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*Original Article*

# MiR-452-5p may serve as an oncogene in colorectal cancer through targeting CDKN1B: a study based on bioinformatics analysis and dual-luciferase reporter assay

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**Abstract:** Despite the relationship between miRNAs and the carcinogenesis of colorectal cancer (CRC) have been extensively studied, the role of miR-452-5p in CRC remained elusive. Hence this study was designed to investigate the clinico-pathological value of miR-452-5p in CRC as well as to explore the molecular basis of miR-452-5p in CRC. The clinico-pathological significance of miR-452-5p in CRC was examined from the data in The Cancer Genome Atlas (TCGA) database. Meta-analysis for GSE datasets from gene expression omnibus (GEO) and an integrated meta-analysis with all the included GSE datasets and TCGA data were conducted to evaluate the expression level of miR-452-5p in CRC. Prognostic value of miR-452-5p in CRC was further examined by the survival curves from Progmir. We then predicted the target genes of miR-452-5p through miRWalk v 2.0. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were conducted for the functional annotation of the target genes. After the determination of target genes, immunostaining results from the Human protein atlas (HPA) database were used to compare cyclin-dependent kinase inhibitor 1B (CDKN1B) expression in CRC and non-cancer tissues. Eventually, the target-ship between miR-452-5p and CDKN1B was identified through dual-luciferase reporter assay. Results from TCGA data mining, GEO meta-analysis and the integrated meta-analysis revealed that miR-452-5p was overexpressed in CRC and was associated with the progression of CRC. Prognostic data from Progmir reported no obvious difference between the survival time of CRC patients with different expression of miR-452-5p. A total of 1156 genes were predicted as the potential target genes of miR-452-5p. GO and KEGG analysis for the potential target genes revealed that these genes were mainly enriched in the following biological process terms and pathway terms: sensory perception of sound, negative regulation of transcription, DNA-templated and positive regulation of cell cycle, pathways in cancer, transcriptional misregulation in cancer and chronic myeloid leukemia. Data from HPA supported the down-regulation of CDKN1B in CRC. Furthermore, the dual-luciferase reporter assay demonstrated that CDKN1B was directly targeted by miR-452-5p. In summary, the overexpressed of miR-452-5p may played an oncogenic role in CRC via targeting CDKN1B.

**Keywords:** CDKN1B, CRC, dual-luciferase reporter assay, miR-452-5p, TCGA, GEO

## Introduction

Colorectal cancer (CRC) ranks as the third most common cancer in the world, posing a serious burden on the health of people with its high incidence and mortality [1-8]. Though great advance has been made in the diagnosis and therapy, CRC patients still suffered from poor prognosis [9]. The pathogenesis of CRC remained far from elucidated and the development of novel biomarker for CRC is required for the improvement of the treatment of CRC. Recently, microRNAs (miRNAs), a class of molecules essential for the progression of cancers, have become a hot spot in the research of cancer [10].

MiRNAs are small non-coding RNA molecules (between 18-25 nucleotides) that modulate the expression of downstream RNAs by binding to the 3'UTR region of target RNAs, which cause either the inhibition of translation or the degradation of RNAs [11-19]. Approximately one third of all human genes were regulated by miRNAs

and more than 50% miRNAs located at the fragile site or regions of cancer-related genes, which implied the association between miRNAs and the carcinogensis [20, 21]. Accumulated evidence suggested that miRNAs served as oncogenic genes or tumor suppressor genes in various human cancers including CRC [22].

MiR-452-5p belongs to the miR-224/miR-452 cluster and played a key role in the biological events of diverse human cancers such as non-small-cell lung cancer, prostate cancer and hepatocellular carcinoma [23-26]. In CRC, limited was known about the functional roles of miR-452-5p in CRC. Only two Chinese doctoral dissertations included in China National Knowledge Infrastructure (http://www.cnki.net/) reported the up-regulation of miR-452-5p in CRC.

Cyclin-dependent kinase inhibitor 1B (CDKN1B), encoding p27 or $p27^{Kip1}$, exerted an inhibitory function on the G1-S transition of cell-cycle by inactivating the cyclin/cyclin-dependent kinase (cyclin-CDK) complexes [27-31]. The decrease of CDKN1B expression was discovered to correlate with the initiation and malignant deterioration of a wide type of epithelial tumors including breast cancer, prostate cancer, and lung cancer [32-34]. Increasing studies have also proved the down-regulation of CDKN1B in CRC and the accompanied poor prognosis of CRC patients [35-38].

With respect to the relationship between miR-452-5p and CDKN1B, miR-452-5p was found to enhance the malignant potential of HCC by targeting CDKN1B in the study of Zheng et al. [39]. Nevertheless, no experiment definite the interaction between miR-452-5p and CDKN1B in CRC up to date and we are the first to investigate the targeting relationship between miR-452-5p and CDKN1B. In this study, we aimed to explore the clinical significance of miR-452-5p and CDKN1B in CRC as well as the underlying molecular mechanism via validating CDKN1B as the target of miR-452-5p in CRC with the combined methods of in silico analysis and dual-luciferase reporter assay.

## Materials and methods

*Investigation of the clinical-pathological significance of miR-452-5p in CRC from the cancer genome atlas (TCGA) database*

TCGA works as a repository that stores abundant molecular and clinical data of various human cancers [40], which brought convenience to our research. We downloaded the miRNA-Seq data of the precursor of miR-452-5p: miR-452 in CRC including expression value and clinico-pathological information from TCGA (http://cancergenome.nih.gov/). A total of 602 CRC patients and 11 non-cancer patients constituted the whole samples. Among the 602 CRC patients, 442 patients were diagnosed as colonic adenocarcinoma (COAD) and the rest were rectal adenocarcinoma (READ) patients. Therefore, we explored the clinico-pathological significance of miR-452 in groups of COAD and READ separately. Then the clinico-pathological significance of miR-452 was evaluated in the group of CRC that integrated all the data from COAD and READ patients.

*Verification of miR-452-5p expression in CRC tissues via meta-analysis for data from gene expression omnibus (GEO) database*

Apart from TCGA database, we verified the expression of miR-452-5p between CRC tissues and non-cancer tissues via performing a meta-analysis for GEO microarray chips containing the expression data of miR-452-5p between CRC and non-cancer tissues. A systematic research of the GSE datasets published before 14th, February 2018 was conducted in GEO database (https://www.ncbi.nlm.nih.gov/gds/). The searching strategy was as follows: (gut OR intestinal OR colorectal OR colonic OR rectal OR colon OR rectum) AND (Cancer OR carcinoma OR adenocarcinoma OR tumour OR tumor OR malignanc* OR neoplas*) AND (MicroRNA OR miRNA OR “Micro RNA” OR “Small Temporal RNA” OR “non-coding RNA” OR ncRNA OR “small RNA”). GSE datasets that covered miR-452-5p expression between CRC and non-cancer tissues were included for meta-analysis.

After determination of eligible studies, the following information was extracted from each GSE dataset: public year, country, sample type, platform, number of CRC cases, mean (M) and standard deviation (SD) of miR-452-5p expression value in CRC group, number of non-cancer cases and M ± SD of miR-452-5p expression value in non-cancer group. The process of calculating standard mean difference (SMD) with 95% confidence interval (95% CI) and the subsequent analysis of heterogeneity was described in previous study [41].

*The integrated meta-analysis*

We collected all the included GSE datasets and downloaded TCGA data to carry out the integrated meta-analysis with the aim of further validating miR-452-5p expression between CRC tissues and non-cancer tissues. Pooling of the overall SMD with 95% CI followed the methods mentioned in the part of GEO meta-analysis.

*Assessment of the prognostic value of miR-452-5p via Progmir*

Progmir is an online tool containing prognostic data of miRNAs in a wide type of human cancers [42]. To fully utilize this resource, we assessed the prognostic value of miR-452-5p in COAD and READ with the available Kaplan-Meier survival curves of overall survival, metastasis-free survival and relapse-free survival from Progmir. All the patients were divided into groups of high and low miR-452-5p expression according to the median of miR-452-5p expression.

