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Adenylate cyclase 9 expression level is associated with hormone receptor-positive breast cancer and predicts patient prognosis

Kayoko Sugino[1], Masahiro Shibata[1,2], Yayoi Adachi[1], Ikumi Soeda[1], Takahiro Ichikawa[1], Takahiro Inaishi[1], Emi Kanaya[3], Mitsuro Kanda[4], Masamichi Hayashi[4] and Norikazu Masuda[1]

[1]*Department of Breast and Endocrine Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[2]*Department of Surgery, Nagoya Ekisaikai Hospital, Nagoya, Japan*
[3]*Department of Surgery and Science, Faculty of Medicine, Academic Assembly, University of Toyama, Toyama, Japan*
[4]*Department of Gastroenterological Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan*

ABSTRACT

Adenylate cyclase family members have recently received attention as novel therapeutic targets. However, the significance of adenylate cyclase 9 (ADCY9) in breast cancer has not been elucidated. Here, we evaluated *ADCY9* expression in breast cancer (BC) cell lines, and polymerase chain reaction array analysis was performed to determine the correlations between *ADCY9* expression levels and 84 tumor-associated genes. The association of *ADCY9* messenger RNA (mRNA) expression levels in clinical breast cancer specimens with patients' clinicopathological factors and prognosis was evaluated. The database of cancer cell line showed that estrogen receptor-positive and progesterone receptor-positive cells expressed higher *ADCY9* mRNA levels. *ADCY9* expression showed positive correlations with several oncogenes, such as *TGFB1, CDKN1A,* and *BAX* in the polymerase chain reaction array analysis. We defined the ratio of *ADCY9* mRNA expression levels in breast cancer and adjacent noncancerous tissues as the "C/N ratio". Among 149 patients with BC, estrogen receptor-positive and progesterone receptor-positive patients exhibited higher C/N ratios than estrogen receptor-negative and progesterone receptor-negative patients, respectively. Patients in the lowest C/N ratio quartile experienced shorter prognosis periods. The C/N ratio of *ADCY9* was found as an independent prognostic factor for disease-free survival. Thus, *ADCY9* expression is high in hormone receptor-positive breast cancer, and its low expression indicates a poor prognosis in patients with breast cancer.

Keywords: breast cancer, ADCY9, estrogen receptor, prognostic marker

Abbreviations:
ADCY: adenylate cyclase
BC: breast cancer
C/N ratio: ratio of mRNA expression levels between cancerous and noncancerous tissues
DFS: disease-free survival
ER: estrogen receptor

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Corresponding Author: Masahiro Shibata, MD, PhD
Department of Breast and Endocrine Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
Tel: +81-52-744-2251, Fax: +81-52-744-2252, E-mail: shibata.masahiro.f1@f.mail.nagoya-u.ac.jp

HER2: human epidermal growth factor receptor 2
mRNA: messenger RNA
PCR: polymerase chain reaction
PgR: progesterone receptor

INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed cancer among women.[1] Medication therapies, including chemotherapy, endocrine therapy, and molecular targeting drugs, have recently been selected based on the expression status of the estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) in primary tumors. Although the prognosis of patients with BC has improved owing to the development of novel therapeutic drugs, metastatic BC remains difficult to cure.[2] An understanding of the oncological functions of unknown molecules and the development of prognostic biomarkers is essential to overcome intractable BC.

The adenylate cyclase (ADCY) superfamily is a group of glycoproteins that regulate intracellular signaling and catalyze the transformation of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP).[3] Recently, overactivation of ADCY has been implicated in modulating cancer progression and resistance to chemotherapy and immunotherapy.[3] Among the members of the ADCY superfamily, the oncological roles of ADCY9 have been poorly studied. Although previous studies have reported the roles of ADCY9 in colon and lung cancer,[4-6] it has been assigned contradictory functions depending on the carcinoma. Only few studies have evaluated the relationship of *ADCY9* messenger RNA (mRNA) expression with patient clinicopathological factors and the prognosis in BC.

In this study, we focused on *ADCY9* and investigated its expression in BC cell lines and patient tumor samples to elucidate its significance and determine whether it could be used as a prognostic marker in BC.

MATERIALS AND METHODS

Sample collection

In this study, a total of 13 BC cell lines (BT-20, BT-474, BT-549, HCC1419, HCC1954, Hs578T, MCF7, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-468, SK-BR-3, and ZR-75-1) and two noncancerous breast epithelial cell lines (MCF-10A and MCF-12A) were used. We purchased BT-549, HCC1419, HCC1954, and Hs578T cell lines from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and BT-474, MCF-7, and MCF-12A were provided from Johns Hopkins University (Baltimore, MD, USA) following the appropriate material transfer agreement. Other cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37 °C.[7] The expression statuses of ER, PgR, and HER2 in each cell line were referred from previous studies that evaluated these expression.[8,9]

Clinical specimens were obtained from patients who had operations at Nagoya University Hospital between December 2002 and November 2009 and had available postoperative surveillance data. They were histologically diagnosed as BC and their cancerous specimens were

resected with a diameter of approximately 1.5 mm and frozen to be stored in −80 °C freezer. The pathological stage of each patient was classified according to the Union for International Cancer Control staging system for BC (8th edition). The postoperative medication therapy was determined at the physician's discretion based on each patient's general condition, pathological features, and BC subtypes.

