The Korean Journal of Pathology 2009; 43: 221-30
DOI: 10.4132/KoreanJPathol.2009.43.3.221

Loss of Heterozygosity on Chromosome 15q15 Near Thrombospondin-1 Gene in Breast Carcinomas

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Received : September 5, 2008
Accepted : February 9, 2009

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Background : Chromosome 15q15 near the thrombospondin-1 (THBS-1) gene may be associated with tumor progression and metastasis. To clarify the potential role of the15q15 region in progression of breast carcinoma, we investigated the loss of heterozygosity (LOH) and the microsatellite instability (MSI) status of chromosome 15q15. **Methods :** LOH and MSI were detected in 84 breast carcinoma specimens using PCR-based microsatellite analysis with three microsatellite markers. **Results :** Of 77 breast carcinomas containing the heterozygous alleles, 25 (32%) showed LOH in at least one microsatellite marker. Partial LOH and total LOH were detected in 14 (18.27%) and 11 (14.3%) cases. The total LOH were inversely correlated with node metastasis. A single LOH at D15S514 was inversely correlated with nuclear grade and a single LOH at the D15S129 allele was associated with increased expression of the THBS-1 gene. MSI-positive breast carcinomas detected in 14 (17%) cases showed no correlation with any clinicopathologic feature. **Conclusions :** These results indicate that loss of the chromosome 15q15 region delays the progression of breast carcinoma because the magnitude of LOH is large and involves the THBS-1 gene and additional genetic elements. The genes located on chromosome 15q15 probably play a tissue-type-dependent role in malignant growth of the tumor.

Key Words : Breast neoplasm; Thrombospondin-1; Loss of heterozygosity; Microsatellite instability

Breast cancer is one of the most common malignant tumors in women, with 15-20% of breast cancers associated with a family history and 25% showing a dominant transmission pattern. However, in 60-85% of cases, breast cancer develops sporadically without family history primarily due to genetically acquired mutations. A multistep tumorigenic process including mutation of oncogenes and tumor suppressor genes has been suggested.[1] With the accumulation of several oncogenes and tumor suppressor gene mutations, the development, progression, and prognosis of tumors are associated with alteration of the function of these genes by activation or inactivation. Therefore, identifying loss of heterozygosity (LOH) may be applied as a means to elucidate the role of genes participating in the progression of cancer and the role of tumor suppressor genes. The analysis of LOH applied microsatellite markers is one of the most accurate methods to study the carcinogenic mechanism, and is of great help for characterization of new tumor suppressor genes.[2] In addition to LOH due to loss of a gene, microsatellite instability (MSI) caused by the insertion or deletion of microsatellites in a wide area has been suggested as the cause of gene mutation involved in the carcinogenic process.[3] Microsatellites are very short repeated DNA fragments that have been used as markers to detect MSI in tumor cells. MSI detected in malignant human tumors is a mutator phenotype that appears due to loss of the original function of a repaired mismatch strand that is generated in association with an abnormality in the DNA mismatch repair system that attempts to correct mismatched DNA.

MSI has been reported in 15-20% of colorectal cancers,[4] and also in uterine endometrial, gastric, esophageal, bladder, and other cancers.[5-8] MSI has been reported in 5-40% of breast cancers.[9] Nonetheless, it is rare, and hence, it is controversial.

It has been reported that LOH of tumor suppressor genes is a genetic mutation that occurs most frequently in solid tumors and gastrointestinal cancers, rather than individual loss of a spe-

cific gene. The range of the overall chromosome loss is more significant.[10] Particularly, LOH of the tumor suppressor gene was detected in 21-51% of primary breast cancers, and it is the most common genetic mutation appearing in breast cancer.[11]

Numerous studies of genes involved in the development of breast cancer have been conducted since the early 1990s. Previously, several reports have demonstrated the onset of cancer to be associated with oncogenes and tumor suppressor genes that include the amplification of c-myc, erb-B2, INT2, genetic mutations of BRCA1, BRCA2, p53.[11]

In 2003, Miller *et al*.[12] collected and analyzed reports regarding LOH. LOH in breast cancer is abundant in the order of 7q, 16q, 13q, 17p, 8p, 21q, 3p, 18q, 2q, and 19p. Similar to solid tumors in other organs, breast cancer is also a complex disease developed by the accumulation of mutations in numerous genes.

