Differential miRNA Expression in Basaloid Squamous Cell Carcinoma of the Oesophagus: miR-3687 Targets *PGRMC2*

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Abstract. *Background/Aim:* Basaloid squamous cell carcinoma of the oesophagus (BSCCE) has poorer prognosis than conventional oesophageal squamous cell carcinoma (ESCC). This study is the first report on highly expressed miRNAs in BSCCE and their target genes. *Materials and Methods:* BSCCE and ESCC patients who underwent esophagectomy were selected for this study. Total RNA was extracted from formalin-fixed paraffin-embedded blocks to examine expression of miRNAs and target genes. miRNA mimic or inhibitor transfected cells were used in validation experiments. miRNA and mRNA quantification were performed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). *Results:* miRNA microarray analysis revealed four candidate miRNAs. Further investigations including cell line experiments demonstrated that miR-3687 was a candidate miRNA and progesterone receptor membrane component2 (*PGRMC2*) was its target gene. *PGRMC2* was found to be related to cell proliferation and local progression. *Conclusion:* miR-3687 may be a candidate miRNA conferring BSCCE aggressiveness, and *PGRMC2* is one of its target genes.

Basaloid squamous cell carcinoma (BSCC) was recently recognized as a variant of squamous cell carcinoma (SCC). As BSCC of the head and neck has been reported to have a poorer prognosis than conventional SCC, it was proposed to be categorized separately from conventional SCC (1). The second edition of the Guidelines for Clinical and Pathologic

Studies on Carcinoma of the Oesophagus (1972), published by the Japanese Society for Oesophageal Diseases, had earlier classified this tumour as basal cell carcinoma, whereas the WHO classification of oesophageal tumours described this entity in 2000 (2). Currently, BSCC of the oesophagus (BSCCE) is considered as a relatively rare tumour accounting for 1.7%-11.3% of all oesophageal carcinomas (2, 3). Most reported incidences pertained to single institutions in the 1990s, while a recent report on the comprehensive registry of oesophageal cancer in Japan revealed an incidence of BSCCE of 2.0%, confirming it as rare (4). While BSCCE as well as BSCC of the head and neck are considered to have a poorer prognosis than conventional ESCC (5-8), recent reports have stated that its prognosis is similar to that of ESCC, if detected at early stages (6, 8, 9).

Several studies have shown the relationship between BSCCE and molecules such as epidermal growth factor receptor (EGFR), cyclin D1, E-cadherin, P16, p53, B-cell lymphoma 2 (*bcl-2*) and vascular endothelial growth factor (VEGF) (7, 8, 10-12). The alterations in these molecules contributing to carcinogenesis and progression of ESCC have been greatly elucidated at the molecular level, and some of them have been targeted for molecular therapy, for instance anti-EGFR antibody drugs. However, few reports have implied the clinical application of some cancer-related molecules in BSCCE (13).

MicroRNAs are small, noncoding, 18-25 nucleotide long RNA molecules, which play an important role in all biological pathways such as differentiation, proliferation, survival, and apoptosis in multicellular organisms including mammals (14). miRNAs function as negative, posttranscriptional regulators of protein expression, interacting with specific mRNAs and inducing their degradation. In recent years, the role of miRNAs in normal cells as well as disease processes including various cancers has been investigated (15). In cancer cells, miRNAs can be up- or down-regulated, acting as oncogenes or tumour suppressors. Therefore, many studies have investigated their

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roles in cancer initiation, differentiation, prognosis, treatment response and therapeutics (16-21). In addition, a huge number of miRNAs along with their predictive target genes and their functions have been identified to be associated with oesophageal carcinoma (22). One of the interesting characteristics of miRNAs is their tumour-type specificity, meaning that they are expressed at specific tissues and act as oncogenes or suppressors. For example, miR-146a-5p acts as an oncogenic miRNA in colorectal cancers and a suppressive miRNA in breast cancers (23, 24). Additionally, some authors have demonstrated that circulating miRNAs, for instance miRNA-18a and -25, may constitute valuable biomarkers for cancer detection and recurrence monitoring in ESCC (25, 26), in which liquid biopsy is considered as a low-invasive and feasible method.

