Diminished Expression of *MiR-15a* Is an Independent Prognostic Marker for Breast Cancer Cases

YOSHIAKI SHINDEN[1,3], SAYURI AKIYOSHI[2], HIROKI UEO[1], SYO NAMBARA[1], TOMOKO SAITO[1], HISATERU KOMATSU[1], MASAMI UEDA[1], HIDENARI HIRATA[1], SHOTARO SAKIMURA[1], RYUTARO UCHI[1], YUKI TAKANO[1], TOMOHIRO IGUCHI[1], HIDETOSHI EGUCHI[1], KEISHI SUGIMACHI[1], YUKO KIJIMA[3], HIROAKI UEO[4], SHOJI NATSUGOE[3] and KOSHI MIMORI[1]

[1]*Department of Surgery, Kyushu University Beppu Hospital, Beppu, Oita, Japan;*
[2]*National Hospital Organization Kyushu Cancer Center, Minamiku, Fukuoka, Fukuoka, Japan;*
[3]*Department of Digestive Surgery, Breast and Thyroid Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, Sakuragaoka, Kagoshima, Japan;*
[4]*Ueo Breast Surgery Hospital, Haya, Oita, Japan*

Abstract. *Background/Aim: MiR-15a targets Cyclin E1 (CCNE1), which regulates the cell cycle and promotes cell proliferation and progression. Herein, we investigated the clinicopathological significance of miR-15a as a prognostic marker in breast cancer (BC) cases. Materials and Methods: We collected primary tumor samples of 230 BC cases, including 68 triple-negative cases. The expression levels of miR-15a in primary tumors were measured by qRT-PCR assay. Results: Low expression of miR-15a in primary tumors was significantly correlated with shorter disease-free survival (p=0.0012) and overall survival (p=0.005) compared to the high miR-15a expression in triple-negative BC cases. Multivariate analysis indicated that low miR-15a expression was an independent prognostic factor for overall survival [RR=2.56(1.03-7.18), p=0.04]. Conclusion: MiR-15a expression levels could be a promising biological and prognostic marker for overall survival especially in triple-negative BC cases.*

Breast cancer (BC) is a leading cause of cancer death among women in industrialized countries. However, despite the advances technologies in diagnosis and treatment of BC, recurrence and metastasis are still serious clinical problems. An accumulation of knowledge over the molecular heterogeneity of BC has led to the consideration of BC as a number of distinct pathological entities. Furthermore, technological advances, particularly in genomics and transcriptomics, have led to the ability to improve diagnosis and treatment by identification of novel molecules that correlate with tumorigenesis and tumor progression (1).

Changes in the levels of miRNA are involved in the initiation and progression of human cancers due to alteration of translation of various target genes (2). The recent rise of interest in miRNAs is described to the breakthrough discovery of their role in many pathological processes, including their involvement in malignant transformation (3). miRNAs are implicated in the pathogenesis of different types of cancers, including leukemias (4), glioblastomas (5) and BC (6).

MiR-15a has been shown to have many important roles in cancer development. For example, *miR-15a* targets *CCNE1*, which regulates the G_0 to G_1 phase transition and promotes cell-cycle progression (7). Luo *et al*. reported that up-regulation of *miR-15a* inhibited cell proliferation and migration (7), and Liu *et al*. reported that up-regulation of several miRNAs, including *miR-15a*, had a tendency to down-regulate *Smurf2*, which is known to play a complex role in tumorigenesis (8). However, the clinical significance of *miR-15a* in BC cases is not yet known.

In the present study, we demonstrated that *miR-15a* is a prognostic marker in BC patients by use of RT-PCR on primary BC tumors.

Materials and Methods

Patients. Patients with BC (n=230) who underwent surgical treatment at 4 Hospitals (National Kyushu Cancer Center Hospital, Kyushu University Beppu Hospital, Oita Prefectural Hospital and Takada-Chuo Hospital) from 2000 to 2005 were enrolled in this study. The average age was 55 years. The patient cohort included 121 estrogen receptor positive cases, 67 HER2/neu-positive cases, 68 triple-negative cases and 101 patients with lymph node metastasis. There were 1 case of stage 0, 64 cases of stage I, 140 cases of stage II, 22 cases of stage III,

Correspondence to: Koshi Mimori, MD, Ph.D., Department of Surgery, Kyushu University Beppu Hospital, 4546 Tsurumihara, Beppu, Oita 874-0838, Japan. Tel: +81 977271650, Fax: +81 977271651, e-mail: kmimori@beppu.kyushu-u.ac.jp

Key Words: miR-15a, breast cancer, prognosis.

