# 金沢大学がん進展制御研究所
# 腫瘍制御（旧：遺伝子診断）研究分野

研究のあゆみと業績 2021

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## Annual Report 2021

**Division of Translational and Clinical Oncology**
**(previously: Division of Diagnostic Molecular Oncology)**
**Cancer Research Institute, Kanazawa University**
**Kanazawa, Japan**

**2021 年 12 月**
**December 2021**

# まえがき

出典：https://jp.quora.com/

今年６月末、オリンピック東京2020がまさに強行されようとしていた頃に、私たちの研究室が開設満20年を迎えようとしていました。本誌のまえに編集した「研究のあゆみと業績 Vicennial Report 2016−2020」にも書いたように、今年7月に生まれた2nd grandkidがやがて成人するくらいの年月に相当します。これがはたして長かったのか、短かったのか？私にはちょうどよかったようです。ただ、昨夏から楽しみにして、７月に予定していた共同研究セミナーと記念会を11月末に延長しても、開催がかないませんでした【附記１】。

毎年、日本漢字能力検定協会（京都市東山区）が全国から募った、その年の世相を漢字1文字で表現する「今年の漢字」を選定するのが年末の恒例です。2021年は「金」が選ばれ、清水寺の森 清範貫主の揮毫により発表されました。私は、もしウィルスあるいはウィルス感染症を一文字で現わせる漢字があれば選ばれたのではないかと変なことを想像し、ネットをみてみました。多くのサイトでは、病毒や濾過性病毒とか書かれています。これではピンとこないと思っていたところ、冒頭のような造字をみかけました。20年の節目に当たる今年はまたしても、隣国が発生源といわれる新たな疫病に振りまわされてしまい、この造字が「金」に重くのしかかる一年でした。

出典：エンタメRBB（https://www.rbbtoday.com/）

記念会ができなかったことは悔やまれるとはいえ、出不精の私にはわりと快適な１年でした。今春は、２名の大学院が学位を獲り【第５頁、附記３】、学位を目指す第一線外科医が９月から研究生に在籍しています。４月には２名のポスドクが採用され、少し活気がでてきました。あと、がん研News Letterの「共同研究者の紹介」欄に、もう長年（2013年～）になる香川大学病理学：松田陽子さんとの交流を交換日記のようなかたちで紹介したことも、殺伐としたこの１年ではほっとできることです【附記２】。私自身は年末になって、日本消化器内視鏡学会から次回（2022年度）の北陸支部セミナーを担当するように指名されました。2008年には消化器・腫瘍外科から身を引いたものの、消化器内視鏡専門医、指導医としてなんとか持ちこたえている自身に課せられた任務と受けとめ、承諾しました。また仲間に助けてもらって、なんとか役目をはたすよう努めます。

この年末には、妙な変異が現れたようで、この先が案じられます。それでも、2020年末の「まえがき」にも云いました。さあ皆さん、元気を出してすばらしい2022年を迎えましょう。それでは、これからもどうぞよろしくお願いします。

2021年12月 年末

源 利成

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HP（更新中）: http://www.kanazawa-u.ac.jp/~ganken/shuyoseigyo/index.html

# 【目次】

研究スタッフ …… 3

共同研究者 …… 4

2021 年のあゆみとできごと …… 5

研究分野と活動の概要 …… 6

研究費 …… 8

研究業績 …… 9
　論文発表
　学会発表
　その他（地域・社会貢献など）

知的財産，関連活動など …… 11

メディア・新聞報道など …… 11

【附記１】腫瘍制御共同研究セミナーと 20 周年記念会（誌上開催） …… 13

【誌上開催：七夕の会】酒呑もおよ！ …… 28

【附記２】金沢大がん研 News Letter（抜粋）：共同研究者紹介 …… 29

【附記３】学位論文: 2021 年3月 修了 …… 32

## 【研究メンバー】(2021 年 12 月現在)

| | | |
|---|---|---|
| 教授 | 源　利成 | 金沢大学附属病院がんセンター(併任) |
| 助教 | 堂本貴寛(たかひろ) | |
| 博士研究員 | 上原将大(まさひろ) | 2021 年 4 月～ |
| | Diliraba Bolidong(ディリレバ ボリドン) | 2021 年 4 月～ |
| 大学院 | 竹中　哲(さとし) | 本学大学院医学系研究科 消化器・腫瘍・再生外科学 |
| (博士課程) | 中西宏佳(ひろよし) | 本学大学院医薬保健学総合研究科 腫瘍制御学(～3 月) |
| | 上原将大(まさひろ) | 本学大学院医薬保健学総合研究科 腫瘍制御学(～3 月) |
| | 太田亮介 | 本学大学院医薬保健学総合研究科 腫瘍制御学 |
| 研究生 | 小竹優範(まさのり) | 厚生連高岡病院 外科(9 月～) |
| 医学類5年 | 守田周平(もりた) | メディカルリサーチトレーニングプログラム(MRT) |
| 同4年 | 石田　岳(たかし) | メディカルリサーチトレーニングプログラム(MRT) |
| 研究支援員 | 浅香敦子 | 研究支援推進員 |
| 研究協力員 | 旭井亮一(あさい) | (株)凸版印刷研究所 |
| | 川島篤弘 | 独立行政法人国立病院機構金沢医療センター 臨床検査科 |
| | 藤沢弘範(ひろのり) | 独立行政法人国立病院機構金沢医療センター 脳神経外科 |
| | 横井健二 | 米国 ヒューストンメソジスト研究所ナノ医療部門 |
| | 島崎猛夫(しまさき) | 金沢医科大学総合医学研究所, 消化器内科 |
| | 東　朋美(ひがし) | 金沢大学医薬保健研究域医学系 環境分子応答学(衛生学) |
| | 笠島里美(かさしま) | 金沢大学医薬保健研究域保健学系 検査技術科学 |
| | 宮下勝吉(かつよし) | 福井県立病院 脳神経外科 |
| | 中島日出夫(なかじま) | 上尾中央総合病院 腫瘍内科 |
| | 下崎真吾 | 医療法人社団 下崎整形外科医院 |
| | 澤田　武 | 名古屋市立大学消化器・内分泌内科学 |
| | 太田亮介 | 金沢大学大学院医薬保健学総合研究科 腫瘍制御学 |
| | Diliraba Bolidong(ディリレバ ボリドン) | 腫瘍制御研究分野(2021 年 1 月～2021 年 3 月) |
| 共同研究者 | 塚　正彦(づか) | 金沢大学医薬保健研究域医学系 法・社会医学(法医学) |

**【共同研究者】（2021 年現在で共同研究が稼動および予定しているもの. 敬称略）**

| | | |
|---|---|---|
| 教授 | 中田光俊（なかだ） | 金沢大学医薬保健研究域医学系 脳機能制御学/脳神経外科学 |
| 助教 | 中村慶史（けいし） | 金沢大学医薬保健研究域医学系 消化器・腫瘍・再生外科学 |
| 教授 | 土屋弘行 | 金沢大学医薬保健研究域医学系 機能再建学／整形外科学 |
| 特任教授 | 山本憲男 | 金沢大学医薬保健研究域医学系 機能再建学／整形外科学 |
| 教授 | 竹村博文 | 金沢大学医薬保健研究域医学系 先進総合外科学 |
| 教授 | Richard Wong | 金沢大学理工学研究域自然システム学系／ナノ生命科学研究所 |
| 教授 | 滝野隆久 | 金沢大学国際基幹教育院 |
| 教授 | 元雄良治（よしはる） | 金沢医科大学 腫瘍内科学（～3 月） |
| 准教授 | 宮下知治 | 金沢医科大学 一般・消化器外科学／腫瘍外科学 |
| 教授 | 伊藤　透 | 金沢医科大学 消化器内視鏡学 |
| 教授 | 石垣靖人 | 金沢医科大学総合医学研究所 |
| 分野長 | 土原一哉（かつや） | 国立がん研究センター 早期・探索臨床研究センター トランスレーショナルリサーチ分野 |
| 教授 | 曽我朋義 | 慶應義塾大学 先端生命科学研究所 |
| 教授 | 竹田　扇（せん） | 帝京大学医学部 解剖学 |
| 講師 | 吉村健太郎 | 山梨大学大学院医学工学総合研究部 解剖学講座 細胞生物学 |
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| 教授 | 佐々木泰史（やすし） | 札幌医科大学 教養教育研究部門 医療人育成センター |
| 准教授 | 久保田英嗣（えいじ） | 名古屋市立大学消化器・内分泌内科学 |
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| 研究員 | 小泉恵太 | 上尾中央総合病院 |
| | 紙（かみ） 健次郎 | （株）ヒューマンメタボロームテクノロジーズ |
| 教授 | Ze'ev Ronai | Sanford Burnham Prebys Medical Discovery Institute, La Jolla, USA |
| 教授 | Andy Giraud | 豪州オーストラリア王立小児病院 |
| | Louise M. Judd | 豪州オーストラリア王立小児病院 |
| | Trevelyan R. Menheniott | 豪州オーストラリア王立小児病院 |
| | Phil Sutton | 豪州オーストラリア王立小児病院 |
| 准教授 | Serge Y. Fuchs | ペンシルヴェニア大学 生物学 |
| 准教授 | Barry Iacopetta | 西オーストラリア大学 腫瘍学 |

## 【2021 年のあゆみとできごと】

2021年 1月　1日　・Dilireba Bolidongさんが研究協力員に採用された。

2021年 1月14日　・源 利成が第 29 回日本癌病態治療研究会 パネルディスカッション：癌治療抵抗性の機序解明に基づく新たな治療法の開発で、基調講演した。
課題：GSK3β のがん生物学とがん治療抵抗性の一隅

2021年 3月 31日　・中西宏佳君と上原将大君が大学院を修了し、医学博士の学位を取得。
【附記3】
課題（中西）：Significance of gene mutations in Wnt signaling pathway in traditional serrated adenomas of the colon and rectum. *PLoS One* 15 (2): e0229262, 2020.
課題（上原）：Glycogen synthase kinase 3β participates in acquired resistance to gemcitabine in pancreatic cancer. *Cancer Sci* 111 (12): 4405-16, 2020.

源と中西宏佳君

上原将大君と源

2021年 4 月 1 日　・上原将大君とDilireba Bolidongさんが博士研究員に採用された。

2021 年 7 月 3 日　・腫瘍制御研究分野共同研究セミナーと 20 周年記念会の開催予定を延期した。
【附記1】

2021年10月　1日　・小竹優範君（厚生連高岡病院 外科）が研究生に在籍。

2021年11月28日　・腫瘍制御研究分野共同研究セミナーと 20 周年記念会の開催を再延期した。
【附記1】

2021年11月28日　・源　利成が、日本消化器内視鏡学会北陸支部評議員会で第 31 回日本消化器内視鏡学会北陸セミナーの会長に指名された。

## 【研究分野と活動の概要】

当研究分野は 1998 年に遺伝子診断の旧称で開設され、その後 23 年間にわたって、消化器がんを中心にがんの多様な生物病態と腫瘍外科学的特性について、基礎と臨床を関係づけるかたちの研究を指向している。そして、その成果を難治がんや希少がんの病態解明と制御に応用するために、学内外のグループと共同研究を進める。

## Division Summary

The mission of our division centers on laboratory and clinical research to develop the novel strategies and modalities for diagnosis and treatment of the gastrointestinal, refractory and rare cancer types including glioblastoma, bone and soft tissue sarcomas. Research projects are based on biological characteristics of individual tumor types that are relevant to their invasive and metastatic potential, resistance to therapy, recurrence and outcome of patients. Our current efforts are focused on (1) research and development of the cancer therapy by targeting aberrant glycogen synthase kinase (GSK)3β; (2) understanding of malignant phenotypes of cancer by investigating pathological metabolic properties (eg., aerobic glycolysis, tumor-promoting autophagy); and (3) biological basis of gastrointestinal and refractory cancer, all for clinical translation. We have been also establishing the tissue material resources of human stomach and colorectal cancer for our own projects described above as well as for studies collaborating with our institutional and many other research groups. In an immediate couple of years, we have obtained the preliminary results indicating the putative roles of tumor GSK3β as a molecular hub that connects the pathways responsible for tumor invasion and resistance to therapy, thus enforcing its potential as a major cancer therapeutic target. We are extending this project toward investigation of the putative roles for GSK3β in promoting esophageal squamous cell carcinoma (ESCC; the major type of esophageal cancer in Asia and Japan) and pancreatic neuroendocrine neoplasms as well as in acquiring chemoresistance in pancreatic cancer. We also have been trying to develop cellular and mouse models predisposing to ESCC by CRISPR-Cas9-based genome editing of the metabolic enzymes including glycogen synthase and GSK3β. In addition to these projects, we have clarified the genetic and epigenetic characteristics of traditional serrated adenoma and duodenal non-ampullary adenoma and adenocarcinoma.

### ＜2021 年の研究成果，進捗状況及び今後の計画＞

#### 1．glycogen synthase kinase（GSK）3β 阻害によるがん治療法の研究、開発

Wnt 経路抑制因子と認識されている GSK3β が固有の分子経路を介して、がんの悪性形質を推進することを系統的に示してきた。そして、GSK3β 阻害のがん治療効果を細胞

レベルと担がん動物で実証した。また，学内外の外科系グループと連携し、膵がん、膠芽腫や骨軟部肉腫などの難治、希少がんで高活性を示す GSK3β が、腫瘍浸潤性と治療（抗がん剤，放射線）不応性の悪性形質を連結することを見出した。一連の研究をもとに、GSK3β 阻害薬品の転用と抗がん剤を併用するがん治療法を開発し、再発膠芽腫（附属病院脳神経外科）と進行膵がん（金沢医科大学病院）を対象とする医師主導臨床研究によりその安全性と抗腫瘍効果を検証した。2021 年には食道扁平上皮がんと抗がん剤耐性獲得膵がんに対する GSK3β 阻害の治療効果とメカニズムを明らかにした。また、GSK3β 阻害によるがん治療の概念実証のため、膵内分泌腫瘍（PNET）の共同研究に加えて、米国で臨床試験中の GSK3β 阻害剤 9-ING-41 を開発した Actuate 社と共同で治療耐性膵がんの前臨床試験を計画した。

### 2. がんの代謝特性にもとづく悪性形質の解析研究

GSK3β はグリコーゲン代謝を制御する。また、特定の中間代謝産物が解糖経路と自食作用の接点になるとが知られている。そこで大腸がんを中心に、がん固有の糖代謝（Warburg 効果）に関わる触媒酵素やがん促進性自食作用に対する GSK3β の統合的解析を進めている。これとは別に、内視鏡的にヨード不染を特徴とする食道の扁平上皮発がん初期の生物学的特性は細胞内グリコーゲンの減少，消失である。そこで、患者由来の正常食道扁平上皮細胞とマウスを対象に、グリコーゲン合成酵素と GSK3β のゲノム編集による食道扁平上皮易発がん状態の誘発を試みる研究を 2018 年に開始し、目的の改変マウスを作出して、経過観察を継続している。また 2021 年には、GSK3β がエネルギーセンサー酵素 AMPK をリン酸化により不活性化するという知見をもとに、食道扁平上皮がんにおける両酵素の関連解析を始めた。

### 3. ヒト消化管がん組織バンクを中心とする大腸がんの分子病理学的研究

消化管がん研究や臨床研究の基礎資源として 2008 年から本事業を開始し、2010 年にこの事業を当研究所ヒトがん組織バンクに継承し現在に至っている。この組織資源の共同利用促進のために、日本医療研究開発機構ゲノム医療支援サイト（http://www.biobank.amed.go.jp/biobank/index.html）に情報公開している。山梨大学：竹田 扇らが開発した大気圧イオン化法-質量分析を用いて、大腸がん質量分析診断法開発の共同研究を継続している。大腸組織の質量分析パターンをもとに特有の統計解析と機械学習を組み合わせて非がん/がんの判別（診断）アルゴリズムを構築し、90%以上の感度と特異度による判別を可能にした（論文作成予定）。現在、島津製作所基盤技術研究所と共同で、大腸がんの質量分析ー内視鏡診断法の内視鏡デバイス開発に着手した。組織バンク検体を利用して、名古屋市立大学（十二指腸腺腫，早期がん）、香川大学（大腸がんテロメア解析）と共同研究を開始した。

**【研究費】（2021 年 1 月以降の新規，継続，分担と連携を含む外部資金の獲得状況）**

| 研究種目・期間（課題番号） | 研究代表者 | 研究分担者 連携研究者 | 研究課題 | 研究経費 |
|---|---|---|---|---|
| 2020年－2022年度 科学研究費補助金（基盤研究Ｃ）(20K09100) | 宮下知治 | 太田哲生，源 利成，ほか | GSK3βを基軸とした食道発癌機構の解明と新規治療戦略の開発 | 直接経費 3,200,000 円 間接経費 960,000 円 |
| 2019年－2021年度 科学研究費補助金（基盤研究Ｂ）(19H03727) | 源 利成 | Richard Wong 宮下知治 | 大腸がんの糖代謝改変と細胞核分裂機構を繋ぐ分子経路の解明とがん制御法開発への応用 | 直接経費 13,400,000 円 間接経費 4,020,000 円 |
| 2019年－2021年度 科学研究費補助金（基盤研究Ｃ）(19K07710) | 堂本貴寛 | | GSK3βによるがん促進的糖代謝特性の解明と制御への応用 | 直接経費 3,400,000 円 間接経費 1,020,000 円 |
| 2019年－2021年度 科学研究費補助金（基盤研究Ｃ）(19K08367) | 太田亮介 | 澤田 武，源 利成 | RNAシーケンスによる大腸鋸歯状腺腫の発癌機構の解明と分子標的治療の基盤確立 | 直接経費 3,400,000 円 間接経費 1,020,000 円 |
| 2019年－2021年度 科学研究費補助金（基盤研究Ｃ）(19K08643) | 澤田 武 | 太田亮介，ほか | 表在性非乳頭部十二指腸腫瘍の発癌機構の解明と，進展を予測する内視鏡診断体系の確立 | 直接経費 3,400,000 円 間接経費 1,020,000 円 |
| 2021年度金沢大学がん進展制御研究所共同研究（一般） | 佐々木泰史 | 澤田 武，源 利成，ほか | 非乳頭部十二指腸腫瘍におけるERBB受容体ファミリーの解析と治療標的の探索（一般：新規） | 350,000 円 |
| 2021年度金沢大学がん進展制御研究所共同研究（若手） | 成澤裕子 | 松田陽子，源 利成，ほか | 2光子励起顕微鏡を用いた膵癌細胞の血行性肝転移と加齢との関連の解明（若手：継続） | 350,000 円 |
| 2021年度金沢大学がん進展制御研究所共同研究（若手） | 北村浩一 | 松下一之，源 利成，ほか | 消化器・難治がんのリボソーム生合成の新規メカニズム解明と診断，治療法への応用（若手：継続） | 350,000 円 |
| 2021年度金沢大学がん進展制御研究所共同研究（一般） | 久保田英嗣 | 澤田 武，源 利成，ほか | 大腸癌における循環腫瘍DNAを用いた抗EGFR抗体薬耐性の検出と病状モニタリングの確立（一般：継続） | 300,000 円 |
| 2021年度金沢大学がん進展制御研究所共同研究 | 宮下知治 | 上原将大，堂本貴寛，源 利成 | GSK3β/STAT3経路を基軸とする膵神経内分泌腫瘍の病態解明と治療法開発（一般：継続） | 300,000 円 |
| 2021年度金沢大学がん進展制御研究所共同研究 | 小泉惠太 | 中島日出夫 堂本貴寛，源 利成 | ハイパーサーミアにおけるFam107B-GSK3βの作用機序解析とハイパーサーミア/GSK3β阻害剤併用の可能性への探求（一般：継続、採択後辞退） | 250,000 円 |
| | | | 期間の総額（間接経費を含む） | 36,490,000 円 |

## 【研究業績】

### Ⅰ．論文発表 [Impact factor 2020/2021]

・英文総説，著書

なし

・英文原著

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2. Kotake M, Bando H, Kaneko M, Takemura H, Minamoto T, Kawakami K. LOH of the thymidylate synthase locus in combination with genotype has prognostic and predictive significance in colorectal cancer. *Mol Clin Oncol* 15: 235, 2021. doi: 10.3892/mco. 2021.2398 [unavailable]

### Ⅱ．学会発表

・国際学会

1. Takahiro Domoto, Masahiro Uehara, Satoshi Takenaka, Dilireba Bolidong, Tatsuhiko Furusawa, Osamu Takeuchi, Tomoharu Miyashita, Toshinari Minamoto. GSK3β interconnects the malignant properties in therapy-resistant pancreatic cancer. The 10th International Conference of the International Society of Gastroenterological Carcinogenesis (ISGC 2021), November 26 (Fri), 27 (Sat), 2021, Gifu, Japan (WEB).
2. Masahiro Uehara, Takahiro Domoto, Satoshi Takenaka, Dilireba Bolidong, Osamu Takeuchi, Tomoharu Miyashita, Toshinari Minamoto. Glycogen synthase kinase (GSK) 3β participates in acquired resistance to gemcitabine in pancreatic cancer. The 10th International Conference of the International Society of Gastroenterological Carcinogenesis (ISGC 2021), November 26 (Fri), 27 (Sat), 2021, Gifu, Japan (WEB).
3. Dilireba Bolidong, Takahiro Domoto, Masahiro Uehara, Hemragul Sabit, Tomoyuki Okumura, Yoshio Endo, Mitutoshi Nakada, Tomoharu Miyashita, Richard W. Wong, Toshinari Minamoto. Potential therapeutic effect of targeting glycogen syntase kinase (GSK)3β in esophageal squamous cell carcinoma (ESCC). The 10th International Conference of the International Society of Gastroenterological Carcinogenesis (ISGC 2021), November 26 (Fri), 27 (Sat), 2021, Gifu, Japan (WEB).

