

RESEARCH ARTICLE

Association of SYK Genetic Variations with Breast Cancer Pathogenesis

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Abstract

Spleen tyrosine kinase (SYK) is a non-receptor type cytoplasmic protein and a known tumor suppressor gene in breast cancer. Polymorphisms in SYK have been reported to be associated with cell invasion/cell mortality and an increased risk of cancer development. In this case control study, all exons of the SYK gene and its exon/intron boundaries were amplified in 200 breast cancer cases and 100 matched controls and then analyzed by single stranded conformational polymorphism. Amplified products showing altered mobility patterns were sequenced and analyzed. Twelve variations were identified in exonic and intronic regions of DNA encoding SH2 domain and kinase domain of the SYK gene. All of these mutations are novel. Among them, 5 missense mutations were observed in exon 15 while one missense mutation was found in exon 8. In addition to these mutations, six mutations were also identified in intronic regions. We found a significant association between SYK mutations and breast cancer and observed that Glu241Arg, a missense mutation is associated with an increase risk of ~7 fold (OR=6.7, 95% CI=1.54-28.8), Thr581Pro (missense mutation) is associated with increased risk of ~16 fold (OR=15.5, 95% CI=2.07-115.45) and 63367 T>G (missense mutation) is associated with increased risk of ~13 fold (OR=12.8, 95% CI=1.71-96.71) for breast cancer. Significant associations were observed for each of these variations with both late menopause ($p < 0.01$) and early menarche ($p < 0.005$) cases when compared to controls. Our findings suggest that the polymorphic gene SYK may contribute to the development of breast cancer in at least the Pakistani population. This study provides an insight view of SYK which may provide a significant finding for the pharmaceutical and biotechnology industry.

Keywords: SYK - tumor suppressor gene - breast cancer - SSCP

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Introduction

Breast cancer is the leading cause of cancer related deaths in females (Jemal et al., 2011). Approximately one in every nine Pakistani women is likely to suffer from breast cancer during her lifetime, which is one of the highest incidence rates in Asia (Naeem et al., 2008). The pathogenesis and progressions of breast cancer are thought to occur through a multistep process, including oncogenes activation and mutation or loss of tumor suppressor genes (Osborn et al., 2004). Protein-tyrosine kinase is an important tumor suppressor family and has been classified into two families: trans-membrane receptor family and non-receptor family. SYK gene encodes 72 kDa non-receptor type cytoplasmic proteins (de Cast, 2011) and consists of three domains; two tandem SH2 domains i.e N-terminal domain (15-109 residues), and C-terminal domain (168-261 residues) and one C terminal kinase domain (de Cast, 2011). The N-terminal SH2 domain along with the other SH2 domains makes a Y-shape molecule (Latour et al., 1998).

SYK normally expresses in hematopoietic cells like B cells and mast cells, however its expression can also be found in non-hematopoietic cells like hepatocytes, fibroblasts and epithelial cells (Latour et al., 1998). Various studies have reported that SYK tyrosine kinase can act as a tumor suppressor gene in epithelial breast cells (Coopman et al., 2000; Yuan et al., 2001) and its expression has been observed in normal human breast tissue as well as in benign breast lesions and low-tumorigenic breast cancer cell lines (Coopman and Mueller, 2006). However in case of invasive breast carcinoma tissues and cell lines, expression of SYK mRNA and protein is seen to be either low or undetectable compared to normal expression. This reduced SYK expression can have significant increased risk for distant metastasis and also cause a poorer prognosis (Coopman and Mueller, 2006). Loss of SYK expression is related with shorter survival in breast cancer patients (Toyama et al., 2002) possibly due to the absence of SYK suppressor function (Coopman et al., 2000; Wang et al., 2003). Polymorphisms and mutations have earlier been reported for SYK in different cancer (Hunter et

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al., 2000; Inayat et al., 2012; Kanwal et al., 2012). But the results about SYK polymorphisms/mutations were controversial and additional studies are needed. The aim of this study to detect polymorphisms and mutations of SYK gene from breast cancer patients and to evaluate the role of these polymorphisms in the etiology of breast cancer in Pakistani population.