This study complied with the Declaration of Helsinki and was approved by the institutional review board (approval number: 2019-0028). All participants provided written informed consent for the use of their clinical samples and data, which have been stored in a locked locker in our office. The collected human specimens were anonymized so that it could be linked and have been stored in a −80 °C freezer in our office. Patient data was also anonymized and the consolidated forms have been stored in a password-locked hard disk and kept locked in our office.

Quantitative real-time reverse transcription polymerase chain reaction

ADCY9 mRNA expression levels in cell lines and clinical samples were evaluated using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). RNA was extracted from the cell lines (8.0×10^6 cells per cell line) and 149 BC specimens. Complementary DNA (cDNA) was synthesized by the way previously described.[10] To normalize *ADCY9* mRNA expression levels, *GAPDH* mRNA levels were used as a house-keeping gene. The primers specific for each gene were as follows: *ADCY9*, forward 5′-TGGTTGCTGACCAGTTGAAA-3′ and reverse 5′-ACCTCAAAGCCAGAAAGCAA-3′, which generated a 106-bp product; *GAPDH*, forward 5′-GAAGGTGAAGGTCG-GAGTC-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3′, which generated a 226-bp product. SYBR Green PCR core reagent kit (Applied Biosystems) was used with these cycling conditions: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 5 s and 60 °C for 60 s, using an ABI StepOnePlus real-time PCR System (Applied Bio-systems, Foster City, CA, USA).[11] Each sample was conducted in triplicate.

Cancer Cell Line Encyclopedia

The mRNA expression levels of *ADCY9* in 58 BC cell lines were obtained from the public database of Cancer Cell Line Encyclopedia (https://sites.broadinstitute.org/ccle/). We accessed it on January 12, 2022.

PCR array analysis

The correlation between the mRNA expression levels of *ADCY9* and cancer-related genes was investigated with the kit of RT^2 Profiler PCR Array Human for Oncogenes & Tumor Suppressor Genes (Qiagen, Hilden, Germany) that can evaluate the expression levels of 84 cancer-related genes. The assay was performed in accordance with the manufacturer's protocol. The relative expression level of each gene was obtained by being normalized with the *GAPDH* level.[11]

Kaplan–Meier survival analysis

The public database of Kaplan–Meier Plotter online software (http://kmplot.com/analysis/index.php?p=background) was employed to analyze relapse-free survival in large number of patients with BC in relation to the expression of *ADCY9* by classifying its expression levels by median.[12] It was accessed on August 13, 2023.

Statistical analyses

Differences in *ADCY9* mRNA levels between the two groups were compared with the Mann–Whitney U test. The correlation between the expression levels of the two genes were analyzed using the Spearman's rank correlation test. The χ^2 test was conducted to analyze the association

between *ADCY9* mRNA expression levels and clinicopathological factors. Prognosis, such as disease-free survival (DFS) and overall survival rates, was evaluated with the Kaplan–Meier method, and survival curves were compared using the log-rank test. To identify prognostic factors, multivariate regression analyses was performed using the Cox proportional-hazards model. These analyses were performed using JMP 12 software (SAS Institute, Inc, Cary, NC, USA). *P* values of < 0.05 were defined as statistically significant.

RESULTS

ADCY9 mRNA expression in BC cell lines

ADCY9 mRNA expression levels varied among the 13 BC cell lines and two non-cancerous mammary cell lines (Fig. 1A). No significant differences were observed in relation to positivity of ER ($P = 0.568$), PgR ($P = 0.758$), or HER2 ($P = 0.884$). To compensate for the small number of cell lines, the Cancer Cell Line Encyclopedia database was used to evaluate *ADCY9* mRNA expression levels in 58 cell lines. ER-positive (n = 17) and PgR-positive (n = 9) cells expressed higher *ADCY9* mRNA levels than ER-negative (n = 41) and PgR-negative (n = 49) cells, respectively (ER, $P = 0.011$; PgR, $P < 0.001$; Fig. 1B). No significant difference was observed between HER2-positive (n = 16) and HER2-negative (n = 31) cells ($P = 0.805$; Fig. 1B).

The correlations between *ADCY9* and the expression levels of 84 cancer-related genes in 13 BC cell lines were evaluated via PCR array analysis, and the expression levels of several oncogenes, including *TGFB1, CDKN1A, BAX*, and *BRCA2*, were positively correlated with that of *ADCY9* (Table 1 and Supplementary Table).

