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DOI: 10.14670/HH-18-362
Article type: ORIGINAL ARTICLE
Accepted: 2021-07-23
Epub ahead of print: 2021-07-23

This article has been peer reviewed and published immediately upon acceptance. Articles in “Histology and Histopathology” are listed in Pubmed.
Pre-print author’s version
D-2-hydroxyglutarate dehydrogenase in breast carcinoma as a potent prognostic marker associated with proliferation

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Keywords: Breast cancer, D-2-hydroxyglutarate dehydrogenase (D2HGDH), Immunohistochemistry, Proliferation, Prognosis

Short running title: D2HGDH in breast cancer
Abstract

**Background:** D-2-hydroxyglutarate dehydrogenase (D2HGDH) catalyzes D-2-hydroxyglutarate to α-ketoglutarate and is involved in the regulation of cellular energy and biosynthetic intermediates. Previously, D2HGDH was reported to decrease 2-hydroxyglutarate level in breast carcinoma cells, but no other report has examined D2HGDH in breast carcinoma, and its significance remains unknown.

**Methods:** We first immunolocalized D2HGDH in 224 invasive breast carcinomas and evaluated its clinicopathological significance. We next examined associations between gene expression of D2HGDH and α-ketoglutarate-dependent dioxygenases in 23 breast carcinoma tissues using the gene expression profile data. Finally, we examined the effects of D2HGDH on the proliferation in three breast carcinoma cells.

**Results:** D2HGDH immunoreactivity was detected in 49% of invasive breast carcinomas, and the immunohistochemical D2HGDH status was positively associated with histological grade, HER2 and Ki-67, while it was inversely associated with estrogen receptor. Moreover, it was significantly associated with worse prognosis of the breast cancer patients, and it turned out to be an independent prognostic factor for both the disease-free and breast cancer-specific survival in these patients. Gene expression profile data revealed that D2HGDH expression was positively associated with the expression of 6 α-ketoglutarate-dependent dioxygenases (KDM3A, PLOD1, EGLN2, ALKBH1,
Consequent in vitro experiments demonstrated that D2HGDH overexpression significantly increased the cell proliferation activity of MCF-7, T47D and MDA-MB-231 cells.

**Conclusion:** These results suggest that D2HGDH plays an important role in the growth of breast carcinoma, possibly through regulating functions of α-ketoglutarate-dependent dioxygenases, and that D2HGDH status is a potent worse prognostic factor in breast cancer patients.
Abbreviations:

α-KG; alpha-ketoglutarate, Cox; proportional hazard model, DCIS; ductal carcinoma in situ of the breast, D2HG; D-2-hydroxyglutarate, D2HGDH; D-2-hydroxyglutarate dehydrogenase, ER; estrogen receptor, FISH; fluorescence in situ hybridization, KDM3A; lysine demethylase 3A, LI; labeling index, PLSD; protected least significant difference, PR; progesterone receptor, pT; pathological T factor, TCA; tricarboxylic acid, WST; 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt.
Introduction

Breast cancer is one of the most common malignancies in women. Although the progress has been made in early detection and better treatment, some breast cancer patients suffer unfavorable clinical outcomes such as recurrence or metastasis. For example, in estrogen receptor (ER)-positive breast cancer patients, recurrence rate was approximately 10% after 5-year endocrine therapy (Djalalov et al., 2015). About 25% of breast cancer patients who received adjuvant chemotherapy developed recurrence (Tevaarwerk et al., 2013). Breast cancer patients with distant metastasis also result in poor prognosis (Davidson et al., 2013). Therefore, it is very important to understand the biological mechanisms of breast cancer to improve the clinical outcome of the patients.