To the best of our knowledge, no study has previously reported the molecular differences between BSCCE and ESCC using miRNAs and their potential target genes. The present study aimed to clarify the differences between BSCCE and ESCC, using miRNA microarray profiling analysis and candidate miRNA validation. In addition, differentially expressed miRNAs were further investigated to identify target genes and their clinicopathological influence, using cell line transfection experiments and samples from ESCC patients.

Materials and Methods

Patients and samples. Four BSCCE patients and ten stage-matched ESCC patients who underwent esophagectomy from 2004 to 2012 at the Yamanashi University Hospital, underwent comprehensive miRNA microarray analysis and candidate miRNAs validation experiments. Postoperatively, all patients were histologically diagnosed as either BSCCE or ESCC by the hospital's Department of Pathology using the criteria of the 10th edition of the Japanese Classification of Oesophageal Cancer. All patients were pathologically classified as stage I or II. Four tumorous specimens from BSCCE patients and ten tumorous and non-tumorous specimens from ESCC patients were collected as formalin-fixed paraffin-embedded (FFPE) tissue samples. Target genes of the candidate miRNAs were searched using predicted target gene mRNA assays and cell line transfection experiments. Subsequently, the relation between the target gene mRNA and clinicopathological features was evaluated using ESCC samples obtained from 84 patients who underwent curative esophagectomy without preoperative chemotherapy or radiotherapy. Patients' clinicopathological characteristics are described on Table I.

This study was approved by the Yamanashi University Ethics Committee (approval number: 1888 and 1959) and followed the Helsinki Declaration and its amendments. All patients provided written informed consent for samples and data use.

Total RNA extraction from FFPE specimens. FFPE tissue samples were cut into 10-µm-thick sections, placed on slides and stained by hematoxylin and eosin to identify tumour areas and guide microdissection. Tumour areas from BSCCE specimens and tumour and adjacent normal areas from ESCC specimens were scratched-off the slides into tubes for RNA extraction. Total RNA was

Table 1. *Patients' clinicopathological features in the microarray experiment, validation study, and target gene assay. ESCC (n=10): microarray experiment and validation study; ESCC (n=84): target gene assay.*

	Basaloid n=4	ESCC n=10	ESCC n=84
Age (y.o., median)	72 (64-79)	68 (59-78)	69 (46-82)
Gender			
M/F	2/2	9/1	71/13
Location			
Ce/Ut/Mt/Lt/Ae	0/2/0/2/0	0/3/5/2/0	4/17/31/28/4
T factor			
1&2/3&4	4/0	10/0	49/35
LN metastasis			
0/1-4	4/0	10/0	39/45
Tumor size (mm, median)	18 (10-20)	45 (9-65)	49 (9-100)
Differentiation			
well/mod/poor	Basaloid	1/4/5	21/48/15
Lymphatic invasion			
ly0/ly1-3	2/2	4/6	31/53
Venous invasion			
v0/v1-3	3/1	3/7	34/50

ESCC: Esophageal squamous cell carcinoma.

extracted using the RNeasy FFPE kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

miRNA microarray analysis. Microarray analyses of BSCCE and ESCC samples were performed using 3D-Gene miRNA oligo chips (Toray Industries, Kamakura, Japan), with 1758 genes mounted onto each DNA chip. Results were compared among EBSCC, ECSCC and normal mucosa cells. Tissue samples from four BSCCE and ten ESCC patients were equally mixed. RNAs were labelled with the 3D-Gene miRNA labelling kit (Toray Industries). Fluorescent signals were scanned using a 3D-Gene scanner 3000 (Toray Industries) and analyzed with the 3D-Gene Extraction software (Toray Industries). The expression levels of each miRNA were normalized to the median signal strength of the entire gene in each chip. The adjusted median signal strength was 25.

miRNA quantification by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). For a validation study, cDNA was prepared from total RNA samples using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems™, Thermo Fisher Scientific Inc, Tokyo, Japan), according to the manufacturer's instructions. miRNA levels were quantified by qRT-PCR using a Human TaqMan MicroRNA Assay kit (Applied Biosystems™, Thermo Fisher Scientific Inc), according to the manufacturer's instructions. Tissue miRNA levels were normalized to UB6 small nuclear RNA (RNU6B) as an internal control. The following primers were used for the TaqMan assay (Applied Biosystems™, Thermo Fisher Scientific Inc): has-miR-3687 (Assay ID: 464645_mat), has-miR-4732-5p (Assay ID: 465097_mat), and RNU6B (Assay ID: 001093). All miRNA's relative expression levels were calculated using the 2^{-ΔΔCT} method.

