Four microRNAs Signature for Survival Prognosis in Colon Cancer using TCGA Data

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This study aims to develop microRNA expression signature for colon cancer survival prognosis based on the Cancer Genomic Common database. miRNAs levels between colon cancer and non-cancer tissues were screened by t-test (p < 0.05). Kaplan-Meier survival method was used to discriminate survival significant miRNAs, followed by miRNAs index accumulation to power the miRNAs-survival reliability. In the end, we test the selected miRNAs in HT126 colon cancer cells to validate its anti-cancer effect. The study identified a 84-miRNAs signature. Of the above 84 miRNAs, we got four miRNAs which were survival associated by using ROC curve method and Kaplan-Meier survival method (p < 0.001). The result showed that low risk group had quite a low death rate, the survival rate was over 80%. The high risk group had survival rate lower than 20%, which was also extremely lower than the overall survival rate. In the HT126 cells study, cell growth assay showed miR-130a sponge inhibited colon cancer cells growth and sensitized the anti-cancer drug effect of 5-FU to blocked cancer cell growth. We developed a prognostic 4-microRNA expression signature for colon cancer patient survival, and validated miR-130a sponge could sensitized 5-FU anti-cancer effect.

Colon cancer is the third most commonly diagnosed cancer in males and the second in females, and the fourth greatest cause of cancer-related deaths worldwide. Nowadays, tumor screening methods include the guaiac-based fecal occult blood test [FOBT], flexible sigmoidoscopy, stool DNA test, computed tomography [CT] colonography, double-contrast barium enema, and colonoscopy. Of these screening options, prognostic survival markers of patients are still under development. Hence, the identification of novel markers, which could indicate high risk or low risk in survival, would greatly optimize the use of therapies and benefit patients. Recently, prognostic microRNAs (miRNAs or miRs) expression signatures have been developed in cancers, and miRNAs signature has been realized as important epigenetic changes in cancer development and therapy.

miRNAs are short 20–22 bp nucleotide, non-coding RNAs which play key roles in biological function. The abnormal expression level of miRNAs is realized as an important issue in cancer development. Therefore, miRNAs therapy is becoming a bright target. These small molecules regulate gene expression through binding to the target mRNA, which influence mRNA stability or suppress translation. It was reported that miRNAs were stable, even in formalin-fixed paraffin-embedded (FFPE) samples. Therefore, miRNAs analysis will not be affected by storage time in tissue samples. Basically, miRNAs regulate the expression of more than 30% of human genes.

It was reported that several miRNAs markers were identified in cancers, such as head and neck squamous cell carcinomas, six-miRNAs signature in bile duct cancer prediction, and 5-miRNAs prognosis in glioma. However, as to the smaller patient number or limited miRNAs number, or different miRNAs-chip platform in colon cancer study, studies lacked a normalized standard. Therefore, a larger patient cohort and normal controls as well as a standard protocol for more specific prognostic classifiers are warranted.

In the current study, we employed a large cohort of colon cancer patients to explore miRNAs expression signature for survival prognosis. We study prognostic value of miRNAs expression in colon cancer, with the aim of developing a multi-miRNAs prognostic expression signature. In this end, we assessed the expression of 1046 miRNAs in 467 patients from Genomic Data Commons Data Portal (https://gdc-portal.nci.nih.gov/). Additionally, we investigated the association of miRNAs expression index with the survival time, and ranked the risk index in colon cancer.
Materials and Methods

Patient cohort and miRNAs data. The results shown here were wholly based upon data generated by the TCGA Research Network: https://gdc.cancer.gov/. The dataset acquired above contained 1046 noted miRNAs expression data. The downloaded clinical data were matched to the miRNAs expression profile. Therefore, some patients were excluded, such as those missing miRNAs expression level, or those without follow up day, or those without most clinical information.

Screening of differentially expressed miRNAs and Hierarchical clustering. Differentially expressed miRNAs between cancer and non-cancer tissues of colon cancer were screened by t-test in Excel file (p < 0.05). Multi-experimental viewer software was used to get hierarchical clustering map (choose hierarchical clustering method). The hierarchical clustering analysis guaranteed cancer and non-cancer specimens were correctly classified by miRNAs levels.