・国内(全国)学会

4. 松田陽子，成澤裕子，葉 娟娟，山川けいこ，向井裕理，谷元ミサ，横平政直，本間尚子，源 利成．長いテロメアを有する腫瘍の頻度と予後との関連．Incidence and prognostic relevance of long telomere cancers. 第110回日本病理学会総会，2021年4月22日(木)－24日(土)，京王プラザホテル，東京．
5. 岩嶋友紀，羽澤勝治，源 利成，Richard Wong．核膜孔複合体による gene-gating 形成機序．日本生化学会北陸支部第39回大会，2021年6月5日(土)，WEB．

6. Takahiro Domoto, Satoshi Takenaka, Masahiro Uehara, Dilireba Bolidong, Tatsuhiko Furukawa, Tomoharu Miyashita, Toshinari Minamoto. Glycogen synthase kinase (GSK) 3β renders pancreatic cancer acquiring resistance to gemcitabine via the STAT3 activation. 堂本貴寛, 竹中 哲, 上原将大, ボリドン ディレレバ, 古川龍彦, 宮下知治, 源 利成. GSK3β は STAT3 の活性化を介して膵がんのゲムシタビン耐性獲得に寄与する. 第 80 回日本癌学会学術総会, 2021 年 9 月 30 日（木）－10 月 2 日（土）, パシフィコ横浜.

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8. Masaharu Hazawa, Toshinari Minamoto, Takeshi Suzuki, Richard Wong. NUP153 drives oncogenic TP63 expression through liquid-liquid phase separation mediated gene-gating in squamous cancer. 羽澤勝治, 源 利成, 鈴木 健之, ウォング リチャード. NUP153 は相分離を介した Gene-gating 機構で TP63 の発現を誘導する. 第 80 回日本癌学会学術総会, 2021 年 9 月 30 日（木）－10 月 2 日（土）, パシフィコ横浜.

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11. ディリレバ ボリドン, 堂本貴寛, 上原将大, アムラ サビット, 奥村知之, 遠藤良夫, 中田光俊, 宮下知治, リチャード ウォング, 源 利成. GSK3β 阻害による食道扁平上皮がんの治療効果とメカニズム. 第 32 回日本消化器癌発生学会総会, 2021 年 11 月 26 日（金）, 27 日（土）, 岐阜市（WEB）.

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O5-2. Takahiro Domoto, Masahiro Uehara, Satoshi Takenaka, Dilireba Bolidong, Tatsuhiko Furusawa, Osamu Takeuchi, Tomoharu Miyashita, Toshinari Minamoto. GSK3β interconnects the malignant properties in therapy-resistant pancreatic cancer.

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松田尚久：内視鏡を大腸がん検診の modality として用いるには

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**・その他（講演, 社会・地域貢献を含む）**

源 利成. 基調講演：GSK3β のがん生物学とがん治療抵抗性の一隅. 第 29 回日本癌病態治療研究会 パネルディスカッション：癌治療抵抗性の機序解明に基づく新たな治療法の開発, 2021 年 1 月 14 日（木）, 15 日（金）, G メッセ群馬, 高崎市.

## Ⅲ. 学会開催

該当なし

## IV. 究成果による知的財産権の出願状況

該当なし

## V. 新聞, 報道など

該当なし

## 【附記1】腫瘍制御共同研究セミナーと20周年記念会：誌上開催

**Division of Translational & Clinical Oncology**
**Cancer Research Institute**
**Kanazawa University**

2021年5月10日(月)

ご関係の皆さま：

皆さんそれぞれにお変わりなく、元気に楽しい毎日を過ごされていることでしょう。いつもお世話になりありがとうございます。

いまからおよそ2か月後の7月3日(土)に開催を予定している共同研究セミナー2021と、同日夕にミキサー形式で併催する「七夕の会2021」を案内したくお便りしました。当研究分野の20周年と、私の研究室主任として20歳(はたち)の誕生日に相当します。詳細は別紙の開催案をご覧ください。それぞれ異なる領域で活躍されている4名の先生方の講演に続いて、金沢医科大学：前学長の勝田省吾先生にご挨拶をいただくプログラムを計画しています。また、時間が許せば、この20年間の研究室の記録や雑感などを講演の合い間に紹介いたします。参加費の暫定額をお知らせします。

参加費　　常勤の方　　15,000円
　　　　　それ以外の方　8,000円
＊当日はお釣りを用意しませんので、ご協力ください。

医療従事者とご高齢の方々を中心にワクチン接種が始まったとはいえ、世のなかが平穏になる兆しは見通せません。このため、皆さんへの案内どころかセミナーやミキサーの開催自体を逡巡しています。このような状況ながら、当日のご参加について皆さんのご意向、ご都合や忌憚のないご意見を、**5月31日(月)**を目途にファクスあるいはメールでご返信ください。ご返信のときに、ファクス番号と、メールを日常的にお使いであればアドレスをお知らせくださると助かります。皆さんのお返事を参考にして、6月中旬までに当日の開催を再考し、開催あるいは中止の方針が決まりしだいお知らせいたします。あやふやな案内になってすみません。状況をご理解のうえ、ご協力いただければありがたく思います。

それでは、皆さんがこれからもお健やかに過ごされるよう願っています。そして、願わくは当日、多くの皆さんがご参加くださることを心待ちにしています。どうぞよろしくお願いします。

源 利成
金沢大学がん進展制御研究所 腫瘍制御
金沢大学附属病院がんセンター（併任）

消滅が危惧されている千里浜海岸

**Toshinari Minamoto, M.D., Ph.D.**
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源 利成
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金沢大学附属病院 がんセンター
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開催延期の案内

**Division of Translational & Clinical Oncology**
**Cancer Research Institute**
**Kanazawa University**

2021年5月31日(月)

ご関係のみなさん：

春の連休前に共同研究セミナーの開催をお知らせして、ひと月以上になります。この間（かん）にパンデミックは変容（変異）し、ワクチン接種が混乱しています。それでも皆さんそれぞれにお変わりなく、お元気に毎日を楽しまれていることでしょう。

先の案内に対して、皆さんから参加の可否とともに温かいお便りとご意見をいただきました。ありがとうございます。皆さんのお返事とパンデミックの現状を勘案し、7月3日（土）に開催を予定している共同研究セミナー2021と、同日夕に併催する「七夕の会2021」を **2021年11月27日（土）に延期**いたします。研究会のプログラムや参加費などは現状と変更はありません。なお、今回のセミナーの適時性は大切でありながら、オンライン開催がセミナーや交流会の趣旨にそぐわないことから、これ以上の延期はありません。9月下旬をめどに当日の開催を再考し、開催あるいは中止の方針が決まりしだい、あらためてお知らせします。また、あいまいな案内になってしましました。とりあえず、11月27日 (土) の午後の予定を確保していただけるとありがたく思います。

このあとも、皆さんがお健やかに過ごされるよう願っています。

源　利成
金沢大学がん進展制御研究所 腫瘍制御
金沢大学附属病院がんセンター（併任）

消滅が危惧されている千里浜海岸

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源　利成
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## 開催中止の案内

Division of Translational & Clinical Oncology
Cancer Research Institute
Kanazawa University

2021 年 10 月 18 日(月)

Re: 共同研究セミナー開催中止と誌上開催の案内

ご関係のみなさん：

５月末に共同研究セミナー開催の延期をお知らせして、４か月以上になり、当地では紅葉を迎えています。奇しくも東京 2020 と歩調を合わせたかのようなパンデミックは休止し、世のなかは平穏になりつつあります。かりにこれが錯覚であるにしても、皆さんそれぞれにお変わりなく、お元気に毎日を楽しまれていることでしょう。

先般、ホテル日航金沢の担当の方と話しました。そしてその内容は、11 月 27 日（土）に先延ばしした共同研究セミナー2021 は開催できるものの、同日夕の「七夕の会 2021」はマスクをして、手酌、黙飲食なら併催可というものでした。これでは楽しくない、面白くない、自分が描いていたセミナーや交流会の趣旨にそぐわないという身勝手な思いから、中止という決断に至りました。当日の予定を確保していただいている皆さんにお詫びします。それでも、セミナーの発表や司会をお引き受けいただいた方々のご協力を無にしないよう、誌上開催として「研究のあゆみと業績 2021」に記録を掲載します。

このあとパンデミックがどうなろうとも、皆さんがお健やかに過ごされるよう願っています。

源 利成
金沢大学がん進展制御研究所 腫瘍制御
金沢大学附属病院がんセンター（併任）

消滅が危惧されている千里浜海岸

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－ 誌上（仮）開催 －

# 金沢大学がん進展制御研究所腫瘍制御学 共同研究セミナー 2021

― 七夕の会 2021 ―

日時：2021 年 11 月 27 日（土）13:30～19:00
場所：ホテル日航金沢
　　　〒920-0853 金沢市本町 2-15-1　電話：076-234-1111
主催：金沢大学がん進展制御研究所腫瘍制御学
共催：七夕の会

事務局：
　堂本貴寛
　金沢大学がん進展制御研究所腫瘍制御学
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# 金沢大学がん進展制御研究所腫瘍制御学共同研究セミナー 2021

日時：2021 年 11 月 27 日（土）13:30〜19:00
場所：ホテル日航金沢　〒920-0853 金沢市本町 2-15-1　電話：076-234-1111
主催：金沢大学がん進展制御研究所腫瘍制御学

| 時間 | プログラム（発表と討論）　敬称略 |
|---|---|
| 13:30- | 受付け |
| | |
| 14:25- | 開会　源　利成　金沢大学がん進展制御研究所 腫瘍制御、七夕の会 |
| | |
| 14:30-14:55 | 骨軟部腫瘍の臨床と研究 |
| 司　会 | 保下崎真吾　医療法人社団 下崎整形外科医院／腫瘍制御学 |
| 発表者 | 阿部健作　金沢大学整形外科学／社会医療法人財団 董仙会 恵寿総合病院 整形外科 |
| | |
| 14:55-15:20 | IgG4 関連疾患の臨床と病理 |
| 司　会 | 松田陽子　香川大学 病理病態・生体防御医学講座 腫瘍病理学 |
| 発表者 | 笠島里美　金沢大学 医薬保健研究域保健学系 病態検査学 |
| | |
| 15:20-15:45 | Non-mass-forming clonal expansion に着目した病理学研究 |
| 司　会 | 中田光俊　金沢大学 医薬保健研究域医学系 脳・脊髄機能制御学 |
| 発表者 | 前田大地　金沢大学 医薬保健系研究域医学系 分子細胞病理学 |
| | |
| 15:45-15:55 | リフレッシュ |
| | |
| 15:55-16:10 | この 20 年間の雑感とこれから |
| 司　会 | |
| 発表者 | 源　利成　金沢大学がん進展制御研究所 腫瘍制御 |
| | |
| 16:10-17:00 | がん研究から志摩半島での地域医療へ |
| 司　会 | 岡田保典　順天堂大学 整形外科 運動器・腫瘍性疾患病態学講座 |
| 発表者 | 江角浩安　国民健康保険 志摩市民病院 |
| | |
| 17:00-17:15 | Closing remarks / 閉会のことば |
| 司　会 | 源　利成　金沢大学がん進展制御研究所腫瘍制御 |
| 発表者 | 勝田省吾　金沢医科大学 前学長 |
| | |
| 17:30- TF | ミキサー：七夕の会 2021 併催 |
| | |

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# 【目 次】

プログラム

骨軟部肉腫の臨床と研究 18

IgG4 関連疾患の臨床と病理 20

Non-mass-forming clonal expansion に着目した病理学研究 22

この 20 年間の雑感とこれから 24

がん研究から志摩半島での地域医療へ

Closing remarks／閉会のご挨拶 26

誌上開催:七夕の会 酒呑もおよ！ 28

# 骨軟部腫瘍の臨床と研究

金沢大学整形外科学／社会医療法人財団 董仙会 恵寿総合病院 整形外科
阿部健作

「骨・軟部腫瘍」分野は，整形外科疾患の中でも、その対応、治療が生命に直接関わる重要分野であり、四肢・脊椎の解剖、機能の理解は当然であるが、画像診断、病理診断、手術手技、化学療法・放射線療法の知識など、とりわけ集学的知識が要求される。骨・軟部腫瘍に対しては、早期に正しい診断を行い、治療計画を経て、適切な治療（あるいは、腫瘍専門病院への紹介）を開始することが治療成績の向上に必須である[1)]。

骨軟部腫瘍の中でも特に悪性腫瘍については、希少性が非常に高く、それにも関わらず組織形態は多彩であるため、新たな治療方法の導入や研究は、他のがん種と比して遅れをとっていると言わざるを得ない。特に化学療法については、近年、悪性骨軟部腫瘍分野においてもいくつかの新たな化学療法薬や分子標的薬が導入されはしたものの、どれも既存の治療効果を上回るものではなく、この数十年，治療内容に大きな変化を認めていない。それゆえ、臨床・研究双方における大学の役割は非常に大きなものとなっている。

悪性骨軟部腫瘍に対する治療の柱は、多くの場合において、手術と化学療法である。当教室においても、これらの研究に力を入れてきている。前者は液体窒素処理骨を用いた再建術や腫瘍脊椎骨全摘術などがあり、これらは保険収載されている。他にも臨床応用に向けて、様々な研究が取り組まれている。後者については、骨肉腫に対する新規シスプラチン製剤による新規治療の開発[2)]や軟骨肉腫に対するザルトプロフェンによる抗腫瘍効果に関する解析[3)]などがあり、臨床応用に向けて研究は続いている。その中で、源先生ご指導のもと、我々は骨軟部肉腫に対する GSK3β を標的とした分子標的治療に関する研究を行ってきた[4, 5)]。

GSK3β に関する研究では、骨肉腫においては、GSK3β 阻害薬の使用により Wnt/β-catanin 経路や NF-κB 経路を介して骨肉腫細胞の増殖を抑制していることが示唆され[4)]、滑膜肉腫と線維肉腫においては、CDK-cyclin 複合体を介した細胞周期の進行による生存・増殖と、MMP-2 を介した浸潤が、GSK3β に依存することが示唆された[5)]。GSK3β 阻害効果を持つ薬剤は実際に臨床の場に出ており、drug repositioning の形で臨床応用されることが今後、期待される。

1) 越智隆弘・吉川秀樹（2007）『最新整形外科学大系　骨・軟部腫瘍および関連疾患』，中山書店．
2) Igarashi K, Yamamoto N, Odani A, Tsuchiya H, et al. Effectiveness of two novel anionic and cationic platinum complexes in the treatment of osteosarcoma. Anticancer Agents Med Chem 15(3):390-9, 2015.
3) Higuchi T, Yamamoto N, Yamamoto Y, Tsuchiya H, et al. Anti-tumor effects of a nonsteroidal anti-inflammatory drug zaltoprofen on chondrosarcoma via activating peroxisome proliferator-activated receptor gamma and suppressing matrix metalloproteinase-2 expression. Cancer Med 7(5):1944-54, 2018.
4) Shimozaki S, Yamamoto N, Domoto T, Minamoto T, Tsuchiya H, et al. Efficacy of glycogen synthase kinase-3β targeting against osteosarcoma via activation of β-catenin. Oncotarget 7 (47): 77038-51, 2016.
5) Abe K, Yamamoto N, Domoto T, Minamoto T, Tsuchiya H, et al. Glycogen synthase 3β as a potential therapeutic target in synovial sarcoma and fibrosarcoma. Cancer Sci 111 (2): 429-40, 2020.

## 阿部健作（あべ　けんさく）

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2010 年 3 月　金沢大学医学部　卒業
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2015 年　4 月　金沢大学　がん進展制御研究所　協力研究員　併任（～2020 年 3 月）
2018 年　4 月　金沢赤十字病院　整形外科　医員
2019 年　4 月　浅ノ川総合病院　整形外科　医長
2020 年　4 月　恵寿総合病院　整形外科　医長
2021 年　4 月　金沢大学　整形外科　協力研究員　併任

所属学会と資格

日本整形外科学会　専門医
中部日本整形外科災害外科学会
日本癌治療学会
日本整形外科学会スポーツ医学会
日本関節鏡・膝・スポーツ整形外科学会
日本人工関節学会

その他　日本スポーツ協会認定スポーツドクター
石川県サッカー協会医事委員
ツエーゲン金沢チームドクター

# IgG4 関連疾患の臨床と病理

金沢大学 医薬保健研究域保健学系 病態検査学　笠島里美

IgG4 関連疾患（IgG4-related disease; IgG4-RD）は、血清の IgG4 高値、組織での多数の IgG4 陽性の形質細胞浸潤と線維増生を特徴とする疾患群であり、2004 年に初めて膵臓で報告され、以後 2000 年代には、全身多数の臓器で IgG4-RD が同時性、異時性に発生することが報告された。我々は、血管外膜肥厚の高度な炎症性腹部大動脈瘤（inflammatory abdominal aortic aneurysm; IAAA）の約半数が IgG4-RD であることを世界で初めて報告し [1)]、その後も、冠動脈瘤, 胸部大動脈疾患の一部、大腿動脈瘤も IgG4-RD が原因であることを明らかにし、血管領域での IgG4-RD の臨床病理学的特徴の解明に寄与してきた. 2010 年代からは, IgG4-RD の病因として、helper T（Th）2 細胞優位の免疫状態やその反応性の制御性 T 細胞の亢進が注目され、Th2 関連サイトカイン（interleukin [IL]-4, IL-5, IL-13）、炎症制御性サイトカイン（IL-10, TGF-β）の検討が各臓器の IgG4-RD で進められた。一方で、一般に動脈疾患では粥腫性動脈硬化症に伴うとされる炎症性サイトカインの上昇、特に IL-6 の上昇が知られる。IgG4 関連血管病変では、動脈硬化性大動脈瘤及び解剖例の正常例と比較して、血清 IL-6 が有意に上昇し、血管外膜では組織球の IL-6 産生が高度であり、動脈硬化性に関わるサイトカイン産生に加えて、“炎症性”大動脈瘤としての特徴を示した [2)]。これは血管病変のない他臓器の IgG4-RD と比較して特徴的な像であった。

IgG4 関連血管病変の外科的治療症例が蓄積されるに従い、血管内治療後には、動脈周囲の線維性肥厚の増悪、発熱や腹痛が術後の持続、瘤径拡大などが多い傾向があり、治療方針選択の際の問題となっている。IgG4 関連血管病変の開腹手術による人工血管置換術ではほぼ全例が完治する一方で、血管内治療例の約半数が術後 2 年以内に何らかの増悪を示した。血管内治療後に血清 IgG4 値が上昇する群は、血管径拡大、血清 IL-6、血清 matrix metalloproteinase（MMP）-9 が上昇しており、血管内治療では残存する血管壁に IgG4-RD の活動性炎症が残ることが, 術後の増悪因子であると想定された [3)]。IgG4 関連動脈瘤では、動脈硬化性大動脈瘤及び解剖例の正常例と比較して、組織でも外膜の MMP-2, MMP-9 産生が亢進しており、リンパ濾胞形成に関わる免疫細胞（濾胞樹状細胞）、抑制型組織球、形質細胞型樹状細胞など多彩な免疫細胞が MMPs 産生に関与していることを報告した [4)]。

現在は、血管外膜のリンパ濾胞の発達が IgG4 関連血管病変の活動性の指標になるとして検討中である。また、IgG4 関連血管病変は, 腹部大動脈やその分枝が好発部位であるが、高安病などの‘いわゆる大血管炎’のよく起こる胸部大動脈, 頸部動脈では頻度が低い理由も長年不明であった。我々は、正常血管の自然免疫細胞の分布に注目し、IgG4 関連血管病変の局在についての解明を進めている。

1) Kasashima S, Zen Y, Kawashima A, et al. Inflammatory abdominal aortic aneurysms: close relationship to IgG4-relared periaortitis. Am J Surg Pathol. 2008;32(2):197-204.
2) Kasashima S, Kawashima A, Zen Y, et al. Upregulated interleukins (IL-6, IL-10, and IL-13) in immunoglobulin G4-related aortic aneurysm patients. J Vasc Surg 2018;67(4):1248-1262.
3) Kasashima S, Kasashima F, Kawashima A, et al. Clinical outcomes after endovascular and open surgery repair of immunoglobulin G4-related or non-related inflammatory aortic aneurysms. J Endovasc Ther 2017;24(6):833-845.
4) Kasashima S, Kawashima A, Kasashima F, et al. Adventitial matrix metalloproteinase production and distribution of immunoglobulin G4-related abdominal aortic aneurysms. J Vasc Surgery Vasc Sci 2020;1:151-165.

