Breast Cancer, Diabetes Mellitus And Glucagon-Like Peptide-1 Receptor Toward Exploring Their Possible Associations

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Abstract

Purpose: Diabetes Mellitus (DM) has been one of the well known risk factors of breast cancer (BC) development and also associated with adverse clinical outcomes of BC patients. Glucagon-like peptide-1 (GLP-1) receptor agonists have been used as antidiabetic therapeutic agents and recent epidemiological studies have reported their use to be correlated with increased BC risks. However, biological or pathological details have remained unknown. Therefore, in this study, we examined the status of GLP-1 receptor (GLP-1R) in BC with and without DM and correlated the findings with the clinicopathological factors of the patients to explore the possible involvement of GLP-1 in BC pathology.

Methods: We immunolocalized GLP-1R in cancer and adjacent non-pathological breast tissues in BC patients with DM (125 cases) and without DM (58 cases). We then compared the status of GLP-1R with that of fibroblast growth factor 7 (FGF7) and fibroblast growth factor receptor 2 (FGFR2), Ki-67 labeling index (Ki-67 LI) and disease free survival (DFS) of the patients and also between cancerous and non-pathological breast tissues.

Results: GLP-1R immunoreactivity was significantly higher ($p=0.044$) in the patients with DM than without in carcinoma tissues. However, this was detected only in invasive carcinoma ($p<0.01$) and not in non-invasive carcinoma nor non-pathological mammary glands. FGF7 was significantly correlated with the status of GLP-1R in BC ($p=0.045$). In addition, in ER positive BC cases, those with GLP-1R positive status tended to have higher Ki-67 LI of more than 14% ($p=0.070$).

Conclusion: These findings all demonstrated the possible association between GLP-1R status and biological features of BC, especially of invasive BC in DM patients.

Introduction

Breast cancer (BC) is the most frequently diagnosed malignancy among women [1]. Among various risk factors of BC reported, the presence of diabetes mellitus (DM) has been generally considered one of the pivotal risk factors of BC development [2]. Results of several epidemiological studies demonstrated that the presence of DM was associated with an increased risk of BC development [3, 4]. For instance, women with DM were reported to have statistically significant increased risks of BC development compared to those without DM [3]. In addition, a past clinical history of DM was also reported to contribute to adverse clinical outcomes of BC patients [5, 6]. For instance, results of meta-analysis revealed that BC patients with DM had a significantly shorter overall survival (OS) as well as disease free survival (DFS) [5]. In addition, the presence of glucose intolerance was also reported to be significantly associated with the OS of BC patients [7]. However, the mechanistic association between DM and BC has remained virtually unknown. Hyperinsulinemia was also reported to decrease the production of sex hormone-binding globulin and increase insulin-like growth factor (IGF)-I in conjunction with DM, and thus proposed to play a pivotal role in BC development [8]. Therefore, the appropriate control of the blood sugar and the

maintenance of suitable body weight after surgery have been generally recommended in postoperative BC patients [7, 9].

Incretins, one of gut peptide hormones, are secreted after intake of food and stimulate insulin secretion. Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are both well known families of incretin hormones secreted from the upper (K cells) and lower (L cells) gut. Therefore, incretins have been clinically used as anti-diabetic therapeutic agents [10]. In particular, several long-lasting GLP-1 analogs with insulinotropic activity have been used in the treatment of type 2 DM [11]. On the other hand, it is also true that GLP-1 related therapy has been reported to increase the risk of developing certain human malignancies [12–14]. For instance, the US Food and Drug Administration (FDA) reported in 2016 that GLP-1 analogues could possibly increase the risk of BC development [13]. They suggested that there were epidemiological “imbalances” reported in the number of BC events among the patients randomized to the GLP-1 analogue liraglutide versus placebo (15 v 3 events; incidence 4.36 v 1.80 per 1000 person years) as results of the randomized controlled trials for weight management [13]. However, there have been very few studies reported on the mechanistic correlation between BC risk and GLP-1.

In addition to the potential adverse effects leading toward BC development, GLP-1 receptor (GLP-1R) agonists have been reported to increase the risks of developing other malignancies such as pancreatic and thyroid cancers [14]. Of particular interest, the cell proliferation of pancreatic intraepithelial neoplasia (PanIN) derived from pancreatic ducts, which is generally considered a premalignant lesion of pancreatic invasive ductal carcinoma, was reported to be stimulated by GLP-1 therapy [14]. In addition, GLP-1 therapy resulted in C-cell hyperplasia of the thyroid glands [14]. Koehler et al. also suggested that GLP-1 analogues could promote tumor cell proliferation through increased *in situ* expression of fibroblast growth factor (FGF) 7 based on their results of *in vivo* and *in vitro* studies [15]. On the other hand, Hicks et al. carefully analyzed the LEADER trial and reported no significant association between increased risk of BC development and the use of GLP-1 analogs [16]. However, they also suggested that the proliferation of breast carcinoma cells could be stimulated by the interaction between GLP-1 analogues and FGF7/FGF receptor (FGFR) 2 axis [16].

Despite some controversies, GLP-1 has been reported to be generally associated with increased risk of cancer development in some human malignancies including BC. However, the status of GLP-1 as well as of the FGF7/FGFR2 axis above have not been studied in BC. Therefore, in this study, we examined the intra-tumoral status of GLP-1R, FGF7, and FGFR2 in BC and adjacent non pathological breast tissues of both DM and non-DM patients in order to further explore the possible involvement of GLP-1 in BC biology.

