KRAS Upregulates miR-181a, miR-200c and miR-210 in a Three-Dimensional-Specific Manner in DLD-1 Colorectal Cancer Cells

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Key words: KRAS; microRNA; colorectal cancer; miR-200c, 3D culture

Running title: Oncogenic KRAS regulates 3D-specific microRNAs in DLD-1 cells
The indicated category: Experimental Study
Abstract. Background: We previously found that oncogenic KRAS induces increased expression of microRNAs (miRNAs), such as miR-200c and miR-221/222, in a three-dimensional (3D)-specific manner in human colorectal cancer (CRC) HCT116 cell, however, the regulation of miRNA expression through oncogenic KRAS in other types of CRC remains unclear. Materials and Methods: The differential expression of 94 cancer-related miRNAs was examined between DLD-1 and DKO-4 cells (DLD-1 cells with a disrupted oncogenic KRAS) in 3D culture (3DC). Results: Increased miR-15b, miR-16, miR-23a, miR-24, miR-103 and miR-222 expression was observed in 3DC and in the two-dimensional culture. Of note, increased miR-181a, miR-200c and miR-210 expression was only observed in 3DC. Furthermore, miR-181a and miR-210 expression was significantly overexpressed in DLD-1 cells in 3DC compared with that in HCT116 cells and was significantly overexpressed in human CRC specimens. Conclusions: Oncogenic KRAS regulates 3D-specific miRNAs that are possibly associated with CRC development in vivo.
Introduction

Cell-cell and cell-extracellular matrix interactions are important in developmental programs and provide in vivo three-dimensional (3D) architectures (1, 2). The deregulation of these interactions is frequently observed in cancer (3). We previously established HKe3 cells, which are human colorectal cancer (CRC) HCT116 cells with a disruption in oncogenic KRAS (4), and analysis using these HKe3 cells have contributed to the understanding of tumor development through in vitro and in vivo oncogenic KRAS signaling (4-8). However, no treatments that target tumors with KRAS mutations have been developed. Systems for the elucidation of the detailed molecular mechanisms underlying the activities of oncogenic KRAS in the 3D microenvironment are essential for the design and development of novel cancer therapies.

We previously investigated the behavior of HKe3 cells in 3D culture (3DC) and reported that the cells make an organized structure resembling a colonic crypt (9). In this model, oncogenic KRAS was found to inhibit luminal apoptosis, cell polarity in 3DC and downregulate DNA repair genes (including TP53) in a 3D-specific manner (4, 9). These results indicated that oncogenic KRAS plays crucial roles in the inhibition of organized structures and accumulation of genetic alterations, resulting in the disruption of the barrier-to-tumor progression in the colonic crypt (4, 9).
Recently, we found that the oncogenic KRAS induces increased expression of microRNAs (miRNAs), such as miR-200c, miR-221 and miR-222, in HCT116 cells compared with that in HKe3 cells in 3DC (10). These miRNAs were significantly overexpressed in human CRC specimens. Of note, protein expression of PTEN, which is a putative target of the miR-200c and miR-221/222 cluster, was reduced under the control of oncogenic KRAS in a 3D-specific manner, suggesting that these miRNAs are associated with the transition from normal colonic mucosa to carcinoma. However, the details on the regulation of miRNA expression through oncogenic KRAS in other types of CRC remain unclear.

In this study, we examined 94 cancer-related miRNAs in DLD-1 and DKO-4 cells (DLD-1 cells with a disrupted oncogenic KRAS) in 3DC. Furthermore, we examined the biological relevance of 3D-specific miRNAs that are regulated by oncogenic KRAS in this model using public datasets of miRNA expression analysis of CRC. We found that 3D-specific miRNA expression was significantly high in CRC specimens.

Materials and Methods

Cell culture. DLD-1 and DKO-4 cells were grown in 2D cultures (2DC) or 3DC described previously (4, 9, 11).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Real-
time qRT-PCR was performed using Cancer microRNA qPCR Array with QuantiMir (System Biosciences, Mountain View, CA, USA) for miRNAs. miRNA expression was normalized to U6 snRNA expression in each cell. Data were analyzed by the $\Delta\Delta\text{Ct}$ method as previously described (12). The relative expression units (REUs) of DLD-1 cells were determined by the REU of miR-181b in DKO-4 cells in 3DC, which was set as 1.0.

