Promoter methylation status of PTEN, SYK and survivin genes in breast cancer tissues derived from Chinese women

Xiaowei Lu¹, Yumei Gu², Dongliang Zhu²,³, Jianxin Tan³, Xiaorong Liu²,³, Xiaoming Zha⁴
¹Department of Breast Surgery, Maternal and Child Health Hospital of Wuxi City, Wuxi 214002; ²Key Laboratory of Human Functional Genomics of Jiangsu Province, ³Department of Cell Biology and Medical Genetics, Nanjing Medical University, Nanjing 210029; ⁴Department of Breast Surgery, The First Affiliated Hospital with Nanjing Medical University, Nanjing 210029, PR China

**Background:** Epigenetic mechanisms of gene transcription, including inactivation of tumor suppressor genes by hypermethylation and activation of oncogenes by hypomethylation, have been shown to contribute to breast tumorigenesis. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) and Spleen tyrosine kinase (SYK) are both tumor suppressor genes and survivin is a novel member of the inhibitor of apoptosis (IAP) family which may promote tumorigenesis.

**Objective:** To investigate the methylation status of PTEN, SYK, and survivin genes in breast cancer derived from Chinese women.

**Materials and methods:** We examined the methylation status of these three genes in 52 paraffin-embedded breast cancer tissues using methylation-specific PCR (MSP) assay in conjunction with sequencing analysis.

**Results:** PTEN and SYK were both methylated in 15.4% (8/52) of breast tumor tissues, while the survivin gene was all demethylated in the examined samples. Random selection of MSP products sequence analyses to three genes all revealed a homogenous methylation status in the CpG sites.

**Conclusion:** The present study showed a moderate methylation status of PTEN and SYK, and an unmethylation status of survivin in breast tumor tissues derived from Chinese women, which suggested methylation mechanisms might be involved in the aberrant expression of these three genes in breast cancer development.

**Keywords:** Breast cancer, methylation status, PTEN, SYK, survivin.

In recent years, the frequency of breast cancer increased greatly in China with change in the life style. Breast cancer is becoming the leading cause of cancer-related deaths among Chinese women. Many studies have demonstrated that the development and progression of this disease involve abnormality of multiple genes through genetic and epigenetic alterations. Epigenetic mechanisms of gene transcription have been a topic of considerable interest in the last few years. Differential methylation is an important epigenetic control mechanism, which has been implicated in the development of breast cancer. Phosphate and tensin homology deleted on chromosome (PTEN) gene at chromosome 10q23.3 is a tumor suppressor gene that is inactivated in several tumor types, including breast [1]. PTEN gene encodes a PIP3 phosphatase and regulates negatively the PI3K/Akt cell survival pathway [2]. Although mutation and homozygous deletion are the most common mechanisms of PTEN inactivation, promoter methylation can also account for PTEN silencing in breast cancer [3]. However, there is a high degree of homology in the promoter region between PTEN and its pseudogene (psiPTEN), which has made analysis of the methylation status of the PTEN promoter quite challenging [4].
Spleen tyrosine kinase (SYK) is a tyrosine kinase involved in cell signaling and is considered a putative tumor suppressor gene found to be silenced through DNA methylation in breast tumor [5]. Recent study indicated that the hypermethylation of SYK occurred at a stage prior to the development of invasion phenotypes, suggesting a potential use of SYK methylation as a valuable biomarker to detect early cancerous lesions of breast [6].

Survivin, a novel member of the inhibitor of apoptosis (IAP) family, was found abundantly expressed in breast cancer [7]. It was reported that apoptosis inhibition by survivin might promote tumorigenesis and predict poor outcome of human common cancers [8, 9]. Furthermore, demethylation might be a crucial contributing factor to the elevated expression of survivin gene in oral carcinogenesis [10].

Although three genes (PTEN, SYK, and survivin) play an important role in the development and progression of breast carcinoma through different mechanisms, their transcription may be all regulated by DNA methylation. To date, the methylation status of PTEN and SYK in breast cancer has been reported in a few studies, but none of which was derived from Chinese women. In addition, no analysis of the methylation status of the survivin has been performed in breast cancer. Given this background, our study was taken to provide an initial insight into the methylation profile of PTEN, SYK, and survivin genes in breast cancer of Chinese women. Using methylation-specific polymerase chain reaction (PCR) assay in conjunction with sequencing analysis, we examined the methylation status of these three genes in 52 paraffin-embedded breast cancer tissues.

**Materials and methods**

**Tissue samples**

Fifty-two paraffin-embedded formalin-fixed blocks of primary breast cancer tissues were obtained from 52 breast cancer patients who underwent breast-conserving operations in Department of Breast Surgery, Maternal and Child Health Hospital of WuXi City, China. The tumors were all determined by pathological examination and all patients were given no preoperative treatments. The use of tissue specimens and clinical information were obtained with patients’ consent and were in accordance with current ethical standards.

