Quinquennial Report 2011-2015

金沢大学がん進展制御研究所腫瘍制御研究分野

研究のあゆみと業績 2015

研究のあゆみと業績 2011～2015

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Annual Report 2015
Quinquennial Report 2011－2015

Division of Translational & Clinical Oncology
Cancer Research Institute
Kanazawa University, Kanazawa, Japan

Quinquennial Activity 2011-2015

まえがき

今年はこの研究室を担当するようになってから 15 年目をむかえました．10 年目をむかえた 2010 年の今ごろには，あとどれくらいもつか気にしていたのがついこの間のように感じます．生まれたての子どもが中学3年生という多感な年ごろをむかえるように，自分の心も体もそうありたいと感じながら過ごしています．今回は，この５年間で少しは世のなかに役立つことができたかなぁと思いながら，本誌の後半に Quinquennial Report としてまとめてみました．ご関心のある方には後半部分（せめて本誌第 54 頁の表紙と 55 頁の駄文だけで）も眺めて，ご意見やご批評をいただければありがたく思います．

2013 年初秋に全国的な学術集会（第 24 回日本消化器癌発生学会総会）を担当したことは以前に紹介しました．これについで，世間並みに節目といわれる 15 年目の晩秋には，当地：金沢市で日本消化器病学会第 121 回北陸支部例会を第 34 回教育講演会と第 10 回専門医セミナーを併催するかたちで担当させていただきました**【附記2】**．地方会とはいえ北陸地域の消化器病学のコアとなる歴史ある学術集会です．私自身，20年来続けてきた消化器外科の手術執刀を終えたのは7年前であり，同時期より当研究所臨床部門の専攻は消化器疾患ではなくなりました．それでも消化器がんの研究と消化器疾患の診療を少しずつ続けてきたことが認められてか，当時の北陸支部長の富山大学消化器総合外科学：塚田一博先生から支部例会を担当するようにお声がけいただきました．私のいまの立場を考えるとありがたいことであり，現支部長の福井大学内科学：中本安成先生はじめ皆さんに支えていただいて何とか務めることができました（図）．

支部例会前日の講師懇談会にて．
前列左より大阪市立大学肝胆膵外科学：久保正二先生（教育講演会 講師），源，東邦大学一般消化器外科学：島田英昭先生（ランチョンセミナー 講師），後列左より石川県立中央病院消化器外科：伴登（ばんどう）宏行先生と金沢医科大学消化器内視鏡学：伊藤 透先生（いずれも教育講演会 講師）．
2015 年 11 月7日（土）

このほかはこれまで通り，研究，診療，学会など日常業務を続けるなかで，つぎの5年間はどうなるのかと漠然と考えながら過ごしています．相変わらず小さなグループです．仲間を増やして皆さんと一緒にいまのスタンスでもう少し仕事を続けてみようと思います．どうぞよろしくお願いします．

2015 年12 月年末

源 利成

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【目次】

【研究スタッフ】（2015 年現在の在籍者）

教授	源　利成	金沢大学附属病院がん高度先進治療センター（併任）
助教	堂本貴寛	テニュアトラック
大学院	金子真美	金沢大学大学院医学系研究科 先進総合外科学
（博士課程）	北村祥貴（ひろたか）	金沢大学大学院医学系研究科 先進総合外科学
	富田泰斗（やすと）	金沢医科大学大学院医学系研究科 一般・消化器外科学
	下崎真吾	金沢大学大学院医学系研究科 整形外科学
	阿部健作	金沢大学大学院医学系研究科 整形外科学（2015 年 1 月～）
	竹中　哲（さとし）	金沢大学大学院医学系研究科 がん局所制御学
（修士課程）	上原将大（まさひろ）	金沢大学大学院医学修士課程（2015 年 3 月～）
	佐々木規雄	金沢大学大学院保健学修士課程（2014 年 4 月～）
博士研究員	Ilia V. Pyko	
	石井浩子	2015 年 6 月～
共同研究員	小竹優範（まさのり）	厚生連高岡病院・外科
卒業研究生	藤井瑞希（みずき）	金沢大学医薬保健学域・保健学類4年（～2015 年 8 月）
	丸山幸奈（ゆきな）	金沢大学医薬保健学域・保健学類4年（～2015 年 8 月）
研究支援員	浅香敦子	研究支援推進員
	阿部尚子	技能補佐員（ヒトがん組織バンク）
研究協力員	旭井亮一（あさい）	（株）凸版印刷
	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）	福井県立病院 脳神経外科
	横井健二	金沢大学 心肺総合外科学；（現）米国メソジスト病院研究所
	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（ひがし）　朋美	金沢大学大学院医学系研究科環境分子応答学／衛生学
	笠島（かさしま）里美	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
	宮下勝吉（かつよし）	金沢大学附属病院 脳神経外科
	中島（なかじま）日出夫	上尾中央総合病院 腫瘍内科
共同研究者	山下　要	金沢大学附属病院がん高度先進治療センター
	塚（づか）　正彦	金沢大学大学院医学系研究科法・社会医学（法医学）

