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RESEARCH ARTICLE

Circulating Plasma MiR-141 Is a Novel Biomarker fo Metastatic Colon Cancer and Predicts Poor Prognos

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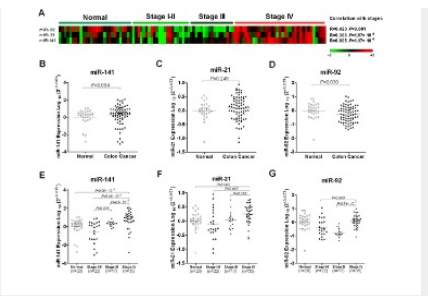
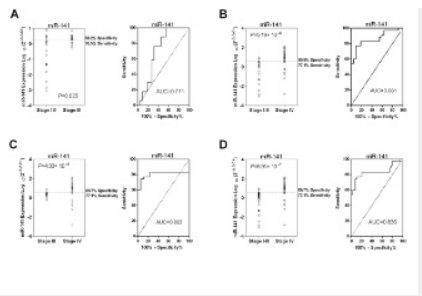
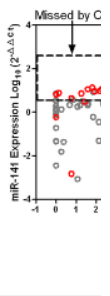
Figures







Abstract

Background

Colorectal cancer (CRC) remains one of the major cancer types and worldwide. Sensitive, non-invasive biomarkers that can facilitate dis staging and prediction of therapeutic outcome are highly desirable rate and help to determine optimized treatment for CRC. The small n microRNAs (miRNAs), have recently been identified as critical regula diseases including cancer and may represent a novel class of canc purpose of this study was to identify and validate circulating microR for use as such biomarkers in colon cancer.

Methodology/Principal Findings

By using quantitative reverse transcription-polymerase chain react circulating miR-141 was significantly associated with stage IV colon 102 plasma samples. Receiver operating characteristic (ROC) analy evaluate the sensitivity and specificity of candidate plasma microRI observed that combination of miR-141 and carcinoembryonic antig marker for CRC, further improved the accuracy of detection. These f in an independent cohort of 156 plasma samples collected at Tianji our analysis showed that high levels of plasma miR-141 predicted p cohorts and that miR-141 was an independent prognostic factor for cancer.

Conclusions/Significance

We propose that plasma miR-141 may represent a novel biomarker in detecting colon cancer with distant metastasis and that high leve plasma were associated with poor prognosis.

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Introduction

Colorectal cancer (CRC) is a worldwide health problem with 655,000
the United States, CRC is the third most common cancer type and th
common cause of cancer-related death [2], with an estimated 51,37
according to the National Cancer Institute. In China, CRC remains the
cancer type and the fourth most common cause of cancer-related d
screening and development of new chemotherapeutic strategies, C
during the past 20 years have not substantially improved. Moreove
is increasing rapidly in recent years in China [4]. Novel biomarkers t
value are thus in urgent need to improve compliance rates. Carcino
(CEA) has been used as a serum marker of CRC, although its sensitiv
different studies [5], [6]. Recently, a family of small regulatory RNAs,
emerged as possible plasma markers for human diseases including
relative stability in the circulation [7].

MicroRNAs are small non-coding RNAs (18–22 nt in length) that regu
target genes by interfering with transcription or inhibiting translatio
demonstrated that microRNAs play a crucial role in almost all cellula
including metabolism, survival, differentiation and apoptosis. Dereg
microRNAs contributes to a variety of diseases, most notably the de
progression of cancer, including CRC. Specifically, microRNA expres
showed that a number of microRNAs, including miR-21, miR-20a and
upregulated in tumor tissues and that higher miR-21 was associate

outcome [9], [10]. The potential of circulating miRNAs in plasma as c
also been evaluated in a few studies. For CRC, a recent study repor
were significantly higher in plasma samples from patients than in h
can be a potential marker for CRC detection [11]. A study of plasma
cancer patients reported that plasma miR-141 levels can be used t
prostate cancer with high sensitivity [12].