Selection of cutoff score for the Kaplan-Meier survival analysis. We selected the cutoff scores based on receiver operating characteristic (ROC) curve analysis 

miRNAs signature index and survival. We assigned high risk miRNAs expression level as one, or else zero, then scored all miRNAs value in the signature. Therefore, each patient would have a score, named signature index. Input their survival status, survival days and the signature index to plot survival curve using Kaplan-Meier survival method (Log-rank). We set index as high risk and low risk into two group based on the index value. Therefore, all patients were divided into high risk or low risk group according to the miRNAs signature index.

Chemicals and cell lines. HT126 cells were cultured with DMEM supplemented 10% FBS (Gibico, Thermo Inc.). miRNAs sponge employed pLVX-shRNA2 vector which is purchased from Clonetech Inc. The complete sponge vector was constructed by Genscript Inc. 5-FU was from Selleck Inc.

Statistics. The levels of miRNAs expression between cancer tissues and non-cancer tissues were analyzed by t-test and p < 0.05 is deemed as significant different. All analysis related with patient survival were testified by Kaplan-Meier survival analysis (Log-rank method). All statistical analyses were performed in Prism 5.0 software.

Results

Identification of a 84-miRNAs signature to discriminate colon cancer from non-cancer. Data of 268 COAD patients and 8 controls were downloaded from Harmonized Cancer Datasets (https://gdc-portal.nci.nih.gov/), including miRNAs expression profile, clinical follow-up information, et al. The overall information of patients were listed in Table 1. We compared all miRNAs expression level to non-cancer level, and got an 84-miRNAs signature cluster map using Multi-experimental viewer in Hierarchical Clustering method (Supplemental Figure s1). In the clustering analysis, 64 miRNAs were up regulated in cancer tissue, and 20 were down regulated in cancer tissue compared to non-cancer tissue (t test, p < 0.001).

Validation of 4 miRNAs were associated with patients survival. Of the above 84 miRNAs, we employed ROC curve method to set the cutoff point to classify 268 patients in two groups: high level group and low level group. If the ROC curve cutoff value was significant, patients were divided into two groups and carried out with Kaplan-Meier survival analysis. Upon the survival analysis, we got 4 miRNAs which were significantly related with patient survival (Fig. 1A, Log-rank method, *p < 0.05). These 4 miRNAs were miR-148a, miR-26a-2, miR-130a and miR-103-1. According to the result, high expression level of miR-148a, miR-26a-2 and miR-130a were regarded as poorer prognostic markers compared to the low level (Fig. 1A–C). As to miR-103-1, contrary, high expression might be a protection factor in patient survival, while low expression showed a shorter survival rate and time (Fig. 1D). We also performed validation of four miRNAs signature in stage 2 and stage 3 respectively. Data showed miRNAs signature-based risk index was also meaningful in stage 2 and stage 3 patients, indicating the robustness of miRNAs signature prognostic biomarker (Supplemental Figure s2).

Four-miRNAs signature index for colon cancer prognosis. We scored the 4-miRNAs signature by value assignment for each miRNAs. For example, higher expression of miR-148a, miR-26a-2 and miR-130a got one score in each patient, and lower expression of miR-103-1 got one score in each patient. Furthermore, we summated the score for each patient. Accordingly, the highest score would be four, and the lowest would be zero (Supplemental Figure s2). We set index above three as high risk, and those below three as low risk. Therefore, all patients were divided into high risk or low risk group according to the miRNAs index score. We analyzed the two groups by Kaplan-Meier survival analysis in Log-rank method. The result showed that low risk group had quite a low death rate which the survival rate was over 80% (Fig. 2A). The high risk group had survival rate lower than 20%, which was also extremely lower than the overall survival rate in Table 1.

KEGG signal pathway and GO annotation of 4-miRNAs predicted genes. In order to further explore the four miRNAs signature in biological function and mechanism. We analyzed those potential targets...
which might be regulated by the four miRNAs through KEGG signal pathway and GO annotation analysis. Firstly, we predicted target genes through online miRNAs prediction software (http://www.microrna.org/microrna/getGeneForm.do), and selected the first 100 predicted targets (Supplemental Figure s3) as input genes for KEGG signal pathway and GO annotation. The results of KEGG pathway analysis were listed in Table 2. The results indicated that cancer related pathways are obviously activated, including glioma, endometrial cancer, thyroid cancer, prostate cancer and leukemia. Several key proteins appealed our interests, such as MDM4, TGFA, CDK19, SHC4 and PTEN. We hypothesized these proteins played vital panel joint in multiple cancers. GO annotation results have three parts: molecular function (Table 3), biological process (Supplemental Figure s4), and cellular component (Supplemental Figure s5). In the molecular function part, we found that many molecular functions were mainly associated with DNA binding and gene transcription. There four miRNAs might be tightly related with gene expression and the cellular and biological function.