【共同研究者】（2015 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）	金沢大学医学系研究科 脳機能制御学／脳神経外科学
医員	古田拓也	金沢大学附属病院 脳神経外科
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准教授	滝野隆久	金沢大学がん進展制御研究所 細胞機能統御学
教授	太田哲生	金沢大学大学院医学系研究科 がん局所制御学／消化器外科学
准教授	伏田幸夫（さちお）	金沢大学大学院医学系研究科 がん局所制御学／消化器外科学
助教	宮下知治	金沢大学大学院医学系研究科 がん局所制御学／消化器外科学
教授	土屋弘行	金沢大学大学院医学系研究科 機能再建学／整形外科学
特任教授	山本憲男	金沢大学大学院医学系研究科 機能再建学／整形外科学
教授	竹村博文	金沢大学大学院医学系研究科 先進総合外科学
教授	Richard Wong	金沢大学理工学域 自然システム学系
教授	元雄良治（よしはる）	金沢医科大学腫瘍内科学
教授	小坂健夫（たけお）	金沢医科大学一般・消化器外科学
講師	藤田秀人	金沢医科大学一般・消化器外科学
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
教授	江角浩安	東京理科大学 生命医科学研究所 臨床研究部門
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
	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	竹田　扇（せん）	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
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
准教授	石渡俊行（いしわた）	日本医科大学・病理学（統御機構・腫瘍学）
医長	松田陽子	東京都健康長寿医療センター病理診断科
副院長	西村元一	金沢赤十字病院 外科
外科部長	伴登宏行（ばんどう）	石川県立中央病院 消化器外科
教授	Andy Giraud	オーストラリア王立小児病院
	Louise M. Judd	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
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
准教授	Vladimir Spiegelman	ウィスコンシン大学 皮膚科学

【2015 年のあゆみとできごと】

2015 年 1 月　・中田光俊先生が本学脳神経外科学教授に就任

2015 年 1 月　・塚 正彦先生（金沢大学第一病理学同門）が本学法・社会医学教授候補に内定

2015 年 1 月　・阿部健作先生（本学整形外科学大学院）が研究に参画
GSK3β を指標にする軟部肉腫の病態解明と新しい治療法の開発研究

2015 年 1 月 23 日　・源　利成：石川県立金沢泉丘高等学校ｽｰﾊﾟｰｻｲｴﾝｽﾊｲｽｸｰﾙで模擬講義
【附記1】　課題：がんのエネルギー代謝
会場：金沢医科大学

2015 年 2 月　・塚 正彦先生（金沢大学第一病理学同門）が本学法・社会医学教授に就任

2015 年 4 月 1 日　・科学研究費補助金基盤研究 B（代表），挑戦的萌芽研究（代表），基盤研究 A（連携），基盤研究 C（連携）などに採択

2015 年 5 月　・竹中 哲君（本学消化器・腫瘍・再生外科学大学院）が研究に参画
仮題：膵がん幹細胞性の生物学

2015 年 6 月 1 日　・石井浩子さんが博士研究員に採用され，研究開始

2015 年 7 月 10 日　・金沢医科大学腫瘍内科学との合同親睦会（七夕の会）
さかなや道場（金沢駅前）

2015 年 7 月 31 日　・本学保健学類の藤井瑞希（みずき）さん，丸山幸奈（ゆきな）さんが卒業研修を修了
課題：大腸がんにおける K-ras 活性化とがん促進因子 GSK3βの発現と関連解析

2015 年 11 月 8 日　・源 利成が学会会長を担当　於：石川県地場産業振興センター新館
【附記2】　日本消化器病学会第 121 回北陸支部例会
第 31 回教育講演会
第 10 回専門医セミナー

2015 年 11 月 17 日～19 日　・源 利成が第 46 回高松宮姫癌研究基金国際シンポジウムに招待
於：パレスホテル東京
課題：Oncometabolomics: A new clue to understand carcinogenesis, cancer biology and to develop novel diagnostics and therapeutics

2015 年 11 月 22 日　・金沢大学がん研究所外科同門会総会・懇親会：ホテル日航金沢

2014 年 12 月 04 日　・腫瘍制御・金沢医科大学腫瘍内科合同忘年会：醍庵分家 海彦（ 金沢駅前）

【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てから一貫して，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，基礎と臨床を密接に関係づける方向で研究を続けている．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ことを検討している．大腸がんの分子病理学的特性は組織バンク資源をもとに外科系大学院の学位研究課題として継続している（後述）．