Materials And Methods

Breast cancer cases

We retrieved archival surgical pathology specimens of BC patients with and without DM, operated at Tohoku University Hospital and Nahanishi Clinic, excluding those who received neoadjuvant

chemotherapy. Clinical information of the patients was retrieved from clinical charts of the patients. DM was ascertained by the medical records, or a random plasma glucose ≥200mg/dL accompanied by HbA1c of >6.5% according to the guideline for diabetes treatment of the Japan Diabetes Society [17]. In all patients examined, periodic clinical follow-up was performed every 3 to 6 months for at least 5 years after surgery. The clinicopathological characteristics of the patients examined in this study are summarized in Table 1. We studied 125 BC cases with DM including 12 cases of ductal carcinoma in situ (DCIS) and 58 BC cases without DM including 5 DCIS. The status of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2) and Ki-67 labeling index (Ki-67 LI) were all retrieved from surgical pathology reports of individual patients. The number of ER positive invasive carcinoma cases were 89 with DM and 38 without DM, respectively. In addition, we also studied GLP-1R immunoreactivity in an additional 31 case of DCIS including 11 with and 20 without DM. Research protocols of this study were approved by the Ethical Committee of Tohoku University School of Medicine (no.2018-1-433) and Nahanishi Clinic (no. NNCEC2017007) Japan.

Immunohistochemistry

Four-micron tissue sections prepared from 10% formalin-fixed and paraffin-embedded tissues of the representative areas of the lesions were prepared in this study. A mouse monoclonal antibody against GLP-1R (ab166987, abcam, Cambridge UK), a rabbit polyclonal antibody against FGF7 (HPA043605, Atlas Antibodies, Stockholm Sweden) and a mouse monoclonal antibody against FGFR2 (ab58201, abcam, Cambridge UK) were used as primary antibodies in this study as summarized in Table 2. After deparaffinization and hydration, the antigen retrieval was performed with citrate buffer using microwave irradiation for 20 minutes in GLP-1R and with autoclave at 121°C for 5 minutes in FGFR2 and FGF7. The slides were then incubated for 18 hours with primary antibodies against GLP-1R, FGF7 and FGFR2. Histofine kit (Nichirei Bioscience, Tokyo, Japan) was used for immunostaining. DAB was used as colorimetric agent for 15 minutes in GLP-1R and FGF7 immunostaining, and for 10 minutes in FGFR2 at room temperature. The tissue slides were finally counterstained with hematoxylin. The positive controls were pancreatic islets of Langerhans in GLP-1R, urinary bladder urothelial carcinoma in FGF7 and pulmonary adenocarcinoma in FGFR2. Negative controls were treated with 1% BSA in PBS instead of the primary antibody. An absorption test with excessive antigens was performed in FGF7 immunostaining.

Evaluation of immunoreactivity

Evaluation of immunoreactivity of GLP-1R, FGF7 and FGFR2 was performed as previously reported [18-20]. Immunoreactivity of GLP-1R and FGFR2 was evaluated by adding the intensity to the distribution scores [18,19]. Relative immunointensity was scored as follows: 0, no immunoreactivity; 1, weak immunoreactivity; 2, moderate immunoreactivity and 3, marked immunoreactivity. The proportion of GLP-1R immunoreactivity was scored as follows: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75% and 4, 76-100%, while that of FGFR2 was as follows: 0, 0%; 1, <10%; 2, 10-49% and 3, 50-100% [18,19]. If the total score was <4, the case was determined as negative for GLP-1R and low for FGFR2. If the total score was ≥4, the case was considered positive for GLP-1R and high for FGFR2 [18,19]. FGF7 immunoreactivity was evaluated

based on the combination of the percentage and intensity of positively stained tumor cytoplasm to generate the H-score=ΣPi(i+1). In this score, i represented immunointensity of immune-positive tumor cells (0 to 3+) and Pi the percentage of positive tumor cells of each intensity with the range from 0% to 100% [20]. Two of the authors (N.H.T and S.K) independently evaluated immunoreactivity and discordant cases were re-evaluated together using multi-headed light microscopy.

Statistical Methods

All the analyses in this study were performed using statistical software (StatMate IV, GraphPad Software, California USA and Stata/ MP 15.1, StataCorp, Texas USA). The associations between GLP-1R or FGFR2 and clinicopathological features of the cases were assessed by the Chi-square test, while those of FGF7 were assessed by t test. The DFS was estimated using Kaplan–Meier analysis and the statistical differences were tested by the log-rank test. The statistically significant difference was defined as $p < 0.05$ in this study.

Results

GLP-1R status between DM and Non-DM groups of BC patients

GLP-1R immunoreactivity was detected in both cell membrane and cytoplasm of carcinoma cells (Fig.1). GLP-1R was also detected in adjacent normal ductal epithelial cells (data not shown). We first compared the status of GLP-1R in BC tissues between DM and non-DM cases (Fig.2-A). GLP-1R immunoreactivity in carcinoma cells was significantly higher ($p=0.044$) in DM (67.5%, 84/125) than non-DM groups (51.7%, 30/57). GLP-1R was also detected in adjacent normal ductal epithelial cells (data not shown) but not in myoepithelial nor stromal cells including inflammatory cells. In the adjacent normal mammary gland tissues, there was no significant differences of GLP-1R immunoreactivity between DM and non-DM groups of the patients (19.5% vs 16.4%, $p=0.62$). GLP-1R was significantly more pronounced in carcinoma cells than in non-neoplastic mammary ductal cells regardless of the presence or absence of DM (DM $p<0.001$, non-DM $p<0.001$).

