Oncogenic mutation in RAS-RAF axis leads to increased expression of GREB1, resulting in tumor proliferation in colorectal cancer

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Abstract

\( \text{BRAF}^{V600E} \) mutation accounts for up to 90% of all \( \text{BRAF} \) mutations in human colorectal cancer (CRC), and constitutively activates the MEK-MAPK pathway. It is recognized that neutralizing mAbs for epidermal growth factor receptor alone are not effective for CRC with \( \text{BRAF}^{V600E} \) mutation. Therefore, there is increasing interest in identification of the possible therapeutic targets in downstream of \( \text{BRAF} \) mutation in CRCs. To address this, we studied genome engineered mouse models for colonic neoplasia that has \( \text{Braf}^{V600E} \) mutation on the basis of \( \text{Apc} \) inactivation, induced in 2 distinct Cre mouse models, \( \text{CDX2P-G22Cre} \) and \( \text{CDX2P-CreERT2} \) mice. We carried out oligonucleotide microarray analysis for colonic neoplasia generated in these mouse models, and compared gene expression profiles among \( \text{Kras}/\text{Braf} \) WT, \( \text{Kras} \)-mutated, and \( \text{Braf} \)-mutated mouse colon tumors to seek new molecular targets corresponding to the KRAS-BRAF-MAPK axis. We found that the expression of the growth regulation by estrogen in breast cancer protein 1 (Greb1) was the most upregulated gene in \( \text{Braf} \)-mutated mouse tumors compared to \( \text{Kras}/\text{Braf} \) WT counterparts. The silencing

Abbreviations: AF, activation factor; Apc, adenomatous polyposis coli; CIMP, CpG island methylator phenotype; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; ER, estrogen receptor; GEMM, genome engineered mouse model; GREB1, growth regulation by estrogen in breast cancer protein 1; HGSC, high-grade serous ovarian cancer; IHC, immunohistochemical; MSI, microsatellite instability; MSS, microsatellite stable; qRT-PCR, quantitative RT-PCR; RIN, RNA integrity number; TAM, tamoxifen.

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of GREB1 significantly reduced the proliferation and tumorigenesis of CRC cell lines, whereas the overexpression of GREB1 promoted cell proliferation. Although GREB1 was first identified as a hormone-responsive gene mediating estrogen-stimulated cell proliferation in endometriosis, breast, and ovarian cancers, these results suggest that RAS-RAF-MAPK signaling upregulates GREB1 expression in CRC, resulting in cellular proliferation. Thus, GREB1 is a possible therapeutic target for CRCs with BrafV600E mutation. Subsequently, we carried out gene expression profiling, and integrated gene expression profiles of Kras/Braf WT, Kras-mutated, and Braf-mutated mouse colon tumors to seek new molecular targets corresponding to the KRAS-BRAF-MAPK axis.

**KEYWORDS**
BRAF mutation, colorectal cancer, ER, GREB1, mouse model

1 | INTRODUCTION

Human CRCs evolve by a series of genetic aberrations accumulated over time.\(^1\)\(^2\) Although the genetic abnormality varies among individual tumors, dysregulation of Wnt/β-catenin and EGFR signaling pathway plays a major role in CRC,\(^1\) thus, are thought to be therapeutic targets. Cetuximab and panitumumab are EGFR-mAbs, clinically approved for the treatment of advanced CRCs.\(^3\) However, 40%-50% of CRCs have KRAS/NRAS mutation, resulting in resistance to these EGFR-mAbs due to constitutive activation of a RAS-RAF-MAPK signaling pathway.\(^3\)\(^5\)

BRAF mutation is a driver gene mutation that constitutively activates the MEK-MAPK signal pathway at the downstream of RAS. Colorectal cancers with BRAF mutation are thus resistant to EGFR-mAbs.\(^6\) As BrafV600E mutation accounts for approximately 90% of all BRAF mutations in CRC,\(^7\) vemurafenib, a specific serine/threonine kinase inhibitor for V600E-mutated BRAF, has been clinically tested.\(^8\)\(^11\) However, the survival benefit is still unsatisfactory.\(^12\)

CpG island methylator phenotype-high frequently presents with BRAF mutation and causes hypermethylation of MLH1 promoter, resulting in MSI-high.\(^13\) In contrast, MSS and CIMP-low CRCs are less frequent, resulting in worse prognosis than the aforementioned CIMP-high/MSI-high/BRAF-mutated CRCs.\(^14\) Those types of CRCs are distinct, and therefore, BRAF mutation in CRCs should be studied in the context of CIMP or microsatellite status.