Figure 1. miRNA expression scatter plot (Basaloid vs. Control). miRNAs' expression in BSCCE and normal epithelium obtained from microarray analysis are shown. The lucent square shows plots demonstrated sufficient (>100) miRNA signal intensity. Four miRNA plots, miR-205-5p, miR-1246, miR-3687, and miR-4732-5p, were below the 95% confidence interval curve.

Target gene prediction. Candidate miRNA's target genes were searched by TargetScan (27). A further pilot study was performed on ESCC samples to identify the correlation between candidate genes and miR-3687. Total RNA was extracted from FFPE tissue samples using the RNeasy FFPE kit (Qiagen, Hilden, Germany). cDNAs were synthesised using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems™, Thermo Fisher Scientific Inc) for the candidate miRNA and the TaqMan high-Capacity cDNA Reverse Transcription kit (Applied Biosystems™, Thermo Fisher Scientific Inc) for a total RNA, according to the manufacturer's instructions. The levels of total RNA and miRNA were quantified using qRT-PCR, according to standard procedures. The following primers were used for the TaqMan assay (Applied Biosystems™, Thermo Fisher Scientific Inc): has-miR-3687 (Assay ID: 464645_mat), RNU6B (Assay ID: 001093), *PGRMC2* (Assay ID: Hs01128872_m1), *FGFRL1* (fibroblast growth factor receptor-like 1) (Assay ID: Hs00222484_m1), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (Assay ID: Hs02786624_g1). Total RNA and miRNA levels were normalized against the endogenous control *GAPDH* and *RNU6B*, respectively. The relative mRNA and miRNA expression levels were assessed using the $2^{-\Delta\Delta CT}$ method.

Cell line transfection experiment. Cell line transfection experiments were performed for the predicted target genes. Human ESCC cell lines TE13 and TE14 were purchased from the Cell Resource Centre for Biomedical Research Institute of Development, Ageing and Cancer (Sendai, Japan) (28). Human ESCC cell lines KYSE 30 and KYSE150 were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) (29). Cells were cultured and either the miR-3687 inhibitor (Anti-miR ID: MH20273) or the negative control inhibitor miRNA (mirVana miRNA Inhibitor Negative Control #1) for downregulation and either the miR-3687 mimic (mirVana miRNA Mimic, Pre-miR ID: MC20273) or the negative control mimic miRNA (mirVana miRNA Mimic Negative Control #1) for enhancement were transfected into the ESCC cells. The transfection experiment was performed as previously described (30). Total RNA from each cell line was extracted using miRNAeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific Inc), according to the manufacturer's instructions. Total RNA levels were quantified using qRT-PCR, according to standard procedures. The TaqMan assay was performed with the following primers (Applied Biosystems™, Thermo

Figure 2. Relative expression levels of candidate miRNAs in control, ESCC, and BSCCE. For both miR-3687 (A) and miR-4732-5p (B) the expression increased in the order of control, ESCC and BSCCE. Noticeable differences in miR-3687 were found between ESCC and BSCCE ($p<0.05$, b), and between control and BSCCE ($p<0.05$, a). miR-4732-5p only differed significantly between control and BSCCE ($p<0.05$, a).

Table II. Top-four microRNAs selected from the microarray experiment.

	Name	ID	Control	Basaloid	Ratio	Ref.
1	hsa-miR-205-5p	MIMAT0000266	277	859	3.10	Ishibashi <i>et al.</i> (31)
2	hsa-miR-4732-5p	MIMAT0019855	187	361	1.93	-
3	hsa-miR-1246	MIMAT0005898	404	759	1.88	Takeshita <i>et al.</i> (32)
4	hsa-miR-3687	MIMAT0018115	160	259	1.63	-

ID: miRbase accession number.

Fisher Scientific Inc): *PGRMC2* (Assay ID: Hs01128872_m1) and *GAPDH* (Assay ID: Hs02786624_g1). Normalization of total RNA levels and assessment of mRNA relative expression were conducted as described in the target gene prediction experiment.