*Prediction of the target genes of miR-452-5p through online software miRWalk2.0*

The target genes of miR-452-5p were predicted by miRWalk2.0, an online software that combined the predictive function of 12 database including: miRWalk, miRDB, PITA, MicroT4, miRMap, RNA22, miRanda, miRNAMap, RNAhybrid, miRBridge, PICTAR2 and Targetscan. Genes predicted simultaneously by at least seven databases were considered as the potential target genes of miR-452-5p. After the determination of the potential target genes, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were carried out in The Database for Annotation, Visualization and Integrated Discovery (DAVID) v.6.8 to acquire the functional annotation of the target genes in three aspects of biological process (BP), cellular component (CC) and molecular function (MF) as well as to analyze the pathways significantly enriched by the potential target genes.

*Immunostaining of CDKN1B from the human protein atlas (HPA) database*

The Human Protein Atlas (HPA) covered a wealth of transcriptome and proteomes data from RNA-sequencing analysis and immunohistochemistry analysis in diversified tissues [43]. In this study, we downloaded the immunostaining results of CDKN1B in normal colon, normal rectum and colorectal tissues to validate the differential expression of CDKN1B in CRC tissues and non-cancer tissues.

*Dual-luciferase reporter assay*

We entrusted Shanghai Bosi Biological Technology Co. Ltd. to perform the whole dual-luciferase reporter assay. The human embryonic kidney (HEK)-293T cells were seeded in a 48-well plate to reach a cell density of 70-80%. The wild and mutant type of the 3'-UTR region of CDKN1B that was predicted to bind to miR-452-5p were synthesized and connected to the psiCHECK-2-Report luciferase reporter plasmid. Co-transfection with miR-452-5p mimics or normal control and co-transfection with psiCHECK-CDKN1B-3'UTR wild type (AAACAGT) or psiCHECK-CDKN1B-3'UTR mutant (GTGACCA) in HEK-293T cells were performed using Lipofectamine® 2000. After incubation at 37°C for 48 h, a Dual-Luciferase Reporter assay (Promega Corporation, Madison, WI, USA) was used to measure the firefly and renilla luciferase activity following the manufacturer's protocol. Firefly luciferase activity was defined as an internal control. Each experiment was repeated three times.

*Statistical analysis*

All the statistical analyses were performed in SPSS 22.0. The primitive expression data of miR-452 and CDKN1B from TCGA was log-2 normalized and all the data are presented in the form of M ± SD. Independent sample's t test was performed to compare miR-452 and CDKN1B expression as well as the relative luciferase activity in two different groups. The receiver operating characteristic (ROC) curves and Kaplan-Meier Survival curves were drawn to evaluate the diagnostic and prognostic value of miR-452 in CRC, respectively.

## Results

*The clinico-pathological significance of miR-452 in CRC from TCGA*

According to the results, miR-452 was significantly overexpressed in COAD, READ and CRC

**Table 1.** The relationship between miR-452 and clinic-pathological parameters of COAD

| Clinical variable | | N | MiR-452-5p relevant expression | | |
|---|---|---|---|---|---|
| | | | M ± SD | t | *P* value |
| Tissue type | Cancer | 442 | 8.240±1.532 | 11.443 | <0.001 |
| | Non-cancer | 8 | 2.010±1.109 | | |
| Age | ≤65 | 185 | 8.614±1.428 | 4.450 | <0.001 |
| | >65 | 257 | 7.970±1.550 | | |
| Gender | Female | 211 | 8.107±1.596 | -1.746 | 0.082 |
| | Male | 231 | 8.361±1.463 | | |
| Lymphatic invasion | No | 243 | 8.355±1.535 | 2.396 | 0.017 |
| | Yes | 156 | 7.978±1.533 | | |
| Residual tumor | R0 | 314 | 8.076±1.564 | 1.478 | 0.147 |
| | R1-R2 | 30 | 7.744±1.133 | | |
| TNM stage | I-II | 241 | 8.085±1.538 | -2.009 | 0.045 |
| | III-IV | 190 | 8.382±1.512 | | |
| Distant metastasis | No | 320 | 8.079±1.571 | -1.796 | 0.073 |
| | Yes | 65 | 8.453±1.324 | | |
| Lymph node metastasis | No | 257 | 8.134±1.534 | -1.712 | 0.088 |
| | Yes | 185 | 8.386±1.520 | | |
| Venous invasion | No | 288 | 8.248±1.538 | -0.050 | 0.960 |
| | Yes | 95 | 8.257±1.531 | | |

Note: N: number; M: mean; SD: standard deviation. The difference of miR-452 expression between two subgroups of opposite clinico-pathological variables was calculated by independent sample's t test in SPSS v 22.0.

**Table 2.** The relationship between miR-452 and clinic-pathological parameters of READ

| Clinical variable | | N | MiR-452-5p relevant expression | | |
|---|---|---|---|---|---|
| | | | M ± SD | t | *P* value |
| Tissue type | Cancer | 160 | 8.803±1.587 | 8.625 | <0.001 |
| | Non-cancer | 3 | 0.862±0.801 | | |
| Age | ≤65 | 80 | 8.798±1.656 | -0.037 | 0.970 |
| | >65 | 80 | 8.808±1.526 | | |
| Gender | Female | 74 | 8.922±1.469 | 0.878 | 0.381 |
| | Male | 86 | 8.700±1.684 | | |
| Lymphatic invasion | No | 79 | 8.923±1.528 | 1.275 | 0.204 |
| | Yes | 63 | 8.605±1.417 | | |
| Residual tumor | R0 | 118 | 8.582±1.618 | 0.362 | 0.718 |
| | R1-R2 | 13 | 8.413±1.388 | | |
| TNM stage | I-II | 77 | 8.449±1.685 | -2.487 | 0.014 |
| | III-IV | 74 | 9.094±1.494 | | |
| Distant metastasis | No | 121 | 8.653±1.644 | -0.487 | 0.627 |
| | Yes | 23 | 8.833±1.454 | | |
| Lymph node metastasis | No | 80 | 8.461±1.658 | -2.696 | 0.008 |
| | Yes | 77 | 9.137±1.471 | | |
| Venous invasion | No | 104 | 8.743±1.449 | -0.921 | 0.359 |
| | Yes | 34 | 9.012±1.580 | | |

Note: N: number; M: mean; SD: standard deviation. The difference of miR-452 expression between two subgroups of opposite clinico-pathological variables was calculated by independent sample's t test in SPSS v 22.0.

compared to the corresponding non-cancer tissues (P<0.001) (**Tables 1-3**). The expression patterns of miR-452 in COAD, READ or CRC and non-cancer tissues were exhibited in **Figure 1**. As for the relationship between miR-452 and other clinico-pathological features of CRC, miR-452 was observed to correlate with age, histological type and the malignant progression of cancer. In the study cohorts of COAD, miR-452 presented obviously higher expression in patients less than 65 years old (8.614±1.428) than in those more than 65 years old (7.970±1.550, t = 4.450, P<0.001) (**Table 1**; **Figure 2A**). Similarly, miR-452 expression in all the CRC patients less than 65 years old was remarkably higher (8.670±1.500) than that in those more than 65 years old (8.169±1.583, t = 3.942, P<0.001) (**Table 3**; **Figure 2B**). As for miR-452 expression in different groups of histological types, READ patients expressed significantly higher levels of miR-452 (8.803± 1.587) than COAD patients (8.240±1.532, t = -3.946, P<0.001) (**Figure 3**). MiR-452 overexpression was also associated with the advanced clinical stage of CRC. COAD patients with higher TNM stage (III-IV) were found to have a significantly higher level of miR-452 expression than patients in the negative groups (P = 0.045) (**Table 1**; **Figure 4A**). In the study cohorts of READ, miR-452 was also observed to be significantly higher in patients with higher TNM stage (III-IV) and

