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RESEARCH ARTICLE

MicroRNA 452 regulates *GTF2E1* expression in colorectal cancer cells

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Abstract. MicroRNAs play important roles in the pathogenesis of human diseases by regulating target gene expression in specific cells or tissues. Previously, we identified microRNA 452 (*MIR452*), which was specifically upregulated in early stage of human colorectal cancer (CRC) tissues. Here, we show the biological role of *MIR452* and general transcription factor IIE subunit 1 (*GTF2E1*) in colorectal cancer. A luciferase reporter system was used to confirm the effect of *MIR452* on *GTF2E1* expression. The expression levels of *MIR452* and the target genes were evaluated by quantitative RT-PCR (qRT-PCR) and western blotting. We verified the association between *MIR452* and the GTF2E1 expression and found that *GTF2E1* transcripts were directly downregulated by *MIR452*. The mRNA and protein levels of *GTF2E1* were also downregulated in CRC cells upon transfection with *MIR452*. GTF2E1 protein expression was decreased in CRC tissues compared to adjacent nontumour tissues. These results suggest that *MIR452* might directly or indirectly regulate the genes transcription related to CRC by downregulating GTF2E1 expression.

Keywords. *MIR452* gene; *GTF2E1*; *GTF2H1*; CDK7; POLR2A; colorectal cancer.

Introduction

MicroRNAs (miRNAs or MiRs) endogenously synthesized small noncoding RNA molecules, involved in the regulation of crucial biological processes such as cell proliferation, differentiation, apoptosis and angiogenesis, by controlling the stability and translation of target mRNAs (Batel 2004; Serocki *et al.* 2018; Aydin *et al.* 2019). They also affect the pathogenesis of various types of cancers by functioning either as oncogenes or tumour suppressor genes (Lu *et al.* 2005; Braga *et al.* 2019). Accumulating evidence suggests that miRNAs are associated with several tumour types including cancers of lung (Wang *et al.* 2019; Zhang *et al.* 2019a, b), liver (Lu *et al.* 2019), gastric (Chen *et al.* 2019; Zhang *et al.* 2019a, b), and colorectal origin (CRC) (Mo *et al.* 2015, 2019; Alam *et al.* 2017).

MicroRNA 452 (*MIR452*, also known as hsa-miR-452) is encoded by the chromosomal region Xq28 in humans and clustered together with *MIR224* within the gamma-aminobutyric acid A receptor epsilon subunit (*GABRE*) gene. Accumulating reports have shown the conflicting results of *MIR452* expression levels in hepatocellular

carcinoma (HCC) (Wang *et al.* 2012; Zheng *et al.* 2014). *MIR452* has been shown to play diverse roles in distinct cancer types or tumourigenesis stage. In our previous study, *MIR452* was upregulated in both early stage CRC tissues (Mo *et al.* 2015, 2019) and in dextran sulphate sodium salt (DSS)-induced colitis tissues of mice (Mo *et al.* 2016), as analysed by differential miRNA expression profiling.

In our previous study, the putative target genes of *MIR452* were identified by comparing the mRNA microarray analysis of *MIR452*-overexpressing human CRC cells with the bioinformatics-predicted candidate target genes. We showed that *MIR452* regulated cell proliferation, migration and angiogenesis by suppressing VEGFA expression in the early stage of CRC progression (Mo *et al.* 2019). We identified 27 putative target genes of *MIR452* in our previous study (Mo *et al.* 2019; Mo and Chae, 2021). One of them, the general transcription factor IIE subunit 1 (*GTF2E1*, also known as *TF2E1* and *TFIIE-A*) was a candidate target gene of *MIR452*. Since *GTF2E1* is a general transcription factor, it was chosen to see that *MIR452* can control the expression of other genes by controlling the general transcription factor.

In this study, we validated the expression levels of *GTF2E1* in *MIR452*-transfected CRC cells, and showed that *GTF2E1* expression was directly downregulated by *MIR452*. The expression levels of genes associated with GTF2E1, such as general transcription factor IIE subunit 2 (*GTF2E2*, also known as *TTD6*; *TF2E2* and *TFIIE-B*), general transcription factor IIH subunit 1 (*GTF2H1*, also known as *P62*; *BTF2*; *TFB1* and *TFIIH*), cyclin dependent kinase 7 (*CDK7*, also known as *CAK1*; *HCAK*; *MO15*; *STK1*; and *CDKN*), and RNA polymerase II subunit A (*POLR2A*, also known as *RPB1*; *RPO2*; *POLR2* and *POLRA*) were also studied.

