Received: 11 February 2019 | Revised: 8 November 2019 | Accepted: 18 November 2019

DOI: 10.1111/cas.14248

ORIGINAL ARTICLE

Cancer Science WILEY

GTF2IRD1 on chromosome 7 is a novel oncogene regulating the tumor-suppressor gene *TGFβR2* in colorectal cancer

Sho Nambara[1] | Takaaki Masuda[1] | Yuta Kobayashi[1] | Kuniaki Sato[1] | Taro Tobo[2] | Kensuke Koike[1] | Miwa Noda[1] | Yushi Ogawa[3] | Yousuke Kuroda[1] | Shuhei Ito[1] | Hidetoshi Eguchi[1] | Keishi Sugimachi[1,4] | Koshi Mimori[1]

[1]Department of Surgery, Kyushu University Beppu Hospital, Beppu, Japan

[2]Department of Pathology, Kyushu University Beppu Hospital, Beppu, Japan

[3]Digestive Disease Center, Showa University Northern Yokohama Hospital, Yokohama, Japan

[4]Department of Gastroenterological Surgery, National Kyushu Cancer Center, Fukuoka, Japan

Correspondence
Koshi Mimori, Department of Surgery, Kyushu University Beppu Hospital, Beppu, Japan.
Email: kmimori@beppu.kyushu-u.ac.jp

Funding information
Priority Issue on Post-K computer, Grant/Award Number: hp160219 and hp170227; JSPS KAKENHI, Grant/Award Number: 15H05707; Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Science Research, Grant/Award Number: JP16K07177, JP16K10543, JP16K19197, JP17K10593, JP17K16454, JP17K16521 and JP17K19608; Eli Lilly Japan K.K. Grant; OITA Cancer Research Foundation; Daiwa Securities Health Foundation; Grant-in-Aid for Scientific Research on Innovative Areas, Grant/Award Number: 15H0912; Japanese Foundation for Multidisciplinary Treatment of Cancer

Abstract

Chromosome 7q (Ch.7q) is clonally amplified in colorectal cancer (CRC). We aimed to identify oncogenes on Ch.7q that are overexpressed through DNA copy number amplification and determine the biological and clinical significance of these oncogenes in CRC. We identified general transcription factor 2I repeat domain-containing protein 1 (*GTF2IRD1*) as a potential oncogene using a CRC dataset from The Cancer Genome Atlas with a bioinformatics approach. We measured the expression of *GTF2IRD1* in 98 patients with CRC using immunohistochemistry and RT-quantitative PCR (RT-qPCR). The biological effects of *GTF2IRD1* expression were explored by gene set enrichment analysis (GSEA). Next, we undertook in vitro cell proliferation and cell cycle assays using si*GTF2IRD1*-transfected CRC cells. We further investigated the oncogenic mechanisms through which *GTF2IRD1* promoted CRC progression. Finally, we assessed the clinical significance of *GTF2IRD1* expression by RT-qPCR. *GTF2IRD1* was overexpressed in tumor cells and liver metastatic lesions. The GSEA revealed a positive correlation between *GTF2IRD1* expression and cell cycle progression-related genes. *GTF2IRD1* knockdown inhibited cell proliferation and induced cell cycle arrest in *Smad4*-mutated CRC. *GTF2IRD1* downregulated the expression of the gene encoding transforming growth factor β receptor 2 (*TGFβR2*), a tumor-suppressor gene in *Smad4*-mutated CRC. On multivariate analysis, high *GTF2IRD1* expression was an independent poor prognostic factor. Clinicopathological analysis showed that *GTF2IRD1* expression was positively correlated with liver metastasis. In conclusion, *GTF2IRD1* promoted CRC progression by downregulating *TGFβR2* and could be a prognostic biomarker on Ch.7q in CRC. *GTF2IRD1* could also be a novel oncogene in CRC.

KEYWORDS

cell cycle, colorectal cancer, *GTF2IRD1*, oncogene, *TGFβR2*

1 | INTRODUCTION

Colorectal cancer (CRC) is a leading cause of tumor-associated morbidity and mortality worldwide, and its incidence continues to rise.[1,2] Despite recent advances in therapeutic approaches, including chemotherapy and molecular targeted therapy, relapse is frequently found among patients with CRC, particularly for those with advanced disease.[3]

Intratumor heterogeneity (ITH), defined as molecular and cellular heterogeneity within a single tumor, is thought to cause the development of resistance against chemotherapy and leads to therapeutic failure because the presence of distinct subpopulations of cells with different sensitivities to chemotherapeutic drugs increases the risk of resistance and recurrence.[4] Therefore, identification of oncogenes expressed in all tumor cells within a single tumor is essential for the discovery of promising therapeutic targets despite ITH.

Amplification of chromosome 7 is frequently found in CRC and colorectal adenoma tissue.[5-7] Moreover, we showed that amplification of the long arm of chromosome 7 (Ch.7q) exists in all regions of a single tumor by undertaking multiregional copy number analysis of tumor tissues in CRC,[8,9] suggesting that amplification of Ch.7q is a fundamental and predominant event and that this region harbors oncogenes that affect the tumorigenesis of CRC.

We recently established a screening system using a bioinformatics approach with public datasets to identify candidate oncogenes in CRC. Using this system, we identified phosphoserine phosphatase (*PSPH*) and eIF5-mimic protein 1 (*5MP1*) as potential oncogenes on Ch.7p in CRC.[10,11] Furthermore, we found that their expression was significantly associated with cell cycle progression genes and was an independent poor prognostic factor in patients with CRC. This system enables us to comprehensively search for oncogenes in CRC.

In this study, we aimed to identify novel potential oncogenes on Ch.7q in CRC using this screening system and to clarify the biological and clinical significance of the identified oncogenes in CRC.

2 | MATERIALS AND METHODS

2.1 | Selection of candidate genes

We obtained RNA sequencing data and DNA copy number data from 615 patients with CRC from The Cancer Genome Atlas (TCGA) from the Broad Institute's Firehose (http://gdac.broadinstitute.org/runs/stddata__2015_08_21/data/COADREAD/20150821/). The RNA sequencing data also included expression profiles from 51 paired normal colon samples. Using these data, we extracted candidate genes from 819 genes on Ch.7q that showed positive correlations between DNA copy numbers and mRNA expression levels (cut-off correlation coefficient, 0.4) (criteria 1) and showed overexpression in tumor tissues compared with normal tissues (more than 2-fold change) (criteria 2).