笠島里美（かさしま さとみ）

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日本臨床検査医学会（臨床検査専門医，臨床検査管理医）
IgG4関連疾患学会（IgG4関連心血管診断基準作成委員）

# Non-mass-forming clonal expansion に着目した病理学研究

金沢大学 医薬保健系研究域 分子細胞病理学　前田大地

私は、2021 年 4 月に金沢大学に着任し、分子細胞病理学分野を担当しております。本セミナーでは、私のこれまでのキャリアと研究内容を振り返りつつ、現在、特に注力しているテーマである”non-mass-forming clonal expansion”について言及したいと考えています。

私は外科病理医で、大学院時代から一貫して婦人科領域の腫瘍性疾患をテーマとし、主に背景遺伝子異常を探索する研究を行ってきました。その間、卵巣癌の領域では、純形態学的アプローチによってその多くが卵管上皮由来であることが実証され、発生母地に関するパラダイムシフトが起こりました。具体的には卵巣 high-grade serous carcinoma 症例の多くにおいて卵管上皮内癌（先行病変）の併存が確認され、両者に共通の *TP53* 変異が存在することが示された訳ですが、同時に正常卵管上皮にも一定頻度で“p53 signature”と呼ばれる微小変異細胞集塊が存在することが知られるようになりました。p53 signature は今まで単なる正常卵管上皮の一部として見過ごされてきた（病変と呼ぶのも憚られる）存在です。ただし、「卵管上皮細胞に *TP53* 変異が生じるだけでは腫瘤を形成せず、悪性化もきたさない」ことを示唆する点が特異です。p53 signature との遭遇をきっかけに、私の興味は、正常組織中に存在する変異細胞集塊へとシフトしました。「正常卵管上皮には *TP53* に限らず、様々な遺伝子変異が生じているのではないか」という仮説を立て、検証した結果、約 2 割に *CTNNB1* 変異細胞集塊が存在することが判明し、β-catenin signature と名付けて報告しました。次世代シーケンスを用いた研究においても、全身諸臓器の非腫瘍部に低 allele frequency で癌関連遺伝子の変異が検出されることが明らかになってきた今、病理組織検体において変異細胞の局在を示す研究系はますます重要になってきています。

さて後半では、non-mass-forming clonal expansion の別の例としてハンナ型間質性膀胱炎（HIC）を取り上げます。私はある一例において軽鎖制限を確認したのをきっかけに、HIC 患者の膀胱で B-cell の clonal expansion が高頻度で起きていることを見出し、以降、継続して研究を行ってきています。HIC の組織像は単なる慢性炎症を示すもので、長期経過を見ても悪性リンパ腫は発生しません。Clonality が証明されたら「腫瘍」と言えるのか。あるいは腫瘍原性遺伝子変異が確認されたら「腫瘍性病変」なのか。従来は、臨床的に腫瘤形成性病変として捉えられ、また組織学的に均一な細胞が集塊を作ってくるような病態をもって「腫瘍」が定義されてきましたが、ポストゲノム時代に入ってその定義は揺らいできています。ただし、「腫瘍なのか非腫瘍なのか」というポイントに拘泥せず、全身諸臓器で生じている特定の細胞の clonal expansion に着目することで、HIC を含め、今まで成因不明とされていた疾患の病態解明の糸口がつかめるのではないか、と私は考えております。今後の研究の展望も含めてお話しさせていただければ深甚に存じます。

1. Maeda D, Akiyama Y, Morikawa T, Kunita A, Ota Y, Katoh H, Niimi A, Nomiya A, Ishikawa S, Goto A, Igawa Y, Fukayama M, Homma Y. Hunner-type (classic) interstitial cystitis: a distinct inflammatory disorder characterized by pancystitis, with frequent expansion of clonal B-cells and epithelial denudation. ***PLoS One***. 10 (11): e0143316, 2015.
2. Koyama K, Maeda D, Kito M, Tamura D, Kudo-Asabe Y, Katoh H, Ishikawa S, Nanjo H, Terada Y, Goto A. Clinicopathological and molecular analyses of linearly expanded epithelial cells with β-catenin alterations, "β-catenin signature", in the normal fallopian tube. ***Histopathology***. 77 (6): 880-9, 2020.

# 前田大地（まえだ　だいち）

金沢大学医薬保健研究域医学系分子細胞病理学分野　教授

学　　歴

2004 年 3 月 東京大学医学部医学科卒業
2010 年 3 月 東京大学大学院医学系研究科博士課程修了

職　　歴

2004 年 4 月　東京大学医学部附属病院初期臨床研修医
2005 年 4 月　三井記念病院初期臨床研修医
2006 年 4 月　三井記念病院病理部医員
2010 年 4 月　東京大学大学院医学系研究科人体病理学・病理診断学分野 助教
2012 年 4 月　東京大学医学部附属病院病理部 助教
2014 年 4 月　秋田大学大学院医学系研究科器官病態学講座 准教授
2018 年 4 月　大阪大学大学院医学系研究科先端ゲノム医療学共同研究講座 特任教授
2021 年 4 月　金沢大学医薬保健研究域医学系分子細胞病理学分野 教授

所属学会と資格

・日本病理学会（専門医、学術評議員、Pathology International 刊行委員、分子病理専門医制度運営委員会委員、分子病理専門医資格審査委員会委員）
・日本婦人科病理学会　（理事）
・日本臨床細胞学会
・日本間質性膀胱炎研究会
・日本婦人科腫瘍学会（代議員、卵巣がん治療ガイドライン 2020 年版評価委員）
・JSAWI (Japanese Society for the Advancement of Women’s Imaging)（世話人）
・日本癌学会

# この20年間の雑感とこれから（仮題）

金沢大学がん進展制御研究所　源　利成

私が 2001 年 7 月の金沢大学がん研究所教授就任を茶崖に報告してから [1]、やがて 20 年が経過します。今回、本誌への寄稿依頼が届いたのは奇遇です。せっかくの機会なので、これまでといま、そしてこれからのことについて認（したた）めます。

私は卒後すぐに、金沢の大学院で病理学を修め、在籍中の2年間は東京医科歯科大学難治疾患研究所に出向しました。この時期にこれまでで唯一、順天堂と交流しました。その後は 2008 年まで金沢大学がん研究所と附属病院（2001 年に金沢大学病院に統合）で消化器・腫瘍外科の修練と研究に従事しました。途中、国立がんセンター研究所と米国マントサイナイ医科大学に出向した期間のほかは、およそ 15 年間に消化器や乳腺、甲状腺のがんを中心に 400 数十人の患者さんを執刀する機会に恵まれました。1998 年春から現在の研究室を併任し始めましたので、2008 年 7 月のがん研外科診療科の廃止までの 10 年間は外科とがん研究の掛けもちでした。それ以降は現在まで、消化器がんや難治・希少がんの橋渡し研究に軸足を移し、大学病院がんセンターで診療を続けています。

外科の恩師からはつぎのような教えを受けました。それは「手術は知識、技量、経験を遺憾なく発揮すべき場であり、外科医を志す医師がその習熟と向上に努力すべきことは言うまでもない。日常診療で外科医が患者さんを診察し、手術し、社会復帰させるまでの一連の過程には、患者さんと医師との信頼関係が最も重要であり、単に手術を行うという技術的な面だけでなく、外科医としての情熱と人間性が要求される。」というものです。また、研究が少し軌道に乗りかけたころ、米国研究者や米国癌学会とトラブルがあったとき、国立がんセンターの恩師から、つぎのような手紙が届きました。その内容は「国際的－international－ということは、各国の歴史、文化が確認して成り立っている美名です。中性の水のようなものではなく、国際化という言葉は商売の駆け引きや民族の勢力争いのとき、最もしばしば使われます。パスツールが云った科学には国境がなくとも、科学者には国境がある、ということです。日本人は人が好いので、結局、西洋人に手玉にとられるのです。西洋にはない仕事を早くして、早く発表することが唯一無二の方策です。元気を出して、日本の科学のためにご健闘ください。」という、叱咤です。これまでの仕事とこれらの助言を踏まえ、臨床と研究に関する自身の考えを駄文にまとめました [2]。ご関心があれば、私か茶崖編集部に電子版別刷りをご請求ください。

人生のおよそ3分の2におよぶ期間、外科、研究ともに大講座ではなく、一貫して小さなグループで活動してきました。それでも多くの仲間に出会い（次頁の写真はそのお一人の人事のお祝いです）、いただいた公的・民間の研究資金は 8 億円を超えています [3]。ありがたいことと国民や仲間のみなさんに感謝しながら、大学で残された3年間をこれからどうしようかと思案しています。

1) 源 利成. 腫瘍外科医の遺伝子診断事始め. 茶崖 105: 6-9, 2002.
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3) 金沢大がん研腫瘍制御 HP：http://ganken.cri.kanazawau.ac.jp/shuyoseigyo/index.html

※本文は、順天堂大学同窓会誌：茶崖（さがい）第 181 号（2021 年 4 月）の寄稿文から転載

源　利成（みなもと　としなり）
金沢大学がん進展制御研究所 腫瘍制御研究分野 教授
金沢大学附属病院がんセンター 併任

学 歴:

1983 年　3 月　順天堂大学医学部　卒業
1987 年　3 月　金沢大学大学院医学研究科
（病理学第一）修了

職 歴:

1987 年　4 月　金沢大学がん研究所附属病院外科　医員
1990 年　1 月　富山逓信病院外科　勤務
1991 年　1 月　金沢大学がん研究所外科部 / 同附属病院外科　助手
1992 年　4 月　国立がんセンター研究所生化学部　研究員
1994 年　4 月　金沢大学がん研究所外科部 / 同附属病院外科　助手（復職）
1995 年 11 月　American Health Foundation, Naylor Dana 疾病予防研究所　博士研究員
1997 年　1 月　ニューヨーク市立大学マントサイナイ医学校がんセンター　客員講師
1997 年 11 月　金沢大学がん研究所腫瘍外科 / 同附属病院腫瘍外科　助手（復職）
1998 年　4 月　金沢大学がん研究所腫瘍制御（旧：遺伝子診断）助教授
金沢大学がん研究所附属病院腫瘍外科　助教授（併任）
2001 年　7 月　金沢大学がん研究所腫瘍制御（旧：遺伝子診断）教授
金沢大学附属病院腫瘍外科（現：がんセンター）教授（併任）

所属学会と資格

基礎系　日本癌学会（評議員）
日本消化器癌発生学会（理事，評議員）
米国癌学会（AACR）：Corresponding Member
国際消化器発がん学会（理事）
日本病理学会（死体解剖認定医：病理解剖），ほか

橋渡系　日本癌病態治療研究会（世話人）
日本分子腫瘍マーカー研究会（世話人）

臨床系　日本外科学会（指導医 2017 年まで，専門医 [認定登録医]）
日本消化器外科学会（指導医，専門医（~2015 年）[認定登録医]，認定医）
日本消化器内視鏡学会（本部評議員，指導医，専門医）
日本消化器病学会（本部学術評議員，指導医，専門医）
日本消化器がん検診学会（代議員，東海北陸支部幹事，指導医，認定医）
日本癌治療学会
日本胃癌学会
大腸癌研究会，ほか

その他　日本がん治療認定医機構 暫定教育医
日本消化器外科学会 消化器がん外科治療認定医
日本がん治療認定医機構 がん治療認定医
石川県予防医学協会：集検事業管理指導委員会 胃がん部会委員，大腸がん部会委員長
石川県成人病予防センター：大腸集団検診指導委員会委員
石川県教育委員会スーパーサイエンスハイスクール（SSH）事業 運営指導委員，ほか

# 源 利成 教授　就任20周年に寄せて

金沢医科大学 元学長　勝田省吾

この度、源 利成先生が教授就任 20 周年を迎えられたことを心からお祝い申し上げます。誠におめでとうございます。

金沢大学医学部第一病理学教室（現分子細胞病理学）の同門の一人として、第一病理と源先生との思い出などを述べたいと思います。

## 1. 第一病理学教室で学んだ「厳しさ」と「競合と調和」

私は臨床の教室に入る前に病理学を学びたいと考え、医学部を卒業後、大学院医学研究科病理学第一に進学しました。病理学第一講座を主宰されていたのは、当時では先端的技術であった電子顕微鏡を用いたコラーゲン線維や結合組織の研究で有名な梶川欽一郎教授でした。私は実験的動脈硬化症の超微構造的研究で学位を取得しました。学位論文の最終稿が出来た時、教授室で梶川先生から教室に残るよう声をかけられ、その場で「はい」と答えたことが懐かしく思い出されます。梶川先生の学問に対して妥協を許さない厳しいご指導は、私の心に一本のしっかりした「芯」を作って下さいました。

1983 年 4 月、梶川教授の後を中西功夫教授が引き継がれ、私は助教授として 11 年間、仕事をさせて頂きました。中西教授は従来の超微構造形態学に加えて、生化学、細胞生物学、分子生物学や遺伝子解析など新しい研究手法を取り入れたダイナミックな先端的医学研究を指向されました。そして、スタッフ個人の研究意欲と素質を大切にしたオーケストラ型の教室を目指され、「競合と調和」を合い言葉に教室運営を心がけられました。これまで全国でご活躍の旧第一病理の皆さんの心には、梶川先生と中西先生のDNA がしっかりと受け継がれていると思っております。

## 2. 源 利成先生のご活躍は病理学第一の誇り

源先生は 1983 年 4 月、中西功夫教授にとって最初の大学院生として病理学第一に入学されました。中西教授の期待を背負って、翌年 4 月より結合組織の生化学的研究の第一人者である東京医科歯科大学難治疾患研究所の永井 裕教授の研究室に 2 年間出向し、結合組織、特にコラーゲンの生化学的研究に従事し、抗コラーゲン抗体の精製など多くの成果を挙げられました。源先生の修得された生化学的研究手法は、その後の第一病理の発展に大きく貢献しました。私自身も源先生が第一病理に導入された生化学的研究の恩恵を受けた一人です。動脈硬化研究のトップジャーナルである、Arteriosclerosis, Thrombosis and Vascular Biology に動脈硬化病変における型別コラーゲンの局在を発表することが出来ました。また、培養平滑筋細胞のコラーゲン合成に関する研究も進めることが出来、私達の研究に新しい分野を開いていただきました。

源先生は 1987 年 4 月、金沢大学がん研究所外科の磨伊正義教授の下に移籍されました。研究と臨床の分野で多くの実績を残され、2001 年 7 月金沢大学がん研究所の教授に就任されました。先生は教授就任後、毎年、年報「研究のあゆみと業績」を発行され、教室の活動状況を届けて下さいました。学内外のグループとの共同研究、研究業績、外部資金の獲得、特許出願など着実に成果を挙げておられ、教室の発展ぶりが分かり、第一病理の同門の一人して大変嬉しく思っておりました。また、毎年「七夕の会」の開催など、いろいろな機会を通して、仲間を大切に絆を深めておられる源先生のご努力に敬意を表します。

今後とも源先生の主な研究テーマ「Wnt 経路抑制因子である glycogen synthase kinase（GSK）3β 阻害によるがん治療法の研究、開発」を発展させ、がん治療に繋がる素晴らしい成果を挙げられることを心より祈念しております。

## 勝田省吾（かつだ しょうご）
金沢医科大学名誉教授、元学長

学 歴：
1971年 9月 金沢大学医学部卒業
1976年 3月 金沢大学大学院医学研究科博士課程修了

略 歴：
1976年 4月 金沢大学医学部助手（病理学第一）
1977年 11月 金沢大学医学部講師（病理学第一）
1979年 4月 金沢大学医療技術短期大学部助教授
1983年 7月 金沢大学医学部助教授（病理学第一）
1989年 5月 文部省在外研究員（米国ワシントン大学病理学教室）
1989年 1月 米国ワシントン大学 Research Fellow（～1990年6月）
1994年 7月 金沢医科大学教授（病理学Ⅱ）
2002年 4月 金沢医科大学副学長（～2004年8月）
2007年 9月 金沢医科大学学長補佐（～2010年8月）
2008年 4月 金沢医科大学国際交流センター長（～2010年8月）
2010年 9月 金沢医科大学学長（～2016年8月）
2013年 9月 金沢医科大学一般教育機構長（～2015年8月）

称号・叙勲：
2014年 10月 ロシア・ヤロスラブリ医科大学名誉博士
2020年 11月 瑞宝中授章受章

所属学会：
2011年 4月 日本動脈硬化学会功労会員
2012年 4月 日本病理学会功労会員

主な原著論文（5編）：

1. Ross R, Masuda J, Raines EW, Gown AM, Katsuda S, Sasahara M, Malden T, Masuko H, Sato H: Localization of PDGF-B protein in macrophages in all phases of atherosclerosis. Science 248: 1009-1012, 1990.
2. Katsuda S, Okada Y, Minamoto T, Oda Y, Matsui Y, Nakanishi I: Collagens in human atherosclerosis: Immunohistochemical analysis using collagen type specific antibodies. Arterioscler Thromb Vasc Biol 12: 494-502, 1992.
3. Katsuda S, Boyd HC, Flinger BC, Ross R, Gown AM: Human atherosclerosis III. Immunocytochemical analysis of the cell composition of lesions of young adults. Am J Pathol 140: 907-914,1992.
4. Katsuda S, Coltrera MC, Ross R, Gown AM: Human atherosclerosis IV. Immunocytochemical analysis of cell activation and proliferation in lesions of young adults. Am J Pathol 142: 1787-1793, 1993.
5. Katsuda S, Okada Y, Okada Y, Imai K, Nakanishi I : Matrix metalloproteinase-9 (92-kd gelatinase/type IV collagenase equals gelatinase B) can degrade arterial elastin. Am J Pathol 145: 1208-1218, 1994.

著書

勝田省吾「金沢医科大学 学生の自信と誇りを育む」 金沢医科大学出版局 2019年3月15日発行

【誌上開催：七夕の会】

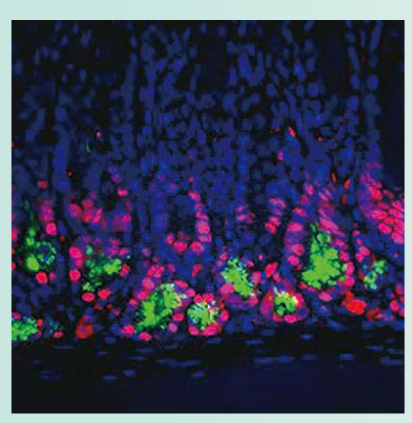
出典：http://kataoka-tsurutaro.com/products/detail.php?product_id=5

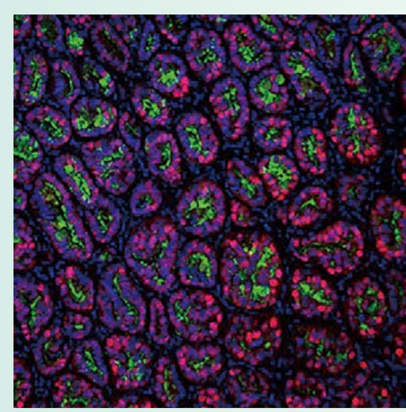
【附記2】 金沢大がん研 News Letter(抜粋):共同研究者紹介

金沢大学がん進展制御研究所
Cancer Research Institute Kanazawa University

# News Letter

Vol.15 October 2021





## Contents


所長挨拶 01

クラウドファンディングに挑戦しました！ 02

シンポジウム・研究会の開催 05

共同研究者の紹介 07
香川大学医学部 松田 陽子 教授
金沢大学がん進展制御研究所 源 利成 教授

高校生へ向けて研究紹介 09
シグナル伝達研究分野 善岡 克次 教授

特別寄稿 宝町から角間へ移転10年 神村 栄吉 先生 11

令和３年度 共同研究採択課題一覧 12

これまでの受賞・表彰／論文業績 13

少し足を延ばして石川歴史探訪 14


写　　真：金沢城玉泉院丸庭園(左)[写真提供:石川県観光連盟]、金沢駅鼓門1(右)「写真提供：金沢市」
研究画像：Lgr5遺伝子陽性のマウス胃正常組織幹細胞(左)と胃がん幹細胞(右)に発現するSox9遺伝子(青；細胞核、緑；Lgr5遺伝子、赤；Sox9遺伝子)[提供:村上和弘助教]

※金沢大学がん進展制御研究所共同利用・共同研究拠点の承諾を得て転載。

松田教授と源教授は2021年度

教　授　松田　陽子
MATSUDA　YOKO

香川大学医学部
腫瘍病理学

## 共同研究からはじまった 源先生との出会い

金沢大学がん進展制御研究所腫瘍制御研究分野の源 利成教授との共同研究のご縁で、このような執筆の機会をいただき厚く御礼申しあげます。私は日本医科大学病理学教室にて、石渡俊行准教授とともに膵癌の新規治療薬開発に関する研究を行っておりました時に、金沢大学がん進展制御研究所の共同研究の公募のお話を耳に挟み、ホームページを拝見した際に、膵臓癌の研究に関する源 利成教授の研究成果に大きな感銘を受けました。当たって砕ける覚悟で源先生にご連絡差し上げたところ、直ぐに温かいお返事をいただき、また私の研究を直接ご相談させていただく機会をいただきました。その後、毎年の共同研究の申請や金沢での成果発表会、及び日本癌学会で多大なご指導をいただきましたこと、この場をお借りして深謝申し上げます。

2015年に私が東京都健康長寿医療センターに移りましてからも、源先生との共同研究を継続させていただきました。2018年には、東京都健康長寿医療センター第4回 老年病理学研究セミナーにて、「GSK3βとがん生物学」について源先生のご講演を賜りました。講演会では、小さな研究から積み重ねて大きな成果を獲得した経緯や、基礎研究から臨床に還元する方法を具体的にご教示いただきました。また、源先生の様々な研究が一見、別々のように見えていても、最終的には一つの方向性を持つ大きな研究を実践されていることに、感銘を受けると同時に、私自身の研究の道筋が開けたことを感じました。2019年に香川大学腫瘍病理学の教授に就任することになった際には、源先生からお祝いと激励のお言葉をいただきました（写真）。香川大学でも、源先生から多大なご指導、ご支援をいただき、膵癌、大腸癌、胃癌の病理研究を継続させていただいております。