Table I. *MiR-15a expression and clinicopathological factors*

	Low expression (n=124)		High expression (n=106)		
Factors	Number	%	Number	%	*p*-Value
Age (mean±SD*1)	56±12		54±11		0.053
Estrogen receptor					0.90
Positive	66	53	55	52	
Negative	58	47	50	48	
Progestrone receptor					0.44
Positive	66	54	51	49	
Negative	57	46	54	51	
HER2					0.40
Positive	32	28	35	33	
Negative	82	72	70	67	
T factors					0.33
Tis-1	78	63	60	57	
T2-4	46	37	46	43	
Lymph node metastasis					0.59
Absent	71	57	58	55	
Present	53	43	48	45	
Nuclear Grade					0.30
1-2	81	69	62	62	
3	37	31	38	38	
Lymphatic invasion					0.62
Absent	68	55	61	58	
Present	56	45	44	42	
Venous invasion					0.38
Absent	114	92	92	88	
Present	10	8	12	12	
Stage					0.29
Stage 0-I	32	25	33	32	
Stage II-IV	92	75	73	68	

*1 SD; Standard deviation.

and 3 cases of stage IV. The stage classification was based on TNM classification on malignant tumor 7th edition by UICC.

Evaluation of miR-15a expression in clinical samples. The resected tumor tissue specimens were immediately frozen in liquid nitrogen and kept at –80˚C until analysis. Total RNA extraction from primary tumors was performed as previously described (9). We synthesized *miR-15a*-specific cDNAs from total RNA using gene-specific primers according to the TaqMan MicroRNA Assays Protocol (Roche Applied Science, Indianapolis, USA). Reverse transcription as well as Real-Time PCR detection were carried-out using hsa-miR-15a-5p (Assay ID: 000389; Applied Biosystems, USA). RNU6B (Assay ID: 001093; Applied Biosystems, USA) was used as a reference gene. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using Applied Biosystems 7500 real-time PCR system, as previously described (9). The raw data of miRNA expression was normalized by RNU6B and calculated as relative quantification of miRNA expression values to that of one case in our samples. Before sample acquisition, each patient provided written informed consent. This study was approved by the ethics committees of Kyushu University.

Table II. *Results of the multivariate analysis between clinicopathological factors and overall survival (Cox proportional hazard model).*

Factors	RR (95% CI)	*p*-Value
Age	3.79 (0.55-27.4)	0.18
ER (positive/negative)	0.50 (0.14-1.93)	0.31
PgR (positive/negative)	7.34 (1.87-29.6)	0.004*
HER2 (positive/negative)	1.15 (0.49-2.84)	0.75
T factor (Tis-1/T2-T4)	3.14 (0.39-19.5)	0.47
Lymph node metastasis (negative/positive)	1.15 (0.13-24.9)	0.09
Lymphatic invasion (negative/positive)	11.4 (0.61-743)	0.86
Venous invasion (negative/positive)	2.59 (0.56-12.7)	0.67
Nuclear Grade (1-2/3)	1.72 (0.75-3.91)	0.20
Stage(0-1/2-4)	3.40 (0.36-76.2)	0.30
miR-15a expression (high/low)	2.56 (1.03-7.18)	0.04*

Statistical analysis. For the analysis of *miR-15a*, differences between clinicopathological factors were analyzed by χ^2 tests for categorial variables. Disease-free survival and overall survival time were measured from the first operation until the date of death or last follow-up. Survival curves were determined using the Kaplan-Meier method and statistical significance between groups was assessed using the wilcoxon test. Multivariate analysis was performed to assess the relative influence of prognostic factors on overall survival, using the Cox proportional hazards model in a forward stepwise procedure. Statistical analysis was performed by JMP® Pro 9.0.2 for Mac OS (SAS Institute).