Materials and Methods

The present study was conducted with a prior approval from ethical committees of both COMSATS Institute of Information Technology Islamabad (CIIT) and hospitals. A total of 200 patients with histologically confirmed breast cancer were recruited from Nuclear Oncology and Radiotherapy Institute (NORI). A total of 100 age, gender and ethnicity matched and cancer free healthy individuals were selected as controls. The inclusion criterion for the controls was absence of prior history of cancer or pre-cancerous lesions. Patients and controls suffering from any other familial disease (diabetes, blood pressure and cardiovascular impairment) were excluded from this study. After obtaining informed consent, all individuals were personally interviewed using the specifically designed questionnaire. Information on age, gender, ethnic group, menopausal status, age at menarche and detailed exposure data on smoking was recorded.

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from white blood cells, using standard phenol-chloroform extraction method (Mahjabeen et al., 2012) and stored at -20°C for further processing. Human SYK exon sequence was taken from Ensembl. Primers were designed using primer 3 software, and checked for specific amplification using BLAST. Whole coding region and their exon intron boundaries of approximately 60 bp sequence of SYK were investigated to identify novel and any splice site variations in parallel to previously reported mutations. Each PCR reaction was carried out for $25\mu\text{l}$ master mixture containing $2\mu\text{l}$ DNA, $1\mu\text{l}$ forward primer, $1\mu\text{l}$ reverse primer, $0.2\mu\text{l}$ dNTPs, and $0.2\mu\text{l}$ Taq DNA polymerase. Program of thermal cyclor was adjusted according to the following conditions, denaturation at 95°C for 5 min, 35 cycles of 94°C for 45 sec, 58°C for 45 sec, 72°C for 45 sec and then a final extension at 72°C for 7 min. PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Mutational screening, sequencing and data analysis

Single stranded conformational polymorphism (SSCP) was used for the mutational analysis of the PCR products. Samples, displaying an altered electrophoretic mobility were re-amplified in another reaction and were analyzed by direct sequencing to confirm and characterize the nature of mutations. Sequencing was carried out by MacroGen (Korea). Control (normal) samples were also sequenced along with cases to check the quality of sequencing.

For each mutation, deviation of the genotype frequencies in the control subjects from those expected under Hardy-Weinberg equilibrium was calculated. We

estimated the cancer risk associated with the alleles, genotypes as odds ratios (ORs) and 95% confidence intervals (CIs) by using unconditional logistic regression with adjustment for age, sex and smoking status in study group. In order to compare the frequency of all the mutations with the menopausal and menarche status logistic regression analysis and t test was used. P-values for trend were calculated by chi-square test.

Results

SSCP and sequencing analysis of SYK gene revealed twelve novel mutations in SYK gene (Figure 1). Among these twelve identified mutations, five missense mutations (Thr581Pro, Ala582Pro, Met583Phe, Glu585Lys, Glu588Lys) were observed in exon 15. First missense mutation observed in exon 15 was Thr581Pro, showing G to A substitution resulting in change in DNA sequence from GTC to TTC and encode the amino acid proline instead of threonine. Second missense mutation observed in exon 15 was Ala582Pro, showing G to C substitution resulting in change of DNA sequence from GCT to CCT and encode the amino acid proline instead of alanine. Third missense mutation Met583Phe reported in exon 15 showings A to T substitution and G to T substitution resulting in change of DNA sequence from ATG to TTT and encode the amino acid phenylalanine instead of methionine. Fourth missense mutation observed in exon 15 was Glu585Lys showing two G to A substitutions in

