

Downregulated miR-495-3p in colorectal cancer targets TGF β R1, TGF β R2, SMAD4 and BUB1 genes and induces cell cycle arrest

Bahram Soltani (✉ soltanib@modares.ac.ir)

tarbiat modares university <https://orcid.org/0000-0003-2468-901X>

Farnoush Kabiri

Tarbiat Modares University

Abdallah Medlej

Tarbiat Modares University

Ali Jason Saleh

Tarbiat Modares University

Nasser Aghdami

Royan Institute for Stem Cell Biology and Technology

Research article

Keywords: miR-495-3p, TGF β signaling, BUB1 gene, Cell cycle, Colorectal cancer

Posted Date: July 25th, 2019

DOI: <https://doi.org/10.21203/rs.2.11910/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

• **Background:** Hsa-miR-495 (miR-495) has been extensively investigated in cancer initiation and progression. On the other hand, our bioinformatics analysis suggested that miR-495 exert its effects through targeting of TGF β signaling components. • **Methods & Results:** In order to investigate such effect, miR-495 precursor was overexpressed in HEK293T and HCT116 cells, that it was followed by downregulation of TGF β R1, TGF β R2, SMAD4 and BUB1 putative target genes, detected by RT-qPCR. Also, luciferase assay supported direct interaction of miR-495 with 3'UTR sequences of TGF β R1, TGF β R2, SMAD4 and BUB1 genes. Furthermore, a negative correlation of expression between miR-495-3p and these target genes was deduced in a set of colorectal and breast cancer cell lines. Then, flow cytometry analysis showed that the overexpression of miR-495 in HCT116 and HEK293T resulted in an arrest at the G1 phase. Consistently, western blotting analysis showed a significant reduction of the Cyclin D1 protein in the cells overexpressing miR-495, pointing to downregulation of TGF β signaling pathway and cell cycle arrest. Finally, microarray data analysis showed that miR-495-3p is significantly downregulated in colorectal tumors, compared to the normal pairs. • **Conclusions:** Overall, results of current study introduced miR-495-3p as a cell cycle progression suppressor, which negatively regulates TGF β R1, TGF β R2, SMAD4 and BUB1 genes. This finding suggests miR-495-3p as a tumor suppressor candidate for further evaluation.

Introduction

MicroRNAs are small non-coding RNAs that regulate gene expression post-transcriptionally [1]. After being transcribed and processed in the nucleus and cytoplasm, miRNAs are loaded into a complex called the miRISC (miRNA-induced silencing complex), and guide it to the 3'UTRs their target transcripts leading to their degradation or translational inhibition [2]. One miRNA can target even hundreds of genes, and a single gene transcript could be targeted by various miRNAs [3]. This characteristic makes the mechanism of regulating genetic expression by miRNAs an extremely complex process and requires the discovery of all the target genes of each miRNA to determine its functions in the various physiological and pathological contexts.

miR-495 is a member of the human 14q32.31 miRNA cluster that encodes a set of important miRNAs [4]. Various studies have been done to investigate the role of miR-495-3p in cancer progression [4]. However, the role of this miRNA in this field is still controversial. Wang et al. reported a downregulation of miR-495-3p expression in breast tumors and showed that this miRNA inhibits G1-S phase transition of breast cancer cells by targeting the BMI-1 oncogene [5]. Li et al. showed that miR-495-3p is downregulated in prostate cancer and regulates migration and invasion in prostate cancer cells by targeting AKT and mTOR transcripts [6]. In contrast, Lin et al. analysis revealed that miR-495-3p expression level is elevated in colon tumors and confirmed an oncogenic role for this miRNA exerted by targeting the WIF1 gene, a Wnt/ β -catenin signaling pathway inhibitor [7]. The various roles of miR-495 in cancer progression have been reviewed recently by Chen et al. [4].

TGF β (transforming growth factor β) signaling pathway plays critical roles in cell biology including cell growth, differentiation, apoptosis, homeostasis and other cellular functions [8]. Canonical TGF β signaling involves the activation of R-SMADs and coSMADs that form heterodimeric complexes and translocate into the nucleus where they regulate the expression of target genes [9]. The non-canonical branch of this pathway includes the activation of Mitogen-activated protein kinase (MAPK) and phosphoinositol 3-kinase (PI3K) signaling pathways [9]. Canonical and non-canonical modes of TGF β signaling exert opposite effects on the cellular fates, with the first inhibits cellular proliferation and survival while the latest activates these processes [10]. The BUB1 protein has been recently introduced as an important TGF β signaling component that is responsible for stabilizing the interaction between TGF β R1 and TGF β R2 receptors and R-SMADs in the cytoplasm [11]. In addition, BUB1 is known to plays an essential role in promoting proper chromosome alignment during cell preparation for division [12].

In the current study, we intended to perform additional investigations concerning the role of miR-495-3p by focusing on its potential uncovered target genes. To this aim, several bioinformatics software was used to predict the potential targets of this miRNA. Additional gene-ontology enrichment analysis of the obtained target lists revealed that a set of the predicted genes represents key components of the TGF β signaling pathway, including the *BUB1* gene. Considering the dual role played by TGF- β signaling during cancer initiation and progression, and its implication in various developmental processes, we selected these genes for further experimental validations as important potential targets for miR-495-3p.

Materials And Methods

- Bioinformatics analysis

Prediction of miR-495-3p target genes was performed using TargetScan [13] and DIANA microT [14, 15] web servers. RNAhybrid web server [16] was used to predict the interaction strength between the miR-495-3p and the selected target genes.

- Vector construction

The genomic region containing pre-miR-495, in addition to the target genes' 3'UTRs regions containing the predicted MREs of miR-495-3p were PCR-amplified using pairs of specific primers (primers listed in table 1). PCR products were cloned into pTG19-T vector, and later sub-cloned into the mammalian expression vector pEGFPC1 (pre-miRNA) or psiCHECK-2 vector (3'UTRs) downstream of the luciferase reporter gene.