Production of cellular energy and biosynthetic intermediates is essential in the proliferation of the cells. D-2-hydroxyglutarate dehydrogenase (D2HGDH) catalyzes D-2-hydroxyglutarate (D2HG) to $\alpha$-ketoglutarate ($\alpha$-KG). D2HG level is accumulated in breast carcinoma tissues through several pathways, including glutaminolysis (Terunuma et al., 2014) and ADHFE1 (Mishra et al., 2018). Considering that D2HGDH regulates $\alpha$-KG levels and functions of $\alpha$-KG-dependent dioxygenases in the malignant lymphoma (Lin et al., 2015), it is possible to speculate that D2HGDH plays an important role in the development of breast carcinoma.
Previously, Matsunaga et al., (2012) showed that D2HGDH knockdown increased the level of 2HG in MCF-7 breast carcinoma cells. However, no other report has examined D2HGDH in breast carcinoma to the best of our knowledge, and the significance of D2HGDH remains unknown in breast carcinoma. Moreover, immunohistochemistry for D2HGDH has not been reported in any human malignancies except for the glioblastoma (Krell et al., 2011). Therefore, in this study, we performed immunohistochemistry for D2HGDH in 224 invasive breast carcinoma tissues and analyzed the clinicopathological significance. Subsequently, \textit{in vitro} studies were examined using breast carcinoma cells to explore the biological function of D2HGDH in breast carcinoma.
Materials and Methods

Patients and tissues

224 specimens of invasive ductal carcinoma of the breast were obtained from Japanese female patients who underwent surgical treatment from 2005 to 2008 at Tohoku University Hospital (Sendai, Japan). All the specimens had been fixed in 10% formalin and embedded in paraffin wax. The 39 patients received neoadjuvant chemotherapy before the surgery, while 105 and 182 patients received adjuvant chemotherapy and endocrine therapy after the surgery, respectively. The median follow-up time was 69 months (range; 2-136 months) in this study.

Research protocols for this study were approved by the Ethics Committee at the Tohoku university school of medicine (approval number 2017-1-149).

Immunohistochemistry

We purchased rabbit polyclonal antibody for D2HGDH (AG4857) from Proteintech (Rosemont, IL, USA) and mouse monoclonal antibody for Ki-67 (MIB1) from DAKO (Carpinteria, CA, USA). We used a Histofine Kit (Nichirei Biosciences, Tokyo, Japan), employing the streptavidin-biotin amplification method, for the immunohistochemistry. The antigen-antibody complex was visualized with 3,3’-diaminobenzidine (DAB) solution with hematoxylin.

Immunohistochemistry for estrogen receptor (ER: CONFIRM anti-ER (SP1)) and
progesterone receptor (PR: CONFIRM anti-PR (1E2); Roche Diagnostics Japan, Tokyo, Japan) was performed with Ventana Benchmark XT (Roche Diagnostics Japan), and that for HER2 was performed by HercepTest (DAKO).

Scoring of immunohistochemistry

D2HGDH was immunolocalized in the cytoplasm of carcinoma cells, and the cases that had more than 10% positive carcinoma cells were considered positive. D2HGDH immunoreactivity was further semi-quantitatively evaluated by modified labeling index (LI) system in this study. Briefly, the percentage of immunoreactivity (LI) was categorized as 0 (no expression), 10 (up to 10%), 20 (11–20%) until 100 (91–100%). ER, PR and Ki-67 were immunolocalized in the nucleus, and their LI was determined in counting more than 1,000 carcinoma cells for each case. Cases with ER or PR LI of more than 1% were considered ER-positive or PR-positive breast carcinoma according to a previous report (Hammond et al., 2010). HER2 immunostaining was scored according to the standardized HercepTest scoring system (score 0-3) (DAKO), and the score 3 was considered positive. HER2 gene amplification was also investigated by fluorescence in situ hybridization (FISH) in the score 2 cases, and the cases showed positive for FISH were also considered positive for HER2 status. Ki-67 LI was dichotomized at 20% in the uni- and multi-variate analyses according to a previous study (Querzoli et al., 2021), although consensus is currently lacking regarding an optimal cut-off for Ki-67 LI in the clinical and research settings.
Laser capture microdissection/microarray analysis

The gene expression profile data of 23 breast carcinomas were used in the present study, which had been assembled in our previous studies (Nagasaki et al., 2007; Takagi et al., 2013) and published to Gene Expression Omnibus (GEO: GSE11965). We focused on gene expression of D2HGDH and 61 α-KG-dependent dioxygenases genes listed in the previous report (Chang et al., 2019). The expression level of α-KG-dependent dioxygenase in each case was divided into two groups (i.e. high or low expression case) according to the median value.

Cell line

The human breast cancer cell lines MCF-7 and T47D were obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and MDA-MB-231 was obtained from American Type Cell Culture (Manassas, VA, USA). These cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Biosera, Nuaille, France).

