The significance of lipid accumulation in breast carcinoma cells through perilipin 2 and its clinicopathological significance

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List of abbreviated words:

PLIN2 perilipin 2

ADRP	adipose differentiation related protein
FFPE	formalin-fixed paraffin-embedded
DCIS	ductal carcinoma in situ
ADH	atypical ductal hyperplasia
IHC	immunohistochemistry

Abstract

Both systemic and intratumoral lipid metabolism have been recently reported to play pivotal roles in both tumor development and progression in various human malignancies including breast cancer. However, its details have remained largely unknown in breast cancer patients. Therefore, in this study, we focused on perilipin 2, which is involved in constituting the intracellular lipid composition. Perilipin 2 was first immunolocalized in 105 cases of breast cancer. The status of perilipin 2 immunoreactivity was significantly positively associated with histological grade, Ki-67 labeling index and HER2 status and negatively with estrogen receptor status of these patients. Subsequent *in vitro* study also revealed that its mRNA expression in triple negative breast carcinoma cells was higher than cells of other subtypes. We then examined the correlation between perilipin 2 immunoreactivity and intracellular lipid droplet evaluated by Oil-red O stating in 13 cases of breast carcinoma tissues. A significantly positive correlation was detected between the status of perilipin 2 and Oil-red O staining. These findings above did indicate that perilipin 2 could represent the status of intracellular lipid droplets in surgical pathology specimens of breast cancer and perilipin 2 was also associated with its more aggressive biological phenotypes.

Keywords

breast cancer, immunohistochemistry, lipid droplets, Oil-red O, perilipin 2

Introduction

Diabetes, hypertension and obesity are all recognized as lifestyle diseases. In particular, obesity and diabetes are both well known to be associated with development of multiple human malignancies including breast cancer, a common cancer in females of both developed and developing countries.[1] Clinical outcome of breast cancer patients has recently been markedly improved due to an introduction of various new modes of therapy,[2-4] but breast cancer is still the major cause of cancer death in women.[1]

Lipid is one of the most important energy sources for living bodies but the correlation between obesity or diabetes or abnormality of lipid metabolism and cancer development has gradually become more evidence. However, it is also true that details of this particular correlation have remained largely unknown at this juncture. Perilipin 2, also known as adipose differentiation related protein (ADRP) or adipophilin, was initially identified as an early induced protein promoting the differentiation of various primitive mesenchymal cells into mature adipocytes.[5-7] Perilipin 2 was then reported to be

involved in accumulation of lipids[8] and protection of intracellular lipid droplets from lipase[9] in various cells other than adipocytes. mRNA expression of perilipin 2 has been subsequently detected in many human tissues such as liver, lung and testis, and immunolocalized on the surface of intracellular lipid droplets.[10] In human malignancies, the number of intracellular lipid droplets was reported to be significantly higher in carcinoma cells compared to normal epithelial cells in colon cancer.[11] Lipid droplet density was also demonstrated to be related to increased tumor cell proliferation in colon cancer, which was inhibited by perilipin 2-silencing or FOXO3-overexpression in carcinoma cells.[12] In breast cancer, both intracellular lipid droplets and perilipin 2 expression were reported to be increased by progestin treatment through progesterone receptor in hormone responsive breast carcinoma cell line T-47D cells.[13, 14] These intracellular lipid droplets were also demonstrated to be involved in docetaxel resistance in T-47D cells.[14] In addition perilipin 2 status was reported to be associated with adverse clinical outcome of patients with phyllodes tumor[15] and special types of breast cancer such as apocrine carcinoma and lipid-rich carcinoma.[16, 17] However, the correlation between perilipin 2 and clinicopathological factors and/or intracellular lipid droplets themselves have remained largely unknown at this juncture. Therefore, in this study, we first immunolocalized perilipin 2 in 105 cases of 10% formalin-fixed

paraffin-embedded (FFPE) breast cancer tissues. We then examined the correlation between perilipin 2 status and the amount of intracellular lipid droplet evaluated by Oil-red O staining in 13 frozen breast carcinoma tissues. We also evaluated perilipin 2 expression in breast carcinoma cell lines to explore its biological significance in breast cancer.