源先生は毎年、「七夕の会」という研究会を主宰され、さらに年次研究成果報告集を刊行されております。そのたびに、私は叱咤激励を受け、いつか源先生のような研究者になりたいと改めて思います。そのため、私も年に一度の研究会を香川で立ち上げました。今後も自分にできることを少しずつ着実に進め、癌の予後改善に少しでも貢献できるよう、病理医の立場からの研究に邁進する所存です。

源先生との共同研究から始まった様々な成果は、源先生をはじめ、教室の皆様、金沢大学がん進展制御研究所の皆様、そして共同研究として御採択いただいたお蔭です。この場を借りて心より感謝申し上げるとともに、これまでの御恩を論文等でお返ししていきたいと思います。最後になりましたが、貴研究所並びに皆様の益々のご発展を心より祈念しております。今後とも御指導・御鞭撻の程何卒お願い申し上げます。

2019年2月金沢にて

## 採択課題で共同研究をすすめています。

# 松田陽子さんとの8年間

教　授　源　利成
MINAMOTO TOSHINARI

金沢大学がん進展制御研究所
腫瘍制御研究分野

第24回日本消化器癌発生学会総会（2013年9月5日、6日:石川県立音楽堂）の開催準備にかまけていた2013年3月11日、当時は日本医科大学病理学教室の講師を務められていた松田陽子さんから丁寧なメールが届きました。それは、膵がんの悪性形質に関する共同研究の提案でした。当研究所の共同研究の募集が始まって間もないころで、はじめてみずしらずの研究者から共同研究の提案が来たわけです。それまでは付きあいの狭かった（いまも狭い）、しかも不愛想な私には新鮮なできごとでした。そして、共同研究課題の採択後の同年6月13日、日本医科大学病理学教室で初めて松田さんに会いました。私自身、大学院で病理学を専攻したこともあって、とても初対面とは思えないほど打ち解けて共同研究の相談や雑談をして、つぎの長津田の東工大へ向かうまでの小一時間があっという間に過ぎました。9月に金沢で担当した学会（上記）に松田さんが参加してくださり、とても楽しい思い出になりました。

翌2014年に松田さんは教室の上司であった石渡（いしわた）俊行氏とともに、東京都健康長寿医療センター病理診断科に異動され、私どもとの共同研究を継続してくださいました。これがご縁になって、当研究分野の開設から満15年にあたる2016年7月9日に開催した共同研究セミナー2016で松田さんに講演していただき（写真）、併催した七夕の会2016で親睦を深めました。ここからさらに交流が続き、私は2018年4月に同センター研究所の協力（特任）研究員を委嘱され、10月12日に老年病理学研究セミナーで講演の機会をいただきました。その半年後、2019年2月27日の共同研究成果報告会に演者として参加されたとき、松田さんが香川大学病理学教室の教授に内定したとの朗報がありました。仲間の活躍は手放しで嬉しいものです。夕刻、居酒屋で少人数の宴席ではあったものの、ささやかなお祝いをしました。いまのパンデミックが始まるちょうど1年前でした。

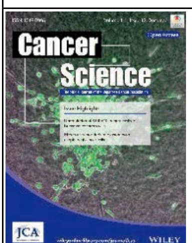
松田さんとの出会いをきっかけに、山梨大学、千葉大学、久留米大学、埼玉医科大学、札幌医科大学、名古屋市立大学、鶴見大学から続々と共同研究の提案が届いています。松田さんと出会ってからこの8年間、私には思いもかけなかったことばかりです。不愛想で人づきあいの悪い私の狭い料簡と視野を見開かせてくれた松田さんは、いまでは私にはかけがえのない仲間のひとりです。

腫瘍制御研究分野開設15周年共同研究セミナー　2016年7月9日（土）　於:ホテル日航金沢

【附記3】 学位論文：2021 年3月修了

**Nakanishi H, Sawada T, Kaizaki Y, Ota R, Suzuki H, Yamamoto E, Aoki H, Eizuka M, Hasatani K, Takahashi N, Inagaki S, Ebi M, Kato H, Kubota E, Kataoka H, Takahashi S, Tokino T, Minamoto T, Sugai T, Sasaki Y. Significance of gene mutations in Wnt signaling pathway in traditional serrated adenomas of the colon and rectum. *PLoS One* 15 (2): e0229262, 2020. doi: 10.1371/journal.pone.0229262.**

Recent studies have shown that colorectal serrated lesions, which include sessile serrated adenomas (SSAs) and traditional serrated adenomas (TSAs), are precursors of colorectal cancer. However, the molecular mechanisms underlying the carcinogenesis, particularly in TSAs, remain largely uncharacterized. To clarify their molecular and clinicopathological characteristics, we performed mutation and methylation analyses of cancer-associated genes in 78 serrated lesions, including TSAs, SSAs and microvesicular hyperplastic polyps. Target exon sequence analysis was performed with 39 genes, including genes known to be frequently mutated in colorectal cancers and/or serrated lesions. We also used bisulfite pyrosequencing to assess the methylation status of various cancer-associated genes and marker genes of the CpG island methylator phenotype (CIMP). The prevalence of mutations in genes associated with Wnt signaling was significantly higher in TSAs than SSAs (65% vs. 28%, $p<0.01$). Among those, RNF43 mutations were observed in 38% of TSAs and 17% of SSAs. In immunohistochemical studies of 39 serrated lesions, the prevalence of abnormal nuclear β-catenin accumulation was significantly higher in TSAs (57%) than SSAs (8%) ($P=0.01$). SMOC1 methylation was detected in 54% of TSAs but in no SSAs ($p<0.01$). Additionally, SMOC1 methylation was more prevalent among TSAs with KRAS mutation (82%) than with BRAF mutation (38%, $p=0.03$). Lesions with CIMP-high or RNF43 mutations were detected only in TSAs with BRAF mutation, suggesting two distinct carcinogenic pathways in TSAs. Mutations in genes associated with Wnt signaling play a greater role in the carcinogenesis of TSAs than SSAs.




**Uehara M, Domoto T, Takenaka S, Bolidong D, Takeuchi O, Miyashita T, Minamoto T. Glycogen synthase kinase 3β participates in acquired resistance to gemcitabine in pancreatic cancer. *Cancer Sci* 111 (12): 4405-16, 2020. doi: 10.1111/cas.14668**

Acquisition of resistance to gemcitabine is a challenging clinical and biological hallmark property of refractory pancreatic cancer. Here, we investigated whether glycogen synthase kinase (GSK)-3β, an emerging therapeutic target in various cancer types, is mechanistically involved in acquired resistance to gemcitabine in human pancreatic cancer. This study included 3 gemcitabine-sensitive BxPC-3 cell-derived clones (BxG30, BxG140, BxG400) that acquired stepwise resistance to gemcitabine and overexpressed ribonucleotide reductase (RR)M1. Treatment with GSK3β-specific inhibitor alone attenuated the viability and proliferation of the gemcitabine-resistant clones, while synergistically enhancing the efficacy of gemcitabine against these clones and their xenograft tumors in rodents. The gemcitabine-resensitizing effect of GSK3β inhibition was associated with decreased expression of RRM1, reduced phosphorylation of Rb protein, and restored binding of Rb to the E2 transcription factor (E2F)1. This was followed by decreased E2F1 transcriptional activity, which ultimately suppressed the expression of E2F1 transcriptional targets including RRM1, CCND1 encoding cyclin D1, thymidylate synthase, and thymidine kinase 1. These results suggested that GSK3β participates in the acquisition of gemcitabine resistance by pancreatic cancer cells via impairment of the functional interaction between Rb tumor suppressor protein and E2F1 pro-oncogenic transcription factor, thereby highlighting GSK3β as a promising target in refractory pancreatic cancer. By providing insight into the molecular mechanism of gemcitabine resistance, this study identified a potentially novel strategy for pancreatic cancer chemotherapy.

RESEARCH ARTICLE

# Significance of gene mutations in the Wnt signaling pathway in traditional serrated adenomas of the colon and rectum

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## Abstract

Recent studies have shown that colorectal serrated lesions, which include sessile serrated adenomas (SSAs) and traditional serrated adenomas (TSAs), are precursors of colorectal cancer. However, the molecular mechanisms underlying the carcinogenesis, particularly in TSAs, remain largely uncharacterized. To clarify their molecular and clinicopathological characteristics, we performed mutation and methylation analyses of cancer-associated genes in 78 serrated lesions, including TSAs, SSAs and microvesicular hyperplastic polyps. Target exon sequence analysis was performed with 39 genes, including genes known to be frequently mutated in colorectal cancers and/or serrated lesions. We also used bisulfite pyrosequencing to assess the methylation status of various cancer-associated genes and marker genes of the CpG island methylator phenotype (CIMP). The prevalence of mutations in genes associated with Wnt signaling was significantly higher in TSAs than SSAs (65% vs. 28%, $p < 0.01$). Among those, *RNF43* mutations were observed in 38% of TSAs and 17% of SSAs. In immunohistochemical studies of 39 serrated lesions, the prevalence of abnormal nuclear β-catenin accumulation was significantly higher in TSAs (57%) than SSAs (8%) ($P = 0.01$). *SMOC1* methylation was detected in 54% of TSAs but in no SSAs ($p < 0.01$). Additionally, *SMOC1* methylation was more prevalent among TSAs with *KRAS* mutation (82%) than with *BRAF* mutation (38%, $p = 0.03$). Lesions with CIMP-high or *RNF43* mutations were detected only in TSAs with *BRAF* mutation, suggesting two distinct carcinogenic

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**Data Availability Statement:** Sequence data has been deposited at the Japanese Genotype-phenotype Archive (JGA, http://trace.ddbj.nig.ac.jp/jga), which is hosted by the DNA DataBank of Japan (DDBJ), under accession number JGAS00000000217.

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**Competing interests:** The authors have declared that no competing interests exist.

**Abbreviations:** A, ascending colon; C, cecum; CGH, comparative genomic hybridization; CIMP, CpG island methylator phenotype; CIMP-H, CpG island methylator phenotype-high; CIN, chromosomal instability; CNV, copy number variation; CRC, colorectal cancer; D, descending colon; FFPE, formalin-fixed, paraffin-embedded; IGV, Integrative Genomics Viewer; InDel, insertion and deletion; LINE, long interspersed nucleotide elements; MSI, microsatellite instability; MSS, microsatellite stable; MVHP, microvesicular hyperplastic polyp; R, rectum; S, sigmoid colon; SNV, single nucleotide variant; SSA, sessile serrated adenoma; T, transverse colon; TSA, traditional serrated adenoma.

pathways in TSAs. Mutations in genes associated with Wnt signaling play a greater role in the carcinogenesis of TSAs than SSAs.

## Introduction

Colorectal cancer (CRC) is a major cause of cancer-related death worldwide. Recent molecular pathological studies, including The Cancer Genome Atlas project, have shown that CRCs are heterogeneous diseases that arise via different molecular pathways [1,2]. Most (80–85%) sporadic CRCs are classified as non-hypermutated or microsatellite stable (MSS) tumors and develop through accumulation of multiple genetic and epigenetic alterations [1,2], including mutation of oncogenes and tumor suppressor genes, as well as chromosomal instability [3,4]. The remaining 15–20% of sporadic CRCs are classified as hypermutated tumors and mainly exhibit microsatellite instability (MSI) and concurrent hypermethylation in multiple loci [2], which is referred to as the CpG island methylator phenotype (CIMP) and is closely associated with *BRAF* mutation [5–7].

Recent studies of the molecular and clinicopathological characteristics of colorectal premalignant lesions have provided insight into the pathogenesis of CRCs as well as clues to prevention and treatment [8–10]. Since establishing the pathological classification of serrated colorectal lesions as hyperplastic polyps, traditional serrated adenomas (TSAs) or sessile serrated adenomas (SSAs) [11–13], a number of studies have demonstrated that SSAs are associated with *BRAF* mutation and CIMP, and that they are precursors of MSI-positive CRCs, which are frequently located in the proximal colon [6,9,10,14–17]. However, less is known about the biological and clinical characteristics of TSAs, although they are also considered to be premalignant lesions and reportedly exhibit *BRAF* or *KRAS* mutations and aberrant DNA methylation [16–21]. In addition, several recent studies have reported *PTPRK-RSPO3* fusion and somatic mutations of *RNF43* in TSAs [22–24]. Because only a small number of analyses have investigated gene mutations in serrated lesions [23,25,26], the molecular mechanisms underlying carcinogenesis, especially in TSAs, are still not well characterized. Although it has been suggested based on immunohistochemical studies that dysregulation of the Wnt signaling pathway contributes to carcinogenesis in serrated lesions [20,21,23,27–29], mutation of individual gene within this pathway have not been investigated.

To clarify the molecular and clinicopathological characteristics of colorectal serrated lesions, we assessed the mutation of genes associated with Wnt signaling as well as other genes reportedly mutated in serrated lesions [26] and advanced CRCs [2]. We also investigated mutations in genes associated with oncogene-induced senescence, which have been reported as germline mutations in patients with multiple SSAs [30], and we performed immunohistochemical studies of β-catenin expression to assess activation of the Wnt signaling pathway. Finally, we investigated DNA methylation of cancer-associated genes including *SMOC1* as well as CIMP marker genes in TSAs because it was reported that *SMOC1* is specifically methylated in TSAs [31].

## Materials and methods

### Patients and tissue samples

Specimens of colorectal serrated lesions (n = 78) were obtained from 78 Japanese patients who underwent endoscopic mucosal resection at Nagoya City University Hospital, Fukui Prefectural Hospital, or Komatsu Municipal Hospital. This study was approved by the

Institutional Review Board at each hospital as well as Kanazawa University and Sapporo Medical University.

## Endoscopic analysis

High-resolution magnifying endoscopes (CF260AZI; Olympus, Tokyo, Japan) were used for all colonoscopic examinations. The morphology of colorectal lesions was determined according to the Paris classification [32]. All lesions detected during colonoscopy were observed at high magnification using indigo carmine dye, after which samples were collected through endoscopic mucosal resection for histological analysis. Tumor locations were defined as proximal colon (cecum, ascending colon, transverse colon) or distal colon (descending colon, sigmoid colon, and rectum).

## Histological analysis

Histological diagnosis of tumors was done at each facility, after which the histological findings for all specimens were reviewed by a board certified pathologist (Sugai T) who was blinded to the clinical and molecular information. Serrated lesions, including microvesicular hyperplastic polyps (MVHPs), SSAs and TSAs, were classified according to WHO classification criteria [33]. Mixed serrated lesion composed of TSA and tubulovillous adenoma was classified as TSA in our analyses. The clinicopathological features of the lesions are summarized in Table 1.

**Table 1. Clinicopathological features of the serrated lesions in this study.**

| | |
|---|---|
| **Patients (n = 78)** | |
| **Age (y, mean ± SD)** | 65.3 ± 10.9 |
| **Sex, n (%)** | |
| Male | 51 (65) |
| Female | 27 (35) |
| **Lesions (n = 78)** | |
| **Location, n (%)** | |
| Proximal | 42 (54) |
| Distal | 36 (46) |
| **Bowel subsites, n (%)** | |
| Cecum | 13 (17) |
| Ascending colon | 22 (28) |
| Transverse colon | 7 (9) |
| Descending colon | 5 (6) |
| Sigmoid colon | 20 (26) |
| Rectum | 11 (14) |
| **Morphology, n (%)** | |
| 0-Ip | 15 (19) |
| 0-Is | 47 (60) |
| 0-IIa | 16 (21) |
| **Histology, n (%)** | |
| MVHP | 23 (30) |
| TSA | 36 (46) |
| TSA + TVA | 1 (1) |
| SSA | 18 (23) |

MVHP, microvesicular hyperplastic polyp; TSA, traditional serrated adenoma; TVA, tubulovillous adenoma; SSA, sessile serrated adenoma.

https://doi.org/10.1371/journal.pone.0229262.t001

## DNA preparation

DNA was isolated from formalin-fixed, paraffin-embedded (FFPE) tissue sections using a QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A TaqMan RNase P Detection Reagents kit (Thermo Fisher Scientific, Waltham, MA) was used to quantify the purified DNA.

## Semiconductor-based next-generation sequencing

A customized panel, encompassing all the exons of 39 cancer-related genes, including genes frequently reported to be involved in advanced CRCs and serrated lesions [2,25,26], was created using the Ion Torrent System with an Ion AmpliSeq Designer (Thermo Fisher Scientific) (S1 Fig). Genes within oncogene-induced senescence pathways detected in patients with multiple serrated polyps were also investigated [30]. The assay design consisted of 1,455 amplicons ranging from 125 to 175 bp in length, covering 94% of the 112.8 kb target sequence.

Library preparation and sequencing with the Ion Torrent sequencer were performed as previously described [34–36]. The templates were sequenced after emulsion PCR was performed with 24 samples per Ion PI chip using the Ion PI HI-Q Chef kit (Thermo Fisher Scientific).

## Identification of somatic mutations and copy number variations (CNVs)

Human genome build 19 (hg19) was used as a reference. Signal processing, mapping to the hg19 reference, and quality control were performed using Torrent Suite version 5.0 (Thermo Fisher Scientific). Somatic mutations (point mutations, insertions, and deletions) were detected using Ion Reporter Software 5.0 (Thermo Fisher Scientific). Because matched normal controls were not available, the control sequence data provided by Thermo Fisher Scientific were used as a control. Pathogenic status of the variant was stated if it was a missense variant with $< 0.1\%$ global minor allele frequency in dbSNP or the 1000 Genomes Project database and/or the variant was registered as pathogenic in ClinVar or COSMIC databases. Variants with allele frequencies between 0.4 and 0.6 or $> 0.9$ were considered germline variants unless listed as a pathogenic variant. Additionally, if the same variants were detected in multiple samples, these variants were considered germline variants unless occurring at a known hotspot variant in databases. Integrative Genomics Viewer (IGV) software (http://software.broadinstitute.org/software/igv/) was used to filter out possible strand-specific errors, such as a mutation that was detected in the forward or reverse DNA strand but not in both strands. CNV detection was also performed with the Ion Reporter Software using an algorithm based on the Hidden Markov Model. Recurrent genomic regions with CNVs were identified using copy numbers greater than 3 and less than 1 for gains and losses, respectively.

## DNA methylation analysis

DNA methylation was analyzed using bisulfite pyrosequencing as described previously [37,38]. Briefly, genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite kit (Qiagen). Pyrosequencing was then carried out using a PSQ 96MA system (Qiagen) with a Pyro Gold Reagent kit (Qiagen), and the results were analyzed using Pyro Q-CpG software (Qiagen). A cutoff value of 15% was used to define genes as methylation-positive. Using five classic CIMP markers (*MINT1*, *MINT2*, *MINT12*, *MINT31* and *MLH1*) and *CDKN2A* (*p16*), tumors were defined as CIMP-positive (three or more loci showed methylation) or CIMP-high (CIMP-H, four or more loci showed methylation). Methylation of *SMOC1*, *GALNT14*, *SFRP1*, *SFRP2*, *IGFBP7*, *SOX5* and long interspersed nucleotide element 1

(LINE-1) was also analyzed using bisulfite pyrosequencing. The primer sequences used were as previously reported [9,31,39].

### Immunohistochemistry

Immunohistochemical studies of β-catenin expression were performed as previously described with 39 serrated lesions, including 14 TSAs, 13 SSAs and 12 MVHPs [40]. A mouse anti-β-catenin monoclonal antibody (1:1000 dilution, Clone 14; BD Biosciences, San Jose, CA) was used. β-catenin expression was semi-quantitatively evaluated in tumor cells with β-catenin-positive nuclei, and positive nuclear accumulation was defined as staining of more than 10% of tumor cell nuclei throughout the lesions, as reported previously [40]. All slides were evaluated by two independent pathologists (YK and TM) who were blinded to the clinical and molecular data.

### Statistical analysis

Continuous data were analyzed using *t*-tests (for two groups) or ANOVA with a post hoc Tukey's HSD test (for more than two groups). Fisher's exact test and logistic regression were used to assess the association between categorical variables. Values of $P < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS 20 (IBM Corporation, Somers, NY) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

## Results

### Clinicopathological characteristics of serrated lesions

The clinicopathological and molecular characteristics of the colorectal serrated lesions analyzed in this study are summarized in Tables 1 and 2. The majority of MVHPs (17/23, 74%) and SSAs (15/18, 83%) were located in the proximal colon, from the cecum to the transverse colon, while TSAs were more prevalent in the distal colon (27/37, 73%), especially the sigmoid colon and rectum (14 cases and 11 cases, respectively). On endoscopic observation, more than one-third (14/37, 38%) of TSAs were protruding, pedunculated (0-Ip) lesions and differed significantly from SSAs, most of which were protruding, sessile (0-Is) lesions (16/18, 88%) (Table 2).

### Targeted amplicon sequencing of colorectal serrated lesions

We performed semiconductor sequencing of all exons in 39 cancer-related genes that were previously detected in 78 colorectal serrated lesions and advanced CRCs. The sequencing overview, including reads, coverage, and uniformity of the read coverage distribution, is shown in S1 Table. Each FFPE sample underwent an average of 2.7 million sequencing reads after quality filtering. A mean coverage depth of 1722.5 reads (100.2–5126.0) per base was observed.

