DNA Methylation Analysis of the Hippo signalling Pathway Core Component Genes in Breast Cancer Cells

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ABSTRACT

Objective: Breast cancer is one of the most frequent malignancies among women. According to the World Health Organization (2020), an estimated 2.3 million women were diagnosed with breast cancer, while 685,000 died worldwide. Therefore, an early diagnosis of cancer is crucial for survival. This study analyses the methylation status of the promoter regions of core component genes of the hippo pathway. The Hippo pathway is a tumor suppressor pathway as this pathway hinders cell growth and cell proliferation and motivates cell death.

Material and Methods: Methylation-sensitive PCR method was used to examine the altered methylation patterns of SAV1, LAST1/2, and MST1/2 in different breast cancer cell lines (MCF7, T47D, HCC1937, and BT-20).

Results: Interestingly, we have found that the promoter regions of the genes being studied are all hemimethylated in all cell lines used in this investigation, apart from the LAST1 gene promoter, which was hypomethylated in T47D and HCC1937 cell lines.

Conclusion: This indicates the importance of hemimethylation, as it is considered an aberrant methylation pattern. Thus, its effect on gene expression must be further considered.

Keywords: Breast Cancer; Hippo pathway; DNA methylation

INTRODUCTION

Breast cancer in female counts for 15.5% of cancer-associated mortality. The Hippo pathway is acknowledged as a tumor suppressor pathway that hinders cell growth, cell division and motivates cell death. The core gene component of the Hippo pathway resampled cascade of kinases includes Salvador homolog-1 protein (SAV1) at the top of the hippo signal pathway, the upstream mammalian STE20-like protein kinase (MST1/2) that activates a downstream large tumor suppressor (LATS1/2) kinase, leading to phosphorylation and inactivation of transcriptional cofactors YAP/TAZ (1,2,3,4). In the Hippo pathway, SAV1 directly binds to protein kinases MST1/2 and induces the kinase cascade that promotes MST1/2, LATS1/2, and YAP/TAZ phosphorylation. Then, the phosphorylated YAP and TAZ are degraded via the ubiquitin–lysosome system (4,5,6,7,8,9,10,11). YAP protein is found at the end of the Hippo signalling pathway and the most important downstream activator, it transfers between the nucleus and cytoplasm. Additionally, the activation of the Hippo pathway causes the YAP to associate with transcriptional factors and functions as a transcriptional co-activator to promote hippo target gene expression (12). The Hippo pathway has the capacity to lead to tumorigenesis. Mutations and altered expression of its core components (SAV1, MST1/2, LATS1/2) promote the migration, invasion, malignancy of cancer cells. Currently, the significance of the biological and deregulation of the Hippo pathway has attracted immense interest among researchers in the past few years. Furthermore, understanding of hippo pathway intensifies the cancer treatment process. Toward the interest of this study, biomarker research related to cancer has shown that not only genetic mutations but also epigenetic alterations such as promoter methylation patterns potentially contributes to malignant growth and could be used in early identification too. Thus, identifying the most significant genes that can be used to detect and tailored therapies of Brest cancer is greatly needed.

Particularly, altered methylation of the promoter region of the Hippo pathway, core genes. Moreover, the hippo core component genes serve a critical role in invasive breast tumor colonization or metastasis through different mechanisms (13,14). Growing evidence underlines the significance of DNA methylation as a reversible epigenetic mechanism, therefore, making it a sought-after target for anticancer treatment in breast cancer. Therefore, to understand the abnormal expression of the Hippo pathway gene component, we studied the methylation status of the promoter region of SAV1, MST1/2, LATS1/2 genes in certain breast cancer cell lines (MCF7, T47D, HCC1937, and BT-20). Methylation sensitive PCR-based technique was used in this investigation.

MATERIAL and METHODS

Cell culture

The study was conducted on four cell lines, namely, MCF7, T47D, HCC1937, and BT-20. Cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). All cell culture media were obtained from ATCC, whereas supplements such as fetal bovine serum (FBS) and trypsin-EDTA were obtained from Gibco (Gaithersburg, MD, USA).