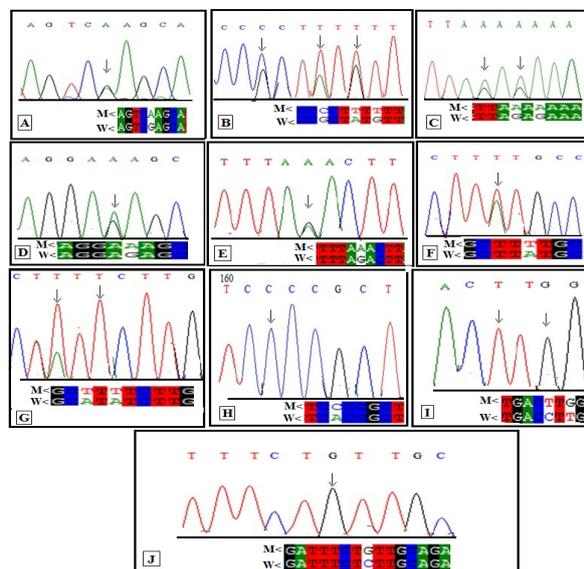


Figure 1. Nucleotide Sequences of Fragments of SYK Gene. (A) Mutant heterozygous codon of Glu241Arg in exon 8. (B) Mutant heterozygous codons of Ala582Pro and Met582Phe in exon 15. (C) Mutant heterozygous codon of Glu585Lys in exon 15. (D) Mutant heterozygous codon of Glu588Lys in exon 15. (E) Mutant heterozygous codon of intronic mutation 63545 G>A. (F) Mutant heterozygous codon of intronic mutation 87307 A>T. (G) Mutant heterozygous codon of intronic mutation 87313 A>T. (H) Mutant homozygous codon of Thr581Pro in exon 15. (I) Mutant homozygous codons of intronic mutations 63365 C>T and 63367 T>G. (J) Mutant homozygous codon of intronic mutation 76377 C>G. (M) variant sequence and (W) wild type sequence

codon 585 resulting in change of DNA sequence GAG to AAA and encode the amino acid lysine instead of glutamic acid. Fifth missense mutation observed in exon 15 was Glu588Lys showing G to A substitution resulting in change of DNA sequence GAG to AAG encodes the amino acid lysine instead of glutamic acid. While one missense (Glu241Arg) mutation was observed in exon 8 showing G to A substitution resulting in change of DNA sequences from GAG to AAG encode the amino acid arginine instead of glutamic acid. In addition to these six mutations (63365 C>T, 63367 T>G, 63545 G>A, 76377C>G, 87307A>T, 87313A>T) were also identified in intronic regions. These mutations are further divided on the basis of whether the mutations are heterozygous or homozygous. Among all the six missense mutations five are heterozygous while one missense mutation is homozygous. Among the six

intronic mutations three are homozygous while other three are heterozygous.

Frequency of SYK heterozygous mutations in cases and controls is shown in Table 1. In case of missense heterozygous mutations ~7 fold increase in breast cancer risk (OR=6.7,95% CI=1.54-28.80) was associated with Glu241Arg, ~3 fold increase (OR=3.2, 95%CI=1.61-6.48) with Glu585Lys and ~4 fold increase (OR=3.6, 95% CI=1.57-8.42) was associated with Gln588Lys. In case of intronic heterozygous mutations ~5 fold increase in breast cancer risk (OR=4.8, 95%CI=1.42-16.38) was associated with 6345 G>A and ~2 fold increase (OR=1.6, 95%CI=0.74-3.63) with 87313A>T. Frequency of SYK homozygous mutations in cases and controls is shown in Table 2. In case of missense homozygous mutations, ~2 fold increase in breast cancer risk (OR=1.5, 95%CI=0.68-3.36) was associated with Thr581Pro. Frequency of reported intronic homozygous mutations showed that ~16 fold increase in breast cancer risk (OR=15.5, 95%CI=2.07-115.45) was associated with Thr581Pro and ~13 fold increase (OR=12.8, 95%CI=1.71-96.71) with 63367 T>G.

Table 1. Allele and Genotype Frequencies of Heterozygous Mutations in SYK Gene and their Associations with Risks for Breast Cancer