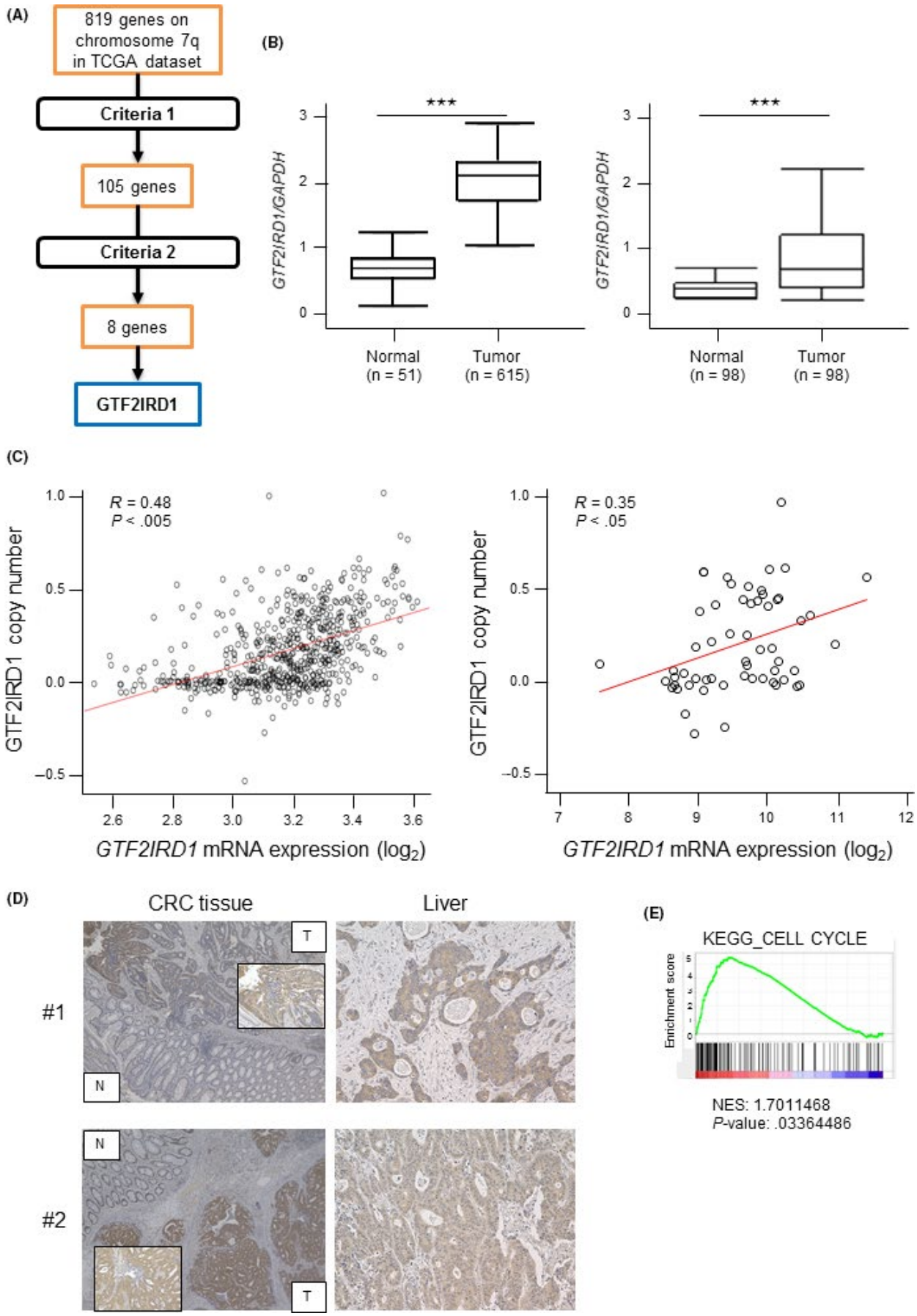
2.2 | Cell culture

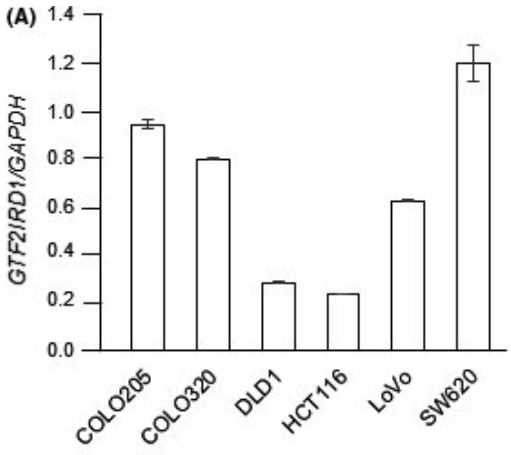
Human CRC cell lines (SW620, COLO205, COLO320, DLD1, HCT116, and LoVo) were purchased from the cell bank at RIKEN BioResource Center (Tsukuba). SW620, COLO205, and DLD1 cells were maintained in RPMI-1640. COLO320 and HCT116 cells were maintained in DMEM. LoVo cells were maintained in Ham's F12 medium. All media contained 10% FBS with 100 U/mL penicillin and 100 U/mL streptomycin sulfate. All CRC cells were cultured in a humidified 5% CO_2 incubator at 37°C.

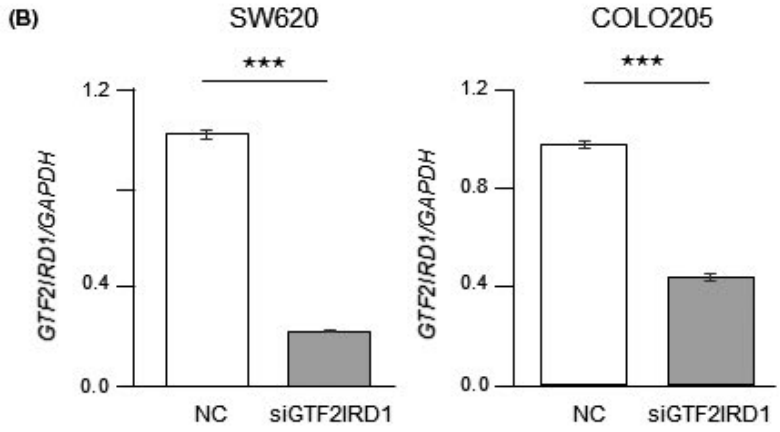
2.3 | Total RNA extraction and RT-quantitative PCR

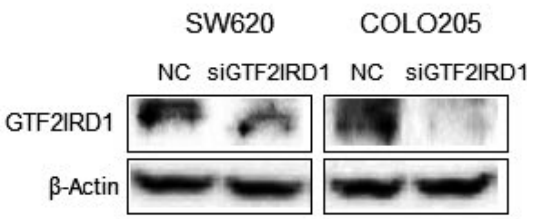
Total RNA from tissues and cell lines was extracted using a modified acid guanidinium thiocyanate-phenol-chloroform extraction (AGPC) method with Isogen (Nippon Gene). Reverse transcription was carried out using 8 μg total RNA with M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Quantitative PCR (qPCR) was undertaken using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) as previously described.[12] The expression levels of general transcription factor 2I repeat domain-containing protein 1 (*GTF2IRD1*) and transforming growth factor β receptor 2 (*TGFβR2*) mRNA were normalized to *GAPDH* mRNA as an internal control. Gene expression was presented as the values relative to the expression level of the cDNA from Human Universal Reference Total RNA (Clontech). The primer sequences for qPCR were as follows: *GTF2IRD1*, forward 5′-GTGCCAGCCAAAGACAGCAG-3′ and reverse 5′-TGGCCATTGCACGAGTGAGA-3′; *TGFβR2*, forward 5′-TGGACCCTACTCTGTCTGTGGA-3′ and reverse 5′-CCCAC TGCATTACAGCGAGAT-3′; *p21*, forward 5′-GCGACTGTGATGCG CTAATG-3′ and reverse 5′-

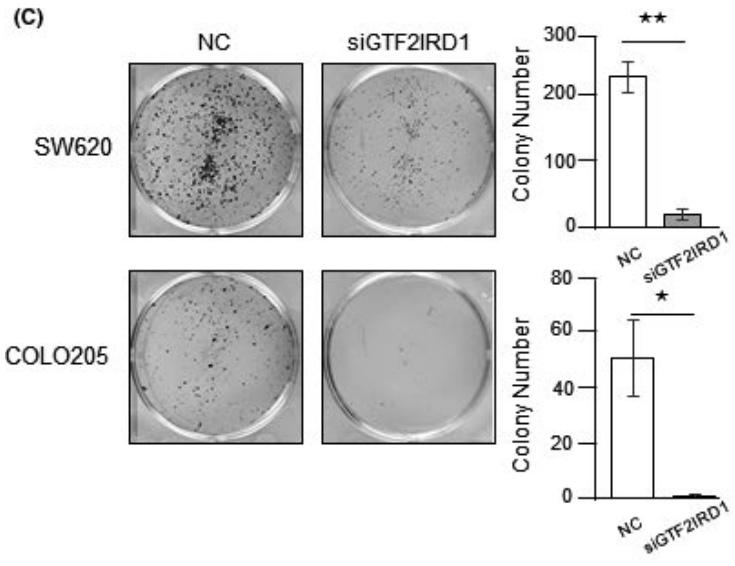
FIGURE 1 Identification of candidate oncogenes on chromosome 7q in colorectal cancer (CRC). A, Schematic diagram of the strategy for candidate oncogene selection. Criteria 1: Positive correlations between DNA copy numbers and mRNA expression levels (cut-off correlation coefficient, 0.4). Criteria 2: Overexpressed in tumor tissues compared with normal tissues (>2-fold change). B, Left, *GTF2IRD1* mRNA expression between 615 CRC tissues and 51 normal colon tissues in The Cancer Genome Atlas (TCGA) dataset. Right, *GTF2IRD1* mRNA expression in 98 CRC tissues and paired normal colon tissues in our dataset by RT-quantitative PCR. ***$P < .0005$. C, Left, Correlation between GTF2IRD1 copy number and *GTF2IRD1* mRNA expression in TCGA dataset. Right, Correlation between GTF2IRD1 copy number and *GTF2IRD1* mRNA expression in the Cancer Cell Line Encyclopedia dataset. *R*, Pearson's correlation coefficient. D, Immunohistochemical staining for GTF2IRD1 in normal colon and tumor tissues (left), and liver metastatic lesion tissues (right) in the same patients. Original magnification, 40× and 200×. E, Gene set enrichment analysis of the expression of *GTF2IRD1* and cell cycle-related genes using reference gene sets in the CRC dataset. KEGG, Kyoto Encyclopedia of Genes and Genomes; N, normal tissue; NES, Normalized Enrichment Score; T, tumor tissue