We then looked at the status of GLP-1R among invasive carcinoma, non-pathological mammary glands and intraductal lesions in invasive cancer cases and compared the findings between DM and non-DM cases (Fig.2-A). The status of GLP-1R in invasive carcinoma was significantly more frequent in DM group than non-DM group of patients (DM 67.6%, 75/111 vs non-DM 46.1%, 24/52, $p<0.01$). In addition, GLP-1R was significantly more frequent in invasive carcinoma than in non-pathological mammary glands regardless of the status of DM. However, the status of GLP-1R in intraductal lesions was not different between DM and non-DM groups of the patients. We also studied GLP-1R expression in DCIS cases, including additional 11 DCIS cases with DM and 20 without DM (Fig.2-B). There were also no significant differences of GLP-1R status between DCIS of DM and non-DM cases (DM 34.8%, 8/23 vs non-DM 40%, 10/25, $p=0.71$). Of particular interest, when we studied only the ER positive invasive carcinoma cases, GLP-1R status was significantly more frequent in the DM group than the non-DM group in the invasive cancer tissue (DM 67.4% vs non-DM 47.4%, $p=0.033$) (Fig.2-C).

FGF7 and FGFR2

Both FGF7 and FGFR2 were detected in the cytoplasm and cell membrane of breast carcinoma cells (Fig.1) and also in adjacent normal ductal epithelial cells (data not shown) but not in myoepithelial nor stromal cells including inflammatory cells. There was no significant correlation between the status of FGF7 and/or FGFR2 and the presence or absence of DM (Fig.3-A, Fig.4-A). Of particular interest, the H-scores of FGF7 were significantly higher in the GLP-1R positive group compared to the negative group both in invasive ductal carcinoma cells ($p=0.045$) and in adjacent non pathological mammary gland tissue ($p<0.001$) in the DM patient group and in adjacent non pathological mammary gland ($p=0.032$) in the non-DM patient group and those of FGF7 tended to be higher in invasive cancer expressing GLP-1R in the non-DM group ($p=0.074$) although the difference did not necessarily reach statistical significance. There were no significant correlations between FGFR2 and GLP1R status in the cases examined in this study ($p=0.15$) (Fig.4-B).

The correlation of GLP-1R with clinicopathological features and clinical outcome in BC cases with DM

There were no significant differences of age, HbA1c levels and Body mass index (BMI) of the patients between GLP-1R positive and negative BC cases regardless of past history of DM (Table.3). There were also no significant differences of recurrence rate between the GLP-1R positive and negative BC cases (Fig.5). The proportion of Luminal B tentatively determined as Ki-67 LI of more than 14% [21] among GLP-1R positive cases tended to be higher than negative cases, although the differences did not reach statistical significance (GLP-1 positive 40% vs negative 20.7%, $p=0.070$) (Fig.6).

Discussion

DM has been reported to be associated with both increased risks of BC development and poor prognosis of the patients with BC as mentioned above based on results of epidemiological studies [2, 3]. Therefore, at present, maintaining the blood glucose levels is generally considered important in the clinical management of BC patients with DM, especially after surgery [6]. However, the potential roles of DM in BC development and prognosis have not necessarily been clarified. Therefore, in this study, we firstly demonstrated the expression of GLP-1R and its related factors, FGF7/FGFR2, in carcinoma cells of invasive ductal carcinoma, DCIS and normal epithelial cells in BC patients with and without DM.

GLP-1, one of the well known hormones of incretin family, is released from the intestine in response to food intake and inactivated by the dipeptidyl peptidase-4 enzyme (DPP4) within a very short circulating half-life (< 2min) [22]. The plasma GLP-1 concentrations were reported higher in the early stages of DM than non-diabetic controls and to be associated with BMI and other factors of metabolic syndrome [23, 24]. It is true that monitoring blood concentration of GLP-1 could provide important information in understanding its potential roles in BC development and progression but this is clinically challenging and may be not feasible due to its extremely short half-life [22]. Therefore, an evaluation of the status of GLP-1R to which GLP-1 specifically binds in BC tissues is considered the feasible approach to understand the potential roles or involvement of GLP-1 in BC patients.

In our present study, we firstly demonstrated that GLP-1R was more abundant in BC patients with DM than without DM. Of particular further interest, significantly higher GLP-1R status in carcinoma cells was detected only in invasive carcinoma but not in non-invasive carcinoma nor adjacent non pathological mammary glands. We also detected abundant FGF7 in the tissues harboring GLP-1R. In addition, the proportion of Luminal B among GLP-1R positive cases tended to be higher than negative cases although the difference did not necessarily reach statistical significance. A significantly positive correlation between GLP-1R and ER was also reported in endometrial cancer patients [25]. ER positive breast carcinoma cell line, MCF-7, was also demonstrated to have GLP-1/GLP-1R intracellular signaling pathway [26]. Therefore, estrogen is reasonably postulated to play a pivotal role in the GLP-1/GLP-1R signaling pathway but further investigation is required for clarification.