Materials and methods

Breast cancer cases

Surgically resected FFPE tissues from 105 patients of invasive carcinoma of the breast were evaluated in this study. All the specimens were retrieved from surgical pathology files from Tohoku University Hospital and Tohoku Kousai Hospital in Sendai, Japan. All the patients were operated between 2000 and 2008. The clinicopathological characteristics of the breast cancer patients were summarized in Table 1. Histological subtypes of 105 patients which we studied were as follows: 103 cases of invasive ductal carcinoma, no special type, 2 cases of invasive micropapillary carcinoma, and 1 case of invasive lobular carcinoma. In this study, we also studied 25 cases of ductal carcinoma in situ (DCIS) and 11 cases of atypical ductal hyperplasia (ADH). Histologically normal

mammary gland contained in breast cancer tissue (126 cases) was evaluated as a normal breast in this particular study. In addition, 13 frozen tissue sections which were anonymized and preserved in Department of Pathology, Tohoku University School of Medicine were used for both Oil-red O stain and immunohistochemistry. Among 13 breast cancer tissues, histological subtypes were as follows: 10 cases of invasive ductal carcinoma, no special type, 2 cases of mucinous carcinoma, and 1 case of DCIS. Research protocols for the present study were approved by the Ethics Committee at the Tohoku University School of Medicine.

Immunohistochemistry

Mouse monoclonal anti-Perilipin 2 antibody diluted at 1:50 was used as the primary antibody (Clone AP 125, PROGEN Biothechnik, Heidelberg, Germany). For antigen retrieval, autoclave was used at 121°C, 5 minutes with buffer at Histofine pH 9 (Nichilei Bioscience, Tokyo, Japan). Hisofine kit (Nichilei Bioscience, Tokyo, Japan) was used for immunostaining. DAB was also used as a colorimetric agent. Human adrenal tissue was used as a positive control of perilipin 2 immunoreactivity.[18] The area ratio of positive tumor cells was quantitatively evaluated under light microscopy. The

patients were tentatively classified into low (<50%) and high (≧50%) groups according to the status of perilipin 2 immunoreactivity.

Oil-red O stain

Serial frozen tissue sections were prepared and Oil-red O stain and immunohistochemistry were performed in these serial frozen tissue sections. Sections were fixed in 10 % formalin, and an Oil-red O staining solution (MUTO PURE CHEMICALS, Tokyo, Japan) and 60 % isopropyl alcohol was used and stained according to the manufacturer's recommended methods. Procedures of perilipin 2 immunohistochemistry were the same as above. Hematoxylin was used as counter-stain. Representative areas were first selected at low magnification, and these positive areas were measured at high power magnification employing image analysis software, LuminaVision (MITANI CORPORATION, Tokyo, Japan). Immunohistochemistry and Oil-red O staining sections were captured in CCD camera and quantitative analysis was performed in these captured images.

Breast carcinoma cell lines

Human breast carcinoma cell lines; MCF-7, MDA-MB-231 and T47D were all commercially obtained from American Type Cell Culture Collection (Manassas, VA, USA). All the cells were grown at 37 °C in a humidified 5 % CO2 incubator.

Quantitative PCR

Cell lines were seeded in 12 well plate, and after 48 hr, TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) was used for total RNA extraction according to the manufacturer's instructions. Reverse transcription was performed in a thermocycler using appropriate amounts of total RNA and following primers. Primer of perilipin 2 were set as follows with reference[19] to the literature; 5'-GTCTGACAGCCTCCTCACTT-3'. mRNA expression was measured with a LightCycler (Roche, Basel, Switzerland). RPL13a was used as a housekeeping gene for mRNA quantification.

Statistical analysis

All statistical analysis was performed in this study using JMP Pro 13 (SAS Institute Inc., Cary, North Carolina, USA). When studying the correlation between Perilipin 2 status

and clinicopathological factors, continuous variables were analyzed using the Mann–Whitney U-test and $\chi 2$-test. For the analysis of quantitative PCR, Tukey's HSD test was used. The over-all survival curve was generated using the Kaplan-Meier method, and the statistical analysis was performed by the log-rank test. The statistically significant difference was defined as $p < 0.05$ in this study.