**Dataset sources.** The Arndt dataset, which comprises the miRNA profiles of human CRC specimens from 58 patients with CRC patients (5 with Dukes’ A, 26 with Dukes’ B, 24 with Dukes’ C and 3 with Dukes’ D) and colonic mucosa specimens from eight healthy control subjects (13), was obtained from the Gene Expression Omnibus (Series GSE10259) using the import module of GenePattern software (14). The differential expression of miRNAs between the two classes was ranked according to a signal-to-noise metric using GenePattern (14, 15). The statistical significance of the differentially expressed genes was determined by the comparative marker selection module of GenePattern (14).

**Statistical analysis.** The data are presented as the means ± standard deviation. The statistical analyses were performed using the unpaired two-tailed Student’s t-test. Differences at $P < 0.05$ were considered to be statistically significant.
miRNA expression in DLD-1 and DKO-4 cells in 2DC and 3DC. To identify miRNAs showing the differential expression levels in CRC cells, we performed qRT-PCR assays for 94 cancer-related miRNAs in DLD-1 and DKO-4 cells in 2DC and 3DC (Fig. 1). We selected miRNAs with an expression level of more than 0.5 (Fig. 1). In 2DC, miR-92 and miR-93 expression in DLD-1 cells was higher than that in DKO-4 cells with a statistically significant difference (Fig. 1). Furthermore, miR-15b, miR-16, miR-23a, miR-24, miR-103 and miR-222 expression in DLD-1 cells in 2DC and 3DC was higher than that in DKO-4 cells in 2DC and 3DC both with a statistically significant difference (Fig. 1). In 3DC, miR-181a, miR-200c and miR-210 expression in DLD-1 cells was higher than that in DKO-4 cells with a statistically significant difference (Fig. 1).

Comparison of overexpressed miRNAs between HCT116 and DLD-1 cells in 3DC. We previously showed the differential expression of miRNAs between HCT116 cells and HKe3 cells (10). We found that miR-23a, miR-125b and miR-191 expression in HCT116 cells in 2DC and 3DC was higher than that in HKe3 cells in 2DC and 3DC (10). In addition, we found that miR-200c, miR-221 and miR-222 expression in HCT116 cells in 3DC was higher than that in HKe3 cells in 3DC (10). In order to identify miRNAs that are commonly upregulated by oncogenic KRAS in both HCT116 and DLD-1 cells, we compared the expression profiles of miRNAs. Among miRNAs upregulated in 2DC and 3DC, miR-23a was commonly upregulated in both HCT116 and
DLD-1 cells. Among miRNAs upregulated in a 3D-specific manner, miR-200c was commonly upregulated in both HCT116 and DLD-1 cells, and miR-181a and miR-210 were specifically upregulated in only DLD-1 cells. These results suggest a common role of miR-200c in 3D structure and a specific role of miR-181a and miR-210 in tumor progression in DLD-1 cells.

3D-specific miRNAs were overexpressed in CRC. To examine if these miRNAs were also expressed in CRC, we analyzed the public microarray expression data for CRC using the GenePattern software (14). We previously showed that 3D-specific miRNAs including miR-200c and miR-221/222 were overexpressed in CRC (10). In this study, upregulation of 3D-specific miRNA expression (miR-181a and miR-210) and expression in both 2DC and 3DC (miR-103, miR-15b, miR-16, miR-23a and miR24) in DLD-1 cells were examined using the Arndt dataset (13). The differential expression of miRNAs between healthy control subjects and tumor specimens in all Dukes’ stage from patients with CRC are shown in Fig. 2, suggesting that 3D-specific miRNA expression (miR-181a and miR-210) in CRC was higher than that in control subjects. These results suggest that 3D-specific miRNAs are potential candidates for diagnostic biomarkers.

Discussion

3D-specific morphological alterations, including the inhibition of cellular polarity and
luminal cavity formation with apoptosis, through oncogenic KRAS signaling (9) may be associated with triggering the 3D-specific miRNA expression. In this study, miR-15b, miR-16, miR-23a, miR-24, miR-103, miR-181a, miR-200c, miR-210 and miR-222 expression was upregulated under the control of oncogenic KRAS in DLD-1 cells grown in 3DC. Interestingly, miR-181a, miR-200c and miR-210 expression was dysregulated by oncogenic KRAS in a 3D-specific manner and was overexpressed in CRC specimens.