We developed 2 different GEMMs for colon cancer, the CDX2P-G22-Cre;Apc\(^{\text{fllox/fox}}\) and CDX2P-Cre-ER\(^T2\);Apc\(^{\text{fllox/fox}}\) models. In these 2 GEMMs, colonic tumors occur with inactivation of both LoxP-flanked Apc alleles induced by CDX2P-driven Cre recombinase. CDX2P-G22-Cre;Apc\(^{\text{fllox/fox}}\) mice express Cre when frame-shift mutation occurs in G22 tracts followed by Cre locus, which appear to recapitulate MSI-high tumors.\(^15\) In contrast, CDX2P-Cre-ER\(^T2\);Apc\(^{\text{fllox/fox}}\) is less similar to an MSI-high model.\(^16\) Therefore, these 2 mouse models are useful to test the effect of Braf mutation in the context of MSI-high and MSS.

As previously described, we have already studied GEMMs that generate proximal colon tumors with or without Kras\(^{G12D}\) mutation induced in the CDX2P-G22-Cre model, and compared the gene expression profiles to seek the possible molecules that are regulated downstream of Kras mutation.\(^17\)\(^18\) Mimicking this strategy, we first established GEMMs that generate colonic tumors with or without BrafV600E mutation. Subsequently, we carried out gene expression profiling, and integrated gene expression profiles of Kras/Braf WT, Kras-mutated, and Braf-mutated mouse colon tumors to seek new molecular targets corresponding to the KRAS-BRAF-MAPK axis.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study was undertaken in strict accordance with the Guide for the Care and Use of Laboratory Animals and the committee of the University of Hiroshima. This study was approved by the Ethical Committee for Epidemiology of Hiroshima University ( Permit Number: Epidemiology-74).

2.2 | Experimental animals

All mice were housed under specific pathogen-free conditions, as described previously.\(^17\) We utilized 2 different kinds of Cre mice, CDX2P-G22Cre and CDX2P-CreER\(^T2\), as described previously and above.\(^15\)\(^18\) These Cre mice were crossed with Apc\(^{\text{fllox/fox}}\) mice and Braf\(^{\text{LSL-V600E/-}}\) mice (C57BL/6J).\(^19\) to obtain CDX2P-G22Cre;Apc\(^{\text{fllox/fox}}\);Braf\(^{\text{LSL-V600E/-}}\), CDX2P-G22Cre;Apc\(^{\text{fllox/fox}}\);CDX2P-CreER\(^T2\);Apc\(^{\text{fllox/fox}}\);Braf\(^{\text{LSL-V600E/-}}\), and CDX2P-CreER\(^T2\);Apc\(^{\text{fllox/fox}}\) mice. Experiments were carried out using these mice and previously described CDX2P-G22Cre;Apc\(^{\text{fllox/fox}}\);Kras\(^{\text{LSL-G12D/-}}\) mice.\(^18\)

Tamoxifen (Sigma-Aldrich) was dissolved in 10 mg/mL corn oil, and single doses of 15 mg/kg TAM were i.p. injected into mice with the CDX2P-CreER\(^T2\) transgene at the age of 6 weeks. Animals were killed and analyzed at various time points after the single injection.