**Isolation of genomic DNA and methylation-specific PCR (MSP)**

The genomic DNA from paraffin-embedded sections was extracted according to the previous literature [11]. Then, 1 μg of genomic DNA from each sample was modified by sodium bisulfite with CpGenome™ DNA Modification Kit (Chemicon). All MSP reactions were performed for 35 cycles with HotStarTaq DNA Polymerase (Qiagen, China). The sequences and annealing temperatures of primers used in this study are listed in Table 1. It should be pointed out that the primers for PTEN gene were specific to PTEN not the PTEN pseudogene. The final PCR products of MSP were selected randomly to be purified and sequenced (Invitrogen). CpGenome™ universal unmethylated DNA and methylated DNA were purchased from Chemicon and used as controls for MSP analysis.

**Table 1.** Summary of primer sequences and PCR amplifications for MSP.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer pair sequences (5’ to 3’)</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN(UF)</td>
<td>TATTAGTTTGGGATTTTTTTTTTGT</td>
<td>60</td>
<td>186</td>
<td>[12]</td>
</tr>
<tr>
<td>PTEN(UR)</td>
<td>CCAACCTCTACTAACACTACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTEN(MF)</td>
<td>GTTTGGGATTTTTTTTTTGC</td>
<td>60</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>PTEN(MR)</td>
<td>AACCCTCTACTAACACTACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SYK(UF)</td>
<td>ATTTGTTGGGTTTGTG</td>
<td>63</td>
<td>141</td>
<td>[5]</td>
</tr>
<tr>
<td>SYK(UR)</td>
<td>ACTTCTACACTACAGGCAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SYK(MF)</td>
<td>CGATATTGCGGTTTTCGCC</td>
<td>67</td>
<td>243</td>
<td></td>
</tr>
<tr>
<td>SYK(MR)</td>
<td>AAAAGGAAACGCCACCAACG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin(UF)</td>
<td>GGTGGGAGGATTATAATTGG</td>
<td>56</td>
<td>168</td>
<td>[13]</td>
</tr>
<tr>
<td>Survivin(UR)</td>
<td>ACCACACCAACCTACAAACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin(MF)</td>
<td>GGGGAGGATTATAATTTCG</td>
<td>53</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td>Survivin(MR)</td>
<td>CCGCACCTCTACCAAGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*UF: unmethylated forward; UR: unmethylated reverse; MF: methylated forward; MR: methylated reverse.*
**DNA sequencing**

Prurified final MSP products were selected randomly to be sequenced using an ABI 3130 Capillary Sequencer (Applied Biosystems, Foster, USA).

**Results**

**Methylation status of PTEN, SYK and survivin genes in paraffin-embedded breast cancer tissues**

In order to explore the methylation status of PTEN, SYK, and survivin genes in breast cancer derived from Chinese women, 52 paraffin-embedded formalin-fixed blocks from primary breast tumors were examined by methylation-specific PCR (MSP). Our results showed that PTEN and SYK tumor suppressor genes were both methylated in 15.4% (8/52) of breast tumor tissues, while no methylation was detected in the survivin gene. To exclude false positive and false negative results, universal unmethylated DNA and universal methylated DNA were used as controls. Representative examples are shown in Fig.1.

**Sequence analysis of three genes in MSP products of breast tumor samples**

To verify the MSP results and observe the methylation status of each CpG site in our PCR products, final methylated products of three tumor samples for PTEN and SYK genes respectively, and unmethylated products of three tumor samples for survivin gene, were selected randomly for purification and sequencing (Fig.2). Sequence analysis of three genes confirmed the MSP data and revealed all methylation occured in the CpG sites. For the PCR products amplified from unmethylated DNA, all C were converted to T. While for the PCR products amplified from methylated DNA, all CG were maintained, and the rest of C were converted to T except for one C in PTEN gene, which may be due to incomplete bisulfite modification. These data not only demonstrated that our MSP method was reliable and sensitive to determine the methylation status of PTEN, SYK, and survivin genes, but also indicated the homogeneity of the methylation alteration of CpG sites in all these genes.

![Sequence analysis of three genes in MSP products of breast tumor samples](image_url)

**Fig. 1** Methylation status of PTEN, SYK and survivin genes in paraffin-embedded breast cancer tissues. One μg control DNA (A) and representative genomic DNA from paraffin-embedded blocks (B) were modified by sodium bisulfite and conducted MSP analysis. U-DNA, universal unmethylated DNA; M-DNA, universal methylated DNA. U and M, PCR product with primers specific for unmethylated and methylated sequences, respectively.
Discussion

Epigenetic mechanisms of gene transcription, including inactivation of tumor suppressor genes by hypermethylation and activation of oncogenes by hypomethylation, have been shown to contribute to breast tumorigenesis. PTEN is an important tumor suppressor implicated in the pathogenesis of a number of familial and sporadic cancers. About 25-50% of women with Cowden disease, a syndrome associated with germ-line mutations of the PTEN gene (at 10q23), develop breast cancer [14, 15], but mutation frequency of PTEN is very low in sporadic breast cancer [1, 16]. However, 29-48% of breast cancer display LOH in 10q23 [17-19], and about 40% of breast cancer show a decrease or absence of PTEN protein levels at the time of diagnosis [1].

