35th Annual International Conference of the IEEE EMBS
Osaka, Japan, 3 - 7 July, 2013

Systemes Analysis of Interactions between microRNAs and Genes in Hepatocellular Carcinoma

Yutaka Fukuoka, *Senior Member, IEEE*, Motoki Tashiro, and Takanori Uchiyama, *Member, IEEE*

Abstract— **MicroRNAs (miRNAs) are short noncoding RNAs and can regulate gene expression at the transcriptional and/or translational levels. There is mounting evidence that miRNAs play an important role in the control of the dynamics of localized gene expression. Expression profiling of miRNA in various cancers revealed that miRNA profiles could discriminate malignancies from their counter parts. In this study, to investigate the localized effect of miRNA in cancer, we analyzed gene and miRNA expressions in hepatocellular carcinoma (HCC) and surrounding nontumor tissues. Based on gene expression levels around miRNAs, we investigated how many miRNAs correlated positively/negatively in expression with genes in the vicinity. Next, the Pearson correlation coefficients were compared between the HCC and nontumor tissues. The results imply that the relationship between the intronic miRNAs and their host genes was altered in HCC, and that feedback loops including the host gene, intronic miRNA, target genes might be formed in HCC.**

I. Introduction

MicroRNAs (miRNAs), short (19-25 nucleotide-long) noncoding single-stranded RNA family, are increasingly implicated in tissue-specific control of gene expression [1]. miRNAs are cleaved from 70-100 nucleotides miRNA precursors, and can regulate gene expression either at the transcriptional or translational levels, based on specific binding to the complementary sequence in coding or noncoding region of mRNA transcripts. There is mounting evidence that small noncoding RNAs including miRNAs play an important role in the control of the dynamics of localized gene expression through heterochromatin formation [2]. We, therefore, have investigated if there is a distance-dependent effect of miRNA [3-5]. We analyzed gene expression levels around the known miRNAs in worms [3], mice [4-5] and humans [5]. The results of these studies indicate that there exist localized effects in gene expression around the miRNAs, and that there might be different classes of miRNAs, i.e., those conserved over various species and those evolved in higher organisms.

Microarray analyses of miRNA expressions in cancer tissues have revealed that miRNA profiles could discriminate malignancies of breast [6], lung [6-7], pancreas [6, 8], and liver [9-12] from their counterparts. Hepatocellular carcinoma (HCC) is one of the most common malignant tumors of the liver and the third most common cause of mortality from cancer in eastern Asia. Since postoperative recurrence and intrahepatic metastases occur frequently, the postoperative 5-year survival rate is low [13].

In this study, to investigate the localized effect of miRNA in cancer, we analyzed gene and miRNA expressions in HCC and surrounding nontumor tissues. First, we analyzed the average gene expression levels around miRNAs. Then, a percentage of the miRNAs whose expressions correlated positively/negatively with gene expression was calculated in the vicinity of miRNA. Next, the Pearson correlation coefficients were compared between the HCC and nontumor tissues. This analysis was repeated for intronic and intergenic miRNAs. Finally, the correlation coefficient between miRNAs and their target genes were compared in both tissues.

II. Materials and Methods

A. Clinical Specimens

Operative specimens of primary HCC were obtained with informed consent from 40 patients in the Department of Hepato-Biliary-Pancreatic Surgery at Tokyo Medical and Dental University Hospital between November 2005 and May 2008 [12, 14]. This research project was approved by the local ethical committee, and all samples were obtained with the patient's informed consent. All the specimens were immediately frozen in liquid nitrogen and then stored at -80 ºC for RNA analysis.

B. RNA Isolation and Expression Profiling

Total RNAs were extracted from frozen specimens using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Small RNA with a miRNA-rich fraction was extracted from the tissue specimens using miRNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The extracted RNAs were quantified with NanoDrop ND-1000. Integrity of obtained RNA was assessed using Agilent Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). All samples had RNA Integrity Number (RIN) $\geq$ 4.0. The extracted RNAs were then analyzed by GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) as well as miRNA microarray, 3D-Gene (Toray Industries, Tokyo, Japan). The GeneChip and 3D-Gene microarrays were scanned using GeneChip Scanner 3000 7G and GenePix 4000B, respectively.