Studies of chromosome 15q have been conducted since 1999. Studies of LOH in colon, breast, ovarian, lung, and bladder cancers, and malignant mesothelioma have shown that the loss was detected on chromosomes 15q11-q13,[13] 15q14,[2] 15q15,[14] 15-q21-22,[15] and 15q26.[16] The tumor suppressor genes considered to be located at the site of LOH on chromosome 15q are the DNA repair gene RAD51, thrombspondin-1 (THBS-1), the TGF-β genes SMAD3, and SMAD6. Nevertheless, in breast cancer, mutation of chromosome 15q is rare, which indicates that genes required for cell proliferation are present more abundantly than tumor suppressor genes. Thus, if chromosome loss is caused by LOH, cancer development may be suppressed or the progression may be delayed.

The THBS-1 gene located on chromosome 15q15 was initially reported as a protein stored in the α particle that is secreted during the activation of platelets. Thus, studies have focused on the gene's function during the aggregation of platelets. Diverse functions, including cell adhesion, migration, proliferation, intercellular contact, cell deposition, activation of enzymes, degradation of extracellular proteins, and suppression of neovascularization were subsequently revealed. All of these functions are involved in maintenance of vascular homeostasis.[17] Neovascularization is an essential process that supplies nutrients and oxygen during wound healing and the tumor growth process, and helps remove wastes. The neovascularization of malignant tumors is an especially important factor for determining the growth and metastasis of tumor cells. In various types of malignant tumors, the level of neovascularization is associated with the progression and metastasis of tumors. The THBS-1 gene is involved in neovascularization, and controls or suppresses the invasion and progression of tumors. Because of the function of the THBS-1 gene in suppressing neovascularization, the role of the THBS-1 gene in tumor suppression and suppression of metastasis has been emphasized.[18] Park *et al*.[15] reported in a study of LOH in colorectal cancers that a tumor suppressor gene is present at the chromosome 15q21.1 site and, thus, THBS-1 may be present in this site. Wick *et al*.[2] reported that a tumor suppressor gene located on chromosome 15q may be involved in the metastasis of breast cancer. On the other hand, in 2003 Wang-Rodriguez *et al*.[19] reported that THBS-1 is involved in the development of breast cancer but is not associated with metastasis.

In the past few years, investigators have developed and used diverse techniques to analyze DNA in tumor tissues. Microdissection methods obtain DNA by collecting cell colonies or a few cells through separation of target lesion areas from normal cells and tissues in the vicinity, and thus the accuracy of testing has increased. Therefore, this method has been widely used as a tool to associate the correlation of histological findings with gene mutations using paraffin embedded tissues.[20]

We extracted DNA in a total of 84 cases of invasive breast cancer from paraffin embedded tissues using micro dissection techniques. D15S129, D15S514, and D15S222 located in the vicinity of chromosome 15q15 were used as markers, and LOH and MSI were examined by performing PCR and immunohistochemical staining for the THBS-1 protein. The role of the loss of chromosome 15q15 was thus examined.

MATERIALS AND METHODS

Patients and specimens

Cases diagnosed as invasive breast cancer at the Holy Family Hospital, Catholic University Medical College from April 2002 to June 2004 were used. Tissue specimens were surgically resected from 84 invasive breast cancer cases not treated with radiation therapy, chemotherapy, or other adjuvant therapies prior to surgery. Normal lymph node tissues were used as a control group.

The age of the subject patients ranged from 31 years to 78 years old, with a mean age of 47.53 years.

Histopathological examination

Patient age, tumor size, the number of lymph node metastasis, histological grade, nuclear grade, presence of estrogen and progesterone receptors were examined by reviewing the medical records and results of pathologic tests. The modified Elston-

Ellis' grade system based on the Bloom and Richardson classification method was applied for histological grade analysis. The classification method proposed by Black and Spear and modified by Fisher *et al*. was used for nuclear grade analysis. The clinical stage was classified according to the TNM disease stage of the American Joint Committee on Cancer (AJCC).

Immunohistochemistry

Four-μm thick sections were obtained from paraffin embedded tissues representing 84 cases. Sections were deparaffinized by a conventional method, and allowed to react with a solution of THBS-1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 1:50 with an antibody dilution solution (Research Genetics, Huntsville, AL, USA). The Streptavidin HRP detection system (Research Genetics) was used with the conventional avidin-biotin complex method (ABC), followed by staining with 3-amino-9-ethylcarbazole (AEC) and counterstaining with Meyer's hematoxylin. Examination was performed using a light microscope.