All single nucleotide variants (SNVs) and insertions and deletions (InDels) detected through bioinformatics analysis underwent visual inspection using the IGV for confirmation. We identified a mean of 2.4 somatic nonsynonymous mutations (range 0–13) per sample (S2 Table). The 11 most commonly mutated genes in serrated lesions are depicted in Fig 1. *BRAF* was the most frequently mutated gene in serrated lesions (68%, 53 of 78 cases), followed by *RNF43* (26%), *KRAS* (21%), and *APC* (10%). *BRAF* V600E mutations were detected in 52 samples, and an N581S mutation was detected in 1 sample accompanied by a *KRAS* Q61H mutation (Patient 60 in Fig 1). The *BRAF* V600E mutation and *KRAS* mutation appeared to be mutually exclusive, as they were never detected in the same sample (Fig 1). *RNF43* mutations

**Table 2. Clinicopathological and molecular characteristics of the respective serrated lesion.**

| | MVHP | SSA | TSA | *p* value (SSA vs TSA) |
|---|---|---|---|---|
| **No. of cases** | 23 | 18 | 37 | |
| **Sex (male/female)** | 16/7 | 9/9 | 26/11 | 0.24 |
| **Age (y, mean ± SD)** | 66 ± 9.1 | 63.3 ± 10.6 | 65.8 ± 11.8 | 0.44 |
| **Tumor location, n (bowel subsite)** | | | | |
| Proximal (C/A/T) | 17 (6/10/1) | 15 (6/5/4) | 10 (1/7/2) | <0.01 |
| Distal (D/S/R) | 6 (1/5/0) | 3 (2/1/0) | 27 (2/14/11) | |
| **Tumor size (mm, mean ± SD)** | 11.3 ± 7.0 | 11.2 ± 4.7 | 12.2 ± 5.4 | 0.5 |
| **Morphology, n (%)** | | | | |
| 0-Ip | 0 (0) | 1 (6) | 14 (38) | 0.01 |
| 0-Is | 13 (57) | 16 (88) | 18 (49) | |
| 0-IIa | 10 (43) | 1 (6) | 5 (13) | |
| **Gene mutation/epigenetic alteration, n (%)** | | | | |
| *BRAF* V600E mutation | 14 (61) | 14 (78) | 24 (65) | 0.37 |
| *KRAS* mutation | 4 (17) | 1 (6) | 11 (30) | 0.08 |
| *RNF43* mutation | 3 (13) | 3 (17) | 14 (38) | 0.13 |
| *APC* mutation | 1 (4) | 0 (0) | 7 (19) | 0.08 |
| WNT signaling associated genes | 9 (39) | 5 (28) | 24 (65) | <0.01 |
| CIMP | 9 (39) | 8 (44) | 16 (43) | 1 |
| CIMP-high | 5 (22) | 4 (22) | 10 (27) | 1 |
| *SMOC1* methylation | 0 (0) | 0 (0) | 20 (54) | <0.01 |

MVHP, microvesicular hyperplastic polyp; SSA, sessile serrated adenoma; TSA, traditional serrated adenoma; C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum; CIMP, CpG island methylator phenotype

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were detected in 38% of TSAs and 17% of SSAs. Nonsense or frameshift mutations in *RNF43* were seen in 30% of TSAs and 11% of SSAs. Most of the mutations were located upstream of the ring finger domain of *RNF43* (Fig 2). We also found *APC* mutations in 7 of 37 TSAs (19%), but none in SSAs. Protein-truncating (nonsense and frameshift) mutations of *APC* were detected in 14% of TSAs (Fig 1). Overall, the frequency of mutations in Wnt pathway components was significantly higher in TSAs than in SSAs (65% vs. 28%, $P < 0.01$) (Fig 3, Table 2). In addition, a *GNAS* R201H mutation was found in 1 TSA, while 1 SSA and 3 MVHPs harbored a *GNAS* V334G mutation, though the biological significance of the latter is unknown (Fig 3A, S2 Table).

## Target amplicon sequencing detects CNVs

We also detected CNVs in segments of the genome that could be duplicated or deleted from the sequencing data (Fig 1). In all samples, the genes most frequently affected by copy number gains were *KRAS* (42%), *CTNNB1* (28%), and *HRAS* (24%), while the genes most frequently affected by copy number losses were *DKK1* (53%), *CDKN2A* (41%), *TP53* (31%), and *XAF1* (31%). In addition, copy number loss at the *APC* gene locus was seen in one TSA (S2 Fig). In total, at least one CNV (loss or gain) affecting a Wnt pathway component was found in 49% of TSAs and 67% of SSAs. When considered together with the gene mutations, at least one genomic abnormality affecting genes associated with Wnt signaling was seen in 84% of TSAs and 78% of SSAs. Among these, the most frequently affected genes associated with Wnt signaling were *CTNNB1* (gain at 3p22.1), which encodes β-catenin, and *DKK1* (loss at 10q21.1), which encodes Dkk-1, a negative regulator of Wnt signaling (S2 Fig).

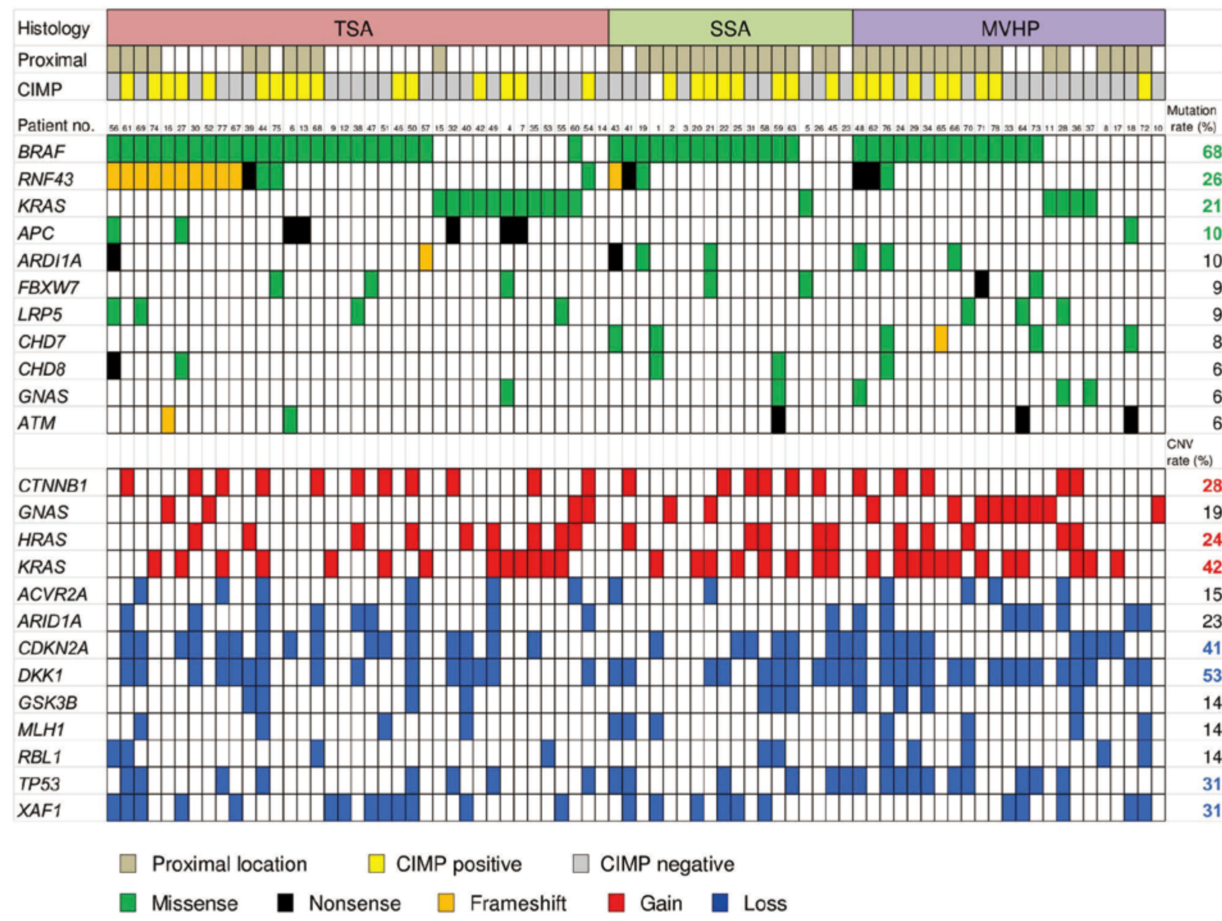


**Fig 1. Summary of somatic mutations and CNVs across 78 serrated lesions.** Profiles of gene mutations and CNVs within individual samples are grouped with respect to histological types. In the upper panel, the top row indicates the histology, the second row indicates the tumor location, and the third row indicates the CIMP status. Columns correspond to the individual cases. In the middle panels, frequently mutated genes, colored to indicate the type of mutation, and their mutational frequency are shown. In the lower panels, CNVs frequently detected in colorectal serrated lesions are shown.

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## Methylation analysis of CIMP markers, cancer-associated genes, and LINE-1

We next assessed methylation of CIMP markers and genes known to be frequently methylated in CRCs (S3 Table) [9]. When comparing TSA and SSA, the prevalence of CIMP statuses (CIMP-positive or CIMP-H) did not significantly differ (Table 2, Fig 3A). Notably, however, *SMOC1* methylation was detected in 20 of 37 TSAs (54%) but in none of the 18 SSAs tested ($P < 0.01$) (Table 2, Fig 3A). By contrast, the frequencies of lesions showing methylation of other cancer-associated genes did not significantly differ between TSAs and SSAs. There were no lesions in which *MLH1* methylation was positive in the present study.

We found that the levels of *SMOC1*, *SFRP1 and SFRP2* methylation were significantly higher in TSAs than in SSAs or MVHPs (S3 Fig). In addition, the level of *SOX5* methylation was significantly higher in TSAs than in MVHPs. Levels of LINE-1 methylation, which was measured to evaluate global DNA hypomethylation in the lesions, did not correlate with the histological types of serrated lesions (S3 Fig).

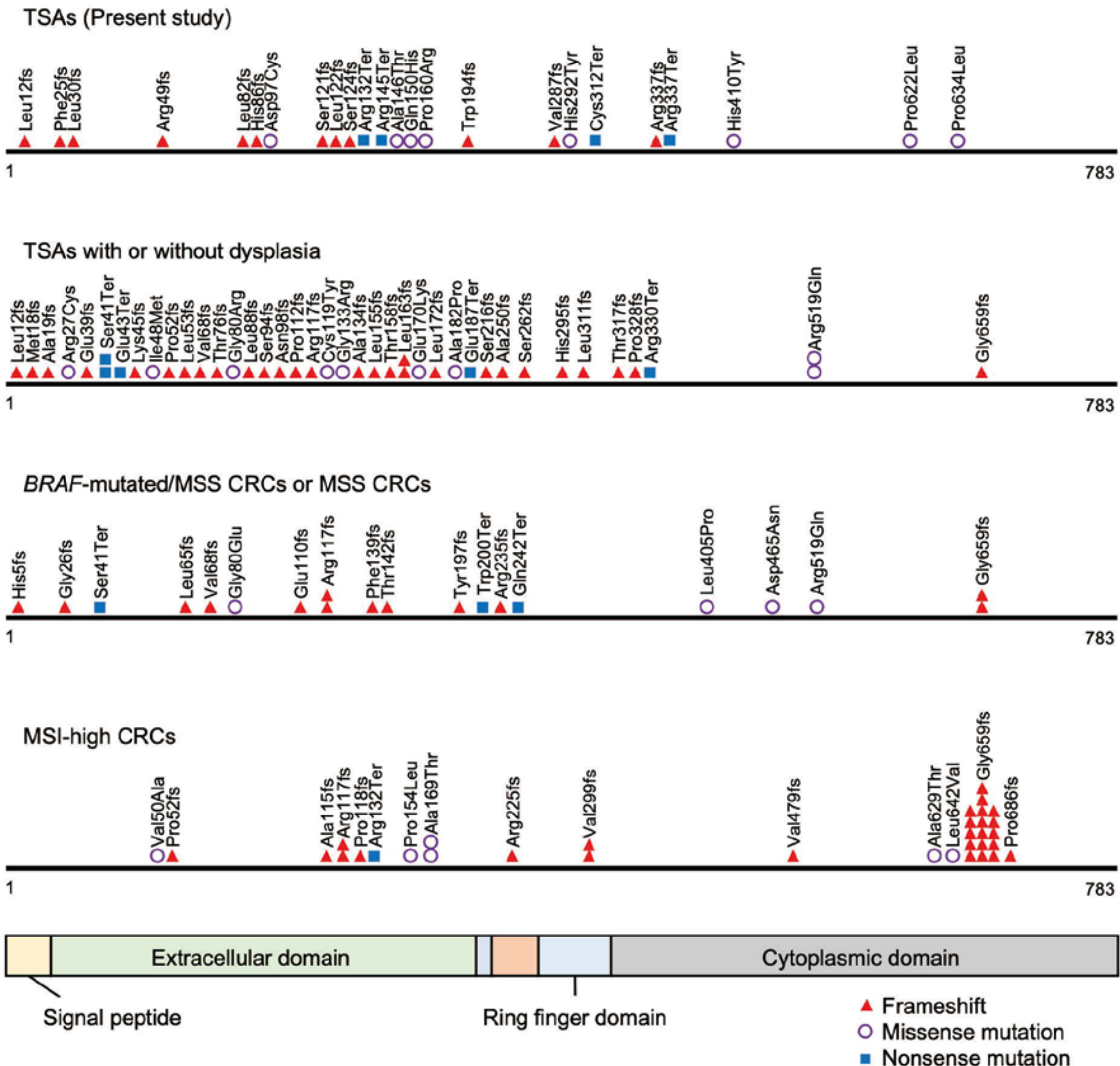


**Fig 2. Distributions of *RNF43* mutations in detected in TSAs in the present study.** Previously reported TSA mutations with or without dysplasia as well as MSS and MSI-high CRCs are shown for reference [23,24,41].

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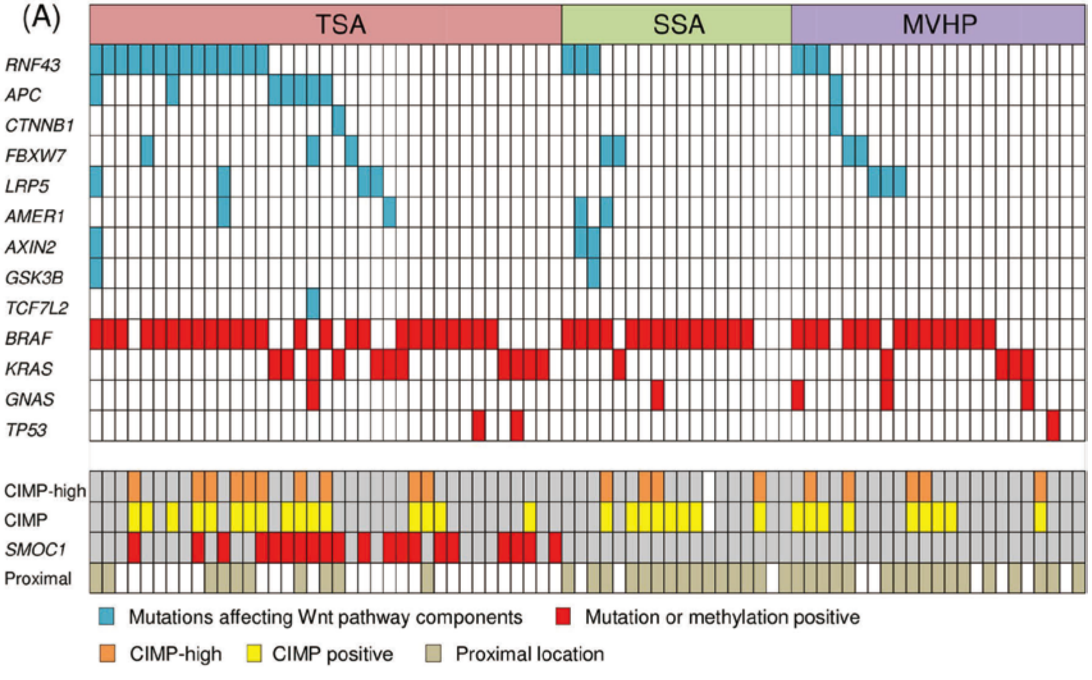
## Clinicopathological and molecular characteristics of TSAs with *KRAS* or *BRAF* mutation

We also compared clinicopathological and molecular characteristics of TSAs with *KRAS* or *BRAF* (V600E) mutation. It appears that TSAs with *BRAF* mutation were more likely to be located in the proximal colon than those with *KRAS* mutation, though the difference was not statistically significant (P = 0.12) (Table 3). Regarding gene mutations, *RNF43* mutation was frequently detected in TSAs with *BRAF* mutation, but not in TSAs with *KRAS* mutation (P < 0.01). Lesions with CIMP-H were also found only in TSAs with *BRAF* mutations (38% vs. 0%, P = 0.03). Although lesions positive for *SMOC1* methylation were seen in both groups, *SMOC1* methylation was significantly more prevalent among TSAs with *KRAS* mutations than those with *BRAF* mutations (82% vs. 38%, P = 0.03) (Table 3). In addition, levels of *SFRP1*



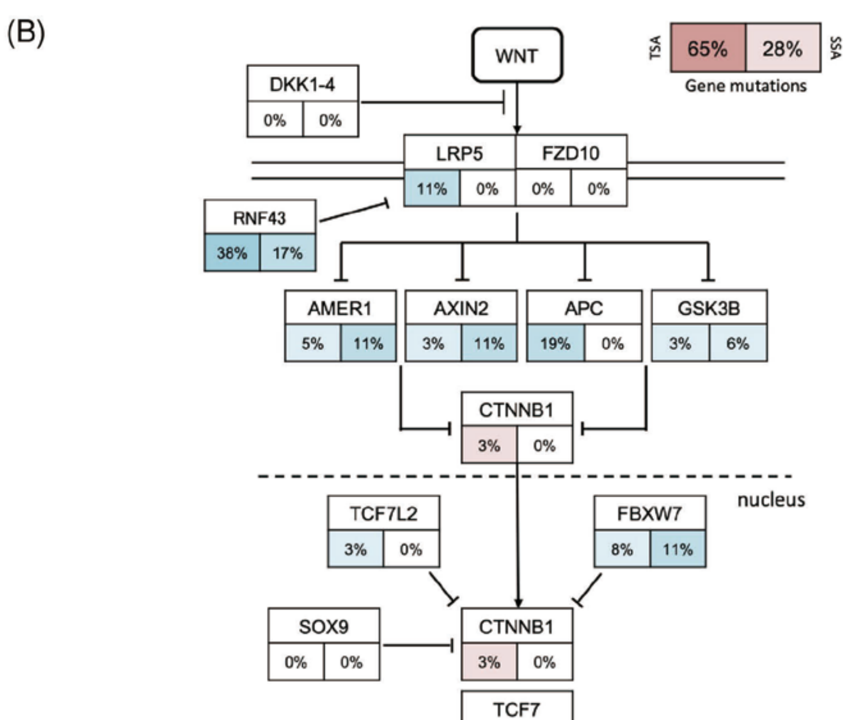




**Fig 3. Gene mutations leading to dysregulation of Wnt signaling pathway in serrated lesions.** (A) Representative mutation and methylation profile across 78 serrated lesions. Blue coloration indicates gene mutations affecting Wnt pathway components, while red coloration indicates positivity for gene mutations or methylation. (B) Frequencies of gene mutations leading to dysregulation of Wnt signaling in TSAs and SSAs. Alteration frequencies are expressed as percentages of all cases. Frequencies of gene mutations in TSAs are shown on the left, while those in SSAs are shown on the right. Red denotes activated genes and blue denotes inactivated genes.

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**Table 3. Clinicopathological and molecular characteristics of the *KRAS*- and *BRAF*- mutant TSAs.**

| | *BRAF* mutant TSA | *KRAS* mutant TSA | *p* value |
|---|---|---|---|
| **No. of cases** | 24 | 11 | |
| **Sex (male/female)** | 16/8 | 9/2 | 0.45 |
| **Age (y, mean ± SD)** | 65.9 ± 8.7 | 63.1 ± 15.9 | 0.61 |
| **Tumor location, n (bowel subsite)** | | | |
| Proximal (C/A/T) | 9 (1/7/1) | 1 (0/0/1) | 0.12 |
| Distal (D/S/R) | 15 (2/9/4) | 10 (0/4/6) | |
| **Tumor size (mm, mean ± SD)** | 11.9 ± 4.5 | 12.1 ± 5.9 | 0.92 |
| **Morphology, n (%)** | | | |
| 0-Ip | 10 (42) | 3 (27) | 0.26 |
| 0-Is | 12 (50) | 5 (46) | |
| 0-IIa | 2 (8) | 3 (27) | |
| **Gene mutation/epigenetic alteration, n (%)** | | | |
| *RNF43* mutation | 13 (54) | 0 (0) | < 0.01 |
| *APC* mutation | 4 (17) | 3 (27) | 0.65 |
| WNT signaling associated genes | 17 (71) | 6 (55) | 0.35 |
| CIMP | 12 (50) | 3 (27) | 0.28 |
| CIMP-high | 9 (38) | 0 (0) | 0.03 |
| *SMOC1* methylation | 9 (38) | 9 (82) | 0.03 |

TSA, traditional serrated adenoma; C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum; CIMP, CpG island methylator phenotype

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methylation were significantly higher in TSAs with *KRAS* mutation than in those with *BRAF* mutation. On the other hand, levels of *IGFBP7* methylation was significantly higher in TSAs with *BRAF* mutation than those with *KRAS* mutation (S4 Fig).