Construction of D2HGDH-overexpressing plasmid

We designed a primer containing a BamH I recognition site on the C-terminal side and a Hind III recognition site on the N-terminal side and amplified the translated region of the human
D2HGDH gene splicing variant 1 (1,566p) by PCR using KOD FX Neo (TOYOBO, Osaka, Japan). After being enzymatically treated with BamH I (Takara Bio, Shiga, Japan) and Hind III (Takara Bio), the PCR product was incorporated into pcDNA3.1 (-) vector (Invitrogen, CA, USA) (pD2HGDH) by ligation reaction using DNA Ligation Kit <Mighty Mix> (Takara Bio).

**Plasmid transfection**

The transfection of empty or pD2HGDH vector was performed at a dose of 0.2 µg per well in 96-well plates using Avalanche®-Everyday Transfection Reagent (APRO Science, Tokushima, Japan).

**Immunoblotting**

SDS-PAGE was performed as described previously (Takagi et al., 2018). Separated protein was subsequently transferred onto Hybond PVDF membranes (GE Healthcare, Buckinghamshire, UK) and exposed with primary antibodies for D2HGDH (Proteintech) as well as β-actin (Sigma-Aldrich), a loading control. Immune complex was then detected using ECL-prime Western blotting detection reagents (GE Healthcare) and visualized by LAS-4000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).
**Cell proliferation assay**

Breast cancer cells were transfected with empty or pD2HGDH vector in 96-well plates. The cell proliferation status was measured by the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) method using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) 0-4 days after the transfection.

**Wound healing assay**

Culture inserts (Platypus Technologies, WI, USA) were placed in 96-well plates. and breast cancer cells were cultured. After removing inserts the next day, empty or pD2HGDH vectors were transfected into the cells. Photographing the gap in each well while 0-3 days after the transfection, we quantified the gap area using ImageJ and evaluated migration ability.

**Statistical analysis**

Associations between immunohistochemical status of D2HGDH and clinicopathological factors were evaluated using Student's t test or a cross-table using the $\chi^2$-test, while associations between D2HGDH LI and clinicopathological factors were evaluated by Mann-Whitney U test or Spearman’s rank correlation coefficient. Disease-free and breast cancer-specific survival curves were generated according to the Kaplan-Meier method, and statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated using a proportional
hazard model (Cox). Microarray data were statistically evaluated by Wilcoxon signed-rank test.

In in vitro experiments, data were presented as mean ± SD (n = 4), and the results were confirmed by other independent experiments. The statistical analyses were carried out using Student’s t-test and Fisher’s protected least significant difference (PLSD) test.

$P$-value < 0.05 was considered significant. The statistical analyses were performed using the StatView 5.0J software (SAS Institute, Cary, NC, USA) in this study.
Results

**D2HGDH immunolocalization in human breast carcinoma**

Immunoreactivity of D2HGDH was detected in the cytoplasm of breast carcinoma cells (Figures 1A and 1B), while it was negative in the non-neoplastic mammary glands or stroma (Figure 1C). Associations between immunohistochemical D2HGDH status and various clinicopathological parameters in the breast carcinoma are summarized in Table 1. The number of D2HGDH-positive cases was 109 out of 224 (49%), and the median value of D2HGDH LI was 10% (range 0–100%). The immunohistochemical D2HGDH status was positively associated with histological grade ($P = 0.039$), HER2 status ($P = 0.016$) and Ki-67 LI ($P = 0.029$), while it was negatively correlated with ER status ($P = 0.0085$). Similarly, D2HGDH LI was positively associated with Ki-67 LI ($P = 0.0007$) and negatively correlated with ER status ($P = 0.018$) in this study (Table 1).

Similar tendencies were detected when cases received neoadjuvant treatment ($n = 39$) were excluded. D2HGDH status was positively associated with pT ($P = 0.032$), HER2 ($P = 0.031$) and Ki67-LI ($P = 0.0055$), while it was negatively correlated with ER status ($P = 0.015$) and PR status ($P = 0.016$) in the cases without neoadjuvant therapy ($n = 185$).
Association between D2HGDH and clinical outcome of breast cancer patients

D2HGDH status was significantly associated with the shorter disease-free survival \((P = 0.0011\) by log-rank test; Figure 2A) and worse breast cancer-specific survival \((P = 0.0022;\) Figure 2B). Similar tendencies were detected in ER-positive cases for both disease-free survival \((P = 0.0054;\) Figure 2C) and breast cancer-specific survival \((P = 0.0020;\) Figure 2D), but not in ER-negative cases \((P = 0.24\) and \(P = 0.54\), respectively).