The results were evaluated independently by two pathologists. Cases in which the cytoplasm of more than 5% of tumor cells was stained were considered to be positive. The results were considered to be negative when less than 10% of the tumor cells were stained. The normal staining pattern of THBS-1 is cytoplasmic. The normal breast tissues were used as a positive control.

Micro-dissection and DNA preparation

Micro-dissection of tumor cells from the 84 formalin-fixed and paraffin-embedded tissue specimens was performed under direct light microscopic visualization. Seven-μm thick histological sections were prepared on glass slides, stained with hematoxylin-eosin, and allowed to air dry. Specific cells of interest were selected from the hematoxylin-stained slides and micro dissected using a disposable 26-gauge needle. We collected normal lymphocytes from lymph node tissues for use as a normal control. For each case, 3,000-10,000 cells were collected. DNA was extracted by incubating each sample in 100 μL of a solution containing 50 mM Tris-HCl, 1 mM EDTA, 0.5% (v/v) Tween-20, and 0.5 mg/mL proteinase K (pH 8.0) in a shaking incubator at 37℃ overnight. Samples were then boiled for 10 min and vortexed. We were able to use this method to obtain both tumor and normal DNA.

Table 1. Microsatellite markers used for the analysis of LOH in breast cancers

Locus	Location	Sequence	End product size
D15S129	15q14-15q15	ACAGCACTTGGTCCTTTCATC-TGTCTGTGCCCTCTTCCCT	208
D15S514	15q15.1-15q21	AGCAACATACATTCCCAGGC-CGTAGTCACCCTTCCCAGAG	188
D15S222	15q21.1	CCTCAGCGTCCTCTCTTG-CTGGTCACTGTCTGTCCTGT	184-198

LOH, loss of heterozygosity.

Assessment of the LOH of chromosome 15q and gene instability by polymerase chain reaction

Three microsatellites (Research Genetic, Huntsville, AL, USA) located on chromosome 15q15 adjacent to the THBS-1 gene were used (Table 1). The THBS-1 gene is located on chromosome 15q15 and, thus, D15S129 and D15S514 located on 15q15.1-15q21, and D15- S222 located on 15q21.1 were used as markers (Fig. 1). The physical map distances were obtained from the genetic location database.

Each PCR reaction was generally performed under standard conditions in a 10-μL reaction mixture containing 1 μL of template DNA, 0.5 μL of each primer, 1.5 mM $MgCl_2$, 1.0 μL of 10 mM dNTP, and 0.1 μL of 0.5U Taq polymerase (Gold Taq, Roche, Indianapolis, IN, USA). The reaction mixture was denatured for 5 min at 94℃ and incubated for 32 cycles (denaturing at 94℃ for 1 min, annealing at 55℃ for 1 min, elongation at 72℃ for 1 min). The final extension was continued 5 min, using the Gene Amp® PCR System 9,700 (Applied Biosystems, Foster, CA, USA). Two μL of the reaction products were then denatured and electrophoresed on a 6% polyacrylamide gel at 8,500

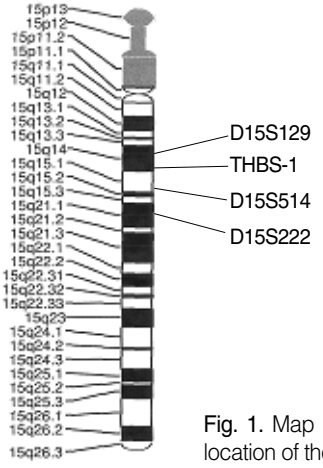

Fig. 1. Map of 15 chromosome with the location of the markers.

V for 4 h. After electrophoresis, silver staining was performed using a Silverstar® Staining Kit (Bioneer Corporation, Daejeon, Korea). The glass plate to which the gel was attached was put on a flat plastic tray to which 10% glacial acetic acid was added, followed by fixation for 30 min with subsequent washing three times in purified water. Stock enhancing solution at 0.1% was added with shaking for 30 min, followed by rinsing several times with purified water. An amount of 1.5 g of silver nitrate ($AgNO_3$) was dissolved in 1.5 L of purified water and 2.25 mL of staining solution containing 37% formaldehyde was added, followed by staining for 30 min. After DNA bands were observed, the gels were washed with purified water, placed in a fix/stop solution with shaking for 4-5 min, and then rinsed. The gels were detached carefully, attached to OHP films, and analyzed using a scanner.

