# Assessment of the expression of microRNAs-221-3p, -146a-5p, -16-5p and BCL2 in oncocytic carcinoma of the breast: A case report

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**Abstract.** Oncocytic carcinoma of the breast is rare and its molecular profiles remain poorly understood. MicroRNAs (miRNAs/miRs) have been identified as contributors to carcinogenesis at the post-transcriptional level; thus, an aberrant expression of miRNAs has attracted attention as a potential biomarker of numerous diseases, including cancer. The present study reports the case of a 76-year-old woman diagnosed with oncocytic carcinoma of the breast. Considering the distinctive feature of oncocytic carcinoma of the breast, which is the presence of granular eosinophilic cytoplasm containing numerous mitochondria, the present study hypothesized that the expression of mitochondria-related miRNAs could be altered in oncocytic carcinomas. Aberrant expression levels of the miRNAs previously reported as mitochondria-related miRNAs, such as miR-221-3p, -146a-5p and -16-5p, were revealed in tissue from specimens of oncocytic carcinoma of the breast, compared with that of a more typical type of invasive ductal carcinoma of the breast. The present study highlights the changes in miRNA expression in oncocytic carcinoma of the breast, suggesting its potential as a biomarker for diagnosis.

## Introduction

Oncocytic carcinomas (OC) are rare malignant tumors composed of oncocytes, which are epithelial cells characterized by a granular eosinophilic cytoplasm containing numerous mitochondria (1). OC of the breast is uncommon, accounting for <1% of all types of breast cancer worldwide (2). Hence, little is known about its pathological molecular features.

MicroRNAs (miRNAs/miRs) are small, non-coding RNAs that play an important role in post-transcriptional gene regulation (3,4). They interact with their target messenger RNAs (mRNAs) to control translation through mRNA degradation or translational repression (5,6). miRNAs are contributors to various biological processes and previous studies have revealed the relevance of miRNA biology in cancer (7-9).

Recently, the presence of miRNAs in mitochondria, known as mitochondrial miRNAs (mito-miRs), have emerged as modulators that target the mitochondria and regulate mitochondrial protein expression and function (10,11). The present study hypothesized that expression of mitochondrial miRNAs could be altered in OC, given its characteristic features. The present study reports the case of a 76-year-old woman diagnosed with oncocytic carcinoma of the breast. To test the aforementioned hypothesis, the present study assessed the expression levels of the miRNAs previously reported to be mito-miRs, such as miR-221-3p, -146a-5p and -16-5p, in tissues from specimens of the patient with OC compared with that in tissues from specimens of a more typical type of invasive ductal carcinoma (IDC) of the breast. The present study also investigated the mRNA expression levels of BCL2, which plays a pivotal role in apoptosis in mitochondria and is the corresponding target of the aforementioned miRNAs.

## Case report

A 76-year-old woman with a left breast tumor was referred to JA Hiroshima General Hospital (Hatsukaichi, Japan) in June 2019. The patient had noticed a gradual enlargement of the mass over the past 3 years. Physical examination revealed a 65-mm, hard, non-mobile mass with a focal cystic appearance in the upper inner quadrant of the left breast (Fig. 1A). Ultrasonography revealed a solid mass with a marked cystic component, potentially indicating hemorrhage and swelling of

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the left axillary lymph nodes (Fig. 1B). Magnetic resonance imaging revealed a solid mass with a cystic component and apparent involvement of the skin and pectoralis major muscle (Fig. 1C). Additionally, positron emission tomography showed abnormal uptake in the mass and axillary lymph nodes (maximum standardized uptake values, 10.2 and 8.0, respectively) but no evidence of systemic metastases (Fig. 1D and E). A core needle biopsy was performed and the mass was diagnosed as an invasive carcinoma. Cytological examination of a specimen obtained with fine-needle aspiration of the lymph node revealed the presence of malignant cells. Subsequently, ~1 month after the first visit, the patient underwent mastectomy and axial lymph node dissection.