Mutations/ Exons	Series	Minor allele frequency	No of heterozygote mutant	No of homozygote wild type	bP value
Glu241Arg	Case patients	0.1	24	176	0.003
	Control subjects	0.04	2	98	
	*OR(95%CI)		6.7(1.54-28.80)	1 (ref)	
Ala582Pro	Case patients	0.06	14	186	0.62
	Control subjects	0.1	5	95	
	*OR(95%CI)		1.4(0.50-4.05)	1 (ref)	
Met583Phe	Case patients	0.11	26	174	0.35
	Control subjects	0.18	9	91	
	*OR(95%CI)		1.5(0.68-3.36)	1 (ref)	
Glu585Lys	Case patients	0.25	57	143	0.0007
	Control subjects	0.21	11	89	
	*OR(95%CI)		3.2(1.61-6.48)	1 (ref)	
Glu588Lys	Case patients	0.19	43	157	0.002
	Control subjects	0.14	7	93	
	*OR(95%CI)		3.6(1.57-8.42)	1 (ref)	
63545 G>A	Case patients	0.11	26	174	0.006
	Control subjects	0.05	3	97	
	*OR(95%CI)		4.8(1.42-16.38)	1 (ref)	
87307A>T	Case patients	0.06	14	186	0.62
	Control subjects	0.1	5	95	
	*OR(95%CI)		1.43(0.5-4.1)	1 (ref)	
87313A>T	Case patients	0.12	28	172	0.26
	Control subjects	0.18	9	91	
	*OR(95%CI)		1.6(0.74-3.63)	1 (ref)	

*OR, odds ratio; CI, confidence interval. *ORs for logistic regression analysis. ^bp>0.05, by χ^2 -test for trend

Correlations were tested among the frequency of SYK gene mutations and menopausal status of breast cancer patients (Table 3). The SYK mutations were

Table 2. Allele and Genotype Frequencies of Homozygous Mutations in SYK Gene and their Association with Risk for Breast Cancer

Mutations/ Exons	Series	Minor allele frequency	No. of homozygous mutants	No. of homozygous Wild type	bP value
63365 C>T	Case patients	0.25	25	175	0.02
	Control subjects	0.27	4	96	
	*OR(95%CI)		3.4(1.16-10.14)	1 (ref)	
63367 T>G	Case patients	0.23	23	177	0.001
	Control subjects	0.07	1	99	
	*OR(95%CI)		12.8(1.71-96.71)	1 (ref)	
Thr581Pro	Case patients	0.26	26	174	0.34
	Control subjects	0.6	9	91	
	*OR(95%CI)		1.5(0.68-3.36)	1 (ref)	
76377C>G	Case patients	0.27	27	173	0.0002
	Control subjects	0.07	1	99	
	*OR(95%CI)		15.5(2.07-115.45)	1 (ref)	

*OR, odds ratio; CI, confidence interval. *ORs for logistic regression analysis. ^bp>0.05, by χ^2 -test for trend

Table 3. Risk of Breast Cancer Associated with Menopausal Status of Breast Cancer Patients

Mutations	Menopause before 51			Menopause after 51		
	Controls (%)	Patients (%)	OR ^a (95%CI),	Controls (%)	Patients (%)	OR ^a (95%CI)
Glu241Arg	1	6	3.06 (0.36-25.78), 0.4	1	14	7.4 (0.96-57.50), 0.02
Ala582Pro	0	4	4.5 (0.56- 36.76), 0.3	0	6	4.5 (0.83-25.46), 0.18
Met583Phe	3	9	1.5 (0.40-5.75), 0.75	0	11	4.7 (1.31-16.89), 0.01
Glu585Lys	5	12	1.21 (0.41-3.54), 0.7	1	20	11.0 (1.45-83.19), 0.003
Glu588Lys	4	13	1.6 (0.52-5.25), 0.4	2	20	5.4 (1.24- 23.77), 0.01
63545 G>A	1	7	3.5 (0.43-29.59), 0.27	2	14	3.6 (0.82-16.55), 0.09
87307A>T	2	2	0.5 (0.06-3.56), 0.6	0	3	4.5 (0.40- 50.33), 0.5
87313A>T	4	4	0.4 (0.11-2.00), 0.4	0	8	4.6 (1.04-20.56), 0.06
Thr581Pro	4	7	0.87 (0.24- 3.0), 1.00	0	10	4.6 (1.23-17.84), 0.03
63367 T>G	0	8	4.6 (1.04-20.56), 0.05	1	12	6.3 (0.80- 49.30), 0.06
63365 C>T	2	7	1.7 (0.36-8.71), 0.72	2	10	2.5 (0.55- 12.00), 0.34
76377C>G	1	5	2.5 (0.29-22.02), 0.6	0	8	4.6 (1.04-20.56), 0.06