## Immunohistochemistry

Of 39 serrated lesions analyzed, 12 (31%) showed positive nuclear accumulation, while 27 (69%) showed membranous expression of β-catenin. The abnormal nuclear accumulation was observed in 8/14 TSAs (57%), 1/13 (8%) of SSAs, and 3/12 (25%) MVHPs (S4 Table, S5 Fig). When compared with SSAs, the nuclear accumulation of β-catenin was more prevalent in TSAs (57% vs. 8%, P = 0.01). Across all lesions, the prevalence of nuclear β-catenin accumulation was significantly higher in lesions with *RNF43* mutations than in those without mutations (83% vs. 21%, P < 0.01). When we focused only on TSAs, the prevalence of nuclear β-catenin accumulation was significantly higher in TSAs with a *BRAF* V600E mutation than those with *KRAS* mutation (7/8 vs. 1/5, P = 0.01).

## Discussion

In the present study, we used targeted next-generation sequencing to assess the mutation of genes associated with Wnt signaling. We found that these mutations occurred with higher frequency in TSAs than SSAs, which is suggestive of the importance of Wnt signaling in the pathogenesis of TSAs. Sekine et al. reported genetic alterations that included *PTPRK-RSPO3* fusions among Wnt pathway components in 71% of TSAs [23]. If *PTPRK-RSPO3* fusions had also been investigated in the present study, the frequency of genetic alterations would have been even higher. *RNF43* encodes E3 ubiquitin ligase, which negatively regulates Wnt

signaling. We detected *RNF43* mutations in 38% of TSAs and 17% of SSAs, which is consistent with earlier reports [23,24,26]. Most mutations, especially protein-truncating mutations, were situated upstream of the ring finger domain without clustering, as was shown in previous studies of TSAs with or without dysplasia [23,24]. Two major frameshift mutations (Arg117fs and Gly659fs) have been reported in CRCs with MSI [41], while CRCs with *BRAF* mutation/MSS had mutation profiles similar to TSAs [24,41]. This finding may indicate that TSAs belong to a carcinogenic pathway that is distinct from the SSA pathway (serrated-neoplasia pathway), making them possible precursors of *BRAF* mutated/MSS CRCs. We also sequenced the entire region of the *APC* gene and found protein-truncating mutations in 14% of TSAs, which is consistent with a report from Sekine et al. (13%) [23], though they only investigated frequently mutated regions of the gene. Protein truncating mutations of *RNF43* and *APC* were found to be mutually exclusive, suggesting the importance of both genes to Wnt signaling during carcinogenesis in TSAs. By contrast, the other genes encoding Wnt pathway components, including *CTNNB1*, *FBXW7*, *LRP5*, *AMER1*, and *AXIN2*, harbored mutations in only small fractions of tumors.

Previous studies have shown that there is wide variation in the rate of β-catenin positivity in both TSAs [21,23,27–29] and SSAs [29]. We showed in the present study that nuclear β-catenin accumulation is significantly more prevalent in TSAs than SSAs. This is likely associated with a high prevalence of mutations in the Wnt signaling pathway, especially in *RNF43*. These results may thus support the greater significance of *RNF43* mutation and Wnt signaling pathway activation in the carcinogenesis of TSAs than SSAs. Alternatively, it is possible that *RNF43* mutation represents subgroups in which Wnt signaling is activated by other genetic or epigenetic mechanisms.

Although an earlier study using a comparative genomic hybridization (CGH) microarray reported that CNVs were found only infrequently in colorectal precursor lesions including serrated lesions [9], CNVs in colorectal serrated lesions have not been thoroughly investigated. The present study demonstrated the frequent occurrence of CNVs at the *CTNNB1* and *DKK1* loci among Wnt signaling pathway associated genes. In addition, copy number losses at 17p (the *TP53* locus) are reportedly associated with progression of tumors from conventional-type adenoma to carcinoma and are frequently found in advanced CRCs [2,3]. This result may indicate the importance of *TP53* to carcinogenesis of serrated lesions. Because recent studies suggest that somatic CNVs at oncogenic loci are not always associated with gene expression [2,42,43], validation of the effect of CNVs through comparison with expression data is needed.

The prevalence of CIMP-positive or CIMP-H lesions did not significantly differ between SSAs and TSAs in this study. CIMP was detected in up to 79% of TSAs in previous studies [16,17]. One recent study reported that the prevalence of CIMP-H was significantly higher in SSAs than TSAs [44], which is inconsistent with our results. This difference likely reflects differences in the sample cohort, or may be due to a difference in the CIMP markers analyzed or the method used for methylation analysis (pyrosequencing vs. *MethyLight*). Interestingly, levels of DNA methylation in *SFRP1* and *SFRP2* were higher in TSAs than in SSAs or MVHPs. Epigenetic inactivation of SFRP family genes, including *SFRP1* and *SFRP2*, occurs early during CRC progression and enables constitutive Wnt signaling in CRCs [45]. It was recently reported that levels of *SFRP1* and *SFRP2* transcription correlate inversely with the methylation levels in samples of gastric mucosa, with or without *H*. *pylori* infection, as well as background mucosa in gastric cancers [46]. It is possible that *SFRP1* and *SFRP2* methylation contributes more significantly to carcinogenesis in TSAs than SSAs, although these phenomena may be influenced by the sample cohort, the degree of contamination by non-neoplastic cells, and methodology. We also analyzed the methylation status of *SMOC1* in a patient cohort different from the one examined in an earlier study [31]. We found that *SMOC1* methylation was highly

specific for TSAs, and the methylation level was significantly higher in TSAs than SSAs. Thus, *SMOC1* methylation may be a potential marker to distinguish TSAs from other serrated polyps.

Recent reports suggest that TSAs belong to a heterogenous category and develop through at least two different neoplastic progression pathways. It is also suggested that lesions with *BRAF* mutation and those with *KRAS* mutation exhibit different clinicopathological and molecular characteristics [18–21]. In the present study, TSAs with *BRAF* mutation were preferentially located in the proximal colon, while those with *KRAS* mutation were preferentially located in the distal colon and rectum. In addition, TSAs with *BRAF* mutation were more likely to be CIMP-positive than those with *KRAS* mutation. CIMP-H positivity was only detected in TSAs with *BRAF* mutation, which is consistent with an earlier study [21]. Lesions with *RNF43* mutation were also found only in TSAs with *BRAF* mutation, while the prevalence of *SMOC1* methylation was significantly higher in TSAs with *KRAS* mutation than with *BRAF* mutation. The level of *IGFBP7* methylation is significantly higher in TSAs with *BRAF* mutation than with *KRAS* mutation, though the frequency of methylation-positive *IGFBP7* cases did not significantly differ between the two groups. *IGFBP7* has been shown to play a central role in *BRAF*-induced senescence and to be a direct target of TP53 [47]. Although methylation of *IGFBP7* has been investigated in specific histological types of serrated lesions [10,48], *IGFBP7* methylation status has not been compared between these two TSA subtypes. In addition to these differences in genetic and epigenetic alterations, the difference in the prevalence of β-catenin expression further supports there being two different neoplastic pathways for TSAs. Determining whether TSAs with *KRAS* or *BRAF* mutation belong to different neoplastic pathways with different malignant potentials will require further clinicopathological and molecular analyses.

The present study has several limitations, including a relatively small sample size, lack of data on fusion genes, and a lack of normal background samples for mutational analysis to rule out single nucleotide polymorphisms. Nonetheless, we were able to make several important observations. First, the mutational status of genes involved in Wnt signaling differs among colorectal serrated polyps, depending on TSA histology, which likely results in the differences in nuclear β-catenin expression. Second, we confirmed that *SMOC1* methylation is very specific to TSAs. Third, we detected significant differences in clinicopathological and molecular variables between TSAs with *KRAS* or *BRAF* mutation, which may indicate the presence of separate carcinogenic pathways among TSAs. By comparing gene expression data from CRCs and SSAs, an earlier study found that a particular subtype of CRCs with a poor-prognosis developed from serrated lesions [49]. Additionally, previous studies have also shown that there are similarities between the gene expression profiles of SSAs and those of MVHPs and MSI CRCs, which supports the concept of a serrated-neoplasia pathway [50,51]. Comprehensive gene expression studies together with analyses of the genetic and epigenetic alterations in TSAs and comparison of those data with other serrated polyps or CRCs could potentially establish the molecular carcinogenesis pathway in TSAs.

## Supporting information

**S1 Fig. List of genes in the custom AmpliSeq gene panel used in this study.**
(PDF)

**S2 Fig. Gene mutations and CNVs in association with Wnt signaling across 78 colorectal serrated lesions.**
(PDF)

**S3 Fig. Levels of methylation of the indicated genes and LINE-1 in the indicated histological types of colorectal serrated lesions.**
(PDF)

**S4 Fig. Levels of methylation of the indicated genes and LINE-1 in TSAs with *BRAF* or *KRAS* mutation.**
(PDF)

**S5 Fig. Representative views of hematoxylin and eosin (left) and β-catenin (right) expression in SSA and TSA.** (A, B) SSA showing membranous localization of β-catenin. (C, D) TSA showing nuclear accumulation of β-catenin.
(TIF)

**S1 Table. Summary of targeted amplicon sequencing data.**
(XLSX)

**S2 Table. Somatic nonsynonymous mutations found in 78 colorectal serrated lesions.**
(XLSX)

**S3 Table. Methylation profiles and CIMP status in colorectal serrated lesions.**
(XLSX)

**S4 Table. Nuclear β-catenin expression in colorectal serrated lesions.**
(XLSX)

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ORIGINAL ARTICLE

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# Glycogen synthase kinase-3β participates in acquired resistance to gemcitabine in pancreatic cancer

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## Abstract

Acquisition of resistance to gemcitabine is a challenging clinical and biological hallmark property of refractory pancreatic cancer. Here, we investigated whether glycogen synthase kinase (GSK)-3β, an emerging therapeutic target in various cancer types, is mechanistically involved in acquired resistance to gemcitabine in human pancreatic cancer. This study included 3 gemcitabine-sensitive BxPC-3 cell-derived clones (BxG30, BxG140, BxG400) that acquired stepwise resistance to gemcitabine and overexpressed ribonucleotide reductase (RR)M1. Treatment with GSK3β-specific inhibitor alone attenuated the viability and proliferation of the gemcitabine-resistant clones, while synergistically enhancing the efficacy of gemcitabine against these clones and their xenograft tumors in rodents. The gemcitabine-resensitizing effect of GSK3β inhibition was associated with decreased expression of RRM1, reduced phosphorylation of Rb protein, and restored binding of Rb to the E2 transcription factor (E2F)1. This was followed by decreased E2F1 transcriptional activity, which ultimately suppressed the expression of E2F1 transcriptional targets including *RRM1*, *CCND1* encoding cyclin D1, *thymidylate synthase*, and *thymidine kinase 1*. These results suggested that GSK3β participates in the acquisition of gemcitabine resistance by pancreatic cancer cells via impairment of the functional interaction between Rb tumor suppressor protein and E2F1 pro-oncogenic transcription factor, thereby highlighting GSK3β as a promising target in refractory pancreatic cancer. By providing insight into the molecular mechanism of gemcitabine resistance, this study identified a potentially novel strategy for pancreatic cancer chemotherapy.

KEYWORDS
acquired resistance, gemcitabine, glycogen synthase kinase-3β, pancreatic cancer, ribonucleotide reductase M1

**Abbreviations:** CDK, cyclin-dependent kinase; dCK, deoxycytidine kinase; dFdC, 2′-2′-difluoro-2′-deoxycytidine; dFdCDP, dFdC diphosphate; E2F1, E2 transcription factor 1; EMT, epithelial-mesenchymal transition; FOLFIRINOX, combination of folate, 5-FU, irinotecan, and oxaliplatin; FU, fluorouracil; GSK3β, glycogen synthase kinase-3β; hENT1, human equilibrative NT1; IHC, immunohistochemistry; IP, immunoprecipitation; nab, nanoparticle albumin-bound; NT, nucleoside transporter; PS, performance status; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; Rb, retinoblastoma gene/tumor suppressor protein; RRM1, ribonucleotide reductase M1; S, serine; TK1, thymidine kinase 1; TP53INP1, tumor protein p53 inducible nuclear protein 1; TS, thymidylate synthase; WB, Western blotting; Y, tyrosine.

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## 1 | INTRODUCTION

Pancreatic cancer is one of the most intractable cancer types, with little improvement in the survival rate over the past decades.[1,2] Patients with locally advanced tumors and those with metastatic tumors account for approximately one-third and one-half, respectively, of all pancreatic cancer cases.[1] Due to the extreme difficulty in obtaining an early diagnosis,[3] only 10%-15% of newly identified pancreatic cancer patients present with resectable or borderline resectable tumor (stage I or II). Most patients have local disease recurrence and/or distant metastasis following surgery. Therefore, most patients undergo nonsurgical therapies including conventional chemotherapy, radiation, and molecular-targeted therapies.[1] More recently, clinical trials with immune checkpoint blockades and precision therapies have also been conducted.[4,5] Despite multimodal combinations of these therapies, the 5-year overall survival rate for pancreatic cancer is at most 3%-15%.[2,6]

Chemotherapeutic agents used for pancreatic cancer patients include gemcitabine alone or in combination with nanoparticle albumin-bound (nab)-paclitaxel, TS-1 (a fluorouracil [FU] prodrug), FOLFIRINOX (sequential combination of folate, 5-FU, irinotecan, and oxaliplatin) and nanoliposomal irinotecan with 5-FU and folate.[7] Currently, gemcitabine-based regimens are the most frequently prescribed in the first-line setting and remain one of the standard therapies for pancreatic cancer.[7,8] However, most patients are initially resistant or soon acquire resistance to gemcitabine, therefore obtaining little survival benefit from this treatment.[9] Therefore, the identification of mechanism-based strategies and therapeutic targets to overcome gemcitabine resistance has become an active area for biological and clinical research.[10]

In the present study we hypothesized that glycogen synthase kinase (GSK)-3β was a candidate molecular target that may affect the efficacy of chemotherapeutic agents. GSK3β is an isoform of the multifunctional GSK3 family serine/threonine kinases and regulates a diverse array of fundamental cellular pathways by phosphorylation and interaction with dozens of structural and functional molecules.[11] GSK3β activity is finely controlled by differential phosphorylation of serine (S)9 (inactive) and tyrosine (Y)216 (active) residues. It is constitutively active in normal cells, but in many circumstances negative regulation of its activity allows cells to maintain vital activity and homeostasis in response to various stimuli.[12] Deregulation of GSK3β expression and activity has been implicated in the pathogenesis and progression of common diseases including diabetes mellitus, neurodegenerative disorders, and various inflammatory and immunological conditions.[12,13] Such diverse roles in normal cells and in diseases have highlighted GSK3β as a potentially attractive drug target and has led to the development of inhibitors.[14] At present, several early-phase clinical trials have been evaluating some of these inhibitors and lithium (an ATP non-competitive and non-specific GSK3 inhibitor) for neurodegenerative disorders and various cancer types, but none of them has been approved for clinical use (reviewed in Ref.15,16).

Based on its known functions against pro-oncogenic pathways (eg, Wnt/β-catenin, hedgehog, Notch signaling) and epithelial-mesenchymal transition (EMT) in untransformed cells, GSK3β has long been hypothesized to suppress tumor development and progression.[13] Previous studies on the putative tumor-suppressive roles of GSK3β have shown that it is inactivated by S9 phosphorylation in various oncogenic pathways. However, there has been no evidence showing that active GSK3β suppresses tumorigenesis, or that GSK3β inhibition promotes tumor development and progression (reviewed in Ref.15). In contrast, many studies by our group and others have demonstrated direct tumor-promoting roles for GSK3β as well as therapeutic effects following its inhibition in at least 25 different cancer types[15-17] including pancreatic cancer.[18] In addition to its therapeutic effect, we and others have shown that GSK3β inhibition sensitizes pancreatic cancer cells to gemcitabine through the impairment of DNA damage repair and cell cycle regulation.[19-21] However, none of the earlier studies modeled the acquired resistance to gemcitabine in pancreatic cancer that is frequently encountered in the clinical setting. To more directly investigate the role of GSK3β in the acquisition of gemcitabine resistance, we established cell clones derived from a gemcitabine-sensitive human pancreatic cancer BxPC-3 cell line that had acquired stepwise resistance to gemcitabine. This system models the development of gemcitabine resistance observed in clinical pancreatic cancer.[22]

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines

This study investigated the human pancreatic cancer cell line BxPC-3 and its derivative clones that acquired stepwise resistance to gemcitabine (BxG30, BxG140, and BxG400 in increasing order of resistance).[22] The cloned cells were confirmed to sustain their resistance without supplementation of gemcitabine in the medium for at least 2 months. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 unit/mL penicillin G and 0.1 mg/mL streptomycin) (Gibco) at 37°C with 5% $CO_2$. All subsequent experiments, except for the mouse xenograft study, were completed within 2 months after initiation from frozen cell cultures.

### 2.2 | Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the cells using ISOGEN (Wako). Complementary DNA was synthesized from total RNA using a Reverse Transcription Kit (Promega). qRT-PCR was performed using the Stratagene Mx3000P system (Agilent Technologies) and SYBR Premix Ex *Taq* (TaKaRa Bio) with sets of sense and anti-sense primers (FASMAC) for the respective genes, as shown in Supporting Information Table S1. Relative mRNA expression of each gene was calculated using the ΔCt method and calibrated against the expression of GAPDH as an internal control.

## 2.3 | Western blotting (WB)

Cellular protein was extracted from cultured cells using a lysis buffer (CelLytic-MT; Sigma-Aldrich) with a mixture of protease and phosphatase inhibitors (Sigma-Aldrich). A 20 μg aliquot of protein extract was subjected to WB for the proteins of interest, as reported previously.[20] The expression of β-actin was monitored as a control for the loading amount of protein sample. Primary antibodies used at their respective dilutions are shown in Table S2.

## 2.4 | Analyses for cell survival, proliferation, and apoptosis

BxPC-3 cells and cells from the gemcitabine-resistant clones (BxG30, BxG140, BxG400) were seeded into 96-well plates and treated with gemcitabine (Sigma-Aldrich) at different concentrations. Two other batches of the same cells were passaged in the absence of gemcitabine for 1 or 2 months, respectively, and then treated with gemcitabine. In these 3 sets of experiments, the relative numbers of viable cells at designated time points following treatment with gemcitabine were determined using the WST-8 assay kit (Cell Counting Kit-8; Dojindo). These cells were also treated with DMSO (Sigma-Aldrich) or with one of the GSK3β inhibitors: AR-A014418 (Calbiochem)[23] or SB-216763 (Sigma-Aldrich)[24] at the indicated final concentration in the medium. The relative number of viable cells at the designated time points was measured as described above. The concentrations of GSK3β inhibitors used in this study are within the reported range of pharmacologically relevant doses.[23,24] After treatment with DMSO or GSK3β inhibitor, the relative numbers of proliferating and apoptotic cells were determined using the Click-iT EdU Proliferation Assay for Microplates kit (Thermo Fisher Scientific) and the Cellular DNA Fragmentation ELISA kit (Roche Diagnostics), respectively. The mean numbers of viable cells, EdU-labeled proliferating cells, and apoptotic cells from triplicate experiments were calculated together with their standard deviations (SDs). They were compared statistically to the same cells treated with gemcitabine at different concentrations, as well as to cells treated with DMSO or GSK3β inhibitors.

## 2.5 | RNA interference (RNAi)

siRNA specific for human GSK3β (GSK3β Validated Stealth RNAi) and negative control siRNA (Stealth RNAi Negative Control Low GC duplex) were obtained from Invitrogen. The specificity of GSK3β-specific siRNA was validated in our previous study.[20] Cells were transfected with 20 nmol/L of either siRNA using Lipofectamine RNAiMAX reagent (Invitrogen). The efficiency of RNA interference was evaluated by WB with an antibody that recognized both GSK3α and GSK3β (Table S2). The effects of GSK3β knockdown on the relative numbers of surviving, proliferating, and apoptotic cells were examined and compared with the same cancer cells transfected with control or GSK3β-specific siRNA for 72 h as described above.

## 2.6 | Effects of GSK3β inhibitor on the susceptibility of cancer cells to gemcitabine

$IC_{50}$ values at 72 h following treatment with gemcitabine and AR-A014418 were measured by WST-8 assay for BxPC-3, BxG30, BxG140, and BxG400 cells. These cells were then treated with gemcitabine at the doses close to the respective $IC_{50}$ in combination with DMSO or AR-A014418 at the concentrations shown in Table S3. The combined effects of gemcitabine and AR-A014418 on the viability of cells were determined as being additive, synergistic, or antagonistic using the isobologram method.[25]

## 2.7 | Immunoprecipitation (IP) analysis

BxG400 cells were treated with DMSO or 25 μmol/L AR-A014418 for 24 h. The nuclear fraction of these cells was isolated using Nuclear Complex Co-IP Kit (Active Motif) and pre-cleaned with protein G magnetic beads slurry (Cell Signaling Technologies). Nuclear extracts from the respective cells were divided into aliquots and immunoprecipitated with a mixture of non-immune mouse and rabbit IgG, mouse anti-Rb, and rabbit anti-E2F1 antibodies, respectively, according to our previous study.[26] An aliquot of nuclear extract (input) and the immunoprecipitated material was analyzed by WB using anti-Rb and anti-E2F1 antibodies. The primary antibodies used at the respective dilutions are shown in Table S2.