Results

Immunohistochemistry of perilipin 2

The cytoplasmic perilipin 2 immunoreactivity was detected in human adrenal cortex as positive control of immunohistochemistry (Figure 1A) and breast carcinoma cells (Figure 1B-1F). Positive immunoreactivity was focally detected in the tumor tissues, yielding marked intra-tumoral heterogeneity. Foamy histiocytes infiltrating inside of tumor nests and non-neoplastic mammary ducts were also positive for perilipin 2. In addition, perilipin 2 immunoreactivity tended to be more pronounced at the luminal side of the tumor nests, especially around the necrotic areas of the tumor, and also the areas of invasion into the surrounding adipose tissues. When correlating the results obtained with clinicopathological factors of the patients, the status of perilipin 2 in carcinoma

cells was significantly associated with results of Nottingham histological grades ($p = 0.0214$), Ki-67 labeling index ($p = 0.0161$) and HER2 status ($p = 0.0003$), and negatively with ER status of the cases examined ($p = 0.0024$) (Table 2). In addition, when studying the results according to the subtypes, perilipin 2 status was significantly higher in HER2 and basal types than luminal type ($p = 0.0081$). The status of perilipin 2 was not associated with age, BMI and diameter of the tumor. There were no significant differences of overall survival rates according to the status of perilipin 2 immunoreactivity (Log-rank test: $p = 0.7835$). When we compared the positive rate among invasive carcinoma, non-tumor breast and lesions diagnosed as DCIS and ADH (Figure 2A-D), a significant difference was detected ($p < 0.0001$). Results of pairwise comparison did demonstrate significant differences between non-tumor breast tissues and ADH ($p = 0.0464$), DCIS ($p = 0.0003$), invasive carcinoma ($p < 0.0001$) (Figure 2E).

Comparison of perilipin 2 immunoreactivity and Oil-red O positive findings in serial frozen tissue sections of breast cancer

Serial tissue sections were prepared in 13 frozen cases of breast cancer and immunohistochemistry of perilipin 2 and Oil-red O stain were both performed in these serial frozen tissue sections in order to directly evaluate the correlation between intracellular lipid contents and perilipin 2 status in the same breast carcinoma cells. Two areas were selected in the Oil-red O stain for each case after careful review of the specimens, and images in the jpeg format were acquired along with those of the same position in immunohistochemistry performed in serial tissue sections (Figure 3). Significantly positive correlation was detected between Oil-red O stain positive lipid and perilipin 2 positive areas ($p < 0.0001$; Correlation coefficient was 0.8162) (Figure 4). In this study, the evaluation of the whole lesion was not performed in these specimens and therefore the analysis of the correlation with clinicopathological factors was not performed.

Quantitative PCR in breast carcinoma cell lines

Results of quantitative PCR targeting mRNA of perilipin 2, of the three cell lines examined, did reveal that perilipin 2 mRNA expression was highest in MDA-MB-231. Significant differences were also detected between MDA-MB-231 and T47D or MCF7

($p < 0.0004$), but no significant differences were detected between MCF-7 and T47D (Figure 5).

Discussion

Perilipin 2 has been proposed to contribute to an accumulation of intracellular lipid droplets in carcinoma cells, which, however, has not been directly verified. Results of our present study did firstly reveal the significant correlation between perilipin 2 and Oil-red O staining using serial frozen tissue section. Therefore, this is the first study demonstrating the correlation between the status of intracellular lipid droplets and perilipin 2 in carcinoma cells in general. Results were also consistent with those of previously reported studies that perilipin 2 was immunolocalized in intracellular lipid droplets.[10] The analysis of intracellular lipid droplets is generally considered pivotal in analyzing the correlation between abnormal lipid metabolism and various biological features in human malignancies but frozen tissues are required for the analysis of lipid or fat in clinical materials. Therefore, perilipin 2 immunohistochemistry could provide important information as to identifying the presence of lipid droplets in FFPE or archival specimens.

In breast cancer, Moritani et al. previously evaluated perilipin 2 immunoreactivity in 26 apocrine carcinomas of breast and 116 non-apocrine breast carcinomas.[16] The frequency of perilipin-positive cases was significantly higher in apocrine carcinomas (92 %) compared to non-apocrine carcinomas (33 %).[16] However, the details of perilipin 2 status and its significance in breast cancer have remained largely unknown. This is the first study evaluating the correlation between perilipin 2 status and various clinicopathological factors in breast cancer patients. In our present study, perilipin 2 was significantly positively associated with Nottingham histological grade, HER2 status, and Ki-67 labeling index, and negatively with ER status of breast cancer patients. In addition, the positive rates of perilipin 2 in HER2 and basal types (triple negative breast cancer) were significantly higher than those in luminal type. In addition, in our present study, perilipin 2 was significantly more abundant in invasive carcinoma and DCIS compared to that of non-tumor tissue. These findings did indicate that perilipin 2 was also associated with malignant phenotypes in breast tissues as reported in colon cancer.[11, 12] The formation of lipid droplets is also well known to depend on perilipin 2 and this accumulated lipid droplets could stimulate the proliferation of colon carcinoma cell lines, in which the loss of FOXO3 was involved.[12] In addition, perilipin 2 was also proposed as a marker for apocrine and lipid-rich phenotypes of breast carcinomas as