In this proof-of-principle study to identify potential biomarkers for CI
whether selected candidate microRNAs could serve as non-invasiv
markers for CRC by analyzing the relative levels of three microRNAs
miR-141) in a cohort of 102 plasma samples from healthy individual
obtained from TexGen, a collaboration of Texas Medical Center Inst
indicated that among these three microRNAs, plasma miR-141 level
elevated in the plasma of colon cancer patients with Stage IV disea
discriminate distant metastasis cases from normal controls and pat
stages. Combination of miR-141 with CEA was complementary and c
the detection accuracy of distant metastasis in colon cancer. These
validated in an independent cohort of 156 plasma samples obtaine
Our further analyses provided supporting evidence that plasma mi
prognostic factor that predicted for poor survival in colon cancer pa
unlike in plasma, miR-141 was not differentially expressed in tumor
Stage IV and Stage I–II colon cancer patients or between tumor tiss
tumor tissues in Stage IV patients, suggesting that elevation of plas
patients might be derived from other systemic responses such as i
in these patients.

Materials and Methods

Ethics statement

Both plasma- and tissue-based studies were approved by the Institu
(IRB) at the MD Anderson Cancer Center and by the Ethics Committe
University Cancer Institute and Hospital. All participants gave writte
information to be stored in the hospital database and used for rese

Clinical samples

Two independent sets of plasma samples were used. A total of 102
age- and gender- matched healthy individuals and CRC patients (St
obtained from TexGen between 2002 and 2008, a collaboration of T
institutions that provides biological samples as well as epidemiolog
(TexGen samples, See Table S1). An independent cohort of 156 pla
age- and gender- matched healthy donors and colon cancer patien
obtained at the Tianjin Medical University Cancer Institute and Hosp
samples) between 2007 and 2009 (See Table S2). Pathologic classif
patients was performed following the International Union Against C
American Joint Committee on Cancer (AJCC) TNM staging system for

established in 2003. Blood samples were collected from all patients
therapy. CEA levels of both TexGen and TCH plasma samples were
enzyme immunoassay as part of routine clinical tests and were acc
database at each institute. Follow-up data of all the recruited colon
both TexGen and TCH were acquired and survival time was calcula
diagnosis to the date of death or last follow-up in June, 2010. All pat
study had not received chemotherapy or radiotherapy prior to the
demographic information of the two sets of samples is summarized
material.

For the tissue-based analysis tumor tissues from 21 colon cancer p
metastatic disease (Stage IV) and 24 colon cancer patients with no
(Stage I and II) were collected between 2007 and 2009 by Tianjin Me
Cancer Institute and Hospital from the same patients whose plasma

RNA isolation and quantitative RT-PCR

Small RNA was enriched from all plasma samples using the mirVana
(Ambion, Austin, TX). Briefly, 250 μL of plasma was thawed on ice an
14,000 rpm for 10 minutes to remove cell debris and other cellular
μL of supernatant was lysed with an equal volume of 2x denaturing
normalization of sample-to-sample variation during the RNA isolatio
of synthetic *C. elegans* miRNA cel-miR-39 was added to each denatu
were then enriched and purified following manufacturer's protocol,
that the enriched small RNAs were eluted in 45 μL of preheated nuc
Standard TRIZOL method (Invitrogen) was used to isolate total RNA

For microRNA based RT-PCR assays, 2.5 μL of enriched small RNAs f
were reverse transcribed using the TaqMan MicroRNA Reverse Tra
Biosystems, San Diego, CA) according to manufacturer's instruction
volume of 7.5 μL. A 1□20 dilution of RT products was used as templa
PCR reaction was performed in triplicates using TaqMan 2x Univers
conditions as described previously [13]. No-template controls for bo
step were included to ensure target specific amplification. The 790
System 2.3 (Applied Biosystems) software defaults were used to co
change in RNA expression by the $2^{-\blacksquare\blacksquare Ct}$ method with 95% confide
for the TexGen specimens were performed at MD Anderson and as
specimens were performed at Tianjin Medical University Cancer Ins

MicroRNA profiling

MicroRNA microarray profiling was performed with RNA isolated fro
using the Ohio State microRNA microarray version 2.0 as previously
Microarray data (including raw and processed data) have been de
Center for Biotechnology Information's (NCBI's) Gene Expression O
GSE7828).