Analysis of PCR results

Bands were analyzed using Densitometer (Image Master® VDS, 2.0 Pharmacia Biotech Inc., San Francisco, USA). Cases in which only one allelic band were observed in normal tissue DNA, loss of heterozygosity could not be assessed and these cases were evaluated as homozygous: non-informative (NI). Cases in which two allelic bands were observed in normal tissue DNA, with one of the two allelic bands lost in more than 50% of tumor tissues were defined as LOH. Cases that exhibited a normal tissue DNA band and an additional band in another site were defined as MSI. Cases, in which the location of bands in normal tissue and tumors were not changed, were defined as the retention of heterozygosity (MSS). Cases in which LOH was detected in all three markers were defined as total LOH, and cases in which LOH was detected in one or two markers were defined as partial LOH.[21] Cases showing MSI in all three markers were defined as total MSI, and cases showing MSI in one or two markers were defined as partial MSI.

Statistics

The Statistical Package for the Social Sciences (SPSS) 12 program® (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Statistical analyses were performed with the chi square test. p-values <0.05 were considered to be statistical significant. Cases showing homozygosity in normal tissues were excluded from the statistical analysis.

RESULTS

Clinical and pathological findings

We studied a total of 84 cases and the clinical and pathological findings of each case were analyzed. Cancers evaluated using the modified Black nuclear grade showed low grade (grade 1) in 11 cases (13.1%), intermediate grade (grade 2) in 50 cases (59.5 %), and high grade (grade 3) in 23 cases (27.4%). 14 cases (16.7 %) were classified as low histological grade (grade I), 61 cases (72.6%) were classified as intermediate grade (grade II), 9 cases (10.7%) were classified as high grade (grade III). Tumor size measurements smaller than 2 cm (T1) was found in 37 cases (44.1%), larger than 2 cm and smaller than 5 cm (T2) was found in 40 cases (47.6%), and larger than 5 cm (T3) was found in 7 cases (8.3%). No metastasis to axillary lymph nodes was found in 48 cases (57.1%) while metastasis was found in 36 cases (42.9%). Estrogen receptor (ER) and progesterone receptor (PR) were positive in the nucleus of epithelial cells of non-tumor tissues and tumor tissues. 47 cases (56.0%) were positive for ER and 37 cases (44.0%) were positive for PR.

Results of immunohistochemical staining

THBS-1 was positive in normal breast tissues (Fig. 2A), and

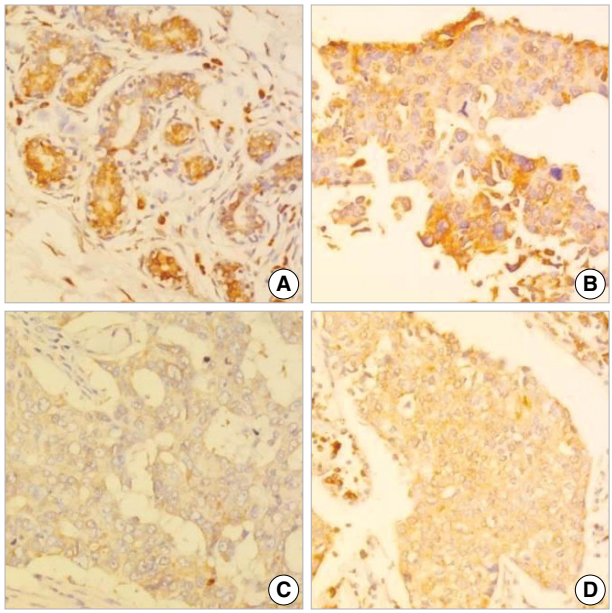

Fig. 2. Photomicrographs of the expression of THBS-1 in normal breast tissue (A) and breast carcinoma cases (B-D). The immune reactivity against THBS-1 is observed in the cytoplasm of tumor cell, vascular endothelial cells, and lymphocytes.

among 84 tumor tissues, 40 cases (47.6%) were positive (Fig. 2B-D), but didn't show a statistically significant correlation with clinical or pathological factors (Table 2).

The frequency of the heterozygote

Heterozygosity of D15S129 was detected in 45 of 84 cases (53.6%), heterozygosity of D15S222 was detected in 58 cases (69.0%), and heterozygosity of D15S514 was detected in 65 cases (77.4%) (Table 3).