*OR, odds ratio; CI, confidence interval. *ORs for logistic regression analysis. ^bp>0.05, by χ^2 -test for trend

Table 4. Risk of Breast Cancer Associated with Menarche status of Breast Cancer Patients

Mutations	Menarche before 12			Menarche after 12		
	Controls (%)	Patients (%)	OR ^a (95%CI),	Controls (%)	Patients (%)	OR ^a (95%CI)
Glu241Arg	2	14	3.6 (0.82-16.55), 0.09	0	10	4.6 (1.23-17.84), 0.03
Ala582Pro	3	9	1.5 (0.40-5.75), 0.75	2	5	1.2 (0.23-6.59), 1.00
Met583Phe	5	17	1.7 (0.63-4.93), 0.35	4	9	1.1 (0.33-3.76), 1.00
Glu585Lys	7	37	3.0 (1.29-7.03), 0.008	4	20	2.6 (0.88-8.02), 0.07
Glu588Lys	4	32	5.6 (1.94-16.26), 0.002	3	23	4.2 (1.23-14.35), 0.01
63545 G>A	2	20	5.4 (1.24-23.77), 0.01	1	6	3.0 (0.36-25.78), 0.4
87307A>T	3	9	1.5 (0.40- 5.75), 0.75	2	5	1.2 (0.23- 6.59), 1.00
87313A>T	5	18	1.8 (0.67-5.21), 0.25	4	9	1.1 (0.33-3.76), 1.00
Thr581Pro	6	17	1.4 (0.55-3.81), 0.49	3	9	1.5 (0.40-5.75), 0.75
63367 T>G	1	17	9.1 (1.20-70.13), 0.008	0	6	4.5 (0.83-25.46), 0.1
63365 C>T	2	20	5.4 (1.24- 23.77), 0.01	2	5	1.2 (0.23 to 6.59), 1.00
76377C>G	1	17	9.1 (1.20- 70.13), 0.008	0	10	4.6 (1.23- 17.84), 0.03

*OR, odds ratio; CI, confidence interval. ^aORs for logistic regression analysis. ^bp>0.05, by χ^2 -test for trend

significantly higher in patients with late menopausal status (menopause after 51 years, $p<0.01$) compared to patients with early menopause. A significantly higher frequency of Glu241Arg heterozygous mutation was observed in patients ($p<0.02$) with late menopause when compared to controls. Similar trend was also observed in patients for other reported heterozygous mutations such as Glu585Lys ($p<0.003$) and Glu588Lys ($p<0.01$) when compared to controls. All these mutations are significantly higher in patients with late menopausal status when compared to controls. In case of homozygous mutations no significant difference was observed in patients with late menopause when compared to controls individuals with late menopause.

As presented in Table 4, frequency of SYK mutations was significantly higher in patients with early menarche (before the age of 12 years, $p<0.005$) as compared to patients with late menarche (after the age of 12 years). A significantly higher frequency of Glu585Lys heterozygous mutation was observed in patients ($p<0.0008$) with early menarche when compared to controls. Similar trend was also observed in patients (with early menarche) for other reported heterozygous mutations such as Glu588Lys ($p<0.002$) and 63545 G>A ($p<0.01$). In case of homozygous SYK mutations, only 76377 C>G mutation was found significantly higher in patients (with early menarche, $p<0.008$) compared to controls.

Discussion

Breast cancer is a main cause of morbidity and mortality among females worldwide (Clamp et al., 2003). The rate of breast cancer is increasing particularly in areas of low incidence such as Asia. In Pakistan, the rate of breast cancer is increasing at an alarming rate, as one in nine women is likely to have suffer from breast cancer in earlier or late stage in her life (Naeem et al., 2008). Various risk factors that can increase the incidence rate of breast cancer, have been identified which can be genetic, hormonal or environmental factors. However, through detection in early age, its mortality rate can be decreased. For this reason, it is necessary that clinical examination of breast should be done regularly (Naeem et al., 2008; Jemal et al., 2011).

