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High Expression of *p62* and *ALDH1A3* Is Associated With Poor Prognosis in Luminal B Breast Cancer

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Abstract. *Background/Aim: p62 (also known as sequestosome 1) is involved in cancer progression, and high expression of p62 indicates poor clinical outcome in several cancer types. However, the association between p62 gene expression and cancer stem cells (CSCs) in breast cancer subtypes remains unclear. Materials and Methods: In the present study, genomic datasets of primary breast cancer (The Cancer Genome Atlas, n=593; and Molecular Taxonomy of Breast Cancer International Consortium, n=2,509) were downloaded. p62 Expression was then examined in normal and breast cancer tissues derived from the same patients. Kaplan–Meier and multivariate Cox regression analyses were employed to evaluate disease-specific survival. Next, the effect on cell viability and in vitro tumor-sphere formation of p62 knockdown using targeted small interfering RNA was assessed by using cells with high activity of aldehyde dehydrogenase 1 (ALDH1^{high}). Results: Patients with normal-like, luminal A or luminal B breast cancer with p62^{high} had poor prognosis. Furthermore, patients with p62^{high} ALDH1A3^{high} luminal B type also exhibited poor prognoses. Knockdown of p62 suppressed viability and tumor-sphere formation by ALDH1^{high} cells of the luminal B-type cell lines BT-474 and MDA-MB-361. These results suggest that p62 is essential for cancerous progression of ALDH1-positive luminal B breast CSCs, and contributes to poor prognosis of luminal B breast cancer. Conclusion: p62 is potentially a prognostic marker and therapeutic target for ALDH1-positive luminal B breast CSCs.*

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Breast cancer has the highest prevalence among cancers of women worldwide, with 2.26 million new cases (24.5% of all cancer cases in women) and 685,000 cancer-associated mortalities (15.5% of all cancer-associated mortalities among women) annually (1). Breast cancer is classified using two parameters: Immunohistochemistry and gene-expression patterns [prediction analysis of microarray 50 (PAM50)] (2-8). Based on its PAM50, breast cancer is classified into at least six subtypes: Normal-like, luminal A, luminal B, human epidermal growth factor receptor type 2 (HER2)-enriched, claudin-low and basal-like (5, 7, 8). Among these, the luminal B type expresses estrogen receptor, and certain luminal B tumors express HER2 and highly express proliferation-related genes such as marker of proliferation Ki-67 (*MKI67*). In addition, the luminal B type has poorer prognosis (7, 9-15). Breast cancer treatment mainly entails surgery, radiotherapy and drug therapy, including chemotherapy, endocrine therapy and molecular targeted therapy. However, there are still numerous

refractory cases, and further stratification and development of prognostic markers and therapeutic targets are required for improving quality of life of patients. Luminal B type can be treated with endocrine therapy and a HER2-targeted antibody, such as trastuzumab (12, 14). Given its poor prognosis, it is necessary to identify effective prognostic markers and molecular targets for the luminal B breast cancer subtype.

Tumor consists of both cancer stem cells (CSCs) and differentiated cancer cells. CSCs exhibit stem cell-like functions such as self-renewal, multipotency and tumorigenicity (16, 17). Since the majority of CSCs are resistant to conventional antitumor treatments, such as chemotherapy and radiotherapy, the development of targeted therapies against CSCs is needed to improve the clinical outcomes of patients (17, 18).

Aldehyde dehydrogenase 1 (ALDH1) is an enzyme that converts aldehydes into carboxylic acids. *ALDH1A1* and *ALDH1A3* are known as CSC markers in several cancer types (19-24). Expression of ALDH1A1 and ALDH1A3 is correlated with tumor grade, metastasis and prognosis of patients with breast cancer (25-27), and ALDH1A3 significantly contributes to ALDH1 activity in breast cancer (25, 28).

p62 (also known as sequestosome 1) is a multifunctional adapter protein that is involved in various physiological functions (29), including nuclear factor-κB signaling (30-33), antioxidant response (34) and autophagy (35-37). p62 is overexpressed (38-48) in several cancer types, and high p62 expression is associated with poor prognosis (41, 44, 46, 49-52) in several types of cancer. In breast cancer, p62 gene and protein are overexpressed (53-55) and high p62 protein expression is associated with poor prognosis of overall and disease-free survival (54, 56). In addition, patients with high *p62* gene expression have shorter recurrence-free (55, 57) and metastasis-free (55) survival. However, the association between *p62* gene expression and CSCs among breast cancer subtypes remains unclear.

The present study examined the association between p62 and ALDH1A3 among breast cancer subtypes. Furthermore, we also examined the role of p62 in ALDH1A3-positive CSCs of luminal B breast cancer.

Materials and Methods

Analysis of The Cancer Genome Atlas dataset (TCGA). TCGA breast cancer dataset (58) was downloaded from Oncomine (https://www.oncomine.org; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (59) on January 5, 2021. This dataset contains mRNA expression data from 61 normal breast tissue and 532 primary breast tumor samples, and the clinicopathological data of these patients had been previously reported (60). The expression of *p62* mRNA (Probe ID: A_23_P81399, A_23_P81401) is presented using the log2 median-centered ratio for both normal and cancer tissues. *p62* mRNA were plotted using a paired comparison of normal *versus* cancer tissue from the same patients (n=60) using the Wilcoxon signed-rank test. Two-sided values of $p<0.05$ were considered to indicate a statistically significant difference. All statistical analyses were carried out using BellCurve for Excel ver. 3.10 (Social Survey Research Information Co., Ltd., SSRI, Tokyo, Japan).

Analysis of the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset. The METABRIC dataset (n=2,509) (61, 62) was downloaded from cBioportal (https:// www.cbioportal.org/) (63, 64) on October 30, 2020. The clinicopathological data from these patients had been previously summarized (60). The METABRIC dataset contains data on both gene alterations (n=2,173) and mRNA expression levels of primary breast cancer samples (n=1,904), and disease-specific survival (DSS) data with mRNA expression levels (n=1,423). The mRNA expression levels were compared among breast cancer subtypes using the Kruskal–Wallis test followed by Steel–Dwass's multiple comparison test for *post-hoc* analysis. The optimal cutoff thresholds to divide patients into groups with high and low expression were defined using receiver operating characteristic curves associating *p62*, *ALDH1A1* or *ALDH1A3* gene expression with DSS. The optimal cutoff threshold was determined using the Youden's index. Survival curves based on DSS were plotted using the Kaplan–Meier method, and curves were compared using the log-rank (Cochran–Mantel–Haenszel) test. A multivariate Cox regression analysis was used to evaluate the influence of gene expression and to estimate adjusted hazard ratios (HRs) using DSS statuses, with age at diagnosis as a confounding factor. Two-sided values of $p<0.05$ were considered to indicate a statistically significant difference. All statistical analyses were carried out using BellCurve for Excel ver. 3.10 (SSRI).

Cell culture and siRNA transfection. The human luminal B type breast cancer cell lines BT-474 and MDA-MB-361, and human normal (non-transformed) mammary epithelial cell line MCF10A were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured at 37˚C in the presence of 5% CO_2. BT-474 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and 0.01 mg/ml insulin (Nacalai Tesque, Inc., Kyoto, Japan). MDA-MB-361 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. MCF10A cells were maintained in mammary epithelial cell growth medium (Lonza, Basel, Switzerland).

p62 knockdown (KD) in BT-474 and MDA-MB-361 cells was achieved using transfection of two siRNAs and two Dicer-Substrate siRNAs (DsiRNAs), which led to long-term suppression of gene expression (65). The sequences were as follows. *p62* siRNA-1: 5'-GUGAACUCCAGUCCCUACATT-3' and *p62* siRNA-2: 5'-GUGACGAGGAAUUGACAAUTT-3') (Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA). *p62* DsiRNA-1: sense strand: 5'-GUGAACUCCAGUCCCUACAGAUGCC-3' and antisense strand: 5'-GGCAUCUGUAGGGACUGGAGUUCACCU-3'; and *p62* DsiRNA-2: sense strand: 5'-GUGACGAGGAAUUGACAAUGGCCAT-3' and antisense strand: 5'-AUGGCCAUUGUCAAUUCCUCGUCACUG-3' (Integrated DNA Technologies, Inc., IDT, Coralville, IA, USA). MISSION siRNA Universal Negative Control (Sigma-Aldrich; Merck KGaA) and Negative Control DsiRNA (sense strand: 5'-CGUUAAUCGCGUAUAAUACGCGUAT-3' and antisense strand: 5'-AUACGCGUAUUAUACGCGAUUAACGAC-3') were used as controls. Transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Inc.). Cells were transfected with 10 nM siRNA and incubated for 24 h, followed by transfection with 10 nM DsiRNA and subsequent incubation for an additional 24 h before assays were performed.

Western blotting. Western blotting of cells was performed as previously described (60, 66-70). The following antibodies were used: Rabbit polyclonal anti-p62 (PM045, 1:10000; Medical & Biological Laboratories, Tokyo, Japan); mouse monoclonal anti-p62 (610833, 1:10,000; BD Biosciences, San Jose, CA, USA); rabbit monoclonal anti-ALDH1A1 (ab52492, 1:3,000; Abcam, Cambridge, UK); rabbit polyclonal anti-ALDH1A3 (PA5-29188, 1:5,000; Thermo Fisher Scientific, Inc.); mouse monoclonal anti-β-actin (60008-I-Ig, 1:10,000; ProteinTech Group, Inc., Rosemont, IL, USA) as the primary antibodies. Goat anti-mouse and anti-rabbit IgG (7076S and 7074S, 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA) were used as secondary antibodies.