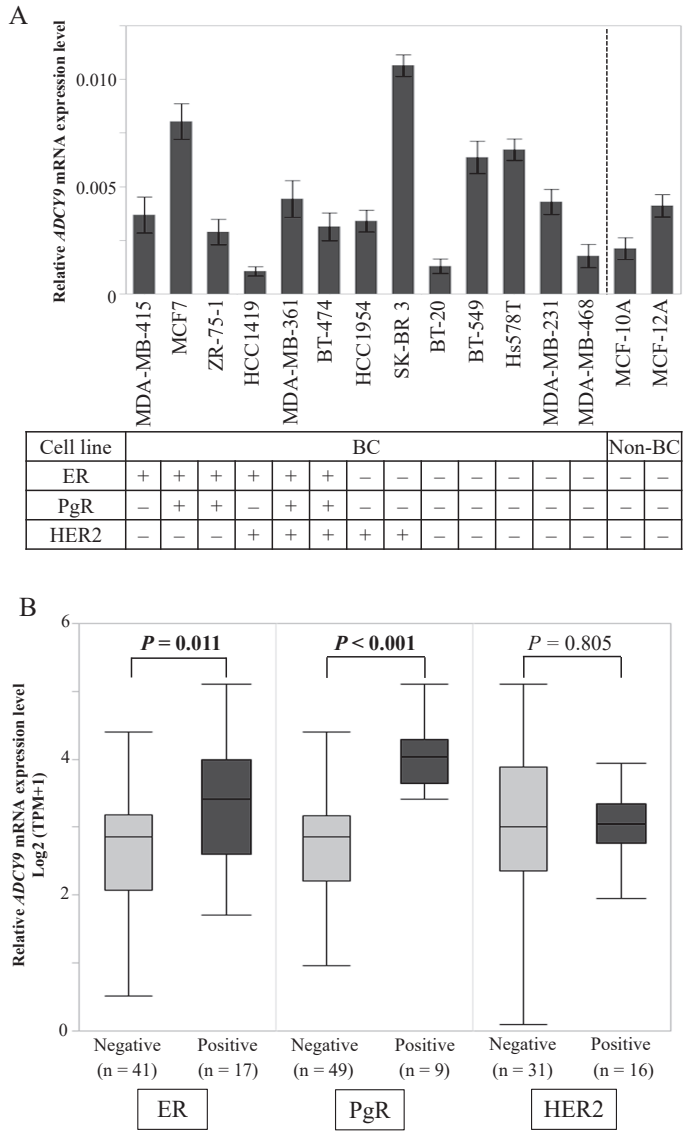

Fig. 1 *ADCY9* mRNA expression levels in BC cell lines

Fig. 1A: Analysis of *ADCY9* mRNA expression. Bar graphs indicate relative *ADCY9* mRNA levels, which were obtained from the *ADCY9* value divided by *GAPDH* value. *ADCY9* mRNA expression levels were heterogeneous among cell lines. The expression status of ER and HER2 was determined from previous studies.[8,9]

Fig. 1B: In the Cancer Cell Line Encyclopedia database, ER-positive and PgR-positive BC cell lines expressed higher *ADCY9* mRNA levels, in comparison with the ER-negative and PgR-negative BC cell lines, respectively. No significant differences were observed between HER2-positive and HER2-negative BC cells.

ADCY9: *adenylate cyclase 9*
BC: breast cancer
ER: estrogen receptor
HER2: human epidermal growth factor receptor 2
PgR: progesterone receptor
mRNA: messenger RNA

Table 1 Correlations between mRNA expression levels of *ADCY9* and cancer-related genes

Genes	Official full names	Correlation coefficients	*P*-values
TGFB1	transforming growth factor beta 1	0.709	0.007
CDKN1A	cyclin dependent kinase inhibitor 1A	0.687	0.010
BAX	BCL2 associated X, apoptosis regulator	0.659	0.014
SMAD4	SMAD family member 4	0.659	0.014
BRCA2	BRCA2 DNA repair associated	0.654	0.015
NRAS	NRAS proto-oncogene, GTPase	0.654	0.015
NFKBIA	NFKB inhibitor alpha	0.648	0.017
RAF1	Raf-1 proto-oncogene, serine/threonine kinase	0.599	0.031
MLH1	mutL homolog 1	0.566	0.044

ADCY9: *adenylate cyclase 9*

Patient characteristics

This study included 149 BC patients. All patients were women with a median age of 52 years (range, 26–78 years). The median duration of patient follow-up was 121.6 months (range, 10–191 months). The characteristics of the patients are summarized in Table 2. Patients who showed positivity for ER, PgR, or HER2 were grouped as "non-triple-negative." As four of the five patients whose HER2 statuses were unknown showed ER-positivity, these patients were categorized as "non-triple-negative"; thus, 15 patients were classified as triple-negative, 133 as non-triple-negative, and 1 as unknown.

Table 2 Clinicopathological characteristics of 149 patients with breast cancer

Clinicopathological parameter	
Age, median (range)	52 (26–78)
≤ 60 years	97 (65.1%)
> 60 years	52 (34.9%)
Histology	
DCIS	6 (4.0%)
IDC	133 (89.3%)
ILC	4 (2.7%)
Others	6 (4.0%)
UICC T factor [a]	
Tis	6 (4.0%)
T1	60 (40.3%)
T2	70 (47.0%)
T3	8 (5.4%)
T4	5 (3.3%)

Node status	
Negative	73 (49.0%)
Positive	76 (51.0%)
UICC pathological stage [a]	
0	6 (4.0%)
I	41 (27.5%)
II	70 (47.0%)
III	31 (20.8%)
IV	1 (0.7%)
ER status	
Positive	113 (75.8%)
Negative	36 (24.2%)
PgR status	
Positive	102 (68.5%)
Negative	47 (31.5%)
HER2 status	
Positive	36 (24.2%)
Negative	108 (72.5%)
Unknown	5 (3.3%)
Triple-negative	
Yes	15 (10.1%)
No	133 (89.3%)
Unknown	1 (0.6%)
Adjuvant therapy	
Endocrine therapy alone	46 (30.9%)
Chemotherapy alone	27 (18.1%)
Chemotherapy and endocrine therapy	60 (40.3%)
None	16 (10.7%)

DCIS: ductal carcinoma in situ
ER: estrogen receptor
HER2: human epidermal growth factor 2
IDC: invasive ductal carcinoma
ILC: invasive lobular carcinoma
PgR: progesterone receptor
UICC: Union for International Cancer Control
[a] Pathological stages were classified using the UICC staging system for breast cancer (8th edition).

