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ORIGINAL ARTICLE

Resveratrol inhibits development of colorectal adenoma via suppression of LEF1; comprehensive analysis with connectivity map

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Abstract

Although many chemopreventive studies on colorectal tumors have been reported, no effective and safe preventive agent is currently available. We searched for candidate preventive compounds against colorectal tumor comprehensively from United States Food and Drug Administration (FDA)-approved compounds by using connectivity map (CMAP) analysis coupled with in vitro screening with colorectal adenoma (CRA) patient-derived organoids (PDOs). We generated CRA-specific gene signatures based on the DNA microarray analysis of CRA and normal epithelial specimens, applied them to CMAP analysis with 1309 FDA-approved compounds, and identified 121 candidate compounds that should cancel the gene signatures. We narrowed them down to 15 compounds, and evaluated their inhibitory effects on the growth of CRA-PDOs in vitro. We finally identified resveratrol, one of the polyphenolic phytochemicals, as a compound showing the strongest inhibitory effect on the growth of CRA-PDOs compared with normal epithelial PDOs. When resveratrol was administered to $Apc^{Min/+}$ mice at 15 or 30 mg/kg, the number of polyps (adenomas) was significantly reduced in both groups compared with control mice. Similarly, the number of polyps (adenomas) was significantly reduced in azoxymethane-injected rats treated with 10 or 100 mg/resveratrol compared with control rats. Microarray analysis of adenomas from resveratrol-treated rats revealed the highest change (downregulation) in expression of LEF1, a key molecule in the Wnt signaling pathway. Treatment with resveratrol significantly downregulated the Wnt-target gene (MYC) in CRA-PDOs. Our data demonstrated that resveratrol can be the most effective compound for chemoprevention of colorectal tumors, the efficacy of which is mediated through suppression of LEF1 expression in the Wnt signaling pathway.

Abbreviations: AOM, azoxymethane; BLI, blue laser imaging; CMAP, connectivity map; CRA-PDOs, colorectal adenoma-patient-derived organoids; DSS, dextran sodium sulfate; FAP, familial adenomatous polyposis; IQR, interquartile range; NBI, narrow binding imaging; NCE-PDOs, normal colorectal epithelia-patient-derived organoids; NSAIDs, nonsteroidal anti-inflammatory drugs.

KEYWORDS
chemoprevention, colorectal adenoma, connectivity map, LEF1, resveratrol

1 | INTRODUCTION

Colorectal cancer is the leading cause of cancer-related deaths worldwide.[1] Colorectal cancer develops mainly from CRA as an adenoma-carcinoma sequence.[2] Therefore, it is important to develop effective chemopreventive agents against CRA as a chemoprevention strategy.[3] Several clinical studies have reported candidate chemopreventive agents, including NSAIDs, aspirin, COX-2 inhibitors, and calcium.[4–8] Although some of these candidate agents significantly inhibited development of CRA or cancer in clinical trials, none of them has been approved as a chemopreventive agent due to their adverse events or poor efficacy.

There are two major reasons why effective chemopreventive agents have not been developed to date. First, previous studies chose candidate drugs for chemoprevention based on epidemiological data. For example, epidemiological studies showed that the incidence of colorectal cancers in patients receiving aspirin was low and, therefore, clinical studies on the preventive effects of aspirin were conducted. However, it is very important to screen for candidate agents much more extensively and comprehensively from a large series of compounds. Second, it has been impossible to evaluate the chemopreventive effects of candidate agents against CRA in vitro because there has been no established CRA cell line for in vitro experiments to date. Taken together, these observations indicate that the development of candidate chemopreventive agents requires a comprehensive large-scale analytic approach coupled with in vitro screening using a relevant CRA cell model.

The CMAP is a database established to connect genes, compounds /drugs, and disease, as reported by Lamb et al.[9] They created a substantial gene-expression profile database from human cultured cells treated with numerous compounds (e.g., FDA-approved drugs). Applying the disease-specific gene signature obtained by DNA microarray analysis of the disease into the CMAP database, the effective compounds that cancel the disease-specific signature can be ranked from highest to lowest. The CMAP analysis has greatly facilitated comprehensive in silico screening of numerous compounds to find candidate drugs for various diseases.[10] Many studies have used CMAP analysis for drug repositioning against common diseases such as diabetes and cancers, including breast cancer[11] and lung cancer.[12] Of these, some drugs have been expected to be put into clinical use. Applying this CMAP analysis method to CRA, we are able to find effective candidate compounds comprehensively among a large library of FDA-approved compounds.

An intestinal organoid culture technology has emerged as a new culture tool and enabled in vitro long-term three-dimensional culture of intestinal cells including colorectal cancer, CRA, and normal epithelia, using biopsy specimens under endoscopy or surgical specimens. This culture method has been widely used for studying stem cell biology, human physiology, and pathology, including tumor cell modeling. Using this organoid culture method, we are able to screen candidate compounds in vitro for their preventive effects on CRA.

Therefore, by combining comprehensive CMAP analysis and in vitro screening with an organoid culture technology,[13] we can identify optimized drugs among numerous chemical compounds as preventive agents against CRA. Therefore, in this study, we first performed a DNA microarray analysis of CRA tissue sets from multiple patients to create a CRA-specific gene signature. Applying the signature into a CMAP database of 1309 FDA-approved compounds, we identified candidate compounds that should cancel the gene signature pattern comprehensively among those compounds. We then screened those candidate compounds in vitro using a CRA patient-derived organoid (PDO) to find the most effective compound against CRA. Since we ultimately identified resveratrol as the most effective candidate compound, we validated its efficacy by using $Apc^{Min/+}$ mice and a chemical carcinogenesis rat model. Moreover, we identified LEF1 as a target of resveratrol, an important transcription factor in the Wnt signaling pathway,[14–17] to clarify the mechanism of action of resveratrol.

2 | MATERIAL AND METHODS

2.1 | Patients and samples

We enrolled three patients with colorectal polyps sized 5–8 mm that were endoscopically diagnosed as adenoma by narrow binding imaging for the signature creation of CRA. We obtained two biopsy specimens from each of the polyps and surrounding normal mucosa. Subsequently, the polyp was endoscopically resected, and a diagnosis of adenoma was histologically confirmed by two pathologists (H.U. and Y.B.). The biopsied samples were immediately frozen at −80°C. Similarly, we obtained three pairs of adenoma and normal mucosal specimens from another three patients to establish organoid cultures. Patient characteristics are provided in Figure S1.