Results

Low miR-15a expression in the primary tumor is a prognostic factor for BC patients. *MiR-15a* expression in the primary tumor was assessed in 230 patients with BC and divided into two groups according to their level of *miR-15a* expression. Analysis of clinicopathological factors in relation to *miR-15a* expression levels revealed no significant correlation (Table I). Patients in the low-*miR-15a* expression group had a significantly shorter term of disease-free survival than those in the high-*miR-15a* expression group (Figure 1a). However, there was no significant correlation between high and low expression levels of *miR-15a* and overall survival. In ER-positive and HER2-positive cases, there was no correlation between *miR-15a* expression and disease-free survival or overall survival (Figure 3). In triple-negative cases, low *miR-15a* expression was significantly correlated with shorter disease-free survival (p=0.0012) and overall survival (p=0.005) (Figure 2).

Multivariate analysis of overall survival showed that the level of *miR-15a* expression was an independent prognostic predictor [relative risk (RR)=2.56; 95% confidence interval (CI), 1.03-7.18; p=0.004] by Cox proportional hazards model] (Table II). PgR was also shown to be an independent prognostic predictor (RR=7.34; 95%CI=1.87-29.6; p=0.004).

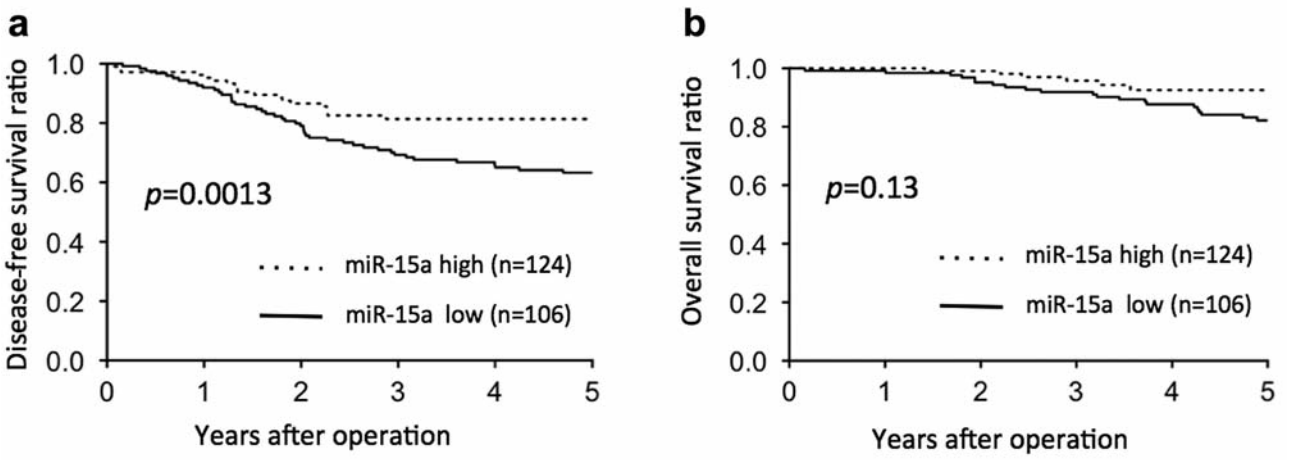

Figure 1. *Kaplan-Meier disease-free survival curve (a) and overall survival curve (b) of breast cancer cases based on the level of miR-15a in primary tumor.*