Isolation of ALDH1^{high} cells. ALDH1^{high} cells were isolated as previously described (60, 66-68, 70, 71) from BT-474 and MDA-MB-361 cells with *p62* KD using ALDEFLUOR™ assay kit (STEMCELL Technologies, Inc., Vancouver, Canada).

WST-8 assay. WST-8 assay was performed as previously described (60, 66, 67, 69, 71). Briefly, after *p62* KD, unsorted cells or sorted ALDH1^{high} cells were seeded into 96-well plates (1×10^5 cells/well) (Thermo Fisher Scientific, Inc.) and incubated for 5 days (BT-474 and unsorted MDA-MB-361 cells) or 9 days (ALDH1^{high} cells derived from MDA-MB-361 cells). Cell viability was then assessed using Cell Counting Reagent SF (Nacalai Tesque, Inc.). The results of unsorted cells were analyzed by Dunnett's multiple comparison test (unpaired). The results of sorted ALDH1^{high} cells were analyzed by Student's *t*-test. Two-sided values of $p<0.05$ were considered to indicate a statistically significant difference. Data are presented as the mean±standard error of the mean of three independent experiments. All statistical analyses were carried out using BellCurve for Excel ver. 3.10 (SSRI).

Tumor-sphere culture. Tumor-sphere formation assay was conducted as previously described (60, 66-71). ALDH1^{high} cells were isolated *via* ALDEFLUOR assay upon *p62* KD. The isolated ALDH1^{high} cells were plated in ultralow-attachment 96-well plates (5×10^4 cells/well) (Greiner Bio-One GmbH, Frickenhausen, Germany) and cultured for 7 days (BT-474) or 15 days (MDA-MB-361) and were captured using a DMIL LED microscope (Leica, Wetzlar, Germany). The number and size of tumor spheres over 1,256 μm^2 (BT-474) and 314 μm^2 (MDA-MB-361) were measured using ImageJ 1.51j8; Java 1.8.0_112 (64-bit) (National Institutes of Health, Bethesda, MA, USA). Statistical significance was determined with Student's *t*-test. Two-sided values of $p<0.05$ were considered to indicate a statistically significant difference. Data are presented as the mean±standard error of the mean of three independent experiments. All statistical analyses were carried out using BellCurve for Excel ver. 3.10 (SSRI).

Results

Kaplan–Meier analyses indicate that clinical outcomes are poor for patients with p62^{high} normal-like, luminal A or luminal B breast cancer. p62 is highly expressed in breast cancer compared with normal mammary epithelium (53, 55). Consistent with this, the present study confirmed that *p62* expression was significantly higher in breast cancer in paired comparison of *p62* mRNA expression between normal and tumor tissues derived from the same patients in TCGA breast cancer dataset (Figure 1A). Next, *p62* expression was examined in different breast cancer subtypes using the METABRIC dataset. Among the subtypes classified based on PAM50, *p62* was highly expressed in the normal-like, luminal A, luminal B and HER2-enriched subtypes than in the claudin-low and basal-like types (Figure 1B).

To assess the role of *p62* expression in PAM50 subtypes, the present study analyzed a METABRIC dataset that included the gene-expression data from patients with breast cancer. The association between *p62* expression and prognosis among the breast cancer subtypes was examined by using the Kaplan–Meier method to compare DSS between patients with *p62*^{high} and *p62*^{low} disease. Firstly, patients with *p62*^{high} status had a poor prognosis in breast cancer overall ($p<0.001$) (Figure 1C). Among the breast cancer subtypes, patients with *p62*^{high} disease had significantly poorer clinical outcomes than those with *p62*^{low} normal-like, luminal A or luminal B disease (Figure 1C). Importantly, there was no significant difference between the survival rates of patients with *p62*^{high} and *p62*^{low} HER2-enriched, claudin-low or basal-like type (Figure 1C). These results suggest that p62 is involved in cancer progression and contributes to poor clinical outcomes in patients with normal-like, luminal A and luminal B breast cancer.

Kaplan–Meier analyses indicate that patients with p62^{high} ALDH1A3^{high} luminal B breast cancer have a poorer clinical outcome. *ALDH1A1* and *ALDH1A3* are known to be CSC markers in several cancer types (19-24). Therefore, the present study next assessed the prognosis of patients with *p62*^{high} *ALDH1A1*^{high} and of those with *p62*^{high} *ALDH1A3*^{high} status. Kaplan–Meier analyses did not show significant differences between the DSS rates of patients with *p62*^{high} *ALDH1A1*^{high} and patients with other statuses in breast cancer overall nor in any subtype (Figure 2A). Kaplan–Meier analyses showed that patients with *p62*^{high} *ALDH1A3*^{high} had poorer clinical outcomes than those with other statuses in breast cancer overall and in those with luminal B breast subtype (Figure 2B). Importantly, there was no significant difference between the survival rates of patients with *p62*^{high} *ALDH1A3*^{high} disease and other patients with normal-like, luminal A, HER2-enriched, claudin-low or basal-like type (Figure 2B). In comparison of DSS among patients according to combined expression of *p62* with *ALDH1A1*, no significant differences were noted in breast cancer overall, nor in any subtype (Figure 3A), whereas DSS of patients with *p62*^{high} *ALDH1A3*^{high} status was significantly poorer only in those with the luminal B breast cancer subtype (Figure 3B). These results suggest that p62 is involved in cancer progression and contributes to poor clinical outcomes of patients with *ALDH1A3*^{high} luminal B breast cancer.

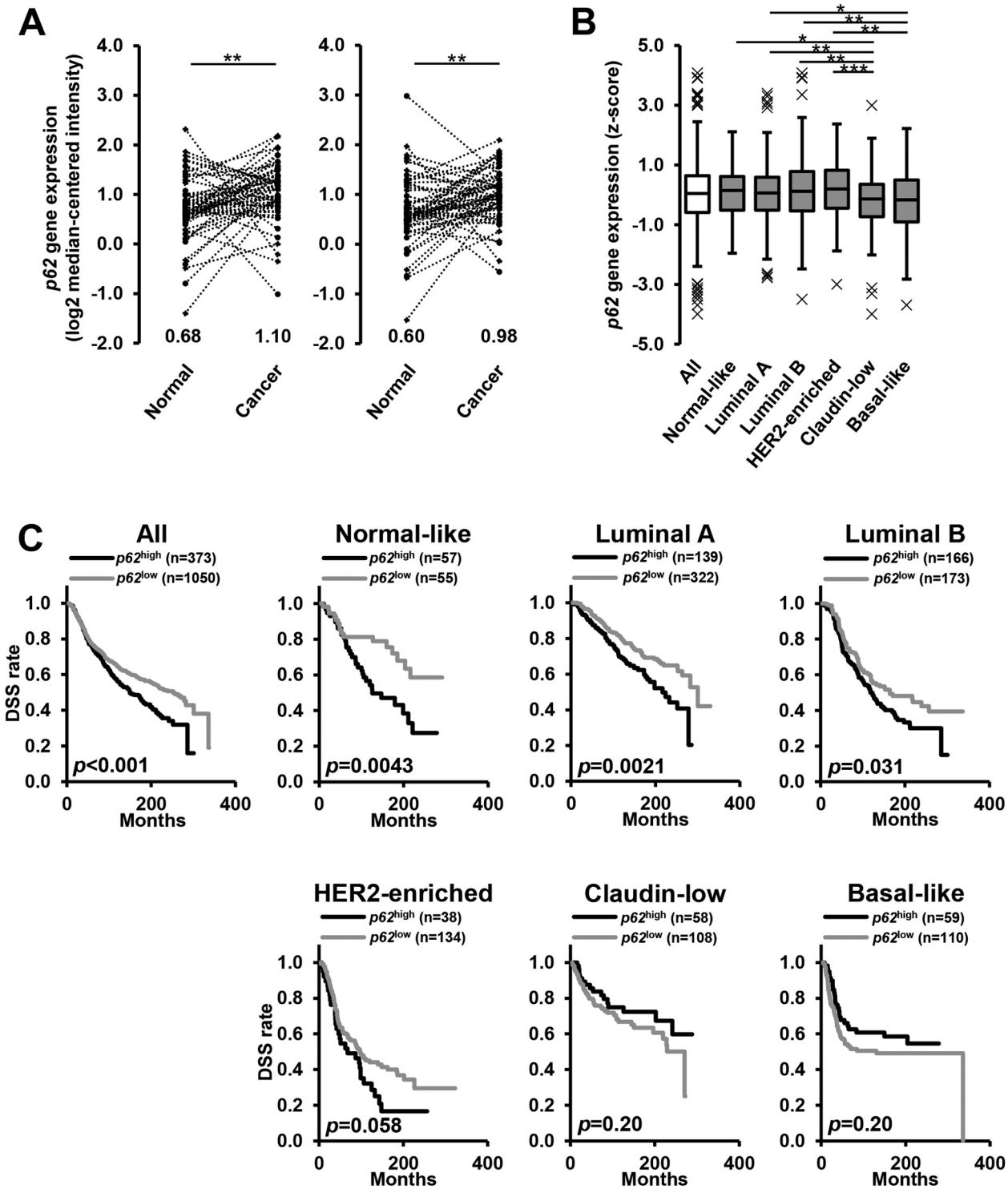

Figure 1. *p62 is highly expressed in breast cancer, and patients with p62^{high} normal-like, luminal A and luminal B breast cancer subtypes exhibit poor clinical outcomes. A: Paired comparison of p62 mRNA expression in normal and tumor tissues derived from the same patients (n=60) in the The Cancer Genome Atlas with Probe IDs A_23_P81399 (left) and A_23_P81401 (right). Median values are shown at the bottom of graphs. Significantly different at **p<0.01 by Wilcoxon signed-rank test. B: p62 mRNA expression by breast cancer subtype. Significantly different at: *p<0.05, **p<0.01 and ***p<0.001 by Kruskal–Wallis test followed by Steel–Dwass's multiple comparison test for post-hoc analysis. C: Kaplan–Meier analyses comparing disease-specific survival (DSS) according to p62 mRNA expression in breast cancer overall and by breast cancer subtype. p-Values were calculated with the log-rank (Cochran–Mantel–Haenszel) test. HER2: Human epidermal growth factor receptor type 2.*