## 2.8 | Luciferase reporter assay

Transcriptional activity of E2F1 was determined by luciferase reporter assay. The respective cancer cells were co-transfected with E2F1 firefly luciferase vector (E2F1(3) Luciferase Reporter Vector, Panomics) and internal control *Renilla* luciferase vector (pRL-SV40 Vector, Promega) in accordance with the manufacturers' protocols. At 72 h after transfection, the cells were treated with DMSO or 25 μmol/L AR-A014418 for 24 h. The cells were then examined for activities of both luciferases using the Dual-Luciferase Reporter Assay System (Promega) and Fluoroscan ascent FL instrument (Dainippon Sumitomo Pharmaceutical). The relative transcriptional activity of E2F1 was determined by normalizing firefly luciferase activity with *Renilla* luciferase activity in the same cells.

## 2.9 | Animal study

The therapeutic effects of gemcitabine and GSK3β inhibitor, either alone or in combination, were examined in gemcitabine-sensitive BxPC-3 cells and in the most gemcitabine-resistant BxG400 cells grown as xenografts in athymic mice. In total, $1 \times 10^6$ BxPC-3 or BxG400 cells suspended in 50 μL of phosphate buffered saline were subcutaneously inoculated into each of 53 athymic mice (Charles River Laboratories, Japan). The mice were randomly assigned to 4

groups and given intraperitoneal injections of 100 μL of 75% DMSO, gemcitabine (20 mg/kg body weight), or AR-A014418 (2 mg/kg body weight) alone or in combination, respectively, twice a week for 7-10 wk. Assuming that total body fluid in mice accounted for *c.* 60% of their body weight, the AR-A014418 dose of 2 mg/kg body weight corresponded to a concentration of *c.* 10 μmol/L in culture medium, which was within the known pharmacological dose range for this agent.[23] The dose of gemcitabine corresponded closely to the standard clinical dose (1000 mg/$m^2$ body surface area). Throughout the experiment, all mice were carefully observed every day for adverse events and their body weight was monitored. Tumors were measured in 2 dimensions, twice a week. Tumor volume ($cm^3$) was calculated using the formula: $0.5 \times S^2 \times L$, where S is the smallest tumor diameter (cm) and L is the largest (cm). The design and protocol of the animal experiment and changes in body weight of mice during treatment are shown in Figure S1. All animal experiments were undertaken in accordance with the Japanese animal ethics guidelines.[27] The protocol was approved by the Institute for Experimental Animal Work, Kanazawa University Advanced Science Research Center.

At necropsy, tumors were removed, fixed in 10% paraformaldehyde and embedded in paraffin for histopathological and immunohistochemical staining. Representative paraffin sections of the tumors were stained with H&E and immunostained using the avidin-biotin-peroxidase complex (ABC) method as described previously.[28] The primary antibodies used at the dilutions for immunohistochemistry (IHC) are shown in Table S2.

### 2.10 | Statistical analysis

The results are presented as mean ± SD. Data were analyzed using Student *t* test in a two-tailed analysis. Statistical significance was defined as values of $P < .05$.

## 3 | RESULTS

### 3.1 | Efficacy of gemcitabine on BxPC-3 cell-derived clones with stepwise acquired resistance to gemcitabine

The $IC_{50}$ values for gemcitabine in BxPC-3 cells and for the gemcitabine-resistant BxG30, BxG140, and BxG400 cells were 1.28 ng/mL, 39 ng/mL, 370 ng/mL, and 2000 ng/mL, respectively (Figure 1A). Consistent with our previous study,[22] the results confirmed that the persistent and stepwise gemcitabine resistance was retained during long-term frozen storage of these resistant clones. We also determined the $IC_{50}$ values of gemcitabine in the same cells following continuous culture in the absence of gemcitabine for 1 or 2 months, respectively (Figure S2). This indicated that gemcitabine resistance was present for at least 2 months after frozen cell culture, thus allowing subsequent experiments. mRNA and protein expression of ribonucleotide reductase M1 (RRM1), a known biomarker

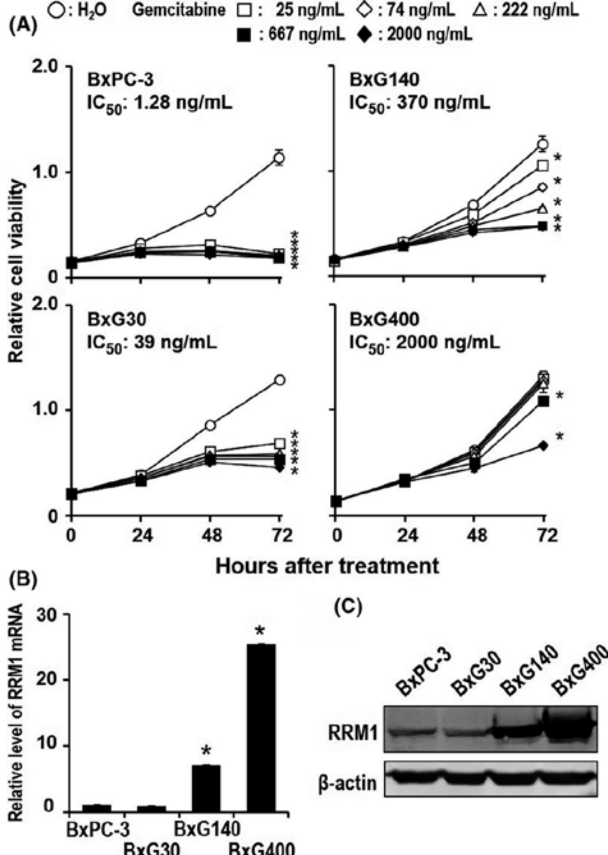



**FIGURE 1** Efficacy of gemcitabine and expression of RRM1 in BxPC-3 cells and cells from gemcitabine-resistant clones (BxG30, BxG140, BxG400). A, Respective cells were treated with $H_2O$ or the indicated concentrations of gemcitabine for the designated times in triplicate. The relative mean number of viable cells at each time point is shown with SDs. *$P < .05$. B, Relative expression of RRM1 mRNA in BxPC-3 and the gemcitabine-resistant clones. Values shown are the means ± SDs of triplicate measurements. *$P < .05$. C, Comparison of RRM1 expression between BxPC-3 cells and the gemcitabine-resistant clones by Western blotting. β-actin expression was monitored as a loading control in each sample

for gemcitabine resistance,[9,29] increased significantly in the BxG140 and BxG400 cells as the level of resistance to gemcitabine increased (Figure 1B,C).

### 3.2 | Effects of GSK3β inhibition on the gemcitabine-resistant clones

We previously reported the constitutive activation of GSK3β in human pancreatic cancer cells, including BxPC-3, and the therapeutic effect of GSK3β inhibition against these cancer cells.[19,20] We therefore evaluated the effects of GSK3β inhibition on 3 gemcitabine-resistant clones. Gemcitabine-resistant cells showed a higher level of cell survival and proliferation and a lower frequency of apoptosis compared with their parent BxPC-3 cells (Figures 2 and S3). The

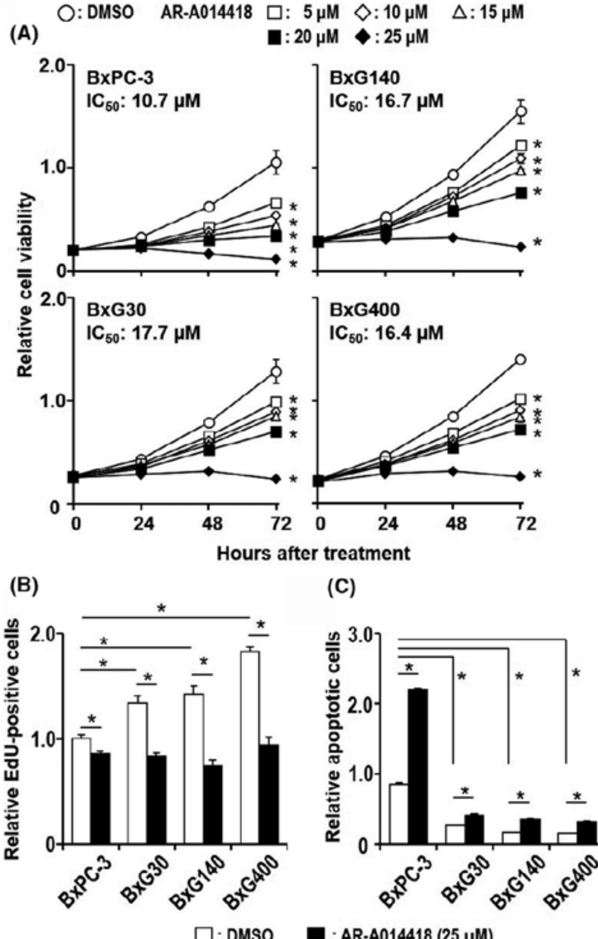



**FIGURE 2** Effects of GSK3β inhibition on viability, proliferation, and apoptosis in BxPC-3 cells and the gemcitabine-resistant clones. A, Respective cells were treated with DMSO or the indicated concentration of AR-A014418 for the designated times. The relative number of viable cells at each time point was measured by WST-8 assay. B,C, EdU-positive proliferating cells (B) and the relative number of apoptotic cells (C) were compared between the respective cells treated with DMSO or AR-A014418. A-C, The mean value from triplicate experiments was scored with SDs. *$P < .05$

GSK3β inhibitors AR-A014418 and SB-216763 reduced the viability of BxPC-3 cells and the gemcitabine-resistant clones in a dose-dependent and time-dependent manner, with similar $IC_{50}$ values irrespective of gemcitabine resistance (Figures 2A and S3A). The GSK3β inhibitors also suppressed the proliferation of all cancer cells and induced their apoptosis (Figures 2B,C and S3B,C). Similar effects were observed in these cancer cells following depletion of GSK3β (Figure S4). These results indicated that GSK3β sustained the survival and proliferation of BxPC-3 cells and of derived gemcitabine-resistant clones.

Compared with the parental BxPC-3 cells that are prone to going apoptosis following inhibition of GSK3β, the effect of GSK3β inhibition on cell proliferation was more prominent than on apoptosis in the BxPC-3-derived gemcitabine-resistant clones (Figures 2B,C, S3B,C, and S4B,C). Such difference suggested that these resistant clones might also acquire the phenotype invulnerable to apoptosis-inducing stimuli. Alternatively, they might have more dependence on the cell proliferation signal mediated by cyclin D1 and cyclin-dependent kinase (CDK)4/6 as we previously observed in pancreatic cancer cells that were primarily unresponsive to gemcitabine.[20] The effects of GSK3β RNAi on cell survival, proliferation, and apoptosis (Figure S4) were less marked than the GSK3β inhibitors (Figures 2 and S3). In many cases, the effect of an enzyme largely depends on its biochemical or catalytic activity rather than its level of expression. The biological effect of RNAi depends on the efficiency of siRNA transfection as well as on the subsequent knockdown of the target molecule. In line with these notions, our previous study using an in vitro kinase assay showed that the GSK3β inhibitor AR-A014418 inactivated GSK3β in human pancreatic cancer cells (including BxPC-3 cells) within 1 h after treatment, while the GSK3β-specific siRNA took longer than 48 h to efficiently, but not completely, deplete GSK3β expression in the same cells.[20] Therefore, it is conceivable that pharmacological GSK3β inhibitors more promptly and efficiently inhibit GSK3β in cells, leading to a more prominent biological effect on cells compared with GSK3β RNAi.

### 3.3 | Combined effect of gemcitabine and GSK3β inhibitor

Molecular-targeted therapeutics are preferably prescribed in combination with conventional chemotherapeutic agents and/or radiation and with other targeted agents. This allows the optimization of therapeutic efficacy and the minimization of undesired effects, as well as preventing the acquisition of therapy resistance.[30] We previously showed that deregulated GSK3β renders pancreatic cancer cells intrinsically unresponsive to gemcitabine via the tumor protein p53 inducible nuclear protein (TP53INP)1-mediated DNA damage repair machinery and the impairment of Rb-mediated cell cycle regulation.[19,20] Therefore, we investigated whether GSK3β inhibition affected cancer cells that had acquired resistance to gemcitabine.

AR-A014418 at 25 μmol/L alone showed adequate and similar therapeutic effects against BxG30, BxG140, and BxG400 cells (Figure 2). We therefore examined the therapeutic effects of all combinations of AR-A014418 at a dose range of 1.56-25 μmol/L and of gemcitabine at different doses in accordance with the $IC_{50}$ of the respective gemcitabine-resistant clones (Table S3). When BxG30, BxG140, and BxG400 cells were treated with gemcitabine at increasing doses, the combination with AR-A014418 reduced the $IC_{50}$ of gemcitabine in a dose-dependent fashion (Figure S5 and Table S4). Analysis of the data using the isobologram method[25] showed that AR-A014418 in combination with gemcitabine was synergistic against cancer cells from all gemcitabine-resistant clones (Figure 3A). Knockdown of GSK3β also significantly enhanced the effects of gemcitabine against these cancer cells (Figure 3B), although the combined effects were not amenable to isobologram

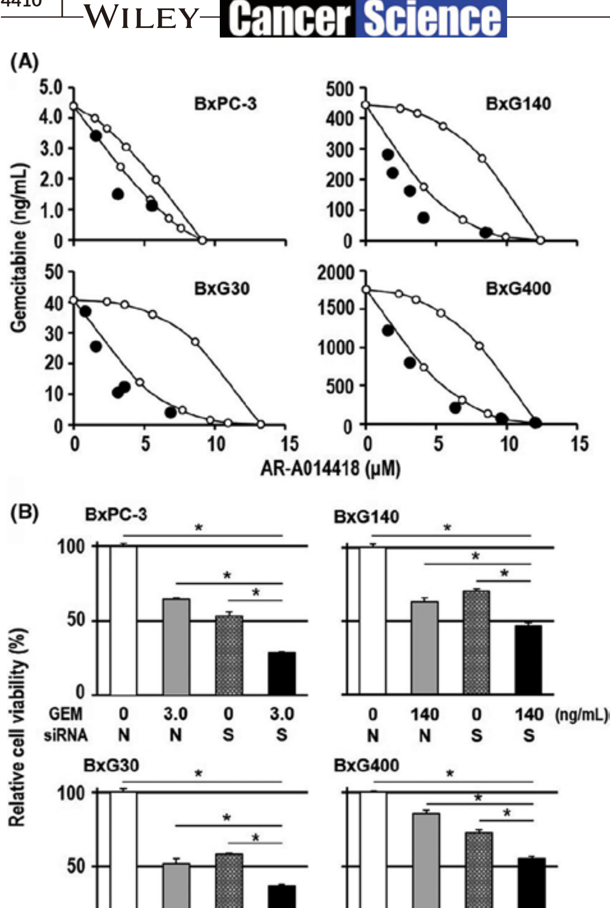



**FIGURE 3** Effect of gemcitabine in combination with GSK3β inhibitor or GSK3β-RNAi on BxPC-3 cells and gemcitabine-resistant clones. A, The respective cells were treated with various combinations of gemcitabine and/or AR-A014418 as shown in Table S3. The combined effect at 72 h after treatment was measured by WST-8 assay and analyzed by the isobologram method at respective doses (closed circle). B, The respective cells were transfected with non-specific (N) or GSK3β-specific siRNA (S) and treated with the indicated concentrations of gemcitabine (GEM) for 72 h. Mean relative number of viable cells in triplicate were scored with SDs and compared between the same cells with different treatments. *$P < .05$

analysis. These results indicated that GSK3β inhibition could circumvent acquired resistance to gemcitabine in pancreatic cancer cells.

## 3.4 | Changes in molecular characteristics of gemcitabine-resistant cancer cells following GSK3β inhibition

To improve the treatment of refractory pancreatic cancer, it is important to clarify the molecular mechanism by which GSK3β inhibition alters the acquired resistance of pancreatic cancer cells to gemcitabine. Strikingly, inhibition of GSK3β expression and activity decreased the expression of RRM1 at both mRNA and protein levels in gemcitabine-resistant cancer cells (Figure 4A,B), although the levels of RRM1 expression in BxG140 and BxG400 cells were still higher than in BxPC-3 and BxG30 cells at 72 h after treatment with AR-A014418 (Figure S6). RRM1 is a transcriptional target for E2F1. The Rb tumor suppressor protein normally traps E2F1, thereby repressing its transcriptional activity.[31,32] We therefore hypothesized that GSK3β may interfere with the Rb-mediated negative regulation of E2F1 during acquisition of resistance to gemcitabine in pancreatic cancer cells.

We have previously shown that expression of cyclin D1 and CDK4 decreased in pancreatic cancer cells (including BxPC-3) concurrently with their sensitization to gemcitabine via GSK3β inhibition.[20] Treatment with AR-A014418 consistently decreased the expression of cyclin D1 and CDK4 in cells from BxPC-3-derived gemcitabine-resistant clones (Figure 4B). As the cyclin D1-CDK4/6 complex negatively regulates the tumor suppressor function of Rb via its phosphorylation, we next investigated the expression and phosphorylation of Rb in these cells. Phosphorylation of Rb ($pRb^{S807/811}$) is known to affect its ability to bind to E2F1[32] and was progressively higher in the gemcitabine-resistant clones compared with BxPC-3 cells. Treatment with AR-A014418 decreased the level of $pRb^{S807/811}$ but did not affect the expression of Rb and E2F1 in the same cells (Figure 5A). This result suggested that GSK3β-mediated loss of Rb function may be involved in the acquisition of resistance to gemcitabine in pancreatic cancer.

## 3.5 | Changes in E2F1 transcriptional activity following GSK3β inhibition in gemcitabine-resistant cancer cells

Further to the results shown above, we next examined whether GSK3β affected the ability of Rb to bind to E2F1, as well as evaluating the transcriptional activity of E2F1 in gemcitabine-resistant cancer cells. Following treatment with DMSO or 25 μmol/L AR-A014418, nuclear extracts from the most resistant BxG400 cells were immunoprecipitated with antibodies to Rb and E2F1. WB of these immunoprecipitates showed that GSK3β inhibition increased binding between Rb and E2F1 in the cell nuclei (Figure 5B). Luciferase reporter assay showed that transcriptional activity of E2F1 in BxG140 and BxG400 cells was significantly higher than in BxPC-3 cells. Treatment with GSK3β inhibitor reduced the transcriptional activity of E2F1 (Figure 5C) and the expression of its target genes including *RRM1* (Figure 4A), *CCND1* (encoding cyclin D1), *TS* and *thymidine kinase 1* (*TK1*) in both BxG140 and BxG400 cells (Figure 5D). These results suggested that GSK3β may alter the functional interaction between Rb and E2F1 during the acquisition of gemcitabine resistance in pancreatic cancer.

## 3.6 | Effects of GSK3β inhibitors on BxPC-3 and BxG400 xenografts in mice

As a prerequisite for the clinical translation of GSK3β-targeted therapy for pancreatic cancer patients with resistance to gemcitabine,

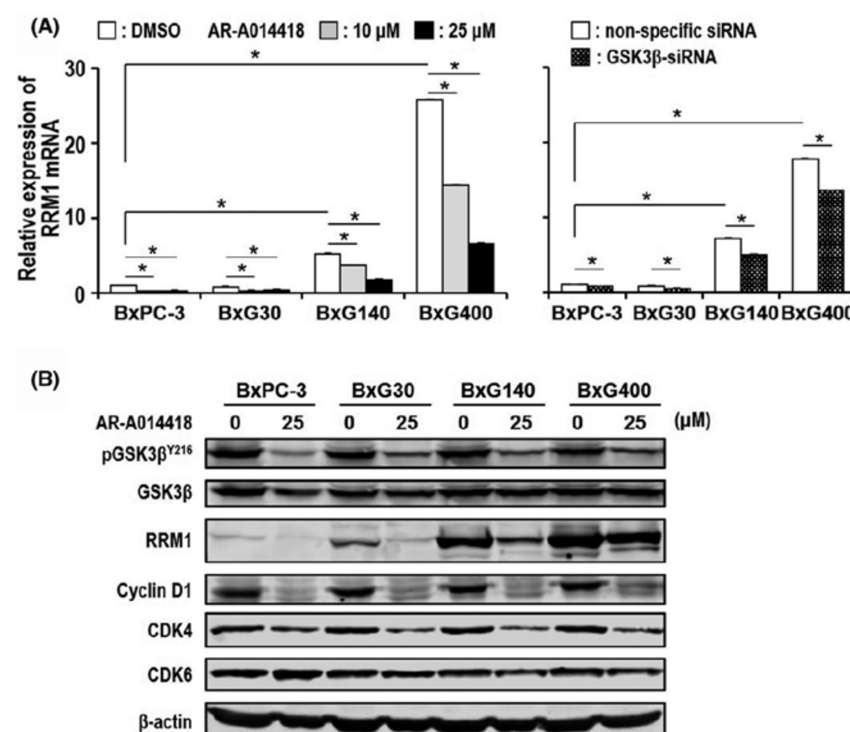



**FIGURE 4** Changes in expression of RRM1, GSK3β and cell cycle regulatory molecules in BxPC-3 cells and gemcitabine-resistant clones following GSK3β inhibition. A, The relative expression of RRM1 mRNA was compared between the respective cells treated with DMSO or AR-A014418 for 24 h (left panel), and between cells transfected with non-specific or GSK3β-specific siRNA (right panel). The mean value from triplicate experiments was scored with SDs. *$P < .05$. B, Western blotting analysis for expression of GSK3β, RRM1 and cell cycle regulatory molecules (cyclin D1, CDK4, CDK6) and for GSK3β phosphorylation (pGSK3β$^{Y216}$) in the respective cells treated with DMSO or AR-A014418 for 24 h. β-actin expression was monitored as a loading control in each sample

we tested the efficacy of gemcitabine and AR-A014418, either alone or in combination, against BxPC-3 and BxG400 cell xenograft tumors in athymic mice (Figure S1A). Xenograft tumors of BxG400 cells grew faster than BxPC-3 tumors and were unresponsive to gemcitabine at 20 mg/kg, which was almost equivalent to the standard clinical dose. Due to animal ethics issues, we euthanized the sham (DMSO)-treated mice with BxG400 xenografts and those treated with gemcitabine at 6 and 8 wk, respectively, after treatment. Compared with treatment with either AR-A014418 or gemcitabine alone, treatment of mice with the 2 agents in combination significantly reduced tumor growth in a time-dependent manner (Figure 6). We observed no serious adverse events in the 4 groups of mice during treatment, and there were no statistically significant differences in mean body weight between the groups (Figure S1B). At necropsy, gross observation and histological examination showed no lesions, primary tumors, or metastatic tumors in the major vital organs of all mice.