2.3 | Tissue harvesting and fixation

Small intestine and colon were harvested and then washed with PBS containing 0.01% Triton-X100 at 4°C for 30-60 minutes on a shaker.
For histological analyses, specimens were fixed in 4% paraformaldehyde and then embedded in paraffin. For RNA extraction from tissue with laser capture microdissection, specimens without any fixation were embedded and frozen in optimal cutting temperature compound (Sakura Finetek Japan), and stored at −80°C.

### 2.4 Laser capture microdissection and gene expression profiling

First, 12 μm frozen sections were prepared, dehydrated, and stained with hematoxylin. Subsequently, cancer tissues were dissected with the LM6500 laser capture microdissection device (Leica Microsystems), from which RNA was immediately extracted with an RNeasy Micro Kit (Qiagen).

The quality of extracted total RNA was evaluated by RIN, which is calculated with Bioanalyzer (Agilent 2100). RNA sample scoring RIN > 6.0 were used for the following microarray analysis.

Gene expression profiling was compared among CDX2P-G22Cre;ApfGfp (L5C-V600E/+ (n = 3), CDX2P-G22Cre;ApfGfp;KrasSLG12D/+ (n = 3), and CDX2P-G22Cre;ApfGfp (n = 3) mouse tumors with a Mouse Gene 1.0 ST Array (Affymetrix). The arrays were scanned using a GeneArray scanner (Affymetrix), and gene expression data were analyzed using GeneSpring GX software version 11 (Agilent Technology). The robust multichip analysis algorithm was used for normalization to remove artifactual differences between arrays, and cut-off values were set at less than 20% to eliminate poorly reproducible entities between chips.

### 2.5 Quantitative RT-PCR

cDNA was generated using a QuantiTect Reverse Transcription Kit (Qiagen) and was analyzed with a Rotor-Gene Q 2PLEX HRM Real-Time PCR system (Qiagen). The PCR primers used for gene analysis are shown in Table S1.

### 2.6 Immunohistochemistry and immunofluorescence

For human tissues, formalin-fixed paraffin-embedded samples were sectioned to 5-μm-thick sections and stained for GREB1 using a Catalyzed Signal Amplification System (Dako Japan), which is based on a streptavidin-biotin-HRP complex formation. After deparaffinization and rehydration, the sections were treated with target retrieval solution (Dako Japan) (pH 9.0) at 96°C for 40 minutes. A rabbit anti-human GREB1 polyclonal Ab (sc-138794; Santa Cruz Biotechnologies) was used at a dilution of 1:100, followed by incubation with peroxidase-labeled anti-rabbit IgG for 60 minutes. Immunocytofluorescence staining for FLAG was done with a primary Ab anti-Flag M2 (clone M2, 1:50; Sigma-Aldrich) and the secondary Ab Alexa 546 Donkey Anti-mouse IgG (diluted 1:500; Thermo Fisher Scientific). Cells were then stained with DAPI (diluted 1:10 000; Thermo Fisher Scientific), and cover-slips were mounted onto microscope slides in the presence of a Mowiol mounting medium. Fluorescence images were captured with an Olympus IX81 microscope.

### 2.7 Western blot analysis

Western blot analysis was carried out as described previously.20, 21 Whole cell lysate was prepared with 1.0% SDS containing protease inhibitors (complete mini protease inhibitor cocktail tablet; Sigma-Aldrich). Anti-GREB1 polyclonal Ab (ab72999, Abcam) and anti-β-actin mAb (clone AC-15; Sigma-Aldrich) were used at 1:1000 dilutions for GREB1.

### 2.8 Surgical specimens

Formalin-fixed paraffin-embedded specimens of human CRC were obtained from 77 patients who had undergone colectomy at Hiroshima University Hospital between 2006 and 2011 and were used for GREB1 IHC analyses. Written informed consent for participation in the study was obtained from all participants.

### 2.9 Plasmid construction

A 2301-bp fragment of the BRAF allele was amplified by PCR using cDNA from colon cancer cells with the BRAF mutation (V600E) and then inserted into the retroviral vector pDON-5 neo (Takara Bio) to generate the pDON-5/BRAFV600E vector. The pDON-5/KRASG12D vector was generated in the same manner.

