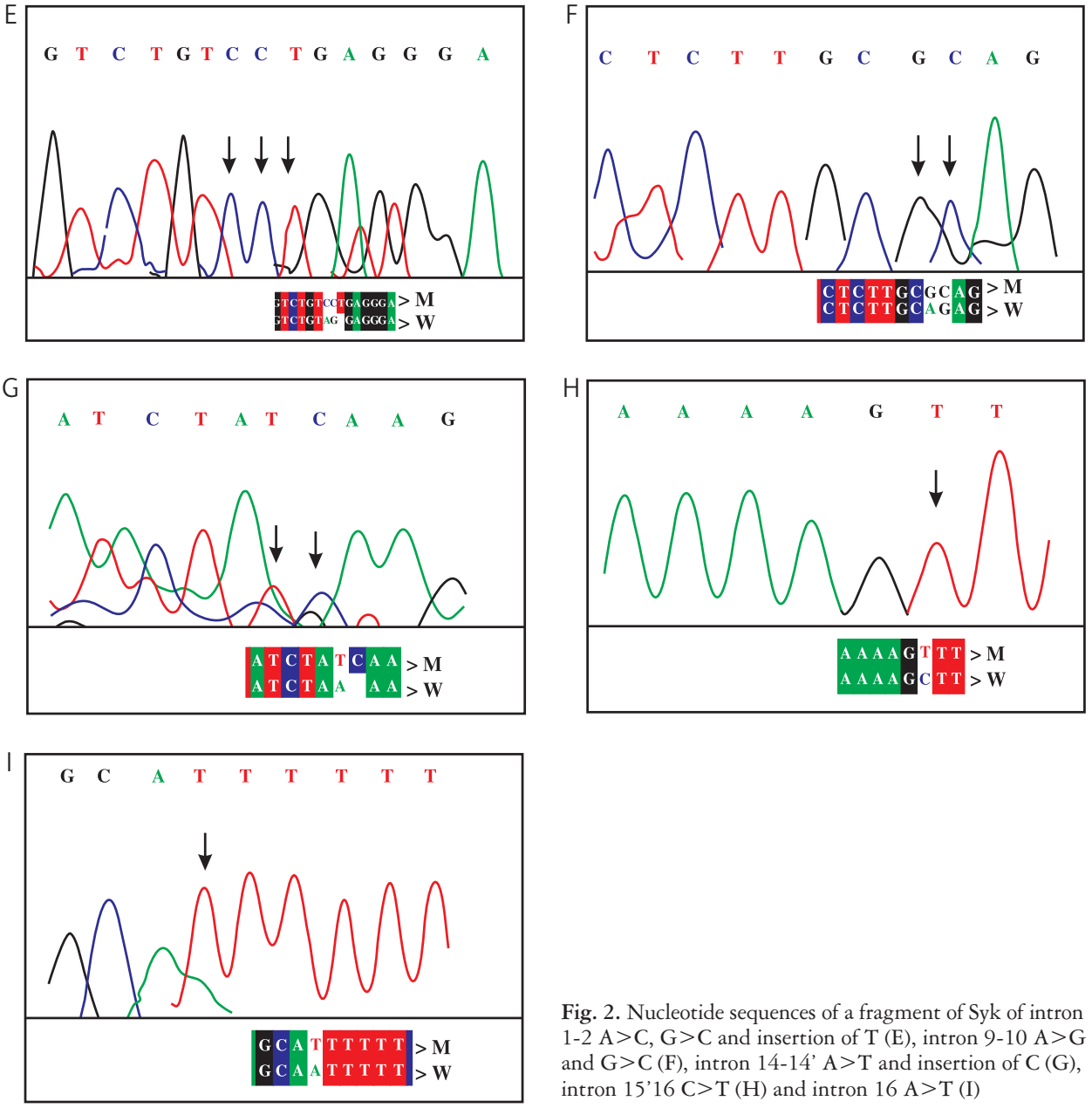

Fig. 2. Nucleotide sequences of a fragment of Syk of intron 1-2 A>C, G>C and insertion of T (E), intron 9-10 A>G and G>C (F), intron 14-14' A>T and insertion of C (G), intron 15'16 C>T (H) and intron 16 A>T (I)

Table II. Sequencing results of intronic variants of the *Syk* gene

REPORTED AND NOVEL INTRONIC VARIANTS IDENTIFICATION IN *SYK* GENE		FUNCTION
INTRON	CHANGE IN NUCLEOTIDE	TYPE OF MUTATION
1-2	A>C ,G>C, insertion of T	–
9-10	GC>AG	splice site mutation
14-14'	A>T, insertion of C (rs1760132)	–
15'-16	C>T	–
16	A>T (rs158689)	–

Four genetic variants in exonic region and 5 in intronic regions were found. Among these nine variants; three have already been reported in the SNP database (http://www.ncbi.nlm.nih.gov/snp), and one has been reported in cosmic somatic mutation database (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Variants found in exonic regions represented the gene part involved in encoding SH2 domains, interdomain B region and kinase domain of the *Syk* protein. Silent mutation in exon 5 (rs35758162) falls in first SH2 domain