The tumor measured 45x12 mm, and four lymph nodes were involved (Fig. 2A and B). Hematoxylin and eosin (HE) staining revealed a granular cytoplasm, ranging from eosinophilic to clear, in ovoid cancer cells (Fig. 2C). In addition, strongly positive immunostaining for antimitochondrial antibodies indicated that the tumor cells were of oncocytic origin (Fig. 2D). Further, immunohistochemical (IHC) staining showed that the tumor cells were positive for estrogen and progesterone receptors but negative for human epidermal growth factor receptor 2 (HER2) and gross cystic disease fluid protein 15 (GCDFP-15; Fig. 2E). The Ki-67 labeling index was 15%. Based on these results, the diagnosis of OC of the left breast was made. The patient underwent postmastectomy radiation therapy 1 month after surgery, receiving 50 Gy in 25 fractions to the chest wall and supraclavicular lymph nodes, sequentially followed by adjuvant endocrine therapy with an aromatase inhibitor. In the 3 years after surgery, there was no cancer recurrence detected by regular 3-month follow-up visits.

For molecular profiling, the present study collected fresh tissue samples from the surgical specimens (both tumor and non-cancerous samples) and stored them at -80˚C until use. The tumor tissues were acquired from the largest cross-section of the surgical specimen of the breast immediately after resection. At the same time, the non-cancerous tissue was acquired from another area ≥5 cm distant from the cancerous region of the surgical specimen that was confirmed grossly as normal breast tissue. The opposite cross section of this non-cancerous sample was microscopically confirmed as normal breast tissue. For comparison, fresh tissue samples of IDC were also collected from a 72-year-old female patient who underwent a consecutive surgery at the same hospital on the same day without planned recruitment with a matching phenotype (positive estrogen and progesterone receptors and negative HER2) who underwent surgery in JA Hiroshima General Hospital in July 2019. Both tumor and non-cancerous samples were also acquired from this patient in the same manner for the patient with oncocytic carcinoma. The present study obtained written permission from this patient with IDC to utilize surgical specimens for research purposes.

Reverse transcription-quantitative PCR (RT-qPCR) was used to evaluate the expression levels of miR-221-3p, -146a-5p and -16-5p as mito-miRs and BCL2 mRNA. MiRNAs were extracted using a miRNeasy Mini kit (QIAGEN, Ltd.) according to the manufacturer's protocol. RT-qPCR was performed using TaqMan MicroRNA Assays (Applied Biosystems; Thermo Fisher Scientific, Inc.), which includes TaqMan MGB probes that contains FAM™ dye (Applied Biosystems; Thermo Fisher Scientific, Inc.) as previously described (12). All primers were obtained from Applied Biosystems: MiR-221 (Assay ID: 000524_mat), miR-146a (000468_mat) and miR-16 (000391_mat). U6 small nuclear RNA and glyceraldehyde 3-phosphate dehydrogenase were used for normalization in RT-qPCR with miRNAs and mRNA, respectively. RT-PCR was performed at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. All experiments were conducted in triplicate. For RT-qPCR, the results were calculated as the mean ± standard deviation of triplicate data, and the expression was illustrated as a fold difference of 1 for non-cancerous samples (normal).

Additionally, the BCL2 protein level was determined using IHC staining using 10% neutral buffered formalin-fixed for 24 h at room temperature, paraffin-embedded samples and assessed H-score, an immunoreactivity semiquantitative scoring system (13). Paraffin-embedded sections with a thickness of 4 $\mu$m were deparaffinized with xylene and washed with serially diluted ethanol for rehydration, antigen retrieval was accomplished using pH 9.0 conditional buffer for 20 min until the temperature reached 98˚C. The endogenous peroxidase activity was blocked with 3-4% (V/V) hydrogen peroxide solution at room temperature for 5 min. Sections were incubated with Ready-To-Use anti-BCL2 antibody (PA0117; Leica Biosystems) at room temperature for 60 min. Ready-To-Use Post Primary Mouse Linker as secondary antibody (DS9800; Leica Biosystems) was applied to the slides at room temperature for 10 min followed by horseradish peroxidase-labeled anti-rabbit IgG antibody (DS9800; Leica Biosystems) at room temperature for 10 min. Application of Leica Bond Polymer Refine Detection at room temperature for 10 min (DS9800; Leica Biosystems) with 3,3'-diaminobenzidine as the chromogen was followed by counterstaining with hematoxylin at room temperature for 5 min. We observed the samples using an light microscope. The scores were all noted for the staining intensity (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining). The BCL2 H-score was then calculated using the following formula: H-score=[1*(% cells 1+) + 2*(% cells 2+) + 3*(% cells 3+)] (14). Notably, the expression level of miR-221-3p was increased, while that of miR-146a-5p and miR-16-5p were decreased in OC compared with that in IDC (Fig. 3A-C). Moreover, the mRNA expression of BCL2 was 2.26-fold higher in OC compared with in IDC (Fig. 3D), which could further explain the data obtained from IHC staining, which showed a higher intensity of BCL2 protein in OC (H-score, 98.4) compared with that in IDC (H-score, 73.6) referred to as non-cancerous sample (Fig. 3E-G).