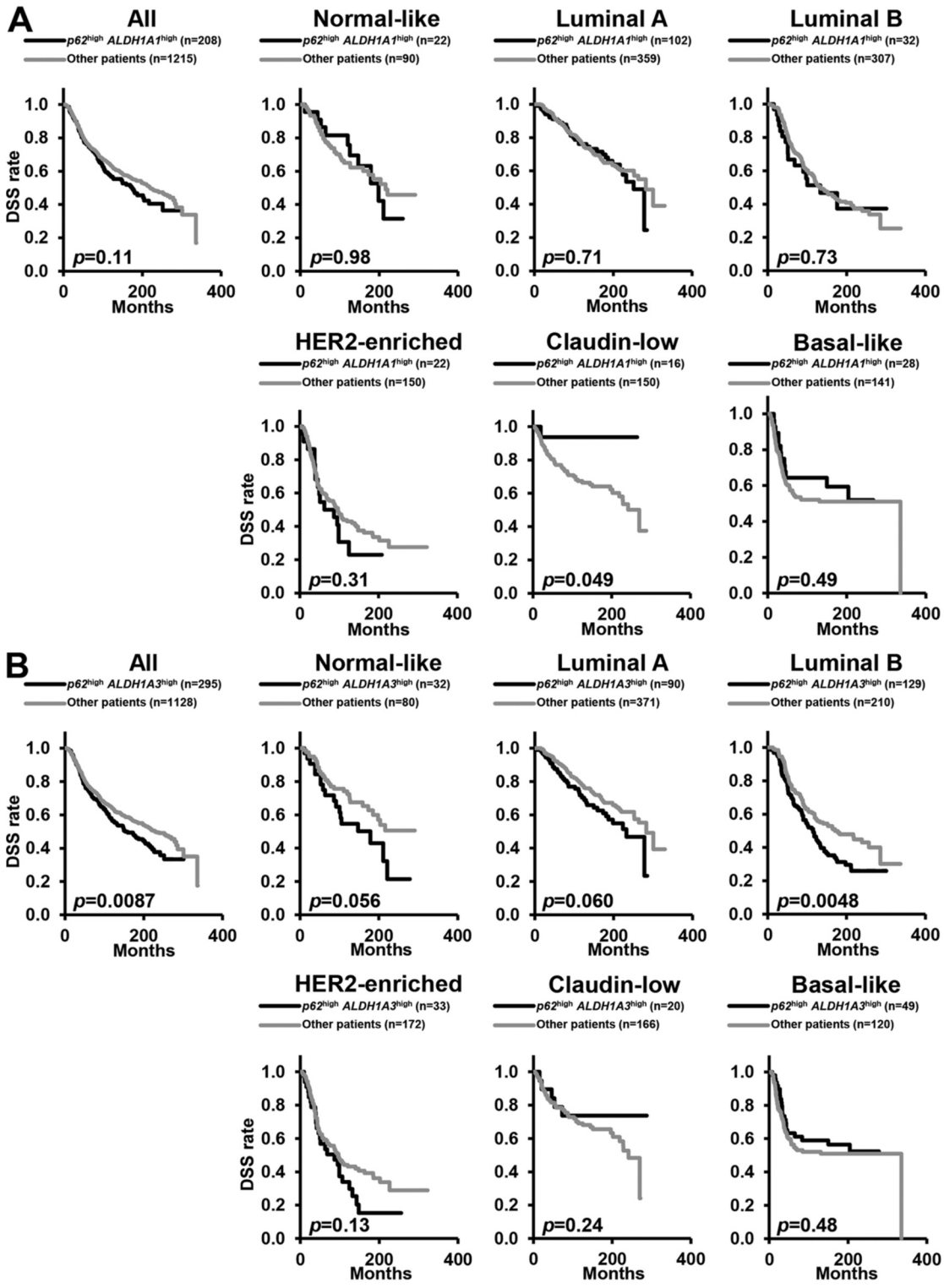

Figure 2. *Patients with luminal B breast cancer with high expression of p62 (p62*^{high}*) and aldehyde dehydrogenase 1A3 (ALDH1A3*^{high}*) exhibit poor clinical outcomes. A: Kaplan–Meier analyses comparing disease-specific survival (DSS) between patients with p62*^{high} *ALDH1A1*^{high} *disease and others (p62*^{high} *ALDH1A1*^{low}*, p62*^{low} *ALDH1A1*^{high} *and p62*^{low} *ALDH1A1*^{low}*) in breast cancer overall and by breast cancer subtype. B: Kaplan–Meier analyses comparing DSS between p62*^{high} *ALDH1A3*^{high} *patients and others (p62*^{high} *ALDH1A3*^{low}*, p62*^{low} *ALDH1A3*^{high} *and p62*^{low} *ALDH1A3*^{low}*) in breast cancer overall and by breast cancer subtype. p-Values were calculated with the log-rank (Cochran–Mantel–Haenszel) test. HER2: Human epidermal growth factor receptor type 2.*

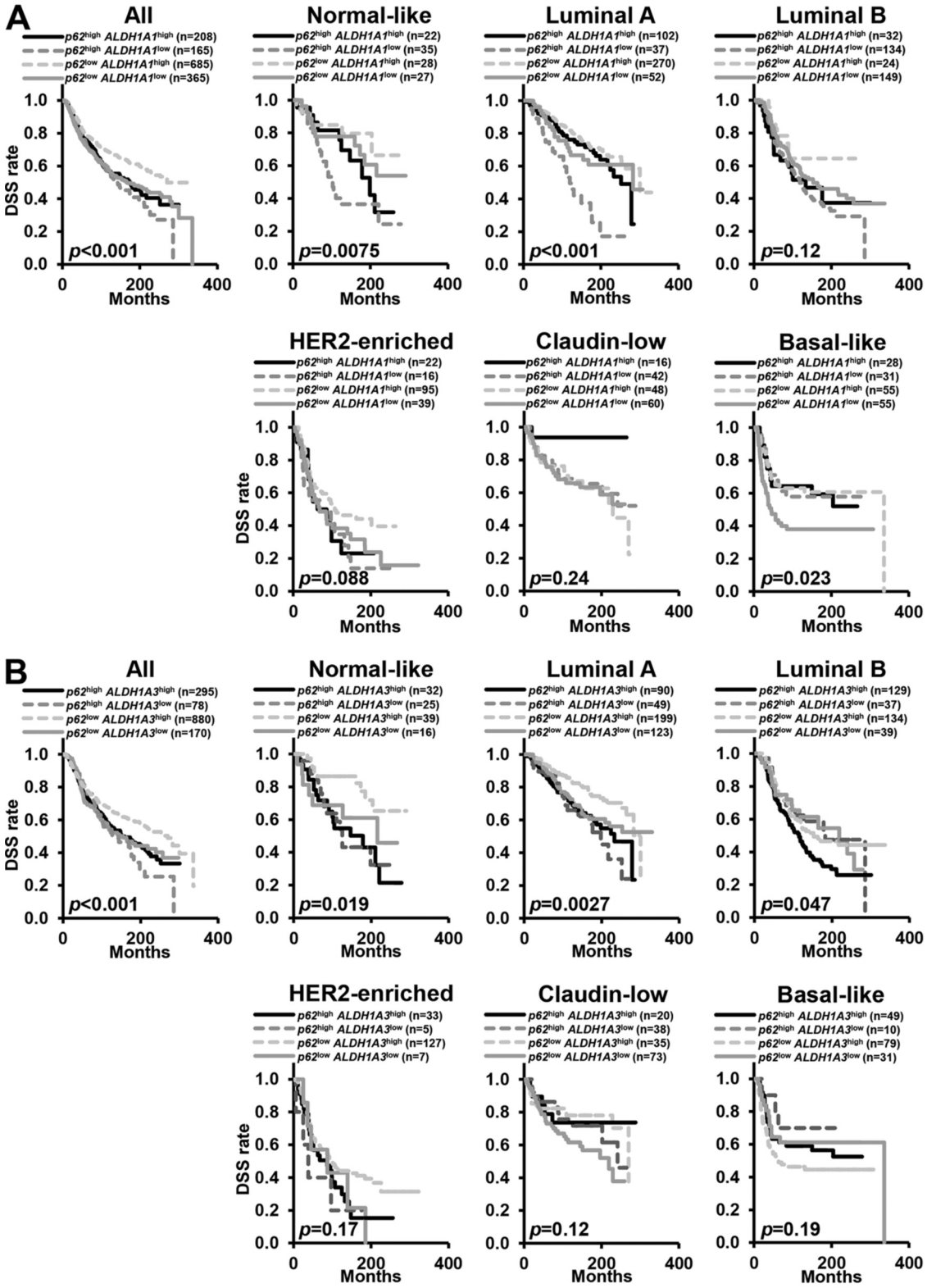

Figure 3. *Patients with luminal B breast cancer with high p62 (p62^{high}) and high aldehyde dehydrogenase 1A3 (ALDH1A3^{high}) expression exhibit poor clinical outcomes. Kaplan–Meier analyses comparing DSS according to combined expression of p62 with ALDH1A1 (A) and with ALDH1A3 (B) in breast cancer overall and by breast cancer subtype. p-Values were calculated with the log-rank (Cochran–Mantel–Haenszel) test. HER2: Human epidermal growth factor receptor type 2.*