Material and methods

Patients and tissue samples

The tissue samples used in this study were provided by the Biobank of Wonkwang University Hospital, a member of the National Biobank of Korea. With approval from the institutional review board (WKIRB-201703-BR-010) and informed consent from patients. We obtained colon cancer tissues from four colon cancer patients (two males and two females) and rectal cancer tissues from six rectal cancer patients (four males and two females). The mean age of the colon cancer patients and rectal cancer patients was 64 years and 71 years, respectively. The human CRC tissue samples and matched normal colon tissue samples were used to analyse GTF2E1 protein expression.

Cell culture

The human CRC cell lines SW48, Caco2, SW480 and HT29 were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea) and American Type Culture Collection (ATCC, Manassas, USA), respectively. SW48, SW480 and HT29 cells were cultured in RPMI-1640 medium (HyClone, Logan, USA) supplemented with 10% FBS and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Caco-2 cells were cultured in Alpha-MEM (HyClone) supplemented with 20% FBS and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

RNA extraction and qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA), as described in our previous reports (Mo *et al.* 2019; Han *et al.* 2019). After digestion with DNase, RNA samples were quantified, aliquoted and stored at −80°C. The total RNA isolated from tissue samples and/or cultured cells was used as a template to synthesize cDNA for qRT-PCR analysis. The miRNA expression levels were validated by the TaqMan qRT-PCR assay (Applied Biosystems) for qRT-PCR. qRT-PCR with the SYBR Green dye (Applied Biosystems) was used to assess mRNA expression. RNU48 (for TaqMan qRT-PCR) and GAPDH served as endogenous controls for qRT-PCR of miRNA and mRNA, respectively. Primers for both qRT-PCR and TaqMan analyses are listed in table 1.

MIR452 transfection

SW48, SW480 and HT29 cells (3×10^5 cells per well) or Caco-2 cells (1.5×10^5 cells per well) were plated in 6-well or 10-cm culture plates and cultured as described above. *MIR452* (hsa-miR-452, pre-miR miRNA Precursor AM17100, product ID: PM12509) was commercially synthesized (Ambion, Austin, USA) and transfected at a concentration of 50 nmol/L using Lipofectamine RNAiMAX (Invitrogen) or siPORT NeoFX transfection reagents (Ambion) according to the manufacturers' recommendations. Cells were harvested at 48 h (for mRNA extraction) or 72 h (for protein extraction) after transfection.

Plasmid construct and luciferase assays

The wild type (WT) 3′ untranslated region (3′UTR) sequences of *GTF2E1* containing the *MIR452* binding site were PCR amplified from human genomic DNA. After amplification, the PCR products were cloned in the pmir-GLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, USA). The corresponding mutant type (MT) 3′UTR sequences of *GTF2E1* were prepared through the deletion of eight nucleotides in the seed region by using site directed mutagenesis PCR. All of the constructs were confirmed by DNA sequencing. The PCR primers used for luciferase assay are described in table 1. Analysis of the luciferase assay results was carried out according to our previously described method (Mo *et al.* 2019; Han *et al.* 2019). Briefly, cells (2×10^4 cells/well) were seeded onto 24-well plates and cotransfected with wild-type or mutant *GTF2E1* constructs (500 ng/well) or with 50 nM of *MIR452* or *MIR1* (negative control) using Lipofectamine 2000 (Invitrogen) or siPORT NeoFX Transfection Agent (Ambion). Firefly and Renilla luminescence were measured 24 h after transfection using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Untransfected cells were used for background subtraction, and the ratio of firefly reporter luminescence to Renilla reporter luminescence (control) was calculated. All experiments were performed in duplicate and repeated at least thrice.

Antibodies and western blot analysis

SW48, SW480, HT29 or Caco2 cells (2×10^5 cells/well) were seeded onto 6-well plates and cultured for 72 h. Whole

Table 1. Primer sequences used for qRT-PCR analysis and luciferase assays in this study.