- Cell culture and transfection

HCT116, SW480, MDA-MB-231, SKBR3 and HEK293T cell lines were cultured at 37 °C in DMEM-HG, DMEM-F12 or RPMI media (Invitrogen, USA), and supplemented with 10 % fetal bovine serum - FBS (Invitrogen, USA) and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma, USA), with 5% CO₂. Transfection of HCT116 and HEK293T cells with pEGFPC1/pre-miR-495-3p, psiCHECK-2/3'UTRs and mocks was done using TurboFect reagent (Fermentas, USA) according to the manufacturer's instructions.

Transfection rates were determined by visualizing GFP expression using inverted fluorescence microscopy (Nikon eclipse Te2000-s).

- ◦ RNA and protein extraction

Total cellular RNA was extracted using RiboX reagent according to the manufacturer's instructions. Protein extraction was performed using the RiboX-precipitated cell extracts according to the protocol recently described by Kopec et al. [17]. Quantitative assignment of the extracted proteins was done using Bradford assay [18].

- ◦ cDNA synthesis and qPCR analysis

Poly-A tailing was performed by incubating 1 µg of total RNA with 2.5 U Poly-A polymerase (NEB, UK) and 2 µl of 10 mM ATP at 37 °C for 30 minutes. Polyadenylated or non-polyadenylated RNA were subjected to DNase I treatment (Takara, Japan) at 37 °C for 30 min, followed by heat inactivation of the enzyme at 72 °C for 10 minutes. After that, cDNA synthesis was performed by utilizing PrimeScript II reverse transcriptase (Takara, Japan) and anchored or universal oligo-dT universal primers (primers shown in Table 1). Real-time PCR was done using the StepOne™ system (Applied Biosystems, USA) and SYBR Premix Kit (Takara Biotechnology, Japan). Primers used in the PCRs are presented in Table 1.

- ◦ Western blotting

Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. Primary antibodies against Cyclin D1 (Santa Cruz, USA, sc-8396) and β-actin (Santa Cruz, USA, sc-130301) and goat anti-mouse secondary antibody (BIORAD, USA, 1721011) were diluted according to the manufacturers' instructions. The expression level of Cyclin D1 protein was normalized against β-actin protein expression.

- ◦ Luciferase assay

HEK293T cells were co-transfected with constructs containing 3'UTR sequences of the *TGFβR1*, *TGFβR2*, *SMAD4*, *BUB1* or *K-RAS* genes and miR-495-3p expressing vector or mock, in 48-well plates. *K-RAS* gene 3'UTR sequence was used as an off-target control. Luciferase activity was measured 48 h after transfection using the Dual-Glo Luciferase System (Promega, USA).

- ◦ Flow cytometry analysis

HCT116 and HEK293T cells untransfected or transfected with pre-miR-495 or mock were harvested and stained with Propidium Iodide (Sigma, USA) 48 hours post-transfection. Cell cycle was analyzed using the automated multicolor flow cytometry system, BD FACS Caliber Flow Cytometer (BD Biosciences, USA). Results were analyzed by Flowing2.5.1 software.

- ◦ Statistical analysis

The results of RT-qPCR were normalized against the expression of *U48* or *GAPDH* genes. Gene expression results were analyzed according to the Delta CT and Delta-Delta CT algorithms by Microsoft Excel. GSE18392 (colon cancer) and GSE10259 (colorectal cancer) microarray data were downloaded from NCBI-GEO database and normalized using R program. GraphPad Prism 7 (San Diego, CA) was used to perform statistical analysis and graph construction. Differences with *p*-values<0.05 were considered statistically significant.

Results

- Important TGFβ signaling components are potential targets of miR-495-3p

Target prediction of miR-495-3p was done using DIANA micro-T and TargetScan and RNAhybrid web servers. Four of the predicted targets grabbed our attention because they represent important components of the TGFβ signaling pathway (Fig. 1A). The four genes are *TGFβR1*, *TGFβR2*, *SMAD4* and *BUB1*. Multiple conserved miRNA responsive elements (MREs) for miR-495-3p were predicted within each of the 3'UTR sequences of the potential target genes (Fig. 1A). The used software showed important interactions between miR-495-3p seed sequence and the predicted MREs (Fig. 1B).

- - Overexpression of miR-495 was followed by the downregulation of the predicted target genes

To investigate the predicted results at the experimental level, pre-miR-495 was cloned in the pEGFPC1 mammalian expression vector and transfected into HEK293T and HCT116 human cell lines. These cells were selected because they are known as TGFβ signaling-active cell lines. RT-qPCR showed that mature miR-495-3p has been successfully overexpressed in both HEK293T and HCT116 cells, in comparison to the cells transfected with mock control vector (Fig. 2A&C).

RT-qPCR against *TGFβR1*, *TGFβR2*, *SMAD4* and *BUB1* putative target genes showed significant reduction in their expression levels in both HEK293T and HCT116 cells overexpressing miR-495-3p, in comparison to the related controls (Fig. 2B & D). Overexpression of this miRNA in HCT116 cells also resulted in reduced cyclin D1 (CCND1) protein level, detected through western blotting analysis (Fig. 2E). Furthermore, dual luciferase assay was performed after co-transfection of HEK293T cells, using the vector ensuring pre-miR-495 overexpression and the vector which carried luciferase ORF fused with one of the candidate genes 3'UTR sequences. The 3'UTR sequence of *K-RAS* gene was used as an off-target to check the accuracy of the test. Luciferase assay results supported direct interaction of miR-495 with 3'UTR sequences of *TGFβR1*, *TGFβR2*, *SMAD4* and *BUB1* putative target genes (Fig 3). Overall, RT-qPCR along with dual luciferase assay results introduced miR-495-3p as a common regulator for the genes that are involved in TGFβ signaling pathway.