Statistical analysis

The statistical significance was determined by using the Wilcoxon s
between groups. Receiver operating characteristic (ROC) curves we
assess the diagnostic accuracy of each parameter, and the sensitiv
the optimum cut-off point were defined as those values that maxim
the ROC curve (AUC). The relative levels of microRNA were quantifie
method, and the data were analyzed as the $\log_{10}$ of the relative qu
microRNA. The statistical analysis was performed with the use of so
version 16.0 (WPSS Ltd., Surrey, United Kingdom) and graphs were
Graphpad Prism 5.0 (Graphpad Software Inc, California). Spearman
was performed to reveal correlation between plasma miR-141 expr
stages. All statistical tests were two-sided, and a P value of 0.05 wa
significant. The correlation between overall survival and plasma mi
using Kaplan-Meier method and Log-rank test. Cox proportional-haz
analysis was used to evaluate whether plasma miR-141 was an ind
factor for colon cancer.

Results

Plasma miR-141 levels are correlated with clinical stage

Three microRNAs (miR-21, miR-92 and miR-141) were selected in ou
their potential to serve as biomarkers for colon cancer. These cand
selected because 1) miR-21 has been found to be upregulated in C
[10], [14]; 2) miR-92 has recently been reported to be a plasma mark
[11]; and 3) miR-141 has been shown to be a potential plasma mark
prostate cancer [12]. Quantitative RT-PCR based microRNA assays c
were performed. We first examined the correlation between these
colon cancer clinical stages. Stage I and Stage II cases were groupe
analysis because only limited Stage I cases were available. Heat-m
that the miR-141 levels clustered according to different stages (Figu
correlation analysis showed that the plasma miR-141 levels were h
colon cancer stages ($r=0.605$, $P=1.17\times10^{-8}$). In contrast, miR-21 an
correlated with clinical stages (Figure 1, A).

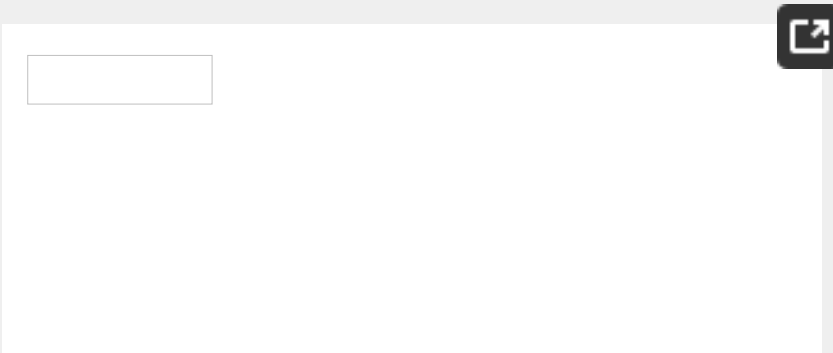

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**Figure 1. Plasma miR-141, miR-21 and miR-92 levels in healthy c
cancer patients.**
(A) Each row corresponds to a plasma miRNA and each column c
each sample. Expression levels for each miRNA are normalized

and shaded in colors such that red denotes high expression an
low expression. Spearman correlation shows that miR-141 is hig
stages (r=0.605, P=1.17×10^{-8}). (B–G) The relative levels of selec
microRNAs were normalized to spike-in control cel-miR-39 and s
of the relative quantity (RQ). The Wilcoxon two-sample tests wer
examine the difference of selected plasma microRNAs between
and colon cancer patients (B–D), or between normal controls an
patients with different clinical stages (E–G).
https://doi.org/10.1371/journal.pone.0017745.g001