This work was financially supported in part by Grant-in-Aids (No. 20510184) from the Ministry of Education, Culture, Sports, Science and Technology in Japan.

Y. Fukuoka and M. Tashiro are with the Department of Electrical Engineering, Kogakuin University, Tokyo, Japan (corresponding author phone: +81-3-3340-3425; fax: +81-3-3348-3486; e-mail: fukuoka@cc.kogakuin.ac.jp).

T. Uchiyama is with the Department of Applied Physics and Physico-Informatics, Keio University, Yokohama, Japan (e-mail: uchiyama@appi.keio.ac.jp).

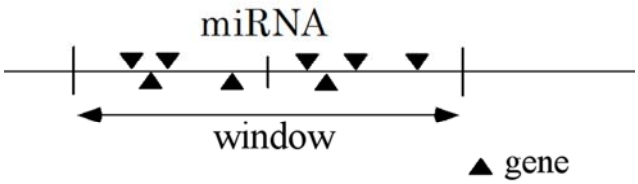

Figure 1. A window centered at a miRNA was set, and the average expression level of the genes was calculated in the window.

C. Gene Expression Data Analysis around miRNAs

We first analyzed the average gene expression levels around miRNAs. In the gene expression data set, the average overall genes in all patients were calculated, and then the expression levels were normalized using the overall average. A window centered at a miRNA was set, and the average of the normalized expression level of the genes was calculated in the window (Fig. 1). This process was repeated for all human miRNAs at miRBase (as of May 2009) [15]. Fifteen different window widths were used: 10 kb, 20 kb, 40 kb, 100 kb, 200 kb, 400 kb, 1 Mb, 2 Mb, 4 Mb, 10 Mb, 20 Mb, 40 Mb, 100 Mb, 200 Mb, and 400 Mb. After this calculation the data for all miRNAs were merged, and the relationship between the average expression level and the window width was investigated.

D. Correlation between expressions of miRNAs and Genes

A percentage of the miRNAs whose expressions correlated positively/negatively with the normalized gene expression was calculated in each width of the windows. When the Pearson correlation coefficient was in a range between -0.4 and 0.4, the miRNA was deemed to have no correlation. Next, the correlation coefficients were compared between the HCC (tumor) and surrounding nontumor tissues. The correlation analysis was repeated for intronic and intergenic miRNAs. Here, an intronic miRNA is encoded in an intron of a gene called a host gene while an intergenic miRNA is encoded between genes. For the intronic miRNA, we calculated the Pearson correlation coefficient between the intronic miRNAs and their host genes. For the intergenic miRNA, the coefficient was calculated between the intergenic miRNAs and the genes in the windows of widths between 10 kb and 100 kb. Finally, the correlation coefficient between miRNAs and their target genes were compared in both tissues.

E. Correlation between expressions of intronic miRNAs and Their Host Genes

We hypothesized that the difference in the Pearson correlation coefficient could be attributable to an altered relationship between intronic miRNAs and their host genes. Accordingly, a pair-wise comparison of the correlation coefficient was made between the HCC and nontumor tissues. Then the target genes of the significantly altered intronic miRNAs were sought using PicTar [16], TargetScan [17], miRanda [18], TarBase [19], and miRTarBase [20]. Finally, protein-protein interaction (PPI) networks of the host and target genes of the intronic mirRNAs were obtained using STRING 9.0 [21].