- - Negative correlation between miR-495-3p and its target genes in various human cell lines

To gain more evidence about the targeting of *TGFβR1*, *TGFβR2*, *SMAD4* and *BUB1* genes by miR-495-3p, we determined the expression levels of this miRNA and the four target genes in SW480 and HCT116 colorectal cancer cells and MDA-MB-231 and SKBR3 breast cancer cell lines (Fig. 4A). A significant

negative correlation was obtained between the expression levels of miR-495-3p and each of the target genes in the four tested cell lines (Fig. 4B). These results provide additional evidence about the targeting of *TGFβR1*, *TGFβR2*, *SMAD4* and *BUB1* genes by miR-495-3p.

- ◦ Overexpression of miR-495-3p arrests cell cycle in HEK293T and HCT116 cells

The impact of miR-495 overexpression on cell cycle status was investigated in HEK293T and HCT116 cells using Propidium Iodide flow cytometry. Results indicated a significant increase of the sub-G1 cell population in both HEK293T and HCT116 cells (~10 % and ~5 %, respectively) overexpressing miR-495, in comparison to untransfected cells or those transfected with mock control (Fig 5A & B). These results suggested a tumor suppressive role for miR-495-3p. Thus, we intended to investigate the expression of this miRNA in some tumors. Analysis of available microarray expression data of colon (GSE18392, 116 cancerous and 29 normal samples) and colorectal (GSE10259 58 cancerous and 8 normal samples) tumors showed that miR-495-3p has been significantly downregulated in the cancerous samples of both groups in comparison to the adjacent normal tissues (Fig. 6). These results suggest miR-495-3p as an important tumors suppressor miRNA in cancer research.

Discussion

MicroRNAs are small non-coding RNAs that are implicated in most biological processes through regulating genetic expression [19]. Although of the various studies that aimed to determine the role of miR-495-3p in cancer initiation and progression, the essential role of miR-495-3p in this field is still not clear [4]. Several studies showed that miR-495-3p inhibits the progression of prostate cancer, colorectal cancer and melanoma [6, 20, 21]. In contrast, recent study provided important evidence about an oncogenic role of miR-495-3p in colon cancer [7].

TGFβ signaling pathway plays critical roles in many developmental and physiological processes [8]. Recent studies revealed that this pathway exerts its effects contextually depending on cell types [22]. For instance, few genes are regulated by TGFβ signaling in pluripotent embryonic cells versus hundreds in differentiated cells [22]. Additional complexity of this pathway is that it can activate or suppress the expression of the same gene depending on the cellular context [22]. Thus, elaborating the molecular regulators of this pathway helps in understanding the mechanisms that leads to diseases. TGFβ signaling pathway has been reported to be regulated by several known miRNAs [23–26].

In the current study, we provided important evidences about the targeting of TGFβ signaling components by miR-495-3p. Bioinformatics analysis revealed the existence of multiple conserved MREs of miR-495-3p within the 3'UTRs of each of the *TGFβR1*, *TGFβR2*, *SMAD4* and *BUB1* genes (Figure 1). Experimental procedures including RT-qPCR and luciferase assay showed a downregulation of the target genes expression in the cells transfected with pre-miR-495 and a direct interaction between this miRNA and the target transcripts, respectively (Figure 2 & 3). In addition, analysis of the expression of miR-495-3p and the tested target genes indicated a negative correlation in a set of colorectal and breast cancer human cell lines (Figure 4).

BUB1 kinase has been added recently to the TGF β signaling components. Nyati et al. showed that BUB1 knockdown reduces TGF β -mediated cancerous cells proliferation [11]. In addition to its role in promoting proper chromosome alignment during cell preparation for division [27], BUB1 is proved to interact with TGF β R1 and TGF β R2 receptors and R-SMADs in the cytoplasm [11]. This interaction stabilizes the heteromeric interaction between TGF β R1 and TGF β R2 receptors and R-SMADs and promotes the activation of TGF β signaling [11]. An important characteristic of BUB1 gene is its short 3'UTR (~266 nucleotides) that results in reducing the chance of its posttranscriptional regulation by miRNAs. However, in the current study, we predicted the presence of an MRE for miR-495-3p in the BUB1 3'UTR sequence, and supported this prediction by experimental evidences. These results revealed additional important aspects of miR-495-3p function and made it important candidate for further research in the field of cancer molecular studies.

TGF β signaling pathway is known to play important role in the control of cell cycle [28]. This notion promoted us to investigate the effect of miR-495-3p on cell cycle status. Flow cytometry analysis showed that the overexpression of miR-495 in HCT116 and HEK293T resulted in an arrest at the G1 phase (Fig. 5). Additional western blotting against Cyclin D1 protein confirmed the flow cytometry analysis (Fig. 2E & F). Moreover, analysis of GSE18392 and GSE10259 colon and colorectal cancer microarray data showed that miR-495-3p is downregulated in tumor samples in comparison to normal adjacent tissues (Fig. 6). Considering the big number of samples that have been analyzed in the microarray data, analysis results represent important evidence about the role of miR-495-3p in cancer progression. These results are consistent with other studies that showed that miR-495-3p is an inhibitor of cell cycle progression [20, 21, 29, 30]. Finally, miR-495-3p represents an important candidate for further studies in the field of cancer and development due to its targeting of essential components of TGF β signaling that plays crucial roles in these processes.

Declarations

Author contributions

FK, AM and AJS performed the experiments. BS designed the experiments and supervised the study. AM, FK and BS wrote the manuscript.

Conflicts of interest

The authors declare that the present research was conducted with no commercial or financial relationships that could be construed as a potential conflict of interest.

Repositories

All datasets on which the conclusions of the paper rely on, are available to readers. Whole datasets are presented in the main manuscript.

Acknowledgments

Authors are thankful for the help and advice of the 4402-laboratory members at the Department of Genetics, TMU. Tehran-Iran.