Treatment of miR-130a sponge enhanced anti-cancer drug therapeutic effect in colon cancer cells. We used HT126 colon cancer cells in the study to explore the anti-cancer effects of miR-130a sponge. In the study, cell growth assay showed miR-130a sponge inhibited colon cancer cells growth in a dose dependent manner in 96 h (Fig. 3A and B). When it combined with anti-cancer drug 5-FU, the miR-130a sponge sensitized the anti-cancer drug effect of 5-FU to block cancer cell growth in 96 h (Fig. 3C).

| Characteristic                     | Cohort (n = 268) |
|-----------------------------------|-----------------|
|                                   | No.  | %     |
| Sex                               |      |       |
| Male                              | 146  | 54.48 |
| Female                            | 122  | 45.52 |
| Age, years                        |      |       |
| Median                            | 61   |       |
| Range                             | 17–85|       |
| Height, cm                        |      |       |
| Median                            | 170  |       |
| Range                             | 80.3–193|     |
| Weight, kg                        |      |       |
| Median                            | 80   |       |
| Range                             | 34–175.3|     |
| Histological classification       |      |       |
| Colon Mucinous Adenocarcinoma     | 40   | 14.93 |
| Colon Adenocarcinoma              | 228  | 85.07 |
| CEA+ level pretreatment           |      |       |
| Median                            | 3.4  |       |
| Range                             | 0.2–1286|     |
| Pathologic stage                  |      |       |
| Stage I                           | 39   | 14.55 |
| Stage II                          | 106  | 39.55 |
| Stage III                         | 81   | 30.22 |
| Stage IV                          | 34   | 12.69 |
| Not known                         | 8    | 2.99  |
| Tissue invasion                   |      |       |
| Vascular invasion                 | 51/237| 21.52 |
| Lymphovascular invasion           | 67/239| 28.03 |
| Perineural invasion               | 43/165| 26.06 |
| Follow-up, days                   |      |       |
| Median                            | 182  |       |
| Range                             | 0–4122|     |
| Mean                              | 534.3|       |
| SD                                | 869.4|       |
| Survival rate                     |      |       |
| 1 year                            | 71   | 82.60 |
| 3 year                            | 44   | 61.11 |
| 5 year                            | 24   | 35.82 |

Table 1. Summary of patient cohort information.
Discussion

miRNAs regulation as a key epigenetic issue, as well as DNA methylation, histone acetylation and methylation, protein modification, would be no doubt becoming important markers in disease diagnosis and prognosis.

Figure 1. Based on ROC curve cutoff point, miRNAs expression level were divided into two groups: high level expression group and low level expression group. Upon the survival analysis, 4-miRNAs, which was significantly related with patient survival (Log-rank method, *p < 0.05). The high expression level of miR-148a, miR-26a-2 and miR-130a were significantly associated with poor clinical outcome. In miR-130a group, 5-year survival rate was 87.5% in low level group, only 56.3% in high level group (A). In miR-26a-2 and miR-148 group, 5-year survival rates were 78.3% and 67.9% in low level group respectively, only 42.8% and 45.2% in high level group (B,C). Reversely, in miR-103-1, 5-year survival rate was 68.7% in high level group, only 40.5% in low level group (D).

Figure 2. Individual patient was scored according to the four-miRNAs signature. Score 2–4 was ranked as high risk group in survival, and score below 2 as low risk group. The result showed that low risk group had quite a low death rate, the survival rate was 82.7% (A). The high risk group had survival rate lower than 20%.