III. Results and Discussion

A. Gene Expression Data Analysis around miRNAs

The relationships between the normalized expression level

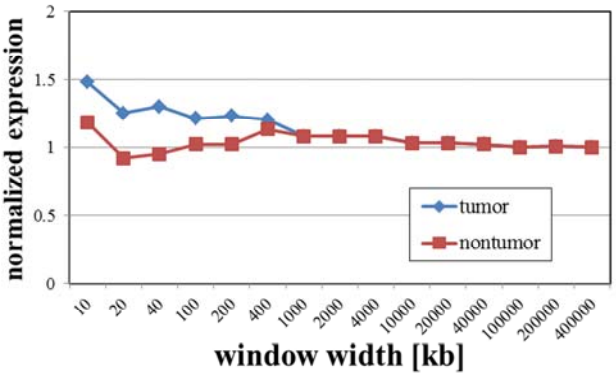

Figure 2. The normalized expression levels in each window width.

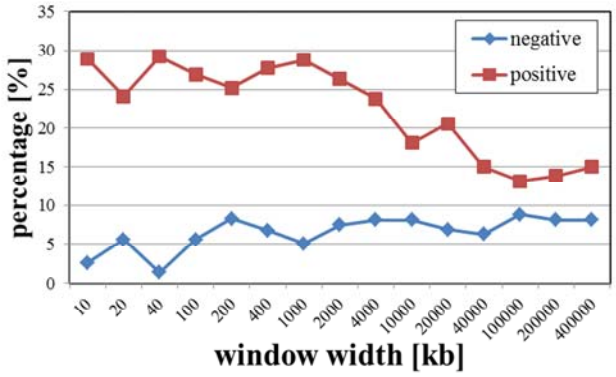

Figure 3. Percentages of the miRNAs whose expressions correlated positively/negatively with the normalized gene expressions in each window width. In smaller windows, more miRNAs tend to have positive correlations with the normalized gene expressions in the window.

TABLE I. Comparison of the Correlation Coefficient between the Tumor and Nontumor Tissues

window width	average correlation coefficient (tumor)	average correlation coefficient (nontumor)	*p* value
10 kb	0.233±0.068	-0.064±0.036	< 0.001
20 kb	0.185±0.086	-0.037±0.047	< 0.001
40 kb	0.226±0.087	-0.033±0.058	< 0.001
100 kb	0.160±0.103	-0.010±0.069	< 0.001
200 kb	0.178±0.120	0.015±0.064	< 0.001
400 kb	0.174±0.125	0.023±0.068	< 0.001
1 Mb	0.171±0.114	0.021±0.062	< 0.001
2 Mb	0.146±0.114	0.009±0.062	< 0.001
4 Mb	0.110±0.113	0.008±0.060	0.001
10 Mb	0.104±0.101	-0.006±0.056	< 0.001
20 Mb	0.114±0.107	0.008±0.054	< 0.001
40 Mb	0.093±0.115	0.019±0.049	0.014*
100 Mb	0.066±0.103	0.013±0.064	0.092
200 Mb	0.058±0.096	0.018±0.048	0.186
400 Mb	0.050±0.098	0.017±0.048	0.284

*The *p* value was smaller than 0.05, but after the Bonferroni correction, this was not significant.

of genes and the window width are plotted in Fig. 2. In the vicinity of miRNA, the normalized gene expression level was increased, in particular, in the tumor tissues in the windows up to 200 kb. This tendency is the same as reported previously [5]. The gene expressions in these windows were larger than that in the nontumor tissues.

B. Correlation between Expressions of miRNAs and Genes

Fig. 3 plots the percentages of the positively/negatively correlated miRNAs with the average gene expression level in each window as a function of the window width. This graph indicates that more miRNAs correlated with neighboring genes positively. To investigate a reason for the results, we compared the correlation coefficients between the tumor and nontumor tissues (TABLE I). There were significant differences in the windows up to 20Mb. The correlation analysis was repeated for intronic miRNAs (TABLE II) and intergenic miRNAs (TABLE III). The correlation coefficient between the intronic miRNAs and their hosts was significantly larger in the tumor tissues than in the nontumor, whereas there was no significant difference between the intergenic miRNAs and genes near them. TABLE IV compares the correlation coefficients between miRNAs and their target genes in both tissues. No significant difference between the tumor and nontumor tissues was found in the relationship between the miRNAs and their targets. These results suggest that the difference in the correlation coefficient between the tumor and nontumor tissues might be attributable to the altered correlation of intronic miRNAs, and that once expressed, miRNAs seemed to function similarly in both tumor and nontumor tissues.