Applications	Primers*	Primer sequence (5′ → 3′)
qRT-PCR	GTF2E1-QF1	AGCCTCACTGGAAGGGAAAT
	GTF2E1-QR1	GCATGACCTCTTCGTTGTCA
	GAPDH-F	CAATGACCCCTTCATTGACC
	GAPDH-R	GACAAGCTTCCCGTTCTCAG
Luciferase assay	GTF2E1 -WF	CAGGAGCTCATGAGGCCATTCCAGCAGA
	GTF2E1 -WR	CAGCTCGAGCAACCCAGGTAAATGAAACATCAC
	GTF2E1 -MF	GTAACCTCAGGTATTTTGGGTTCT
	GTF2E1 -MR	AGAACCCAAAATACCTGAGGTTAC
Taq-Man analysis	RNU48	GATGACCCCAGGTAACTCTGAGTGTGTCGC
		TGATGCCATCACCGCAGCGCTCTGACC
	hsa-mir-452	AACUGUUUGCAGAGGAAACUGA

*QF, quantitative forward primer; QR, quantitative reverse primer; WF, forward primer for wild-type constructs; WR, reverse primer for wild-type constructs; MF, forward primer for mutate-type constructs; MR, reverse primer for mutate-type constructs.

cell lysates were prepared by culturing cells in RIPA buffer supplemented with a protease inhibitor mixture for 30 min at 4°C. Proteins were isolated by centrifugation at 12,000 rpm for 30 min at 4°C. Equal amounts of protein (50 μg; as determined by the Bradford assay) were boiled in Laemmli buffer, and separated by either 12.0% or 15.0% SDS-PAGE, and then transferred onto PVDF membranes. The membranes were incubated with blocking buffer (5% BSA in TBS containing 0.1% Tween-20 (TBS-T)) for 2 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies to GTF2E1 (sc-374014), GTF2E2 (sc-238), GTF2H1 (sc-292), POLII (sc-899) and CDK7 (sc-7344; Santa Cruz Biotechnology), and washed thrice (10 min per wash) with TBS-T. Membranes were then incubated with HRP-conjugated rabbit or mouse IgG secondary antibodies for 1 h at room temperature. After washing thrice for 10 min in TBS-T, proteins were detected using ECL reagent (Millipore Corporation, Billerica, USA) and FluorChem E System (Cell Biosciences, Santa Clara, USA). After protein detection, some membranes were stripped using stripping buffer for 1 h at room temperature and reprobed with either GAPDH (sc-47724), which was used as a loading control. Protein expression was evaluated using ImageJ software (v. 1.44; http://rsbweb.nih.gov/ij/index.html).

Statistical analysis

We obtained consistent results from all experimental replicates. All data were represented as mean ± standard deviation (SD). Statistical differences were analysed by Student's *t*-test or two-sided *t*-test, and results with *P* values less than 0.05 ($P < 0.05$) were regarded as statistically significant.

Results

GTF2E1 is direct targets of MIR452 in CRC cells

To determine whether *MIR452* directly regulates *GTF2E1* mRNA, we used a luciferase reporter system. The predicted *MIR452* recognition sites in the *GTF2E1* 3′ UTRs were cloned into a pmirGLO Dual-Luciferase miRNA Target Expression Vector (pmirGLO-*GTF2E1* WT). We also generated mutated (MT) *MIR452* binding sites by site-directed mutagenesis (pmirGLO- *GTF2E1* MT) (figure 1a). The base pairing between the *MIR452* seed sites and the *GTF2E1* mRNA targets is depicted in figure 1a. Subsequently, the vectors were cotransfected into Caco-2 cells with pre-*MIR452* or pre-*MIR1*. *MIR452* inhibited luciferase activity in cells transfected with pmirGLO-*GTF2E1* WT ($\sim$32%, vs controls, $P < 0.05$) (figure 1b). In contrast, *MIR452* did not reduce luciferase activity in cells transfected with pmirGLO-*GTF2E1* MT vector construct (figure 1b).
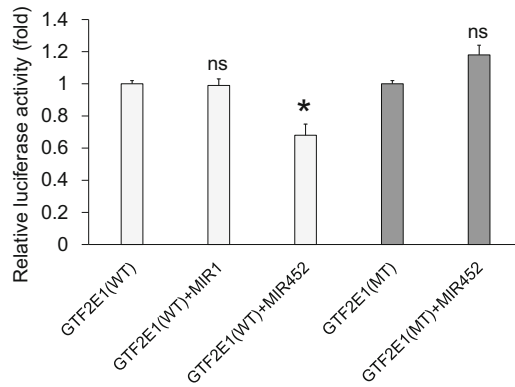
MIR452 regulates GTF2E1 expression in CRC cells