(A)
819 genes on chromosome 7q in TCGA dataset
Criteria 1
105 genes
Criteria 2
8 genes
GTF2IRD1
(B)
GTF2IRD1/GAPDH
***
Normal (n = 51)
Tumor (n = 615)
Normal (n = 98)
Tumor (n = 98)
(C)
R = 0.48
P < .005
R = 0.35
P < .05
GTF2IRD1 copy number
GTF2IRD1 mRNA expression (log2)
(D)
CRC tissue
Liver
#1
#2
T
N
(E)
KEGG_CELL CYCLE
Enrichment score
NES: 1.7011468
P-value: .03364486


(A)
GTF2IRD1/GAPDH
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
COLO205
COLO320
DLD1
HCT116
LoVo
SW620


(B)
SW620
COLO205
***
***
GTF2IRD1/GAPDH
1.2
0.8
0.4
0.0
NC
siGTF2IRD1


SW620
COLO205
NC
siGTF2IRD1
NC
siGTF2IRD1
GTF2IRD1
β-Actin


(C)
NC
siGTF2IRD1
SW620
COLO205
Colony Number
300
200
100
0
**
NC
siGTF2IRD1
Colony Number
80
60
40
20
0
*
NC
siGTF2IRD1

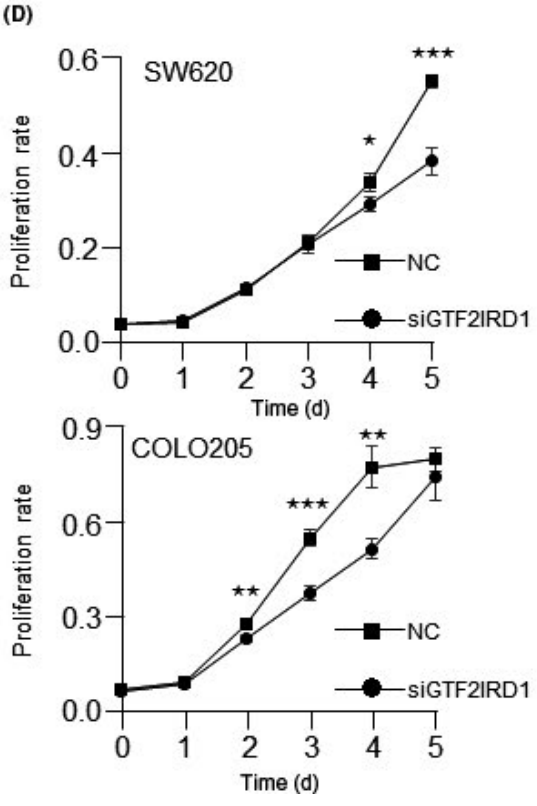

(D)
SW620
Proliferation rate
0.6
0.4
0.2
0.0
***
*
NC
siGTF2IRD1
0
1
2
3
4
5
Time (d)
COLO205
Proliferation rate
0.9
0.6
0.3
0.0
**
***
**
NC
siGTF2IRD1
0
1
2
3
4
5
Time (d)

FIGURE 2 Effects of *GTF2IRD1* knockdown on cell proliferation in colorectal cancer (CRC) cells. A, RT-quantitative PCR (RT-qPCR) analysis of *GTF2IRD1* mRNA expression in 6 CRC cell lines. B, Left, RT-qPCR for *GTF2IRD1* mRNA expression in *GTF2IRD1* siRNA-transfected SW620 and COLO205 cells and control siRNA-transfected cells. ***$P < .0005$. Right, Immunoblotting for total protein expression of GTF2IRD1 in *GTF2IRD1* siRNA-transfected SW620 and COLO205 cells and control siRNA-transfected cells. C, Left, Colony formation assays. SW620 and COLO205 cells were cultured after *GTF2IRD1* siRNA or control siRNA transfection for 14 d. Right, Total number of colonies. *$P < .05$, **$P < .005$. D, MTT proliferation assays. Proliferation rates of *GTF2IRD1* siRNA-transfected SW620 and COLO205 cells were compared with those of control siRNA-transfected cells (NC). *$P < .05$, **$P < .005$, ***$P < .0005$

GAAGGTAGAGCTTGGGCAGG-3′; and *GAPDH*, forward, 5′-TTGGTATCGTGGAAGGACTCTA-3′ and reverse, 5′-TGTCATATTTGGCAGGTT-3′.

2.4 | Protein extraction

For total protein extraction, cells were lysed in lysis buffer (25 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl, 0.2 mmol/L EDTA, 0.1% NP-40, 5% glycerol, and proteinase inhibitor cocktail).

2.5 | Immunohistochemical analysis

Immunohistochemistry for GTF2IRD1 in CRC cases with liver metastasis was carried out on formalin-fixed, paraffin-embedded tissues as previously described.[13] The primary Ab against GTF2IRD was used at a dilution of 1:100. Rabbit polyclonal Abs to GTF2IRD were purchased from Sigma-Aldrich. Tumor histology was independently reviewed by an experienced research pathologist (T.T.) at Kyushu University.

2.6 | Immunoblotting analysis

Immunoblotting analysis was carried out as previously described.[14] Briefly, equal amounts of protein (35 μg) were electrophoresed on 4%-20% Tris-glycine gels and then electroblotted onto Immobilon-P Transfer Membranes (Merck Millipore) at 70 V for 4 hours at room temperature. Nonspecific binding sites were blocked with blocking buffer (TBS and 0.1% Tween-20 with 5% nonfat milk powder) for 1 hour at room temperature, and the blots were incubated with specific primary Abs in blocking buffer (anti-GTF2IRD1, anti-TGFβR2, and anti-phospho-cyclin-dependent kinase [CDK] 2 [Tyr15] Abs at a 1:250 dilution; anti-phospho-Smad2, anti-Smad2, anti-p21, anti-phospho retinoblastoma (pRb), anti-bone morphogenetic protein receptor type 1B (BMPR1b), and anti-β-actin Abs at a 1:1000 dilution) at 4°C overnight. After washing, the blots were incubated with an appropriate secondary Ab conjugated with HRP for 1 hour at room temperature. After washing, the detection was undertaken using an ImageQuant LAS 4000 Mini system (GE Healthcare Japan). Rabbit polyclonal Abs targeting GTF2IRD1 were purchased from Atlas Antibodies. Rabbit polyclonal Abs targeting TGFβR2 and mouse mAbs to β-actin were purchased from Santa Cruz Biotechnology. Rabbit polyclonal Abs targeting phospho-Smad2, Smad2, p21, and pRb were purchased from Cell Signaling Technology. Rabbit polyclonal Abs targeting BMPR1b were purchased from GeneTex. Rabbit polyclonal Abs targeting phospho-CDK2 (Tyr15) were purchased from Abcam. Protein concentrations were quantified using Bradford protein assays.

2.7 | *GTF2IRD1* siRNA transfection

GTF2IRD1-specific siRNA (Silencer Predesigned siRNA: sense CAUCGUCCAUGACAAGUCATT and antisense UGACUUGUCAUGGACGAUGGA) and negative control siRNA (Silencer Negative Control 1 siRNA) were purchased from Ambion. Small interfering RNA oligonucleotides were transfected into SW620 or COLO205 cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

2.8 | Colony formation assay

Cells (1000 cells/well) were seeded in 6-well plates. After incubation for 24 hours followed by siRNA transfection, the cells were cultured for an additional 14 days, and the colonies were stained using a Differential Quik Stain Kit (Sysmex) according to the manufacturer's instructions. Visible colonies were photographed using a Chemiluminescence Imaging FUSION SOLO S (VILBER). Colony counts were determined using ImageJ software (NIH).