GLP-1R has been reported to be expressed in many tissues throughout the body [27] and its activation is also well known to exert clinically beneficial effects on diabetes-related complications and comorbidities such as reducing weight, and modulation of circulatory systems apart from 'incretin' function and others [11, 28]. GLP-1R expression in BC tissue was upregulated among DM patients in our present study. Therefore, even the small degrees of serum GLP-1 increase in DM patients could result in more pronounced effects on breast carcinoma cells but it awaits further investigations for confirmation of this interesting hypothesis.

FGF7 has been known as a paracrine growth factor produced and secreted in breast stromal fibroblasts and to exert proliferative and morphogenic effects on both epithelial and myoepithelial cells in normal breast as well as BC [29]. The activation of GLP-1R signaling was also reported to promote the tumor growth in the models of gut hyperplasia and tumorigenesis through increasing *in situ* FGF7 levels in intestinal tumor [15]. FGFR2, the receptor of FGF7, has also been reported to be involved in cell growth, invasiveness, mortality, and angiogenesis [30–32]. For example, FGFR2 was reported to maintain the stem-like tumor-initiating cells (TICs) by promoting cellular self-renewal and maintaining the biopotency of the TICs, which could result in facilitating the development of BC [32]. Various variants of FGFR2 have been also reported to be significantly associated with the risks of BC development and progression [33]. In addition, FGF7 and FGF2R signals were reported to promote motility and proliferation of breast carcinoma cell lines [34–36]. In our present study, we did demonstrate relatively abundant FGF7 in BC cases positive for GLP-1R. This finding also implicated that the pathways among GLP-1R, FGF7 and FGFR2 similar to those above are considered to be involved in tumor growth and progression in BC.

The incidence of ER positive BC was reported to be higher among women with DM than those without [37]. In our present study, among ER positive cases, the status of GLP-1R tended to be associated with higher Ki-67 LI, although this tendency did not reach statistical significance. FGF7/FGFR2 signal was also reported to exert suppressive or inhibitory effects on the actions of selective ER modulators [38]. Therefore, the increased GLP-1R in BC tissue of DM patients detected in our present study is reasonably postulated to be associated with adverse clinical outcome of the patients through FGF7/FGFR2 pathway influencing tumor growth and progression or the treatment resistance to endocrine therapy but this awaits further investigation for clarification.

In this study, we firstly demonstrated that GLP-1R activation could be associated with the development, progression and therapeutic resistance of BC in DM patients, by comparing GLP-1R status between DM and non-DM patients. This finding is consistent with increasing risks of development and progression of BC in DM patients using GLP-1R related medication [12, 13]. Maintaining appropriate weight and controlling glucose tolerance have been clinically considered essential for preventing adverse clinical outcomes of the patients [7, 9]. Results of our present study also did shed new insights into the clinical management of DM patients with past history of BC. In particular, results of our present study regarding tumor proliferation and treatment resistance associated by GLP-1R activation could also provide the important information as to the postoperative management of BC patients with DM.

Conclusion

We firstly demonstrated the possible association between GLP-1R activation and the development/progression of BC in DM patients, which is also consistent with previously reported clinical and epidemiological findings. Appropriate management of DM including control of glucose tolerance, weight and selection of appropriate anti-diabetic drugs could be important in BC patients with DM.

Declarations

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Conflicts of interest/Competing interests: There are no conflicts of interest that needs to be disclosed.

Availability of data and material: The datasets generated during and/or analyzed during the current study are not publicly available due personal information protection but are available from the corresponding author on reasonable request.

Code availability: Not applicable

Authors' contributions: NHT and HS devised the project and the main conceptual ideas. NHT worked out almost all of the experiments and performed the analysis. SK and KT supervised the evaluation of immunohistochemistry. EI, YM, SK, KT interpreted the pathological results. SK and HS supervised the pathological examinations. NT and TI managed and provided the clinical data. YK, KU, SeT, ShT, NT, AK, and MM collected clinical data, selected appropriate cases, and evaluated the results. NT, TI, and HS interpreted and supervised the all results. NHT wrote the draft manuscript. YM, TK, NT, and HS reviewed and edited the manuscript. All authors discussed the results and commented on the manuscript.

Ethics approval: Research protocols of this study were approved by the Ethical Committee of Tohoku University School of Medicine (no.2018-1-433) and Nahanishi Clinic (no. NNCEC2017007) Japan.

Consent to participate: Informed consent was obtained from all participants.

Consent for publication: It was included in the approval of the Tohoku University Ethics Committee.

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Tables

Due to technical limitations, table 1, 2 and 3 is only available as a download in the Supplemental Files section.