Clinical and prognostic significance of ADCY9 mRNA levels

The *ADCY9* mRNA levels in the BC tissues 89 (59.7%) of the 149 patients were lower than those in the adjacent normal tissues. We calculated the ratio of *ADCY9* mRNA expression levels in the BC and adjacent noncancerous tissues and defined it as the "C/N ratio". The C/N ratio of *ADCY9* did not significantly differ between patients with Tis/T1 (n = 66), T2/T3/T4 (n = 83; $P = 0.055$), negative and positive lymph node metastases (n = 73 and 76, respectively; $P = 0.348$), Union for International Cancer Control (UICC) stage 0/I/II (n = 117), and stage III/IV (n = 32, $P = 0.513$; Fig. 2A) cancers.

In assessments of the relevance of conventional biomarkers (Fig. 2B), the *ADCY9* C/N ratio in ER-positive specimens (n = 113) was significantly higher than that in ER-negative specimens (n = 36; $P < 0.001$); the *ADCY9* C/N ratio in PgR-positive specimens' (n = 102) was also higher than that in PgR-negative specimens (n = 47; $P < 0.001$); and HER2-positive specimens (n = 36) expressed a lower C/N ratio of *ADCY9* than HER2-negative specimens (n = 108; $P = 0.023$; missing HER2 data for five patients). Additionally, the *ADCY9* C/N ratio was lower in triple-negative BC (n = 15) than that in non-triple-negative BC (n = 133; $P < 0.001$; missing data for one patient; Fig. 2B).

In the subsequent analyses, the patients with the lowest quartile of *ADCY9* C/N ratio were designated as the "low *ADCY9* group" (n = 38), while the remaining patients were designated as the "high *ADCY9* group" (n = 111). While these two groups showed no significant associations with the T factor ($P = 0.065$), node status ($P = 0.324$), and pathological stage ($P = 0.703$), the low *ADCY9* group showed a significant association with the ER-negative ($P < 0.001$), PgR-negative ($P < 0.001$), HER2-positive ($P = 0.014$), and triple-negative status ($P = 0.013$; Table 3).

The low *ADCY9* group showed a significantly shorter DFS than the high *ADCY9* group (5-year DFS rates, low *ADCY9* group: 65.8%, high *ADCY9* group: 85.6%; $P = 0.002$; Fig. 3A). The overall survival of the low *ADCY9* group was also shorter than that in the high *ADCY9* group (5-year overall survival rates: low *ADCY9* group, 76.3%; high *ADCY9* group, 94.6%; $P < 0.001$; Fig. 3B). Multivariate analysis of DFS identified T factor T2/T3/T4 (hazard ratio: 2.24; 95% confidence interval: 1.02–5.65, $P = 0.044$), positive lymph node metastasis (hazard ratio: 4.02; 95% confidence interval: 1.79–9.99, $P < 0.001$), and the low *ADCY9* group (hazard ratio: 2.00; 95% confidence interval: 1.01–3.88, $P = 0.047$) as independent prognostic factors (Table 4). To compensate for the small sample size of our cohort, the prognostic impact of *ADCY9* expression was investigated using Kaplan–Meier Plotter. Similarly, when patients were assigned to either the lowest quartile (low *ADCY9* group) or to other quartiles, the low *ADCY9* group (n = 1233) exhibited worse relapse-free survival than the high *ADCY9* group (n = 3696; hazard ratio = 0.77; $P < 0.001$; Fig. 3C).

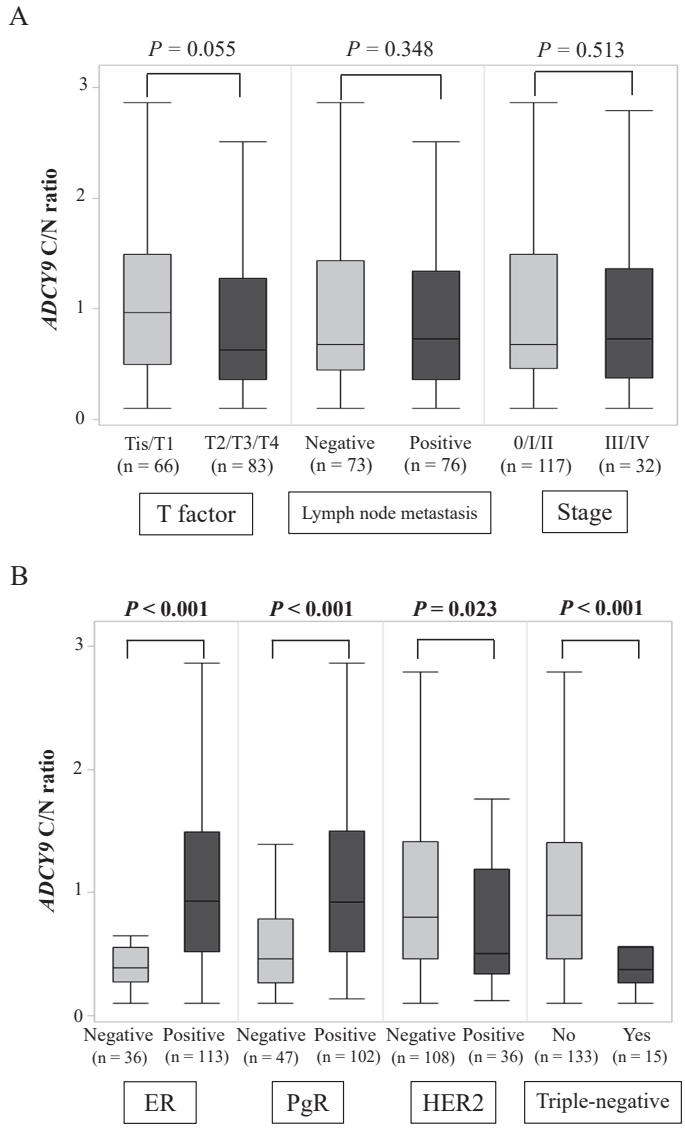

Fig. 2 *ADCY9* C/N ratio in breast cancer tissues

Fig. 2A: Relationship between the *ADCY9* C/N ratio and pathological factors. No significant differences were observed in relation to the T factor, lymph node metastasis, or stage.