References

1. Treiber, T., N. Treiber, and G. Meister, *Regulation of microRNA biogenesis and its crosstalk with other cellular pathways*. Nat Rev Mol Cell Biol, 2019. 20(1): p. 5–20.
2. Gebert, L. F. R. and I. J. MacRae, *Regulation of microRNA function in animals*. Nature Reviews Molecular Cell Biology, 2019. 20(1): p. 21–37.
3. Helwak, A., et al., *Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding*. Cell, 2013. 153(3): p. 654–65.
4. Chen, H., et al., *Expression, regulation and function of miR-495 in healthy and tumor tissues*. Oncol Lett, 2017. 13(4): p. 2021–2026.
5. Wang, L., et al., *Downregulated miR-495 [Corrected] Inhibits the G1-S Phase Transition by Targeting Bmi-1 in Breast Cancer*. Medicine, 2015. 94(21): p. e718-e718.
6. Li, J. Z., et al., *MicroRNA-495 Regulates Migration and Invasion in Prostate Cancer Cells Via Targeting Akt and mTOR Signaling*. Cancer Invest, 2016. 34(4): p. 181–8.
7. Lin, L., et al., *MiR-495-3p facilitates colon cancer cell proliferation via Wnt/ β -catenin signaling pathway by restraining Wnt inhibitory factor*. Tropical Journal of Pharmaceutical Research, 2017. 16(9): p. 2113–2120.
8. Yan, X. and L. Zhang, *A special issue on TGF-beta signaling: regulation, crosstalk, and biology*. Acta Biochim Biophys Sin (Shanghai), 2018. 50(1): p. 1–2.
9. Gotovac, J. R., et al., *TGF-beta signaling and its targeted therapy in gastrointestinal cancers*. Discov Med, 2018. 26(142): p. 103–112.
10. Papageorgis, P., *TGF β 2; Signaling in Tumor Initiation, Epithelial-to-Mesenchymal Transition, and Metastasis*. Journal of Oncology, 2015. 2015: p. 15.
11. Nyati, S., et al., *The kinase activity of the Ser/Thr kinase BUB1 promotes TGF-beta signaling*. Sci Signal, 2015. 8(358): p. ra1.

- 12.Klebig, C., D. Korinth, and P. Meraldi, *Bub1 regulates chromosome segregation in a kinetochore-independent manner*. The Journal of cell biology, 2009. 185(5): p. 841–858.
- 13.Agarwal, V., et al., *Predicting effective microRNA target sites in mammalian mRNAs*. eLife, 2015. 4: p. e05005.
- 14.Paraskevopoulou, M. D., et al., *DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows*. Nucleic Acids Res, 2013. 41(Web Server issue): p. W169–73.
- 15.Reczko, M., et al., *Functional microRNA targets in protein coding sequences*. Bioinformatics, 2012. 28(6): p. 771–6.
- 16.Rehmsmeier, M., et al., *Fast and effective prediction of microRNA/target duplexes*. Rna, 2004. 10(10): p. 1507–17.
- 17.Kopec, A.M., et al., *Optimized solubilization of TRIzol-precipitated protein permits Western blotting analysis to maximize data available from brain tissue*. J Neurosci Methods, 2017. 280: p. 64–76.
- 18.Bradford, M. M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. Anal Biochem, 1976. 72: p. 248–54.
- 19.Vidigal, J. A. and A. Ventura, *The biological functions of miRNAs: lessons from in vivo studies*. Trends in cell biology, 2015. 25(3): p. 137–147.
- 20.Yan, L., J. Yao, and J. Qiu, *miRNA–495 suppresses proliferation and migration of colorectal cancer cells by targeting FAM83D*. Biomed Pharmacother, 2017. 96: p. 974–981.
- 21.Chen, G. and Y. Xie, *miR–495 inhibits proliferation, migration, and invasion and induces apoptosis via inhibiting PBX3 in melanoma cells*. Onco Targets Ther, 2018. 11: p. 1909–1920.
- 22.Massagué, J., *TGFβ signalling in context*. Nature Reviews Molecular Cell Biology, 2012. 13: p. 616.
- 23.Jafarzadeh, M., et al., *Experimental evidences for hsa-miR–497–5p as a negative regulator of SMAD3 gene expression*. Gene, 2016. 586(2): p. 216–21.
- 24.Abedini Bakhshmand, E., et al., *Hsa-miR–5582–3P regulatory effect on TGFbeta signaling through targeting of TGFbeta-R1, TGFbeta-R2, SMAD3, and SMAD4 transcripts*. 2018. 119(12): p. 9921–9930.
- 25.Jahangiri Moez, M., H. Bjeije, and B. M. Soltani, *Hsa-miR–5195–3P induces downregulation of TGFβR1, TGFβR2, SMAD3 and SMAD4 supporting its tumor suppressive activity in HCT116 cells*. The International Journal of Biochemistry & Cell Biology, 2019. 109: p. 1–7.
- 26.Dokanehiifard, S. and B. M. Soltani, *TrkC-miR2 regulates TGFbeta signaling pathway through targeting of SMAD3 transcript*. J Cell Biochem, 2018.

27. Johnson, V. L., et al., *Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression*. J Cell Sci, 2004. 117(Pt 8): p. 1577–89.
28. Zhang, Y., P. B. Alexander, and X. F. Wang, *TGF-beta Family Signaling in the Control of Cell Proliferation and Survival*. Cold Spring Harb Perspect Biol, 2017. 9(4).
29. Mao, Y., et al., *MiR-495 inhibits esophageal squamous cell carcinoma progression by targeting Akt1*. Oncotarget, 2016. 7(32): p. 51223–51236.
30. Wang, L., et al., *Downregulated miR-495 Inhibits the G1-S Phase Transition by Targeting Bmi-1 in Breast Cancer*. Medicine, 2015. 94(21): p. e718.