Results of univariate analysis of disease-free survival using Cox (Table 2), pathological T factor \((pT)\), Ki-67 status, D2HGDH status and lymph node metastasis were demonstrated to be significant prognostic factors. Multivariate analysis revealed that \(pT\) \((P = 0.0035)\), Ki-67 status \((P = 0.013)\) and D2HGDH status \((P = 0.016)\) were independently worse prognostic factors for disease-free survival. On the other hand, as shown in Table 3, univariate analysis for breast cancer-specific survival revealed Ki-67 status, D2HGDH status, \(pT\), lymph node status and histological grade as significant prognostic variables. Subsequent multivariate analysis demonstrated that \(pT\) \((P = 0.034)\) and D2HGDH \((P = 0.035)\) were independent worse prognostic markers.

When D2HGDH LI and Ki-67 LI were used as continuous values in the Cox analyses, D2HGDH LI was similarly demonstrated as an independent worse prognostic factor for both disease-free survival \((P = 0.0026\) in univariate analysis and \(P = 0.0063\) in multivariate analysis) and breast cancer-specific survival \((P = 0.010\) in univariate analysis and \(P = 0.046\) in multivariate analysis).
Associations between gene expression levels of D2HGDH and α-KG-dependent dioxygenases in breast carcinoma tissues

We also examined associations between gene expression of D2HGDH and α-KG-dependent dioxygenases in 23 breast carcinoma tissues using the gene expression profile data which were assembled in our previous studies (Nagasaki et al., 2007; Takagi et al., 2013) and published to Gene Expression Omnibus (GEO: GSE11965). As shown in Table 4, among the 61 α-KG-dependent dioxygenases (Wai et al., 2019), 6 genes (KDM3A, PLOD1, EGLN2, ALKBH1, ASPH and ALKBH7) were positively associated, while 4 genes (JMJD1C, EGLN3, PLOD2 and HR) were negatively associated, with D2HGDH expression in the breast carcinoma. To confirm these results, when we performed immunohistochemistry for KDM3A (lysine demethylase 3A) in the immunohistochemical cohort set, KDM3A immunoreactivity was significantly ($P < 0.0001$) associated with D2HGDH immunoreactivity (Figure 3).

Effects of D2HGDH expression on cell proliferation in breast carcinoma cells

Our present study revealed that D2HGDH immunoreactivity was significantly associated with Ki-67 LI in the breast carcinoma tissues. To examine the biological functions of D2HGDH in human breast carcinoma cells, we transfected D2HGDH plasmid into MCF-7, T47D and MDA-MB-231 cells. As shown in Figure 4A, D2HGDH protein level levels were markedly increased in
these cells transfected with D2HGDH plasmid compared to those transfected with control plasmid.

As shown in Figure 4B, proliferation activity was significantly increased in MCF-7 cells transfected with D2HGDH plasmid from 1 to 4 days after the transfection compared to the control cells transfected with control plasmid. A similar tendency was also detected in T47D (Figure 4C) and MDA-MB-231 (Figure 4D) cells under the same conditions.

When we performed a wound healing assay in these cells, no significant association was detected between MCF-7 (Figure 5A), T47D (Figure 5B) and MDA-MB-231 (Figure 5C) cells transfected with D2HGDH plasmid compared to their controls.
Discussion

This is the first study to demonstrate the clinical significance of D2HGDH in breast carcinoma tissues. In this study, D2HGDH immunoreactivity was detected in 49% of breast carcinomas, but it was negative in the normal mammary gland. Moreover, D2HGDH status was significantly associated with worse prognosis of the patients, and it turned out to be an independent prognostic factor for both disease-free and breast cancer-specific survival. Very recently, Wang et al. (2020) showed that six-gene signature including D2HGDH is an independent prognostic factor in head and neck squamous cell carcinoma patients, and Liu et al. (2020) reported that expression level of D2HGDH mRNA was significantly associated with better prognosis of ovarian cancer patients. However, association between D2HGDH and prognosis of human carcinoma is largely unknown, and especially no information has been available at the protein level. Considering that progression of the carcinoma needs more cellular energy and various metabolites, it is suggested that D2HGDH is aberrantly expressed in the breast carcinoma tissues and plays an important role in the aggressive growth.