A 5850-bp fragment of the GREB1 allele containing a Flag tag at the 3′-end was amplified by PCR using cDNA from MCF-7 cells and then inserted into the retroviral vector pDON-5 neo to generate the pDON-5/GREB1 vector. Hairpin-loop oligonucleotides targeting GREB1 and a nonsilencing sequence were synthesized and inserted into pSUPER.retro. neo + gfp (OligoEngine) to generate pSUPER/GREB1 shRNA and pSUPER/non-silencing shRNA (Table S2).

### 2.10 Cell lines and retroviral infections

All cell lines, SW48 (PRID: CVCL_1724), Caco2 (PRID: CVCL_0025), RKO (PRID: CVCL_0504), Hct116 (PRID: CVCL_0291), MCF-7 (PRID: CVCL_0031), and Colo320 (PRID: CVCL_1989) were obtained from ATCC. AmphiPack-293 (Takara Bio, PRID: CVCL_WI47) cells were transfected with retroviral constructs; and supernatants containing nonreplicating amphotropic virus were harvested.

For induction of BRAFV600E and KRASG12D mutations, SW48 and Caco2 cells, in which KRAS and BRAF are known to be
WT, were infected with virus containing pDON-5/Braf\textsuperscript{V600E}, KRAS\textsuperscript{G12D} and empty vectors. For GREB1 overexpression, RKO cells were infected with virus containing pDON-5/GREB1 and pDON-5 vectors. For GREB1 silencing, Hct116 cells, in which endogenous GREB1-mRNA was confirmed to be high, were infected with virus containing pSUPER/GREB1 shRNA and pSUPER/nonsilencing shRNA. Cells were selected with neomycin (500 μg/mL for RKO and Hct116 cells) for 2-5 weeks, as previously described.\textsuperscript{21}

2.11 | Cell proliferation assays

Cellular proliferation was measured using a brightfield image label-free high-content time-lapse assay with the IncuCyte Zoom system (Essen BioScience) according to the manufacturer’s instructions. Cell viability was also measured with MTS-based CellTiter 96 AQueous One Solution Reagent (Promega), as described previously.\textsuperscript{17,21}

2.12 | Soft agar colony formation assays

The ability of knockdown GREB1 or nonsilencing cells (Hct116) to form macroscopically visible colonies in soft agar was determined essentially as described previously.\textsuperscript{22}

2.13 | In vivo tumorigenesis assays

Female BALB/cJcl-nu/nu mice (CLEA Japan) were used at 5 weeks of age. A total of 5.0 × 10\textsuperscript{6} Hct116 cells (GREB1 knockdown or nonsilencing) and RKO cells (GREB1 overexpression or empty) were s.c. injected into the right flanks of 8 nude mice. The tumors were removed and weighed on day 14.

2.14 | Braf\textsuperscript{V600E} mutant and Braf WT patient cohort

The effect of Braf\textsuperscript{V600E} mutation on GREB1 expression in human CRC was analyzed using The Cancer Genome Atlas dataset (accession number: phs000178) for colorectal adenocarcinoma (COADREAD).

2.15 | Statistical analysis

The statistical significance of differences was determined by Mann-Whitney U test, \( \chi^2 \) test, unpaired t test, or Fisher’s exact test. Differences with a P value of less than .05 were considered statistically significant. All statistical analyses were undertaken using JMP 12 software (SAS Institute).