Table

Due to technical limitations, table 1 is only available as a download in the supplemental files section

Figures

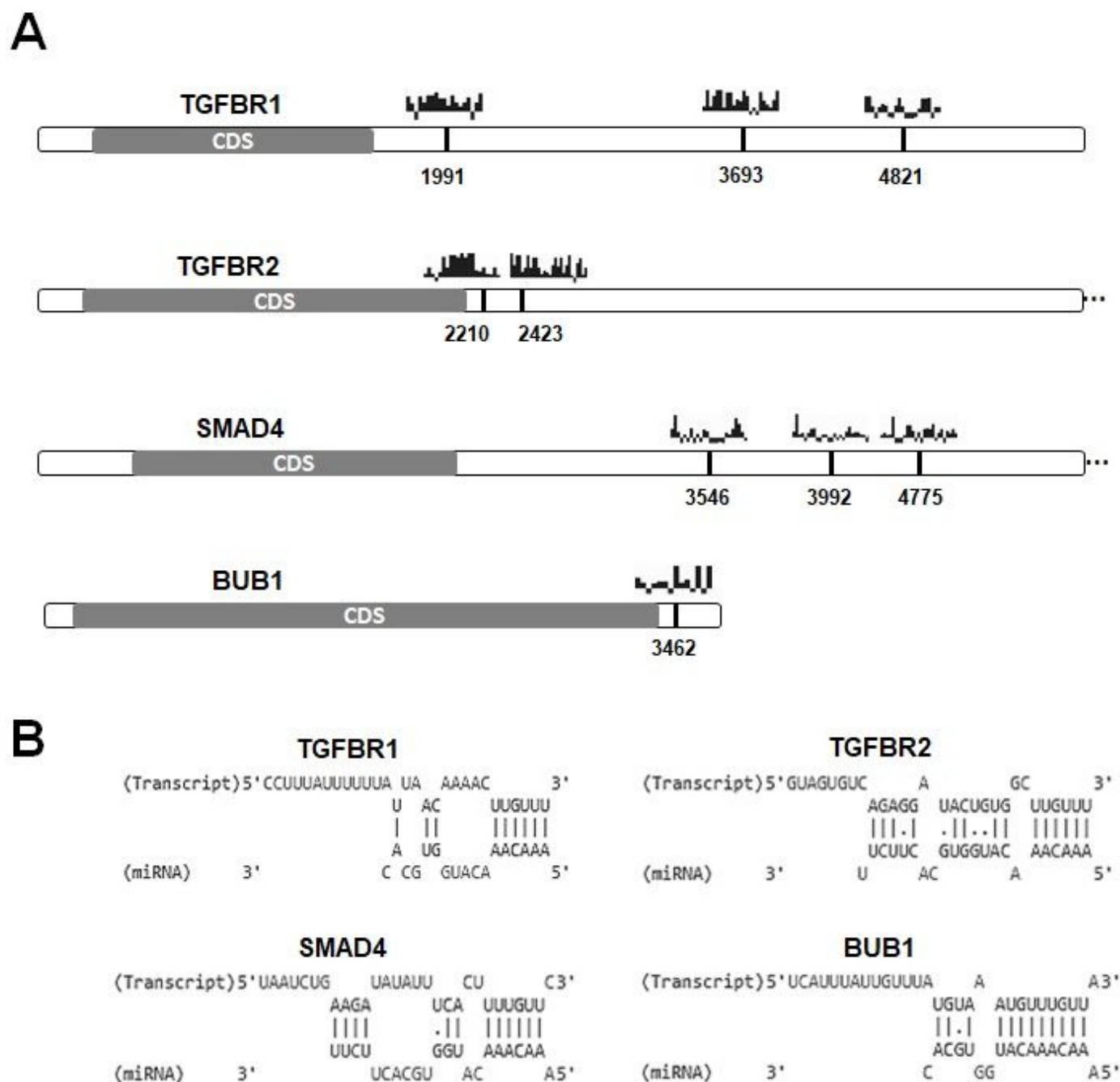


Figure 1

Bioinformatics tools predicted TGF β R1, TGF β R2, SMAD4 and BUB1 genes as potential targets of miR-495-3p. (A) Multiple miRNA responsive sites (MREs) of miR-495-3p were predicted within each of the 3'UTR sequences of TGF β R1, TGF β R2, SMAD4 and BUB1 genes, using DIANA microT, Targetscan and RNAhybrid servers. The conservation status of each of the MREs sequences is shown above their positions in each gene. The numbers indicate the position of each MRE according to the transcription initiation site. (B) Interaction status between the seed sequence of miR-495-3p and its MREs within the 3'UTRs of the four predicted target genes (one miRNA/MRE interaction of each gene is shown).