Cell lines

Cells were routinely passaged by mild trypsin-EDTA detachment and cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Human breast adenocarcinoma MCF7 cells (ATCC HTB 22): Are originally mammary gland epithelial cells of breasts obtained from metastatic site pleural effusion adenocarcinoma, MDA-MB231 is ER-negative and p53 mutant while MCF-7 is ER-positive and p53 wild-type (Cailleau et al., 1974; Landers et al., 1997. Pratt and Pollak, 1993). MCF7 cells were cultured in phenol red-free DMEM supplemented with 10% FCS, 0.1 mM nonessential amino acids, 110 g/ml sodium pyruvate, and 1% glutamine.

Human breast carcinomaT-47D cells (ATCC HTB 133): Were cultured in a-MEM containing 10% FCS, 0.1 mM nonessential amino acids, 6 @Lg/m1 insulin, and 1% glutamine (GIBCO).

Human breast carcinoma BT-20 cells (ATCC HTB 19): Were established from a pleural effusion in the 1950s, and many of the most studied cell lines (e.g., MDA-MB-231) were established in the 1970s. These tumor cell lines were derived before the routine evaluation of hormone receptor expression and HER-2/Neu amplification in clinical samples.

BT-20 cells were cultured in EMEM with the omission of any added insulin.

HCC1937 cell line: This cell line was initiated from a primary ductal carcinoma on October 13, 1995. This took 11.5 months to be established. The tumor was classified as TNM Stage IIB, and grade 3 BRCA1 analysis revealed that the cell line is homozygous for the BRCA1 5382C mutation.

The lymphoblastoid cell line derived from the same patient is heterozygous for the same mutation. Besides, cells were cultured in RPMI 1640 medium (Life Technologies, Paisley, UK) with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10%.

DNA extraction

The cells were harvested by trypsinization and were transferred into 1.5ml centrifuge tubes. Genomic DNA extraction was performed using the Wizard Genomic DNA purification Kit (Promega, Madison, WI, USA). Briefly, the cells were lysed using nuclei lysis buffer, and the RNase digestion step was included at this time.

The cellular proteins are then removed by a salt precipitation step, which precipitates the proteins but leaves the high molecular weight genomic DNA in solution. Finally, genomic DNA is concentrated and desalted by isopropanol precipitation. The DNA concentration and purity (260/280) were checked using a Nano-drop spectrophotometer.

Bisulphite conversion.

Bisulphite conversion of DNA takes advantage of the bisulphited-mediated chemical conversion of unmethylated cytosine residues into uracil. Methylated cytosine residues were remain unchanged, while the sequence-specific PCR primers can distinguish unmethylated genomic regions after bisulphite conversion (Herman and Braylin 2003).

Aliquots of 2 µg of each DNA sample were subjected to Bisulphite modification using Methyl Edge Bisulphite Conversion System (Promega) following the manufacturer's instructions. Briefly, 2 µg of DNA was mixed with 130 µl of bisulphite conversion reagent.

The bisulphite conversion was performed using the following thermal cycling program: denaturation at 98°C for 8 min followed by another incubation at 54°C for 60 min. After cycling, the bisulphite-converted DNA was purified on a spin column, eluted in a volume of 20-µl, and stored at -20˚C.

Methylation analysis.

The methylation status of the promoter regions of SAV1, MST1, MST2, LATS1, and LATS2 was analysed using methylation-specific PCR (MS-PCR). One hundred nanograms of bisulphate- treated DNA was PCR amplified in a 20-µl reaction buffer containing 10x Taq hot start master mix (New England Biolabs, Ipswich, MA). Primer sequences and reaction conditions are listed in Table 1. PCR reactions of the genes were performed using Touchdown (TD) PCR method. TD-PCR offers a simple and rapid means to optimize PCRs, increasing specificity, sensitivity, and yield without the need for lengthy optimizations and/or the redesigning of primers. TD-PCR employs an initial annealing temperature above the projected melting temperature (Tm) of the primers being used, then progressively transitions to a lower, more permissive annealing temperature throughout successive cycles. Any difference in Tm between correct and incorrect annealing will produce an exponential advantage of the two-fold per cycle (Korbie and Mattick 2008). The cycling program for TD-PCR involves two separate phases.