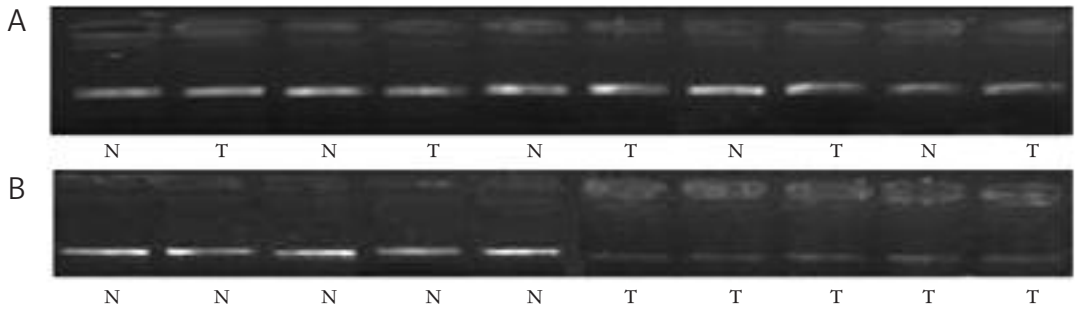

Fig. 3. Agarose gel (2%) electrophoresis image showing GAPDH mRNA expression in control tissue and tumor tissue (A). *Syk* mRNA expression in control tissue and *Syk* mRNA expression in tumor tissue (B) detected by semi quantitative RT-PCR. Image B indicates downregulation or partially lost mRNA expression of *Syk* in tumor tissues as compared with controls. T indicates breast cancer samples and N indicates control tissue. The *Syk* gene RT-PCR products normalized to the level of GAPDH mRNA

Table III. Correlation between the expression of Syk and clinical classification of breast cancer

Variables	Normal Syk expression	Low Syk expression	P-value
age (years)			
≤ 25	1 (1)	0 (1)	1.000
≤ 50	3 (14)	11 (14)	0.236
≤ 75	1 (10)	9 (10)	0.140
grade			
I	5 (5)	0 (5)	0.181
II	1 (8)	7 (8)	0.282
III	0 (12)	12 (12)	0.013*
stage			
I	5 (5)	0 (5)	0.181
II	1 (8)	7 (8)	0.282
III	0 (12)	12 (12)	0.013*
type of carcinoma			
invasive ductal	0 (15)	15 (15)	0.002**
invasive lobular	2 (4)	2 (4)	1.000
medullary	2 (3)	1 (3)	1.000
others	1 (3)	2 (3)	1.000
tumor size			
≤ 2 cm	5 (5)	0 (5)	0.1810
> 2 cm and < 5 cm	0 (9)	9 (9)	0.0294*
≥ 5 cm	0 (11)	11 (11)	0.0294**

**represents significant value*

region and no change in amino acid sequence occurred (CAG>CAA; Gln>Gln). Missense mutation in exon 7 GAC>AAC falls in second SH2 domain, changing amino acid aspartic acid to asparagine. This mutation at position 219 amino acid may affect structural stability because the conversion of aspartic acid to asparagine may alter conformation due to the negative charge.

Frame shift mutation (insertion of A) in exon 10 falls in interdomain B region. Interdomain B region encoded by amino acids from 261 to 376, 290 position is close to tyrosine at position 296, which is important for phosphorylation. Interdomain B has putative autophosphorylation sites also at position 323, 348 and 352. This linker segment acts as a docking region that provides phosphotyrosyl residues for binding to SH2 domains of other signaling molecules [3]. Missense mutation in exon 15' (COSM48799) falls in kinase domain (GAT>AAT) at position 603 and was adjacent to tyrosine at position 602 as important tyrosine residues for phosphorylation.

Although Syk has been identified as a potent modulator of breast epithelial cell growth, its role in breast

cancer progression and metastasis still needs to be elucidated [21]. In the absence of any reported genetic variations in somatic breast cancer patients, the role of these genetic changes remains as a question mark and detailed studies with a larger set of data is required before making any conclusion regarding these changes. Nevertheless, these changes emphasize the role of the *Syk* gene in the pathogenesis of breast cancer and need to be further explored.

The results from the current data indicated downregulation of the *Syk* gene in breast tissues. Additionally, the inverse relationship of *Syk* mRNA expression with stages of breast cancer was also observed. Stage I tumor samples did not show any detectable loss of expression as compared with normal control (from normal epithelium) but partial to complete loss of expression was found in stage II and stage III (p-value = 0.013) samples. The common type of carcinoma was invasive ductal carcinoma, all patients with invasive ductal carcinoma showed loss of expression of the *Syk* gene (highly significant p-value of 0.002**) and was in either stage II or stage III. Thus, it is possible that the *Syk* expression is lost progressively during the development of breast cancer.

Earlier studies have shown that loss of expression of *Syk* is associated with increased mammary tumor invasiveness and metastasis [6]. It has also been reported that *Syk* mRNA was downregulated (measured by quantitative reverse transcriptase-PCR) at least 2-fold in 30% of breast tumors, and the downregulation was associated with a poorer prognosis [19]. Previous studies have also indicated the epigenetic inactivation of the *Syk* gene in breast tumors, supporting its role in metastasis suppression [11]. Further studies with a larger number of clinical samples will provide a better insight, which may correlate with different etiological factors and clinicopathological parameters.

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Authors declare that there is no conflict of interest.

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