We next analyzed whether these candidate microRNAs could serve
cancer markers by comparing their plasma levels between cancer
controls. Surprisingly, unlike the previous report that upregulation o
colon cancer biomarker, the Wilcoxon two-sample test showed that
microRNAs, plasma miR-92 was significantly decreased in the TexG
cancer patients (p=0.03) (Figure 1, D). However, the decrease in pla
observed in Stage I–II and Stage III and the level increased to a simi
normal controls in Stage IV colon cancer (Figure 1, G). Plasma levels
141 did not show significant difference between controls and canc
& C). After stratification of the cancer patients according to their clin
miR-141 was significantly upregulated in Stage IV colon cancer (Fig
less extent, plasma miR-21 was also shown to be elevated in Stage
1, F). Based on these observations, we sought to focus on miR-141 f
characterization.

High plasma miR-141 levels are associated with Stage IV
complement with CEA in diagnosis

The above results demonstrated that among the three selected mic
significantly correlated with colon cancer stages. The detailed Wilc
showed that the plasma miR-141 was significantly elevated in Stag
compared with Stage I–II, Stage III and Stage I–III combined. The ROC
identify a cut-off value that could distinguish stage IV colon cancer
ROC curve analysis showed that at the optimal cut-off, plasma miR-
sensitivity and a 77.1% specificity in separating Stage IV cases from
an AUC of 0.861 (Figure 2, B), a 77.1% sensitivity and a 89.7% specifi
Stage IV and Stage III cases with an AUC of 0.803 (Figure 2, C), and a
89.7% specificity in separating the Stage IV and combined Stage I–I
0.836 (Figure 2, D). Our analysis also revealed that in this cohort, pla
significantly elevated in Stage III patients compared with early stag
(Figure 2, A). These results suggest that circulating miR-141 might b
metastatic colon cancer.

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Figure 2. Higher plasma miR-141 is significantly associated with cancer in the training set.
(A) Small RNA was isolated from plasma samples and miR-141 w quantitative RT-PCR assays. The Wilcoxon two-sample test was evaluate differences of miR-141 levels between the Stage III an ROC analysis was performed to determine the sensitivity and sp value of AUC in the right panel. The Wilcoxon two-sample tests b and Stage I–II, (C) Stage IV and Sage III, and (D) Stage IV and Stag performed to evaluate the association of plasma miR-141 with S status.
https://doi.org/10.1371/journal.pone.0017745.g002

Because the blood CEA test is widely used marker for CRC patients, the performance of miR-141 with CEA as a biomarker. We examined combination of miR-141 and CEA was more sensitive than either ma individually. Our analysis showed that they were indeed compleme specificity was set at 100%, miR-141 identified seven Stage IV colon missed by CEA alone and CEA identified four Stage IV metastatic col were missed by miR-141 alone (Figure 3).

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Figure 3. Combination of CEA and miR-141 identifies additional the training cohort.
Two-parameter (expression of miR-141 and CEA in plasma) class discriminate distant metastatic colon cancer. The cut-off value f and for miR-141 is 16.77 defined from the ROC curve. The corres values are marked by grey lines.
https://doi.org/10.1371/journal.pone.0017745.g003

Validation of plasma miR-141 as a colon cancer marker population

Results from the above studies provided evidence that high levels could be a potential biomarker that complements CEA for metastati

further validate the potential utility of miR-141 in the clinical manage
we performed a validation study with an independent set of plasma
samples) (See Table S2 for demographic information of TCH cases).
were made independently following the same experimental proced
TexGen samples.