described above.[16, 17] However, there were no significant differences in overall survival rate of breast cancer patients in our present study. We also studied the expression levels of perilipin 2 mRNA and its mRNA was also elevated in basal type carcinoma cells, MDA-MB-231 than in luminal type ones, T47D and MCF-7.

ER-negative breast carcinoma cell lines were also known to accumulate more lipids compared to ER-positive cell lines.[20] Those lipid droplets were considered to induce cell proliferation of ER-negative, not ER-positive, breast carcinoma cell lines.[20] However, in ER-positive T47D cells, lipid droplets were also reported to be involved in development of therapeutic resistance to the anti-proliferative effects of docetaxel[14] and tamoxifen.[21] Therefore, the roles of lipid droplets in breast carcinoma cells may be different depending on intrinsic subtypes but further investigations such as prognostic analysis in each subtype are required to clarify the clinical or biological significance of perilipin 2 in breast carcinomas.

Results of our present study also demonstrated that perilipin 2 was markedly present in carcinoma cells located in the areas around the necrotic tissue, especially in the center of the tumor nests and also in those infiltrating into the surrounding adipose tissue (Figure 1D-1F). Moritani et al., also reported similar results.[16] perilipin 2 mRNA was also reported to be enhanced in cultured cells under hypoxic conditions[22, 23] and the

amounts of intracellular lipid droplets to be increased in parallel with perilipin 2 expression in those cells.[24] Results of our present study were also consistent with those findings above. In addition, the removal of lipid from the culture medium was reported to decrease cell chemotaxis in MDA-MB-231 cell line[25] and increased intracellular lipid as a result of perilipin 2 activation was also demonstrated to increase tumor cell proliferation.[10] In our present study, perilipin 2 immunoreactivity was markedly detected at the sites of carcinoma infiltration into adjacent adipose tissues in which tumor cells and adipose tissue directly contacted each other *in situ*. These findings also indicated that the increased lipid concentration in the tissue microenvironment of the tumor as a result of the direct contact above could also influence the biological behavior of carcinoma infiltration. Increased lipid utilization is considered to provide advantageous for tumors such as tumor growth and survival rate under hypoxic environment and/or chemotherapy.[14, 22] In this study, perilipin 2 was demonstrated to be correlated not only with intracellular lipid but also with multiple adverse clinicopathological factors. The increase in perilipin 2 and intracellular lipid droplet was also considered to be related to poor prognosis. Therefore, perilipin 2 could represent a novel prognostic marker related to lipid metabolism or a new therapeutic target factor manipulating lipid metabolism but further investigations are required for clarification.

Disclosure statement

None declared.

Author contributions

SK: conception and design of the study, acquisition and analysis of data, drafting the manuscript and figures. YM: conception and design of the study, drafting the manuscript. AS: acquisition and analysis of data. EI: acquisition and analysis of data. KO: acquisition and analysis of data. YO: acquisition and analysis of data. HH: acquisition and analysis of data. TI: acquisition and analysis of data. NY: revision of the draft. HS: revision of the draft.

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Figure legends

Figure 1 Immunohistochemistry of perilipin 2

A: Adrenal gland as positive control. B to F: breast carcinoma. B: Weak immunoreactivity was detected in the cytoplasm of breast carcinoma cells. C: Marked immunoreactivity in the cytoplasm of breast carcinoma cells. D: Positive findings on luminal side of carcinoma cells forming tumor nest. E and F: Marked immunoreactivity in carcinoma cells at the sites of infiltration into adjacent adipose tissue.

Figure 2 Perilipin 2 immunoreactivity and positive rate of perilipin 2 according to the lesions

A and B: DCIS. A: Hematoxylin and eosin stain. B: Perilipin 2 immunohistochemistry. C and D: ADH. C: Hematoxylin and eosin stain. D: Perilipin 2 immunohistochemistry. E is positive rate of perilipin 2 according to the lesions. There was a significant difference ($p < 0.0001$) according to Kruskal-Wallis test. By pairwise comparison, significant difference was detected between non-tumor tissue and ADH ($p = 0.0464$), DCIS ($p = 0.0003$), invasive carcinoma ($p < 0.0001$).