## Discussion

Although miRNAs have been explored as important biomarkers in numerous fields, there are few studies on OC of the breast because of its rarity. The present report presents a case of OC of the breast, along with expression analysis of three mito-miRNAs (miR-221-3p, -146a-5p and -16-5p) in tissue samples from OC compared with those of IDC. Additionally, the present study assessed the expression level of BCL2 mRNA using the same samples. The differential expression of these miRNAs and mRNA in this study may
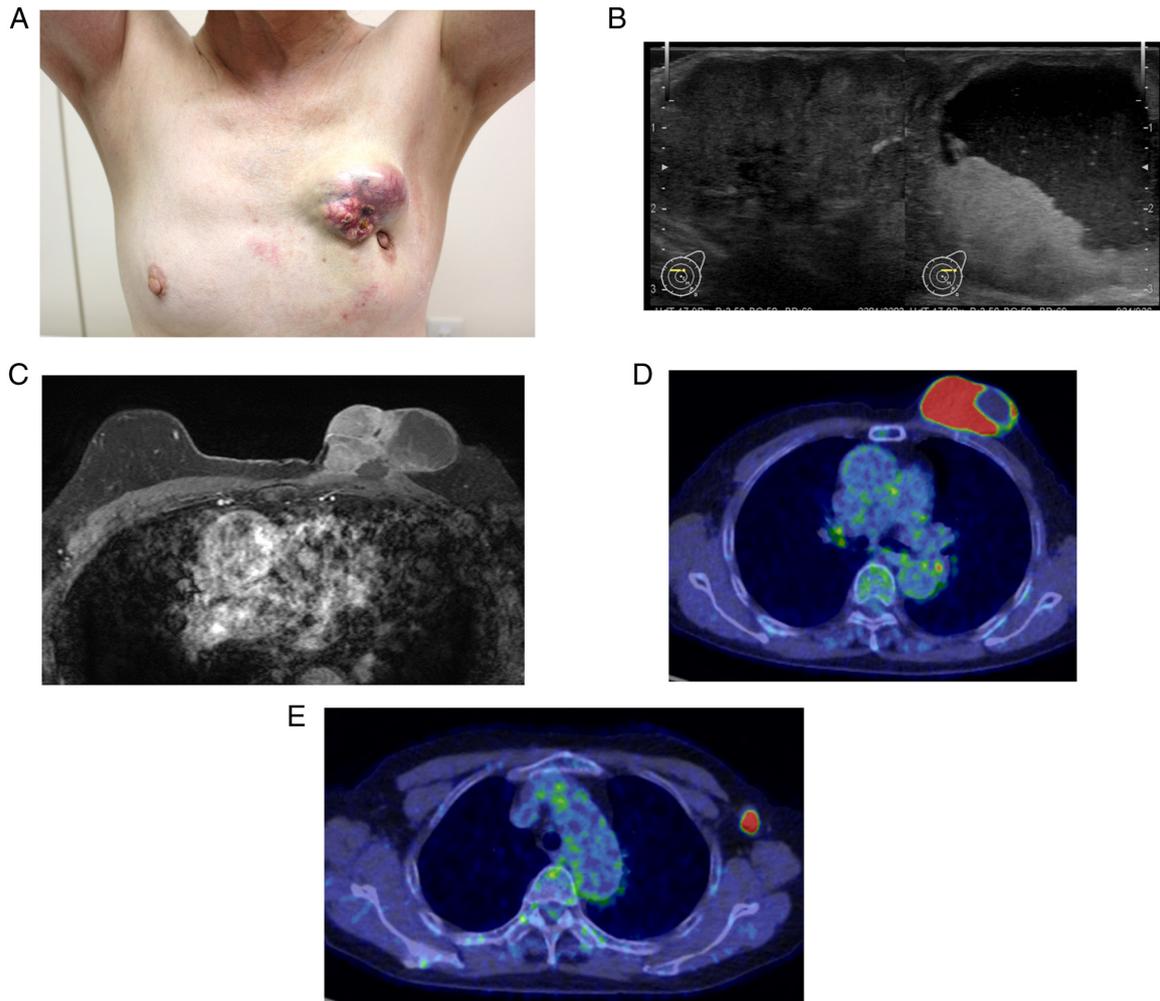
Figure 1. Preoperative imaging studies. (A) Gross appearance of the left breast. (B) Ultrasonographic view of a mass with uncircumscribed margin, showing a complex cystic and solid echo pattern. (C) Magnetic resonance image of a partially enhanced, heterogeneous mass indicating invasion into the skin and pectoralis major muscle. Positron emission tomography-computed tomography images showing 18-F fluorodeoxyglucose uptake values of (D) 10.2 for the breast tumor and (E) 8.0 for the axial lymph node.