2.2 | CRA-specific gene signature and CMAP analysis

The CRA-specific gene signature was estimated as described previously.[18,19] To identify the CRA-specific signatures from the two sets of gene-expression data (normal mucosa and adenoma), we estimated the difference in gene expression between the two sets as follows. Following the outlier test for all values of genes, we calculated a *z*-score for each gene by using the average and the variance of the gene values except for outliers. The *z*-score of each gene was

then transformed into probability, and then each difference in gene probability between the two sets, p_k^d, was calculated as follows:

$$p_k^d = p(z_k^a) - p(z_k^b) = \frac{1}{\sqrt{2\pi}}\int_{-\infty}^{z_k^a} e^{-\frac{z}{2}}\,dz - \frac{1}{\sqrt{2\pi}}\int_{-\infty}^{z_k^b} e^{-\frac{z}{2}}\,dz$$

where the k-th gene between the two gene sets, a and b, was compared. Using the above formula, we can estimate the difference of each gene in the two gene sets, a and b, regardless of the differing dynamic ranges of the two gene sets in respective measurement conditions. In this analysis, we set $|p_k^d| > 0.2$ as a threshold to estimate the gene signature.

To characterize the gene signature above, pathway analysis was performed, as we described previously.[18,19] Subsequently, an enrichment analysis was applied for the gene signatures to the canonical pathway gene sets (c2.cp.v3.0. entrez) in the molecular signature database (http:// www.broadinstitute.org/gsea/msigdb). We selected significant pathways with a false discovery rate <5%.

Based on the significant pathways estimated above, we further narrowed down the genes that belonged only to the significant pathways, to estimate another gene signature of differentially and coordinately expressed genes between the two sets. Finally, we estimated the compounds from the CMAP database in which microarray-based whole gene profiles of 1309 FDA-approved compounds were compiled (http://www.broadinstitute.org/cmap/). Based on the 2 CRA-specific gene signatures, we identified potentially effective compounds with a threshold of $p < 0.05$.

2.3 | Patient-derived organoid culture

Patient-derived organoid culture of CRA and colorectal normal epithelium was performed as previously described.[20,21]

2.4 | Effect of resveratrol on intestinal tumorigenesis in Apc$^{Min/+}$ mice

Male C57BL/6-Apc$^{Min/+}$ mice (Apc-deficient mice) were purchased from Jackson Laboratory. In total, 21 mice were randomly assigned to three groups and were given 0, 15, or 30 mg/kg of resveratrol in drinking water as an admixture, as we described previously.[22] The mice were sacrificed at 8 weeks after administration and whole colorectums were carefully removed. Tumor number and size were determined using a stereomicroscope.

2.5 | Effect of resveratrol on azoxymethane-induced colorectal tumors in rats

Male F344 rats were purchased from CLEA Japan, Inc. In total, 30 rats were divided into a vehicle group, a 10 mg/kg group, and a 100 mg/kg group. AOM (20 mg/kg) was administered intraperitoneally on day 1 and day 4, and rats were given free access to drinking water containing 1% DSS from day 7 to day 13. The dose of resveratrol was determined by weighing rats twice a week, suspending the compound in sterile water, and administering directly to the stomach with a gastric sonde once daily. At 15 weeks after administration, we observed left colon polyps using a thin veterinary endoscope system (AVS Co., Ltd.) in all rats to confirm that the colorectal lesion was successfully produced and avoid meaningless sacrifice. Resveratrol administration was continued for 16 weeks, followed by sacrifice and counting the number of colorectal polyps under a stereomicroscope. Four polyps in each group were excised and frozen for transcriptome analysis. Immediately after sacrifice, blood was collected from the aorta of three animals in each group for analysis.

Detailed methods on PDO culture, compounds, cell viability assay, microarray analysis, bromodeoxyuridine (BrdU) assay, real-time PCR, western blotting, and statistics are provided in Appendix S1.