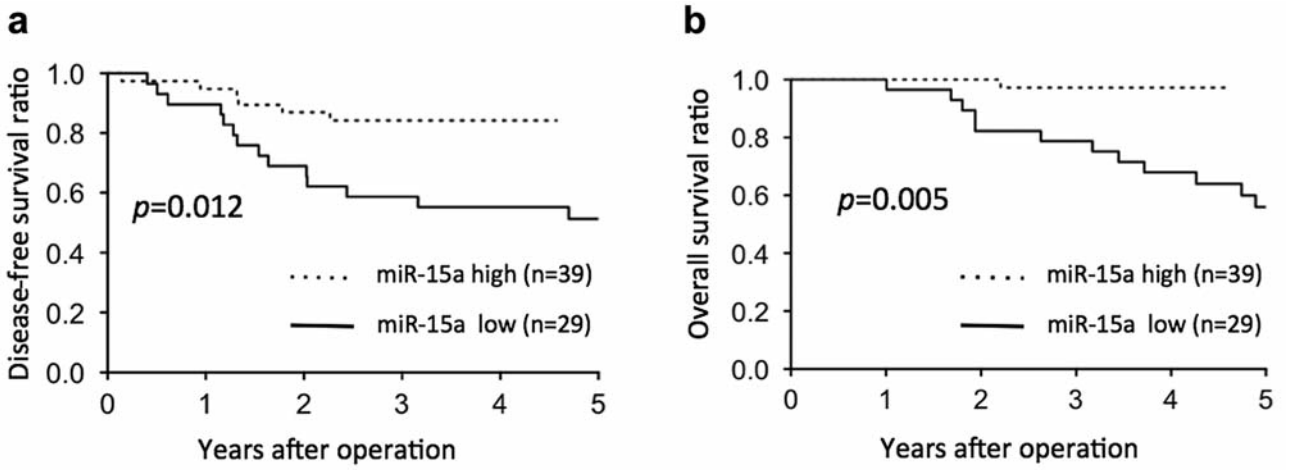

Figure 2. *Kaplan-Meier disease-free survival curve (a) and overall survival curve (b) of triple-negative breast cancer cases based on the level of miR-15a in primary tumor.*

Discussion

In the present study, we demonstrated that low *miR-15a* expression correlated with poor prognosis for patients with BC, particularly in triple-negative cases. It is known that *miR-15a* has many targets and is involved in several cancer pathways. Luo *et al*. reported that *miR-15a* targets *CCNE1*, which regulates the G_0 to G_1 phase transition and promotes cell-cycle progression. They reported that the up-regulation of *miR-15a* led to an increased number of cells in the G_0/G_1 phase, reduced cells in the S and G_2/M phases, and inhibited cell proliferation and migration (7). They also demonstrated the tumor suppressive activity of *miR-15a* in a breast cancer cell line. In addition, Liu *et al*. reported that the up-regulation of several miRNAs, including *miR-15a*, led to the down-regulation of *Smurf2*, which is known to play a complex role in tumorigenesis (8). However, Kodahl *et al*. reported that measurement of the expression of a combination of several miRNAs, including *miR-15a*, was able to discriminate between ER-positive BC patients and healthy controls (10). Interestingly, they found *miR-15a* expression was high in BC patients compared to healthy controls, suggesting perhaps an oncogenic role for *miR-15a*. The results of the present study support the hypothesis that *miR-15a* has a tumor-suppressive effect in breast cancer cases. With regard to specific subtypes of breast cancer, we