provide valuable insights into the molecular features of OC of the breast.

The World Health Organization classification defines OC of the breast as a tumor in which >70% of all cells have oncocytic features (2). The distinctive feature of oncocytes is the enlargement of cells containing abundant eosinophilic granules in their cytoplasm due to an increase in altered mitochondria (15). OC morphologically resembles apocrine carcinoma, which also has an abundant eosinophilic granular cytoplasm (16). Consequently, distinguishing OC from apocrine carcinoma solely based on HE staining is challenging. Accurate diagnosis of OC necessitates IHC staining using mitochondrial markers and GCDFP-15, a marker for apocrine differentiation, which must be absent for the diagnosis of OC. Ragazzi et al (17) reported that oncocytic breast carcinoma shows a trend for shorter survival based on the analysis of 104 cases, including not otherwise specified invasive breast carcinomas, using mitochondrial immunostaining. Therefore, oncocytic breast carcinomas should be considered in differential diagnosis for cytoplasmic granular eosinophilia. In the present case, almost all tumor cells showed a positive staining for antimitochondrial markers and

a negative staining for GCDFP-15 following the diagnosis as OC of the breast.

Increasing evidence in recent years has indicated that miRNAs participate in regulating gene expression at the post-transcriptional level (4). Consequently, the aberrant expression of miRNAs has garnered attention as a potential biomarker in disease development, including cancer (9). A previous study revealed that miR-221-3p is induced by docosahexaenoic acid (18). MiR-146a-5p is expressed in various immune cells, plays a role in regulating inflammatory response and is a potential biomarker for vascular complications of diabetes (19,20). Furthermore, miR-16-5p is associated with diabetes and Alzheimer's disease progression (21). Additionally, differential expression of mito-miRs has been identified in numerous diseases, such as heart failure, indicating their specific function in the pathogenic process (22,23). Despite advancements in understanding the underlying mechanisms involving mito-miRs, the contribution of mito-miRs in oncocytic tumorigenesis remains unclear. Using gene and miRNA expression analyses focusing specifically on mitochondrial function and its interactions in follicular thyroid tumors, miRNAs were found to be involved