The results validated that the plasma miR-141 level was significantl
patients than in normal controls, Stage I–II and Stage III patients (Fig
the ROC curves showed that a cut-off for plasma miR-141 could be
66.7% sensitivity and a 80.8% specificity for separating the Stage IV
I–II cases (AUC=0.756) (Figure 4, B), a 66.7% sensitivity and a 89.7%
separating from Stage III cases (AUC=0.779) (Figure 4, C), and a 66.7
84.0% specificity for separating from Stage 1–III cases (AUC=0.764)
TexGen plasma samples, we however failed to detect a significant
levels between Stage III and Stage I–II cases (not shown), suggestin
miR-141 is more associated with distant and less with lymph-node
cancer.

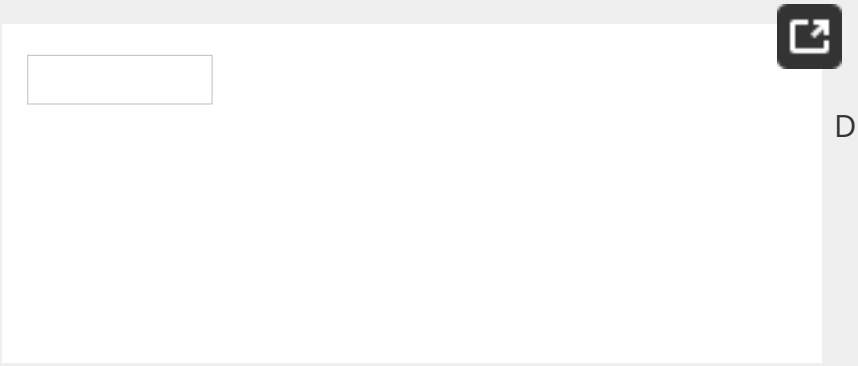

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Figure 4. Higher plasma miR-141 level is associated with Stage IV patients in the validation data set.
Small RNA isolation and miR-141 quantitative RT-PCR assays wer
same way as for the training cohort. (A) The Wilcoxon two-sampl
performed to compare miR-141 levels between normal controls
cancer patients with different clinical stages. (B–D) The same an
performed to compare miR-141 levels between Stage IV cases a
(B), between Stage IV and Stage III cases (C), and between Stage
cases (D), respectively. ROC analysis was performed to determin
and specificity with the value of AUC in the right panel. (E) Combi
miR-141 identified additional metastatic patients that were miss
alone. See Figure 3 for the definition of the cut-off values for CEA
https://doi.org/10.1371/journal.pone.0017745.g004

We also evaluated the CEA data in the TCH dataset. Similar to what
TexGen samples, combination of miR-141 and CEA identified additio
that were otherwise missed by either marker used alone (Figure 4,
validated that the combination of these two biomarkers was a more
for detecting Stage IV CRC patients.

Plasma miR-141 is correlated with poor survival in colon

To further evaluate whether plasma miR-141 levels can predict prog performed a survival analysis on TexGen and TCH cases. Kaplan-M showed that higher expression of plasma miR-141 was significantly survival both in TexGen (P=0.004, log-rank test) and Tianjin (P=0.00 cohorts (Figure S1, A and B). Univariate Cox regression analysis der plasma miR-141 was a significant prognostic indicator of the colon 3.80, 95%CI=1.46−91) and Tianjin cases (HR=4.83, 95%CI=2.06−11. 1). To avoid any potential bias between the TexGen cohort and Tian performed the univariate and multivariate survival analyses for all c Cox proportional hazard regression analysis showed that plasma m independent prognostic marker in colon cancer patients when we m from both centers (HR=2.40, 95%CI=1.18−4.86) (Table 1).