**Table 3.** The relationship between miR-452 and clinic-pathological parameters of CRC

| Clinical variable | | N | MiR-452-5p relevant expression | | |
|---|---|---|---|---|---|
| | | | M ± SD | t | *P* value |
| Tissue type | Cancer | 602 | 8.389±1.565 | 14.108 | <0.001 |
| | Non-cancer | 11 | 1.697±1.130 | | |
| Histological type | COAD | 442 | 8.240±1.532 | -3.946 | <0.001 |
| | READ | 160 | 8.803±1.587 | | |
| Age | ≤65 | 265 | 8.670±1.500 | 3.942 | <0.001 |
| | >65 | 356 | 8.169±1.583 | | |
| Gender | Female | 285 | 8.318±1.602 | -1.053 | 0.293 |
| | Male | 317 | 8.453±1.531 | | |
| Lymphatic invasion | No | 322 | 8.494±1.551 | 2.493 | 0.013 |
| | Yes | 219 | 8.158±1.524 | | |
| Residual tumor | R0 | 432 | 8.214±1.593 | 1.316 | 0.193 |
| | R1-R2 | 43 | 7.946±1.238 | | |
| TNM stage | I-II | 318 | 8.173±1.580 | -3.147 | 0.002 |
| | III-IV | 264 | 8.582±1.538 | | |
| Distant metastasis | No | 441 | 8.237±1.610 | -1.722 | 0.086 |
| | Yes | 88 | 8.553±1.361 | | |
| Lymph node metastasis | No | 337 | 8.212±1.568 | -3.082 | 0.002 |
| | Yes | 262 | 8.607±1.542 | | |
| Venous invasion | No | 392 | 8.379±1.529 | -0.492 | 0.623 |
| | Yes | 129 | 8.456±1.573 | | |

Note: N: number; M: mean; SD: standard deviation. The difference of miR-452 expression between two subgroups of opposite clinico-pathological variables was calculated by independent sample's t test in SPSS v 22.0.

lymph node metastasis (P = 0.014, P = 0.008) (**Table 2**; **Figure 4B**, **4C**). Aggregating the clinico-pathological data of COAD and READ, miR-452-5 showed significantly higher expression in patients with higher TNM stage (III-IV) and lymph node metastasis (P = 0.002, P = 0.002) (**Table 3**; **Figure 4D**, **4E**).

To evaluate the prognostic value of miR-452 in CRC, we divided all the patients into two groups of miR-452 high and low expression based on the average of miR-452 expression, and plotted Kaplan-Meier Survival Curves for the study cohorts of COAD, READ and CRC. The cutoff values of miR-452-5p expression were 8.2396, 8.8028 and 8.3893 for COAD, READ and CRC. However, all the survival analysis revealed no significant influence of miR-452 on the prognosis of CRC patients (Data not shown).

With respect to the diagnostic significance of miR-452 in CRC, three ROC curves in **Figure 5** indicated the strong diagnostic capacity of miR-452 for COAD (AUC = 1.000, P<0.001, cutoff value = 3.857, sensitivity = 1, specificity = 1, positive predictive value = 1, negative predictive value = 1), READ (AUC = 1.000, P<0.001, cutoff value = 2.377, sensitivity = 1, specificity = 1, positive predictive value = 1, negative predictive value = 1) and CRC (AUC = 1.000, P<0.001, cutoff value = 3.857, sensitivity = 1, specificity = 1, positive predictive value = 1, negative predictive value = 1).

*Verification of miR-452-5p expression in CRC tissues via meta-analysis for data from GEO database*

According to the searching strategy, a total of 464 GEO datasets came as initial records. Through scanning titles and abstracts, 43 GSE datasets were reserved. Finally, a total of 29 GEO datasets with 820 CRC tissues and 571 non-cancer tissues that endured the rigid inspection of full-text were enrolled for the meta-analysis. Extracted information and data of all included GSE datasets were displayed in **Table 4**. Forest plot in **Figure 6A** indicated an overall SMD with 95% CI of 0.22 (-0.11-0.55), which reflected a trend of up-regulation for miR-452-5p in CRC. Since the heterogeneity between included studies was significant ($I^2$ = 84.2%, P<0.001), random-effect model was applied to pool the overall SMD with 95% CI from all included GSE datasets. Then, we conducted subgroup analysis based on sample types to trace the origin of heterogeneity. The results suggested that miR-452-5p from the subgroup of tissue presented overexpression in CRC (SMD = 0.38, 95% CI = 0.08-0.67) while miR-452 = 5p from sera presented down-regulation in CRC (SMD = -0.16, 95% CI = -0.66, 0.34). The heterogeneity between studies from the subgroup of tissue remained sig-

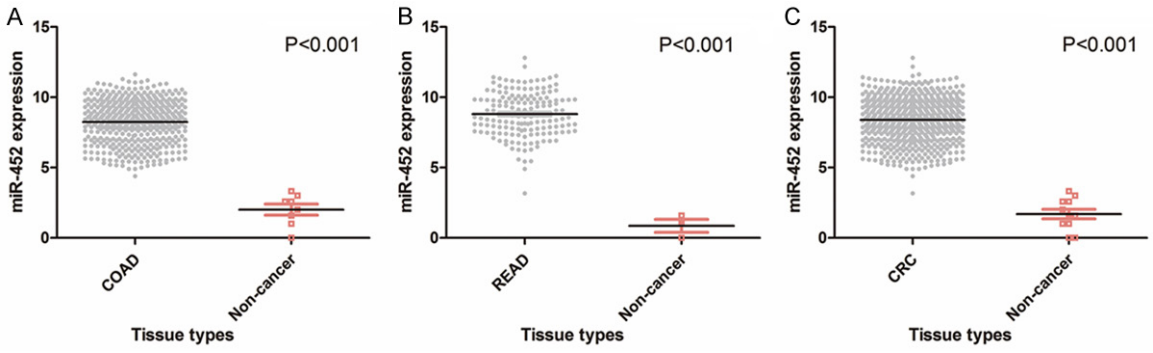


**Figure 1.** The difference of miR-452 expression in CRC and non-cancer tissues from TCGA. A: miR-452 expression between COAD and non-cancer tissues; B: miR-452 expression between READ and non-cancer tissues; C: miR-452 expression between CRC and non-cancer tissues All the scatter plots reflected that miR-452 was significantly over-expressed in COAD, READ and CRC tissues compared with the corresponding non-cancer tissues (P<0.001). The line in the bar means mean value.

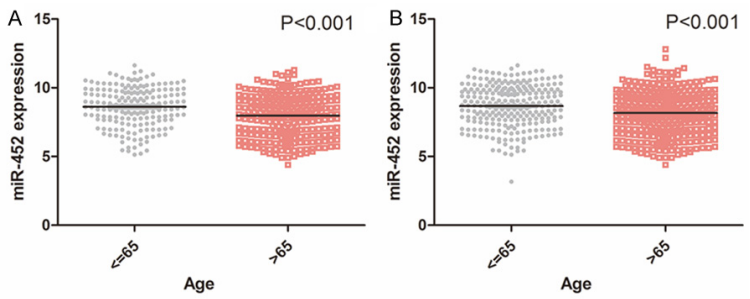


**Figure 2.** The difference of miR-452 expression in subgroups of age from TCGA. A: miR-452 expression between young patients (≤65) and old patients (>65) in the study cohorts of COAD (P<0.001); B: miR-452 expression between young patients (≤65) and old patients (>65) in the study cohorts of CRC (P<0.001) Both scatter plots suggested that miR-452 presented obviously higher expression in young patients (P<0.001). The line in the bar means mean value.

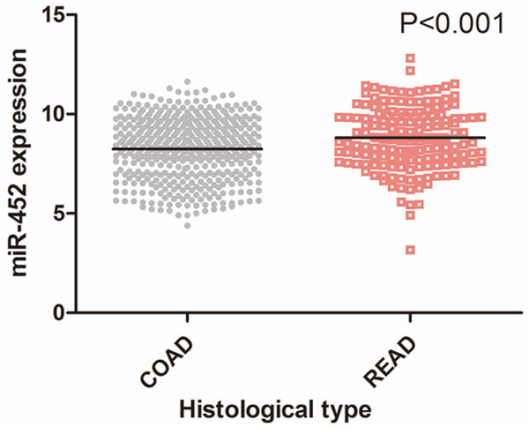


**Figure 3.** The difference of miR-452 expression in different histological types of CRC from TCGA. READ patients expressed significantly higher levels of miR-452 than COAD patients (P<0.001). The line in the bar means mean value.

nificant ($I^2$ = 75.2%, P<0.001) (**Figure 6B**). Then sensitivity analysis was conducted to further investigate the influence of a single GSE dataset on the pooled effect of the whole study cohorts. However, the results revealed that the removal of any GSE dataset induced no significant bias to the overall pooled effect (**Figure 6C**). No publication bias was detected by Begg's test and Egger's test (P = 0.329) (**Figure 6D**).