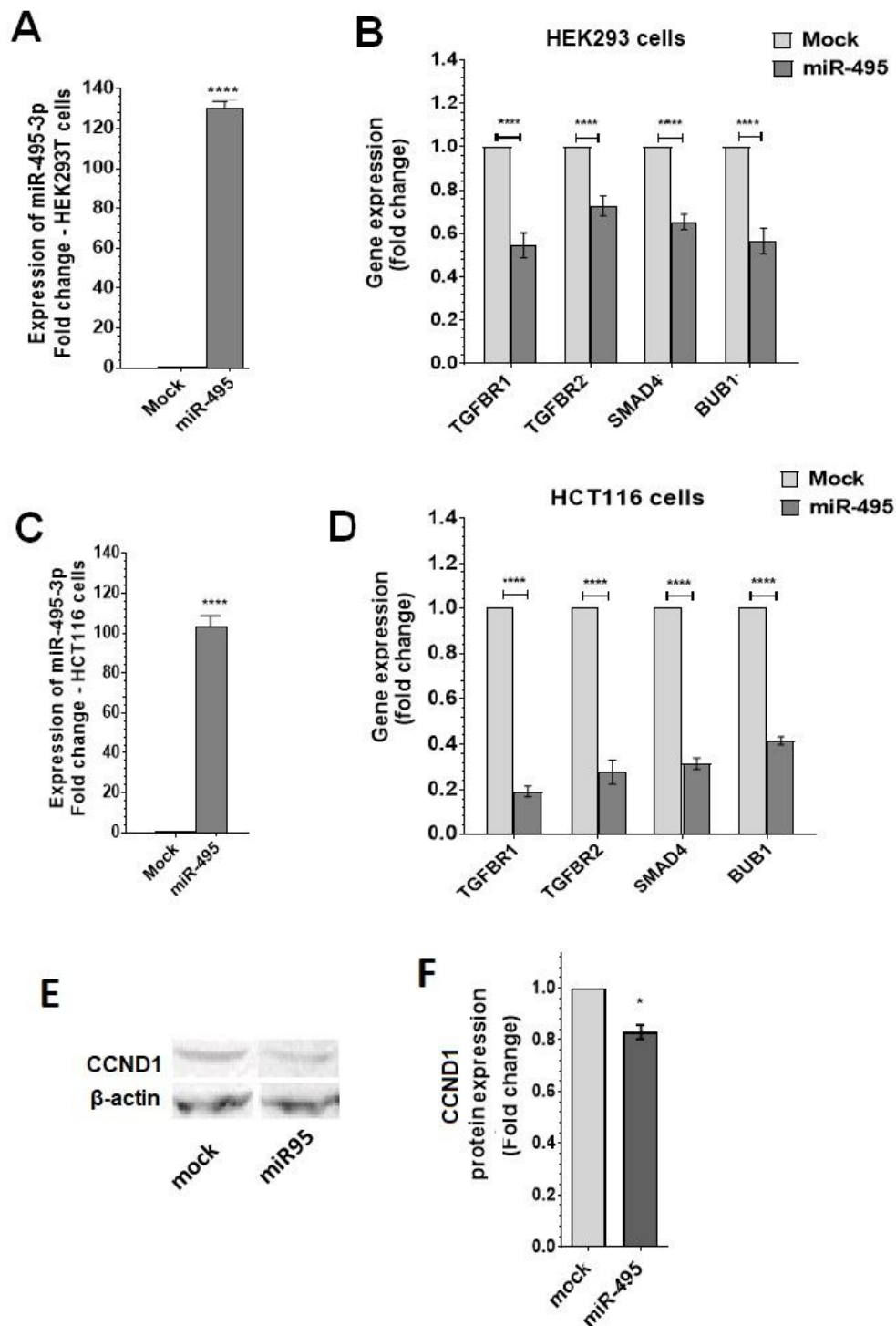


Figure 2

Overexpression of miR-495-3p in HEK293T and HCT116 cells resulted in reduction of TGFβR1, TGFβR2, SMAD4 and BUB1 genes expression in both cell lines. (A&C) HEK293T and HCT116 cells transfected with pEGFPc1/pre-miR-495 construct or pEGFPc1 showed significant overexpression of miR-495-3p in both cell lines compared to the related controls. (B &D) Significant reduction of TGFβR1, TGFβR2, SMAD4 and BUB1 genes expression level, following the transfection of pre-miR-495 in both HEK293T and HCT116

cells, compared with the related controls. (E & F) Western blotting analysis against cyclin D1 (CCND1) protein indicated a significant reduction in the expression of this protein in the cells overexpressing pre-miR-495, in comparison to control. β -actin was used to normalize western blot results. RT-qPCR results were normalized according to the expression of GAPDH and U48 genes. Asterisks (***) and ****) represent p-values<0.001 and <0.0001, respectively.

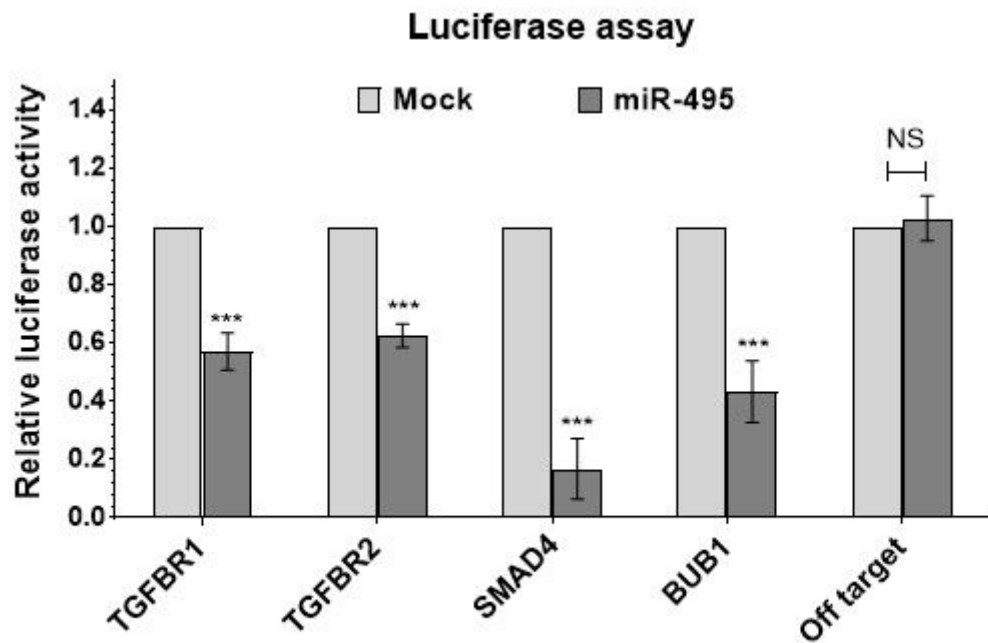


Figure 3

Direct interaction of miR-495-3p with 3'UTR sequences of putative target genes. Luciferase activity was significantly reduced in the HEK293T cells co-transfected with the pEGFPc1/pre-miR-495 vector ensuring miR-495 expression and the pScheck vector harboring luciferase gene ORF, fused with candidate 3'UTR sequence. Data were compared to the cells co-transfected with mock and the 3'UTRs of the target genes. Cells co-transfected with pEGFPc1/pre-miR-495 vector and the pScheck vector harboring 3'UTR of K-RAS gene was also used as an off-target and showed no variation with the control sample.