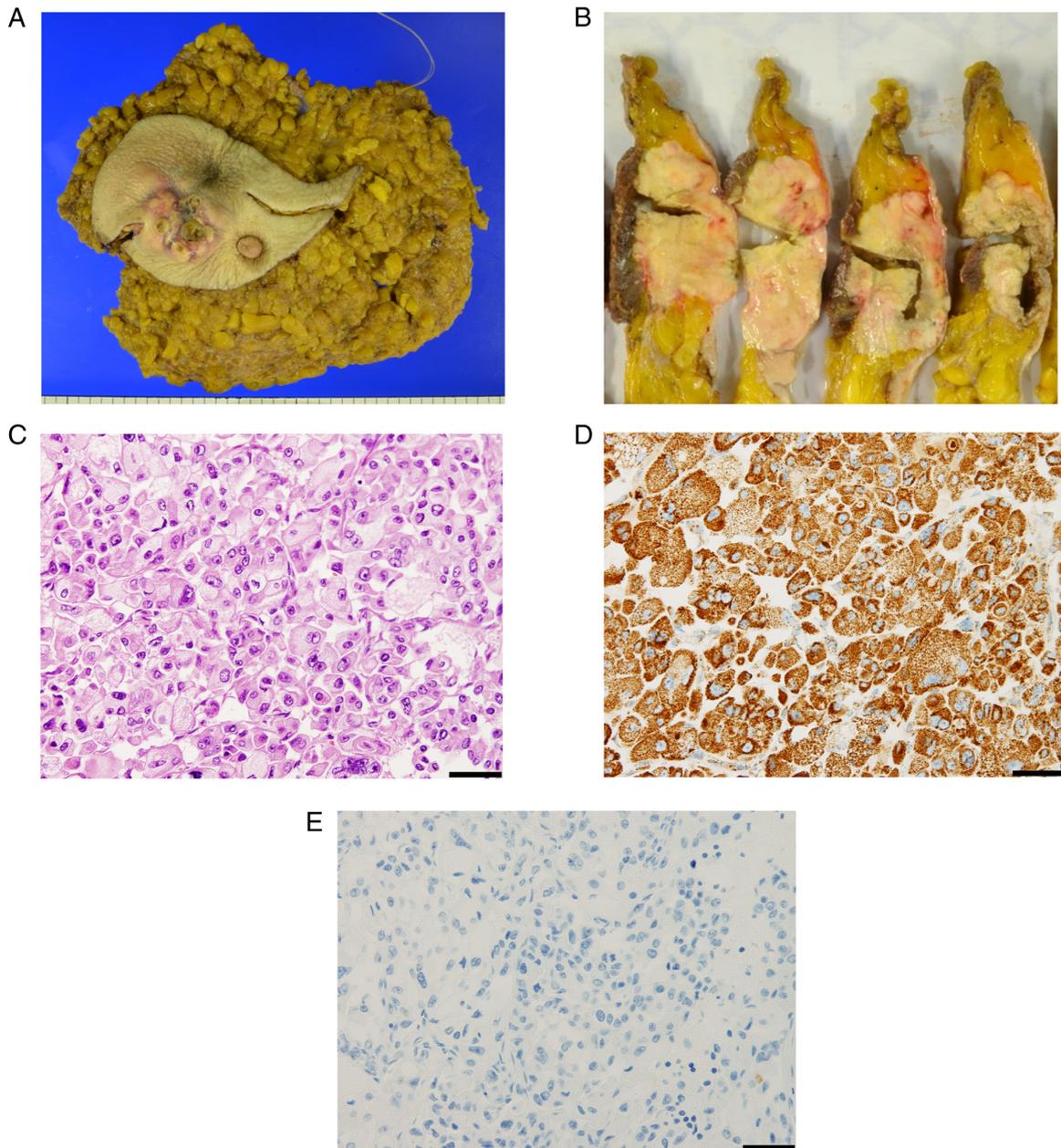
Figure 2. Pathological findings in the surgical specimen from the left breast. (A) Macroscopic appearance. (B) A slice of the specimen showing involvement of the skin and pectoralis major muscle. (C) Hematoxylin and eosin staining showing tumor cells with abundant acidophilic granular cytoplasm (magnification, x400; scale bar, 50 $\mu$m). Immunohistochemical staining showing (D) positive antimitochondrial antibody and (E) negative gross cystic disease fluid protein 15 (magnification, x400; scale bar, 50 $\mu$m).

in the development of oncocytic variants. However, to the best of our knowledge, there are few reports of oncocytic breast carcinoma and no study has explored the relationship between mito-miRs and oncocytic variants (24). Considering mitochondria accumulation as the hallmark of OC, the present study hypothesized that the expression of mito-miRs would be altered in OC in contrast to IDC. A previous study identified the diagnostic utility of multiple mi-RNAs combination using miR-221, -146-b and -375 for Hürthle cell (oncocytic) thyroid tumors (25). Additionally, the upregulation of miR-221 is a characteristic of the OC of the thyroid and kidney (26). Similarly, the present data showed that expression of miR-221-3p was significantly elevated in OC compared with that in IDC. The present study also observed the differential

expression of miR-146a-5p and -16-5p. A previous study that used cultured breast cancer cells derived from a hormone receptor negative and HER2 negative phenotype different from the present study showed that miR-146a is poorly expressed in cancer cells compared with non-cancer breast cells (27). In addition, Chen *et al* (28) reported that miR-146a is relatively less expressed in lung cancer lesions compared with normal lung tissues. However, these observations have been poorly described, and the miRNA expression of OC of the breast has not been investigated in OC of the breast due to its rarity.