Figures

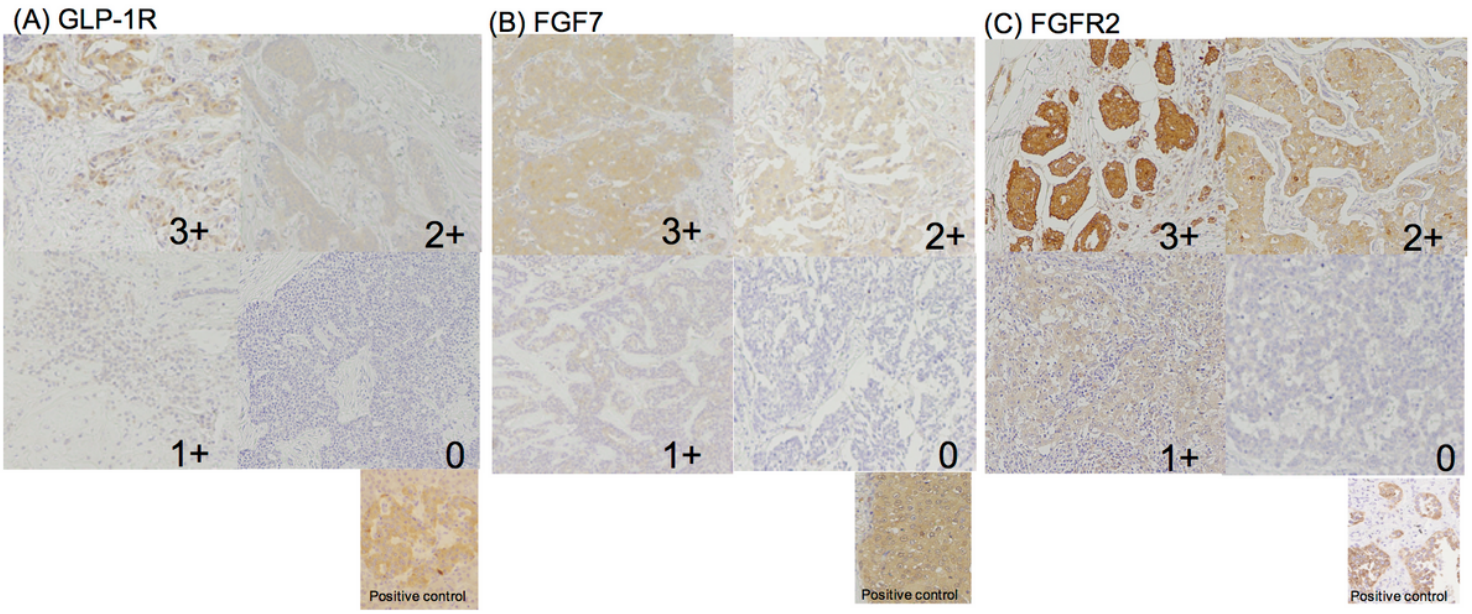

Figure 1

Representative illustration of immunochemical findings in breast carcinoma cases examined in this study 0, 1+, 2+ and 3+ correspond to the intensity score of immunoreactivities obtained. The positive controls were pancreatic islets of Langerhans with positive expression of GLP-1R (A), bladder cancer tumor with that of FGF7 (B) and lung cancer tumor with that of FGFR2 (C).