Phase 1 is the touchdown phase, which comprises 10 cycles with annealing temperature above Tm of the primers being used and transitions to a lower annealing temperature throughout successive cycles. Phase 2 is a generic amplification stage of 20 or 25 cycles using the final annealing temperature reached in Phase 1.

Phase 1 annealing temperature of 630C to 530C (MST2-USP {unmethylation specific primers}; SAV1-USP), 680C to 580C (MST2-MSP {methylation specific primers}, SAV1-MSP), 600C to 500C (LATS1-USP), 650C to 550C (LATS1-MSP, LATS2-MSP, LATS2-USP) for 10 cycles followed by phase 2 with an annealing temperature of 530C (MST2-USP; SAV1-USP), 580C (MST2-MSP, SAV1-MSP), 500C (LATS1-USP) and 550C (LATS1-MSP, LATS2-MSP, LATS2-USP) for 30 cycles. The extension was performed at 680C for 30 sec for all genes. PCR products were separated on 2.5% agarose gel and visualized with ethidium bromide.

RESULTS

Each experiment has been repeated three to six times to ensure reproducibility. TD-PCR products were visualized on 2.5% agarose gel. Looking at the TD-PCR of the MST1/2 genes in all Cell lines (MCF7, T47D, HCC1937, and BT-20). The gel image shows that MST1/2 genes are hemimethylated in MCF7, T47D, HCC1937, and BT-20 as PCR products were observed with both MSP and USP primers. For SAV1, the gene was also hemimethylated in MCF7, T47D, HCC1937, and BT-20 cell lines. Whereas LAST1 gene promoter was unmethylated in T47D and HCC1937 cell lines, but it was hemimethylated in MCF7 and BT-20 cell lines. Finally, the LAST2 gene promoter was hemimethylated in all study cell lines (Fig 1).

Table 1: Primers and conditions for Methylation-Specific PCR

Gene	CpG island Promoter size (bp)	transcription start	Primer	Product size (bp)	Tm (C⁰)	TD-PCR Annealing Temp (C⁰)
MST1	442	-70 to +50	MSP-F: GCGGGGCGGGTTTAGGAGGTTC	120	67	58
			MSP-R: CCAATAACCCCTCACCGACGC		62	
		-73 to +52	USP-F: AACCAATAACCCCTCACCAACACAACAA	125	61	56
			USP-R: CGGGAGGGAGATTCGTCGCG		64	
MST2	755	-18 to +81	MSP-F: CGGGAGGGAGATTCGTCGCG	99	64	58
			MSP-R: AAACCGAAACACCGACCGACCG		63	
		-25 to +83	USP-F: TTTTAAGTGGGAGGGAGATTTGTTGTGG	108	59	53
			USP-R: AAAAACCAAAACACCAACCAACCAAACC		59	
SAV1	909	-183 to -67	MSP-F: GATAGTCGTAGTTCGGCGGGG AC	116	61	58
			MSP-R: GCAACGCGAACCGCCG		63	
		-187 to -56	USP-F: TGAGGATAGTTGTAGTTTGGTGGGGAT	131	60	53
			USP-R: AAAAACTCAACACAACACAAACCACCA		58	
LATS1	473	-39 to +87	MSP-F: GAACGATTAGAGTTGCGGGCGAC	126	61	56
			MSP-R: AAC ATTTCCCGACGTCGCTTACG		61	
		-40 to +88	USP-F: TGAATGATTAGAGTTGTGGGTGATGT	128	56	50
			USP-R: AAACATTTCCCAACATCACTTACACA		55	
LATS2	1414	-123 to +14	MSP-F: TTCGTTCGGATTGGTATGCGGTC	137	60	55
			MSP-R: CCATCTTCCCGAAACGCTCACG		62	
		-128 to +13	USP-F: GGTGTTTTGTTTGGATTGGTATGTGGTT	141	58	55
			USP-R: CATCTTCCCAAAACACTCACACCACA		60	