Discussion

miRNAs regulation as a key epigenetic issue, as well as DNA methylation, histone acetylation and methylation, protein modification, would be no doubt becoming important markers in disease diagnosis and prognosis.
miRNAs signature have gradually shown its unique and meaningful effects in cancer early diagnosis and survival prognosis. Although the function of miRNAs constituting signature is being increasingly recognized, the
mechanism is quite complicated. The survival prognostic miRNAs signature meets many crucial standards. Firstly, the signature must be specific in cancer and non-cancer; secondly, the signature is correlated with patient survival; last, the signature has synergized effect in patient survival prognosis. Several diagnostic and predicted miRNAs signature have revealed by scientists worldwide. It was reported that several miRNAs markers were identified for cancer prediction and prognosis, such as head and neck squamous cell carcinomas, bile duct cancer prediction, and glioma. However, up to date, the miRNAs expression patterns in the survival prognosis of colon cancer have not been investigated systematically. In the present study, we got four miRNAs which were survival associated by using ROC curve method and Kaplan-Meier survival method (p < 0.001) from 467 colon cancer database. We scored the miRNAs signature to form an index by assignment value for each miRNAs. The result showed that low risk group had quite a low death rate, the survival rate of which was over 80%. The high risk group had a survival rate lower than 20%, which was also extremely lower than the overall survival rate. Recently, Gao et al. developed 8 cancer hallmark-based gene signature sets which in combination can predict prognostic of recurrence for patient receiving fluorouracil-based chemotherapy of stage 2 colon cancer. In agreement with their study, and enlightening by their idea, we performed validation of four miRNAs signature for stage 2 and stage 3 prognostic analysis, due to the heterogeneous of cancers. To our surprise, not all single miRNA expression level works well for stage 2 and stage 3 patients (data not shown). The combined miRNAs expression level-based index showed association of risk factor with patient survival rate in stage 2 and stage 3 colon cancer patients. The reason might be related with the different potential targets with different weighting effects on cancer hallmark of stage 2 and stage 3, additionally, discrepant regulation on genes expression of each miRNA in different cancer stage.

Wang et al. reported that cancer hallmark network framework play important roles in predicting tumor clinical phenotypes. Therefore, we performed GO Terms and KEGG pathway analysis. We also found many predicted genes participated in cancer related hallmark and acted oncogene functions. Many genes were involved in the cancer hallmarks, such as cell death (more than 25 genes, p < 0.004, Supplemental Tables s4 and s5), sustained angiogenesis and vessel morphogenesis (more than 12 genes, p < 0.001, Supplemental Table s4), and cellular metabolic processes (more than 47 genes, p = 0.018, Supplemental Table s4). Furthermore, many genes participated in transcription activity (more than 50 genes, Table 3), mRNA processing (more than 5 genes, Table s5), RNA polymerase II transcription factor activity (more than 10 genes, Table 3). These genes were associated with limitless replicative potential, inflammation cytokines release, and immune system. Additionally, we performed KEGG pathway analysis, and found that all pathways were associated with cancer, such as p53 pathway (7 genes, p = 0.002, Table 2), glioma pathway (8 genes, p = 0.001, Table 2), pathway in cancer (14 genes, p = 0.009 Table 2), et al. Taken together, the four miRNAs signature potentially regulated cancer related genes, and influenced cancer hallmarks. The vital impacts of these four miRNAs on cancer still need further exploration in the future.

Our observations indicated that the four miRNAs signature may play a critical role in cancer cell growth after anti-cancer drug treatment. To further testify such hypothesis, we constructed miR-130 sponge expression vector and treated cancer cells with anti-cancer drug 5-FU. Results showed miR-130a sponge inhibited HT126 cells growth and sensitized the anti-cancer drug effect of 5-FU to block cancer cell growth. The latest study about...
gastric cancer reported that miR-130 was an oncogene by directly targeting TGFbetaR2, and as a result, it promotes cancer growth\textsuperscript{19}. Egawa H. et al. also indicated that the miR-130 family has a crucial role in malignant progression of bladder cancer\textsuperscript{20}. The authors suggested the miR-130 family could be a promising therapeutic target for invasive bladder cancer. Some other studies revealed miR-130 also participated in cardiac development and pulmonary hypertension\textsuperscript{21,22}. So far, rare study focused on miR-130a in colon cancer. In agreement with the previous study of miR-130 as an oncogene, our study further revealed that miR-130a sponge vector could suppressed colon cancer cell growth. The miR-130a sponge also sensitized 5-FU drug anti-cancer effect. The influence of miR-130a expression on regulation of cell growth and anti-cancer drug efficacy might be through targeting some vital signal pathway members. In the miR-130a targets prediction, many potential genes play important roles in cell growth (MDM4, KLF7, ACVR1, IRF1, DNKL2 et al.), transcription activity (MDM4, MYBL1, ACVR1, MAF et al.), mRNA processing (KLF7, CPEB1, IRF1, RPS6KA5 et al.), protein kinase activity (PAN3, TGFBR2, CDK19, RPS6KA5 et al.) and metabolic process (PAN3, CPEB1, RPS6KA5, SBF2 et al.). Therefore, we hypothesized that miR-130a might act as a anti-cancer target, as well as a prognostic biomarker for colon cancer.