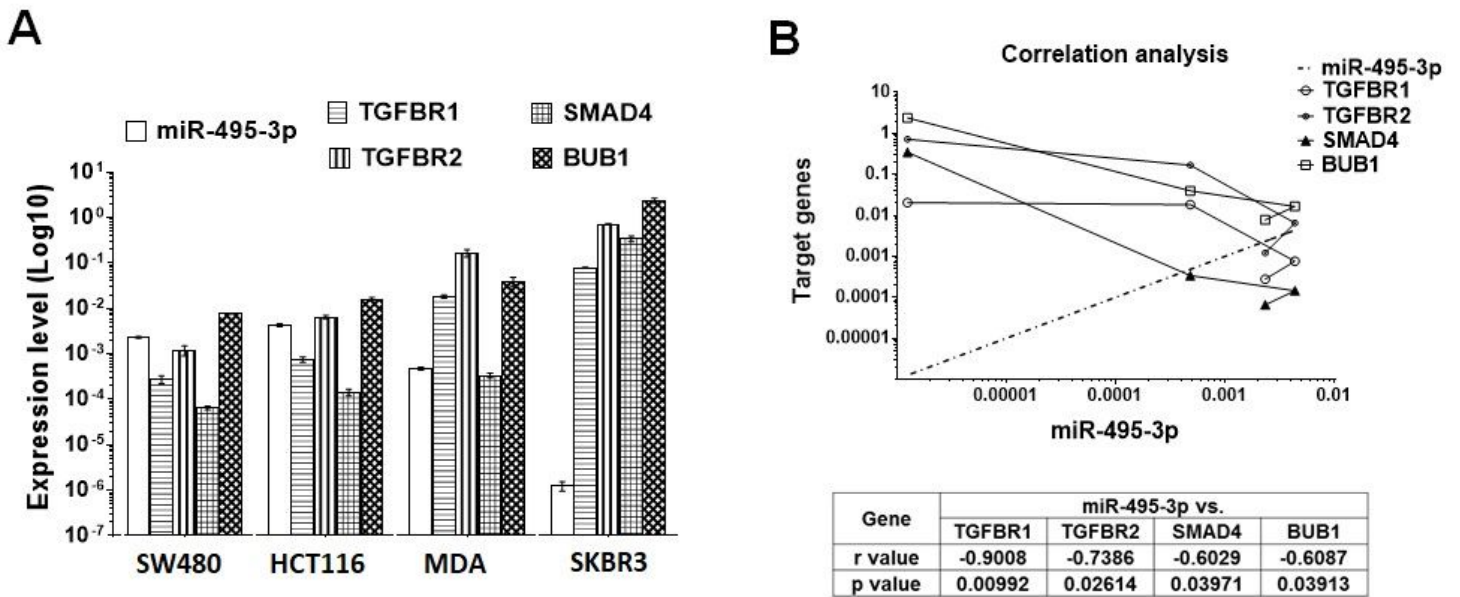


Figure 4

Hsa-miR-495-3p exhibited negatively correlated expression patterns with its putative target genes in tested set of human cell lines. (A) Expression of miR-495-3p and TGF β R1, TGF β R2, SMAD4 and BUB1 targets genes was quantified in the SW480 and HCT116 colorectal cancer cell lines and MDA-MB-231 and SKBR3 breast cancer cell lines using RT-qPCR (log₁₀ values of the expression levels is shown). RT-qPCR results were normalized according to the expression of GAPDH and U48 genes. (B) Correlation analysis of the expression patterns showed significant negative correlation between miR-495-3p and its putative target genes expression in the used cell lines. The table under the correlation analysis graph shows the r- and p-values of correlation between the miRNA and each of its target genes.