C. Correlation between expressions of intronic miRNAs and Their Host Genes

There were 31 intronic miRNA-host gene pairs which showed significantly altered correlations between the tumor and nontumor tissues. Of these, TABLE V indicates those pairs in which the correlation coefficient was larger in the tumor tissue than that in the nontumor. The reason we focused on these pairs was because this phenomenon might be explained by a hypothesis that an independent promoter for the intronic miRNA was damaged and thus, the promoter could not control the transcription of the intronic miRNA. According to this hypothesis, it is highly probable that the intronic miRNA would be transcribed with its host gene because of the damaged independent promoter. Fig. 4 depicts an example of PPI network of the host and target genes of an intronic miRNA. As shown in the figure, the host gene had some interactions with target genes, implying that there might be a feedback loop including the host gene-intronic miRNA-target genes.

TABLE II. CORRELATION COEFFICIENT BETWEEN INTRONIC MIRNAS AND THEIR HOST GENES

average correlation coefficient (tumor)	average correlation coefficient (nontumor)	*p* value
0.266±0.105	0.012±0.072	< 0.001

TABLE III. CORRELATION COEFFICIENT BETWEEN INTERGENIC MIRNAS AND GENES NEAR THEM

window width	Average correlation coefficient (tumor)	average correlation coefficient (nontumor)	*p* value
10 kb	0.172±0.109	-0.074±0.0.029	0.029*
20 kb	0.099±0.108	-0.048±0.037	0.147
40 kb	0.115±0.057	-0.069±0.078	0.047*
100 kb	0.078±0.069	0.008±0.056	0.265

*The *p* values were smaller than 0.05, but after the Bonferroni correction, these were not significant.

TABLE IV. CORRELATION COEFFICIENT BETWEEN INTRONIC MIRNA AND THEIR TARGET GENES

average correlation coefficient (tumor)	average correlation coefficient (nontumor)	*p* value
0.198±0.092	0.145±0.083	0.072

TABLE V. SIGNIFICANTLY ALTERED CORRELATION COEFFICIENT BETWEEN INTRONIC MIRNAS AND THEIR HOST GENES

intronic miRNA	host gene	correlation coefficient (tumor)	correlation coefficient (nontumor)
mir-107	PANK1	0.289	-0.389
mir-10b	HOXD3	0.865	0.001
mir-1231	NAV1	0.552	-0.296
mir-1249	C22orf9	0.333	-0.185
mir-1909	REXO1	0.439	-0.052
mir-30e	NFYC	0.517	0.084
mir-335	MEST	0.316	-0.352
mir-33a	SREBF2	0.925	0.180
mir-599	VSP13B	0.993	0.316
mir-602	EHMT1	0.850	-0.527
mir-626	MGA	0.983	-0.109
mir-634	PRKCA	0.522	-0.364
mir-660	CLCN5	0.549	-0.248
mir-95	ABLIM2	0.773	0.087

IV. CONCLUSION

In this study, we analyzed gene and miRNA expressions in HCC and surrounding nontumor tissues. First, we analyzed the average gene expression levels around miRNAs. Then, a percentage of the miRNAs whose expressions correlated positively/negatively with gene expression was calculated in the vicinity of miRNA. Next, the Pearson correlation coefficients were compared between the tumor and nontumor tissues. This analysis was repeated for intronic and intergenic miRNAs. The correlation coefficient between miRNAs and their target genes were compared in both tissues. Finally, PPI networks of the host and target genes of intronic miRNAs were investigated.