2.9 | Cell proliferation assay

Colorectal cancer cell proliferation was assessed using an MTT assay kit (Roche Applied Science) as previously described.[15] After incubation for 24 hours, followed by siRNA transfection, the cells were cultured for an additional 0-5 days, and the absorbance of the samples was measured.

2.10 | Cell cycle assay

Nocodazole (an inhibitor of tubulin assembly; 5 μg/mL) was added 48 hours after siRNA transfection, and the cells were incubated for an additional 16 hours. Cells were harvested, washed with PBS, and fixed in ice-cold 70% ethanol at −20°C overnight. Samples were then washed with PBS and stained with propidium iodide containing RNase A for 20 minutes at 37°C. Cell cycle distribution was measured by flow cytometry (Sony).

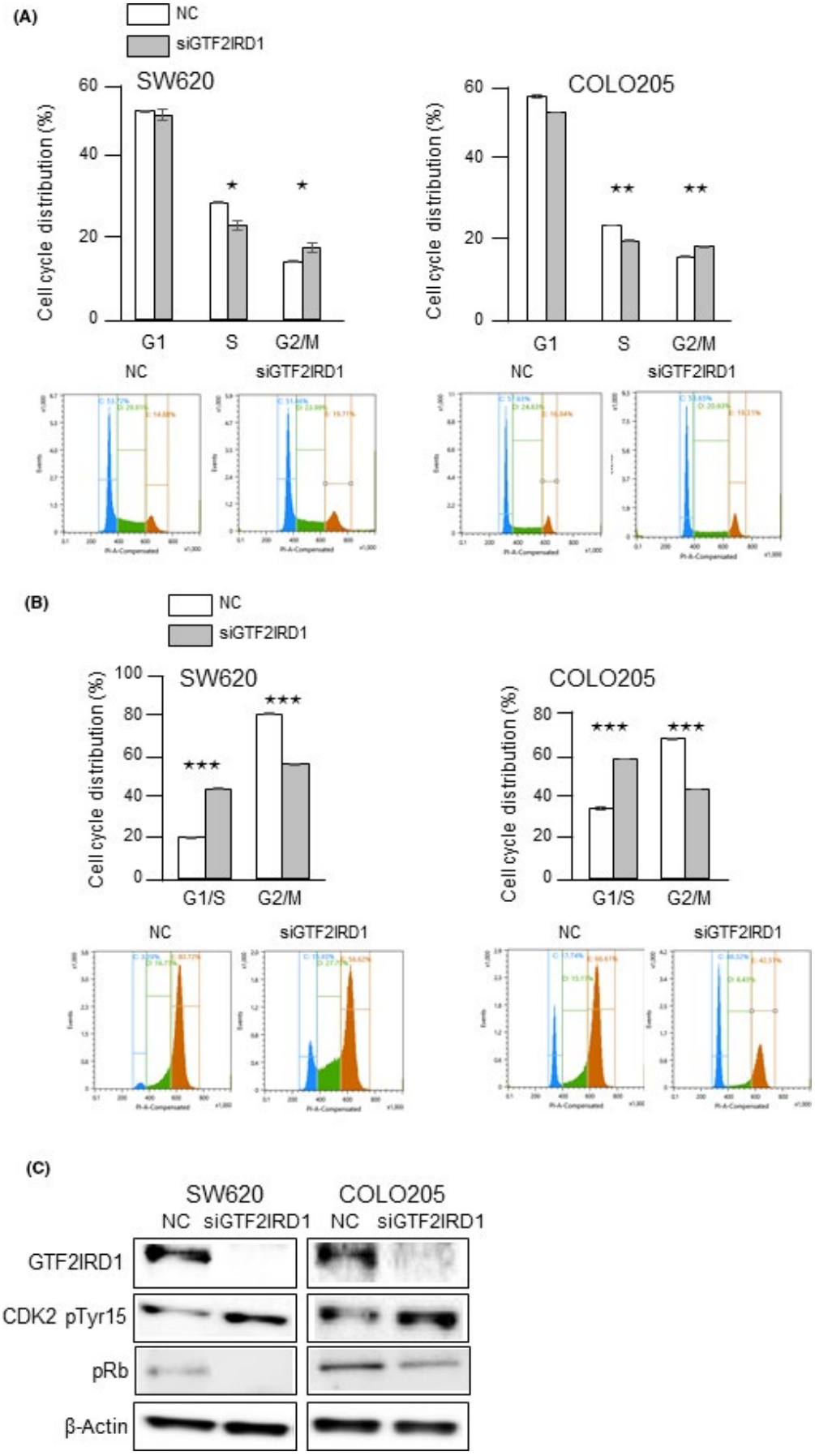

(A)
NC
siGTF2IRD1
SW620
COLO205
Cell cycle distribution (%)
60
40
20
0
G1
S
G2/M
NC
siGTF2IRD1
(B)
NC
siGTF2IRD1
SW620
COLO205
Cell cycle distribution (%)
100
80
60
40
20
0
G1/S
G2/M
NC
siGTF2IRD1
(C)
SW620
COLO205
NC
siGTF2IRD1
NC
siGTF2IRD1
GTF2IRD1
CDK2 pTyr15
pRb
β-Actin

FIGURE 3 Effects of *GTF2IRD1* knockdown on the cell cycle in colorectal cancer cells. A, Cell cycle distributions were analyzed by flow cytometry. *P < .05, **P < .005. B, Cell cycle distributions in the presence of nocodazole were analyzed by flow cytometry. ***P < .0005. C, Immunoblotting for phospho-cyclin-dependent kinase 2 (CDK2) (Tyr15) and pRb in *GTF2IRD1* siRNA-transfected SW620 and COLO205 cells and control siRNA-transfected cells (NC)

2.11 | In vitro invasion assay

Cell invasion capacities were assessed using the BD BioCoat Tumor Invasion System, 24 Multiwell (BD Bioscience) as previously described.[15]

2.12 | Patients with CRC and collection of clinical samples

Primary CRC samples and paired normal tissues were obtained from 98 patients who underwent surgery at Kyushu University Beppu Hospital and affiliated hospitals from 1992 to 2007. All patients had a histological diagnosis of CRC and were closely followed at 3-month intervals. The median follow-up period was 3.0 years. All patients were treated in accordance with the Japanese Society of Cancer of the Colon and Rectum Guidelines for the Treatment of Colorectal Cancer. Written informed consent was obtained from all patients, and the Institutional Review Board of our university approved this study. Sample collection was carried out as previously described.[14] Data on patient age, sex, histology, tumor depth of invasion, lymph node metastasis, lymphatic invasion, venous invasion, liver metastasis, and clinical stage were obtained from clinical and pathological records.

2.13 | Cancer Cell Line Encyclopedia data analysis

We obtained normalized mRNA expression data and DNA copy number data from 58 available CRC cell lines from the Cancer Cell Line Encyclopedia (CCLE) dataset (http://www.broadinstitute.org/ccle/home). Candidate gene mRNA expression and DNA copy number data were extracted from this reference.

2.14 | Gene set enrichment analysis

The correlations between *GTF2IRD1* expression and previously annotated gene expression signatures were analyzed by applying gene set enrichment analysis (GSEA).[16] We acquired CRC expression profiles from the NCBI's Gene Expression Omnibus database (accession code GSE7963) and analyzed the expression profiles using GSEA. Gene sets of *GTF2IRD1* targets were extracted from C2 curated gene sets in the Broad Institute database (http://www.broadinstitute.org/gsea/msigdb/collections.jsp).

2.15 | The Cancer Genome Atlas data analysis

Paired RNA sequencing and survival data of 620 available patients with CRC were obtained from TCGA (http://cancergenome.nih.gov/). *GTF2IRD1* mRNA expression, *Smad4* mutation status, and survival data were extracted from this reference.