IHC examination of the tumors removed from sham (DMSO) and gemcitabine-treated mice showed higher levels of active GSK3β (pGSK3β$^{Y216}$) and RRM1 expression in BxG400 tumors than in BxPC-3 tumors. Treatment with AR-A014418 alone or in combination with gemcitabine decreased the pGSK3β$^{Y216}$ level and RRM1 expression (Figure 7). Similar to the results from cell culture studies (Figures 4B and 5A), cyclin D1 expression and Rb phosphorylation (pRb$^{S807/811}$) in BxG400 tumors treated with DMSO or gemcitabine alone were higher than in BxPC-3 tumors treated with the same agents, but were reduced following treatment with AR-A014418 alone or in combination with gemcitabine (Figure S7).

## 4 | DISCUSSION

Current first-line chemotherapy for locally advanced and metastatic pancreatic cancers consisted of 2 combination protocols, FOLFILINOX and nab-paclitaxel with gemcitabine. Both have been shown to improve the efficacy of gemcitabine monotherapy.[33,34] The only approved second-line regimen for patients who failed gemcitabine-based therapy is nanoliposomal irinotecan with 5-FU and folate.[35] Importantly, however, no study has yet shown a significant improvement in outcome from any of the combination regimens over gemcitabine alone in patients with poor (≥2) PS as defined by the Eastern Cooperative Oncology Group. As the vast majority of pancreatic cancer patients present with a PS ≥ 2, gemcitabine therefore remains the standard of treatment for pancreatic cancer.[7,8] This in turn has attracted growing attention to the problem of gemcitabine resistance.[9,10]

Putative biochemical mechanisms of gemcitabine resistance include the decreased expression of human equilibrative nucleoside transporter-1 (hENT1) that is indispensable for cellular uptake of gemcitabine,[36] the inactivation of deoxycytidine kinase (dCK) that is a late-limiting enzyme for metabolic activation of gemcitabine,[37] and the overexpression of RRM1 that sustains DNA synthesis, thus counteracting the pharmacological action of gemcitabine.[29] These molecular alterations found in the tumors were associated with poor survival of pancreatic cancer patients undergoing treatment with gemcitabine in various clinical settings.[38,39] Recent experimental approaches aimed at overcoming the acquired resistance to gemcitabine include targeting of hENT1 expression by TS inhibitor and bypassing nucleoside transporters by prodrugs. Other approaches

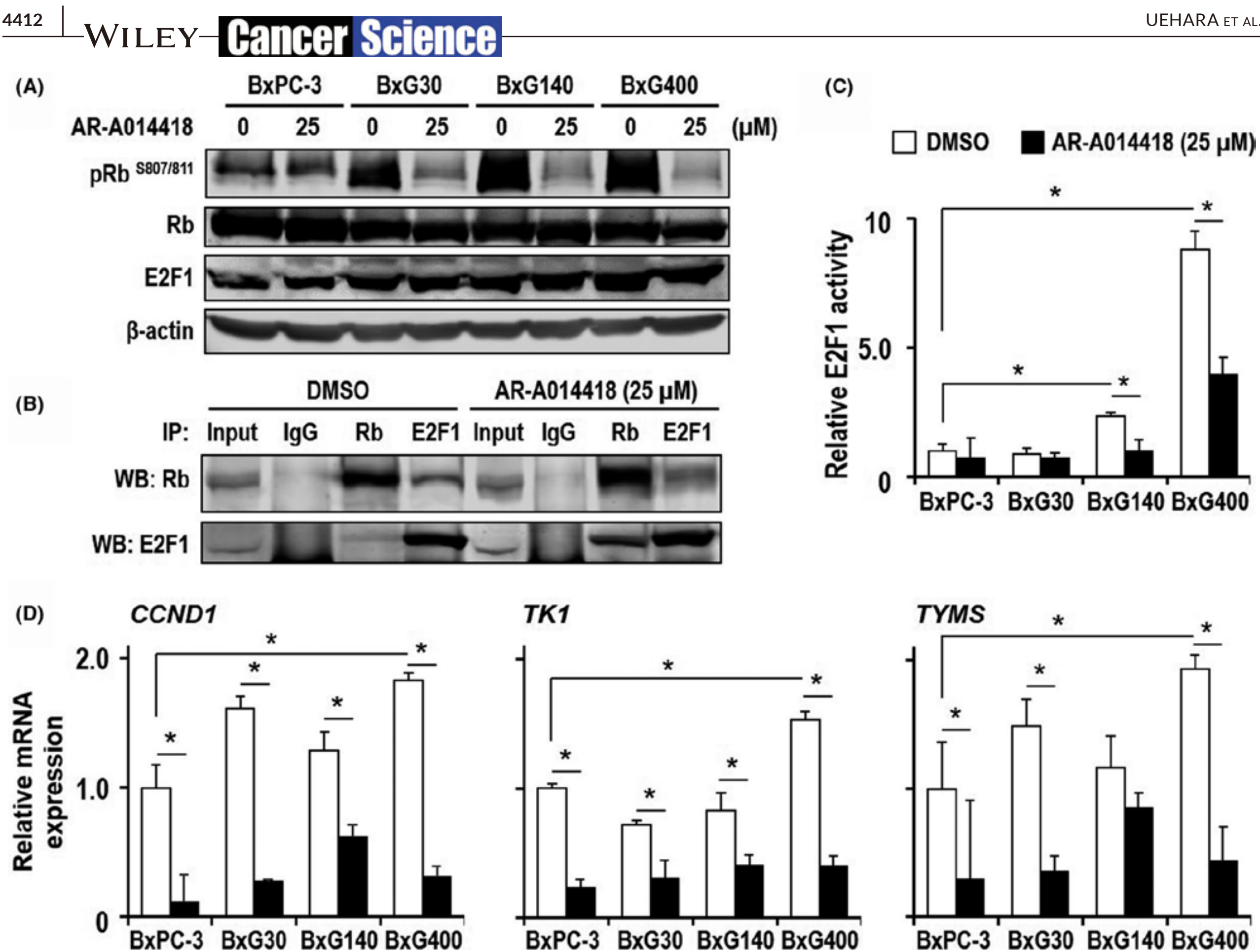



**FIGURE 5** Effects of GSK3β inhibitor on the expression and phosphorylation of Rb, its binding to E2F1 and the transcriptional activity of E2F1. A, Western blotting (WB) analysis for expression of Rb, E2F1 and phosphorylated of Rb (pRB$^{S807/811}$) in cells treated with DMSO or AR-A014418 for 24 h. β-actin expression was monitored as a loading control in each sample. B, Immunoprecipitates (IP) from nuclear extracts of DMSO-treated or AR-A014418-treated BxG400 cells with non-immune mouse/rabbit IgG and the antibody to Rb or E2F1 were analyzed by WB with the indicated antibodies. C, Relative transcriptional activity of E2F1 in the respective cells treated with DMSO or AR-A014418 was measured by luciferase reporter assay. D, Relative expression of CCND1 (cyclin D1), TK1, and TS mRNA in the respective cells treated with DMSO or AR-A014418. C, D, Data are the mean values with SDs of triplicate experiments. *$P < .05$

included improving the intracellular delivery of gemcitabine by conjugating it with nanocarriers, as well as molecular targeting of the pro-oncogenic Wnt, hedgehog and Notch signaling that are reactivated during acquired resistance to gemcitabine (reviewed in Ref. 9,10). However, there have been few attempts at targeting RRM1 to enhance the efficacy of gemcitabine, even though it is recognized as a potentially strong target for overcoming gemcitabine resistance.[40]

The gemcitabine-resistant clones (BxG30, BxG140, BxG400) used in this study were established from gemcitabine-sensitive pancreatic cancer BxPC-3 cells and were characterized by overexpression of RRM1, but only minor alterations in the expression of hENT1, dCK, RRM2, and Bcl2.[22] This suggested that there may be dependency of these resistant clones on RRM1 for the acquisition of resistance to gemcitabine. We showed that GSK3β inhibitors alone exerted therapeutic effects against BxPC-3 cells as well as against gemcitabine-resistant clones with a similar $IC_{50}$. Notably, when combined with various concentrations of gemcitabine, the GSK3β inhibitor synergistically enhanced the efficacy of gemcitabine against all of the resistant clones in culture and against the most resistant BxG400 xenografts in mice. These effects of GSK3β inhibition were associated with decreased expression of RRM1, suggesting that impairment of the transcriptional activity of E2F1 may be responsible for the "resensitization" of resistant clones to gemcitabine. GSK3β inhibition consistently attenuates E2F1 transcriptional activity, resulting in decreased expression of its transcriptional targets including *RRM1*, *CCND1*, *TS*, and *TK1*. As we previously reported,[20] GSK3β inhibition decreases the expression of cyclin D1 and CDK4 and phosphorylation of Rb, thereby restoring the binding of Rb to E2F1 in tumor cell nuclei. Consequently, our results suggest that the mechanism whereby GSK3β confers acquired resistance to gemcitabine in pancreatic cancer is via disturbance of the physiological (tumor-suppressive) interaction between Rb and E2F1.

The levels of RRM1 expression in the gemcitabine-resistant BxG140 and BxG400 cells decreased during longer treatment

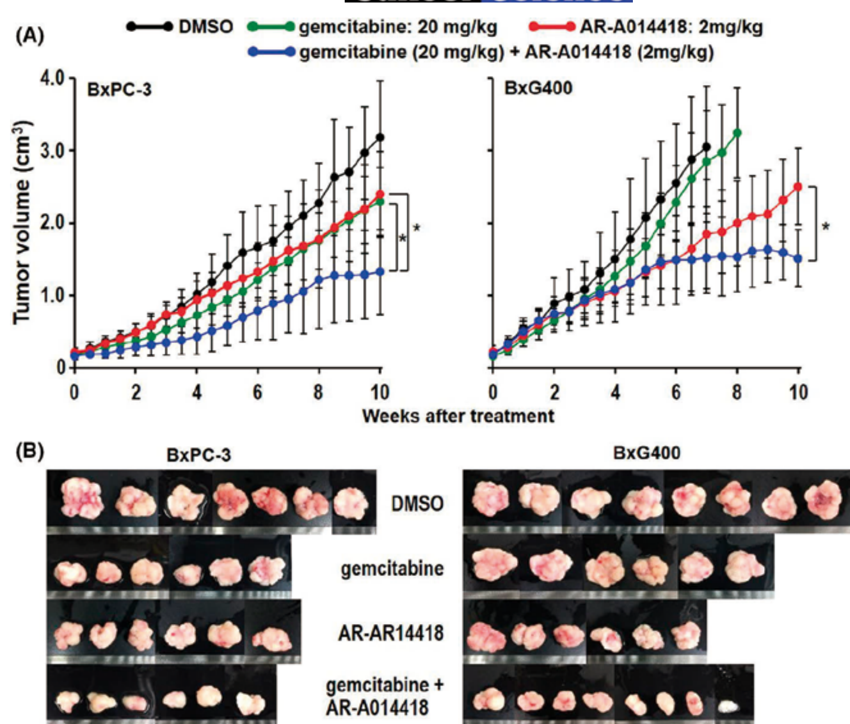
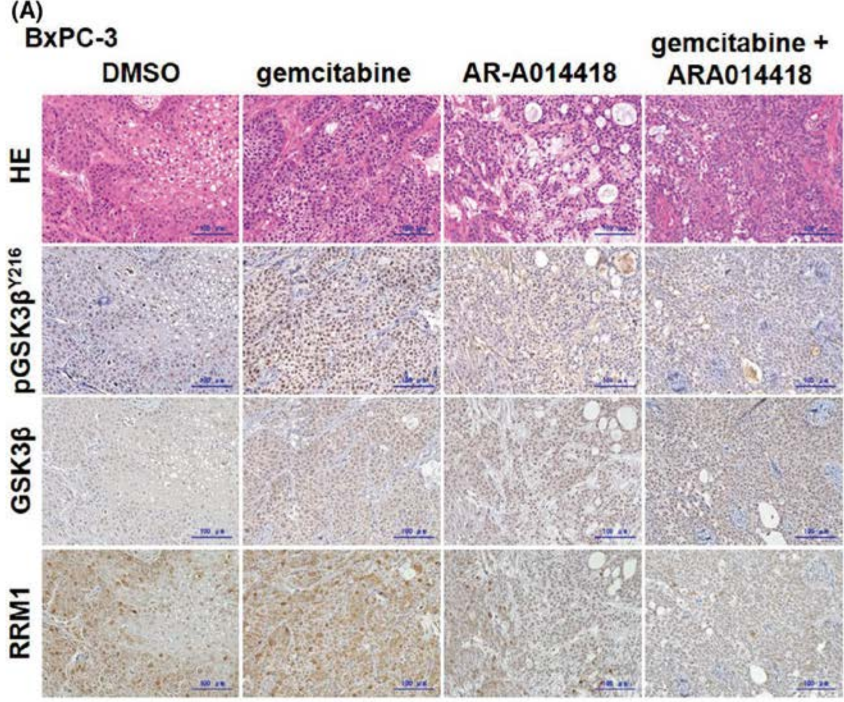



**FIGURE 6** Efficacy of gemcitabine and GSK3β inhibitor on BxPC-3 and BxG400 xenograft tumors in athymic mice. A, Time course of mean xenograft tumor volume with SDs in mice treated with DMSO, gemcitabine alone, AR-A014418 alone, or the gemcitabine/AR-A014418 combination. Mice with the BxG400 xenograft and treated with DMSO or gemcitabine were euthanized at 6 and 8 wk, respectively, after treatment because of animal experiment ethical issue. *$P < .05$. B, Gross appearance of xenograft tumors removed at autopsy from the mice

with AR-A014418, but they were still higher than in BxPC-3 and BxG30 cells (Figure S6). Isobologram analysis showed synergistic therapeutic effects of gemcitabine and AR-A014418 in combination against both resistant clones (Figure 3A). Nevertheless, AR-A014418 could substantially, but not completely, reverse the resistance to gemcitabine in BxG140 and BxG400 cells (Figure S5) and BxG400 xenograft tumors in rodents (Figure 6). An intermediate metabolite of gemcitabine, dFdCDP (2′-2′-difluoro-2′-deoxycytidine diphosphate), potently binds to and inhibits RRM1, thereby exerting its therapeutic effect via decrease of competing deoxyribonucleotide pools necessary for DNA synthesis (reviewed in Ref.9). Our results may therefore imply that the amount of RRM1 in the resistant clones treated with both gemcitabine and AR-A014418 exceeded the dose of gemcitabine. Collectively, it is suggested that the remaining RRM1 in the resistant clones after treatment with GSK3β inhibitor may still contribute to gemcitabine resistance. As we previously reported,[22] resistance to gemcitabine in these clones was not only dependent largely on RRM1, but also on the other known factors (such as hENT1, dCK, RRM2, and Bcl2) and probably on unknown factors. Accordingly, future systematic analysis of RRM1 and these factors is necessary to clarify whether any remaining RRM1 in the resistant clones (BxG140, BxG400) following the treatment with GSK3β inhibitor contributes to the gemcitabine resistance in these cells.

As previously reported, the gemcitabine-resistant clones examined in this study were also resistant to 5-FU, cisplatin, irinotecan (CPT-11), and docetaxel.[22] As TS and TK1 are known biomarkers for the efficacy of 5-FU and ionizing radiation, respectively, GSK3β may participate in cross-resistance to multiple chemotherapeutic agents. Furthermore, combination with GSK3β inhibitor may potentially enhance the efficacy of FORFIRINOX, nab-paclitaxel with gemcitabine, nanoliposomal irinotecan with 5-FU, and folate, as well as ionizing radiation. In addition to pancreatic ductal adenocarcinoma, a previous study reported that GSK3β participates in tumor progression and resistance to everolimus, an inhibitor of mechanistic target of rapamycin complex 1, in pancreatic neuroendocrine neoplasm.[41] Collectively, GSK3β may potentially play broader pathologic roles in pancreatic malignancy.

The proposed biological mechanisms for gemcitabine resistance in cancer cells include pro-invasive capacity and cancer stemness phenotypes (reviewed in Ref. 9,10). Previous studies have shown that gemcitabine-resistant pancreatic cancer cells acquire a pro-invasive phenotype such as EMT,[42] thus contributing to acquired resistance.[43,44] Based on the notion of an interconnection between cancer invasion and therapy resistance,[45] we previously showed that GSK3β facilitates both pro-invasive capacity and resistance to chemotherapy in pancreatic cancer[20] and glioblastoma.[46,47] It has also been reported that gemcitabine treatment promotes pancreatic cancer stemness through a distinct molecular pathway.[48] In light of the mounting evidence for tumor-promoting roles of GSK3β, we propose that GSK3β functions as a molecular hub that integrates therapy resistance, pro-invasive capacity, and the cancer stemness phenotype in refractory cancer, as represented by pancreatic cancer.[15,16] This cancer type is also characterized biologically by a desmoplastic and immunosuppressive tumor microenvironment that has emerged as a robust barrier to various therapeutic agents and radiation.[49,50] Recent evidence has suggested that GSK3β plays an active role in establishing the



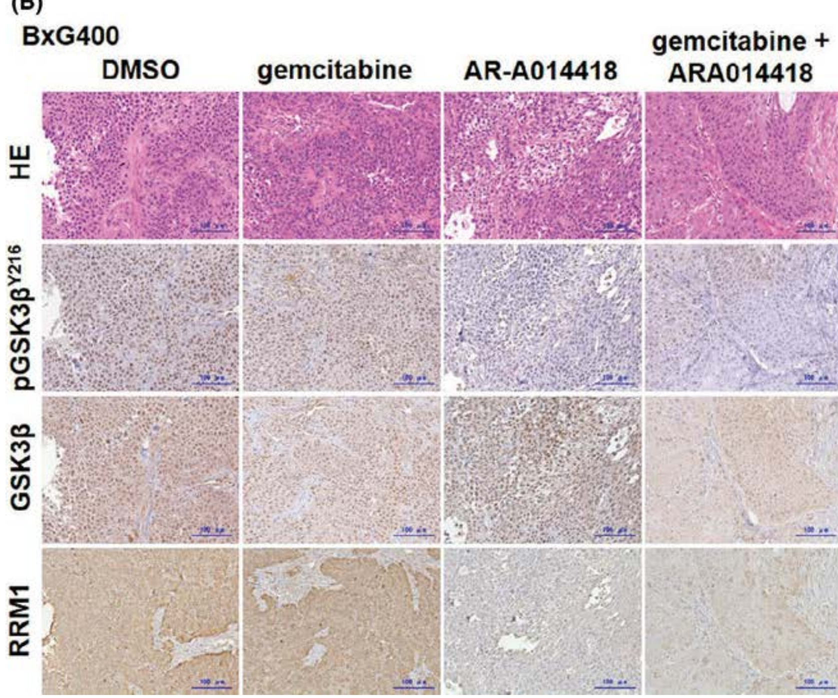




**FIGURE 7** Histological and immunohistochemical findings of xenograft tumors of BxPC-3 (A) and BxG400 (B) cells in mice treated with DMSO, gemcitabine alone, AR-A014418 alone or the gemcitabine/AR-A014418 combination. Serial sections of the tumors were stained with H&E and immunostained for GSK3β, pGSK3β$^{Y216}$, and RRM1. Scale bars, 100 μm

immunosuppressive tumor environment (reviewed in Ref. 16). Therefore, further research in this area is important for understanding the broader biological mechanisms of GSK3β-mediated therapy resistance in pancreatic cancer.

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## DISCLOSURE

The authors have no conflict of interest.

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## SUPPORTING INFORMATION

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

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