The present study performed molecular profiling focused on BCL2 as the potential target gene of miR-221-3p, -146a-5p and -16-5p. Although it has not been proven that miR-221-3p directly regulates BCL2, miR-221-3p has been reported to
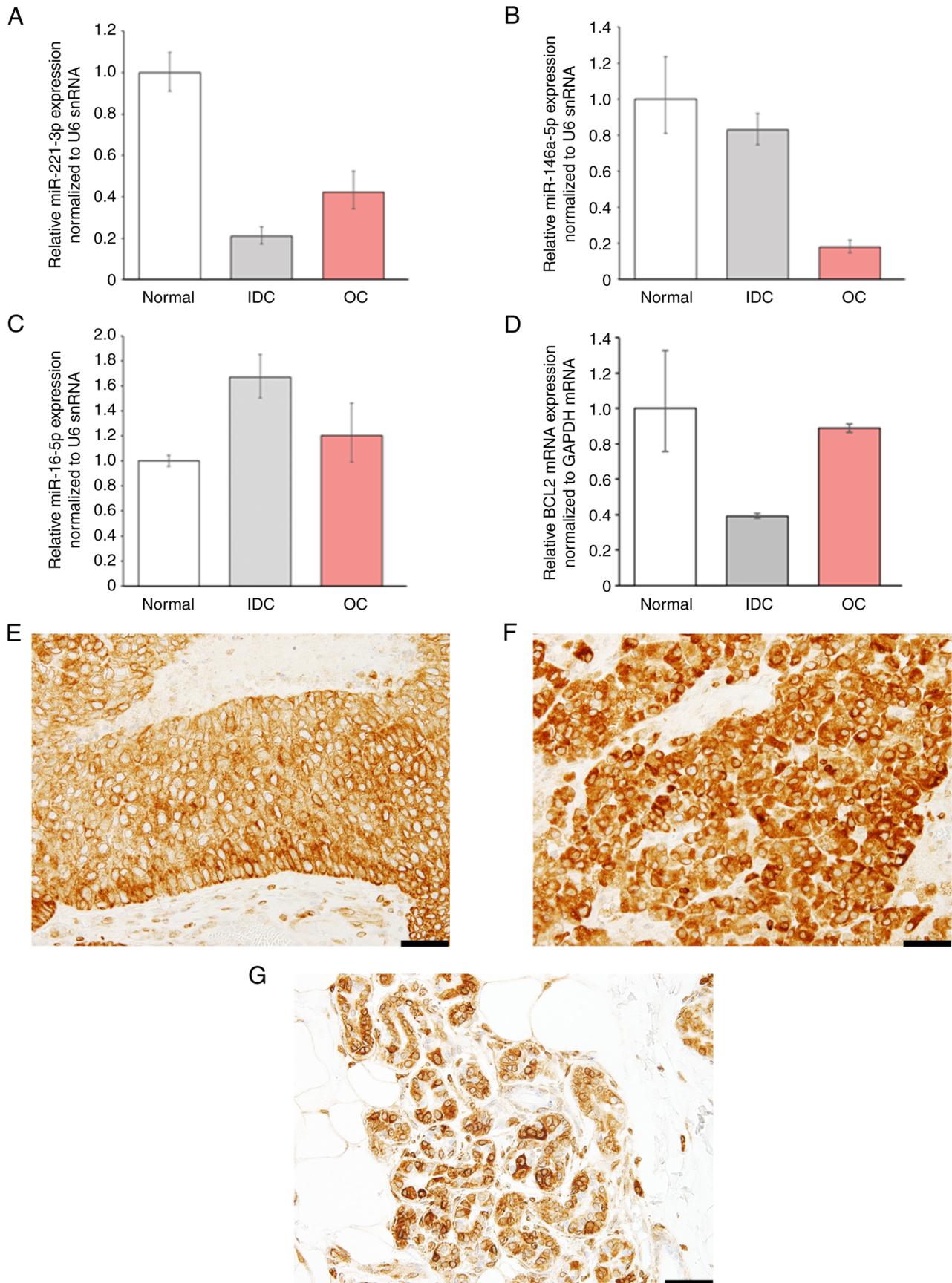
Figure 3. Differential expression of miR-221-3p, miR-146a-5p, miR-16-5p and BCL2 in OC and IDC. The relative expression level, examined via RT-qPCR, of (A) miR-221-3p, (B) miR-146a-5p, (C) miR-16-5p and (D) BCL2 messenger RNA in OC and IDC compared with non-cancerous tissue (normal). Immunohistochemical staining of BCL2 in (E) IDC, (F) OC and (G) normal (scale bar, 50 $\mu$m). The intensity of staining was stronger in OC compared with in IDC (magnification, x400). All RT-qPCR experiments were conducted in triplicate. Data are represented as mean ± standard deviation, presented as the fold-change compared with non-cancerous tissue. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; miRNA, microRNA; OC, oncocytic carcinoma; IDC, invasive ductal carcinoma; RT-qPCR, reverse transcription-quantitative PCR.