*The integrated meta-analysis*

A total of 1422 CRC tissues and 582 non-cancer tissues were included in the integrated meta-analysis. The forest plot generated all included GSE datasets and TCGA data also reflected an upward tendency of miR-452-5p expression in CRC (SMD = 0.39, 95% CI = -0.03-0.82) with enormous heterogeneity among studies ($I^2$ = 91.3%, P<0.001) (**Figure 7A**). Subgroup analysis based on sample type demonstrated up-regulated expression of tissue-derived miR-452-5p expression in CRC and down-regulated expression of sera-derived miR-452-5p in CRC (**Figure 7B**). The heterogeneity among studies from the subgroup of tissue was still obvious ($I^2$ = 89.5%, P<0.001). Subsequent sensitivity analysis and publication detection both reported no positive results (**Figure 7C** and **7D**).

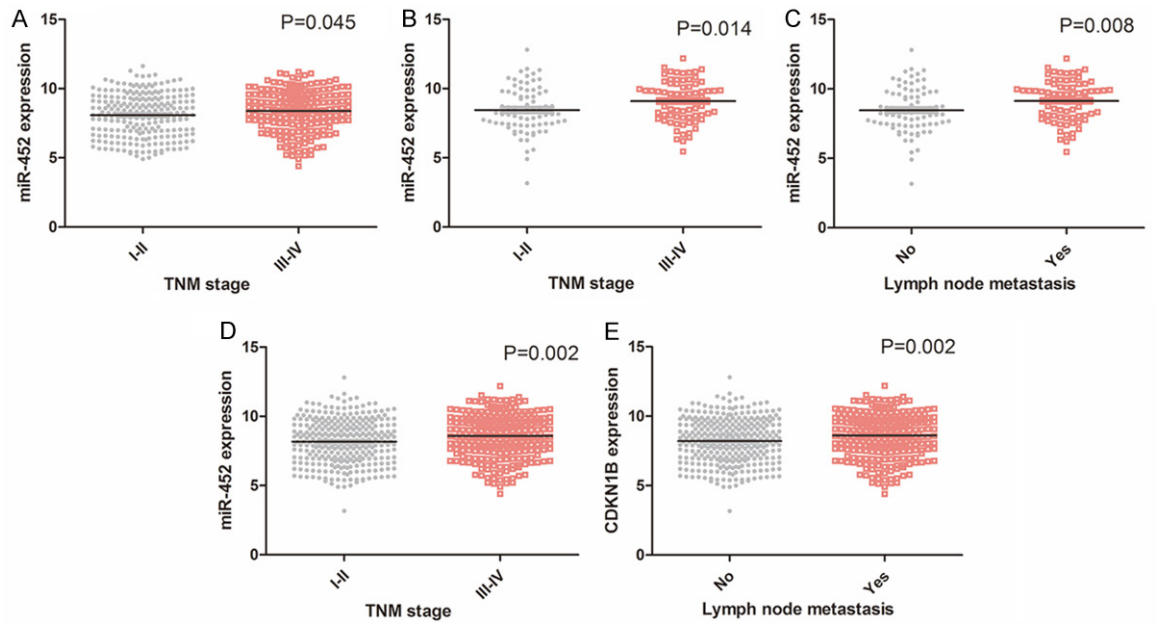


**Figure 4.** The relationship between miR-452 expression and the progression of CRC from TCGA. A: miR-452 expression between different subgroups of TNM stage in the study cohorts of COAD; B and C: miR-452 expression between different subgroups of TNM stage and lymph node metastasis in the study cohorts of READ; D and E: miR-452 expression between different subgroups of TNM stage and lymph node metastasis in the study cohorts of CRC The results revealed that miR-452 exhibited remarkably higher expression in patients with advanced TNM stage (III-IV) and lymph node metastasis than in patients of negative groups (all P<0.05). The line in the bar means mean value.

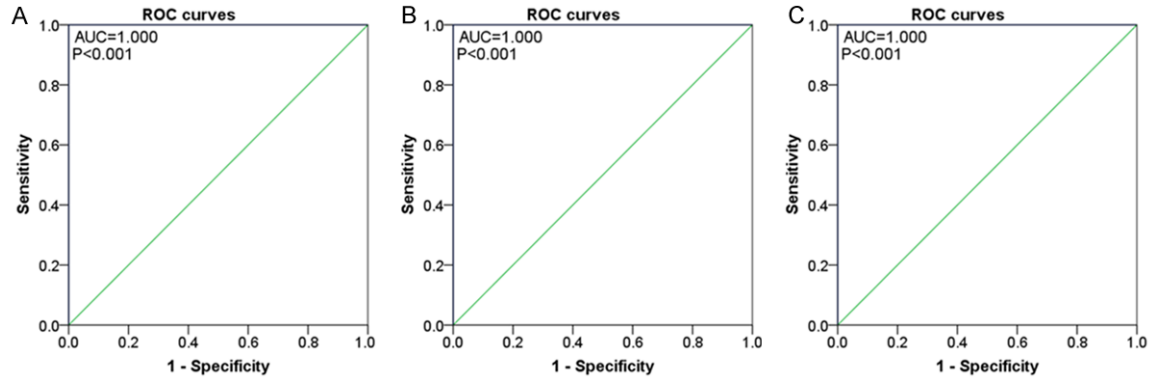


**Figure 5.** The diagnostic value of miR-452 in CRC from TCGA. A: miR-452 showed strong capacity of diagnosing COAD (AUC = 1.000, P<0.001); B: miR-452 could significantly distinguish READ from non-cancer tissues (AUC = 1.000, P<0.001); C: miR-452 possessed significant diagnostic value for CRC (AUC = 1.000, P<0.001).

*Assessment of the prognostic value of miR-452-5p via progmir*

To further study the prognostic properties of miR-452-5p in CRC, we downloaded the overall survival, metastasis-free survival and relapse-free survival curves for COAD and READ. Unfortunately, only the metastasis-free survival curves for READ depicted a trend of longer survival time in patients with lower miR-452-5p expression (P = 0.7678) (**Figure 9B**). The impact of miR-452-5p on the prognosis of COAD or READ patients was indefinite in other survival curves (all P>0.05) (**Figures 8**, **9**).

*Prediction of the target genes of miR-452-5p through online software miRWalk2.0*

The prediction results from miRWalk2.0 shows that a total of 15509 genes were predicted to be the target genes of miR-452-5p and 1156 genes including CDKN1B appeared in the