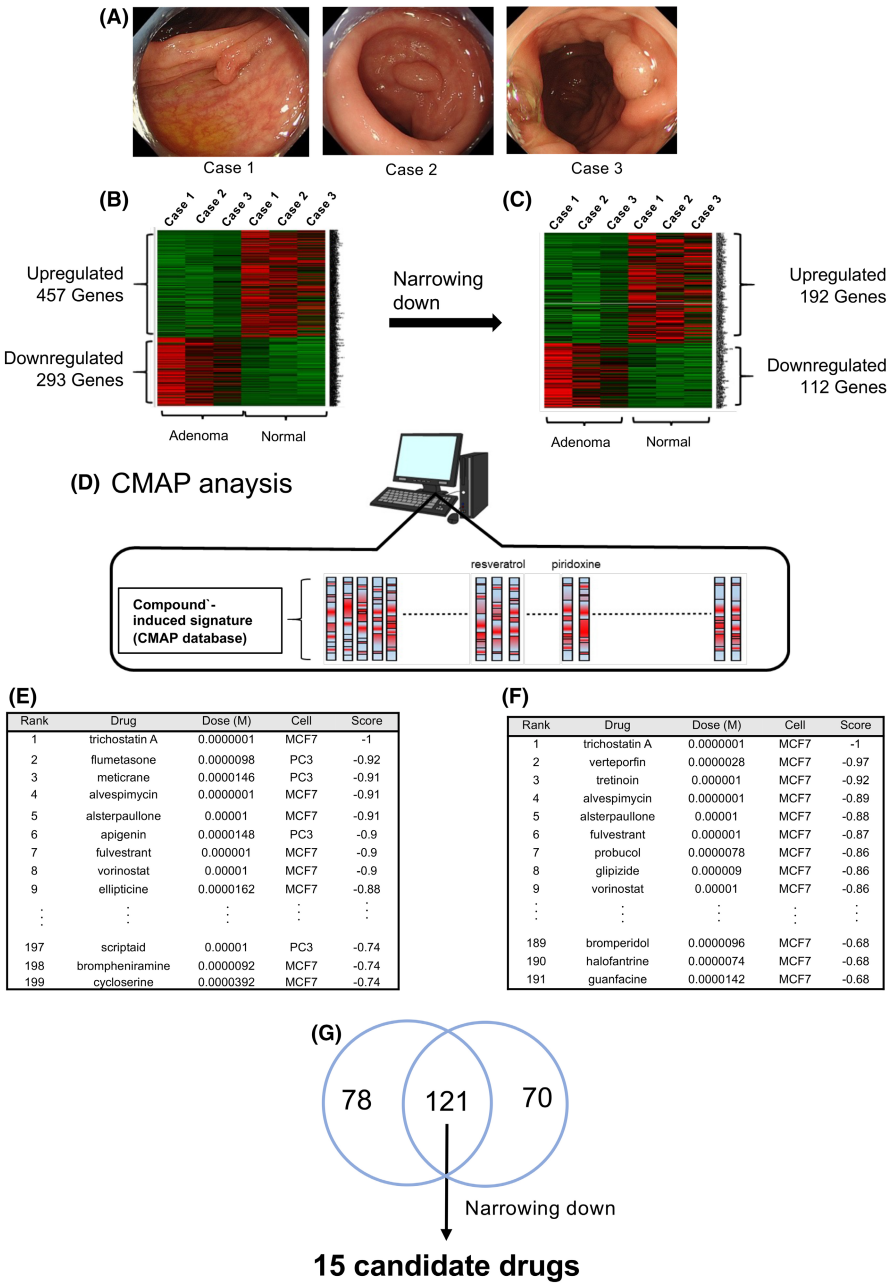
3 | RESULTS

3.1 | Generation of gene-expression signature of human colorectal adenoma

To identify the candidate chemopreventive compounds by in silico analysis with CMAP, we first investigated the gene-expression profile of human CRA in comparison with the corresponding normal epithelial tissues in each pair from three CRA cases by microarray analyses (Figure 1A). Among 31,042 genes, we identified 750 genes that were differentially expressed between the CRA and normal epithelia (Figure 1B; Table S1); 457 upregulated and 293 downregulated genes. We then selected 304 genes (192 upregulated and 112 downregulated genes) by applying gene set enrichment analysis (GSEA) for the signaling pathway (Figure 1C; Tables S2 and S3).

3.2 | Identification of putative preventive compounds for human colorectal adenoma applying the gene signatures to CMAP analysis

To discover candidate compounds for prevention of CRA, we applied the two gene sets of CRA (Figure 1B,C) to the CMAP database in which the differentially expressed genes among whole human genes in cancer cell lines under the treatment with each of 1309 FDA-approved compounds is compiled (Figure 1D). By CMAP analysis, we identified the compounds that canceled the gene expression of the CRA-specific signature to those of normal tissue; the CMAP analysis with 750 differentially expressed genes (Figure 1B) identified 199 compounds that were ranked in the top 5% of the most effective compounds (Figure 1E; Table S4). Similarly, the CMAP analysis with 304 genes (Figure 1C) identified 191 compounds (Figure 1F; Table S5). We selected 121 compounds that existed commonly in the two Venn diagrams of 199 and 191 compounds, respectively,



(E)

Rank	Drug	Dose (M)	Cell	Score
1	trichostatin A	0.0000001	MCF7	-1
2	flumetasone	0.0000098	PC3	-0.92
3	meticrane	0.0000146	PC3	-0.91
4	alvespimycin	0.0000001	MCF7	-0.91
5	alsterpaullone	0.00001	MCF7	-0.91
6	apigenin	0.0000148	PC3	-0.9
7	fulvestrant	0.000001	MCF7	-0.9
8	vorinostat	0.00001	MCF7	-0.9
9	ellipticine	0.0000162	MCF7	-0.88
⋮	⋮	⋮	⋮	⋮
197	scriptaid	0.00001	PC3	-0.74
198	brompheniramine	0.0000092	MCF7	-0.74
199	cycloserine	0.0000392	MCF7	-0.74

(F)

Rank	Drug	Dose (M)	Cell	Score
1	trichostatin A	0.0000001	MCF7	-1
2	verteporfin	0.0000028	MCF7	-0.97
3	tretinoin	0.000001	MCF7	-0.92
4	alvespimycin	0.0000001	MCF7	-0.89
5	alsterpaullone	0.00001	MCF7	-0.88
6	fulvestrant	0.000001	MCF7	-0.87
7	probucol	0.0000078	MCF7	-0.86
8	glipizide	0.000009	MCF7	-0.86
9	vorinostat	0.00001	MCF7	-0.86
⋮	⋮	⋮	⋮	⋮
189	bromperidol	0.0000096	MCF7	-0.68
190	halofantrine	0.0000074	MCF7	-0.68
191	guanfacine	0.0000142	MCF7	-0.68

FIGURE 1 Generation of human colorectal adenoma signature, connectivity map (CMAP), and compound ranking based on CMAP. (A) Endoscopic images of the three cases of adenomatous polyps. (B, C) A heat map from microarray analysis of the top 750 genes differentially expressed between human colorectal adenoma (CRA) and surrounding normal epithelia in each of the three cases. (C) Heat map of 304 genes narrowed down from (B) by applying gene set enrichment analysis for the signaling pathway. (D) Conceptual diagram of CMAP. (E) A list of the top 5% compounds identified from gene signature (B) using CMAP analysis. The top 5% originally included 305 entries (Table S4), from which 199 compounds were selected, excluding overlaps of different concentrations. (F) Top 5% compounds identified from gene signature (C) using CMAP analysis. Similarly, the list originally included 305 entries (Table S5) and 191 compounds were selected. The connectivity score was calculated as described previously,[9] ranging from −1 to 1; a negative score denotes an inhibitory effect of compounds on the CRA-specific signature to the normal epithelia ranking with the strongest compound designated as −1. (G) Venn diagram shows 121 overlapping compounds between (E) and (F), which narrowed down to 15.

as putative preventive candidates (Table S6). Of these, we further selected 15 compounds (Figure 1G) considering oral bioavailability, adverse events, long-term administration, and cost for suitability of chemopreventive agents.

3.3 | Selection of candidate compounds using human colorectal adenoma organoids

To select the most effective compounds against CRA from the 15 candidates, we first established PDOs from CRA (CRA-PDOs) and NCE-PDOs from three CRA cases and examined the inhibitory effects of each compound on the growth of CRA-PDO. The IC_{50} values of all 15 compounds on CRA- and CRN-PDOs are summarized in Figure 2A. The IC50 of resveratrol on CRA-PDO was significantly lower than that of NCE-PDO (45.49 μM [95% CI: 38.06–53.04] vs 84.27 μM [73.98–94.72], $p = 0.005$; Figure 2B). Similarly, the IC_{50} of pyridoxine on CRA-PDO was significantly lower than that of CRN-PDO (2552 μM [95%CI: 2218–2942] vs. 1444 μM [1219–1704], $p = 0.0018$; Figure 2C). However, the remaining 13 compounds did not show statistically significant differences in IC50 values between CRA-PDOs and NCE-PDOs Figure (S2).