target PUMA (p53 upregulated modulator of apoptosis), a BCL2 family member also called BCL2 binding component 3, showing that downregulation of BCL2 is observed after miR-221 reduction (29). Current research has suggested that miR-146a-5p functions as a tumor suppressor that regulates apoptosis via the mitochondrial pathway by targeting anti-apoptotic proteins in aging-related diseases, including cancer (30). Apoptosis is a specific mechanism for programmed cell death, and its dysregulation can induce carcinogenesis. BCL2 is one of the fundamental anti-apoptotic factors involved in maintaining homeostasis between cell formation and death and controlling mitochondrial function (31-33). Although the underlying mechanisms have not been elucidated, a recent study demonstrated that mito-miRs appear to have a close relationship with mitochondrial protein expression (34). The higher expression of BCL2 and lower expression of miR-146a-5p in OC of the breast when compared with that in IDC in the present study suggests that miR-146a-5p may be involved in the altered expression of BCL2 through mitochondrial metabolism. Direct interaction between miR-16 and BCL2 has been reported in chronic lymphocytic leukemia, and loss of miR-16 is involved in overexpression of BCL2 (35). Although its interaction in breast cancer has not been shown, the overexpression of miR-16 has been reported to inhibit the activation of BMI1, a member of the BCL2 family, resulting in apoptosis. The present findings are consistent with these reports, as a lower expression of miR-16-5p and higher expression of BCL2 was observed in OC compared with in the more common IDC (36,37).

The present study has several limitations. First, only one sample of OC was investigated; multiple OC samples could not be obtained because of its rarity. We acknowledge that our results would be insufficient to generalize to all cases of OC; however, providing descriptions of molecular profiling in a fresh sample is important and beneficial for patients with rare tumors, from both clinical and translational standpoints (38). In addition, the current analysis was conducted using tissue samples; hence, there is no proof that the current study observed expression differences directly related to miRNAs, specifically inside the mitochondrial compartment. Further, the present study was unable to identify the mechanisms underlying the association between the three miRNAs and BCL2 in OC.

Nevertheless, the present findings may help improve the understanding of this rare tumor, given the current lack of knowledge regarding the expression and function of miRNA in OC of the breast.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

RT collected the clinical data. YK wrote the manuscript. KK, YK, and MO acquired and interpreted clinical data. YD performed the pathological examination. YY, SF, and HT performed and analyzed the experimental examination. MO revised the manuscript. YK and MO confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

This study was approved by the Ethical Committee for Human Genome Research of Hiroshima University (approval no. IRINHIM129). Written informed consent was obtained from the patient before enrollment.

## Patient consent for publication

Written informed consent for the publication of the clinical data, including photos and images, was obtained from the patient.

## Competing interests

The authors declare that they have no competing interests.

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