NOTE, DCIS; ductal carcinoma in situ, ADH; atypical ductal hyperplasia, IC; invasive carcinoma.

Figure 3 Comparison of Oil-red O stain and immunohistochemistry

Left: Oil-red O stain and Right: immunohistochemistry of perilipin 2. Scale bar is 50 micrometers. 4 cases are shown as representative in 13 cases. The positive areas were overlapped between these two stains.

Figure 4 Comparison of positive area of Oil-red O stain and perilipin 2 immunohistochemistry

A regression line was prepared by plotting the positive area in Oil-red O stain on the vertical axis and immunohistochemistry on the horizontal axis.

Figure 5 Comparison of mRNA of perilipin 2 in breast carcinoma cell lines

The amounts of mRNA of perilipin 2 in MDA-MB-231 were significantly higher than those of other cell lines (MDA-MB-231 vs MCF7: $p = 0.0001$, MDA-MB-231 vs T47D: $p = 0.0004$).

Table 1 Characteristics of patient in this study

Patient characteristics	
Age (years)	
range	27 - 87
median	57
Body mass index	
range	16.4 - 32.4
median	23.2
Histrogical subtype†	
Invasive ductal carcinoma, no special type	102
Invasive micropapillary carcinoma	2
Invasive lobular carcinoma	1
Tumor invasion size (mm)	
range	1 - 67
median	17.4
Lymphatic invasion†	
positive	65
negative	40
Venous invasion†	
positive	39
negative	66
Nottingham histological grade†	
1	34
2	43
3	24
ER†	
positive	78
negative	28

Table 2 Perilipin 2 immunohistochemistry and clinicopathological parameters in 105 breast cancer cases

Clinicopathological parameters		Perilipin 2 immunohistochemistry positive ratio		p value
		Low cases (%)	High cases (%)	
Nottingham histological grade	I	33 (97)	1 (3)	0.0214*
	II	35 (81)	8 (19)	
	III	17 (71)	7 (29)	
Ki-67 Labeling index		85 (88)	12 (12)	0.0161*
median ± SD		9 ± 12.3 %	20 ± 15.6 %	
ER status	positive	71 (91)	7 (9)	0.0024*
	negative	18 (67)	9 (33)	
HER2 status	positive	14 (61)	9 (39)	0.0003*
	negative	74 (89)	9 (11)	
Subtype	luminal	74 (90)	8 (10)	0.0081*
	HER2	7 (58)	5 (42)	
	basal	8 (73)	3 (27)	
Age		89 (85)	16 (15)	0.1521
median ± SD		56 ± 13.3 years	53 ± 11.2 years	
Body mass inedex		87 (86)	14 (14)	0.5619
median ± SD		23.1 ± 3.8	21.9 ± 3.1	
Tumor invasive size		85 (86)	14 (14)	0.2239
median ± SD		15 ± 12.6 mm	17 ± 9.4 mm	
Lymphatic invasion	positive	54 (83)	11 (17)	0.5403
	negative	35 (88)	5 (12)	
Venous invasion	positive	31 (79)	8 (21)	0.2476
	negaive	58 (88)	8 (12)	
pT	1	55 (87)	8 (13)	0.6910
	2	16 (80)	4 (20)	
	3	0	0	
	4	8 (89)	1 (11)	
Lymph node metastasis	positive	28 (80)	7 (20)	0.3528
	negative	60 (87)	9 (13)	