We then assessed the effects of resveratrol and pyridoxine on organoid size by analyzing phase-contrast microscopy images (Figure 2D,E). The mean size of five randomly selected organoid clusters of CRA-PDO treated with resveratrol (100 μM, which is close to the IC50) was significantly smaller than that with vehicle alone, whereas the mean size of five organoid clusters of NCE-PDO did not show any significant difference between resveratrol- and vehicle-treated groups (Figure 2F). A similar trend was observed

(A)

Drug/ Compound	Description	IC 50 (μM) NCE-PDO	IC 50 (μM) CRA-PDO	P value
Resveratrol	A type of stilbenoid polyphenol	84.27	45.49	0.0050
Pyridoxine	A form of vitamin B6	2552	1444	0.0018
Lansoprazole	Proton pump inhibitor	51.65	45.93	0.1080
Omeprazole	Proton pump inhibitor	150.1	142.0	0.1172
Luteolin	A type of flavonoid	52.29	53.01	0.8166
N-deacetyl Colchiceine	A colchicine analog	9.051	9.226	0.0036
Lorglumide	CCKA receptor antagonist	144.8	154.2	0.1668
Mecrofenoxate	Anti-vertigenous drug	1542	1850	0.3181
Tretinoin	A type of vitamin A derivative	138.9	150.8	0.2958
Apigenin	A type of flavonoid	207.8	305.8	0.3100
Piperlongmine	A type of alkaloid from Long pepper	12.17	25.18	0.0008
Trolox C	Water-soluble analog of vitamin E	> 10000	> 10000	ND
Ginkgolide A	A type of terpenoid in ginkgo leaves	> 10000	> 10000	ND
N-acetyl-L-leucine	Amino acid related substances	> 10000	> 10000	ND
Tomatidine	Alkaloids contained in tomato	> 10000	> 10000	ND

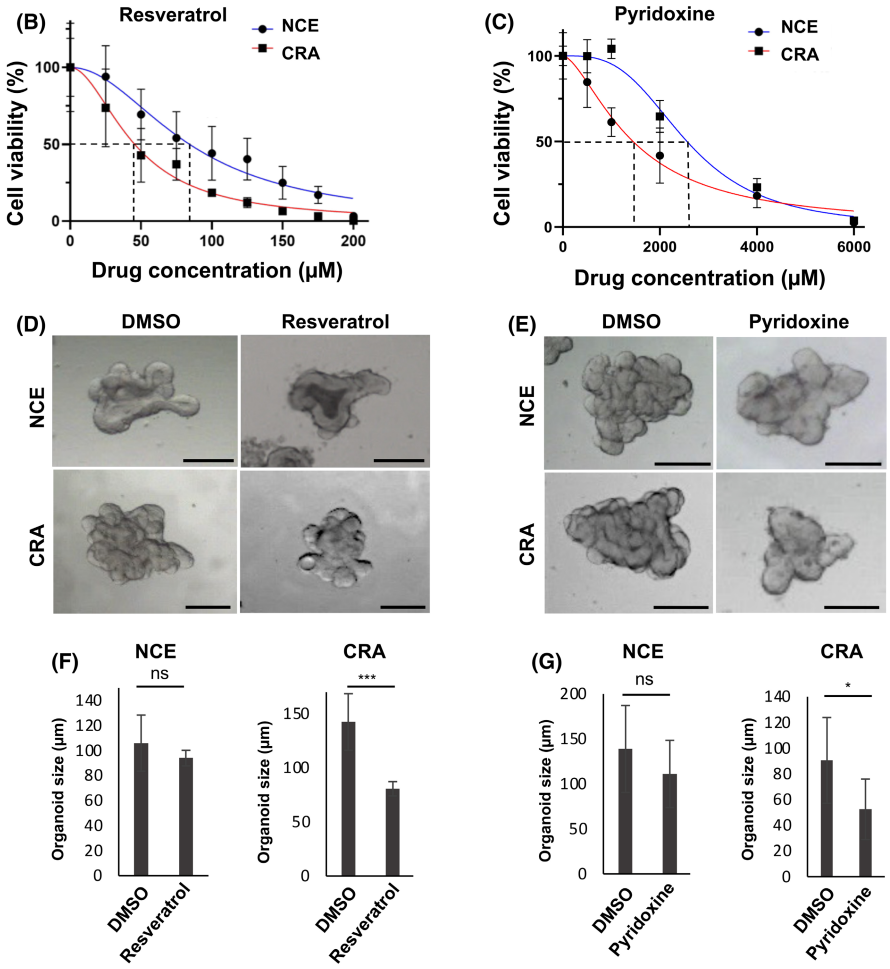


FIGURE 2 Screening of candidate compounds using organoids from colorectal adenoma and normal mucosa. (A) IC_{50} values of 15 compounds for human CRA or normal colorectal epithelia (NCE) of patient-derived organoid (PDO). Each organoid was treated with each compound or vehicle (DMSO) alone for 72 h, and CellTiter-Glo assay was performed to calculate IC_{50} values. ND, not detected. (B, C) The viability of CRA-PDOs and NCE-PDOs treated with various concentrations of resveratrol (B) and pyridoxine (C) were determined by CellTiter-Glo assay. IC_{50} values were calculated by nonlinear regression analysis. Error bars, ±SD. (D, E) Representative bright-field images of CRA-PDOs and NCE-PDOs cultured for 72 h with resveratrol (D) or pyridoxine (E). Scale bars, 50 μm. (F, G) Changes in the size of PDOs treated with resveratrol (100 μM) or pyridoxine (1000 μM) for 72 h. Average organoid size was calculated as the average of the data from nine representative images per treatment group. Error bars, ±SD. $^{*}p < 0.05$, $^{***}p < 0.001$. ns, not significant.

when organoids were treated with pyridoxine (Figure 2G). However, since pyridoxine is included in the normal diet, we considered resveratrol to be more appropriate as a candidate drug. Accordingly, we used resveratrol for further experiments.

3.4 | Inhibitory effect of resveratrol on polyp development in $Apc^{Min/+}$ mice

We next investigated the inhibitory effects of resveratrol on intestinal polyp development in the $Apc^{Min/+}$ mouse, which has characteristics resembling human FAP and is a well established model to investigate the mechanism of early stages of spontaneous intestinal tumorigenesis.[22] We administered resveratrol (15 or 30 mg/kg) or vehicle alone to $Apc^{Min/+}$ mice and evaluated the number and size of polyps at 8 weeks (Figure 3A). There was no significant difference in body weight (Figure S3), dietary intake, or clinical symptoms between the three groups. Representative images of the intestinal polyps are shown in Figure 3B. The number of polyps in mice treated with resveratrol (30 mg/kg) was noticeably lower than in control mice. The histological findings of polyps from both mice were adenomas (Figure 3B). All the polyps examined in all mice were histologically adenomas, consistent with previous study.[23] The median number of polyps (IQR) in the 15 and 30 mg/kg resveratrol-treated groups was 57 (52–69) and 61 (50–66), respectively, both of which were significantly lower than in the vehicle group (85 [68–97]; $p < 0.05$ and $p < 0.01$, respectively; Figure 3C). No significant differences were observed between