We investigated whether *MIR452* regulated *GTF2E1* mRNA and protein levels in Caco-2 cells. *MIR452* mimic was transfected into Caco-2 cells, and qRT-PCR analysis was performed using cells harvested 48 h after transfection. *GTF2E1* mRNA levels were significantly downregulated in Caco-2 cells transfected with *MIR452* (figure 1c; $P < 0.05$). *MIR452* mimic or anti-*MIR452* were transfected into Caco-2 cells, and western blot analyses were performed using cells harvested 72 h after transfection. GTF2E1 protein expression levels were significantly downregulated in Caco-2 cells transfected with *MIR452* (figure 1d; $P < 0.05$). On the contrary, GTF2E1 protein expression levels were upregulated in Caco-2 cells transfected with anti-*MIR452*
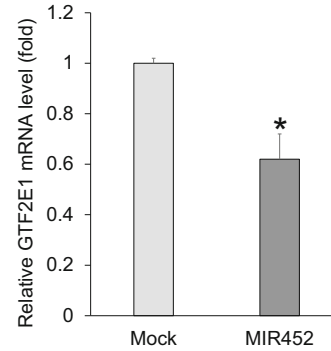
(a)

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GTF2E1 3'UTR (WT) 5'-TACCATTGAGCATTGTAACCTCAGGAAACAGTTTATTTTGGGTT--3'
MIR452            3'-----------AGUCAAAGGAGACGUUUGUCAA-5'
GTF2E1 3'UTR (MT) 5'-TACCATTGAGCATTGTAACCTCAGG-------TATTTTGGGTT--3'
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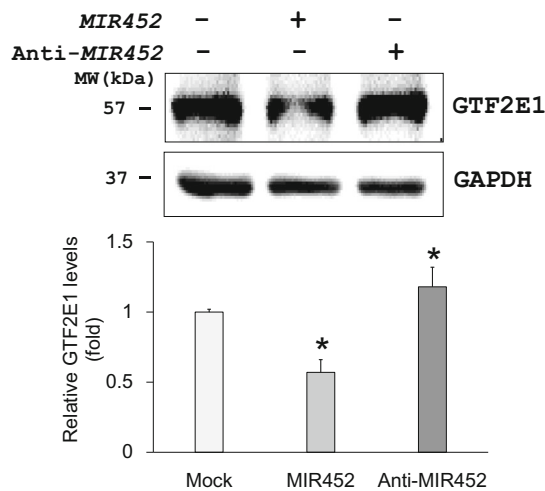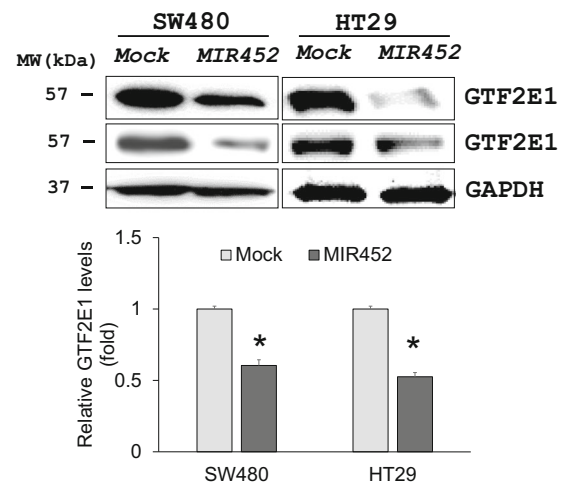
(b)

(c)

(d)

(e)