Table I. *Multivariable Cox regression analysis of disease-specific survival according to expression of p62, aldehyde dehydrogenase 1 (ALDH1A1) and ALDH1A3 by breast cancer subtype.*

Comparison		Hazard ratio*	95% CI	*p*-Value
p62^{high} *vs.* *p62*^{low}	**All breast cancer**	**1.31**	**1.10-1.55**	**0.0021**
	Normal-like	**2.30**	**1.25-4.23**	**0.0072**
	Luminal A	**1.47**	**1.05-2.06**	**0.024**
	Luminal B	1.31	0.98-1.76	0.072
	HER2-enriched	1.50	0.98-2.31	0.062
	Claudin-low	0.71	0.40-1.29	0.26
	Basal-like	0.74	0.46-1.19	0.21
p62^{high} *ALDH1A1*^{high} *vs.* other	All breast cancer	1.19	0.96-1.48	0.11
	Normal-like	0.98	0.49-1.97	0.95
	Luminal A	1.04	0.71-1.52	0.85
	Luminal B	1.04	0.62-1.74	0.88
	HER2-enriched	1.32	0.77-2.25	0.31
	Claudin-low	0.15	0.021-1.10	0.062
	Basal-like	0.80	0.43-1.48	0.48
p62^{high} *ALDH1A3*^{high} *vs.* other	**All breast cancer**	**1.26**	**1.05-1.52**	**0.014**
	Normal-like	1.71	0.95-3.09	0.073
	Luminal A	1.36	0.94-1.98	0.11
	Luminal B	**1.46**	**1.09-1.95**	**0.012**
	HER2-enriched	1.42	0.90-2.22	0.13
	Claudin-low	0.61	0.24-1.56	0.31
	Basal-like	0.85	0.52-1.39	0.51

CI: Confidence interval; HER2: Human epidermal growth factor receptor type 2. *Adjusted by age at diagnosis, as estimated using the Cox proportional hazard model. Significant differences are shown in bold.

Multivariate Cox regression analysis indicates that patients with p62^{high} ALDH1A3^{high} luminal B breast cancer have a poorer clinical outcome. To confirm the results obtained by Kaplan–Meier analyses, a multivariate Cox regression analysis of DSS was performed with age at diagnosis as a confounding factor using the same dataset (Table I). Patients with *p62*^{high} status had significantly poorer clinical outcomes considering breast cancer overall, and normal-like or luminal A subtype than those with *p62*^{low} disease (Table I). Patients with *p62*^{high} luminal B, HER2-enriched, claudin-low or basal-like subtypes did not have poorer clinical outcomes than those with *p62*^{low} status (Table I). Multivariable Cox regression analysis also showed that *p62*^{high} *ALDH1A1*^{high} status was not predictive of poorer clinical outcomes than other *p62 ALDH1A1* statuses in breast cancer overall nor in any subtype (Table I). However, *p62*^{high} *ALDH1A3*^{high} disease was predictive of significantly poorer clinical outcomes compared with other *p62 ALDH1A3* statuses in breast cancer overall and in patients with luminal B breast subtype (Table I) but not in those with other breast cancer subtypes (Table I). These results suggest that p62 is involved in cancer progression and contributes to poor prognosis of ALDH1A3^{high}, *i.e.*-CSC-enriched, luminal B breast cancer.

p62 siRNA KD suppresses cell viability and in vitro tumor-sphere formation by ALDH1^{high} cells. Based on the above results, the present study next examined the possibility that p62 may serve as a therapeutic target of ALDH1A3-positive CSCs in luminal B breast cancer. The luminal B type breast cancer cell lines BT-474 and MDA-MB-361 were used, which overexpress p62 protein compared with the normal human (non-transformed) mammary epithelial cell line MCF10A (Figure 4A). *p62* siRNA significantly suppressed p62 protein expression in BT-474 and MDA-MB-361 cells (Figure 4B). WST-8 cell viability assays with BT-474 and MDA-MB-361 cells showed that *p62* KD suppressed cell viability (Figure 4C). These findings suggest that p62 contributes to cell survival/proliferation in luminal B breast cancer.

ALDH1A1 protein was slightly expressed in BT-474 and MDA-MB-361 cells. ALDH1A3 protein was overexpressed in BT-474 compared with MCF10A and MDA-MB-361 cells (Figure 4A). Next, to assess the role of p62 in ALDH1-positive luminal B CSCs, ALDEFLUOR assays were used to examine the effects of *p62* depletion on cell viability and *in vitro* tumor-sphere formation in ALDH1^{high} BT-474 and MDA-MB-361 cells. *p62* KD led to suppression of cell viability by ALDH1^{high} BT-474 and MDA-MB-361 cells (Figure 4D). This result suggests that p62 is essential for survival/proliferation. In addition, *p62* siRNA suppressed the *in vitro* area of tumor spheres formed by ALDH1^{high} BT-474 and MDA-MB-361 cells but it did not suppress the number of *in vitro* tumor spheres (Figure 4E, respectively). These results

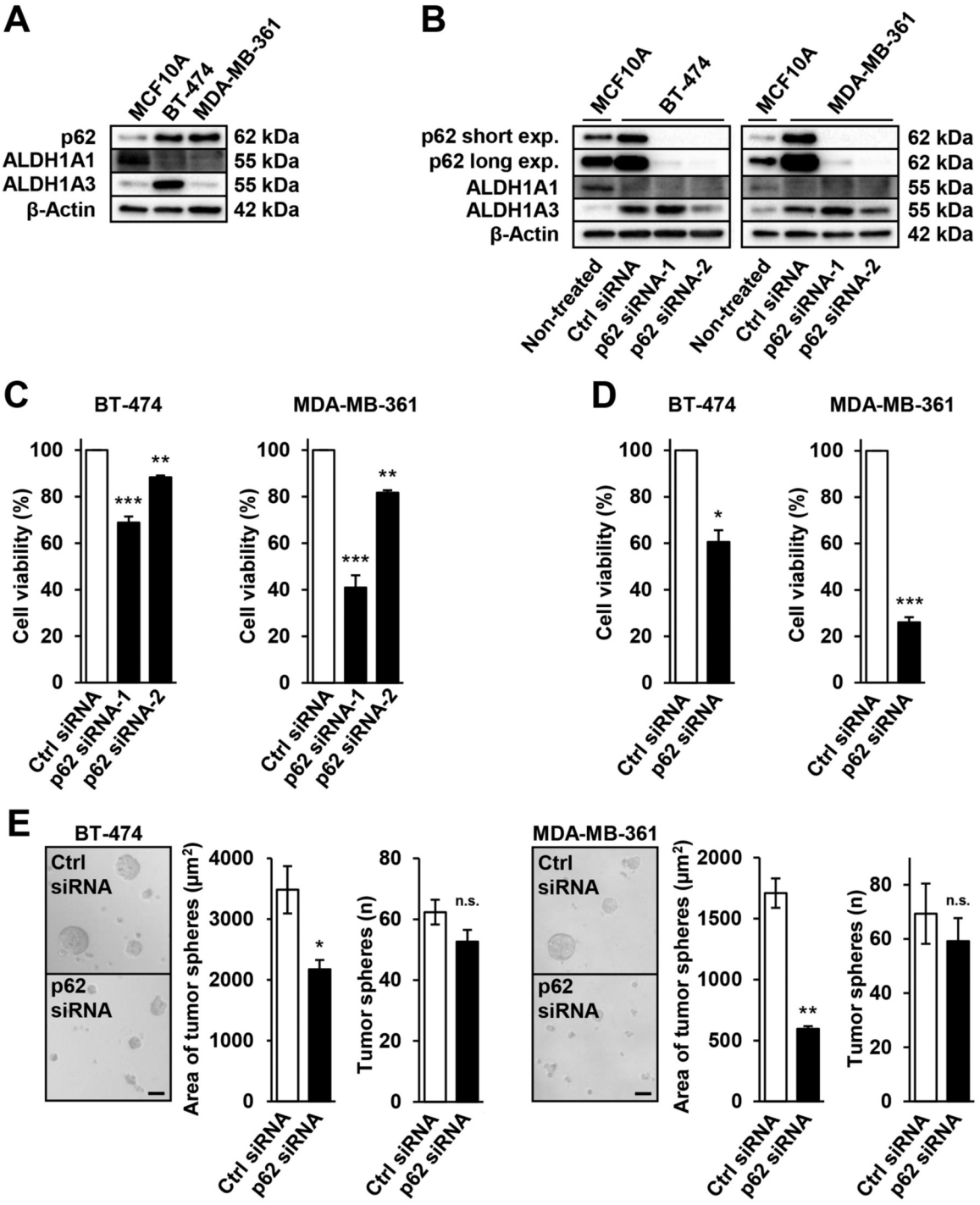

Figure 4. *Knockdown of p62 with siRNA leads to the suppression of cell viability and in vitro tumor-sphere formation by luminal B breast cancer cells with high aldehyde dehydrogenase 1 (ALDH1) expression. A: Western blotting of p62, ALDH1A1 and ALDH1A3 expression in native MCF10A, BT-474 and MDA-MB-361 cells. B: Western blotting of p62, ALDH1A1 and ALDH1A3 in BT-474 and MDA-MB-361 cells with and without knockdown of p62 using targeted siRNA-1/2 and DsiRNA-1/2. Non-treated MCF10A cells acted as a positive control. C: WST-8 assays assessing cell viability of BT-474 and MDA-MB-361 cells after p62 knockdown using targeted siRNA-1/2 and DsiRNA-1/2. D: WST-8 assays assessing cell viability of ALDH1^{high} BT-474 and MDA-MB-361 cells after p62 knockdown using targeted siRNA-1 and DsiRNA-1. E: In vitro tumor sphere-formation culture was performed to assess tumor-sphere formation by ALDH1^{high} BT-474 and MDA-MB-361 cells following p62 knockdown via targeted siRNA-1 and DsiRNA-1. Representative images are shown; scale bar, 50 μm. Data represent the mean±standard error of the mean of three independent experiments. Significantly different at: *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test or Dunnett's multiple comparison (unpaired). n.s.: Not significantly different.*

suggest that p62 is also essential for tumor formation by ALDH1-positive breast CSCs in luminal B breast cancer. Furthermore, p62 may be a therapeutic target as well as a prognostic marker in ALDH1-positive luminal B breast cancer.