MSP-F – Methylation-specific primer forward; USP-F – Unmethyaltion-specific forward
MSP-R – Methylation-specific primer reverse; USP-R – Unmethyaltion-specific reverse
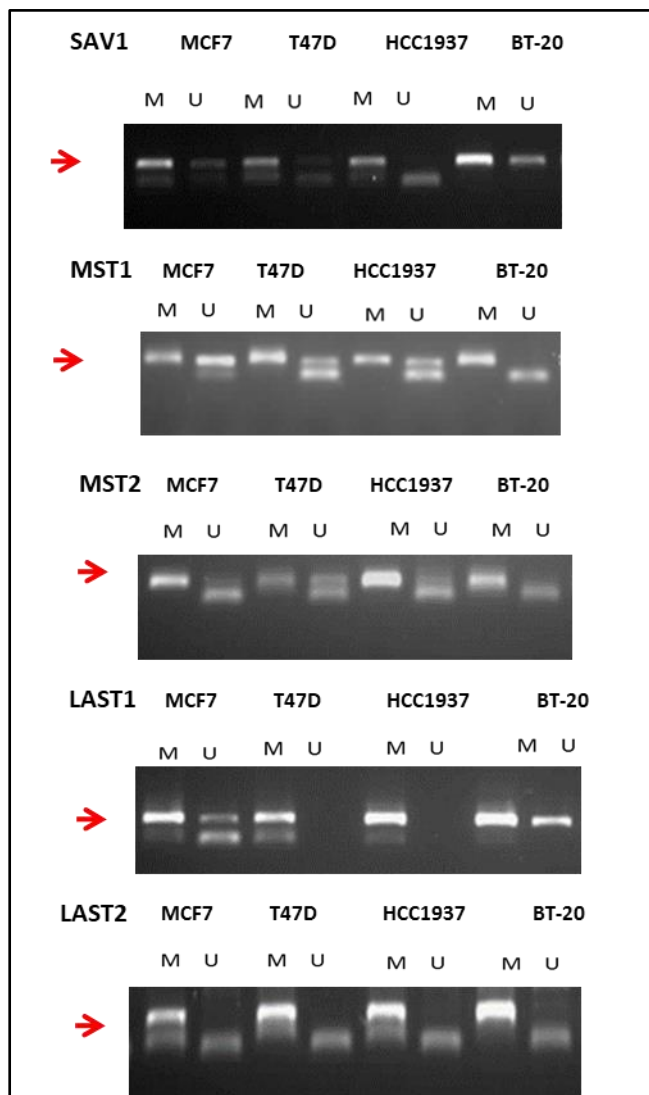
Fig 1: Gel images for hippo pathway methylated (M) and unmethylated (U) candidate genes. SAV1, MST1/2, and LATS1/2 were examined via MSP (M) and USP (U). Cell lines: MCF7, 2-T47D, 3-HCC1937, 4-BT-20. Separate MSP reactions were conducted for both methylated and unmethylated DNA sequences using primer sets specific for each reaction. The PCR products were separated by electrophoresis on 2.5% agarose gel mixed with ethidium bromide staining to visualize bands under UV light. For each run, we included positive control, which is a fully methylated DNA (EpiTect Control DNA (human); Qiagen), while nuclease-free sterile water used as a negative control.

DISCUSSION

Altered DNA methylation patterns in cancer tissues were first reported almost 40 years ago. This was accomplished when global methylation analysis by chromatographic techniques indicated a reduction of DNA methylation levels in numerous types of tumors from which the tumors originated compared to normal tissue [15,16,17]. Southern blotting techniques were then used to evaluate the hypomethylation changes for specific genes and at repetitive sequences [18,19].

It is well established that active genes in somatic cells maintain most of the CpG islands in their promoter regions unmethylated to ensure gene transcription. Furthermore, DNA methylation could prevent gene transcription by shifting transcription factor binding affinity to a gene promoter, stopping the binding of methylation-specific recognition factors to promoter or gene bodies, and condense the chromatin structure to limit the accessibility of transcription factors and/or other DNA binding proteins.