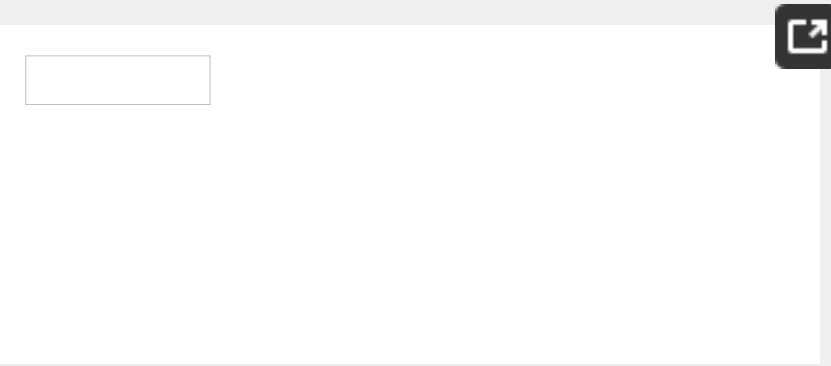

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Table 1. Plasma miR-141 is an independent prognostic factor by analysis.

https://doi.org/10.1371/journal.pone.0017745.t001

MiR-141 is not differentially expressed in colon cancer ti

We sought to determine whether the elevated miR-141 in plasma fr patients reflected differential expression in the tumor tissues from stages. We first compared miR-141 levels in tumor tissues between in the TCH cohort, which surprisingly showed that miR-141 was not cases (p=0.495) (Figure 5, A). We next analyzed our previously des microarray profiling data (GEO, GSE7828). Our analysis also confirm level was not significantly higher in tumor tissues than in adjacent n IV patients, as well as in patients of other stages (Figure 5, B).

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Figure 5. MiR-141 in tumor tissues is not associated with colon c
(A) Total RNA was isolated from tumor tissues of metastatic or no
patients from the Tianjin cohort, and the miR-141 levels were me
quantitative RT-PCR using RNU6B as an endogenous control. Th
sample test was performed to compare miR-141 expression in tu
between these two groups. A $P<0.05$ is considered significant. (
expression levels were measured using microRNA profiling data
cohort of CRC samples. The Wilcoxon matched-pairs tests were
compare the miR-141 levels between tumor tissues and adjacen
these CRC patients.
https://doi.org/10.1371/journal.pone.0017745.g005

Discussion

In this study, we took a focused approach and examined three can
their potential value as plasma biomarkers for colon cancer. Result
revealed that plasma miR-141 was a sensitive marker and complem
detecting Stage IV colon cancer. This result was independently vali
samples from colon cancer patients in Tianjin, China. Thus, this colla
carried out by two independent teams of researchers at two differe
ethnic patient populations provides strong evidence that miR-141 is
marker that complements CEA in determining stage IV colon cancer
also demonstrated that higher plasma miR-141 was associated with
both cohorts and that miR-141 was an independent prognostic indi
We believe that the identification of miR-141 may represent a key a
search for valuable plasma markers for colon cancer that have the
translated into clinical applications including prognosis, monitoring
and detecting disease recurrence. Further validation in a larger coh
prospective study will be needed to determine conclusively whethe
circulating marker for late stage colon cancer.

Apart from the potential impact on clinical diagnosis and prognosis,
revealed some intriguing aspects regarding the origin of serum miR
from this study and the previous study reporting miR-141 elevation
cancer [12] link miR-141 to metastasis, we thus tested a straightforw
the elevated plasma miR-141 reflected the elevated miR-141 level i
colon cancer patients. However surprisingly, we did not observe a s
in the miR-141 expression levels between tumor tissues and adjace
among different stages of CRC or in tumor tissues between non-me
metastatic Stage IV. Therefore, the elevation of miR-141 in plasma is
indication of elevated miR-141 in corresponding tumor tissues at th
possible that miR-141 is only elevated in the metastases at the seco
belongs to the miR-200 family, which promotes the mesenchymal-to
(MET) by inhibiting the expression of the E-cadherin transcriptional s
ZEB2 [15], [16]. Thus it is consistent that we did not observe elevate
primary site of Stage IV colon cancer where EMT instead of MET is b
resulting in cells with higher potential for cell migration and metasta

mesenchymal-like metastatic cells extravosate at distant site, a tran
(MET) occurs, which may explain the surge of miR-141 in metastatic
hypothesis will need to be tested with matching primary tumors and
future.