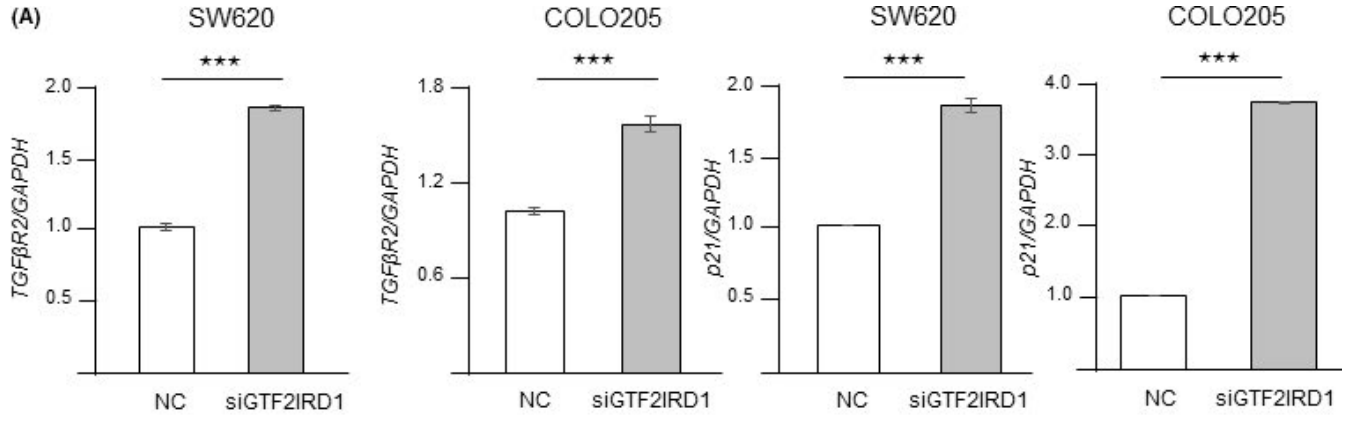
2.16 | Statistical analysis

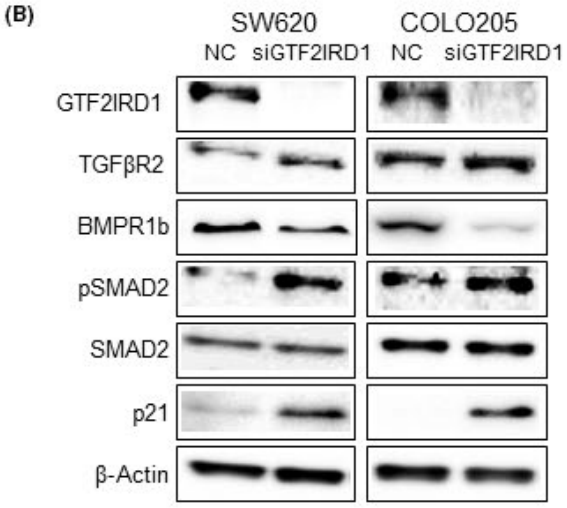
For continuous variables, data are expressed as mean ± SD, and statistical analyses were carried out using Student's t tests. The degree of linearity was estimated by Pearson's correlation coefficient. Categorical variables were compared using χ^2 tests or Fisher's exact tests. Overall survival was estimated using the Kaplan-Meier method, and survival curves were compared using log-rank tests. Based on the levels of *GTF2IRD1* mRNA expression in our dataset, cases were divided into 2 groups by the minimum P value approach, a comprehensive method to find the optimal risk separation cut-off point in continuous gene expression measurement.[17] Data analyses were undertaken using JMP 12 software (SAS Institute) and R software version 3.1.1 (The R Foundation for Statistical Computing).[18] Clinicopathological factors and clinical stages were classified using the TNM system of classification.

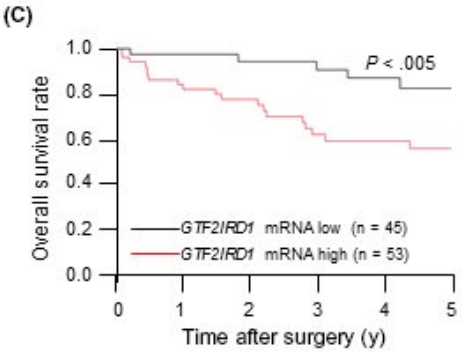
3 | RESULTS

3.1 | *GTF2IRD1* is a potential oncogene in CRC

We identified 8 genes that satisfied the criteria described above. Among the 8 genes, we focused on *GTF2IRD1* (Figure 1A) because this gene has been reported to promote mammary tumor growth.[19] *GTF2IRD1* mRNA expression in tumor tissues was 4.57-fold higher than that in normal tissues (P < .0005; Figure 1B). *GTF2IRD1* mRNA expression and copy numbers were positively correlated in TCGA dataset (R = .48, P < .001; Figure 1C). Consistent with this result, there was a significant positive correlation between *GTF2IRD1* mRNA expression and copy numbers in the CCLE dataset (R = .35, P < .05; Figure 1C). Next, we undertook immunohistochemical analysis to confirm GTF2IRD1 protein expression in CRC tumor cells from our hospital. GTF2IRD1 was stained more intensely in the nuclei and cytoplasm of CRC tumor cells than in those of normal colon cells (Figure 1D). Notably, GTF2IRD1 showed similar strong staining in tumor cells from liver metastatic lesions from the same patients (Figure 1D). Moreover, RT-qPCR analysis also showed that *GTF2IRD1* mRNA expression in tumor tissues was significantly higher than that in paired normal tissues (P < .0005; Figure 1B). Indeed, *GTF2IRD1* mRNA expression levels in tumor tissues were higher than those in normal tissues in 91.3% of 98 patients with CRC. In addition, GSEA revealed a positive correlation between *GTF2IRD1* mRNA expression and the expression of a gene set involved in cell cycle progression (P < .05; Figure 1E). These findings suggested that *GTF2IRD1* was a novel oncogene in CRC.


(A)
SW620
COLO205
SW620
COLO205
***
TGFβR2/GAPDH
p21/GAPDH
NC
siGTF2IRD1


(B)
SW620
COLO205
NC siGTF2IRD1 NC siGTF2IRD1
GTF2IRD1
TGFβR2
BMPR1b
pSMAD2
SMAD2
p21
β-Actin


(C)
P < .005
Overall survival rate
GTF2IRD1 mRNA low (n = 45)
GTF2IRD1 mRNA high (n = 53)
Time after surgery (y)

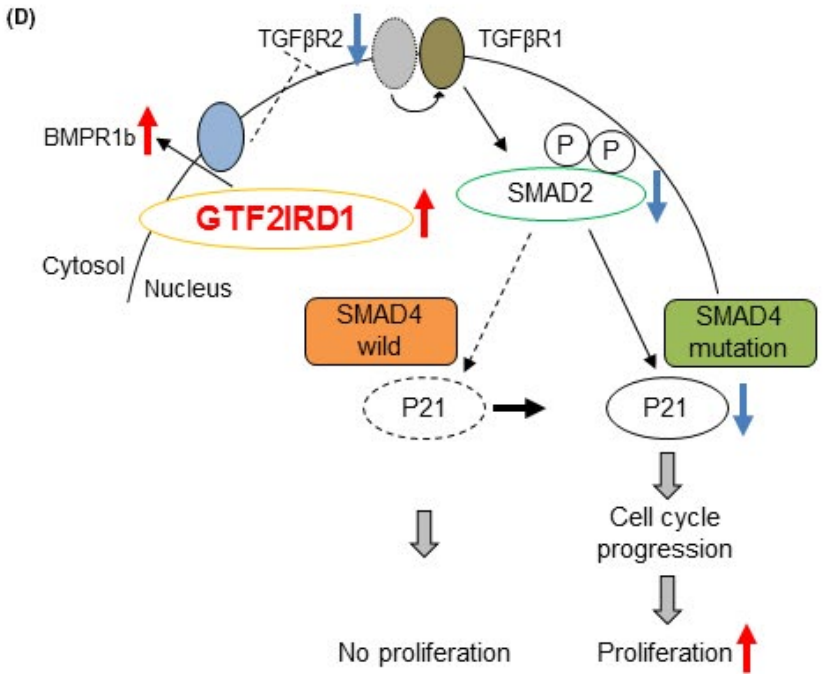

(D)
TGFβR2
TGFβR1
BMPR1b
P
P
SMAD2
GTF2IRD1
Cytosol
Nucleus
SMAD4 wild
SMAD4 mutation
P21
P21
Cell cycle progression
No proliferation
Proliferation