This study was devoted to studying the methylation status of the promoter regions of genes which, are recognized as the core component of the Hippo pathway that is linked to Brest cancer development and mortality rate. We have studied the methylation of SAV1, MST1/2, and LAST1/2 gene promoters in MCF7, T47D, HCC1937, and BT-20 breast cancer cell lines. SAV1 is at the front of the hippo signal pathway and is needed to activate the pathway. Remarkably, SAV1 plays a vital role in tumor suppression via hippo pathway-dependent and -independent mechanisms (20).

Moreover, recent research studies findings revealed low expression levels of MST1/2 in breast cancer and were closely associated with the poor prognosis of patients. Therefore, it was suggested that MST1 is an independent risk factor for breast cancer. However, overexpression of MST1 significantly inhibited the proliferation and migration while promoting the apoptosis of breast cancer cells. Moreover, the overexpression of MST1 significantly activated the Hippo signalling pathway (21).

With regard to LAST1 and LAST2, which are part of the hippo pathway, they were shown to participate in the regulation of human breast cell fate by direct interaction between hippo and estrogen receptor-α (ERα) signalling. The absence of LATS stabilizes ERα and the hippo effectors YAP and TAZ, which together control breast cell fate through intrinsic and paracrine mechanisms (22). Our study showed that SAV1, MST1/2, and LAST2 promoter regions were all hemimethylated in MCF7, T47D, HCC1937, and BT-20 cell lines.

This proves that linking specific DNA methylation changes with tumorigenesis in a cause-and-effect relationship has been challenging. However, Chunbo Shao et al. suggested that hemimethylated CpG dyads are intermediates in active demethylation during carcinogenesis and not just due to a failure of maintenance methylation during replicative DNA synthesis (23). Using openly available reduced representation bisulphite-sequencing (RRBS) data (GSE27003) of 7 breast cancer cell lines, namely (BT20, BT474, MCF7, MDAMB231, MDAMB468, T47D, and ZR751), Shuying Sun et al., has identified

hemimethylated sites for genes are involved or known to be involved in breast cancer (24).

Research findings were mainly obtained using statistical and bioinformatics data analysis to detect the hemimethylated sites of genes (25). Our findings, though, would be more meaningful because we investigated our genes of interest by examining the methylation status of their promoters' regions using bisulphite conversion and MSP in a wet lab. For LAST1, we observed that the promoter region was hypomethylated in both T47D and HCC1937 cell lines, whereas it was hemimethylated in MCF7 and BT-20 cell lines.

To the best of our knowledge, we are the first to show that the core component genes in the Hippo pathway about breast cancer, SAV1, MST1/2, and LAST 2 are hemimethylated in the breast cancer cell lines we studied. Thus, it is also significant to examine hemimethylated genes' expression levels and how these levels are related to the methylation and hemimethylation patterns of these genes.

Although DNA methylation controls genes by affecting gene expression, the relationship of hemimethylated patterns and gene expression in Brest cancer is not well studied yet. Some research findings suggested that it is important to study the relationship between hemimethylation patterns and gene expression in cancers.

Because, many of the previous methylation studies are conducted by assuming symmetric methylation, i.e., not considering hemimethylated (26). Therefore, identifying the hemimethylated profile for each single cell line or each breast cancer subtype is critical because some genes that contain hemimethylated CpG sites may play a role in tumor growth or suppression. Additionally, some of the hemimethylated genes associated with breast cancer are connected through biological pathways.

Lastly, these results suggest that certain genes in breast cancer cells may undergo active methylation or demethylation, which results in genome-wide hemimethylated and may indicate a transition between different stages of breast cancer. This transition may occur before tumour development. Thus, further study of hemimethylation may serve as a method to identify breast cancer in earlier stages and increase the chances of patient survival.

CONCLUSION

Because of the existence of hemimethylation, its impact on transcription must be studied in-depth. Also, it is constructive if research article states which DNA strand is analyzed after bisulfite conversion. We also suggest in the future that it would be optimal if a methylation analysis is carried out on both DNA strands separately.

The net result of some of these cancer-associated DNA hemimethylation could be abnormal modulation of transcription and even some aberrant post-transcriptional processing of transcripts and increases in DNA recombination, thereby contributing to tumor formation and progression.

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