Figure 1. MIR452 directly downregulates GTF2E1 expression. (a) Sequence alignment of *MIR452* seed region and the putative binding sites in the *GTF2E1* 3'UTRs. The wild-type (WT) or mutant (MT) 3'UTR of the *GTF2E1* gene containing the *MIR452* target sequence was cloned into pmirGLO Dual-Luciferase miRNA Target Expression Vector. (b) Each luciferase reporter construct was cotransfected into Caco-2 cells with either *MIR1* mimic as a negative control or *MIR452* mimic. After 24 h, luciferase activity was measured in the cell extracts. Transfection with *MIR452* mimic inhibited luciferase activity in the presence of WT *GTF2E1* 3'UTR (*$P < 0.05$; two-sided *t*-test). Inhibition was not observed when cells were transfected with reporter constructs containing the MT 3'UTR target sequences or with *MIR1* (ns, not significant). (c) *GTF2E1* mRNA levels in *MIR452*- or mock-transfected Caco-2 cells. Total RNA was isolated 48 h after transfection, and *GTF2E1* mRNA levels were examined by qRT-PCR (*$P < 0.05$; two-sided *t*-test). (d) Western blot analysis of *MIR452*- or anti-*MIR452* or mock-transfected Caco-2 cells. The GTF2E1 bands were quantified relative to the appropriate loading controls using ImageJ software and are shown relative to the protein level in mock-transfected cells. The GTF2E1 protein levels were reduced upon *MIR452* transfection in Caco-2 cells (* $P < 0.05$). The data are representative of three independent experiments.

(figure 1d; $P < 0.05$). The ani-*MIR452* used in this experiment was purchased for commercial use and could not see the perfect *MIR452* suppression effect. These results suggest that the expression of *GTF2E1* is directly regulated by *MIR452* in CRC cells.

We also investigated GTF2E1 expression levels by *M1R452* transaction using SW480 and HT29 cell lines. GTF2E1 protein expression levels were significantly downregulated in both SW480 and HT29 cells transfected with *MIR452* (figure 1e; $P < 0.05$).
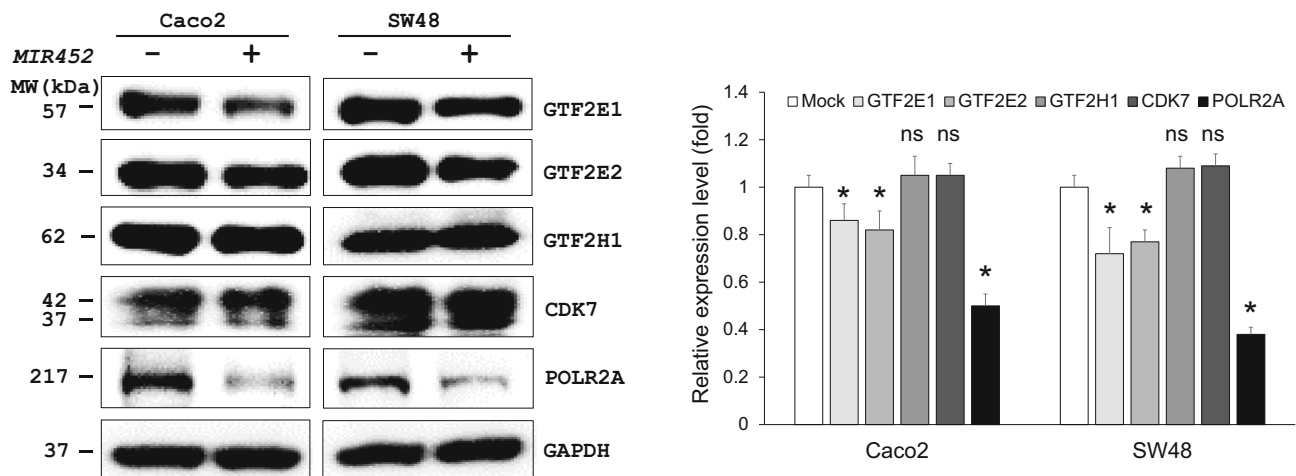
Figure 2. *MIR452* regulate GTF2E1 associated molecules in CRC cells. The expression levels of GTF2E2, GTF2H1, CDK7 and POLR2A in Caco-2 and Sw48 cells upon *MIR452* mimic or mock- transfection. The results on the right indicate the relative expression levels of each protein. Five independent experiments were performed in duplicates, and the statistical differences were calculated using student's *t*-test or two-sided *t*-test (ns, not significant; $*P < 0.05$).