Determining the relationship between the target gene and clinicopathological indicators. To elucidate the clinicopathological roles of the candidate gene, another investigation was performed. Total RNA extraction from FFPE samples of 84 ESCC patients, cDNA synthesis, quantification of *TGRMC2* mRNA by qRT-PCR and assessment of its relative expression levels were conducted as previously described in the target gene prediction experiment. Then, 84 ESCC patients were divided into two groups and the relationship between *PGRMC2* mRNA expression and clinicopathological indicators, such as age, gender, tumour size, T factor, LN metastasis, differentiation, lymphatic invasion, and venous invasion, was analysed statistically.

Statistical analysis. All statistical analyses were performed using the R software (version 3.5.2, R Core Team, 2014) and the GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). Results from the miRNA validation study were analysed using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc*

test. The correlation between miR-3687 and *PGRMC2* was analyzed in a pilot study using the Pearson's correlation analysis. Paired data on cell line experiments were analyzed using the one-sided paired *t*-test. The correlations between *PGRMC2* mRNA expression levels and clinicopathological indicators were analyzed using either the *t*-test, Chi square test or Fisher's exact probability test. A level of $p<0.05$ was considered statistically significant.

Results

Identifying differential expression of miRNAs using a comprehensive miRNA array-based approach in BSCCE tissue. The expression levels of each miRNA were compared between BSCCE and normal epithelium. Four miRNAs were selected from 1758 candidates: miR-205-5p, miR-1246, miR-3687, and miR-4732-5p, whose expression was at least 1.5-fold higher in BSCCE than in normal epithelium samples and their signal intensity was high (more than 100). The obtained scatter-plots diagram showed that all of the candidate miRNA plots were below the 95% confidence interval curve (Figure 1).

Figure 3. Correlations among *PGRMC2*, *FGFR1*, and *miR-3687* expression. The mRNA assay revealed that both *PGRMC2* (A) and *FGFR1* (B) inversely correlated with *miR-3687*. The correlation was only significant between *PGRMC2* and *miR-3687* ($p<0.0160$).

miRNA expression levels in BSCCE and ESCC validation study. Two of the four candidate miRNAs, *miR205-5p* and *miRNA-1246*, have been previously reported to be related to oesophageal carcinoma. Therefore, the residual miRNAs, *miR-3687* and *4732-5p*, were selected for the validation study (Table II) (31, 32). The selected miRNAs expression levels were investigated by qRT-PCR in BSCCE, ESCC, and normal tissue samples. The results of the validation study are illustrated in Figure 2. Both *miR-3687* and *4732-5p* showed increased expression levels, in the order of normal epithelium, ESCC and BSCCE. Both differed significantly between BSCCE and normal epithelium; the former differed significantly between BSCCE and ESCC. Since the difference was much more obvious for *miR-3687* than for *miR-4732-5p*, we selected *miR-3687* for further analysis to explore the target gene and its influence on the clinicopathological indicators of ESCC patients.

Selection of a potential target gene for miR-3687. The miRNA target analysis tool, TargetScan (27) was searched for target gene prediction. Two among numerous potential target genes, *PGRMC2* and *FGFR1*, were selected based on their previously reported relation to malignant neoplasia. A pilot study performed on samples from 11 ESCC patients revealed that both *PGRMC2* and *FGFR1* were inversely correlated with *miR-3687*. However, the correlation was only statistically significant for *PGRMC2* (Figure 3; $r=-0.4933$, $p=0.016$).

Relationship between miR-3687 and PGRMC2 in ESCC cell lines. Since Hagio *et al.* have reported that the expression of *miR-3687* was high in the KYSE30 and TE13 cell lines and low in the KYSE150 and TE14 cell lines (30), the *miR-3687* inhibitor was transfected into the former cell lines whereas the *miR-3687* mimic was transfected into the latter cell lines. For each cell line, *PGRMC2* mRNA expression was investigated three to four times. In both, KYSE30 and TE13 cells, the expression of *PGRMC2* mRNA was increased in inhibitor

Table III. Correlation between clinicopathological features and *PGRMC2* mRNA expression.