FIGURE 4 *GTF2IRD1* downregulated the *TGFβR2* gene in colorectal cancer (CRC) cells. A, RT-quantitative PCR for *TGFβR2* and *p21* mRNA expression in *GTF2IRD1* siRNA-transfected SW620 and COLO205 cells and control siRNA-transfected cells (NC). ***$P < .0005$. B, Immunoblotting for TGFβR2, BMPR1b, phospho-SMAD2, SMAD2, and p21 protein expression in *GTF2IRD1* siRNA-transfected SW620 and COLO205 cells and control siRNA-transfected cells. C, Overall survival rate in patients with CRC according to *GTF2IRD1* mRNA expression in tumor tissues in our dataset. D, Schema indicating that overexpression of GTF2IRD1 promotes cell cycle progression and tumor proliferation

TABLE 1 Univariate and multivariate analysis of clinicopathological factors affecting overall survival rate in colorectal cancer patients

	Univariate analysis			Multivariate analysis		
Factor	RR	95% CI	*P* value	RR	95% CI	*P* value
Age, >65/≤65 years	1.853	0.843-4.178	.124	–	–	–
Sex, male/female	2.027	0.856-5.568	.112	–	–	–
Histology grade: well, moderate/others	0.188	0.071-0.651	.012*	0.275	0.091-1.059	.059
T factor, ≤SS/≥SE	0.193	0.078-0.438	<.001*	0.512	0.181-1.389	.189
Lymph node metastasis, absent/present	0.189	0.069-0.449	<.001*	0.581	0.187-1.669	.314
Lymphatic invasion, absent/present	0.349	0.154-0.768	.009*	0.633	0.235-1.703	.362
Venous invasion, absent/present	0.225	0.101-0.534	.001*	0.392	0.144-1.072	.068
Liver metastasis, absent/present	0.091	0.041-0.219	<.001*	0.229	0.074-0.691	.009*
GTF2IRD1 mRNA expression, low/high	0.269	0.089-0.664	.003*	0.336	0.103-0.914	.032*

Abbreviations: CI, confidence interval; RR, relative risk; SE, serosa; SS, subserosa.
*Statistically significant.

3.2 | *GTF2IRD1* promotes proliferation of CRC cells

The results of GSEA motivated us to investigate whether *GTF2IRD1* regulated cell cycle progression and consequent tumor proliferation. Accordingly, RT-qPCR analysis was undertaken to quantify *GTF2IRD1* mRNA expression in several CRC cell lines. Endogenous *GTF2IRD1* mRNA expression was higher in SW620 and COLO205 cells (Figure 2A). Therefore, SW620 and COLO205 cells were selected for subsequent experiments. To examine the biological roles of *GTF2IRD1* in CRC, we carried out knockdown experiments using siRNA. si*GTF2IRD1* induced significant downregulation of *GTF2IRD1* mRNA expression in SW620 and COLO205 cells (Figure 2B). Immunoblotting analysis confirmed a substantial decrease in GTF2IRD1 protein in si*GTF2IRD1*-transfected SW620 and COLO205 cells (Figure 2B).

The effects of *GTF2IRD1* on proliferation in CRC were examined by colony formation assays and MTT assays. GTF2IRD1 knockdown significantly reduced colony formation (Figure 2C). The MTT assays showed that GTF2IRD1 knockdown significantly inhibited cancer cell proliferation (Figure 2D). In contrast, GTF2IRD1 knockdown did not affect invasion potential (Figure S1).

3.3 | *GTF2IRD1* promoted cell cycle progression in CRC cells

Next, we undertook cell cycle analysis. GTF2IRD1 knockdown significantly decreased the S phase fraction (Figure 3A). Furthermore, there was a significant increase in the G_1/S phase fraction in GTF2IRD1 knockdown cells in the presence of nocodazole, which prevents reentry of cells into G_1 phase (Figure 3B). Immunoblotting analysis showed a substantial increase in phosphorylation of CDK2 at Tyr15 and decrease in phosphorylation of Rb, indicating G_1/S arrest, in GTF2IRD1-knockdown cells (Figure 3C). Taken together, these data indicated that *GTF2IRD1* promoted cell proliferation by promoting cell cycle progression in CRC.

3.4 | *GTF2IRD1* downregulates TGFβR2

Expression of *TGFβR2*, a well-known tumor suppressor gene in CRC, is downregulated by *GTF2IRD1* in mouse embryonic fibroblasts.[20,21] We initially evaluated the expression of this gene using si*GTF2IRD1*-transfected SW620 and COLO205 cells. *TGFβR2* mRNA and protein expression levels were significantly higher in si*GTF2IRD1*-transfected cells than in mock-transfected cells (Figure 4A,B). To further test whether *TGFβR2* was a downstream target of *GTF2IRD1*, we measured phospho-Smad2 levels in GTF2IRD1-knockdown cells. Immunoblotting analysis showed that phospho-Smad2 levels were significantly higher in si*GTF2IRD1*-transfected cells. Furthermore, there was a strong increase in *p21* mRNA and protein expression (Figure 4A,B), which is upregulated by activation of TGFβ. These data indicated that GTF2IRD1 downregulated membrane TGFβR2 levels, resulting in reduced TGFβ activity.

Bone morphogenetic protein (BMP) is a member of the TGFβ superfamily, and *BMPR1b* is a BMP signal receptor. It is well known that TGFβ and BMP signals play an opposing role in kidney disease[22] and bone formation.[23] These motivated us to assess the expression of BMPR1b by GTF2IRD1-knockdown experiments. Levels of BMPR1b expression were significantly lower in si*GTF2IRD1*-transfected cells than in mock-transfected cells (Figure 4B). This result suggested that GTF2IRD1 upregulated membrane BMPR1b levels.

3.5 | *GTF2IRD1* promotes proliferation of *Smad4*-mutated CRC cells

It is well known that the Smad signaling pathway is composed of the TGFβR2, TGFβR1, and Smad proteins. The cytoplasmic Smad2 and Smad3 proteins are phosphorylated by TGFβ signal through TGFβR2 and TGFβR1, which allows them to form a heteromeric complex with Smad4. This Smad complex is translocated into the nucleus and activates gene transcription, including *p21*. Our experimental data showed that GTF2IRD1 downregulated TGFβR2 expression, resulting in the downregulation of phospho-Smad2 and p21 expression. However, SW620 and COLO205 harbor loss of function mutation of *Smad4*,[24] indicating that the TGFβ signal pathway is inactivated in these cells. Thus, we undertook the cell proliferation assay and immunoblotting analysis using *Smad4*-wild CRC cells, COLO320, whose endogenous *GTF2IRD1* mRNA expression was near to SW620 and COLO205 cells (Figure 2A). Unexpectedly, GTF2IRD1 knockdown did not upregulate colony formation (Figure S2A). The MTT assays showed that GTF2IRD1 knockdown did not promote cancer cell proliferation (Figure S2B). The TGFβR2 and phospho-Smad2 protein expression levels in *Smad4*-wild cells was higher as well as *Smad4*-mutated cells, but p21 protein expression levels were lower in si*GTF2IRD1*-transfected WT cells than in mock-transfected WT cells (Figure S2c), indicating that GTF2IRD1 downregulated TGFβR2 expression, resulting in reduced p21 expression in *Smad4*-mutated CRC, but not in *Smad4*-wild CRC.

3.6 | High expression of *GTF2IRD1* mRNA predicts poor prognosis in CRC patients

Because our experimental data suggested that *GTF2IRD1* was associated with tumor aggressiveness in CRC, we assessed the prognostic and clinical significance of *GTF2IRD1* mRNA expression in CRC. First, we evaluated the survival rates according to *GTF2IRD1* mRNA expression in patients with CRC. The overall survival rate in patients in the high *GTF2IRD1* mRNA expression group was significantly lower than that in patients in the low expression group in our dataset ($P < .0005$; Figure 4C). In the univariate analysis, poor histology, higher T factor (≥SE), lymph node metastasis, lymphovascular invasion, liver metastasis, and high *GTF2IRD1* mRNA expression were significantly associated with a lower overall survival rate (Table 1). The multivariate analysis indicated that liver metastasis ($P < .05$) and high *GTF2IRD1* mRNA expression ($P < .05$) were independent poor prognostic factors in CRC (Table 1). Also, the survival rates according to *Smad4* mutation status in patients with CRC were examined using TCGA dataset because our experimental data suggested that *GTF2IRD1* showed malignant potential in *Smad4*-mutated CRC. As expected, the overall survival rate in patients in the high *GTF2IRD1* mRNA expression group was significantly lower than that in patients in the low expression group in *Smad4*-mutated CRC cases (Figure S3A). In *Smad4*-wild CRC cases, there was no significant difference in the overall survival rate between high and low *GTF2IRD1* mRNA expression group (Figure S3B). These results imply that GTF2IRD1 acts as an oncogene in the *Smad4*-mutated CRC case, but not in the *Smad4*-wild CRC case.