MIR452 regulates proteins related to GTF2E1

To determine the functional interaction between *MIR452* and GTF2E1 in Caco-2 and SW48 cells, we analysed the expression levels of proteins associated with the functions of GTF2E1 by western blot analysis. GTF2E2 and POLR2A protein expression levels were reduced upon transfection with *MIR452* mimic in both Caco-2 and SW48 cells, respectively (figure 2; $P < 0.05$). However, GTF2H1 and CDK7 protein expression levels were slightly upregulated when transfected with *MIR452* mimic in both Caco-2 and SW48 cells, however, the results were not statistically significant (figure 2; ns, not significant). These results indicated that *MIR452* regulates the expression of GTF2E1–GTF2E2–POLR2A axis in CRC cells.

MIR452 expression level in human CRC tissues

We have previously found that *MIR452* is upregulated in human CRC tissues. To confirm this result, we compared the *MIR452* levels in five human CRC tissue samples with those in matching healthy colon tissues by qRT-PCR. *MIR452* levels were increased in CRC tissues (four out of five, figure 3a).

GFT2E1 expression levels in human CRC tissues

On the basis of the findings described above, we evaluated GTF2E1 expression in 10 human CRC tissues and their matched normal colorectal tissues by western blotting. GTF2E1 protein expression was decreased in CRC tissues (nine out of 10 pairs) compared to adjacent nontumourous tissues ($P < 0.05$; figure 3b).

Discussion

MiRNAs are important regulators of gene expression and their aberrant expression has been implicated in the development of various diseases (Bartel 2004; Lu *et al.* 2005; Garzon *et al.* 2006). Accumulating evidence suggests that miRNAs act as tumour suppressors or oncogenes by targeting genes involved in cell proliferation, survival, apoptosis and metastasis (Brennecke *et al.* 2003; Ma *et al.* 2007; Su *et al.* 2009). Therefore, characterization of the correlations between miRNAs and their target genes in colon cancer may have a substantial diagnostic, prognostic and therapeutic value. We identified *MIR452* as an associated microRNA in both early stage human CRC tissues and DSS-induced colitis tissues of mice, and showed that *MIR452* expression was upregulated in both these types of tissues compared to matched healthy CRC and normal mouse colon tissues, respectively (Mo *et al.* 2016, 2019). In this study, we validated the expression levels of *GTF2E1* in CRC tissues and *MIR452*-transfected CRC cells to confirm that it indeed is the target gene of *MIR452*.

GTF2E1 is a subunit gene of *GTF2E* (also known as *TFIIE*) that is a member of the general transcription factors (GTFs). The transcription of protein-encoding genes in eukaryotes is performed by RNA polymerase II (also known as *RNAP II* and *POL II*) and GTFs (Roeder 1996; Bentley 2014). Five GTFs such as TFIIB, TFIID, TFIIE, TFIIF and TFIIH are required for transcription initiation by Pol II (Orphanides *et al.* 1996; Roeder 1996). GTF2E is composed of two subunits α (GTF2E1) and β (GTF2E2) with a molecular
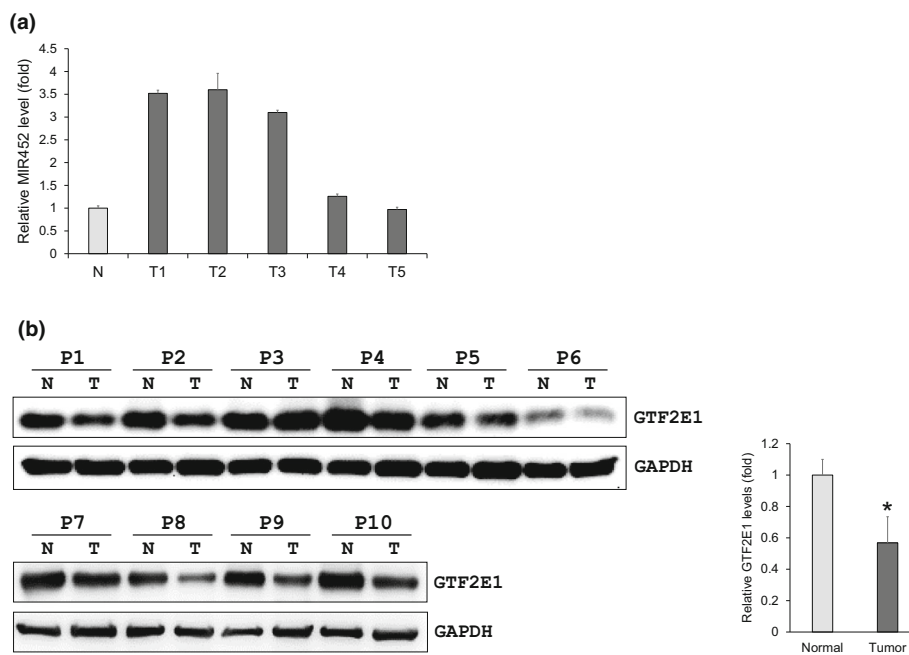
Figure 3. Endogenous *MIR452* and GTF2E1 expression levels in human CRC tissues. (a) The expression of *MIR452* was validated using 10 CRC tissue samples and matched with normal colon tissue samples. MiRNAs levels were normalized to colon specific RNU48. Values are presented as the fold-change in tumour tissue relative levels (*ΔΔ*CT) to normal tissue. (b) GTF2E1 expression in 10 pairs of human CRC tissue samples and adjacent normal colon tissue samples. The GTF2E1 levels were quantified by ImageJ software (* $P < 0.05$; two-sided *t*-test). N and T indicate normal colorectal and tumour colorectal tissues, respectively. The data are representative of three independent experiments.