Table 2. Clinicopathologic factors and the expression of THBS-1 in 84 patients with breast carcinomas

Clinico-pathologic factors	No. of cases (%)	THBS-1 expression		p-value
		Positive (n=41)	Negative (n=43)	
Age (years)				NS
<50	57 (67.9)	27	30	
≥50	27 (32.1)	14	13	
Size				NS
≤2 cm	37 (44.1)	19	18	
2-5 cm	40 (47.6)	18	22	
>5 cm	7 (8.3)	4	3	
Lymphnode metastasis				NS
0	46 (59.7)	24	24	
1-4	18 (23.4)	8	13	
>5	13 (16.9)	8	6	
Histologic grade				NS
I	14 (16.7)	7	7	
II	61 (72.6)	29	32	
III	9 (10.7)	5	4	
Nuclear grade				NS
I	11 (13.1)	6	5	
II	48 (57.2)	25	23	
III	25 (29.8)	10	15	
Stage				NS
I	26 (31.0)	13	13	
II	40 (47.6)	16	24	
III	18 (21.4)	12	6	
IV	0 (0)	0	0	
Estrogen receptor				NS
Negative	37 (44.0)	21	16	
Positive	47 (56.0)	20	27	
Progesterone receptor				NS
Negative	47 (56.0)	28	19	
Positive	37 (44.0)	13	24	

THBS-1, thrombospondin-1; NS, not significant.

Table 3. Heterozygote rate of microsatellite markers used in 84 breast carcinomas

Normal	Tumor	D15S129	D15S222	D15S514
Heterozygote		45 (53.6%)	58 (69.0%)	65 (77.4%)
Loss of heterozy-gosity (LOH)		15	9	15
Microsatellite instability (MSI)		9	10	5
Retention of hetero-zygosity (MSS)		21	39	45
Homozygote				
Non-informative (NI)		39 (46.4%)	26 (31.0%)	19 (22.6%)

The result of LOH of the THBS-1 gene and correlation with clinicopathological factors

LOH of at least one marker was detected in 25 of 84 cases, LOH of D15S129 was found in 15 of 45 cases (33.3%), LOH of D15S222 was found in 9 of 59 cases (15.5%), and LOH of D15S514 was observed in 15 of 65 cases (23.1%) (Table 3).

The relationship of the frequency of LOH of each marker to clinicopathological factors was examined (Table 4).

The frequency of LOH of D15S129 showed a statistically significant correlation with expression of the THBS-1 gene (p=0.025). Statistical significance for other factors was not detected. While LOH of D15S222 showed a statistically significant inverse correlation with lymph node metastasis (p=0.023), statistical significance for other correlative factors was not detected. The frequency of LOH of D15S514 showed a statistically significant correlation to lymph node metastasis and nuclear grade (p=0.026, p=0.003), but statistical significance for other correlative factors was not detected.

Partial LOH, showing heterozygocity, was detected in 14 of 77 cases (18.3%), total LOH was detected in 11 cases (14.3%), and a statistically significant inverse correlation with lymph node metastasis was shown (p=0.047). Statistical significance for other correlative factors was not detected (Fig. 3, 5).

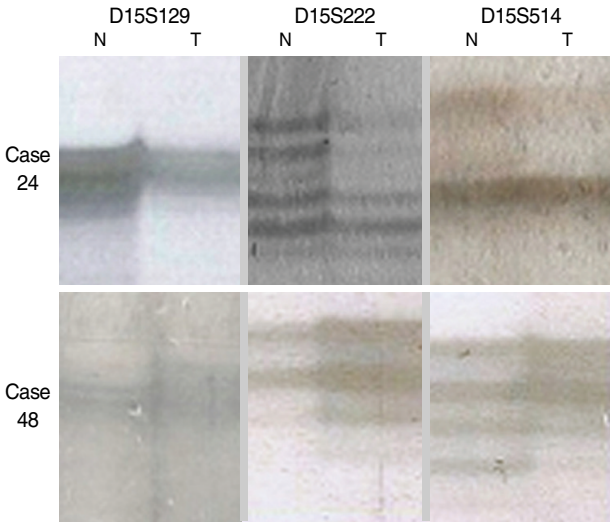

Fig. 3. Examples of total LOH in two breast carcinoma cases.