The results imply that the relationship between the intronic miRNAs and their host genes is altered in HCC, and that feedback loops including the host gene, intronic miRNA,

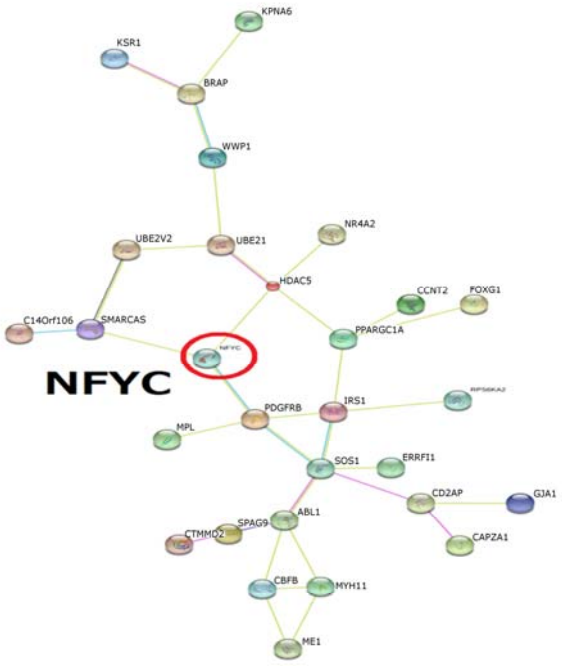

Figure 4. A protein-protein interaction network of the host (NFYC) and target genes of an intronic miRNA miR-634. The pair showed a significant alteration between the tumor and nontumor tissues. The host gene has some interactions with some target genes. This implies that a feedback loop including the host gene-intronic miRNA-target genes might be formed.

target genes might be formed in HCC. These suggest an important role of intronic miRNAs in HCC.

ACKNOWLEDGMENT

The authors thank Mr. T. Iwamura for his work, which is a preliminary version of the study and contributes greatly to the current work.

REFERENCES

[1] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanisms, and function," *Cell*, vol. 116, pp. 281–297, 2004.

[2] E. Bernstein, and C. D. Allis, "RNA meets chromatin," *Genes Dev.*, vol. 19, pp. 1635–1655, 2005.

[3] H. Inaoka, Y. Fukuoka, and I. S. Kohane, "MicroRNA has genome-wide localized effect on gene expression in *C. elegans* germline," *BMC Bioinformatics*, vol. 7, Art#112, 2006.

[4] H. Inaoka, Y. Fukuoka, and I. S. Kohane, "Evidence of spatially-bound gene regulation in *M. musculus*: Decreased gene expression proximal to microRNA genomic location," *Proc. Natl. Acad. Sci. USA*, vol. 104, pp. 5020-5025, 2007.

[5] H. Inaoka, Y. Fukuoka, and M. Noshiro, "Genome-wide analysis of expression of genes locate near miRNAs in humans and mice," *Sensors & Materials*, 24, pp. 303-310, 2012.

[6] S. Volinia, G. A. Calin, C. G. Liu, S. Ambs, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Roldo, M. Ferracin, R. L. Prueitt, N. Yanaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C. C. Harris, and C. M. Croce, "A microRNA expression signature of human solid tumors defines cancer gene targets," *Proc. Natl. Acad. Sci. USA*, vol. 103, pp. 2257-2261, 2006.

[7] N. Yanaihara, N. Caplen, E. Bowman, M. Seike, K. Kumamoto, M. Yi, R. M. Stephens, A. Okamoto, J. Yokota, T. Tanaka, G. A. Calin, C. G. Liu, C. M. Croce, and C. Harris, "Unique microRNA molecular profiles in lung cancer diagnosis and prognosis," *Cancer Cell*, vol. 9, pp. 189–198, 2006.

[8] E. J. Lee, Y. Gusev, J. Jiang, G. J. Nuovo, M. R. Lerner, W. L. Frankel, D. L. Morgan, R. G. Postier, D. J. Brackett, and T. D. Schmittgen, "Expression profiling identifies microRNA signature in pancreatic cancer," *Int. J. Cancer*, vol. 120, pp. 1046-1054, 2007.