3 | RESULTS

3.1 | CDX2P-G22Cre:Apc\textsuperscript{flox/flox},Braf\textsuperscript{LSL-V600E/+} and CDX2P-CreER\textsuperscript{TK2}:Apc\textsuperscript{flox/flox},Braf\textsuperscript{LSL-V600E/+} mice developed polyoid lesions, including well to moderately differentiated adenocarcinoma, in the proximal colon

In the G22Cre mouse model, genetic analysis showed evidence of recombination of the Apc\textsuperscript{flox} and Braf\textsuperscript{LSL-V600E} alleles in the tumor tissue, but not in the small intestine or normal tissue of colon (Figure 1A). Median survival time of CDX2P-G22Cre:Apc\textsuperscript{flox/flox},Braf\textsuperscript{LSL-V600E/+} and CDX2P-G22Cre:Apc\textsuperscript{flox/flox} mice was 8.5 weeks and 19.1 weeks, respectively (Figure 1B). Histologically, CDX2P-G22Cre:Apc\textsuperscript{flox/flox},Braf\textsuperscript{LSL-V600E/+} and CDX2P-G22Cre:Apc\textsuperscript{flox/flox} mice generated high-low and low-grade adenoma, respectively, but no invasions to the submucosa, lymph node metastases, or distant metastases were observed (Figure 1C-H). Body weights of both CDX2P-CreER\textsuperscript{TK2}:Apc\textsuperscript{flox/flox},Braf\textsuperscript{LSL-V600E/+} and CDX2P-CreER\textsuperscript{TK2}:Apc\textsuperscript{flox/flox} mice were measured every week after TAM injections. CDX2P-CreER\textsuperscript{TK2}:Apc\textsuperscript{flox/flox},Braf\textsuperscript{LSL-V600E/+} mice showed significantly lower body weights from 9 week after the injection of TAM (Figure 1I). Similarly, in comparison to CDX2-G22Cre mice, the median survival time of CDX2P-CreER\textsuperscript{TK2}:Apc\textsuperscript{flox/flox},Braf\textsuperscript{LSL-V600E/+} and CDX2P-CreER\textsuperscript{TK2}:Apc\textsuperscript{flox/flox} mice was 13.0 weeks and 26.5 weeks, respectively (Figure 1J). Results consistent with the CDX2-G22Cre mouse model were also observed for CDX2P-CreER\textsuperscript{TK2} mouse model (Figure 1K-P). We carried out IHC staining of Ki67, Cdx2, β-catenin, and pS3 (Figure S1).

3.2 | Identification of candidate genes whose expression was altered in response to Braf mutation by microarray analysis

In gene expression profiles from the oligonucleotide microarray, between CDX2P-G22Cre:Apc\textsuperscript{flox/flox},Braf\textsuperscript{LSL-V600E/+} (Braf MT) and CDX2P-G22Cre:Apc\textsuperscript{flox/flox},Braf\textsuperscript{LSL-V600E/+} (Braf WT) mice, differentially expressed genes of the tumor (P < .05) were identified as those having a fold change of at least 2.0 (upregulated gene) or −4.0 (downregulated gene). Five genes were significantly upregulated, and 8 genes were significantly downregulated in the tumors of CDX2P-G22Cre:Apc\textsuperscript{flox/flox},Braf\textsuperscript{LSL-V600E/+} mice. Similar results were obtained for all candidate genes in the comparison between CDX2P-G22Cre:Apc\textsuperscript{flox/flox},Kras\textsuperscript{LSL-G12D/+} and CDX2P-G22Cre:Apc\textsuperscript{flox/flox} mice. It was suggested that these genes might be regulated by the RAS-RAF axis (Table 1).

Quantitative RT-PCR was subsequently carried out to validate expression changes in these 13 candidate genes (Figure S2). The genes that were indicated to be increased or decreased in qRT-PCR both in G22Cre and CreER\textsuperscript{TK2} mouse model were Greb1, Abcb1a, Cyp2s1, and Bex1. Of these candidates, we decided to further investigate Greb1, because the fold change of Greb1 was the largest among the 4 candidates.
3.3 | \( \text{BRAF}^{V600E} \) and \( \text{KRAS}^{G12D} \) mutation led to an increase of mRNA levels of \( \text{GREB1} \) in CRC cell lines

To investigate the impact of mutant \( \text{BRAF} \) and mutant \( \text{KRAS} \) on the expression of \( \text{GREB1} \) in vitro, SW48 and Caco2 in which \( \text{BRAF}^{V600E} \) and \( \text{KRAS}^{G12D} \) were analyzed in terms of \( \text{GREB1} \)-mRNA expression by qRT-PCR, and induction of \( \text{BRAF}^{V600E} \) and \( \text{KRAS}^{G12D} \) resulted in higher expression level of \( \text{GREB1} \) in both cell lines (Figure 2).