TABLE 2 Correlation between *GTF2IRD1* mRNA expression of tumor tissues and clinicopathological factors in colorectal cancer

Factors	Low (n = 45) Number (%)	High (n = 53) Number (%)	*P* value
Age (years)			
<65	16 (35.6)	24 (45.3)	.329
≥65	29 (64.4)	29 (54.7)	
Sex			
Male	32 (71.1)	31 (58.5)	.194
Female	13 (28.9)	22 (41.5)	
Histology			
Well/moderate	42 (93.3)	49 (92.4)	.866
Others	3 (6.7)	4 (7.6)	
Depth of invasion			
≤SS	28 (62.2)	32 (60.4)	.852
≥SE	17 (37.8)	21 (39.6)	
Lymph node metastasis			
Absent	29 (64.4)	27 (50.9)	.178
Present	16 (35.6)	26 (49.1)	
Lymphatic invasion			
Absent	30 (66.7)	31 (58.5)	.405
Present	15 (33.3)	22 (41.5)	
Venous invasion			
Absent	38 (84.4)	43 (81.1)	.666
Present	7 (15.6)	10 (18.9)	
Liver metastasis			
Absent	44 (97.8)	44 (83.0)	.016*
Present	1 (2.2)	9 (17.0)	
UICC TNM stage			
I, II	27 (60.0)	25 (47.2)	.205
III, IV	18 (40.0)	28 (52.8)	

Abbreviations: SE, serosa; SS, subserosa.
*Statistically significant.

3.7 | Clinicopathological significance of *GTF2IRD1* mRNA expression in CRC

Next, we examined the association between *GTF2IRD1* mRNA expression and clinicopathological factors in patients with CRC from our hospital (Table 2). The high *GTF2IRD1* mRNA expression group (n = 53) had a higher frequency of liver metastasis ($P < .05$) compared with the low expression group (n = 45), suggesting that CRC cells with high expression of GTF2IRD1 should grow faster than with the low expression at metastatic regions, including liver, resulting in early clinical detection of metastasis of GTF2IRD1-high expressed CRC cells because GTF2IRD1 can facilitate cell proliferation of CRC. There were no significant associations between *GTF2IRD1* mRNA expression and age, sex, poor histology, depth of invasion, lymph node metastasis, lymphatic invasion, venous invasion, or clinical stage.

4 | DISCUSSION

In this study, we identified *GTF2IRD1* as a driver gene on Ch.7q of CRC using our bioinformatics approach and determined the biological and clinical significance of *GTF2IRD1* expression in CRC. To the best of our knowledge, this is the first study to provide evidence that *GTF2IRD1* might act as a novel oncogene in CRC.

GTF2IRD1 is a member of the *GTF2I* gene family, which encodes a set of multifunctional transcription factors. *GTF2IRD1* is located in the Williams-Beuren syndrome critical region 7q11.23.[25,26] This syndrome is a genetic disorder associated with multiple systemic abnormalities, including craniofacial dysmorphology and several symptoms, such as hypertension and anxiety.[27] Additional studies have reported that there is a positive correlation between the expression of *GTF2IRD1* and that of cell cycle progression-related genes and genes involved in the noncanonical Wnt-calcium pathway, which is known to modulate migration.[28,29] Consistent with these data, our GSEA revealed that *GTF2IRD1* expression in CRC was positively correlated with cell cycle-related gene sets, and in vitro analysis showed that *GTF2IRD1* promoted cell cycle progression and consequently accelerated cell proliferation.

Our clinical study showed that *GTF2IRD1* was overexpressed due to copy number amplification at Ch.7q in CRC. The expression of *GTF2IRD1* was positively associated with the malignant pathological phenotype. Furthermore, high expression of *GTF2IRD1* was an independent poor prognostic factor in CRC. Huo et al revealed that high *GTF2IRD1* expression correlated with poor overall survival in patients with breast cancer, lung cancer, and ovarian cancer.[19] These clinical findings strongly supported that *GTF2IRD1* is a tumor-promoting oncogene in human cancers, including CRC. Moreover, high *GTF2IRD1* expression could be a novel biomarker of poor prognosis in patients with CRC.

Next, we assessed the mechanisms through which *GTF2IRD1* promoted CRC progression. We found that GTF2IRD1 downregulated TGFβR2 expression, resulting in reduced TGFβ activity in *Smad4*-mutated CRC (Figure 4D). Our schema was strongly supported by the fact that suppression of TGFβ activity accelerated cell cycle progression and consequent cell proliferation.[30,31] Mutational inactivation of *TGFβR2* occurs in approximately 30% of CRCs and promotes the formation of CRC by inhibiting the tumor-suppressor activity of the TGFβ signaling pathway.[21,32] In a recent paper, *GTF2IRD1* was reported to repress obesity-associated adipose tissue fibrosis and improve systemic glucose homeostasis by suppressing the TGFβ signaling pathway.[33]

Loss of chromosome 18q21 including *Smad4* has been detected in up to 60% of CRC, and *Smad4* was reported to harbor loss of function mutation frequently in CRC,[34,35] suggesting that *Smad4* is dysfunctional in the majority of CRC. Ijichi et al reported that p21 was upregulated through Smad2/3-dependent transcriptional activation through a *Smad4*-independent manner in Smad4-null pancreatic cancer cells.[36] This report suggested a novel mechanism of p21 regulation that Smad4 is not involved. In our study, p21 expression was downregulated in *Smad4*-mutated CRC cells, but not in *Smad4*-wild CRC cells, suggesting that *GTF2IRD1* acts as an oncogene possibly in a *Smad4*-independent manner in *Smad4*-mutated CRC cases, but not in *Smad4*-wild CRC cases. Survival analysis according to *Smad4* mutation status supported our hypothesis described above. Our results and the reports suggested that downregulation of *TGFβR2* could be an important pathway in *Smad4*-mutated CRC formation and progression, but not in *Smad4*-wild CRC, resulting in the difference of malignant phenotype between *Smad4*-mutated and *Smad4*-wild CRC. Further studies will be required to confirm this.

Recently, some reports have focused on the cross-talk between TGFβ signal and the BMP signaling pathway in the progression of cancers. BMP7 attenuates TGFβ-mediated tumor suppression in proliferation or invasion in prostate cancer or breast cancer respectively.[37,38] In our study, GTF2IRD1 downregulated TGFβR2, but upregulated BMPR1b expression in CRC cells, suggesting that the upregulation of BMPR1b by GTF2IRD1 might indirectly decrease TGFβR2 expression, resulting in suppression of TGFβ signal (Figure 4D).

Some limitations exist in this study. First, only COLO320 was available as *Smad4*-wild CRC cells for in vitro analysis. Second, we could not identify the novel signal mediator regulating *p21* instead of *Smad4*. Further in vitro and in vivo experiments are required to clarify these mechanisms.

In summary, our study identified *GTF2IRD1* as a novel oncogene on Ch.7q and showed that GTF2IRD1 promoted cell cycle progression by downregulation of *TGFβR2* in CRC. Furthermore, because overexpression of *GTF2IRD1* due to copy number amplification of Ch.7q is considered to be a universal driver event present in all CRC cells, *GTF2IRD1* could be a therapeutic target to overcome ITH in patients with CRC.

ACKNOWLEDGMENTS

This study used the super-computing resource provided by the Human Genome Center, Institute of Medical Science, University of Tokyo (http://sc.hgc.jp/shirokane.html). We thank M. Oshiumi, M. Utou, K. Oda, M. Kasagi, S. Sakuma, N. Mishima, and T. Kawano for their excellent technical assistance. This work was supported in part by the

following grants and foundations: Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Science Research (grant nos. JP16K07177, JP16K10543, JP16K19197, JP17K16454, JP17K16521, JP17K10593, and JP17K19608); OITA Cancer Research Foundation; Daiwa Securities Health Foundation; Grant-in-Aid for Scientific Research on Innovative Areas (grant no. 15H0912); Priority Issue on Post-K computer (grant nos. hp170227, hp170227, and hp160219); JSPS KAKENHI (grant no. 15H05707); Eli Lilly Japan KK Grant; and the Japanese Foundation for Multidisciplinary Treatment of Cancer.