We previously showed that increased miR-200c, miR-221 and miR-222 expression was observed only in HCT116 cells in a 3D-specific manner, and these miRNAs were significantly overexpressed in human CRC specimens (10). Of note, miR-200c was upregulated in both HCT116 and DLD-1 cells, suggesting the 3D-specific role of miR-200c in CRC with oncogenic KRAS. Indeed, recent studies have shown that miR-200c, which is abundantly expressed in clinical CRC specimens (16, 17), and further induces chemoresistance in esophageal cancers (18).

Interestingly, miR-181a and miR-210 were specifically upregulated by oncogenic KRAS in DLD-1 cells in a 3D-specific manner. miR-181 is reported to be a biomarker for cancer stem cells (CSCs) (19) and CSCs are associated with aggressive and metastatic CRC (20). In addition, miR-210 is a sensor for hypoxic stress during tumorigenesis (21), suggesting that the expression of miR-210 represents the hypoxic state of the innermost region of the 3D structure. Thus, these results suggest that oncogenic KRAS promotes cancer development through 3D-specific miRNAs in DLD-1 cells.
Furthermore, analysis of a public dataset strongly indicated that all 3D-specific miRNAs, including miR-200c, miR-211/222 (10), miR-181 and miR-210, reflected the *in vivo* status of CRC and suggested a strong correlation between the miRNAs that are dysregulated by oncogenic KRAS in a 3D colonic-crypt model and in clinical CRC specimens.

In summary, we found increased miR-181a, miR-200c and miR-210 expression in DLD-1 cells grown in 3DC and in human CRC specimens. Further elucidation of the precise molecular mechanisms of miRNAs that are regulated by oncogenic KRAS using this 3D colonic-crypt model will lead to a better understanding of CRC development *in vivo* and provide a novel approach for cancer therapy.

Acknowledgments

We thank Takami Danno and Yumiko Hirose for their technical assistance. This study was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Clinical Research Foundation.
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Figure legends

Figure 1. Expression of 94 cancer-related miRNAs in two-dimensional and three-dimensional cultures (3DC and 2DC). The expression profiles of cancer-related miRNAs in DKO-4 cells in 2DC (black bar), in DLD-1 cells in 2DC (dark gray bar), in DKO-4 cells in 3DC (light gray bar) and in DLD-1 cells in 3DC (white bar). Relative expression units (REUs) of DLD-1 cells and DKO-4 cells were determined by the REU of miR-181b in DKO-4 cells grown in 3DC, which was set as 1.0. *P < 0.05.

Figure 2. Differential expression of miRNAs in HCT116 cells and DLD-1 cells in 3DC. (A) Venn diagrams show upregulated miRNA expression in HCT116 cells compared with that in HKe3 cells in 2DC and 3DC (dark gray circle) and upregulated miRNA expression in DLD-1 cells compared with that in DKO-4 cells both in 2DC and 3DC (light gray circle). (B) Venn diagrams show upregulated miRNA expression in HCT116 cells compared with that in HKe3 cells in 3DC (dark gray circle) and upregulated miRNAs in DLD-1 cells compared with those in DKO-4 cells in 3DC (light gray circle).

Figure 3. Expression of the seven miRNAs, which are 3D-specifically upregulated in DLD-1 cells, in human CRC specimens and control specimens in the Arndt dataset. Rows represent
miRNAs and score. Columns represent normalized expression of the seven miRNAs selected from human CRC specimens from 58 patients with CRC compared with colonic mucosa specimens from eight healthy control subjects.
A. Upregulated miRNAs in 2DC and 3DC

HCT116 / HKCe3
- miR-125b
- miR-191

DLD-1 / DKO-4
- miR-15b
- miR-16
- miR-24
- miR-103
- miR-222

miR-23a

B. Upregulated miRNAs in 3DC

HCT116 / HKCe3
- miR-221
- miR-222

DLD-1 / DKO-4
- miR-181a
- miR-210

miR-200c