[9] Y. Murakami, T. Yasuda, K. Saigo, T. Urashima, H. Toyoda, T. Okanoue, and K. Shimotohno, "Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues," *Oncogene*, vol. 25, pp. 2537-2545, 2006.

[10] Y. Laderio, G. Couchy, C. Balabaud, P. Bioulac-Sage, L. Pelletier, S. Rebouissou, and J. Zucman-Rossi,"MicroRNA profiling in hepatocellular tumor is associated with clinical features and oncogene/tumor suppressors gene mutations," *Hepatology*, vol. 47, pp. 1955-1963, 2008.

[11] W. Li, L. Xie, X. He, K. Tu, L. Wei, J. Wu, Y. Guo, X. Ma, P. Zhang, Z. Pan, X. Hu, Y. Zhao, H. Xie, G. Jiang, T. Chen, J. Wang, S. Zheng, J. Cheng, D. Wan, S. Yang, Y. Li, and J. Gu, "Diagnostic and prognositic implications of microRNAs in human hepatocellular carcinoma," *Int. J. Cancer*, vol. 123, pp. 1616-1622, 2008.

[12] Y. Katayama, M. Maeda, K. Miyaguchi, S. Nemoto, M. Yasen, S. Tanaka, H. Mizushima, Y. Fukuoka, S. Arii, and H. Tanaka, "Identification of pathogenesis-related microRNAs in hepatocellular carcinoma by expression profiling," *Oncology Lett*, vol. 4, pp. 817–823, 2012.

[13] H. Blum, "Hepatocellular carcinoma: therapy and prevention," *World J. Gastroenterol*., vol. 11, pp. 7391-7400, 2005.

[14] M. Yasen, H. Mizushima, K. Mogushi, G. Obulhasim, K. Miyaguchi, K. Inoue, I. Nakahara, T. Ohta, A. Aihara, S. Tanaka, S. Arii, and H. Tanaka, "Expression of Aurora B and alterative variant forms in hepatocellular carcinoma and adjacent tissue," *Cancer Sci*, vol. 100, pp. 472-480, 2009.

[15] S. Griffiths-Jones, H. K. Saini, S. van Dongen, and A. J. Enright, "miRBase: tools for microRNA genomics," *Nucleic Acids Res*., vol. 36, pp. D154-D158, 2008.

[16] A. Krek, D. Grün, M. N. Poy, R. Wolf, L. Rosenberg, E. J. Epstein, P. MacMenamin, I. da Piedade, K. C. Gunsalus, M. Stoffel, and N. Rajewsky, "Combinatorial microRNA target predictions," *Nat. Genet.*, vol. 37, pp. 495-500, 2005.

[17] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," Cell, vol. 120, pp. 15-20, 2005.

[18] B. John, A. J. Enright, A. Aravin, T. Tuschl, C. Sander, and D. S. Marks, "Human microRNA targets," *PLoS Biol.*, vol. 2, e363, 2004.

[19] G. L. Papadopoulos, M. Reczko, V. A. Simossis, P. Sethupathy, A. G. Hatzigeorgiou, "The database of experimentally supported targets: a functional update of TarBase," *Nucleic Acids Res.*, vol. 38, pp. D155-D158, 2009.

[20] S. D. Hsu, F. M. Lin, W. Y. Wu, C. Liang, W. C. Huang, W. L. Chan, W. T. Tsai, G. Z. Chen, C. J. Lee, C. M. Chiu, C. H. Chien, M. C. Wu, C. Y. Huang, A. P. Tsou, and H. D. Huang, "miRTarBase: a database curates experimentally validated microRNA-target interactions," *Nucleic Acids Res.*, vol. 39, pp. D163-D169, 2010.

[21] D. Szklarczyk, A. Franceschini, M. Kuhn, M. Simonovic, A. Roth, P. Minguez, T. Doerks, M. Stark, J. Muller, P. Bork, L. J. Jensen, C. von Mering, "The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored," Nucleic Acids Res., vol. 39, pp. D561-D568, 2011.