**Table 4.** Basic information of all included GSE datasets

| ID | First author | Public year | Country | Experiment type | Sample type | Platform | Cancer N | Cancer M | Cancer SD | Non-cancer N | Non-cancer M | Non-cancer SD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GSE108153 | ZL Zeng | 2017 | China | Non-coding RNA profiling by array | Tissue | GPL19730 | 21 | 4.783196 | 0.608005 | 21 | 4.158101 | 0.790354 |
| GSE101502 | YS Huang | 2017 | China | Non-coding RNA profiling by array | Tissue | GPL21439 | 3 | 0.517359 | 0.20857 | 3 | 0.650236 | 0.353548 |
| GSE81581 | Jose, María, Sayagués | 2017 | Spain | Non-coding RNA profiling by array | Tissue | GPL16384 | 23 | 6.375622 | 1.623039 | 9 | 5.799779 | 0.758203 |
| GSE98406 | Amiee, Bell, Potter | 2017 | USA | Non-coding RNA profiling by array | Tissue | GPL16384 | 14 | 5.519721 | 0.159361 | 7 | 5.52961 | 0.166723 |
| GSE53592 | Yanyang, zhao | 2016 | China | Non-coding RNA profiling by array | Tissue | GPL8786 | 3 | 4.561645 | 0.461335 | 3 | 4.651158 | 0.39958 |
| GSE77380 | Toyoki, Yoshimoto | 2016 | Japan | Non-coding RNA profiling by array | Tissue | GPL16770 | 3 | 4.000035 | 0.893793 | 5 | -3.32193 | 4.97E-16 |
| GSE41655 | Xiaoyu, Shi | 2015 | China | Non-coding RNA profiling by array | Tissue | GPL11487 | 33 | 1.186618 | 0.951628 | 15 | 1.107647 | 0.782306 |
| GSE73178 | Tomokazu, Fuji | 2015 | Japan | Non-coding RNA profiling by array | Tissue | GPL20712 | 2 | 0.260972 | 0.234405 | 2 | 0.005453 | 0.002712 |
| GSE54632 | Wen-Jian, Meng | 2015 | China | Non-coding RNA profiling by array | Tissue | GPL8786 | 5 | 3.966088 | 0.078213 | 5 | 3.947117 | 0.092137 |
| GSE68377 | Wen-Jian, Meng | 2015 | China | Non-coding RNA profiling by array | Tissue | GPL8786 | 7 | 3.99312 | 0.07817 | 7 | 3.986304 | 0.203663 |
| GSE48267 | Yuanhao, Zhang | 2015 | USA | Non-coding RNA profiling by array | Tissue | GPL10850 | 61 | -6.44782 | 2.934263 | 61 | -6.45108 | 2.939148 |
| GSE68306 | Yong, Huang | 2015 | USA | Expression profiling by array | Tissue | GPL20111 | 11 | 5.328668 | 0.818544 | 16 | 4.872463 | 0.301598 |
| GSE61741 | Andreas, Keller | 2014 | Germany | Non-coding RNA profiling by array | Blood | GPL9040 | 29 | -0.57619 | 3.819473 | 94 | 4.491538 | 2.199242 |
| GSE39833 | Naoto, Tsuchiya | 2014 | Japan | Non-coding RNA profiling by array | Sera | GPL14767 | 88 | 1.352523 | 17.30548 | 11 | -0.54077 | 0.466071 |
| GSE54088 | Mohammed, Abba | 2014 | Germany | Non-coding RNA profiling by array | Tissue | GPL8178 | 9 | 10.54184 | 0.915713 | 10 | 9.375982 | 0.713133 |
| GSE35834 | Stefania, Bortoluzzi | 2014 | Italy | Non-coding RNA profiling by array | Tissue | GPL8786 | 31 | 2.867091 | 1.228467 | 23 | 2.863878 | 1.238642 |
| GSE39845 | Yong, Fung, Lin | 2013 | Malaysia | Non-coding RNA profiling by array | Tissue and blood | GPL14613 | 17 | 0.653242 | 1.840985 | 9 | 1.059501 | 1.738345 |
| GSE49246 | Zhenhua, Chen | 2013 | China | Non-coding RNA profiling by array | Tissue | GPL17496 | 40 | 9.795297 | 0.350539 | 40 | 9.595772 | 0.146713 |
| GSE45349 | Yujuan, Dong | 2013 | Hong Kong | Expression profiling by RT-PCR | Tissue | GPL16850 | 4 | 0.000533 | 0.000467 | 4 | 0.001489 | 0.000482 |
| GSE25609 | María Dolores, Giráldez | 2013 | Spain | Non-coding RNA profiling by array | Sera | GPL8179 | 30 | 4.342722 | 0.909328 | 20 | 4.779856 | 1.354848 |
| GSE35982 | Jihong, Fu | 2013 | China | Non-coding RNA profiling by array | Tissue | GPL14767 | 8 | 5.826321 | 6.595839 | 8 | 5.7314 | 6.629549 |
| GSE38389 | Rolf, Søkilde | 2012 | Sweden | Non-coding RNA profiling by array | Tissue | GPL11039 | 69 | 7.870894 | 0.229904 | 71 | 7.840194 | 0.195286 |
| GSE35602 | Koshi, Mimori | 2012 | Japan | Non-coding RNA profiling by array | Tissue | GPL8227 | 17 | 2.038869 | 1.457246 | 8 | 0.619577 | 0.631975 |
| GSE28364 | James, F, Reid | 2012 | Italy | Other | Tissue | GPL13328 | 40 | 3.490646 | 0.369141 | 40 | 3.623344 | 0.115501 |
| GSE30454 | Juanjo, Lozano | 2011 | Spain | Non-coding RNA profiling by array | Tissue | GPL8179 | 54 | 8.453735 | 1.230978 | 20 | 8.915024 | 0.301402 |
| GSE18392 | Aaron, Sarver | 2010 | USA | Non-coding RNA profiling by array | Tissue | GPL8178 | 116 | 3.619553 | 0.054189 | 29 | 3.54074 | 0.041417 |
| GSE14985 | Roy, Navon | 2009 | Israel | Non-coding RNA profiling by array | Tissue | GPL8227 | 3 | 4.444394 | 0.371818 | 3 | 3.929105 | 0.084355 |
| GSE10259 | Jack, Yu | 2009 | USA | Non-coding RNA profiling by array | Tissue | GPL4411 | 59 | 6.943626 | 0.396972 | 7 | 6.63916 | 0.195237 |
| GSE83924 | Wichmann, Barna | 2016 | Hungary | Non-coding RNA profiling by array | Tissue | GPL16384 | 20 | 6.619178 | 0.682027 | 20 | 6.179945 | 0.500718 |

Note: N: number; M: mean; SD: standard deviation.

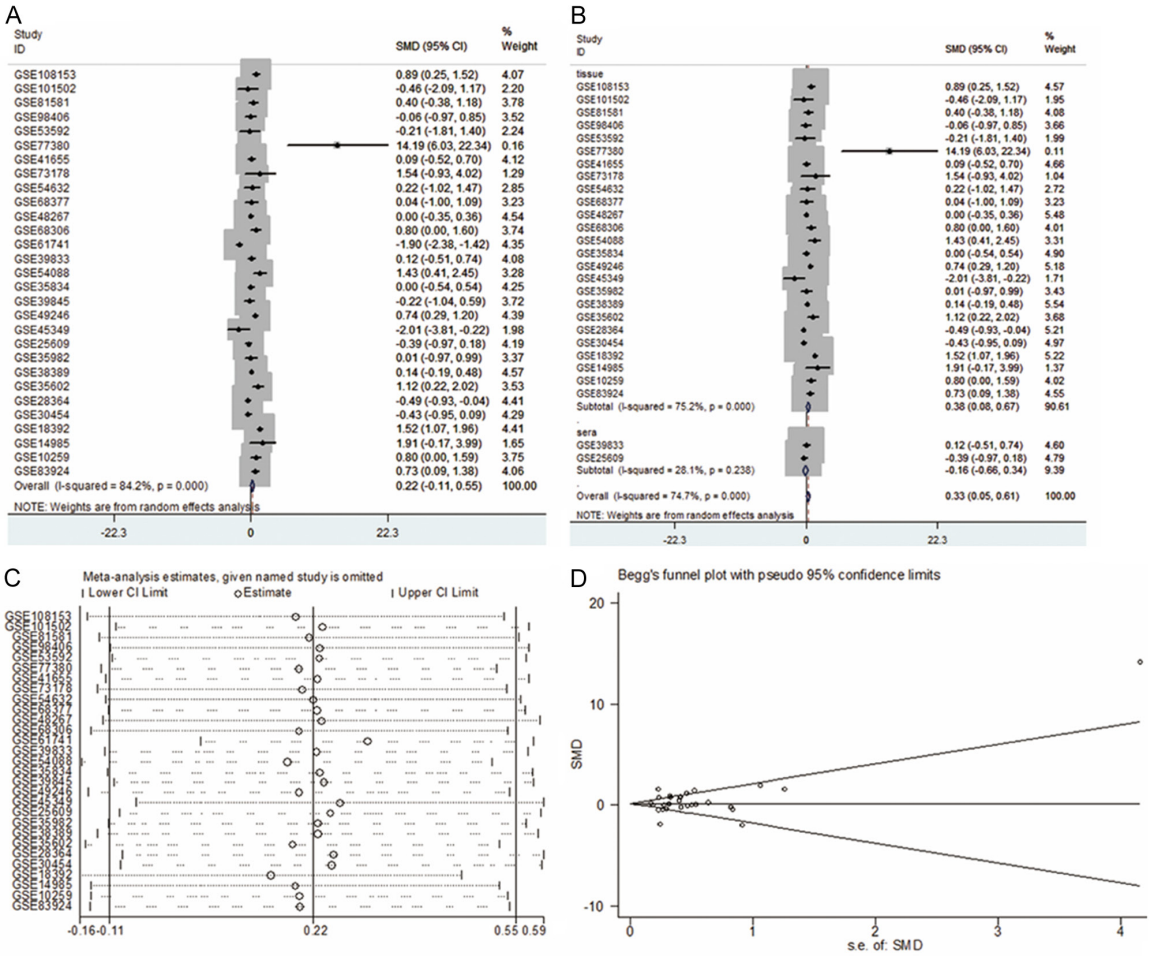


**Figure 6.** GEO meta-analysis for miR-452-5p expression in CRC. A: The forest plot of overall SMD with 95% CI for GEO meta-analysis. B: Subgroup analysis based on sample type. C: Sensitivity analysis. D: Begg's Funnel plot.