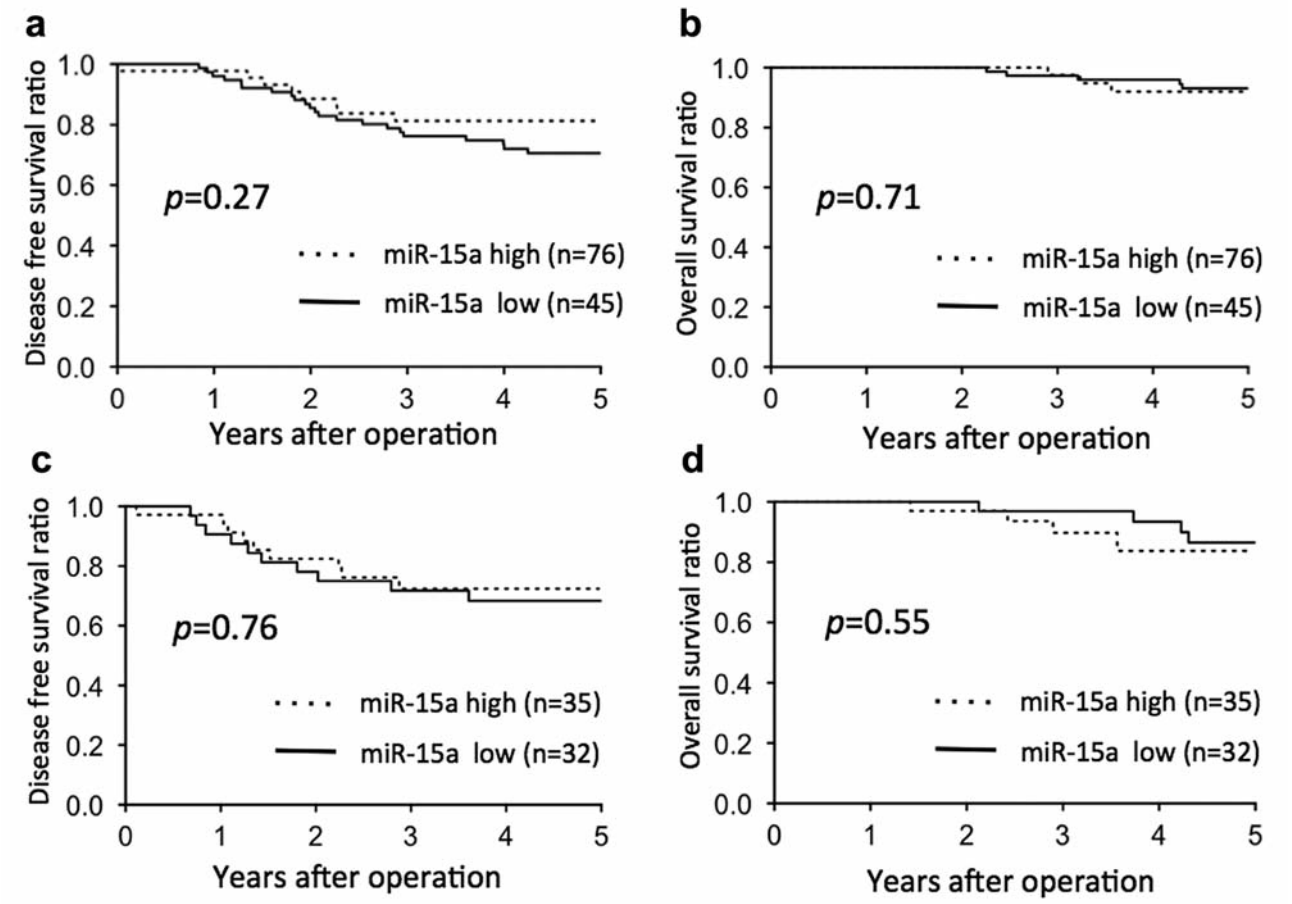

Figure 3. *Kaplan-Meier disease-free survival curve (a) and overall survival curve (b) in ER-positive breast cancer cases based on the level of miR-15a in primary tumor. Disease-free survival curve (c) and overall survival curve (d) in ER-negative breast cancer cases based on the level of miR-15a in primary tumor.*

found a significant correlation between *miR-15a* expression and poor prognosis in triple-negative cases. The relationship between *miR-15a* and *Smurf2* in triple-negative BC (8) has also been demonstrated by Liu *et al*., and it was also suggested that genes like *Smurf2* targeted by *miR-15a* have critical roles in triple-negative BC.

In the present study, we found that in addition to *miR-15a* expression, the PgR expression was an independent prognostic predictor (RR=7.34; 95%CI=1.87-29.6; p=0.004). Although it is well-known that PgR-negative cases have a poorer prognosis than PgR-positive cases in luminar BC (11, 12), the significance of PgR expression status seemed to be overestimated in our study. Although it is possible that the nature of our samples produced some bias, the significant correlation between *miR-15a* and poor prognosis seems to be independent of PgR due to finding no significant correlation between the expression levels of *miR-15a* and PgR expression status (Table I).

In conclusion, *miR-15a* expression in BC primary tumors was an independent prognostic factor for overall survival; low *miR-15a* in the primary tumor predicted a poor prognosis for BC patients. In triple-negative patients, a low level of *miR-15a* expression was significantly correlated with shorter disease-free survival and overall survival.

Acknowledgements

The Authors would like to thank K. Oda, M. Kasagi, and S. Kono for their technical assistance. This work was supported in part by the Japan Society for the Promotion of Science (25830102).

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Received August 26, 2014
Revised October 5, 2014
Accepted October 10, 2014