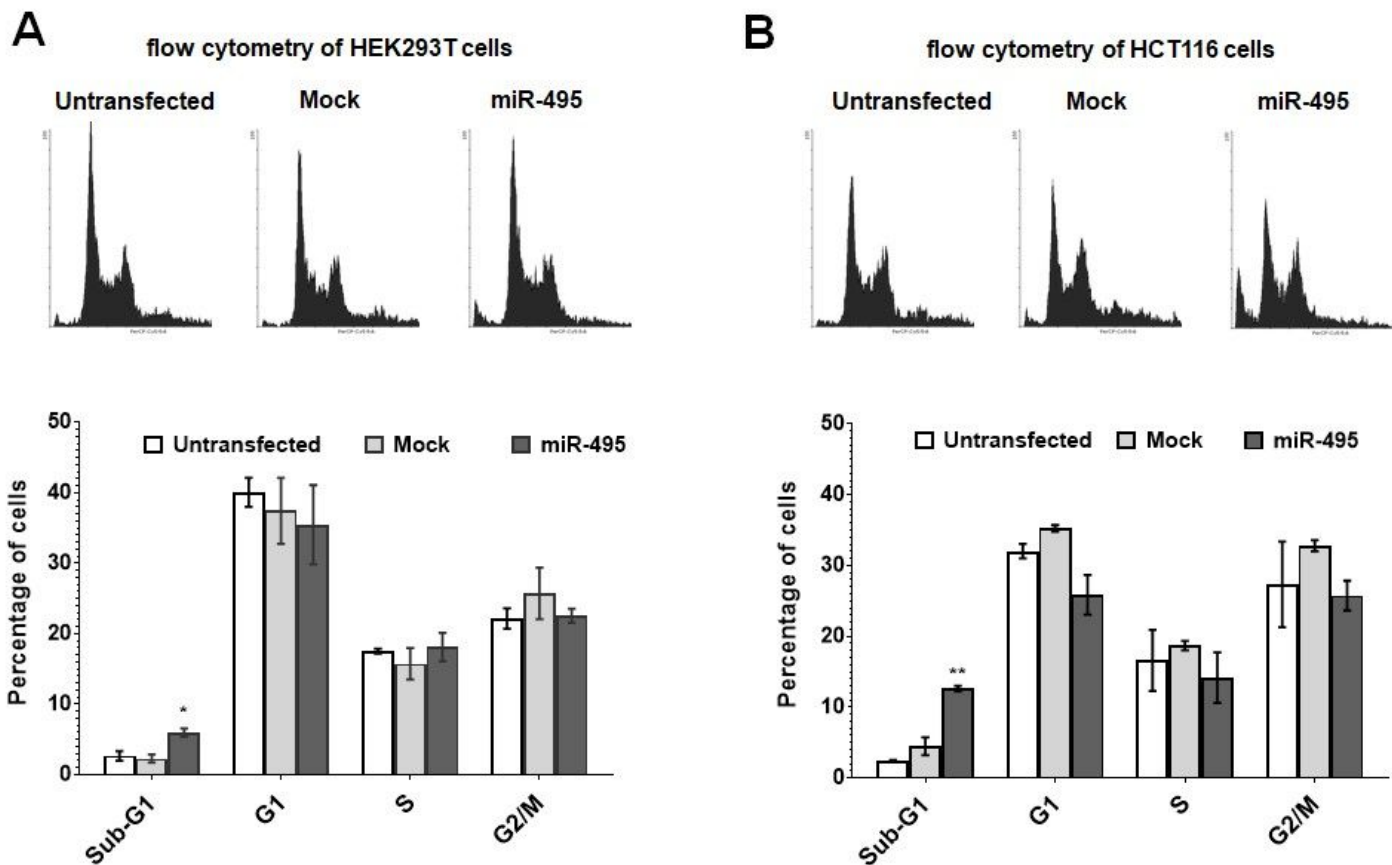


Figure 5

Overexpression of miR-495-3p in HEK293T and HCT116 cells arrested cell cycle progression of both cell lines. (A&B) HEK293T and HCT116 cell cycle analysis by PI flow cytometry. Results showed increased sub-G1 cell population of the cells transfected with pEGFPc1/pre-miR-495 vector in comparison to the controls in both cell lines. However, the impact of miR-495 overexpression was more intense in HCT116 cells (~10 % increase in sub-G1 population of HCT116 cells in comparison to ~4% increase in HEK293T cells, in comparison to the related controls).

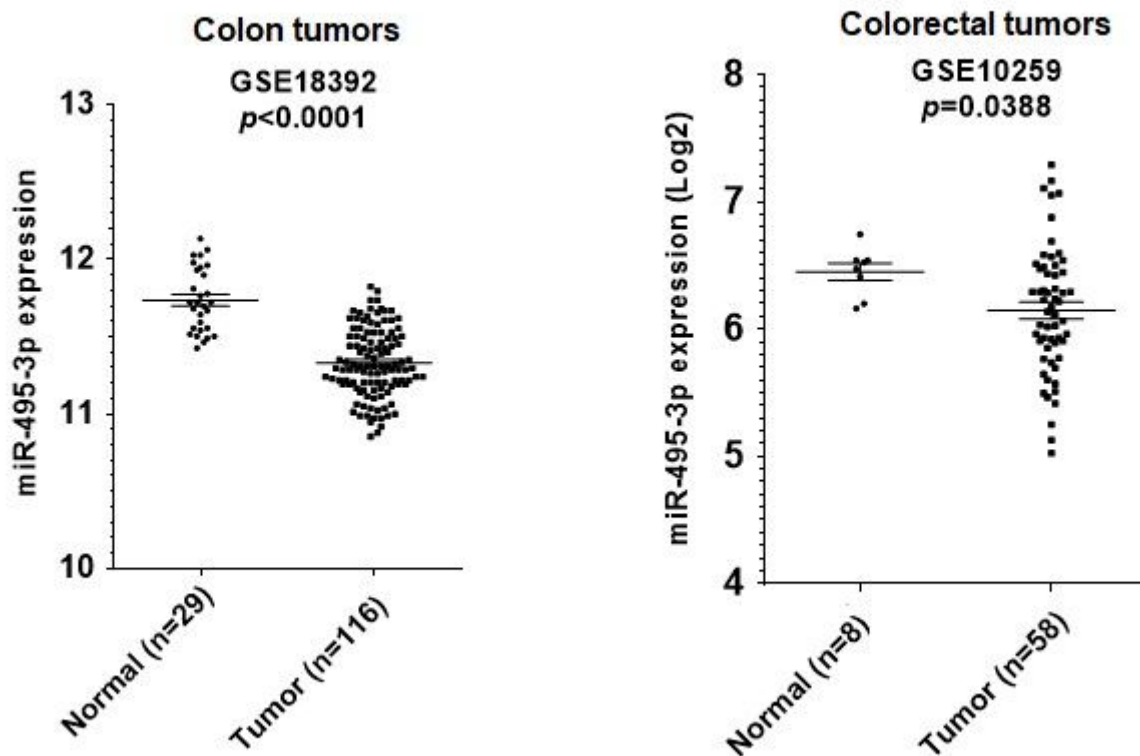


Figure 6

Analysis of microarray data for the expression of miR-495. GSE18392 (colon cancer) and GSE10259 (colorectal cancer) microarray data indicated a significant reduction of miR-495-3p expression in the colorectal tumor samples in comparison to colorectal normal adjacent tissues. Asterisks (*, **) represent p-values < 0.05 and < 0.01 , respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplement1.tif](#)