records of at least seven databases. GO analysis for the 1156 potential target genes suggested that these genes were significantly assembled in 151, 46 and 50 terms of BP, CC and MF, respectively (all P<0.05). Specifically, several BP terms including sensory perception of sound, negative regulation of transcription, DNA-templated and positive regulation of cell cycle were found to be associated with CDKN1B (**Table 5**). Additionally, CDKN1B was related to CC terms such as cytoplasm, nucleoplasm and nucleus. CDKN1B might participate in molecular functions including protein binding and kinase activity (**Table 5**). Moreover, KEGG pathway analysis demonstrated that the 1156 potential target genes may be involved in 37 pathways (P<0.05) and CDKN1B may act as a component in eight pathways including Pathways in cancer, Transcriptional misregulation in cancer and Chronic myeloid leukemia (P<0.05) (**Table 6**).

*Immunostaining of CDKN1B from the human protein atlas (HPA) database*

There were three normal colon samples and two normal rectum samples included in the immunohistochemistry with antibody CAB021888 from HPA and the immunostaining of CDKN1B in all the five normal colorectal samples were medium. Conversely, only four samples in the total 12 CRC samples exhibited medium CDKN1B immunostaining with antibody CAB021888. Staining intensity of CDKN1B in the rest of the eight CRC samples was low or not detected. Four immunostaining pictures in **Figure 8** represented the expression patterns of CDKN1B in normal colon (**Figure 10A**), normal rectum (**Figure 10B**) and CRC tissues (**Figure 10C** and **10D**).

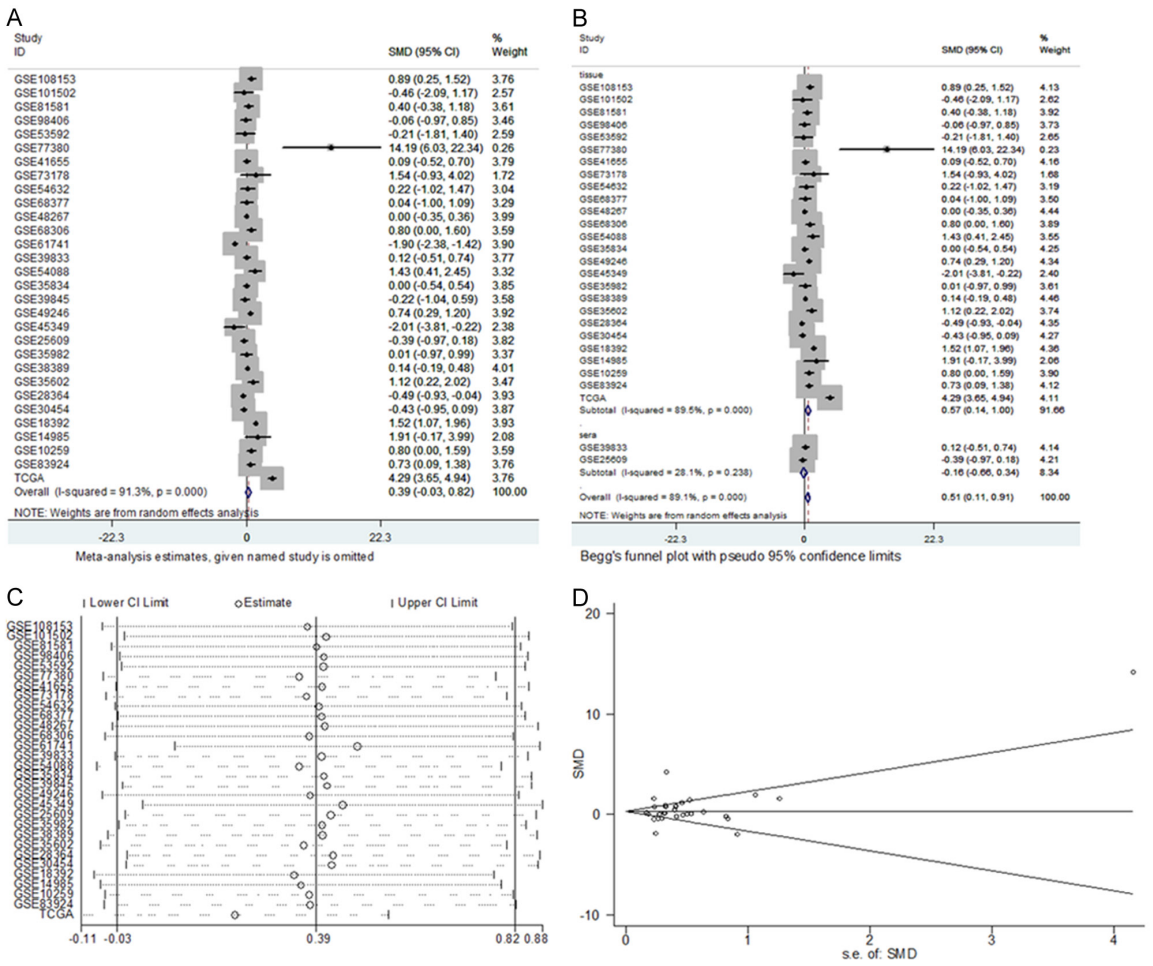


**Figure 7.** The integrated meta-analysis for miR-452-5p expression in CRC. A: The forest plot of overall SMD with 95% CI for the integrated meta-analysis. B: Subgroup analysis based on sample type. C: Sensitivity analysis. D: Begg's Funnel plot.

*Dual-luciferase reporter assay*

The detection results from Luciferase reporter assay indicated that the fluorescence activity of psiCHECK-CDKN1B was remarkably inhibited by miR-452-5p ($P<0.05$); while the fluorescence activity of psiCHECK-CDKN1B-mut was not significantly affected by the transfection of miR-452-5p ($P>0.05$) (**Figure 11**).

## Discussion

With the rapidly increasing interests in the functions of miRNAs in cancer, a series of miRNAs have been claimed in previous studies to engage in the initiation and development of CRC. Song et al. reported that miR-582 promoted the progression of CRC through targeting phosphatase and tensin homologue [44]. In the study of Alam KJ et al., down-regulated miR-375 enhanced the proliferation and migration of CRC cells via modulating CTGF-EGFR signaling pathway [45]. MiR-146b-5p was also found to accelerate cell growth, invasion, and metabolism in CRC through regulating PDHB [46]. Nevertheless, there is rare research on the clinic-pathological significance and the molecular function of miR-452-5p in CRC.