3.4 | Effects of \( \text{GREB1} \) knockdown on cell growth

We analyzed the effects of \( \text{GREB1} \) expression on proliferative ability by \( \text{GREB1} \) knockdown in CRC cell lines. Quantitative RT-PCR confirmed that the shRNA significantly blocked \( \text{GREB1} \) expression compared with that in cells transfected with non-silencing RNA (Figure 3A; \( P < .05 \)).
Effects of GREB1 overexpression on cell growth

RKO cells were transfected with a vector encoding human full-length GREB1 (pDON-5/GREB1) or an empty vector (pDON-5). The efficiency of GREB1 overexpression is shown in Figure 3I, J.

The proliferation assays carried out using the IncuCyte Zoom system revealed that cell proliferation was significantly increased in the GREB1 overexpression group compared with that in the empty vector group (Figure 3K; *P < .05). We also undertook a skin

### Table 1: Gene expression profiling using microarray analysis for Braf-mutated (MT), Kras-MT, and Braf/Kras WT mouse models

| Gene symbol | Function                                             | Braf MT vs Braf/Kras WT, fold | Kras MT vs Braf/Kras WT, fold | Validation (G22Cre), P value | Validation (CreERT2), P value |
|-------------|------------------------------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| **Upregulated genes**                                      |                                      |                               |                               |                               |                               |
| Greb1       | Promote cell proliferation                           | 4.5                           | 3.9                           | 0.009                         | 0.016                         |
| Eda2r       | Activate protein of NFKB and JNK                     | 4.0                           | 3.5                           | 0.009                         | 0.075                         |
| Abcb1a      | Intestinal epithelial barrier function               | 3.3                           | 2.1                           | 0.016                         | 0.028                         |
| Cyp2s1      | Metabolic enzyme                                     | 3.1                           | 1.7                           | 0.047                         | 0.009                         |
| Nox1        | Enzymes function to generate physiological levels of ROS | 2.6                           | 1.4                           | 0.117                         | 0.117                         |
| **Downregulated genes**                                   |                                      |                               |                               |                               |                               |
| Lrg1        | Regulator of angiogenesis                            | −14.3                         | −3.5                          | 0.009                         | 0.347                         |
| Gkn3        | Regulator of cell proliferation                      | −7.6                          | −4.8                          | 0.009                         | 0.601                         |
| Ltf         | Iron-binding glycoprotein of the transferrin         | −7.3                          | −4.0                          | 0.009                         | 0.174                         |
| Muc6        | Secreted glycoprotein                               | −6.6                          | −3.3                          | 0.075                         | 0.028                         |
| Lcn2        | Protein associated with neutrophil gelatinase        | −6.1                          | −1.7                          | 0.009                         | 0.174                         |
| Lgr5        | Receptors for glycoprotein hormones                  | −5.3                          | −1.9                          | 0.028                         | 0.117                         |
| Bex1        | Regulator of cell cycle                              | −4.0                          | −3.5                          | 0.009                         | 0.028                         |
| Lum         | Interacts with collagen and limits growth of fibrils  | −4.0                          | −2.4                          | 0.624                         | 0.250                         |

Fold change Braf MT vs Braf/Kras WT: Fold indicates a gene expression ratio of the tumors of CDX2P-G22Cre;Apc^flox/flox;Braf^V600E/+ mice to those of CDX2P-G22Cre;Apc^flox/flox mice.

Fold change Kras MT vs Braf/Kras WT: Fold indicates a gene expression ratio of the tumors of CDX2P-G22Cre;Apc^flox/flox;Kras^G12D/+ mice to those of CDX2P-G22Cre;Apc^flox/flox mice.