	PGRMC2 mRNA expression		p-Value
	High	Low	
n	42	42	
Age (y.o., median)	70(46-82)	68.5(52-78)	0.772
Gender			
M/F	36/6	35/7	0.762
Tumor size (mm, median)	32.5(9-80)	52.5(9-100)	0.010*
T factor			
0/1/2/3/4	20/7/13/2	9/13/19/1	0.059
LN metastasis			
0/1/2/3/4	22/7/8/1/4	17/6/10/3/6	0.673
Differentiation			
well/mod/poor	10/23/9	11/25/6	0.694
Lymphatic invasion			
ly 0/1/2/3	20/11/7/4	11/18/10/3	0.173
Venous invasion			
v 0/1/2/3	20/16/5/1	14/16/11/1	0.346

* $p<0.05$, *t*-test.

transfected cells compared to control. In both, KYSE150 and TE14 cells, the expression of *PGRMC2* mRNA was significantly decreased in mimic transfected cells compared to control (KYSE150; $p<0.001$, TE14; $p<0.01$) (Figure 4). *PGRMC2* may thus be a target gene for *miR-3687*.

Relationship between PGRMC2 mRNA expression and clinicopathological variables in ESCC patients. A total of 84 ESCC samples were used for a further study. Patients were divided into high and low *PGRMC2* mRNA expression groups, based on the median expression level. A significant difference was found only in tumour size ($p=0.01$), but a strong correlation with T factor was suspected ($p=0.059$) (Table III),

Figure 4. *PGRMC2* mRNA expression after transfection of miR-3687 inhibitor or mimic. A: miR-3687 high-expression cell lines. B: miR-3687 low-expression cell lines. In group A, *PGRMC2* mRNA expression increased inhibitor transfected cell lines compared to control. In group B, *PGRMC2* mRNA expression decreased significantly mimic transfected cell lines compared to control (KYSE150 $p < 0.001$, TE14 $p < 0.01$).

indicating that *PGRMC2* mRNA expression in ESCC may be related to cell proliferation and local progression.

Discussion

Although a number of molecular and genetic oesophageal carcinoma-related changes has been investigated, no previous study has examined the difference between BSCCE and ESCC using a miRNA approach except for our colleague's report.

The present study aimed first, to identify differentially expressed miRNAs in BSCCE. Microarray profiling analysis revealed four candidate miRNAs, of which miR-3678 and miR-4732-5p were further selected for the validation study and finally miR-3687 proceeded to a target gene experiment. Hagio *et al.* have reported the relationships between miR-3687 and oesophageal cancer's clinicopathological features and

showed that is associated with poor prognosis, migration and invasion leading to tumour aggressiveness (30). A literature search on miR-3687 retrieved only a few articles related to malignant neoplasia. One study has referred to invasive ability and poor prognosis of oesophageal cancer, another to increasing local recurrence of conjunctival melanoma and a third to telomerase activity of a breast cancer cell line (30, 33, 34). The present study was the first to investigate both miR-3687 as a differentially expressed miRNA in BSCCE and its putative target genes in oesophageal carcinoma. It is widely known that miRNAs show tissue-type specificity. Therefore, miR-3687 may have different characteristics and target genes in ESCC and BSCCE. However, Hagio *et al.*, have shown that highly expressed miR-3687 is related to poor prognosis in ESCC, therefore, high miR-3687 expression in BSCCE may also be related to poor prognosis.

The *PGRMC2* gene was selected in the validation experiment and was shown to correlate inversely with miR-3687 and to relate with tumour size. *PGRMC2* has been reported to be involved in ovarian cancer, cervical adenocarcinoma and breast cancer, and inversely correlated with migration and metastasis (35-37). The present study suggests that *PGRMC2* may be related to cell proliferation and local progression in ESCC. Further research is needed to clarify its molecular biological roles in carcinogenesis and progression, to help understand the higher incidence of oesophageal cancer in males than in females.

In conclusion, *miR-3687* may constitute a candidate marker of BSCCE aggressiveness, and *PGRMC2* is one of target genes of miR-3687 with a relation to cell proliferation and local progression. Although the present study included a small number of cases, it provides the first investigation of differentially expressed miRNAs and predictive target genes in BSCCE patients. Further research based on a large-scale collection of BSCCE samples may provide detailed molecular, biological, and clinicopathological information.

Conflicts of Interest

The Authors declare no conflicts of interest with respect to this study.

Authors' Contributions

Jiro Nakamura performed the majority of experiments and wrote the manuscript. Shinji Furuya and Daisuke Ichikawa designed the research and helped to draft the manuscript. All other Authors have contributed to data collection and interpretation, and critically reviewed the manuscript.

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