Discussion

The present study demonstrated that p62 was essential for survival/proliferation and tumor formation by ALDH1^{high} breast CSCs in luminal B breast cancer, and was involved in cancer progression and contributed to poor prognosis of ALDH1A3^{high} luminal B breast cancer.

p62 gene amplification and enhancement of gene expression were detected in clear-cell renal cell carcinoma (72). Therefore, *p62* gene alterations were assessed using the METABRIC and TCGA datasets in this study. *p62* gene amplification was detected in 1.7% and 1.0% of breast cancer cases, respectively (METABRIC: 37/2,173; and TCGA: 10/996), *p62* mutation was detected in 0.0 and 0.50% (METABRIC: 0/2,173; TCGA: 5/996), and deletion was detected in 0.092 and 0.20% (METABRIC: 2/2,173; TCGA: 2/996), respectively. Thus, the high level of *p62* mRNA expression observed in breast cancer compared with that of normal tissues may reflect the transcriptional activation of *p62*. It has been reported that *p62* is a target gene in cancer, which is located downstream of nuclear factor-E2-related factor 2, nuclear factor-κB and activator protein-1 (34, 73, 74). A detailed mechanism of *p62* gene expression in ALDH1-positive luminal B CSCs will need to be investigated.

High p62 expression is associated with poor prognosis in several cancer types, including breast cancer (38-48, 53-55). However, few reports have mentioned an association between *p62* gene expression and prognosis, and none have mentioned breast cancer subtypes. The present results revealed that patients with *p62*^{high} normal-like, luminal A and luminal B breast cancer exhibited poor prognosis (Figure 1C), and patients with *p62*^{high} *ALDH1A3*^{high} luminal B breast subtype had poor clinical outcomes (Figure 2B and Figure 3B; Table I). These results suggest that p62 is involved in cancer progression and contributes to poor prognosis of *ALDH1A3*^{high} patients, that is, patients who have luminal B ALDH1A3-positive breast CSCs. Among the breast cancer subtypes, p62 mostly appears to contribute to the poor prognosis of luminal B type. In normal-like and luminal A breast cancer, p62 might be associated with CSC markers other than ALDH1A1 and ALDH1A3. ALDH1A1 contributes to adhesion, migration, extravasation, initial colonization steps (28) and poor prognosis (26); however, the present results suggest that p62 contributes to survival/proliferation of and tumor formation by ALDH1A3-positive (not ALDH1A1-positive) CSCs in luminal B breast cancer (Figure 2A and Figure 3A; Table I). Thus, p62-targeted therapies may be suitable for ALDH1A3-positive CSC-enriched luminal B breast cancer.

The characteristics of luminal B type compared with the luminal A type are as follows: Luminal B is often HER2-positive and exhibits high expression of proliferation-related genes such as *MKI67*, as well as higher histological grade and worse prognosis (7, 9-15). As far as we are aware, there have been no reports to date on the association between p62 and luminal B breast cancer by PAM50 classification; however, it has been reported that the expression of p62 protein is correlated with that of HER2 in breast cancer (75, 76), and that p62 facilitates HER2-induced mammary tumorigenesis through multiple signaling pathways (77). On the other hand, one of the characteristics of luminal B type compared with HER2-enriched type is high estrogen receptor expression (4, 5, 8). Thus, the contribution of p62 to luminal B type ALDH1A3-positive CSCs may be associated with HER2 and estrogen receptor.

As shown in Figure 4C, p62 contributes to cell survival/proliferation in luminal B breast cancer cells. Xu *et al*. reported that colony numbers were suppressed by *p62* depletion *via* short hairpin RNA, and that tumor xenografts derived from stably transfected, *p62*-depleted MDA-MB-231 cells exhibited significantly reduced growth rates and numbers of Ki67-positive cells (54). Nozaki *et al*. reported that MDA-MB-453 and MFM-223 cells transfected with *p62* siRNA exhibited significantly reduced cell proliferative activity according to the results of 5-bromo-2-deoxyuridine incorporation assay (78). Notably, the results of our study revealed that *p62* KD led to stronger suppression of the viability of sorted ALDH1^{high} cells than of unsorted cells (Figure 4C and D). This suggests that the contribution of p62 to cell survival/proliferation is greater in ALDH1-positive CSCs than in other differentiated cells.

In vitro, *p62* KD suppressed the size of tumor spheres, but did not significantly reduce the number of tumor spheres formed by ALDH1^{high} cells (Figure 4E). These results indicate that p62 is involved in cell proliferation rather than in cell survival in ALDH1-positive luminal B breast CSCs.

It has been reported that p62 contributes to the characteristics exhibited by CSCs, including survival/proliferation, tumor formation (54, 78), infiltration, metastasis (55), and chemotherapy and radiotherapy resistance (47, 52, 79-81) in various cancer types, including breast cancer. p62 participates in maintaining breast cancer stem-like properties by stabilizing MYC proto-oncogene bHLH transcription factor mRNA (54), and nuclear fctor-E2-related factor 2 activation (82). It is necessary to investigate the mechanism by which p62 is involved in these properties exhibited by ALDH1-positive luminal B breast CSCs.

p62 protein interacts with atypical protein kinase C (PKCλ), and this interaction is involved in several biological functions (29, 83, 84). PKCλ is also essential for the survival and *in vitro* tumor-sphere formation of ALDH1-positive breast CSCs, and high expression of PKCλ and ALDH1A3

is associated with poor prognosis at late tumor-stage of breast cancer (68). Therefore, the association between p62 and PKCλ in ALDH1-positive luminal B breast CSCs should be further investigated.

The present study revealed that p62 contributes to survival/proliferation and tumor formation in ALDH1-positive luminal B breast cells, which leads to cancer progression and contributes to poor prognosis of ALDH1A3-positive, *i.e.* CSC-enriched, luminal B breast cancer. Overall, it can be concluded that p62 is potentially a prognostic marker and therapeutic target for ALDH1-positive luminal B breast CSCs.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

A.O., C.O., A.M., T.S. and Ke.S. performed bioinformatics; A.O., S.T., Ya.H. and A.M. performed the experiments; A.O., M.K., C.M., A.M., N.M., Yu.N. and Y.X. performed analyzed the data; Y.M., Yo.N., T.H., Yo.H., Ka.S. and S.O. supplied experimental materials and resources; A.O., H.M. and K.A. conceived the study; A.O. and K.A. drafted the article; all Authors contributed to discussion and review of the final article; and approved it.

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References

1 Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin *71(3)*: 209-249, 2021. PMID: 33538338. DOI: 10.3322/caac.21660

2 Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lønning PE, Børresen-Dale AL, Brown PO and Botstein D: Molecular portraits of human breast tumours. Nature *406(6797)*: 747-752, 2000. PMID: 10963602. DOI: 10.1038/35021093

3 Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lønning PE and Børresen-Dale AL: Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA *98(19)*: 10869-10874, 2001. PMID: 11553815. DOI: 10.1073/pnas.191367098

4 Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, Rasmussen KE, Jones LP, Assefnia S, Chandrasekharan S, Backlund MG, Yin Y, Khramtsov AI, Bastein R, Quackenbush J, Glazer RI, Brown PH, Green JE, Kopelovich L, Furth PA, Palazzo JP, Olopade OI, Bernard PS, Churchill GA, Van Dyke T and Perou CM: Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol *8(5)*: R76, 2007. PMID: 17493263. DOI: 10.1186/gb-2007-8-5-r76

5 Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, Davies S, Fauron C, He X, Hu Z, Quackenbush JF, Stijleman IJ, Palazzo J, Marron JS, Nobel AB, Mardis E, Nielsen TO, Ellis MJ, Perou CM and Bernard PS: Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol *27(8)*: 1160-1167, 2009. PMID: 19204204. DOI: 10.1200/JCO.2008.18.1370

6 Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, Cheang MC, Gelmon K, Nielsen TO, Blomqvist C, Heikkilä P, Heikkinen T, Nevanlinna H, Akslen LA, Bégin LR, Foulkes WD, Couch FJ, Wang X, Cafourek V, Olson JE, Baglietto L, Giles GG, Severi G, McLean CA, Southey MC, Rakha E, Green AR, Ellis IO, Sherman ME, Lissowska J, Anderson WF, Cox A, Cross SS, Reed MW, Provenzano E, Dawson SJ, Dunning AM, Humphreys M, Easton DF, García-Closas M, Caldas C, Pharoah PD and Huntsman D: Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS Med *7(5)*: e1000279, 2010. PMID: 20520800. DOI: 10.1371/journal.pmed.1000279