Validation: Results of quantitative RT-PCR between Braf MT and Braf/Kras WT mouse tumor in each Cre mouse model.

Abbreviations: JNK, c-Jun N-terminal Kinase; NFKB, nuclear factor-κB; ROS, reactive oxygen species.

**Figure 2**: Expression levels of GREB1 mRNAs in SW48 and Caco2 cells (quantitative RT-PCR). SW48 and Caco2 cells overexpressing BRAF^V600E or KRAS^G12D showed significantly higher expression of GREB1 compared with control cells. Representative data from 5 independent experiments are shown. *P < .05. Data are expressed as mean ± SD
xenograft in vivo tumorigenesis assay GREB1-overexpressed and empty vector-induced control cells. Tumor volumes and weight were larger and heavier in the GREB1-overexpressed group than in the control group (Figure 3L-N; size, 131.1 ± 41.5 vs 65.3 ± 36.6 mm$^3$, respectively, $P < .05$; weight, 108.7 ± 40.7 vs 64.8 ± 31.1 mg, respectively, $P < .05$).

3.6 Strong GREB1 expression predicts poor prognosis in CRC patients

We analyzed the expression of GREB1 protein in human colorectal cancer (CRC) specimens (Figure 4A-D). Immunohistochemical analyses for surgical specimens of human CRCs showed that 37 (48.1%) of the 77 CRC cases showed positive results for GREB1 (cut-off, greater than 30%), and the expression of GREB1 protein was higher in tumor tissues than in adjacent normal tissue (Figure 4B-D). The expression of GREB1 mRNA was significantly increased in BRAF$^V600E$ tumor tissues compared with BRAF$^WT$ tumor tissues (Figure 4E).

In terms of prognosis, we found GREB1-positive CRCs showed lower overall survival rates than GREB1-negative ones (Figure 4F). In this study, a significant difference was detected at cut-off of 30%. The other cut-offs (10%, 50%, and 80%) did not show a significant difference, although a tendency was recognized (data not shown). In multivariate analyses for prognosis, GREB1, as well as lymphatic...
invasion, was revealed as the independent prognostic factor in CRC (Table 2).

4 | DISCUSSION

In this study, we generated a CRC mouse model in which there is biallelic Apc inactivation and oncogenic gain-of-function mutations in Braf in the colon epithelium; these mutations induce the formation of adenocarcinomas in the proximal colon. We then identified Greb1 as a novel gene upregulated by either KrasG12D or BrafV600E mutation induced by 2 distinct Cre mouse models for mouse colonic neoplasia.

Greb1 was partially discovered from the human brain cDNA library encoding large proteins located in the short arm of chromosome 12 (p25.1), where it spans roughly 108 kb, and a complete sequence of GREB1 cDNA was identified as a primary target for ER regulation in estradiol-stimulated breast cancer cell line MCF-7. GREB1 transcript codes for a putative 1949-aa protein with at least 4 transmembrane domains and an N-myristoylation domain. Although GREB1 was first identified as a hormone-responsive gene, it stands out as an oncogene, which mediates estrogen-stimulated cell proliferation in endometriosis, breast, and ovarian cancers. Therefore, GREB1 has been considered as a candidate clinical marker for response to endocrine therapy as well as a potential therapeutic target. Similarly, GREB1 might play an important role as an oncogene in prostate cancer growth and could be a prognostic marker as well as predictive marker of androgen responsiveness. These results highlight the important role of GREB1 as a hormone-responsive gene and functions as an oncogene.

In the current study, our data indicated that GREB1 promoted proliferation and tumorigenesis in CRC cell lines in vitro as well as in a mouse xenograft model. Although little is known about the contribution of GREB1 to CRC, it was suggested that high expression of GREB1 protein might be a poor prognostic factor in our CRC cases (Figure 4).