Another possibility is that selected cellular miRNAs contribute more
circulating miRNAs in cancer patients. The *in vitro* studies also show
expression profiles in conditioned medium were different from those
secreted miRNAs may represent a class of signaling molecules in m
communication [17], although the mechanisms control microRNA pa
are largely unknown. In cancer patients especially at late stage, the
changes including inflammatory response that can involve immune
organs. It is conceivable that these systemic responses may be a s
circulating miRNAs in cancer patients. Along this line, in addition to c
cancers, circulating miR-141 level has also been associated with ot
conditions including ovarian cancer [18] and pregnancy [19]. The d
plasma between Stage IV and Stage I–II CRC may be related to diffe
response in CRC and this possibility will need to be further investiga

Among our candidate miRNAs, miR-21 is an oncogene that is altered
regulating the expression of multiple cancer-related target genes s
[20], [21]. Previous studies showed that expression of miR-21 was u
tumor tissues and was gradually elevated during tumor progressio
late stage as compared with matched non-tumor tissues, suggestin
good circulating marker for CRC detection if this same elevation tren
plasma. However, our analysis did not reveal a difference of plasma
cancer patients and healthy controls. Although circulating miR-21 w
elevated in patients with distant metastasis, the sensitivity and spec
lower than those for miR-141. Similarly, miR-92, a previously identifie
CRC in the Hong Kong cohorts did not represent a useful marker in
the TexGen cohort. We reason that the genetic variations among di
well as environmental factors and diets may contribute to these co

Although the origin of the plasma miR-141 is yet to be resolved, our
the ongoing search for plasma miRNA markers can be a fruitful proc
other nucleotide molecules such as DNA and mRNA, miRNAs are res
RNase activity and, thus, are relatively stable in the circulation. In ac
here, there are increasing examples of plasma miRNAs as potential
leukemia patients, the plasma miR-92 level is dramatically reduced
observed with our CRC in this study), and the ratio of miR-92a/miR-6
potential biomarker for this disease [22]. In addition, plasma levels o
miRNAs (miR-21, miR-210, miR-155, and miR-196a) were found to be
pancreatic cancer [23], and miR-31 upregulation in plasma may be a
cancer [24]. These studies showed the potential of using circulating
biomarkers, but they are of limited value at present because of a la
validations. In our study, the fact that plasma miR-141 was shown to
in two independent cohorts consisting of two different ethnic popul
compelling evidence that miR-141 may emerges as a valuable mark
significance.

Although biomarkers for advanced cancer can be potentially used
therapeutic outcome, biomarkers that can detect early stage disea
metastasis are expected to represent more clinically relevant endp
overall survival rate. Future efforts are still needed to identify circul
biomarkers that can accurately detect CRC at its early stage.

Supporting Information

Figure S1.

Higher miR-141 predicts poor prognosis in both cohorts. Kaplan-M
colon cancer patients in both cohorts. The survival data were comp
rank test and miR-141 expression levels in patients defined as high
median. P-value of log-rank test is 0.004 and 0.002 in TexGen (A) an
respectively. Higher plasma levels of miR-141 were associated with
in colon cancer patients.
https://doi.org/10.1371/journal.pone.0017745.s001
(JPG)

Table S1.

https://doi.org/10.1371/journal.pone.0017745.s002
(DOC)

Table S2.

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Author Contributions

Conceived and designed the experiments: HC KC SRH WZ. Performe
DEC HZ AJS. Analyzed the data: LZ MN. Contributed reagents/materi
KC. Wrote the paper: HC LZ KC SRH WZ.

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