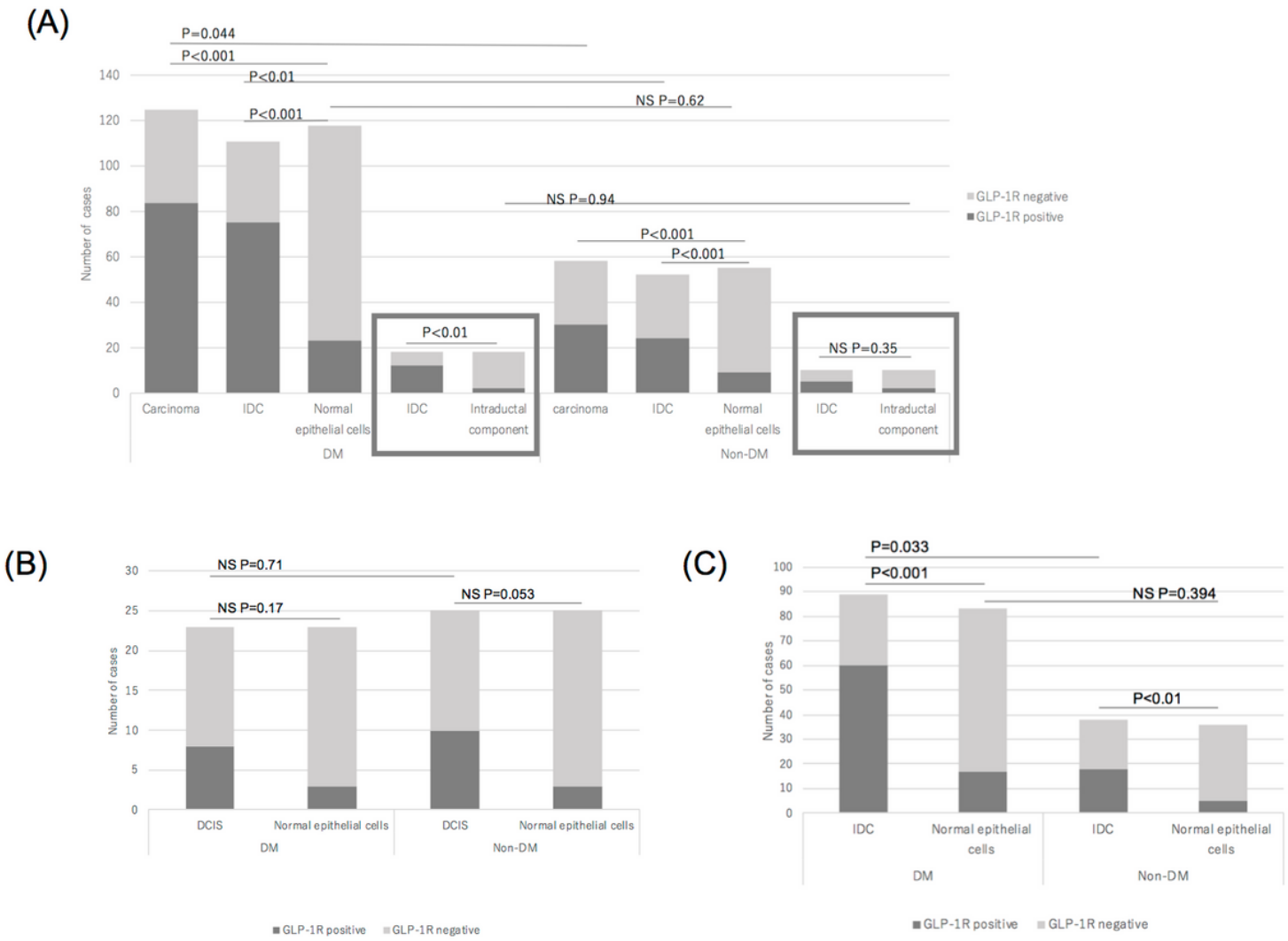

Figure 2

Summary of GLP-1R A GLP-1R expression In carcinoma tissues, GLP-1R immunoreactivity was significantly higher (p=0.044) in DM. GLP-1R immunoreactivity was significantly more pronounced in carcinoma cells than that of non-neoplastic mammary ducts regardless of the presence of DM (DM p<0.001, non-DM p<0.001). Inside the boxes, the detectable intraductal lesions in invasive cancer cases were analyzed. B GLP-1R in DCIS C GLP-1R in invasive ductal carcinoma tissues among ER positive cases

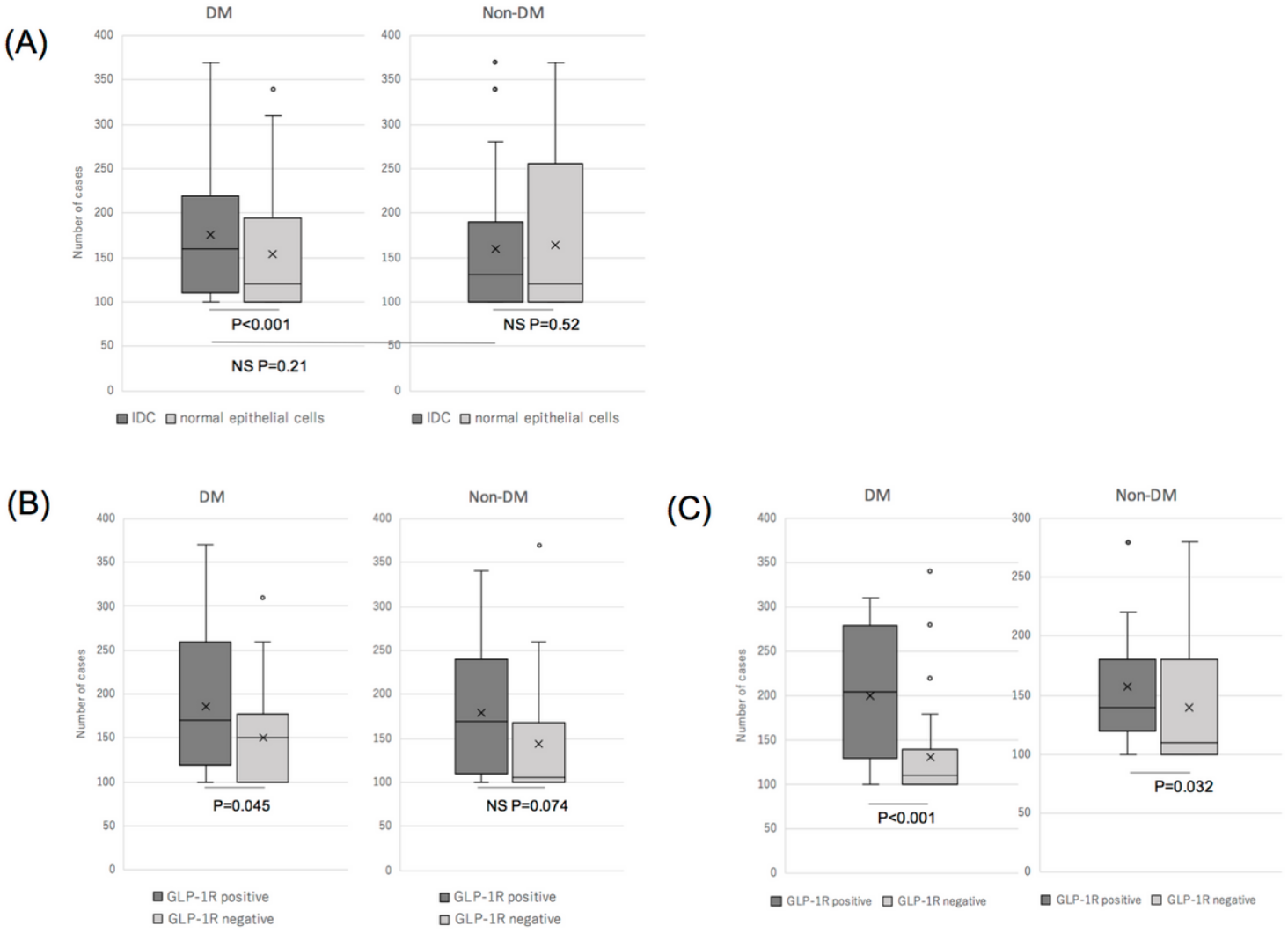

Figure 3

Summary of FGF7 in our present study A FGF7 in all the cases examined B FGF7 in invasive ductal carcinoma comparing between GLP-1R positive and negative cases C FGF7 in adjacent non pathological ductal epithelial cells comparing between GLP-1R positive and negative case