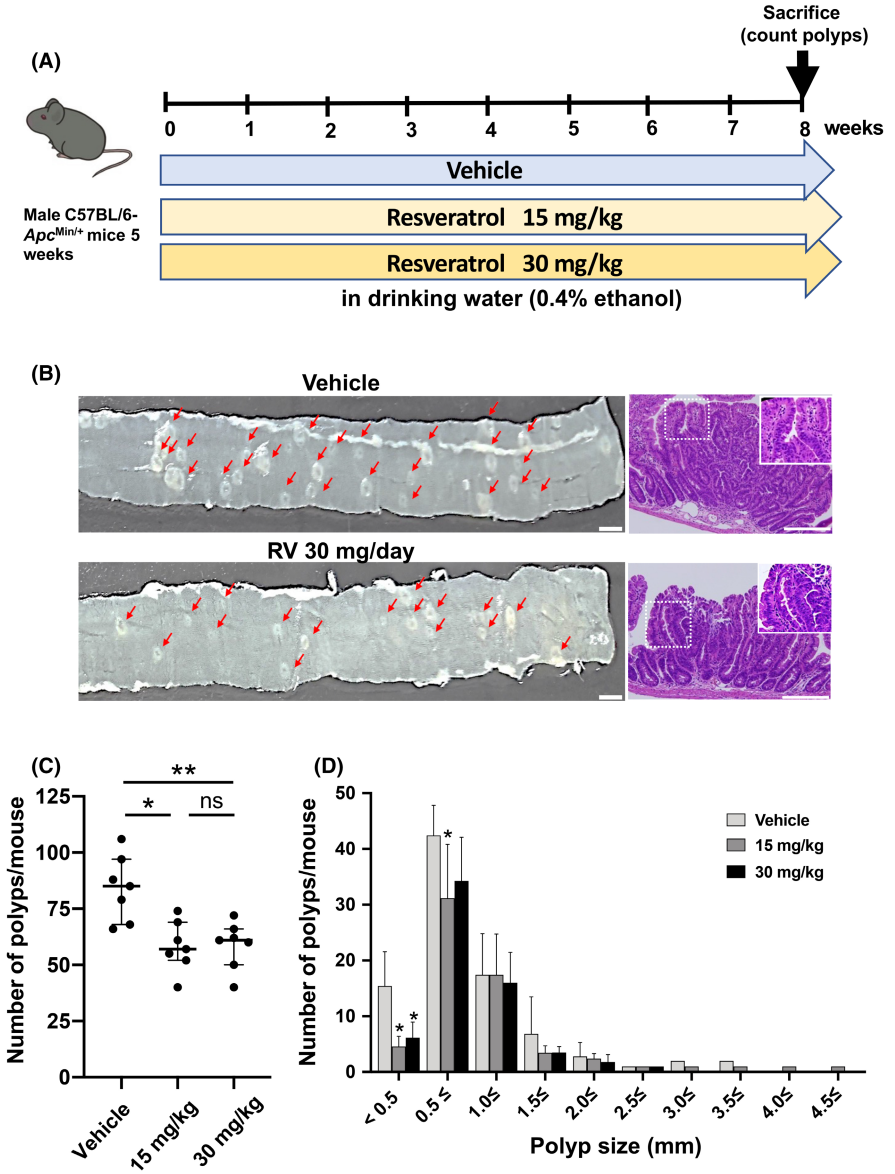


FIGURE 3 Effect of resveratrol on development of intestinal tumors in $Apc^{Min/+}$ mice. (A) Experimental protocol. Male C57BL/6-$Apc^{Min/+}$ mice were randomly assigned to three groups and were given 0, 15, or 30 mg/kg of resveratrol in drinking water as an admixture for 8 weeks. (B) Left panels show representative macroscopic views of intestinal mucosa of mice treated with vehicle alone or resveratrol (30 mg/kg), respectively. Scale bars, 1 mm. Arrows indicate polyps. Right panels show representative histologic findings (H&E staining) of intestinal polyps in both mice. Insets show magnification of the squared areas, findings compatible with adenoma. Scale bars, 200 μm. (C) The number of intestinal polyps in mice treated with resveratrol and vehicle alone. $^{*}p < 0.05$, $^{**}p < 0.01$. (D) The number of intestinal polyps analyzed by size in mice treated with resveratrol and vehicle alone. $^{*}p < 0.05$ vs. vehicle.

the 15 and 30 mg/kg groups, suggesting that the 15 mg/kg dose of resveratrol was sufficiently effective. In addition, there was a trend that small polyps were more strongly inhibited by resveratrol treatment (Figure 3D).

3.5 | Inhibitory effect of resveratrol on polyp development in AOM-injected rats

We further verified the effect of resveratrol on polyp development in an AOM-induced chemical carcinogenesis rat model, which elicits similar symptoms and pathology to human colorectal carcinogenesis.[23] We administered resveratrol (10 or 100 mg/kg) or vehicle alone into AOM-injected rats and compared the number and size of polyps between the resveratrol and control groups (Figure 4A). At 15 weeks after administration of resveratrol, we confirmed the formation of polyps in the left-side colorectum using a veterinary endoscopy. Representative endoscopic images of rats in each group are shown in Figure 4B. The median number of polyps in the resveratrol-treated groups (10 and 100 mg/kg) was significantly lower than in the control group (Figure 4C). We then sacrificed the rats at 16 weeks, and observed the entire colorectum under a stereomicroscope to precisely count all of the colorectal polyps. The representative colorectal appearance is shown in Figure 4D. The histological findings of polyps from both rats were adenomas. All the polyps examined in all rats were histologically adenomas. The number of polyps in the 10 and 100 mg/kg groups were significantly lower than in the control group (median 6.5, [IQR 5.75–9.25] vs. 2.0 [1.0–3.5]; $p < 0.01$ and 3.0 [1.0–3.5]; $p < 0.01$ respectively; Figure 4E). However, no significant difference was observed between the two treatment groups. In addition, there was a tendency that small polyps were more strongly inhibited (Figure 4F).

No apparent weight loss and no decreased oral intake or clinical symptoms in each rat group was observed throughout the experiment. A common blood test revealed no abnormal findings (Table S7).