NOTE, *; $p < 0.05$

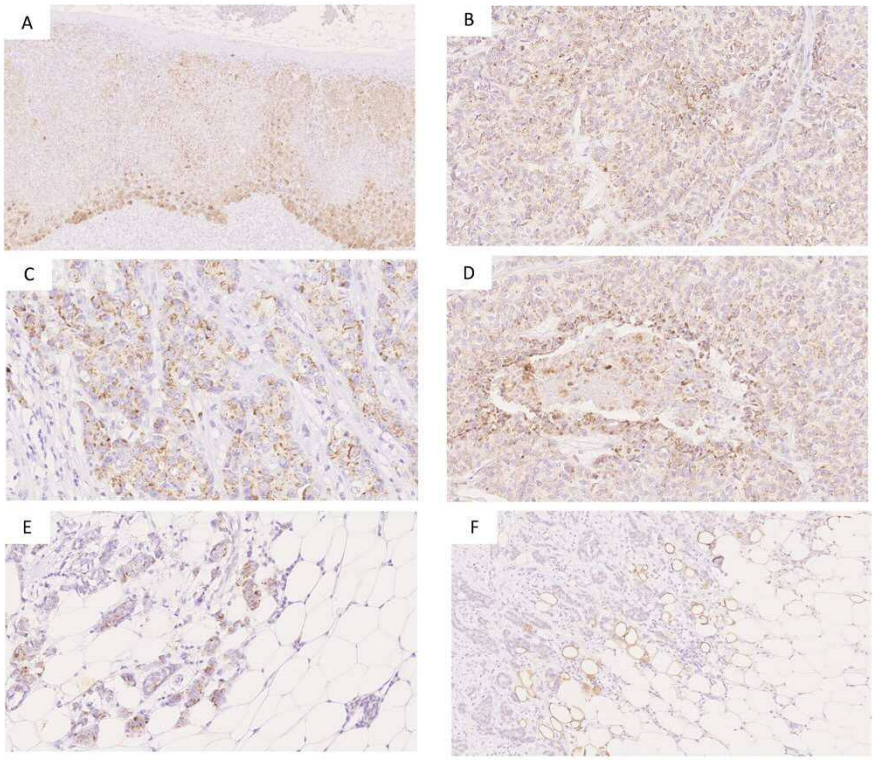

Figure 1 Immunohistochemistry of perilipin 2

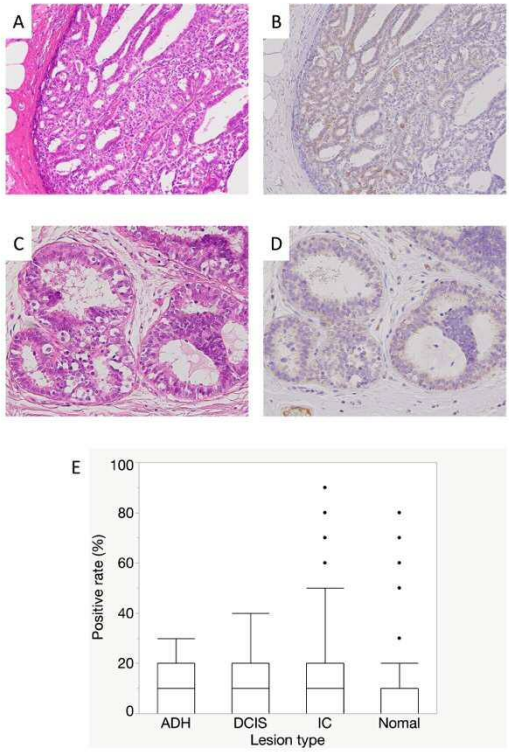

Figure 2 Perilipin 2 immunoreactivity and positive rate of perilipin 2 according to the lesions