In current study, it was observed that breast cancer is more prevalent in the age group 39-51 years (77%) followed by 25-38 years (23%). Various previous studies conducted on breast cancer patients including Malaysian, Iranian and Pakistani women have also reported similar results (Ahmed 2003; Abbasi et al., 2009). These findings support the fact that Asian women suffer from this disease almost a decade earlier than the western women where this disease is most commonly found at the age of 60 (Harrison et al., 2010). In present study 53% patients were diagnosed with breast cancer in their left breast while 41% patients had cancer in their right breast. However, only 6% had bilateral breast cancer. These findings are similar to percentage reported earlier in Pakistani population (Lakhani et al., 1995).

In this study, we evaluated possible associations between variants of the tumor suppressor gene SYK and the risk of developing breast cancer. We identified potentially functional polymorphisms and genetic markers in the candidate gene by SSCP and sequencing from a subset of 200 breast cancer patients and 100 control subjects. We identified twelve novel mutations located in exon 8, 15 and intronic region. Data analysis shows that out of twelve variations five non-synonymous variations (Glu241Arg, Ala582Pro, Met583Phe, Gln585Lys, and Gln588Lys) were heterozygous, however the non-synonymous mutation Thr581Pro is a homozygous mutation. In case of intronic mutations, three mutations are heterozygous (63545 G>A, 87307A>T, 87313A>T) while remaining three are homozygous (63365 C>T, 63367 T>G, 76377C>G). The analysis of data of all these mutations indicate that the ratio of all these mutations was higher in breast cancer patients when compared to their respective controls.

One missense mutation (Glu>Arg) was observed in exon 8 at position 241 which encodes SH2 domain. One silent mutation (rs35758162, Glu>Glu) and one missense mutation (Asp>Asn) in SH2 domain has already been reported in Pakistani population and may affect structural stability of SYK gene in breast cancer patients (Inayat et al., 2012). SH2 domains interact to tyrosine-phosphorylated immunoreceptor tyrosine-based activation motifs and this binding activates SYK (Latour et al., 1998; Wang et al., 2003; Coopman et al., 2000; Coopman and

Mueller, 2006). Deregulation of SYK has been implicated in both immune, (T-cell lymphomas) and non-immune human cancers (breast cancer) (Liu et al., 2006).

Five missense mutations (Thr581Pro, Ala582Pro, Met583Phe, Gln585Lys, and Gln588Lys) have been found in exon 15 of SYK which is encoded by kinase domain. The kinase domain of SYK is involved in various signaling pathways. An intact kinase domain is required for efficient downstream signaling (Hunter et al., 2000). As exon 15 is present in kinase domain of SYK gene, any mutation or alteration in sequence of these exons can produce crucial effect on signaling pathways of SYK (Hunter et al., 2000). Kinase domain is highly polymorphic domain as numbers of mutations in SYK domain has already reported in Pakistani population (Inayat et al., 2012), but due to the lack of the available data related to progression of breast cancer the exact role of these variations remain unpredictable. Further studies exploring different domains of SYK can be helpful in determining the role of these variations and their effect on the development of breast cancer.

The data analysis showed that all observed mutations in present study were significantly higher in breast cancer patients with late menopause when compared to patients and controls with earlier menopause. Mc Pherson et al. (2000) reported the similar correlation between late menopausal status and breast cancer. In the present study, a positive association was observed between the patients who experienced menarche at an early age and SYK gene polymorphism, which suggests that menarche at an early age is a risk factor of breast cancer. This finding is in line with results reported earlier in different populations (Hu et al., 1997; Kruk 2007). Nevertheless, some studies have reported a non significant relationship between menarche and breast cancer (Minami et al., 1997; Kanwal et al., 2012).

Here we report the significant presence of different genetic variations of SYK gene in breast cancer patients. The data suggests that a combination of missense mutations, or another mutation linked to it in the same gene or gene in vicinity, could conceivably play a role in the process of developing breast cancer in a Pakistani population. This demonstrates that SYK could prove to be a good candidate of better diagnosis, treatment and prevention of breast cancer but more detailed studies are needed for a clearer picture of SYK gene and protein in pathogenesis of breast cancer.

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