In the present study, we confirmed overexpression of miR-452-5p in CRC with samples from TCGA, GEO meta-analysis and the integrated meta-analysis. We believed that the overexpression of miR-452-5p in CRC identified in this study was convincing because of the large size of samples from the integrated meta-analysis (1422 CRC tissues and 582 non-cancer tissues). The result of our study is in alignment

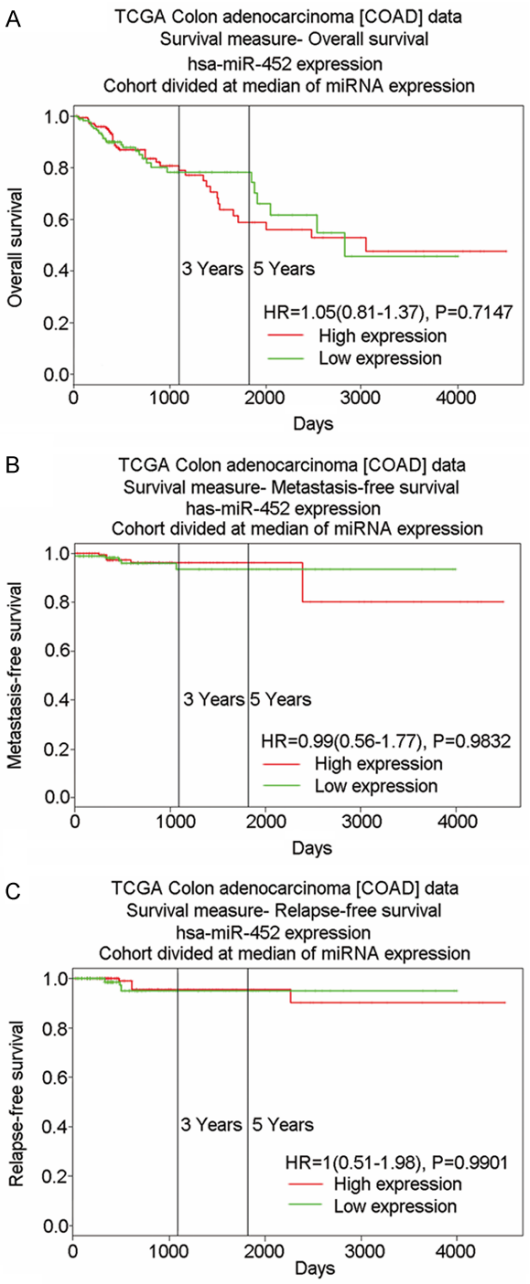


**Figure 8.** The prognostic significance of miR-452-5p in COAD from Progmir. A: Kaplan-Meier survival curves for the overall of COAD patients with high miR-452-5p expression and patients with low miR-452-5p expression; B: Kaplan-Meier survival curves for the metastasis-free of COAD patients with high miR-452-5p expression and patients with low miR-452-5p expression; C: Kaplan-Meier survival curves for the relapse-free survival of COAD patients with high miR-452-5p expression and patients with low miR-452-5p expression. The division of patient groups was based on the median of miR-452-5p expression. MiR-452-5p expression had no significant impact on the overall survival, metastasis-free survival and relapse free survival of COAD patients (all P>0.05).

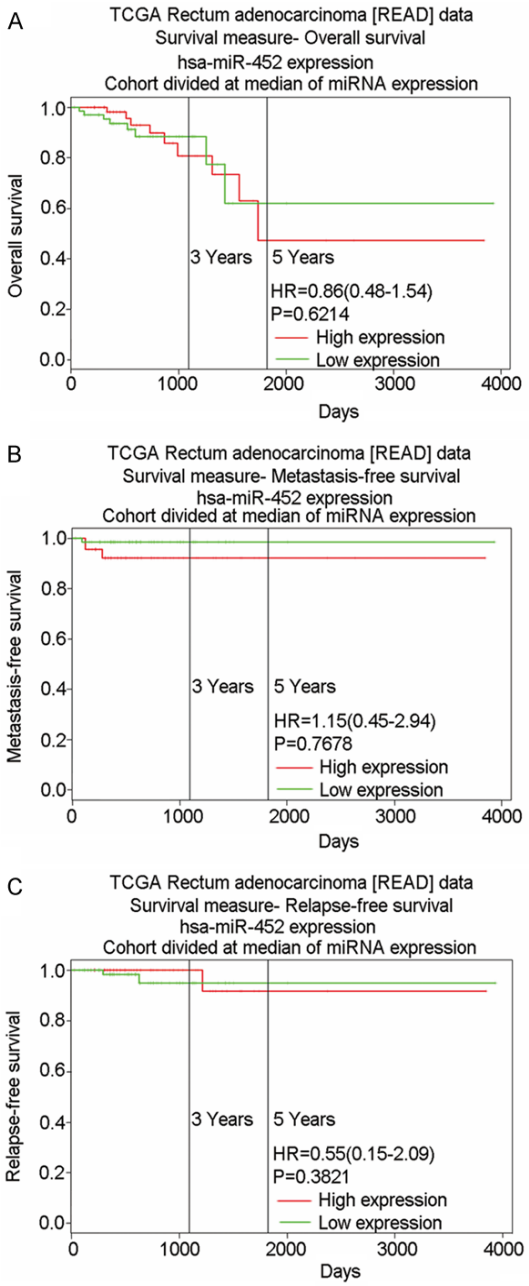


**Figure 9.** The prognostic significance of miR-452-5p in READ from Progmir. A: Kaplan-Meier survival curves for the overall of READ patients with high miR-452-5p expression and patients with low miR-452-5p expression; B: Kaplan-Meier survival curves for the metastasis-free of READ patients with high miR-452-5p expression and patients with low miR-452-5p expression; C: Kaplan-Meier survival curves for the relapse-free survival of READ patients with high miR-452-5p expression and patients with low miR-452-5p expression. The division of patient groups was based on the median of miR-452-5p expression. MiR-452-5p expression had no significant impact on the overall survival, metastasis-free survival and relapse free survival of READ patients (all P>0.05).

**Table 5.** Significant GO terms related to CDKN1B from GO analysis

| Category | ID | Term | Count | *P* value |
|---|---|---|---|---|
| GOTERM_BP_DIRECT | GO:0007605 | Sensory perception of sound | 20 | 3.94E-04 |
| GOTERM_BP_DIRECT | GO:0045892 | Negative regulation of transcription, DNA-templated | 44 | 0.011758683 |
| GOTERM_BP_DIRECT | GO:0045787 | Positive regulation of cell cycle | 7 | 0.014937248 |
| GOTERM_BP_DIRECT | GO:0008284 | Positive regulation of cell proliferation | 41 | 0.015681933 |
| GOTERM_BP_DIRECT | GO:0043066 | Negative regulation of apoptotic process | 40 | 0.017284523 |
| GOTERM_BP_DIRECT | GO:0045732 | Positive regulation of protein catabolic process | 9 | 0.026947082 |
| GOTERM_BP_DIRECT | GO:0051271 | Negative regulation of cellular component movement | 3 | 0.046354975 |
| GOTERM_CC_DIRECT | GO:0005737 | Cytoplasm | 393 | 5.87E-09 |
| GOTERM_CC_DIRECT | GO:0005654 | Nucleoplasm | 232 | 1.37E-08 |
| GOTERM_CC_DIRECT | GO:0005634 | Nucleus | 395 | 2.47E-07 |
| GOTERM_CC_DIRECT | GO:0043234 | Protein complex | 43 | 3.87E-04 |
| GOTERM_CC_DIRECT | GO:0005829 | Cytosol | 223 | 0.018147159 |
| GOTERM_MF_DIRECT | GO:0005515 | Protein binding | 639 | 9.32E-12 |
| GOTERM_MF_DIRECT | GO:0016301 | Kinase activity | 28 | 0.001593199 |

Note: GO: Gene Ontology; BP: biological process; CC: cellular component; MF: molecular function.