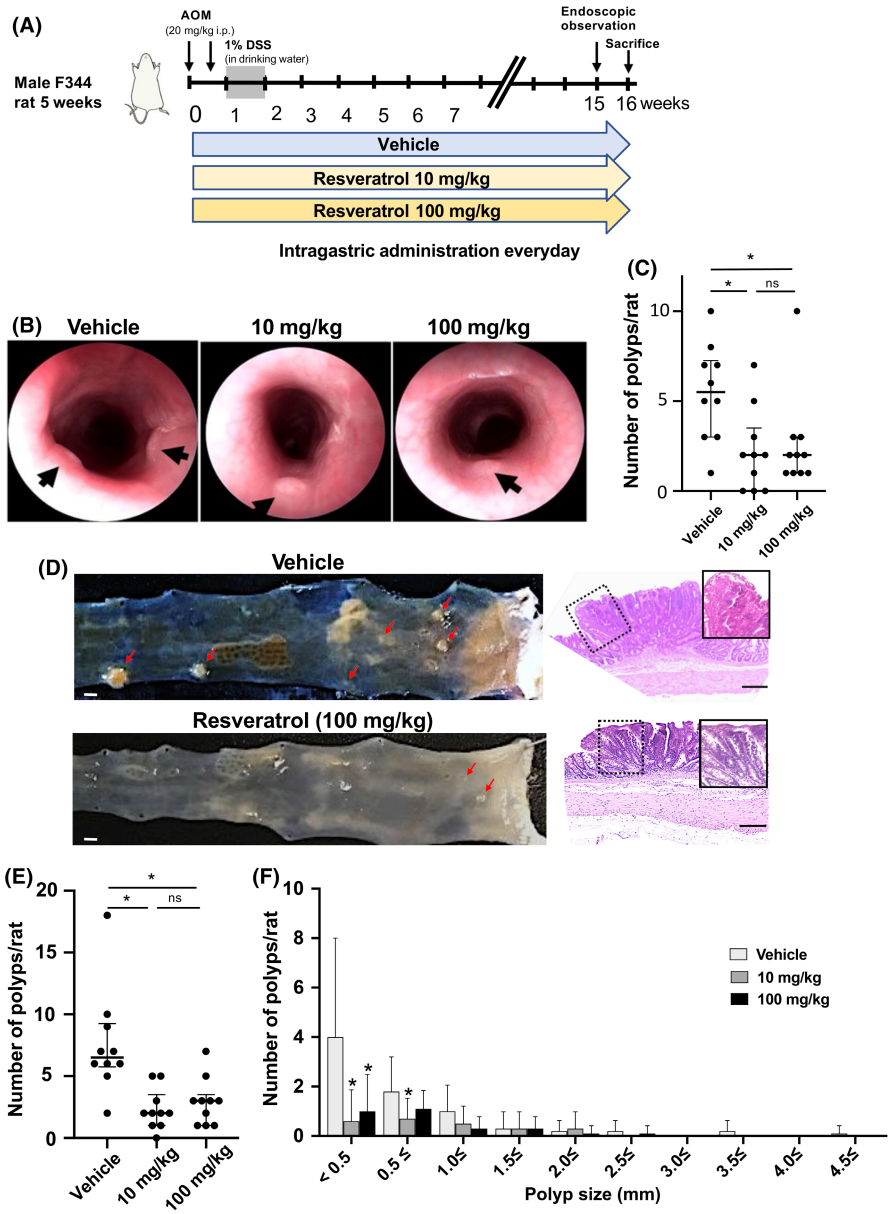


FIGURE 4 Inhibitory effect of resveratrol on polyp development in azoxymethane (AOM)-injected rats. (A) Experimental protocol. Rats in each group received intraperitoneal injections of AOM (20 mg/kg) twice a week. At 1 week later, the rats were treated with dextran sodium sulfate. Rats were given resveratrol (10 or 100 mg/kg) or vehicle alone by intragastric administration for 16 weeks. (B) Representative endoscopic image of polyps in the left-side colon of each group at 15 weeks. Arrows indicate polyps. (C) The number of polyps was counted by endoscopy. $^*p < 0.05$. (D) Left panels show representative macroscopic views of colorectal mucosa of rats treated with vehicle alone or resveratrol (100 mg/kg). Scale bars, 2 mm. Arrows indicate polyps. Right panels show representative histologic findings (H&E staining) of respective polyps. Insets show magnification of the squared areas, findings compatible with adenoma. Scale bars, 500 μm. (E) The number of polyps in rats treated with vehicle alone and resveratrol. $^*p < 0.05$. (F) The number of polyps analyzed by size in each group. $^*p < 0.05$ vs. vehicle.

3.6 | Mechanism of the inhibitory effect of resveratrol on polyps

To investigate the mechanism of the inhibitory effect of resveratrol on AOM-induced rat polyps, we performed a microarray analysis on the four adenomatous polyps in control rats and two polyps in resveratrol-treated rats (Figure 5A). Among 31,042 genes, we identified 652 whose absolute p_k^d value was higher than 0.2; 473 upregulated and 179 downregulated genes (Table S8). Additionally, we selected 894 genes, which were reportedly upregulated or downregulated in colorectal tumors from the three databases including adenoma organoid data,[24] the Molecular Signatures Database (MSig DB) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Table S9). We then detected 35 genes that were common to both groups, consisting of six upregulated and 29 downregulated genes. Among these, the gene with the highest volatility was LEF1, and its p_k^d value was −0.56 (Figure 5B).

3.7 | LEF1 expression was strongly associated with the inhibitory effect of resveratrol on adenoma organoid proliferation

Because LEF1 is reportedly an essential transcription factor of the Wnt signaling pathway, which is associated with cell proliferation in colon tumors,[17,25] we hypothesized that modulation of LEF1 might underlie the inhibitory effect of resveratrol on adenoma proliferation. We first examined the inhibitory effect of resveratrol on cell proliferation in CRAs using a BrdU assay. Resveratrol treatment significantly decreased BrdU incorporation to 35.6 ± 5.1%, 36.3 ± 8.2%, 39.3 ± 4.3%, respectively, in each of the three CRA-PDOs ($p < 0.01$ for each; Figure 6A). We next examined the effect of resveratrol on the LEF1 mRNA levels in CRA. The LEF1 mRNA level was reduced by more than 50% by resveratrol treatment in all 3 PODs from different cases ($p < 0.01$ for each; Figure 6B). Moreover, the mRNA levels of MYC, which is reportedly a target of