Our results demonstrated that the four-miRNAs signature is a potential marker for colon cancer patient survival prognosis. The suppressing effect of colon cancer cell growth of miR-130a sponge vector supplies a brand new insight into the therapeutic strategy against colon cancer, especially for those patients with poor predicted survival rate.

References
1. Torre, L. A. et al. Global cancer statistics, 2012. CA Cancer J Clin. 65(2), 87–108 (2015).
2. Khare, S. & Verma, M. Epigenetics of colon cancer. Methods Mol Biol. 863, 177–85 (2012).
3. Osaki, M., Okada, F. & Ochiya, T. miRNA therapy targeting cancer stem cells, a new paradigm for cancer treatment and prevention of tumor recurrence. Ther Deliv. 6(3), 323–37 (2015).
4. Fabian, M. R., Sonenberg, N. & Filipowicz, W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem. 79, 351–79 (2010).
5. Szafranska, A. E. et al. Accurate molecular characterization of formalin-fixed, paraffin-embedded tissues by microRNA expression profiling. J Mol Diagn. 10(5), 415–23 (2008).
6. Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 120(1), 15–20 (2005).
7. Wong, N. et al. Prognostic microRNA signatures derived from The Cancer Genome Atlas for head and neck squamous cell carcinomas. Cancer Med. 5(7), 1619–28 (2016).
8. Wang, M. et al. A six-microRNA set as prognostic indicators for bile duct cancer. Int J Clin Exp Med. 8(10), 17261–70 (2015).
9. Yan, W. et al. MicroRNA expression patterns in the malignant progression of gliomas and a 5-microRNA signature for prognosis. Oncotarget. 5(24), 12908–15 (2014).
10. Zlobec, I. et al. Selecting immunohistochemical cut-off scores for novel biomarkers of progression and survival in colorectal cancer. J Clin Pathol. 60(10), 1112–6 (2007).
11. Zhu, Z. H. et al. Three immunomarker support vector machines-based prognostic classifiers for stage IB non-small-cell lung cancer. J Clin Oncol. 27(7), 1091–9 (2009).
12. Guo, S. et al. Identification and Construction of Combinatory Cancer Hallmark-Based Gene Signature Sets to Predict Recurrence and Chemotherapy Benefit in Stage II Colorectal Cancer. JAMA Oncol. 2(1), 37–45 (2016).
13. Wang, E. et al. Predictive genomics: a cancer hallmark network framework for predicting tumor clinical phenotypes using genome sequencing data. Semin Cancer Biol. 30, 4–12 (2015).
14. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. Cell. 144(5), 646–74 (2011).
15. Hainaut, P. & Plymoth, A. Targeting the hallmarks of cancer: towards a rational approach to next-generation cancer therapy. Curr Opin Oncol. 25(1), 50–1 (2013).
16. Cho, M., So, I., Chun, J. N. & Jeon, J. H. The antitumor effects of geraniol: Modulation of cancer hallmark pathways (Review). Int J Clin Pathol. 21(3), 297–308 (2012).
17. Engstrom, W. et al. The potential for chemical mixtures from the environment to enable the cancer hallmark of sustained proliferative signalling. Carcinogenesis. 36 Suppl 1, S38–60 (2015).
18. Ward, P. S. & Thompson, C. B. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. Cancer Cell. 21(3), 297–308 (2012).
19. Duan, J. et al. Onco-miR-130 promotes cell proliferation and migration by targeting TGFbetaR2 in gastric cancer. Oncotarget. 6(3), 63–73 (2015).
20. Egawa, H. et al. The miR-130 family promotes cell migration and invasion in bladder cancer through FAK and Akt phosphorylation by regulating PTEN. Sci Rep. 6, 20574 (2016).
21. Lopez-Sanchez, C. et al. Negative Fgf8-Bmp2 feed-back is regulated by miR-130 during early cardiac specification. Dev Biol. 406(1), 63–73 (2015).
22. Bertero, T. et al. Matrix Remodeling Promotes Pulmonary Hypertension through Feedback Mechanosactivation of the YAP/TAZ-miR-130/301 Circuit. Cell Rep. 13(5), 1016–32 (2015).
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