DISCLOSURE

No potential conflicts of interest were disclosed.

ORCID

Sho Nambara https://orcid.org/0000-0001-7643-3636
Takaaki Masuda https://orcid.org/0000-0002-6341-2438
Kuniaki Sato https://orcid.org/0000-0001-6014-1911
Keishi Sugimachi https://orcid.org/0000-0001-8768-5388
Koshi Mimori https://orcid.org/0000-0003-3897-9974

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011;61:69-90.
2. Takahashi Y, Sugimachi K, Yamamoto K, et al. Japanese genome-wide association study identifies a significant colorectal cancer susceptibility locus at chromosome 10p14. *Cancer Sci*. 2017;108:2239-2247.
3. DeSantis CE, Lin CC, Mariotto AB, et al. Cancer treatment and survivorship statistics, 2014. *CA Cancer J Clin*. 2014;64:252-271.
4. McGranahan N, Swanton C. Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. *Cancer Cell*. 2015;27:15-26.
5. Douglas EJ, Fiegler H, Rowan A, et al. Array comparative genomic hybridization analysis of colorectal cancer cell lines and primary carcinomas. *Cancer Res*. 2004;64:4817-4825.
6. Herbergs J, Arends JW, Bongers EM, Ramaekers FC, Hopman AH. Clonal origin of trisomy for chromosome 7 in the epithelial compartment of colon neoplasia. *Genes Chromosomes Cancer*. 1996;16:106-112.
7. Zarzour P, Boelen L, Luciani F, et al. Single nucleotide polymorphism array profiling identifies distinct chromosomal aberration patterns across colorectal adenomas and carcinomas. *Genes Chromosomes Cancer*. 2015;54:303-314.
8. Uchi R, Takahashi Y, Niida A, et al. Integrated multiregional analysis proposing a new model of colorectal cancer evolution. *PLoS Genet*. 2016;12:e1005778.
9. Saito T, Niida A, Uchi R, et al. A temporal shift of the evolutionary principle shaping intratumor heterogeneity in colorectal cancer. *Nat Commun*. 2018;9:2884.
10. Sato K, Masuda T, Hu Q, et al. Phosphoserine phosphatase is a novel prognostic biomarker on chromosome 7 in colorectal cancer. *Anticancer Res*. 2017;37:2365-2371.
11. Sato K, Masuda T, Hu Q, et al. Novel oncogene 5MP1 reprograms c-Myc translation initiation to drive malignant phenotypes in colorectal cancer. *EBioMedicine*. 2019;44:387-402.
12. Masuda TA, Inoue H, Nishida K, et al. Cyclin-dependent kinase 1 gene expression is associated with poor prognosis in gastric carcinoma. *Clinical Cancer Res*. 2003;9:5693-5698.
13. Nambara S, Iguchi T, Oki E, Tan P, Maehara Y, Mimori K. Overexpression of CXCR7 is a novel prognostic indicator in gastric cancer. *Dig Surg*. 2017;34:312-318.
14. Hirata H, Sugimachi K, Komatsu H, et al. Decreased expression of fructose-1,6-bisphosphatase associates with glucose metabolism and tumor progression in hepatocellular carcinoma. *Cancer Res*. 2016;76:3265-3276.
15. Kurashige J, Hasegawa T, Niida A, et al. Integrated molecular profiling of human gastric cancer identifies DDR2 as a potential regulator of peritoneal dissemination. *Sci Rep*. 2016;6:22371.
16. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. 2005;102:15545-15550.
17. Mizuno H, Kitada K, Nakai K, Sarai A. PrognoScan: a new database for meta-analysis of the prognostic value of genes. *BMC Med Genomics*. 2009;2:18.
18. Tsutsumi S, Saeki H, Nakashima Y, et al. Programmed death-ligand 1 expression at tumor invasive front is associated with epithelial-mesenchymal transition and poor prognosis in esophageal squamous cell carcinoma. *Cancer Sci*. 2017;108:1119-1127.
19. Huo Y, Su T, Cai Q, Macara IG. An in vivo gain-of-function screen identifies the Williams-Beuren syndrome gene GTF2IRD1 as a mammary tumor promoter. *Cell Rep*. 2016;15:2089-2096.
20. Chimge NO, Mungunsukh O, Ruddle F, Bayarsaihan D. Expression profiling of BEN regulated genes in mouse embryonic fibroblasts. *J Exp Zool B Mol Dev Evol*. 2007;308:209-224.
21. Biswas S, Chytil A, Washington K, et al. Transforming growth factor beta receptor type II inactivation promotes the establishment and progression of colon cancer. *Cancer Res*. 2004;64:4687-4692.
22. Meng XM, Chung AC, Lan HY, et al. Role of the TGF-beta/BMP-7/Smad pathways in renal diseases. *Clin Sci*. 2013;124:243-254.
23. Wu M, Chen G, Li Y, et al. TGF-β and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. *Bone Res*. 2016;4:16009.
24. Woodford-Richens KL, Rowan AJ, Gorman P, et al. SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway. *Proc Natl Acad Sci USA*. 2001;14:9719-9723.
25. Franke Y, Peoples RJ, Francke U. Identification of GTF2IRD1, a putative transcription factor within the Williams-Beuren syndrome deletion at 7q11.23. *Cytogenet Cell Genet*. 1999;86:296-304.
26. Tassabehji M, Hammond P, Karmiloff-Smith A, et al. GTF2IRD1 in craniofacial development of humans and mice. *Science*. 2005;310:1184-1187.
27. Game X, Panicker J, Fowler CJ. Williams-Beuren syndrome. *N Engl J Med*. 2010;362:1449.
28. Bikle DD, Xie Z, Tu CL. Calcium regulation of keratinocyte differentiation. *Expert Rev Endocrinol Metab*. 2012;7:461-472.
29. Corley SM, Canales CP, Carmona-Mora P, et al. RNA-Seq analysis of Gtf2ird1 knockout epidermal tissue provides potential insights into molecular mechanisms underpinning Williams-Beuren syndrome. *BMC Genom*. 2016;17:450.
30. Massague J. TGFbeta in cancer. *Cell*. 2008;134:215-230.
31. Colak S, Ten Dijke P. Targeting TGF-beta signaling in cancer. *Trends Cancer*. 2017;3:56-71.
32. Biswas S, Trobridge P, Romero-Gallo J, et al. Mutational inactivation of TGFBR2 in microsatellite unstable colon cancer arises from the cooperation of genomic instability and the clonal outgrowth of transforming growth factor beta resistant cells. *Genes Chromosomes Cancer*. 2008;47:95-106.
33. Hasegawa Y, Ikeda K, Chen Y, et al. Repression of adipose tissue fibrosis through a PRDM16-GTF2IRD1 complex improves systemic glucose homeostasis. *Cell Metab*. 2018;27:180-194.e6.
34. Thiagalingam S, Lengauer C, Leach FS, et al. Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nat Genet*. 1996;13:343-346.

35. The Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012;487:330-337.
36. Ijichi H, Otsuka M, Tateishi K, et al. Smad4-independent regulation of p21/WAF1 by transforming growth factor-beta. *Oncogene*. 2004;23:1043-1051.
37. Yang S, Zhong C, Frenkel B, et al. Diverse biological effect and Smad signaling of bone morphogenetic protein 7 in prostate tumor cells. *Cancer Res*. 2005;65:5769-5777.
38. Naber HP, Wiercinska E, Pardali E, et al. BMP-7 inhibits TGF-beta-induced invasion of breast cancer cells through inhibition of integrin beta(3) expression. *Cell Oncol*. 2012;35:19-28.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Nambara S, Masuda T, Kobayashi Y, et al. *GTF2IRD1* on chromosome 7 is a novel oncogene regulating the tumor-suppressor gene *TGFβR2* in colorectal cancer. *Cancer Sci*. 2020;111:343–355. https://doi.org/10.1111/cas.14248