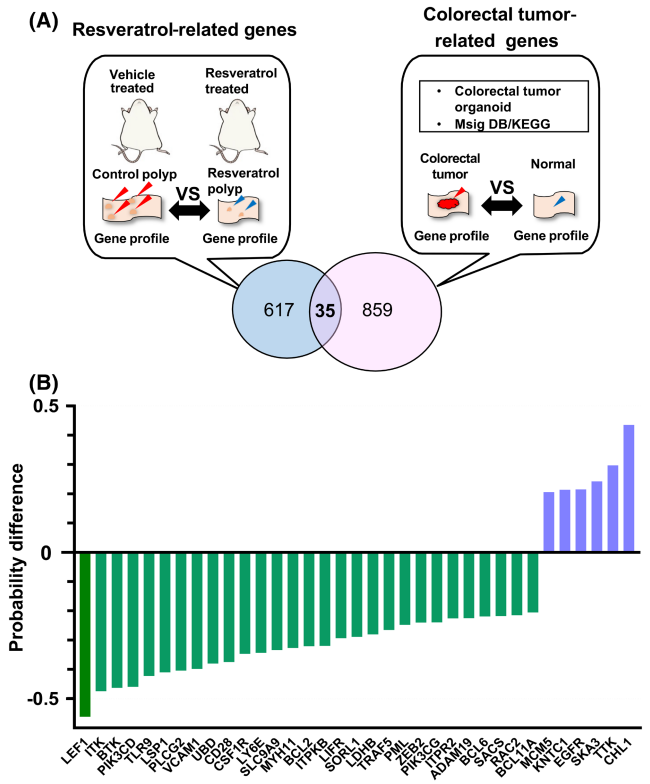


FIGURE 5 Effect of resveratrol on gene expression in colorectal adenomas. (A) Schema of gene-expression analysis. Adenomatous polyps were excised from resveratrol- or vehicle-treated rats, and 652 genes with significantly altered expression were identified by DNA microarray analysis. In addition, 894 genes were selected from three databases containing information relevant to the development of colorectal tumor organoid data[24] and colorectal tumors (MSig DB/KEGG). The Venn diagram shows 35 overlapping genes between both groups. (B) Probability difference (p_k^d) of the 35 genes. p_k^d value was calculated as described in Materials and Methods Section 2.2.

LEF1,[16] was decreased to less than half in all three CRAs-PDOs by resveratrol treatment ($p < 0.01$ for each; Figure 6C). Conversely, resveratrol did not induce any significant change in the mRNA levels of β-catenin (Figure 6D), a co-transcriptional factor of LEF1. Moreover, resveratrol did not change the nuclear β-catenin protein levels and cytoplasmic β-catenin protein levels, respectively (Figure S5). It was also confirmed that LEF-1 and MYC protein levels in CRA-PDOs were obviously downregulated by resveratrol treatment (Figure 6E).

To further clarify the mechanism of action of resveratrol against tumor development, we examined the mitogen-activated protein (MAP) kinase and Akt signaling pathways, both of which are involved in cell growth of colorectal tumors.[25,26] However, most MAP kinase relevant components, including phosphorylated MEK, ERK, and Akt in CRA-PDOs were unchanged by resveratrol treatment (Figure 6F). These results strongly suggest that resveratrol downregulates LEF1, thereby inhibiting transcription of Wnt-target genes such as MYC, leading to growth inhibition in CRA.

4 | DISCUSSION

In this study using CMAP analysis, we found 15 candidate compounds as preventive agents against CRA among 1309 FDA-approved compounds, and identified resveratrol as the most effective preventive compound by using in vitro screening with CRA-PDO. We also showed clear inhibitory effects of resveratrol on CRA development in vivo using both an Apc-deficient mouse model and a chemical carcinogenesis rat model. Finally, we clarified the underlying mechanism of the inhibitory effect of resveratrol, which targets LEF-1, a key molecule in the Wnt signal pathway. This is the first study to identify an effective preventive compound (resveratrol) against CRA comprehensively among a large number of compounds by using microarray-based CMAP analysis and in vitro screening with PDO. Because CMAP is based on data of pre-existing compounds, the chosen compound (resveratrol) can be immediately used for clinical chemopreventive studies following the drug repositioning paradigm. In fact, we are going forward with a chemopreventive trial of resveratrol against CRA in humans.

Resveratrol is a polyphenolic phytochemical found in various plants, and it is a constituent of red grape skin. Resveratrol has been reported to show anti-cancer effects and antioxidant effects in vitro experiments. Chemopreventive effects of resveratrol against breast cancer and prostate cancer as well as colorectal cancers in vitro and in vivo using animal models have been studied, although some showed contradictory results.[27] Regarding chemopreventive studies for colorectal cancer in animals, a few studies have shown that resveratrol inhibited the number of CRA in Apc-deficient mice by 27% to ~70%. However, a contradictory study has been reported showing that resveratrol had no antitumor effects in the same strain of Apc-deficient mice. This may be explained by the physicochemical properties of resveratrol; it is photo-degradable, very insoluble in water, and rapidly metabolized in plasma. However, we observed a significant inhibitory effect of resveratrol on the development of CRA not only in Apc-deficient mice but also in an AOM-induced rat carcinogenesis model. These effects may have been facilitated by administering resveratrol every day in water and by using an oral administration tube to carefully avoid light exposure.

We administered 15 mg or 30 mg of resveratrol to $Apc^{Min/+}$ mice in accordance with previous studies, with very careful handling of resveratrol. We then administered 10 and 100 mg/kg doses of resveratrol to AOM-treated rats. All doses were significantly effective, but the inhibition rate was roughly similar among the doses. These doses of resveratrol in mice and rats correspond to a human dose of 73–146 mg/60 kg/day (1.22–2.44 mg/kg/day) and 97–968 mg/60 kg/day (1.61–16.13 mg/kg/day), respectively, based on calculation of body surface area (BSA).[28] However, it has been noted that simplistic BSA scaling without careful consideration of interspecies differences including absorption, distribution, metabolism, etc. may lead to negative consequences or dangerous outcomes.[29,30] Moreover, with respect to resveratrol,