Table 4. Relationship between clinic pathologic features and LOH detected in 84 patients with breast carcinomas

Clinico-pathologic factors	No. of cases (%)	D15S129	p	D15S222	p	D15S514	p	Partial LOH	Total LOH	p
No. of patients		15		9		15		14	11	
Age (years)			NS		NS		NS			NS
<50	57 (67.9)	10		4		9		11	6	
≥50	27 (32.1)	5		5		6		3	5	
Size (cm)			NS		NS		NS			NS
<2	37 (44.1)	6		5		7		6	4	
2-5	40 (47.6)	7		2		5		7	7	
>5	7 (8.3)	2		2		3		1		
Node metastasis			NS		0.023		0.026			0.047
0	48 (57.1)	11		9		14		10	10	
1-3	21 (25.0)	3		0		0		3	0	
≥4	15 (17.9)	1		0		1		1	1	
Histologic grade			NS		NS		NS			NS
I	14 (16.7)	1		1		0		2	0	
II	61 (72.6)	12		6		12		10	9	
III	9 (10.7)	2		2		3		2	2	
Nuclear grade			NS		NS		0.003			NS
I	11 (13.1)	3		2		6		2	4	
II	50 (59.5)	8		6		7		9	6	
III	23 (27.4)	3		1		2		3	1	
Stage			NS		NS		NS			NS
I	26 (31.0)	7		5		5		6	4	
II	40 (47.6)	7		4		9		7	6	
III	18 (21.4)	1		0		1		1	1	
IV	0 (0)	0		0		0				
ER			NS		NS		NS			NS
–	37 (44.0)	6		4		8		4	7	
+	47 (56.0)	9		5		7		10	4	
PR			NS		NS		NS			NS
–	47 (56.0)	9		7		10		8	8	
+	37 (44.0)	6		2		5		6	3	
THBS-1			0.025		NS		NS			NS
–	43 (51.2)	10		5		6		10	4	
+	41 (48.8)	5		4		9		4	7	

LOH, loss of heterozygosity; NS, not significant; ER, estrogen receptor; PR, progesterone receptor; THBS-1, thrombospondin-1.

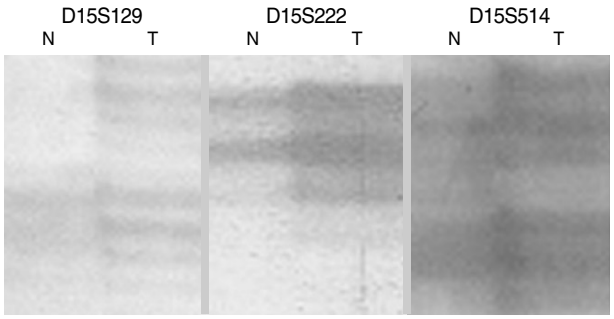

Fig. 4. Examples of MSI in three breast carcinoma cases.

Analytical results of MSI of the 15q gene and correlation with clinicopathological factors

In 14 of 84 cases, MSI was detected in more than one marker. In D15S129, MSI was detected in 9 of 45 cases (20.0%), in D15S222, MSI was detected in 10 of 58 cases (17.2 %) and in D15S514, and MSI was detected in 5 of 65 cases (7.7 %) (Table 3, Fig. 4, 5).

Partial MSI was detected in 8 of 84 cases (9.5%), and total MSI was detected in 6 cases (7.2%). The relationship of the frequency of total MSI with clinicopathological factors was examined (Table 5). Five cases were detected in the younger than age 50 group (83.3%) and 1 case was detected in the older than 50 group (16.7%). The frequency of total MSI was lower in the older group, while total MSI was not detected in the tumor larger than 5 cm group. In addition, 4 cases of total MSI were detected in the no lymph node metastasis group (66.7%), and 1 case was detected in each of the 1-3 cm and the more than 4 cm groups (16.7%). Moreover, more lymph node metastasis trended with