In our present study, D2HGDH immunoreactivity was significantly associated with Ki-67 LI and histological grade in breast carcinoma. Ki-67 LI well reflects the proliferative activity of breast carcinoma (de Azambuja et al., 2007), while histological grade is evaluated by the mitotic rate, nuclear atypia and tubule formation of breast carcinoma. Moreover, the results of in vitro
experiments demonstrated that overexpression of D2HGDH was significantly associated with the proliferation activity of breast carcinoma cells, but not with the migration property.

It is generally considered that proliferating carcinoma cells preferentially use anaerobic glycolysis for energy production (i.e., Warburg effect), and indeed, Sato-Tadano et al. (2013) reported that immunoreactivity of hexokinase II, which plays a rate-limiting step of glycolysis, was the potent worse prognostic factor. However, emerging evidence demonstrates that certain cancer cells rely heavily on the tricarboxylic acid (TCA) cycle for energy production and macromolecule synthesis. Aberrant TCA cycle function is implicated in tumorigenesis (Anderson et al., 2018). Considering that D2HGDH increased α-KG by catalyzing D2HG (Achouri et al., 2004), it is suggested that D2HGDH expression causes aberrant TCA function and contributes to the most suitable metabolic environment to the growth of breast carcinoma cells.

Since D2HGDH converts D2HG to α-KG, it increases α-KG level in the breast carcinoma cells. α-KG is known as a key intermediate in the TCA cycle (Chen et al., 2020), and recent studies have demonstrated that α-KG induced lung metastasis of the breast carcinoma (Elia et al., 2019). α-KG-dependent dioxygenases consume α-KG as obligate cosubstrate (Baksh and Finley 2021), and more than 60 α-KG-dependent dioxygenases are known (Chang et al., 2019). Recently, Chang et al. (2019) showed some α-KG-dependent dioxygenases were associated with good or poor prognosis of carcinoma patients, but breast carcinoma has not been studied in this study.

In our present study, D2HGDH expression was significantly associated with expression of
a part of $\alpha$-KG-dependent dioxygenases in the breast carcinomas, and especially, D2HGDH was the most positively associated with lysin demethylase KDM3A (Table 4). Interestingly, KDM3 was included in the signature associated with poor prognosis in 9 cancer types by the report of Chang et al. (2019), and previous reports demonstrated that KDM3A promoted growth, invasion, chemoresistance and endocrine therapy-resistance in breast carcinoma (Wade et al., 2015; Ramadoss et al., 2017). Therefore, D2HGDH may be associated with aggressive phenotype of breast carcinoma through activation of certain $\alpha$-KG-dependent dioxygenases such as KDM3A. The significance of $\alpha$-KG-dependent dioxygenases remains largely unclear in breast carcinoma, and further examinations are required to clarify the biological functions of D2HGDH in breast carcinoma.

In summary, D2HGDH immunoreactivity was detected in 49% of invasive breast carcinomas, while it was negative in the normal breast tissues. Immunohistochemical D2HGDH status was significantly associated with histological grade, HER2 status and Ki-67 LI. The D2HGDH status was significantly associated with worse prognosis of the breast carcinoma patients and it turned out to be an independent prognostic factor. Subsequent in vitro experiments demonstrated that D2HGDH overexpression significantly increased the cell proliferation activity of MCF-7, T47D and MDA-MB-231 cells. These results suggest that D2HGDH plays an important role in the growth of breast carcinoma and immunohistochemical D2HGDH status is a potent prognostic factor in breast cancer patients.
Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgement

This work was partly supported by JSPS KAKENHI Grant Number 19K09065 and 19K07410.