Fig. 2B: Relationship between the *ADCY9* C/N ratio and the conventional biomarkers. *ADCY9* C/N ratio in the ER-positive and PgR-positive BC groups were significantly higher than those in the ER-negative and PgR-negative groups, respectively. The HER2-positive group showed a lower *ADCY9* C/N ratio than the HER2-negative group. The triple-negative group showed a lower *ADCY9* C/N ratio than the non-triple-negative group.

ADCY9: *adenylate cyclase 9*
C/N ratio: ratio of mRNA expression levels between cancerous and noncancerous tissues
ER: estrogen receptor
HER2: human epidermal growth factor receptor 2
PgR: progesterone receptor

Table 3 Associations between *ADCY9* mRNA expression and clinicopathological characteristics of 149 patients with breast cancer

Clinicopathological parameters	Low *ADCY9* group (n = 38)	High *ADCY9* group (n = 111)	*P*-values
Age			
≤ 60 years	22	75	0.284
> 60 years	16	36	
Histology			
DCIS	1	5	
IDC	35	98	0.785
ILC	0	4	
Others	2	4	
UICC T factor [a]			
Tis/T1	12	54	0.065
T2/T3/T4	26	57	
Node status			
Negative	16	57	0.324
Positive	22	54	
UICC pathological stage [a]			
0/I/II	29	88	0.703
III/IV	9	23	
ER status			
Positive	19	94	**< 0.001**
Negative	19	17	
PgR status			
Positive	15	87	**< 0.001**
Negative	23	24	
HER2 status			
Positive	15	21	**0.014**
Negative	22	86	
Triple-negative			
Yes	8	7	**0.013**
No	29	104	
Adjuvant therapy			
Endocrine therapy alone	5	41	
Chemotherapy alone	13	14	**0.003**
Chemotherapy and endocrine therapy	14	46	
None	6	10	

ADCY9: *adenylate cyclase 9*
DCIS: ductal carcinoma in situ
ER: estrogen receptor
HER2: human epidermal growth factor 2
IDC: invasive ductal carcinoma
ILC: invasive lobular carcinoma
PgR: progesterone receptor
UICC: Union for International Cancer Control
[a] Pathological stages were classified using the UICC staging system for breast cancer (8th edition).

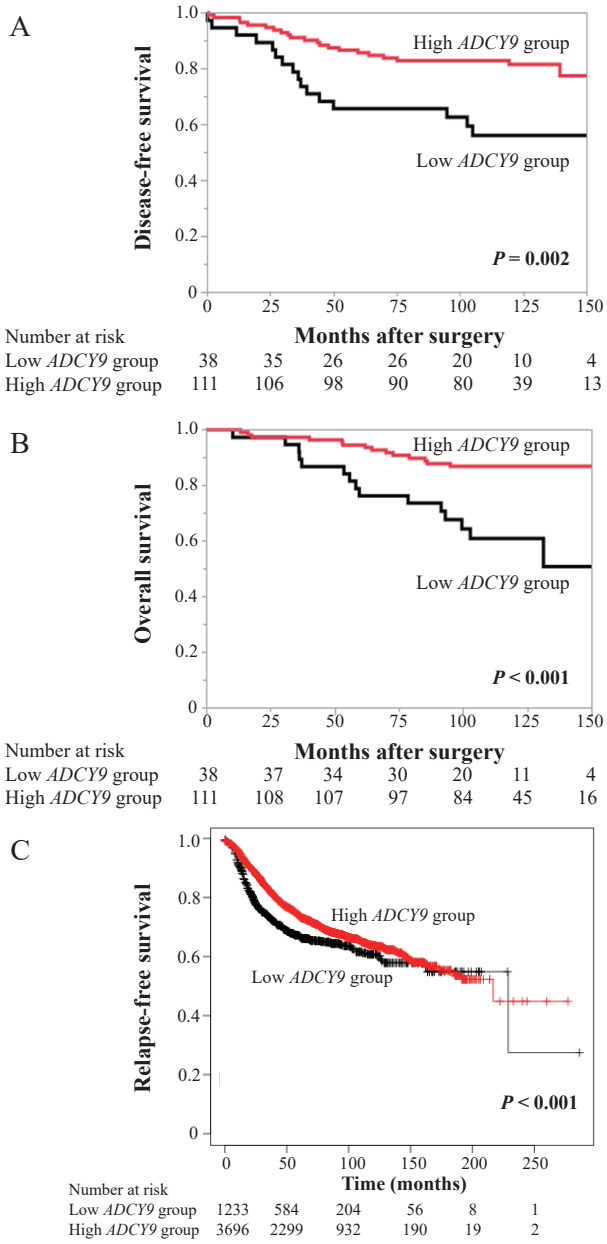

Fig. 3 Relationship between the *ADCY9* C/N ratio and prognosis

Fig. 3A, B: In the cohort that evaluated our samples, the low *ADCY9* group showed shorter disease-free survival (A) and overall survival (B) than the other patients.