Table 5. Relationship between MSI and clinicopathologic findings

Clinico-pathologic factors	No. of cases (%)	D15S129	p	D15S222	p	D15S514	p	Partial MSI	Total MSI	p
No. of patients		9		10		5		8	6	
Age (years)			NS		NS		NS			NS
<50	57 (67.9)	6		7		4		5	5	
≥50	27 (32.1)	3		3		1		3	1	
Size (cm)			NS		NS		NS			NS
<2	37 (44.1)	5		4		2		3	2	
2-5	40 (47.6)	4		4		2		4	4	
>5	7 (8.3)	0		2		1		1	0	
Node metastasis			NS		NS		NS			NS
0	48 (57.1)	5		4		3		3	4	
1-3	21 (25.0)	2		2		1		1	1	
≥4	15 (17.9)	2		4		1		4	1	
Histologic grade			NS		NS		NS			NS
I	14 (16.7)	1		1		2		0	2	
II	61 (72.6)	7		9		2		7	4	
III	9 (10.7)	1		0		1		1	0	
Nuclear grade			NS		NS		NS			NS
I	11 (13.1)	1		2		1		0	2	
II	50 (59.5)	6		5		3		6	3	
III	23 (27.4)	2		3		1		2	1	
Stage			NS		NS		NS			NS
I	26 (31.0)	2		2		1		2	2	
II	40 (47.6)	5		4		3		2	3	
III	18 (21.4)	2		4		1		4	1	
IV	0 (0)	0		0		0		0	0	
ER			NS		NS		NS			NS
−	37 (44.0)	4		4		2		2	3	
+	47 (56.0)	5		6		3		6	3	
PR			NS		NS		NS			NS
−	47 (56.0)	7		6		4		5	4	
+	37 (44.0)	2		4		1		2	2	
THBS-1			NS		NS		NS			NS
−	43 (51.2)	3		4		1		3	2	
+	41 (48.8)	6		6		4		5	4	

MSI, microsatellite instability; NS, not significant; ER, estrogen receptor; PR, progesterone receptor; THBS-1, thrombospondin-1.

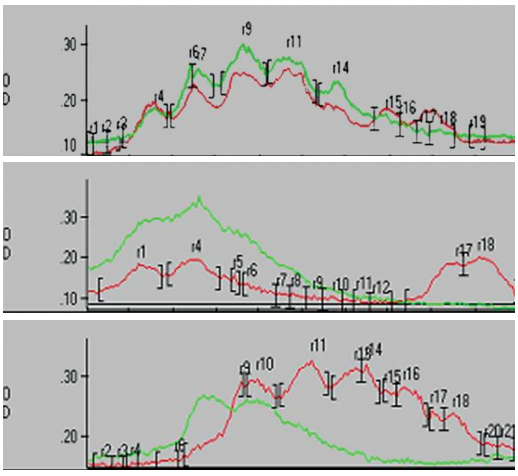

Fig. 5. Examples of graphs of allelic ratio of normal DNA (green line) and tumor DNA (red line).

the lower frequency of total MSI. However, statistical significance could not be established.

DISCUSSION

Development of malignant tumors is complex and occurs by means of diverse processes that involve the mutation of numerous genes and not the mutation of a single chromosome.[22] It has been revealed by molecular biological studies that activation of oncogenes by amplification and the loss of tumor suppressor genes by mutation are involved in this process. The deletion site of a chromosome observed frequently in tumor cells corresponds to the location of tumor suppressor genes and, thus, the detection of LOH using microsatellites is a useful method to assess the loca-

tion of tumor suppressor genes.

MSI was discovered during investigations seeking genes associated with hereditary colon cancer.[23] It is a highly variable phenotype caused by impairment of the mismatch repair system to correct mismatched DNA. The simple repeated sequence of MSI has been detected in low frequency in different cancer types.[24] Microsatellites, which is a very short repeated DNA fragment that is distributed abundantly and evenly in genes, are similar to LOH in that they can be used as markers to detect MSI. Microsattelites can be readily detected by polymerase chain reaction. According to recent molecular biological studies, MSI plays the most important role in the multiple steps of the tumorigenic process in humans. However, it has been reported to be different from the typical mechanism of tumor development by activation of oncogenes and inactivation of tumor suppressor genes. It is not yet clear when and how MSI reacts during the tumorigenic processes.

Diverse and complex gene mutations are involved in the development and progression of breast cancer and studies of LOH and MSI of several genes have been conducted. In a study reported by Miller *et al*.[12] LOH in breast cancer is abundant in the order of chromosome 7q, 16q, 13q, 17p, 8p, 21q, 3p, 18q, 2q, and 19p. The importance of LOH in the development and progression of breast cancer was emphasized. On the other hand, Halford *et al*.[25] reported that the frequency of MSI in sporadic breast cancer is low, and MSI may not be involved in the development and progression of breast cancer.