Author contribution

Study design: KT, YM, TS. Management of pathological samples and their clinicopathological information; NH-S, MM, HS. Acquisition of data: CH, AS, MY, HM. Data analysis and interpretation; KT, TS. Manuscript preparation: CH. Manuscript review and editing: TS. Final approval of the version submitted: CH, KT, AS, MY, HM, YM, NH-S, MM, HS, TS.
References


IDH1 and IDH2 have critical roles in 2-hydroxyglutarate production in D-2-hydroxyglutarate dehydrogenase depleted cells. Biochem. Biophys. Res. Commun. 423, 553-556.


Figure legends

Figure 1. Immunohistochemistry for D2HGDH in invasive breast carcinoma. A: D2HGDH was immunolocalized in the cytoplasm of breast carcinoma cells. B: D2HGDH-negative case. C: D2HGDH immunoreactivity was negative in the normal breast tissue. Bar = 50 μm, respectively.

Figure 2. Disease-free (A, C) and breast cancer-specific survival (B, D) of breast cancer patients according to D2HGDH status. A, B: D2HGDH status in whole cases (n = 224), C, D: ER-positive cases (n = 184). The solid line shows D2HGDH-positive group, and the dashed line shows D2HGDH-negative group. P-values < 0.05 were considered significant and are shown in bold.

Figure 3. Immunohistochemistry for KDM3A in invasive breast carcinoma. Mouse monoclonal antibody for KDM3A (1E12) was purchased from GeneTex (Hsinchu, Taiwan, R.O.C.), and the antigen-antibody complex was visualized with DAB solution with hematoxylin. A: KDM3A was immunolocalized in the breast carcinoma cells. B: KDM3A-negative case. C: KDM3A immunoreactivity was negative in the normal breast tissue. Bar = 50 μm, respectively. D: A significant association between immunohistochemical KDM3A and D2HGDH status in 224 breast carcinomas. KDM3A was positive in 115 out of 224 (51%) cases in this cohort set.
**Figure 4.** Effects of D2HGDH on cell proliferation and migration properties in breast carcinoma cells. **A:** D2HGDHE immunoreactivity in MCF-7, T47D and MDA-MB-231 cells transfected with D2HGDH plasmid (pD2HGDH) or control plasmid (pcDNA3.1; VC) by immunoblotting. b-actin immunoreactivity is shown as an internal control of mitochondrial protein. **B-D:** Proliferation activity of MCF-7 (B), T47D (C) and MDA-MB-231 (D) cells transfected with D2HGDH plasmid. The cells were transfected with D2HGDH plasmid (pD2HGDH; black circle and solid line) or control plasmid (VC; open triangle and dashed line). Relative cell viability was calculated as the ratio of that in the nontreatment cells (day 0) using the WST-8 assay. Data were presented as mean ± SD (n = 4). *; $P < 0.05$, **; $P < 0.01$ and ***; $P < 0.001$ compared to the cells transfected with control plasmid at the same time after treatment.

**Figure 5.** Effects of D2HGDH on migration properties in breast carcinoma cells. Wound healing assays in MCF-7 (A), T47D (B) and MDA-MB-231 (C) cells. The cells were transfected with D2HGDH plasmid (pD2HGDH; black circle and solid line) or control plasmid (VC; open triangle and dashed line). The relative migration area (%) was evaluated as a ratio compared to that at 0 hour after removal of culture insert (day 0). Data were presented as mean ± SD (n = 4).
Table 1. Association between immunohistochemical D2HGDH status and clinicopathological factors in 224 breast carcinomas.

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<th>- (n = 115)</th>
<th>P value</th>
<th>D2HGDH LI</th>
<th>P value</th>
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<td>12.9±1.2</td>
<td>0.029*</td>
<td>0.0007 (ρ = 0.24)</td>
<td></td>
</tr>
</tbody>
</table>