Fig. 3C: According to the Kaplan–Meier Plotter, patients in the lowest-quartile *ADCY9* group exhibited shorter relapse-free survival than the high *ADCY9* group.

ADCY9: *adenylate cyclase 9*

C/N ratio: ratio of mRNA expression levels between cancerous and noncancerous tissues

Table 4 Prognostic factors for disease-free survival in 149 patients with breast cancer

Variables	n	Univariate			Multivariate		
		Hazard ratios	95% CI	*P*-values	Hazard ratios	95% CI	*P*-values
Age (> 60 years)	52	1.62	0.83 – 3.10	0.146			
UICC T factor (T2/T3/T4)	83	3.81	1.77 – 9.46	**< 0.001**	2.24	1.02 – 5.65	**0.044**
Lymph node metastasis (positive)	76	4.12	1.97 – 9.68	**< 0.001**	4.02	1.79 – 9.99	**< 0.001**
ER status (negative)	36	2.03	1.00 – 3.95	**0.049**	1.23	0.48 – 2.80	0.649
PgR status (negative)	47	1.84	0.95 – 3.52	0.071			
HER2 status (positive)	36	1.68	0.81 – 3.30	0.157			
Triple-negative (yes)	15	2.56	1.03 – 5.53	**0.043**	2.63	0.86 – 7.93	0.088
Low *ADCY9* expression	38	2.64	1.36 – 5.05	**0.005**	2.00	1.01 – 3.88	**0.047**

ADCY9: *adenylate cyclase 9*
CI: confidence interval
ER: estrogen receptor
HER2: human epidermal growth factor 2
PgR: progesterone receptor
UICC: Union for International Cancer Control

DISCUSSION

In this study, *ADCY9* mRNA expression was positively associated with ER and PgR expression in both BC cell lines and clinical specimens. Moreover, a low C/N ratio of *ADCY9* was associated with poor survival and was found to be an independent prognostic factor. These results implied that activated ADCY9 plays certain roles in BC despite lacking the function analyses.

The activation of ADCY proteins drives the production of cAMP from ATP, leading to elevated intracellular cAMP levels that play important roles in cancer cell proliferation, apoptosis, invasion, angiogenesis, and immune escape.[3] Several recent studies have shown that interventions targeting the steps upstream and downstream of the cAMP signaling pathway show anticancer effects and enhance the sensitivity of conventional therapeutic drugs[3,13,14]; thus, ADCY is considered a potential target of anticancer therapies. The ADCY family consists of 10 subtypes, each of which is encoded by an independent gene.[3] The expression of ADCY subtypes varies in various cancers; some are highly expressed in tumors and participate in tumor formation and development as oncogenes, while others may show low or even no expression.[3] Among them, *ADCY4* mRNA expression is significantly downregulated in BC tissues in comparison with normal tissues, and high *ADCY4* expression is correlated with improved survival in patients with BC.[15] In contrast, downregulation and hypermethylation of *ADCY6* has been associated with a better prognosis in patients with BC.[16]

Among the ADCY family members, the oncological roles of ADCY9 have been poorly studied, and several studies have reported inconsistent findings regarding its roles in various malignancies. A cohort study of *ADCY9* polymorphisms in colon cancer showed that the *ADCY9* expression level was higher in cancerous tissues than that in adjacent tissues, and low *ADCY9* expression in cancer tissues was associated with a longer DFS.[4] Immunohistochemical analyses showed that ADCY9 is highly expressed in advanced colon cancer and is a poor prognostic marker,[5] which implies that ADCY9 performs a tumor-progressive role. In contrast, a recent study showed tumor-suppressive activities of *ADCY9* in lung adenocarcinoma.[6] Accordingly, *ADCY9* expression was downregulated in cancerous tissues, and high *ADCY9* expression led to a better

prognosis and was an independent prognostic factor.[6] In addition, overexpression of *ADCY9* was shown to restrain the proliferation, invasion, and migration of lung adenocarcinoma cell lines via micro-RNA.[6] The possible reason why the significance of *ADCY9* expression differs among the cancer types is because the expression level of *ADCY9* varies depending on the tissue,[3] and the involved signaling-pathways and their weights on cellular function also differ. In BC, although computational analysis identified *ADCY9* as a hub gene in the cisplatin-responsive network using the MCF-7 cell line,[17] no studies have reported the relationship between *ADCY9* expression and patients' clinicopathological features and prognosis.

In this study, we found an association between the *ADCY9* C/N ratio, hormone receptor status, and prognosis. As these results were based on observational data, the underlying mechanism was difficult to explain. Nevertheless, based on the results of our PCR array analysis in BC cells and those of previous studies, several possible explanations can be inferred. Regarding the relationship between *ADCY9* and hormone receptors, *ADCY9* was identified as the most differentially expressed gene in the ER signature based on the results of gene expression analysis in Chinese patients with BC, and it was reported to be located in the calcium- and gonadotropin-releasing hormone signaling pathways.[18] Another study reported that *ADCY9* is associated with the ER signaling pathway in non-small cell lung cancer, which may improve prognosis.[19] Although *ADCY9* levels in our cell lines were heterogenous even among same subtypes, the Cancer Cell Line Encyclopedia database showed the positive relation between *ADCY9* levels and hormone receptor positive status, which was validated in clinical samples. The results of the PCR array analysis showed a positive correlation between the expression levels of *ADCY9* and *CDKN1A*, which encodes p21. Expression of p21 has been reported to correlate with ER-positive status in BC.[20] These results together imply that *ADCY9* may be involved in the ER signaling pathway in BC. However, *BRCA2,* whose expression level positively correlated with that of *ADCY9* in the PCR array analysis, was highly expressed in ER-negative BC patients.[21] Therefore, further pathway analyses are required.