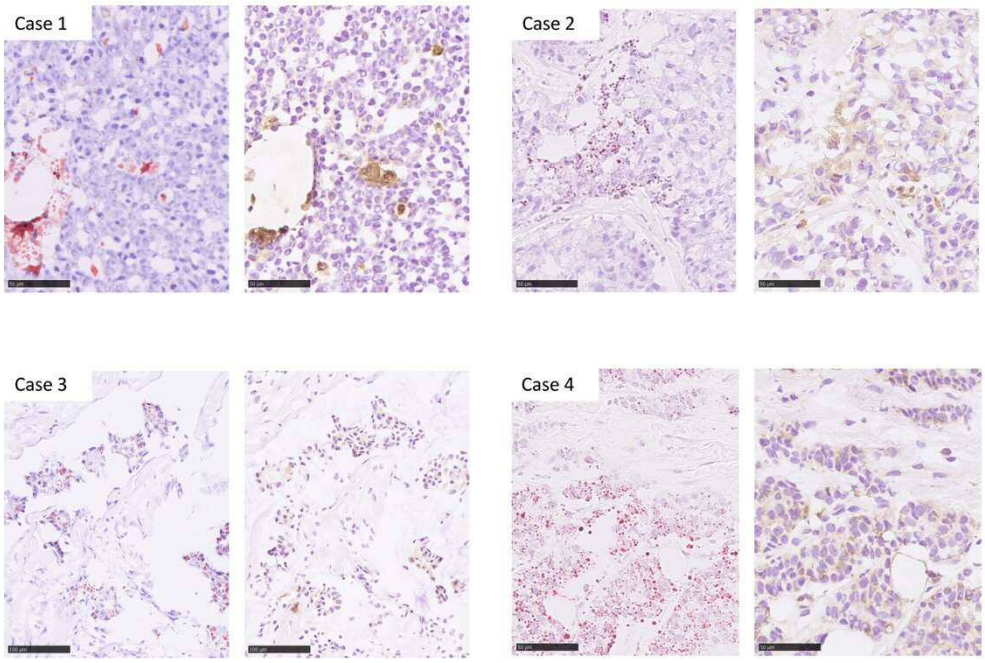

Figure 3 Comparison of Oil-red O stain and immunohistochemistry

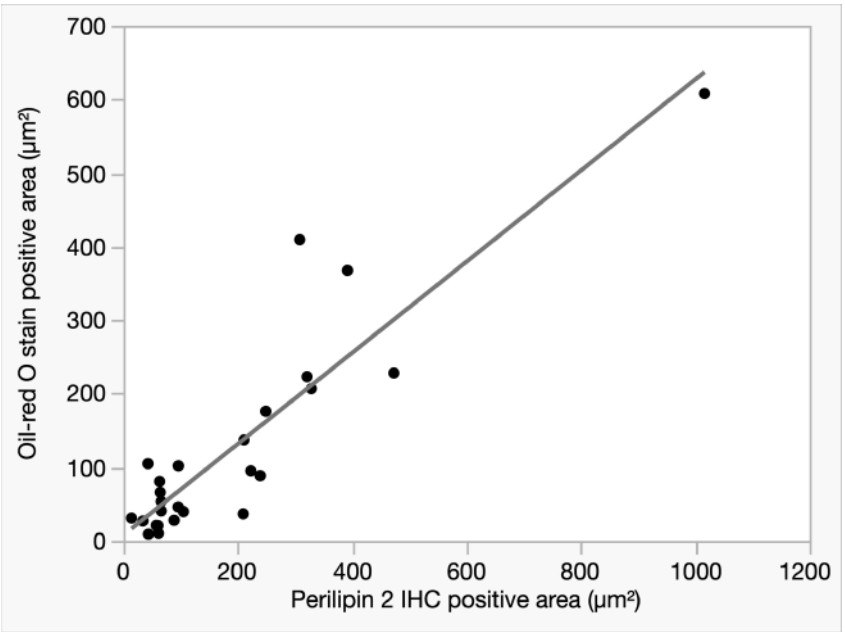

Figure 4 Comparison of positive area of Oil-red O stain and perilipin 2 immunohistochemistry

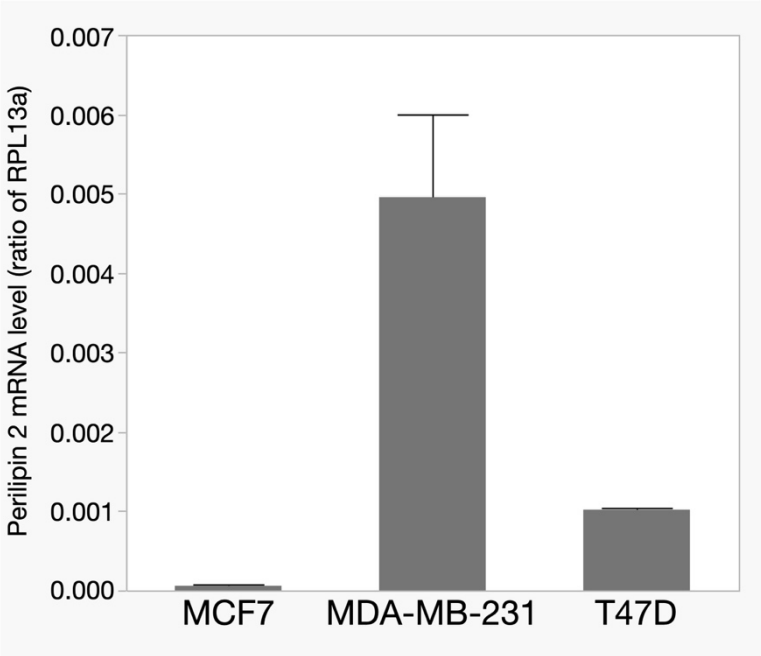

Figure 5 Comparison of mRNA of perilipin 2 in breast carcinoma cell lines