Promoter hypermethylation has been identified as an alternative mechanism of tumor suppressor gene inactivation [3]. However, studies of the PTEN promoter methylation have been restricted due to the strong homology with a highly conserved PTEN processed pseudogene, located on chromosome 9p21,
which shares over 98% homology with PTEN and this sequence identity extends 841 base pairs into the promoter region [20]. Zysman et al. [4] showed that it is the PTEN pseudogene, and not the PTEN gene, that is predominantly methylated in endometrial, breast, and colon cancer cell lines, as well as in a panel of primary endometrial tumors. It was also shown that PTEN displayed an unmethylation status in astrocytoma, colorectal and hepatocellular carcinomas [13, 21, 22]. However, other studies have reported that the promoter of PTEN, not PTEN pseudogene was hypermethylated in 30%-50% of breast cancer [3, 23]. In our study, with careful consideration of the critical nucleotide differences between PTEN and PTEN pseudogene, we found PTEN gene was methylated in 15.4% (8/52) of breast tumor tissues of Chinese women, which supported the results that PTEN promoter was methylated in some breast tumor tissues and suggested that methylation might be a regulator of PTEN expression in breast cancer.

Similar to PTEN gene, SYK was also found methylated in 15.4% (8/52) of breast tumor tissues in this study. However, the methylation frequency of PTEN and SYK were both lower in our results than that in the previous reports (Table 2), in which the methylation rate of two genes were both more than 30% [3, 5]. It might be genetic background that contributed to variant regulation mechanisms of PTEN and SYK genes. Evidence that some genes express differentially in different ethnic groups has accumulated, e.g. progesterone receptor A in uterine leiomyoma [24] and cyclin D1 [25] and BCSG1 [26] in breast cancer. According to our study, only moderate methylation of PTEN and SYK occurred in the population of China. Besides promoter methylation, other genetic and epigenetic events may regulate their expression, such as germline mutations or LOH in 10q23 for PTEN [27, 28], and aberrant RNA alternative splicing for SYK [29].

The role of methylation in the survivin gene has been suggested by a few of studies [10, 13, 22], in which survivin was in a uniformly unmethylated status in tumors. However, little information has been published on the methylation status of survivin in breast tumor. In our study, no methylation was found in all the breast tumor tissues (0/52), which implied that demethylation might be responsible for elevated expression of the survivin gene in breast carcinogenesis. Our study is the first one to review that the survivin gene is unmethylated in breast tumor, which is in accordance with previous studies in other tumors.

Our present study provided an initial insight into the methylation status of PTEN, SYK, and survivin genes in breast tumor tissues derived from Chinese women. The methylation profile may represent a potential new biomarker of risk prediction in breast cancer of Chinese women. In the future work, we will investigate the relationship between promoter methylation and gene expression in breast tissues of different pathology to determine better the role of DNA methylation in regulation of these genes. Moreover, we will make further studies to see the value of the methylation profile as a new biomarker in predicting risks in breast cancer.

**Conclusion**

Our present study showed a moderate methylation status of PTEN and SYK, and an unmethylation status of survivin in breast tumor tissues derived from Chinese women, which suggested methylation mechanisms might be involved in the aberrant expression of these three genes in breast cancer development.

### Table 2. Frequency of promoter methylation of PTEN and SYK genes in breast cancer tissues from different races.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Frequency of promoter methylation (%)</th>
<th>Tissues from race</th>
<th>Data source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN</td>
<td>48 (43/90)</td>
<td>Spaniards</td>
<td>[3]</td>
</tr>
<tr>
<td>PTEN</td>
<td>34 (15/44)</td>
<td>American</td>
<td>[23]</td>
</tr>
<tr>
<td>PTEN</td>
<td>15.4 (8/52)</td>
<td>Chinese</td>
<td>This study</td>
</tr>
<tr>
<td>SYK</td>
<td>32 (12/37)</td>
<td>American</td>
<td>[5]</td>
</tr>
<tr>
<td>SYK</td>
<td>15.4 (8/52)</td>
<td>Chinese</td>
<td>This study</td>
</tr>
</tbody>
</table>
Acknowledgements

All authors made significant contributions to the work. The authors would like to thank Prof. Yujie Sun (Key Laboratory of Human Functional Genomics of Jiangsu Province, Nanjing Medical University) for her help in methods.

This study was supported by grants from the National Natural Science Foundation of China (No. 30772490). The authors have no conflict of interest to report.

References