The most novel finding of this study was that patients with a low *ADCY9* C/N ratio had a poor prognosis; thus, the *ADCY9* C/N ratio was identified as a prognostic factor. Several possible explanations can be provided for this observation. First, the low *ADCY9* group contained more patients with ER-negative status, who are more likely to show a worse prognosis than patients with ER-positive status.[22] However, as the multivariate analysis of DFS identified the *ADCY9* C/N ratio as a prognostic factor independent of hormone receptor status, this was not considered the only reason. The second possible reason can be determined from the PCR array results. The expression levels of *TGFB1* and *SMAD4*, downstream targets of *TGFB1*, showed high correlation coefficients with those of *ADCY9. TGF-β1,* a multifunctional cytokine, performs contradictory roles in BC. Although it is linked to increased tumor progression in the late stages, it inhibits cellular proliferation and promotes apoptosis in the early stages.[23] *CDKN1A,* which also showed high correlation with *ADCY9,* encodes p21, a downstream mediator of TGF-β1 and p53.[20,24,25] Patients with BC and low p21 expression levels have a poor prognosis,[20,24] supporting our results. Moreover, the expression level of *BAX,* which encodes a pro-apoptotic protein,[26] was correlated with that of *ADCY9*. *BAX* is a key gene involved in apoptosis and is regulated by p53 and cAMP.[3,26] Overexpression of *ADCY9* in cancer cell lines leads to increased apoptosis in lung adenocarcinomas.[6] On the basis of these observations, it is suggested that the low C/N ratio of *ADCY9* may reflect the downregulation of these tumor-suppressive pathways, especially those related to pro-apoptosis, which creates a favorable environment for cancer cell survival.

This study had some limitations. The oncological functions of *ADCY9* and its regulatory mechanisms in BC cells have not been evaluated. Therefore, basic functional analyses based on *ADCY9* inhibition are needed. Although we have described a possible explanation based on

the results of the PCR array analysis, further experiments can yield a better understanding of the pathways through which *ADCY9* affects the survival of BC cells. Moreover, our clinical data were retrospective in nature. Adjuvant therapeutic interventions such as endocrine therapy, chemotherapy and molecular targeted therapy, may have affected patient prognosis. In addition, status of drug usage such as glucocorticoids and contraceptives, which can affect the expression status of hormone receptors,[27-29] were unknown. However, because it is unlikely that many Japanese patients have taken these drugs, their impact on our results is considered limited.

Although we validated our data using a large public database to compensate for this limitation, validation using prospectively collected data would have been more desirable. Despite these limitations, this is the first study to demonstrate a relationship between *ADCY9* mRNA expression and BC prognosis. Our findings can help clarify the molecular mechanisms underlying BC and may have possible clinical applications. For example, assessment of *ADCY9* mRNA levels in resected samples may be helpful for evaluating each patient's prognosis and selecting adjuvant medication therapy regimens. Further mechanistic studies are required to overcome these limitations.

CONCLUSION

This study suggests that *ADCY9* mRNA expression is high in hormone receptor-positive BC, and its low expression in cancerous tissue indicates a poor prognosis in patients with BC.

AUTHOR CONTRIBUTIONS

Conceptualization: M.S. and M.K.; experiments: K.S., Y.A., I.S., T.I. (Takahiro Ichikawa), and T.I. (Takahiro Inaishi); data analysis: K.S., M.S., Y.A., I.S., and E.K.; resources (cell lines): M.H.; data and sample collection: K.S., M.S., Y.A., I.S., T.I. (Takahiro Ichikawa), and T.I. (Takahiro Inaishi); writing the manuscript: K.S., and M.S.; supervision: N.M. The final manuscript has been approved by all the authors. All the authors have read and agreed to the published version of the manuscript.

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DISCLOSURE STATEMENT

All authors declare that we have no conflicts of interest.

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SUPPLEMENTARY INFORMATION

Supplementary Table Correlations between mRNA expression levels of *ADCY9* and 84 cancer-related genes