7 Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, He X and Perou CM: Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res *12(5)*: R68, 2010. PMID: 20813035. DOI: 10.1186/bcr2635

8 Romero A, Prat A, García-Sáenz JA, Del Prado N, Pelayo A, Furió V, Román JM, de la Hoya M, Díaz-Rubio E, Perou CM, Cladés T and Martín M: Assignment of tumor subtype by genomic testing and pathologic-based approximations: implications on patient's management and therapy selection. Clin Transl Oncol *16(4)*: 386-394, 2014. PMID: 23907291. DOI: 10.1007/s12094-013-1088-z

9 Chia SK, Bramwell VH, Tu D, Shepherd LE, Jiang S, Vickery T, Mardis E, Leung S, Ung K, Pritchard KI, Parker JS, Bernard PS, Perou CM, Ellis MJ and Nielsen TO: A 50-gene intrinsic subtype classifier for prognosis and prediction of benefit from adjuvant tamoxifen. Clin Cancer Res *18(16)*: 4465-4472, 2012. PMID: 22711706. DOI: 10.1158/1078-0432.CCR-12-0286

10 Dowsett M, Sestak I, Lopez-Knowles E, Sidhu K, Dunbier AK, Cowens JW, Ferree S, Storhoff J, Schaper C and Cuzick J: Comparison of PAM50 risk of recurrence score with oncotype DX and IHC4 for predicting risk of distant recurrence after endocrine therapy. J Clin Oncol *31(22)*: 2783-2790, 2013. PMID: 23816962. DOI: 10.1200/JCO.2012.46.1558

11 Prat A, Cheang MC, Martín M, Parker JS, Carrasco E, Caballero R, Tyldesley S, Gelmon K, Bernard PS, Nielsen TO and Perou CM: Prognostic significance of progesterone receptor-positive tumor cells within immunohistochemically defined luminal A breast cancer. J Clin Oncol *31(2)*: 203-209, 2013. PMID: 23233704. DOI: 10.1200/JCO.2012.43.4134

12 Ades F, Zardavas D, Bozovic-Spasojevic I, Pugliano L, Fumagalli D, de Azambuja E, Viale G, Sotiriou C and Piccart M: Luminal B breast cancer: molecular characterization, clinical management, and future perspectives. J Clin Oncol *32(25)*: 2794-2803, 2014. PMID: 25049332. DOI: 10.1200/JCO.2013.54.1870

13 Gnant M, Filipits M, Greil R, Stoeger H, Rudas M, Bago-Horvath Z, Mlineritsch B, Kwasny W, Knauer M, Singer C, Jakesz R, Dubsky P, Fitzal F, Bartsch R, Steger G, Balic M, Ressler S, Cowens JW, Storhoff J, Ferree S, Schaper C, Liu S, Fesl C, Nielsen TO and Austrian Breast and Colorectal Cancer Study Group: Predicting distant recurrence in receptor-positive breast cancer patients with limited clinicopathological risk: using the PAM50 Risk of Recurrence score in 1478 postmenopausal patients of the ABCSG-8 trial treated with adjuvant endocrine therapy alone. Ann Oncol *25(2)*: 339-345, 2014. PMID: 24347518. DOI: 10.1093/annonc/mdt494

14 Prat A, Pineda E, Adamo B, Galván P, Fernández A, Gaba L, Díez M, Viladot M, Arance A and Muñoz M: Clinical implications of the intrinsic molecular subtypes of breast cancer. Breast *24(Suppl 2)*: S26-S35, 2015. PMID: 26253814. DOI: 10.1016/j.breast.2015.07.008

15 Pu M, Messer K, Davies SR, Vickery TL, Pittman E, Parker BA, Ellis MJ, Flatt SW, Marinac CR, Nelson SH, Mardis ER, Pierce JP and Natarajan L: Research-based PAM50 signature and long-term breast cancer survival. Breast Cancer Res Treat *179(1)*: 197-206, 2020. PMID: 31542876. DOI: 10.1007/s10549-019-05446-y

16 Reya T, Morrison SJ, Clarke MF and Weissman IL: Stem cells, cancer, and cancer stem cells. Nature *414(6859)*: 105-111, 2001. PMID: 11689955. DOI: 10.1038/35102167

17 Visvader JE and Lindeman GJ: Cancer stem cells: current status and evolving complexities. Cell Stem Cell *10(6)*: 717-728, 2012. PMID: 22704512. DOI: 10.1016/j.stem.2012.05.007

18 Nguyen NP, Almeida FS, Chi A, Nguyen LM, Cohen D, Karlsson U and Vinh-Hung V: Molecular biology of breast cancer stem cells: potential clinical applications. Cancer Treat Rev *36(6)*: 485-491, 2010. PMID: 20231058. DOI: 10.1016/j.ctrv.2010.02.016

19 Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS and Dontu G: ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell *1(5)*: 555-567, 2007. PMID: 18371393. DOI: 10.1016/j.stem.2007.08.014

20 Jiang F, Qiu Q, Khanna A, Todd NW, Deepak J, Xing L, Wang H, Liu Z, Su Y, Stass SA and Katz RL: Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. Mol Cancer Res *7(3)*: 330-338, 2009. PMID: 19276181. DOI: 10.1158/1541-7786.MCR-08-0393

21 Tanei T, Morimoto K, Shimazu K, Kim SJ, Tanji Y, Taguchi T, Tamaki Y and Noguchi S: Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers. Clin Cancer Res *15(12)*: 4234-4241, 2009. PMID: 19509181. DOI: 10.1158/1078-0432.CCR-08-1479

22 Landen CN Jr, Goodman B, Katre AA, Steg AD, Nick AM, Stone RL, Miller LD, Mejia PV, Jennings NB, Gershenson DM, Bast RC Jr, Coleman RL, Lopez-Berestein G and Sood AK: Targeting aldehyde dehydrogenase cancer stem cells in ovarian cancer. Mol Cancer Ther *9(12)*: 3186-3199, 2010. PMID: 20889728. DOI: 10.1158/1535-7163.MCT-10-0563

23 Su Y, Qiu Q, Zhang X, Jiang Z, Leng Q, Liu Z, Stass SA and Jiang F: Aldehyde dehydrogenase 1 A1-positive cell population is enriched in tumor-initiating cells and associated with progression of bladder cancer. Cancer Epidemiol Biomarkers Prev *19(2)*: 327-337, 2010. PMID: 20142235. DOI: 10.1158/1055-9965.EPI-09-0865

24 Kim MP, Fleming JB, Wang H, Abbruzzese JL, Choi W, Kopetz S, McConkey DJ, Evans DB and Gallick GE: ALDH activity selectively defines an enhanced tumor-initiating cell population relative to CD133 expression in human pancreatic adenocarcinoma. PLoS One *6(6)*: e20636, 2011. PMID: 21695188. DOI: 10.1371/journal.pone.0020636

25 Marcato P, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M, Helyer L, Pan L, Leidal A, Gujar S, Giacomantonio CA and Lee PW: Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. Stem Cells *29(1)*: 32-45, 2011. PMID: 21280157. DOI: 10.1002/stem.563

26 Liu Y, Lv DL, Duan JJ, Xu SL, Zhang JF, Yang XJ, Zhang X, Cui YH, Bian XW and Yu SC: ALDH1A1 expression correlates with clinicopathologic features and poor prognosis of breast cancer patients: a systematic review and meta-analysis. BMC Cancer *14*: 444, 2014. PMID: 24938375. DOI: 10.1186/1471-2407-14-444

27 Marcato P, Dean CA, Liu RZ, Coyle KM, Bydoun M, Wallace M, Clements D, Turner C, Mathenge EG, Gujar SA, Giacomantonio CA, Mackey JR, Godbout R and Lee PW: Aldehyde dehydrogenase 1A3 influences breast cancer progression via differential retinoic acid signaling. Mol Oncol *9(1)*: 17-31, 2015. PMID: 25106087. DOI: 10.1016/j.molonc.2014.07.010

28 Croker AK, Rodriguez-Torres M, Xia Y, Pardhan S, Leong HS, Lewis JD and Allan AL: Differential functional roles of ALDH1A1 and ALDH1A3 in mediating metastatic behavior and therapy resistance of human breast cancer cells. Int J Mol Sci *18(10)*: 2039, 2017. PMID: 28937653. DOI: 10.3390/ijms18102039

29 Moscat J and Diaz-Meco MT: p62 at the crossroads of autophagy, apoptosis, and cancer. Cell *137(6)*: 1001-1004, 2009. PMID: 19524504. DOI: 10.1016/j.cell.2009.05.023

30 Puls A, Schmidt S, Grawe F and Stabel S: Interaction of protein kinase C ζ with ZIP, a novel protein kinase C-binding protein. Proc Natl Acad Sci USA *94(12)*: 6191-6196, 1997. PMID: 9177193. DOI: 10.1073/pnas.94.12.6191

31 Sanchez P, De Carcer G, Sandoval IV, Moscat J and Diaz-Meco MT: Localization of atypical protein kinase C isoforms into lysosome-targeted endosomes through interaction with p62. Mol Cell Biol *18(5)*: 3069-3080, 1998. PMID: 9566925. DOI: 10.1128/MCB.18.5.3069

32 Sanz L, Sanchez P, Lallena MJ, Diaz-Meco MT and Moscat J: The interaction of p62 with RIP links the atypical PKCs to NF-kappaB activation. EMBO J *18(11)*: 3044-3053, 1999. PMID: 10356400. DOI: 10.1093/emboj/18.11.3044