To elucidate the mechanism by which oncogenic BRAF upregulates GREB1 expression, it is important to understand how ER modulates the signaling pathway from the upper molecule to its downstream targets. The molecular structure of ER has 2
TABLE 2  Results of univariate and multivariate analyses of prognostic factors for overall survival of 5 years in patients with colorectal cancer

|                             | Univariate analysis | Multivariate analysis |
|-----------------------------|---------------------|-----------------------|
|                             | n = 77              |                       |
|                             | p value | HR  | 95% CI  | p value |
| GREB1 <30                   | 40 .03   | 13  | 1.61-312.4 | .01 |
| GREB1 ≥30                   | 37       |     |         |       |
| Age (y) <64                 | 41 .37   | 0.63 | 0.13-2.96 | .55 |
| Age (y) ≥64                 | 36       |     |         |       |
| Gender Male                 | 52 .55   | 0.44 | 0.10-1.77 | .24 |
| Gender Female               | 25       |     |         |       |
| pT <3                       | 7 .94    | 1.99 | 0.13-110  | .65 |
| pT ≥3                       | 70       |     |         |       |
| pN <1                       | 39 .08   | 2.38 | 0.53-10.9 | .24 |
| pN ≥1                       | 38       |     |         |       |
| Tumor location Right sided | 11 .6    | 0.62 | 0.02-4.71 | .69 |
| Tumor location Left sided   | 66       |     |         |       |
| Histologic type Well/moderate | 73 .14   | 35.6 | 0.73-2713 | .06 |
| Histologic type Poorly      | 4        |     |         |       |
| Ly <Ly2                     | 60 .03   | 16.1 | 1.11-278  | .04 |
| Ly ≥Ly2                     | 12       |     |         |       |
| V <V2                       | 60 .99   | 1.06 | 0.06-12.8 | .96 |
| V ≥V2                       | 11       |     |         |       |

Abbreviations: CI, confidence interval; HR, hazard ratio; Ly, lymphatic invasion; V, venous invasion.

activation domains, the ligand-independent AF-1 domain, and the ligand-dependent AF-2 domain. Estrogen receptor has been classically thought to be activated by the ligand-dependent pathway.

Recently, a ligand-independent pathway has also been reported. In response to the activation of the MAPK pathway, phosphorylation occurs on Ser118, which is located in the AF-1 domain of ER, and its phosphorylation provides an important mechanism that regulates AF-1 activity. Moreover, activation of RAS-RAF-MAPK signaling by this ligand-independent pathway has been reported as a mechanism of TAM resistance in ER-positive breast cancer as well as HGSOC. In ER-positive breast cancer, there was a correlation between TAM resistance and expression of phosphorylated RAF (ser338). In the case of ER-positive HGSOC, TAM resistance was correlated with phosphorylated MAPK expression, which was reversed by a MEK inhibitor (selumetinib). Based on these findings, phosphorylating ER-Ser118 by the ligand-independent ER pathway might play an important role in carcinogenesis as the downstream pathway of activated RAF.

As GREB1 was upregulated by either BRAFV600E or KRASG12D mutation in GEMMs and human CRC cell lines, it was expected that activation of the KRAS-BRAF-MAPK axis might induce estradiol-independent ER activation, resulting in oncogenic GREB1 upregulation. Although we have tried to demonstrate ER phosphorylation by BRAFV600E, the Abs for ER and phospho-ER are not capable of detecting endogenous proteins, as the ER-mRNA expression levels in CRC cell lines are 2000 times lower than in breast cancer cell lines by qRT-PCR (Figure S3).

In conclusion, we found that BRAFV600E mutation led to upregulation of GREB1 expression, which promoted tumor cell proliferation in colorectal tumorigenesis. Although the mechanism of GREB1 expression was reported to be controlled by estradiol-independent phosphorylation through BRAFV600E mutation in some breast cancer cell lines, we could not determine a similar mechanism in human CRC cell lines. However, even though this question should be addressed by other methods, the GREB1 knockdown in vivo transplantation model showed remarkable reduction of tumors and is thus a potential candidate molecular target for the treatment of BRAFV600E-mutated CRCs.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

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SUPPORTING INFORMATION

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

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