Genes	Official full names	Correlation coefficients	*P*-values
ABL1	ABL proto-oncogene 1, non-receptor tyrosine kinase	0.3297	0.2713
AKT1	AKT serine/threonine kinase 1	0.4615	0.1124
APC	APC regulator of WNT signaling pathway	0.4066	0.168
ATM	ATM serine/threonine kinase	0.2033	0.5053
BAX	BCL2 associated X, apoptosis regulator	0.6593	0.0142
BCL2	BCL2 apoptosis regulator	0.5495	0.0518
BCL2L1	BCL2 like 1	–0.2363	0.4371
BCR	BCR activator of RhoGEF and GTPase	0.2692	0.3737
BRCA1	BRCA1 DNA repair associated	0.4451	0.1275
BRCA2	BRCA2 DNA repair associated	0.6538	0.0153
CASP8	caspase 8	0.0934	0.7615
CCND1	cyclin D1	0.4011	0.1744
CDH1	cadherin 1	0.0989	0.7479
CDK4	cyclin dependent kinase 4	0.5495	0.0518
CDKN1A	cyclin dependent kinase inhibitor 1A	0.6868	0.0095
CDKN2A	cyclin dependent kinase inhibitor 2A	–0.1758	0.5656
CDKN2B	cyclin dependent kinase inhibitor 2B	0.3631	0.2226
CDKN3	cyclin dependent kinase inhibitor 3	0.5165	0.0707
CTNNB1	catenin beta 1	0.1703	0.578
E2F1	E2F transcription factor 1	0.1099	0.7208
EGF	epidermal growth factor	–0.2802	0.3538
ELK1	ETS transcription factor ELK1	0.0659	0.8305
ERBB2	erb-b2 receptor tyrosine kinase 2	–0.2418	0.4262
ESR1	estrogen receptor 1	0.2308	0.4481
ETS1	ETS proto-oncogene 1, transcription factor	–0.0055	0.9858
FHIT	fragile histidine triad diadenosine triphosphatase	–0.1484	0.6286
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	0.0769	0.8028
FOXD3	forkhead box D3	–0.0934	0.7615
HGF	hepatocyte growth factor	–0.4088	0.1654
HIC1	HIC ZBTB transcriptional repressor 1	0.0604	0.8445
HRAS	HRas proto-oncogene, GTPase	0.3846	0.1944
IGF2R	insulin like growth factor 2 receptor	0.1923	0.5291
JAK2	Janus kinase 2	0.4011	0.1744
JUN	Jun proto-oncogene, AP-1 transcription factor subunit	0.2802	0.3538
JUNB	JunB proto-oncogene, AP-1 transcription factor subunit	–0.0055	0.9858
JUND	JunD proto-oncogene, AP-1 transcription factor subunit	–0.0604	0.8445
KIT	KIT proto-oncogene, receptor tyrosine kinase	0.1044	0.7343
KITLG	KIT ligand	–0.0055	0.9858
KRAS	KRAS proto-oncogene, GTPase	0.2308	0.4481
MCL1	MCL1 apoptosis regulator, BCL2 family member	0.0934	0.7615
MDM2	MDM2 proto-oncogene	0.3681	0.2159
MEN1	menin 1	0.1868	0.5411

MET	MET proto-oncogene, receptor tyrosine kinase	0.1703	0.578
MGMT	O-6-methylguanine-DNA methyltransferase	0.1099	0.7208
MLH1	mutL homolog 1	0.5659	0.0438
MOS	MOS proto-oncogene, serine/threonine kinase	–0.0989	0.7479
MYB	MYB proto-oncogene, transcription factor	0.1648	0.5905
MYC	MYC proto-oncogene, bHLH transcription factor	0.3626	0.2233
MYCN	MYCN proto-oncogene, bHLH transcription factor	–0.2473	0.4154
NF1	neurofibromin 1	0.2253	0.4593
NF2	neurofibromin 2	0.2253	0.4593
NFKB1	nuclear factor kappa B subunit 1	0.4341	0.1383
NFKBIA	NFKB inhibitor alpha	0.6484	0.0165
NRAS	NRAS proto-oncogene, GTPase	0.6538	0.0153
PIK3C2A	phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 alpha	–0.0055	0.9858
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	0.4615	0.1124
PML	PML nuclear body scaffold	0.3077	0.3064
PRKCA	protein kinase C alpha	0.5385	0.0576
RAF1	Raf-1 proto-oncogene, serine/threonine kinase	0.5989	0.0306
RARA	retinoic acid receptor alpha	0.2857	0.344
RASSF1	Ras association domain family member 1	–0.1319	0.6676
RB1	RB transcriptional corepressor 1	0.4066	0.168
REL	REL proto-oncogene, NF-kB subunit	0.2198	0.4706
RET	ret proto-oncogene	0.0165	0.9574
ROS1	ROS proto-oncogene 1, receptor tyrosine kinase	–0.2637	0.3839
RUNX1	RUNX family transcription factor 1	0.3297	0.2713
RUNX3	RUNX family transcription factor 3	–0.3242	0.2799
S100A4	S100 calcium binding protein A4	0.1648	0.5905
SERPINB5	serpin family B member 5	–0.3956	0.1809
SH3PXD2A	SH3 and PX domains 2A	–0.0495	0.8725
SMAD4	SMAD family member 4	0.6593	0.0142
SRC	SRC proto-oncogene, non-receptor tyrosine kinase	0.011	0.9716
STAT3	signal transducer and activator of transcription 3	0.1593	0.6031
STK11	serine/threonine kinase 11	0.3681	0.2159
TGFB1	transforming growth factor beta 1	0.7088	0.0067
TNF	tumor necrosis factor	0.033	0.9149
TP53	tumor protein p53	0.4066	0.168
TP73	tumor protein p73	0.2747	0.3637
TSC1	TSC complex subunit 1	0.4066	0.168
VHL	von Hippel-Lindau tumor suppressor	0.3846	0.1944
WT1	WT1 transcription factor	–0.3681	0.2159
WWOX	WW domain containing oxidoreductase	0.3956	0.1809
XRCC1	X-ray repair cross complementing 1	0.3736	0.2086
ZHX2	zinc fingers and homeoboxes 2	0.1538	0.6158

ADCY9: *adenylate cyclase 9*
mRNA: messenger RNA