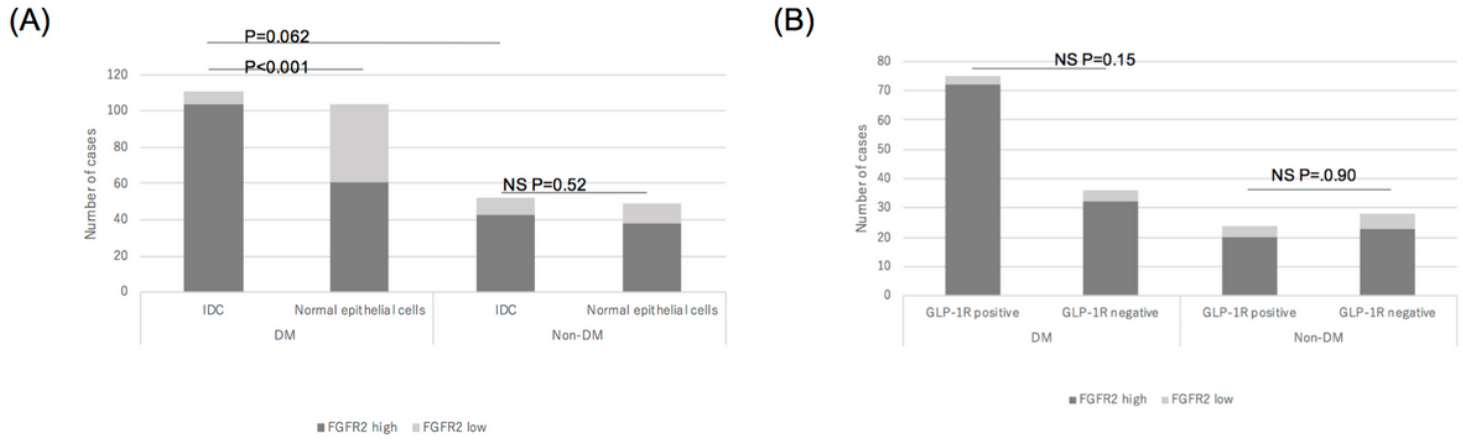

Figure 4

Summary of FGFR2 in our present study A FGFR2 in all the cases B FGFR2 in invasive ductal carcinoma cells comparing between GLP-1R positive and negative cases