†; Data are presented as mean±SEM. All other values represent the number of cases.
*; P-value < 0.05 was considered significant.
pT; Pathological T factor.
Associations between immunohistochemical status of D2HGDH and clinicopathological factors
were evaluated using Student's t test (age and Ki-67 LI) or a cross-table using the $\chi^2$-test (other values), while associations between D2HGDH LI and clinicopathological factors were evaluated by Spearman’s rank correlation coefficient (age and Ki-67 LI) or Mann-Whitney U test (other values).
### Table 2. Univariate and multivariate analyses of disease-free survival in 224 breast cancer patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
<th>Relative risk (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT (pT2-4 / pT1)</td>
<td>(&lt;0.0001^\dagger)</td>
<td>(0.0035^*)</td>
<td>3.01 (1.44-6.32)</td>
</tr>
<tr>
<td>Ki-67 status (≥ 20% / &lt;20%)</td>
<td>(0.0004^\dagger)</td>
<td>(0.013^*)</td>
<td>2.30 (1.19-4.45)</td>
</tr>
<tr>
<td>D2HGDH status (positive / negative)</td>
<td>(0.0020^\dagger)</td>
<td>(0.016^*)</td>
<td>2.43 (1.18-5.00)</td>
</tr>
<tr>
<td>Lymph node metastasis (positive / negative)</td>
<td>(0.018^\dagger)</td>
<td>0.99</td>
<td>1.00 (0.48-2.08)</td>
</tr>
<tr>
<td>Histological grade (3 / 1, 2)</td>
<td>0.060</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER status (negative / positive)</td>
<td>0.077</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2 status (positive / negative)</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\^\dagger; P-value < 0.05 was considered significant in the univariate analysis, and the factor was subsequently examined in the multivariate analysis.

\^\*; The factor showed P-value < 0.05 was considered as an independent factor in the multivariate analysis.

95% CI; 95% confidence interval.
Table 3. Univariate and multivariate analyses of breast cancer-specific survival in 224 breast cancer patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P value</td>
<td>P value</td>
</tr>
<tr>
<td>Ki-67 status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(≥ 20% / &lt;20%)</td>
<td>0.0073†</td>
<td>0.082</td>
</tr>
<tr>
<td>D2HGDH status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(positive / negative)</td>
<td>0.0086†</td>
<td>0.035*</td>
</tr>
<tr>
<td>pT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pT2-4 / pT1)</td>
<td>0.0019†</td>
<td>0.034*</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(positive / negative)</td>
<td>0.015†</td>
<td>0.53</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3 / 1,2)</td>
<td>0.041†</td>
<td>0.56</td>
</tr>
<tr>
<td>ER status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(negative / positive)</td>
<td>0.099</td>
<td></td>
</tr>
<tr>
<td>HER2 status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(positive / negative)</td>
<td>0.84</td>
<td></td>
</tr>
</tbody>
</table>

†; P-value < 0.05 was considered significant in the univariate analysis, and the factor was subsequently examined in the multivariate analysis.

95% CI; 95% confidence interval.
**Table 4.** Genes of $\alpha$-KG-dependent dioxygenases significantly associated with D2HG expression in 23 breast carcinoma tissues.

<table>
<thead>
<tr>
<th></th>
<th>Positive association</th>
<th></th>
<th>Inverse association</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene symbol</td>
<td>$P$ value</td>
<td></td>
<td>Gene symbol</td>
<td>$P$ value</td>
</tr>
<tr>
<td>KDM3A</td>
<td>0.016</td>
<td></td>
<td>JMJD1C</td>
<td>0.0050</td>
</tr>
<tr>
<td>PLOD1</td>
<td>0.016</td>
<td></td>
<td>EGLN3</td>
<td>0.0090</td>
</tr>
<tr>
<td>EGLN2</td>
<td>0.020</td>
<td></td>
<td>PLOD2</td>
<td>0.025</td>
</tr>
<tr>
<td>ALKBH1</td>
<td>0.029</td>
<td></td>
<td>HR</td>
<td>0.045</td>
</tr>
<tr>
<td>ASPH</td>
<td>0.036</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALKBH7</td>
<td>0.038</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

61 genes were examined as $\alpha$-KG-dependent dioxygenases based on the report by Wai et al. (2019).
A. Disease-free survival

D2HGDH: - (n = 115)

D2HGDH: + (n = 109)

\[ P = 0.0011 \]

B. Breast cancer-specific survival

D2HGDH: - (n = 115)

D2HGDH: + (n = 109)

\[ P = 0.0020 \]

C. ER+ Disease-free survival

D2HGDH: - (n = 102)

D2HGDH: + (n = 82)

\[ P = 0.0054 \]

D. ER+ Breast cancer-specific survival

D2HGDH: - (n = 102)

D2HGDH: + (n = 82)

\[ P = 0.0020 \]
A  MCF-7

B  T47D

C  MDA-MB-231

Days after transfection

Days after transfection

Days after transfection