33 Sanz L, Diaz-Meco MT, Nakano H and Moscat J: The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. EMBO J *19(7)*: 1576-1586, 2000. PMID: 10747026. DOI: 10.1093/emboj/19.7.1576

34 Jain A, Lamark T, Sjøttem E, Larsen KB, Awuh JA, Øvervatn A, McMahon M, Hayes JD and Johansen T: p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. J Biol Chem *285(29)*: 22576-22591, 2010. PMID: 20452972. DOI: 10.1074/jbc.M110.118976

35 Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H and Johansen T: p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol *171(4)*: 603-614, 2005. PMID: 16286508. DOI: 10.1083/jcb.200507002

36 Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Øvervatn A, Bjørkøy G and Johansen T: p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem *282(33)*: 24131-24145, 2007. PMID: 17580304. DOI: 10.1074/jbc.M702824200

37 Ichimura Y, Kumanomidou T, Sou YS, Mizushima T, Ezaki J, Ueno T, Kominami E, Yamane T, Tanaka K and Komatsu M: Structural basis for sorting mechanism of p62 in selective autophagy. J Biol Chem *283(33)*: 22847-22857, 2008. PMID: 18524774. DOI: 10.1074/jbc.M802182200

38 Kitamura H, Torigoe T, Asanuma H, Hisasue SI, Suzuki K, Tsukamoto T, Satoh M and Sato N: Cytosolic overexpression of p62 sequestosome 1 in neoplastic prostate tissue. Histopathology *48(2)*: 157-161, 2006. PMID: 16405664. DOI: 10.1111/j.1365-2559.2005.02313.x

39 Ellis RA, Horswell S, Ness T, Lumsdon J, Tooze SA, Kirkham N, Armstrong JL and Lovat PE: Prognostic impact of p62 expression in cutaneous malignant melanoma. J Invest Dermatol *134(5)*: 1476-1478, 2014. PMID: 24270664. DOI: 10.1038/jid.2013.497

40 Kuo WL, Sharifi MN, Lingen MW, Ahmed O, Liu J, Nagilla M, Macleod KF and Cohen EE: p62/SQSTM1 accumulation in squamous cell carcinoma of head and neck predicts sensitivity to phosphatidylinositol 3-kinase pathway inhibitors. PLoS One *9(3)*: e90171, 2014. PMID: 24599075. DOI: 10.1371/journal.pone.0090171

41 Liu JL, Chen FF, Lung J, Lo CH, Lee FH, Lu YC and Hung CH: Prognostic significance of p62/SQSTM1 subcellular localization and LC3B in oral squamous cell carcinoma. Br J Cancer *111(5)*: 944-954, 2014. PMID: 24983366. DOI: 10.1038/bjc.2014.355

42 Ren F, Shu G, Liu G, Liu D, Zhou J, Yuan L and Zhou J: Knockdown of p62/sequestosome 1 attenuates autophagy and inhibits colorectal cancer cell growth. Mol Cell Biochem *385(1-2)*: 95-102, 2014. PMID: 24065390. DOI: 10.1007/s11010-013-1818-0

43 Deng D, Luo K, Liu H, Nie X, Xue L, Wang R, Xu Y, Cui J, Shao N and Zhi F: p62 acts as an oncogene and is targeted by miR-124-3p in glioma. Cancer Cell Int *19*: 280, 2019. PMID: 31708690. DOI: 10.1186/s12935-019-1004-x

44 Lei C, Zhao B, Liu L, Zeng X, Yu Z and Wang X: Expression and clinical significance of p62 protein in colon cancer. Medicine (Baltimore) *99(3)*: e18791, 2020. PMID: 32011477. DOI: 10.1097/MD.0000000000018791

45 Li T, Jiang D and Wu K: p62 promotes bladder cancer cell growth by activating KEAP1/NRF2-dependent antioxidative response. Cancer Sci *111(4)*: 1156-1164, 2020. PMID: 31967368. DOI: 10.1111/cas.14321

46 Yang W, Niu L, Zhao X, Duan L, Li Y, Wang X, Zhang Y, Zhou W, Liu J, Zhao Q, Han Y, Fan D and Hong L: Development and validation of a survival model based on autophagy-associated genes for predicting prognosis of hepatocellular carcinoma. Am J Transl Res *12(10)*: 6705-6722, 2020. PMID: 33194067.

47 Kobayashi T, Ishida M, Miki H, Matsumi Y, Fukui T, Hamada M, Tsuta K and Sekimoto M: p62 is a useful predictive marker for tumour regression after chemoradiation therapy in patients with advanced rectal cancer: an immunohistochemical study. Colorectal Dis *23(5)*: 1083-1090, 2021. PMID: 33316131. DOI: 10.1111/codi.15486

48 Yu F, Ma R, Liu C, Zhang L, Feng K, Wang M and Yin D: SQSTM1/p62 promotes cell growth and triggers autophagy in papillary thyroid cancer by regulating the AKT/AMPK/mTOR signaling pathway. Front Oncol *11*: 638701, 2021. PMID: 33937040. DOI: 10.3389/fonc.2021.638701

49 Inoue D, Suzuki T, Mitsuishi Y, Miki Y, Suzuki S, Sugawara S, Watanabe M, Sakurada A, Endo C, Uruno A, Sasano H, Nakagawa T, Satoh K, Tanaka N, Kubo H, Motohashi H and Yamamoto M: Accumulation of p62/SQSTM1 is associated with poor prognosis in patients with lung adenocarcinoma. Cancer Sci *103(4)*: 760-766, 2012. PMID: 22320446. DOI: 10.1111/j.1349-7006.2012.02216.x

50 Iwadate R, Inoue J, Tsuda H, Takano M, Furuya K, Hirasawa A, Aoki D and Inazawa J: High expression of SQSTM1/p62 protein is associated with poor prognosis in epithelial ovarian cancer. Acta Histochem Cytochem *47(6)*: 295-301, 2014. PMID: 25859063. DOI: 10.1267/ahc.14048

51 Iwadate R, Inoue J, Tsuda H, Takano M, Furuya K, Hirasawa A, Aoki D and Inazawa J: High expression of p62 protein is associated with poor prognosis and aggressive phenotypes in endometrial cancer. Am J Pathol *185(9)*: 2523-2533, 2015. PMID: 26162509. DOI: 10.1016/j.ajpath.2015.05.008

52 Arai A, Chano T, Ikebuchi K, Hama Y, Ochi Y, Tameno H and Shimada T: p62/SQSTM1 levels predicts radiotherapy resistance in hypopharyngeal carcinomas. Am J Cancer Res *7(4)*: 881-891, 2017. PMID: 28469960.

53 Thompson HG, Harris JW, Wold BJ, Lin F and Brody JP: p62 overexpression in breast tumors and regulation by prostate-derived Ets factor in breast cancer cells. Oncogene *22(15)*: 2322-2333, 2003. PMID: 12700667. DOI: 10.1038/sj.onc.1206325

54 Xu LZ, Li SS, Zhou W, Kang ZJ, Zhang QX, Kamran M, Xu J, Liang DP, Wang CL, Hou ZJ, Wan XB, Wang HJ, Lam EW, Zhao ZW and Liu Q: p62/SQSTM1 enhances breast cancer stem-like properties by stabilizing MYC mRNA. Oncogene *36(3)*: 304-317, 2017. PMID: 27345399. DOI: 10.1038/onc.2016.202

55 Li SS, Xu LZ, Zhou W, Yao S, Wang CL, Xia JL, Wang HF, Kamran M, Xue XY, Dong L, Wang J, Ding XD, Bella L, Bugeon L, Xu J, Zheng FM, Dallman MJ, Lam EWF and Liu Q: p62/SQSTM1 interacts with vimentin to enhance breast cancer metastasis. Carcinogenesis *38(11)*: 1092-1103, 2017. PMID: 28968743. DOI: 10.1093/carcin/bgx099

56 Luo RZ, Yuan ZY, Li M, Xi SY, Fu J and He J: Accumulation of p62 is associated with poor prognosis in patients with triple-negative breast cancer. Onco Targets Ther *6*: 883-888, 2013. PMID: 23888115. DOI: 10.2147/OTT.S46222

57 Yokota A, Hiramoto M, Hino H, Tokuhisa M, Miyazaki M, Kazama H, Takano N and Miyazawa K: Sequestosome 1 (p62) accumulation in breast cancer cells suppresses progesterone receptor expression via argonaute 2. Biochem Biophys Res Commun *531(2)*: 256-263, 2020. PMID: 32800344. DOI: 10.1016/j.bbrc.2020.07.058

58 Cancer Genome Atlas Network: Comprehensive molecular portraits of human breast tumours. Nature *490(7418)*: 61-70, 2012. PMID: 23000897. DOI: 10.1038/nature11412

59 Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A and Chinnaiyan AM: ONCOMINE: a

cancer microarray database and integrated data-mining platform. Neoplasia *6(1)*: 1-6, 2004. PMID: 15068665. DOI: 10.1016/s1476-5586(04)80047-2

60 Tamori S, Nozaki Y, Motomura H, Nakane H, Katayama R, Onaga C, Kikuchi E, Shimada N, Suzuki Y, Noike M, Hara Y, Sato K, Sato T, Yamamoto K, Hanawa T, Imai M, Abe R, Yoshimori A, Takasawa R, Tanuma SI and Akimoto K: Glyoxalase 1 gene is highly expressed in basal-like human breast cancers and contributes to survival of ALDH1-positive breast cancer stem cells. Oncotarget *9(92)*: 36515-36529, 2018. PMID: 30559934. DOI: 10.18632/oncotarget.26369