mass of 56 kDa and 34 kDa, respectively (Ohkuma *et al.* 1990). These two subunits interact with each other (Okamoto *et al.* 1998), and act as key factors to recruit the TFIIH kinase module (CAK) of GTF2H (Compe *et al.* 2019). Accumulating reports suggest that GTF2E plays a pivotal role in transcription by directly influencing the transition from initiation to elongation (Peterson *et al.* 1991; Watanabe *et al.* 2003). GTF2H1 and CDK7 are subunit genes of GTF2H (TFIIH), and POLR2A is a member of POL II.

We showed that *GTF2E1* is a direct target of *MIR452* in CRC cells (figure 1b). The *GTF2E1* mRNA and protein expression levels were downregulated upon transfection with *MIR452* mimic in Caco-2 cells (figure 1, c&d). On the contrary, GTF2E1 protein levels were upregulated in Caco-2 cells when transfected with anti-*MIR452* construct (figure 1d). Thus, these results suggest that the upregulated levels of *MIR452* in CRC cells may regulate GTF2E1 expression levels during colorectal carcinogenesis.

These results led us to study whether the expression of GTF2E1 reduced by *MIR452* in CRC cells affected genes related to transcription initiation. The expression levels of GTF2E1, GTF2E2 and POLR2A were significantly downregulated when transfected with *MIR452* mimic in the two CRC cell lines (figure 2). These results suggest that the expression level of *GTF2E2* was directly downregulated by *MIR452*, while *POLR2A* was indirectly regulated by *MIR452* transfection in CRC cells. Although GTF2H1 and CDK7 expression levels were slightly increased upon *MIR452*

mimic transfection in both CRC cell lines, there were no significant associations (figure 2). Although these results suggest that the functions of GTF2E and GTF2H might be differing from each other during transcription initiation due to *MIR452* regulation, the reason for this difference is unclear at this point.

In this study, we also showed that the protein expression levels of GTF2E1 were downregulated in CRC tissues compared to adjacent nontumourous tissues (figure 3b). Thus, these results suggest that the upregulated expression of *MIR452* in CRC tissues might directly regulate the transcription of other genes by inhibition of GTF2E1 during CRC pathogenesis. GTF2E1 is connected to the driver and tumour suppressor genes, and affect cancer-related functions such as transmembrane transport and transformation factors (Chang *et al.* 2020).

Although we did not study in great detail the signalling mechanisms of GTF2E1 mediated by the regulation of MIR452, our results in CRC cells suggest that *GTF2E1* was a direct target gene of *MIR452*. Therefore, the upregulated expression of *MIR452*, as observed in CRC tissues, might regulate the expression levels of GTF2E1 during CRC carcinogenesis. Down regulated GTF2E1 by *MIR452* during early CRC carcinogenesis plays an important role in transcription by directly influencing the transition from initiation to elongation. Our results could provide a valuable resource for further functional studies of genes associated with *GTF2E1* in the pathogenesis of various organs.

Acknowledgements

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