Inoue *et al*.,[26] reported that in solid tumors, including gastrointestinal cancers, the prognosis of tumors with aneuploid DNA or high grade LOH is poor and the prognosis of tumors with diploid DNA or MSI-positive tumors is good. These findings support Choi *et al*.,[4] who claimed that MSI is a good prognostic factor. By summarizing the results of these studies pertinent to LOH and MSI, depending on types of tumors, the type and site of the chromosome where LOH and MSI frequently occur can be assessed. However, for breast cancer, discrepancies among results exist regarding the site and function of the loss of chromosome 15q.[27] Rhiem *et al*.,[27] reported the possibility that the gene in the chromosome 15q21.1 site may be THBS-1, but the probability that the gene was CYP19 was higher. Jaffe *et al*.,[28] reported that THBS-1 is located on chromosome 15q15 and mutation of the THBS-1 gene occurs in the advanced disease and metastatic stages rather than in the development stage. However, loss of 15q has been reported to be either rare or not associated with progression of breast cancer.[12,19]

In our study, three markers located on chromosome 15q15 adjacent to THBS-1 were used to examine whether these genes are associated with the progression and metastasis of breast cancer. Together, LOH and MSI were examined, and the expression of THBS-1 protein was compared with the LOH and MSI analysis to assess whether the gene located in this location is indeed the THBS-1 gene.

The frequency of LOH on chromosome 15q15 was observed to be approximately 33%, the frequency of LOH on the marker D15S129 showed a statistically significant correlation with expression of the THBS-1 protein ($p=0.025$), and the frequency of the markers D15S222 and D15S514 did not show a correlation with expression of the THBS-1 protein. Lymph node metastasis showed an inverse correlation with the frequency of LOH ($p=0.023$, 0.026) and expression of the THBS-1 protein was not associated with the level of lymph node metastasis, indicating absence of a direct association. Total LOH (LOH in all three markers) was detected frequently in breast cancer without lymph node metastasis, and the frequency of individual LOH also showed an inverse correlation. Thus, we believe that LOH on chromosome 15q15 where the THBS-1 gene is located plays a role in suppression of or delay of breast cancer progression, including lymph node metastasis.

In regard to the frequency of LOH on marker D15S514, it was demonstrated that the lower the nuclear grade of the tumor, the higher the frequency of LOH. Thus, similar to lymph node metastasis, there is a statistically significant correlation that also indicates that LOH of 15q15 adjacent to THBS-1 has the function of suppressing or delaying breast cancer progression. In addition to the THBS-1 gene in the chromosome 15q15 site, the DNA repair gene RAD51, the TGFβ gene SMAD3, and SMAD6 are adjacent to this site. Thus, individual loss of these genes may be required for the development of cancer. However, if several genes are lost simultaneously, the normal gene functions are excessively weakened, resulting in poor cell survival.

Maacke *et al*.,[29] reported, in an immunohistochemical staining study of the RAD51 gene located on chromosome 15q15.1, that the greater progression of breast cancer correlated with the stronger the expression of the RAD51 protein, and that the RAD51 gene is involved in progression and metastasis of breast cancer. On the other hand, Rapakko *et al*.[30] reported, in an LOH study of the RAD51 gene, that RAD51 did not correlate with the progression and metastasis of breast cancer. We performed LOH and MSI analyses in parallel of D15S1007 where the RAD51 gene is located. Mutation was not detected so an assessment of whether RAD51 is involved in the progression and metastasis of breast cancer was not made. RAD51 is located in the vicin-

ity of THBS-1 on chromosome 15q. Hence, immunohistochemical staining studies of RAD51 may be useful for elucidating the role of RAD51 and differentiating THBS-1.

MSI observations in samples taken from younger patients showed that a high frequency of total MSI correlated with smaller tumor size and less lymph node metastisis. Although this correlation was not statistically significant, MSI was thought to be a good prognostic factor. Thus, MSI of THBS-1 located on chromosome 15q15 was not involved in the development and progression of breast cancer.

In summary, loss of chromosome 15q15 plays a role in delaying the progression of breast cancer, and since the range of loss was broad, loss of the THBS-1 gene and several other genes are thought to be involved. Therefore, loss of genes in a wide area located on chromosome 15q15 plays a positive role in delaying the progression of cancer. On the other hand, MSI on chromosome 15q15, which is a good prognostic factor, is not involved in the progression of breast cancer. In addition, expression of the THBS-1 protein was associated with the loss of a single gene, but not associated with lymph node metastasis. Thus, they appear not to be associated. However, this is a contradictory result regarding the role of the THBS-1 gene as a metastasis suppression gene. More study is needed of the role of chromosome 15q15 to determine whether it is independent of THBS-1 or includes THBS-1, and the role of chromosome 15q15 in breast cancer.

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