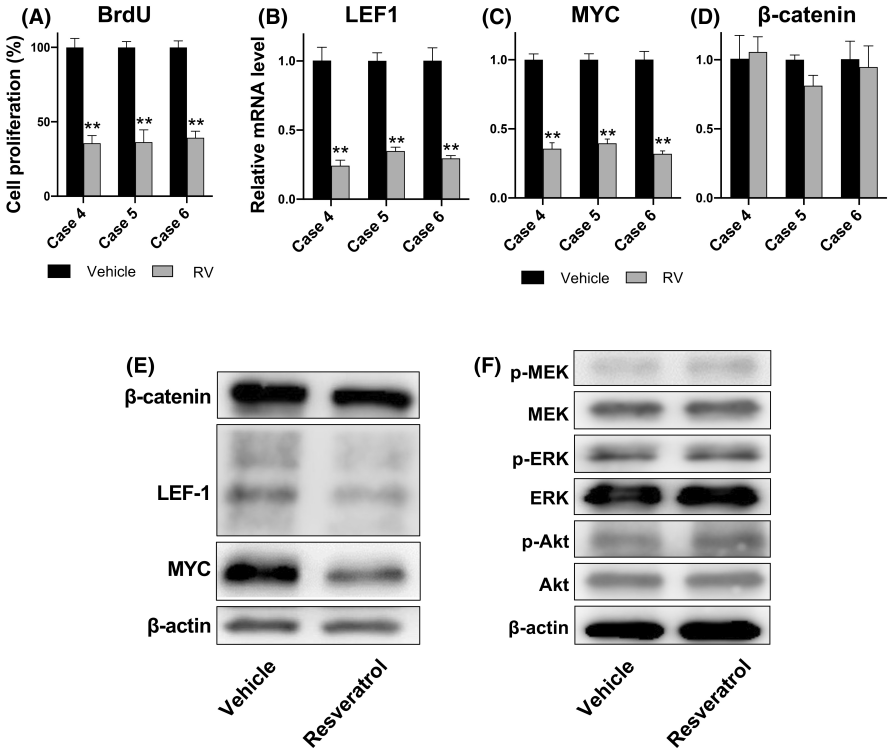


FIGURE 6 Resveratrol inhibits the proliferation of colorectal adenomas by suppressing LEF1. (A) Evaluation of cell proliferation activity by BrdU assay. Resveratrol (100 μM) was added to human CRA organoids from three cases, and BrdU uptake was examined after 6 h incubation. (B–D) The mRNA levels of LEF1, MYC, and β-catenin in CRA organoids were examined after resveratrol treatment by RT-PCR. All experiments were performed in triplicate. Error bar, ±SD. (E) The expression of LEF-1, MYC, and β-catenin in CRA organoids. (F) Phosphorylation of MEK/ERK/Akt protein in CRA organoids. CRA organoids were treated with resveratrol (100 μM) or vehicle alone for 6 h, and western blotting was performed. $^{**}p < 0.01$.

multiple studies in humans have already reported its safety and pharmacokinetic parameters. In previous clinical trials of resveratrol for Alzheimer and other diseases,[31–33] 200–500 mg/day was given orally for 6–12 months, and no severe adverse events were reported. Considering our data and previous studies, and the efficacy and cost in the trial, it would be acceptable to use a dose of 200–500 mg/day for 6–12 months. We would like to target post-polypectomy patients for chemoprevention because they have a very high risk of additional metachronous adenoma.

We found that LEF-1 was the most downregulated gene with the lowest p_k^dvalue in adenoma specimens of resveratrol-treated rats. LEF-1 directly interacts with β-catenin and serves as transcription factor LEF-1/TCF, which promotes transcription of Wnt-responsive target genes, indicating the pivotal role of LEF1 in the Wnt/β-catenin signaling pathway in CRA. LEF-1 is reported to be induced by Wnt-signal activation including Apc and β-catenin mutations (accumulation) during colorectal carcinogenesis.[34,35] Moreover, the K-RAS mutation reportedly induces LEF-1 expression in human and murine colorectal tumors.[36] Because K-RAS mutation and/or β-catenin mutation are involved in the AOM/DSS-rat model,[37,38] it appears that LEF-1 expression was induced by these gene mutations, and played an important role in adenoma formation in the rat model (Figure S6). While in $Apc^{Min/+}$ mice, LEF1 expression was induced by Wnt-signal activation. The assumed mechanisms of adenoma formation and resveratrol effect in Apc $^{Min/+}$ mice, AOM/DSS rats, and humans are summarized in Figure S6. Similarly, LEF1 expression is induced in human adenoma mainly due to Wnt-signal activation. Our results clearly indicated that resveratrol inhibited LEF1 mRNA and protein levels and reduced transcription of Wnt-related genes (MYC). Considering previous studies,[16,17,34,39–42] we suggest that resveratrol inhibits LEF1 expression and thereby suppresses the transcription of Wnt-related genes, leading to the inhibition of adenoma formation, although there is a contradictory study regarding LEF1 function.[35]

It is generally accepted that in the early step of CRA development, Apc mutations (two-hit) first occur, followed by additional gene alterations including RAS. That is, the smaller adenomas have fewer gene mutations in addition to the Apc mutation. This may explain our observation that resveratrol inhibited smaller polyps more efficiently than larger polyps in animal experiments. It has been reported that resveratrol inhibited COX-2 expression in colorectal mucosa, which attenuates the development of CRA.[43] Since COX-2 is a downstream player in the Wnt/β-catenin signaling pathway, we infer that the inhibitory effects of resveratrol on LEF-1 are responsible for downregulation of COX-2 and subsequent tumor growth inhibition.

We focused our mechanistic studies exclusively on LEF-1 as it had the lowest p_k^d between the adenomas in resveratrol- and vehicle-treated rats. However, we identified other genes with relatively high absolute p_k^d values, including IL2-inducible T-cell kinase (ITK), Bruton's tyrosine kinase (BTK), and phosphatidyl-inositol-4,5-bisphosphate 3-kinase catalytic subunit delta (PIK3CD). All these genes are reportedly overexpressed in colorectal cancer and involve colorectal carcinogenesis. Inhibitors of these gene products are currently under preclinical/clinical investigation for cancer treatment. Therefore, inhibition of these genes might contribute to the chemopreventive effects of resveratrol.

LEF1 is a key gene in the Wnt/β-catenin signaling pathway because it binds accumulated β-catenin in the nucleus and promotes Wnt-responsive gene transcription, driving cell growth and malignant progression. The Wnt/β-catenin signaling pathway is reportedly activated in many tumors other than CRA. Therefore, it is expected

that resveratrol would be an effective preventive agent for many these cancers.

In conclusion, resveratrol was identified as the most effective compound for chemoprevention against CRA using CMAP analysis and in vitro screening with CRA-PDO. Resveratrol effectively inhibited CRA development in Apc-deficient mice and in chemical carcinogenesis rat models by suppressing the expression of LEF1 in the Wnt signaling pathway.

AUTHOR CONTRIBUTIONS

H.W., Y.S., K.O., and T.T. designed the research. H.W., K.O., S.F., M.B., T.K., and Y.M. performed experiments. H.W., N.M., K.H., Y.S., and M.M. analyzed the data. H.W., K.H., H.M. and Y.S. performed statistical analysis. H.W., Y.S., and T.T. drafted the manuscript. T.T. obtained funding. All authors contributed to the writing and approved the final manuscript.

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DISCLOSURE

The authors have no conflict of interest.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Tokushima University Hospital (Approval number; 2250).

INFORMED CONSENT

Written informed consent was obtained from all patients.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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