**Table 6.** Significant pathways related to CDKN1B from KEGG pathway analysis

| Category | ID | Term | Count | *P* value |
|---|---|---|---|---|
| KEGG_PATHWAY | Hsa05200 | Pathways in cancer | 44 | 1.38E-04 |
| KEGG_PATHWAY | Hsa05202 | Transcriptional misregulation in cancer | 22 | 0.001449251 |
| KEGG_PATHWAY | Hsa05220 | Chronic myeloid leukemia | 12 | 0.004267639 |
| KEGG_PATHWAY | Hsa04151 | PI3K-Akt signaling pathway | 33 | 0.012546233 |
| KEGG_PATHWAY | Hsa04068 | FoxO signaling pathway | 16 | 0.017538851 |
| KEGG_PATHWAY | Hsa05161 | Hepatitis B | 16 | 0.033063391 |
| KEGG_PATHWAY | Hsa04012 | ErbB signaling pathway | 11 | 0.0405767 |
| KEGG_PATHWAY | Hsa05215 | Prostate cancer | 11 | 0.043377456 |

Note: KEGG: Kyoto Encyclopedia of Genes and Genomes.

with two Chinese doctoral dissertations included in China National Knowledge Infrastructure (http://www.cnki.net/). Furthermore, we discovered for the first time that miR-452-5p exhibited significant different expression between COAD and READ, which implied the potential value of miR-452-5p in identifying the subtypes of CRC. Additionally, overexpressed miR-452-5p could induce the deterioration of CRC based on the positive relationship between miR-452-5p expression and advanced TNM stage and lymph node metastasis of CRC. In addition, high AUC values from the ROC curves indicated the potential diagnostic significance of miR-452-5p in CRC.

After investigating the clinico-pathological significance of miR-452-5p in CRC, we further explored the mechanism of miR-452-5p in CRC. Now that the biological functions of miRNAs were achieved by suppressing the expression of target genes [47], seeking the target genes of miR-452-5p might shed light on the mechanism of miR-452-5p in CRC.

In this study, we employed online software to predict the target genes of miR-452-5p. Among the selected potential target genes of miR-452-5p, we focused on the target-ship between miR-452-5p and CDKN1B, a cell-cycle regulator that was frequently reported to be down-regulated in CRC. Immunohistochemistry analysis from HPA yielded a concordant result with prior studies that CDKN1B was down-expressed in CRC, which indirectly provide a hint on the target-ship between miR-452-5p and CDKN1B. To verify CDKN1B as the target gene of miR-452-5p, we further conducted dual-luciferase reporter assay. The notable inhibitory effect of miR-452-5p on the fluorescence activity of psi-CHECK-CDKN1B further proved that CDKN1B is a direct target of miR-452-5p.

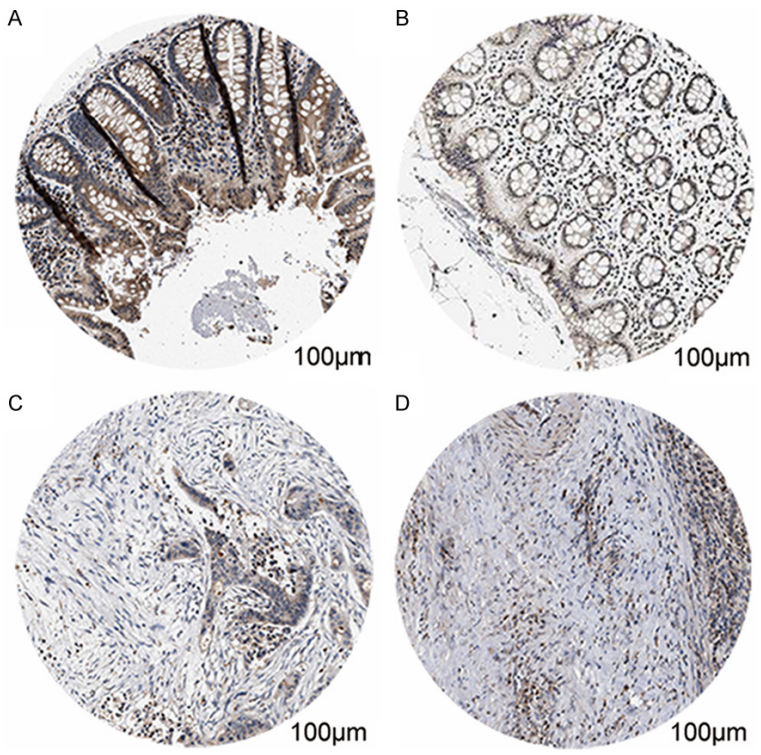


**Figure 10.** Immunostaining patterns of CDKN1B in CRC tissues and normal tissues from HPA. A: A medium staining intensity of CDKN1B was observed in the cytoplasm, membrane and nucleus of normal colon cells; B: A medium staining intensity of CDKN1B was observed in the cytoplasm and membrane of normal rectum cells; C: CDKN1B expression was not detected in CRC cells; D: Low CDKN1B expression was detected in the cytoplasm and membrane of CRC cells. Antibody CAB021888 was used for the immunohistochemistry.

To facilitate a thorough understanding of miR-452-5p and CDKN1B in the development of CRC, we probed into the function annotation of CDKN1B. GO analysis from DAVID implicated that CDKN1B was significantly involved in biological processes such as regulation of transcription, regulation of cell cycle, regulation of cell proliferation and regulation of apoptotic process. There is evidence that CDKN1B interacted with miRNAs to influence the proliferation and apoptosis of tumor cells in non-small-cell lung cancer and CRC [48, 49]. Thus, we postulated that miR-452-5p might target CDKN1B to stimulate tumor growth and restrain apoptosis of CRC cells. Apart from GO analysis, KEGG pathway analysis also offered insights into the molecular basis of CDKN1B in CRC. According to the results, CDKN1B might participate in pathways including pathways in cancer, Transcriptional misregulation in cancer, PI3K-Akt signaling pathway, FoxO signaling pathway and ErbB signaling pathway, which were all relevant to cancers. Particularly, the expression of CDKN1B was under the control of the three signaling pathways that regulated multiple cellular processes in various human cancers: PI3K-Akt signaling pathway, FoxO signaling pathway and ErbB signaling pathway [50-55]. The activation of FoxO proteins could induce the expression of DKN1B [56-58]. In cervical cancer, PI3K-Akt signaling pathway mediated the degradation of CDKN1B [59]. Decreased ErbB3 inactivated PI3K-Akt signaling pathway, subsequently causing up-regulated nuclear CDKN1B [54]. Therefore, we hypothesized that decreased CDKN1B expression by the up-regulation of miR-452-5p may impact these signaling pathways to function in the pathogenesis of CRC.

Despite the interesting findings in this study, limitation of our research needed to be pointed out. Both sensitivity analysis and subgroup analysis failed to find out the exact source of heterogeneity. We speculated that the significant heterogeneity in GEO meta-analysis and the integrated meta-analysis might originate from sample types and platforms of different studies.

In conclusion, miR-452-5p was overexpressed in CRC and was indicative of the malignant progression of CRC. MiR-452-5p may play an oncogenic role in CRC by directly targeting CDKN1B. Future in vivo or in vitro studies were warranted to further investigate the impact of miR-452-5p and CDKN1B on the biological events of CRC cells.

**Acknowledgements**

Thanks are due to The Cancer Genome Atlas (TCGA) for providing the expression data of miR-452 and CDKN1B in CRC, to Gene Expression Omnibus (GEO) for providing GSE datasets pertaining to miR-452-5p expression

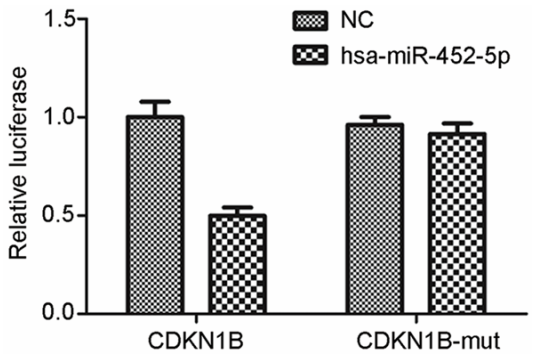


**Figure 11.** The effect of miR-452-5p on the luciferase activity of wild and mutant type of CDKN1B. The expression of wild CDKN1B gene in HEK-293T cells transfected with miR-452-5p was significantly down-regulated compared with the control group ($P<0.05$) while the expression of mutant CDKN1B gene in HEK-293T cells was not significantly influenced by the transfection of miR-452-5p compared with the control group ($P>0.05$). NC: normal control.

data between CRC and non-cancer tissues, to Progmir for providing the prognostic data of miR-452-5p in CRC, to miRWalk2.0 for providing the predicted target genes of miR-452-5p and to the Human protein atlas (HPA) for providing the immunohistochemical results of CDKN1B in normal tissues and CRC tissues.

## Disclosure of conflict of interest

None.

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