61 Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, Gräf S, Ha G, Haffari G, Bashashati A, Russell R, McKinney S, METABRIC Group, Langerød A, Green A, Provenzano E, Wishart G, Pinder S, Watson P, Markowetz F, Murphy L, Ellis I, Purushotham A, Børresen-Dale AL, Brenton JD, Tavaré S, Caldas C and Aparicio S: The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature *486(7403)*: 346-352, 2012. PMID: 22522925. DOI: 10.1038/nature10983

62 Pereira B, Chin SF, Rueda OM, Vollan HK, Provenzano E, Bardwell HA, Pugh M, Jones L, Russell R, Sammut SJ, Tsui DW, Liu B, Dawson SJ, Abraham J, Northen H, Peden JF, Mukherjee A, Turashvili G, Green AR, McKinney S, Oloumi A, Shah S, Rosenfeld N, Murphy L, Bentley DR, Ellis IO, Purushotham A, Pinder SE, Børresen-Dale AL, Earl HM, Pharoah PD, Ross MT, Aparicio S and Caldas C: The somatic mutation profiles of 2,433 breast cancers refines their genomic and transcriptomic landscapes. Nat Commun *7*: 11479, 2016. PMID: 27161491. DOI: 10.1038/ncomms11479

63 Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C and Schultz N: The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov *2(5)*: 401-404, 2012. PMID: 22588877. DOI: 10.1158/2159-8290.CD-12-0095

64 Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C and Schultz N: Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal *6(269)*: pl1, 2013. PMID: 23550210. DOI: 10.1126/scisignal.2004088

65 Kim DH, Behlke MA, Rose SD, Chang MS, Choi S and Rossi JJ: Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat Biotechnol *23(2)*: 222-226, 2005. PMID: 15619617. DOI: 10.1038/nbt1051

66 Nozaki Y, Tamori S, Inada M, Katayama R, Nakane H, Minamishima O, Onodera Y, Abe M, Shiina S, Tamura K, Kodama D, Sato K, Hara Y, Abe R, Takasawa R, Yoshimori A, Shinomiya N, Tanuma SI and Akimoto K: Correlation between c-Met and ALDH1 contributes to the survival and tumor-sphere formation of ALDH1 positive breast cancer stem cells and predicts poor clinical outcome in breast cancer. Genes Cancer *8(7-8)*: 628-639, 2017. PMID: 28966724. DOI: 10.18632/genesandcancer.148

67 Motomura H, Nozaki Y, Onaga C, Ozaki A, Tamori S, Shiina TA, Kanai S, Ohira C, Hara Y, Harada Y, Takasawa R, Hanawa T, Tanuma SI, Mano Y, Sato T, Sato K and Akimoto K: High expression of *c-Met*, *PKCλ* and *ALDH1A3* predicts a poor prognosis in late-stage breast cancer. Anticancer Res *40(1)*: 35-52, 2020. PMID: 31892551. DOI: 10.21873/anticanres.13924

68 Nozaki Y, Motomura H, Tamori S, Kimura Y, Onaga C, Kanai S, Ishihara Y, Ozaki A, Hara Y, Harada Y, Mano Y, Sato T, Sato K, Sasaki K, Ishiguro H, Ohno S and Akimoto K: High PKCλ expression is required for ALDH1-positive cancer stem cell function and indicates a poor clinical outcome in late-stage breast cancer patients. PLoS One *15(7)*: e0235747, 2020. PMID: 32658903. DOI: 10.1371/journal.pone.0235747

69 Motomura H, Ozaki A, Tamori S, Onaga C, Nozaki Y, Waki Y, Takasawa R, Yoshizawa K, Mano Y, Sato T, Sasaki K, Ishiguro H, Miyagi Y, Nagashima Y, Yamamoto K, Sato K, Hanawa T, Tanuma SI, Ohno S and Akimoto K: Glyoxalase 1 and protein kinase Cλ as potential therapeutic targets for late-stage breast cancer. Oncol Lett *22(1)*: 547, 2021. PMID: 34093768. DOI: 10.3892/ol.2021.12808

70 Motomura H, Tamori S, Yatani MA, Namiki A, Onaga C, Ozaki A, Takasawa R, Mano Y, Sato T, Hara Y, Sato K, Xiong Y, Harada Y, Hanawa T, Tanuma SI, Sasaki K, Ohno S and Akimoto K: GLO 1 and PKCλ regulate ALDH1-positive breast cancer stem cell survival. Anticancer Res *41(12)*: 5959-5971, 2021. PMID: 34848450. DOI: 10.21873/anticanres.15415

71 Onaga C, Tamori S, Motomura H, Ozaki A, Matsuda C, Matsuoka I, Fujita T, Nozaki Y, Hara Y, Kawano Y, Harada Y, Sato T, Mano Y, Sato K and Akimoto K: High *SLC20A1* expression is associated with poor prognoses in claudin-low and basal-like breast cancers. Anticancer Res *41(1)*: 43-54, 2021. PMID: 33419798. DOI: 10.21873/anticanres.14750

72 Li L, Shen C, Nakamura E, Ando K, Signoretti S, Beroukhim R, Cowley GS, Lizotte P, Liberzon E, Bair S, Root DE, Tamayo P, Tsherniak A, Cheng SC, Tabak B, Jacobsen A, Hakimi AA, Schultz N, Ciriello G, Sander C, Hsieh JJ and Kaelin WG Jr: SQSTM1 is a pathogenic target of 5q copy number gains in kidney cancer. Cancer Cell *24(6)*: 738-750, 2013. PMID: 24332042. DOI: 10.1016/j.ccr.2013.10.025

73 Duran A, Linares JF, Galvez AS, Wikenheiser K, Flores JM, Diaz-Meco MT and Moscat J: The signaling adaptor p62 is an important NF-kappaB mediator in tumorigenesis. Cancer Cell *13(4)*: 343-354, 2008. PMID: 18394557. DOI: 10.1016/j.ccr.2008.02.001

74 Ling J, Kang Y, Zhao R, Xia Q, Lee DF, Chang Z, Li J, Peng B, Fleming JB, Wang H, Liu J, Lemischka IR, Hung MC and Chiao PJ: $Kras^{G12D}$-induced IKK2/β/NF-κB activation by IL-1α and p62 feedforward loops is required for development of pancreatic ductal adenocarcinoma. Cancer Cell *21(1)*: 105-120, 2012. PMID: 22264792. DOI: 10.1016/j.ccr.2011.12.006

75 Rolland P, Madjd Z, Durrant L, Ellis IO, Layfield R and Spendlove I: The ubiquitin-binding protein p62 is expressed in breast cancers showing features of aggressive disease. Endocr Relat Cancer *14(1)*: 73-80, 2007. PMID: 17395976. DOI: 10.1677/erc.1.01312

76 Choi J, Kim DH, Jung WH and Koo JS: Metabolic interaction between cancer cells and stromal cells according to breast cancer molecular subtype. Breast Cancer Res *15(5)*: R78, 2013. PMID: 24020991. DOI: 10.1186/bcr3472

77 Cai-McRae X, Zhong H and Karantza V: Sequestosome 1/p62 facilitates HER2-induced mammary tumorigenesis through multiple signaling pathways. Oncogene *34(23)*: 2968-2977, 2015. PMID: 25088198. DOI: 10.1038/onc.2014.244

78 Nozaki F, Hirotani Y, Nakanishi Y, Yamaguchi H, Nishimaki H, Noda H, Tang X, Yamamoto H, Suzuki A, Seki T and Masuda S: p62 regulates the proliferation of molecular apocrine breast cancer

cells. Acta Histochem Cytochem *49(4)*: 125-130, 2016. PMID: 27682016. DOI: 10.1267/ahc.16013

79 Yan XY, Zhang Y, Zhang JJ, Zhang LC, Liu YN, Wu Y, Xue YN, Lu SY, Su J and Sun LK: p62/SQSTM1 as an oncotarget mediates cisplatin resistance through activating RIP1-NF-κB pathway in human ovarian cancer cells. Cancer Sci *108(7)*: 1405-1413, 2017. PMID: 28498503. DOI: 10.1111/cas.13276

80 Wang Z, Zhang J, Li M, Kong L and Yu J: The expression of p-p62 and nuclear Nrf2 in esophageal squamous cell carcinoma and association with radioresistance. Thorac Cancer *11(1)*: 130-139, 2020. PMID: 31755241. DOI: 10.1111/1759-7714.13252

81 Xu L, Xu F, Kong Q, Yang T, Tan D, Zhang X, Li N, Zhao S, Zhao J and Li M: Inhibition of p62/SQSTM1 sensitizes small-cell lung cancer cells to cisplatin-induced cytotoxicity by targeting NEDD9 expression. Mol Carcinog *59(8)*: 967-979, 2020. PMID: 32424979. DOI: 10.1002/mc.23215

82 Ryoo IG, Choi BH, Ku SK and Kwak MK: High CD44 expression mediates p62-associated NFE2L2/NRF2 activation in breast cancer stem cell-like cells: Implications for cancer stem cell resistance. Redox Biol *17*: 246-258, 2018. PMID: 29729523. DOI: 10.1016/j.redox.2018.04.015

83 Suzuki A, Akimoto K and Ohno S: Protein kinase C λ/ι (PKCλ/ι): a PKC isotype essential for the development of multicellular organisms. J Biochem *133(1)*: 9-16, 2003. PMID: 12761193. DOI: 10.1093/jb/mvg018

84 Reina-Campos M, Diaz-Meco MT and Moscat J: The dual roles of the atypical protein kinase Cs in cancer. Cancer Cell *36(3)*: 218-235, 2019. PMID: 31474570. DOI: 10.1016/j.ccell.2019.07.010

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