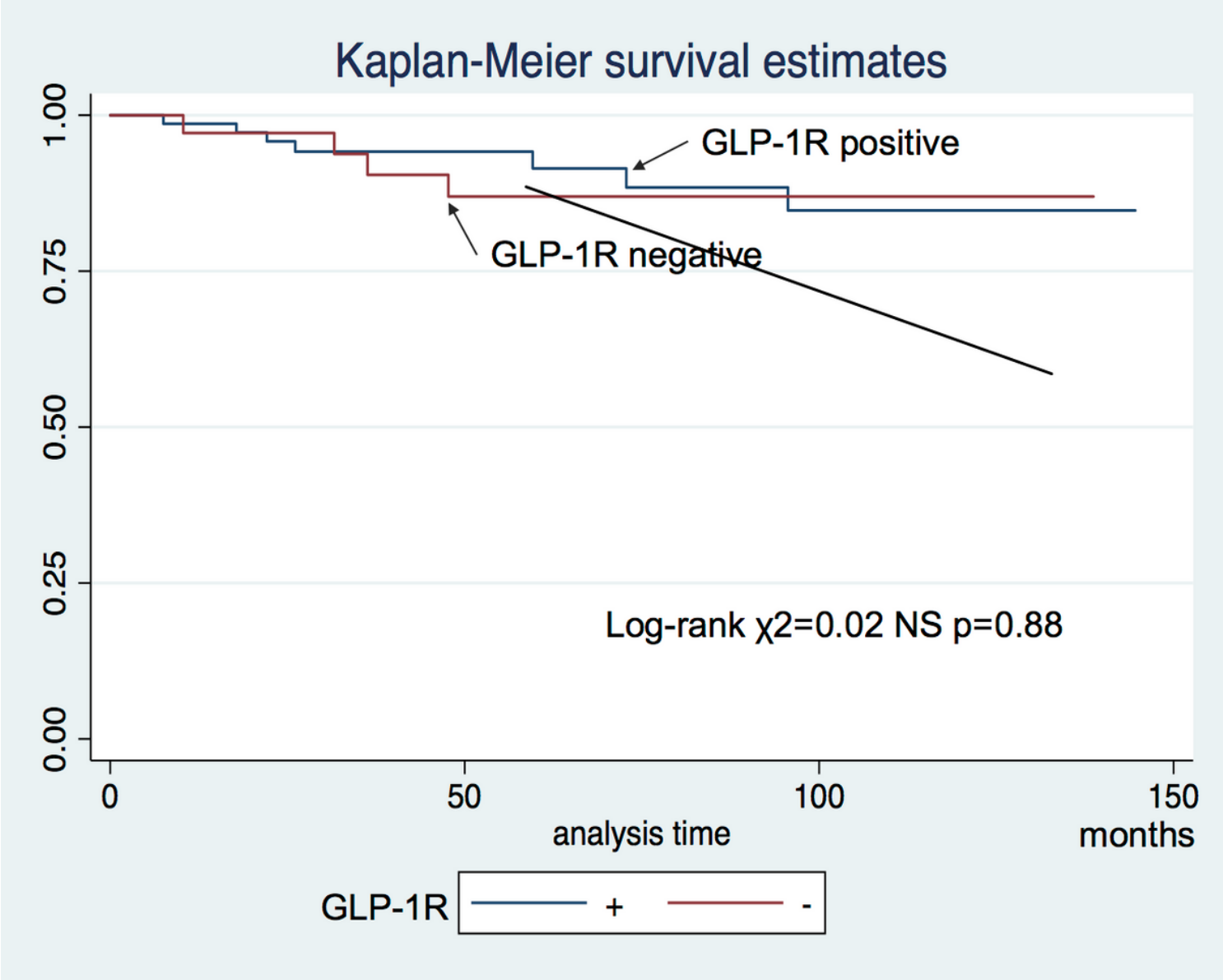

Figure 5

DFS comparing BC cases with GLP-1R positive and negative BC in the DM group

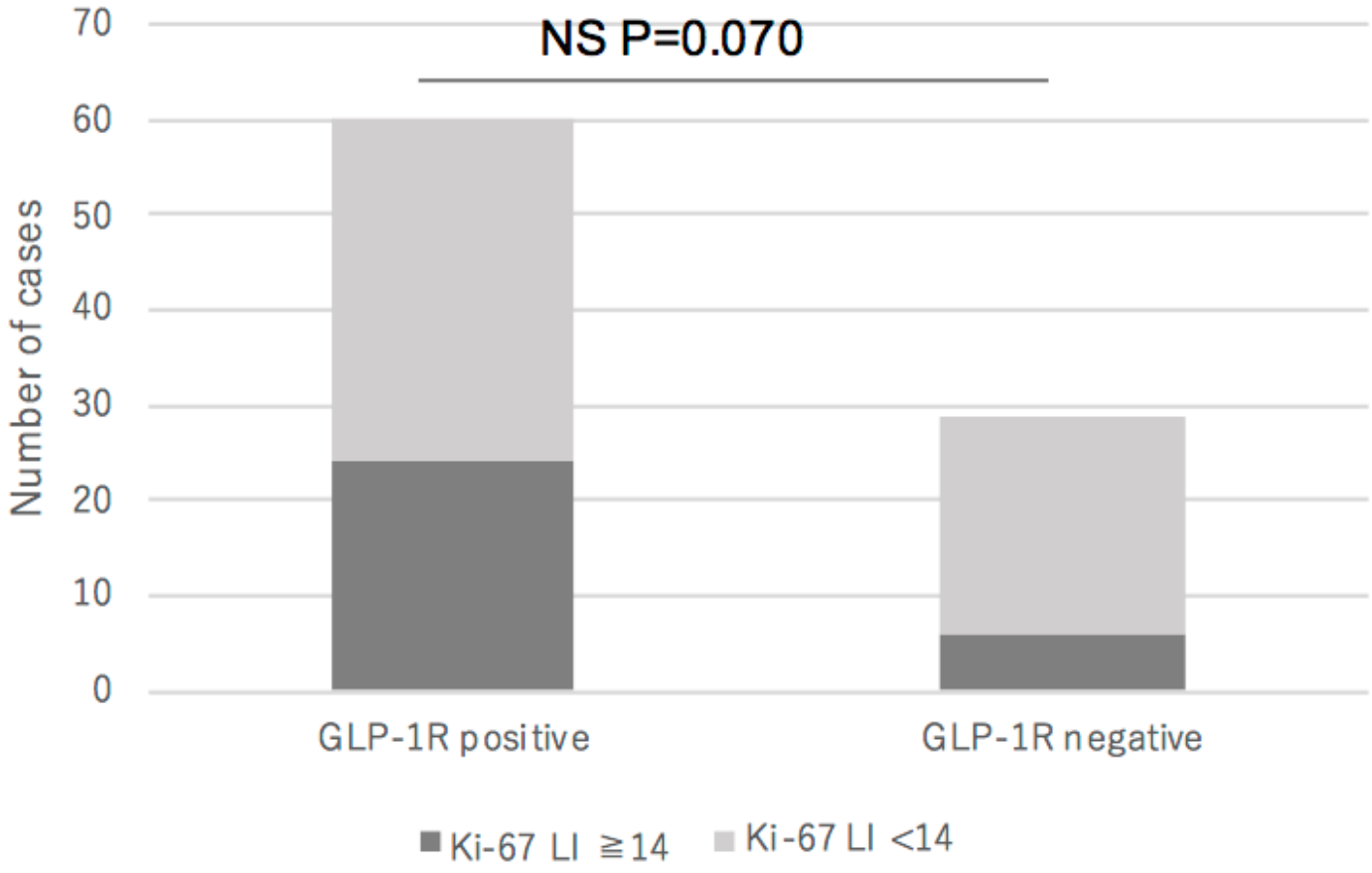

Figure 6

High and low Ki-67 LI cases among ER positive cancer with DM with comparison between DM and non-DM cases According to the 12th St Gallen International Breast Cancer Conference 2011, the recommended Ki-67 LI cut off point for classification of Luminal A and Luminal B was 14% [21].

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.tiff
- Table2.tiff
- Table3.tiff