1．Wnt 経路に関わる新しい分子細胞機構の検討

Wnt 経路固有のがん化シグナルを理解する目的で，大腸がんの腫瘍-宿主境界の腫瘍環境で活性化される β-カテニンを機軸とするがん化経路の病理作用を明らかにしてきた．とくに β-カテニンが転写誘導する CRD-BP（coding region determinant-binding protein）に着目し，大腸がん病態との関連を検討している．がんにおける β-カテニン活性化の仕組みについて，その分解複合体構成因子やユビキチン経路の調節異常を明らかにしてきた．β-カテニンの核移入はその活性化に必須であるが，核局在構造を持たない β-カテニンが細胞質と核を往来する仕組みは明らかではない．本学 Wong 教授とともに，核-細胞質間分子移送を担う核膜孔複合体因子（nucleoporins: Nups）の検索を始めた．これまでに，大腸がんではある種の Nup が β-カテニンや Tcf7L2（Tcf-4）の核内発現を調節していることを見出した．

この課題は大腸がんの Wnt 経路の病理作用の理解と，個体発生や分化など多様な生命現象の研究へ応用が期待される．これまでの研究経過を振り返ると，一連の成果は国際的評価が高い科学誌に掲載されてきた．しかし，その引用回数（インパクト）は低く，臨床応用には程遠い状況であり，今後の研究継続の是非や方向性を慎重に検討している．

2．glycogen synthase kinase (GSK) 3β 阻害によるがん治療法の開発研究と応用

大腸がんにおける Wnt/β-カテニン経路の研究過程で思いがけず，同経路の抑制因子としてひろく認識されていた GSK3β が固有の分子経路を誘導して，がん細胞の生存，不死化，増殖を推進することを発見した．そして，GSK3β 阻害の強力で特異的ながん治療効果を細胞レベルと非臨床試験で実証した．大腸がん研究と並行して，本学脳神経外科学，整形外科学，消化器・腫瘍・再生外科，金沢医科大学腫瘍内科学，総合医学研究所などと連携し，GSK3β の「がん促進作用」は膵がんや膠芽腫，骨軟部肉腫などの難治，希少がんにも観察され，腫瘍細胞に高度の浸潤性と治療（抗がん剤，放射線）不応性などの悪性形質を賦与することを見出した．そして，GSK3β 阻害医薬品の転用（repurposing/repositioning）と抗がん剤を併用するがん治療法を共同開発し，再発膠芽腫（本学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型臨床研究によりその安全性と抗腫瘍効果を試験している（UMIN000005111，UMIN000005095）．

がんにおける GSK3β の生物学を理解するため，がん固有のエネルギー代謝（Warburg 効果）に関わる代謝酵素や自食作用（オートファジー）に着目して機能解析を進め，新しい知見が得られつつある．また，遅ればせながら，抗がん剤耐性化膵がんや原発膠芽腫スフェアを対象に，GSK3β によるがん幹細胞形質の制御について研究を開始した．消化器がんの臨床に即した課題として，内視鏡的にヨード不染で認識される食道発がん初期病巣（前がん病変）の発生機構に関して，がん代謝の視点から今後アプローチしたい．

GSK3β 阻害によるがん治療法開発の基盤として，国内製薬企業が保有する阻害剤や GSK3β 制御性 micro-RNA の配列をもとに東工大生命理工学科と共同開発するアンチセンスオリゴ核酸のがん治療効果の解析，cell-based ELISA による新規阻害剤スクリーニング技術の開発などの準備を進めている．

3. エピジェネティクスを指標にするがん診断・治療法の開発

大腸発がん経路をジェネティック・エピジェネティックな変化により説明・細分類し，診断・治療に応用することを目的に，本学外科学大学院生と共同研究員が継続している．

4. ヒト消化管がん組織検体資源化プロジェクト

がんの生物学的解析から観察される現象を実際の病巣で検証してはじめて，がんの臨床に橋渡しできる．この目的で，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．この組織資源をもとに国内外の機関と共同研究により，胃がんや大腸がんにおける Wnt 経路の分子病理特性（Nature 2006, Cancer Res 2009）やレトロポゾン，トレフォイル因子，RUNX3 などを対象にエピジェネティック変化の解析による発がん，進行の分子機構を明らかにしてきた（J Clin Invest 修正投稿中，Oncogene 2015, Gastroenterology 2011, Clin Cancer Res 2010, Gastroenterology 2010）．これらの研究に加えて 2014 年より，山梨大学が開発した大気圧イオン化法−質量分析装置を用いて，大腸組織検体のマススペクトルデータベースをもとに統計解析手法により非がん/がんの判別（診断）システムを構築し，生体分子の発現量を解析することでがん病態解明の基盤を構築することを目的に，同大解剖学を主体に本学消化器・腫瘍・再生外科学と共同研究を始めた．

七夕の会 2015（於：さかなや道場 金沢駅前） 2015 年 7 月 10 日

【研究費】（2015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
2015－2017 年度科学研究費補助金（基盤研究B）：15H04928	源　利成	宮下知治 太田哲生 曽我朋義， 清尾康志	GSK3β 経路を標的とする大腸がんの病態解明と治療法開発の基盤形成	14,240,000 円
2015－2016 年度科学研究費補助金（挑戦的萌芽）：15K15493	源　利成	Richard Wong 石垣靖人 太田哲生 宮下知治	大腸がんにおける β-カテニン核移送に作用する核膜孔複合体因子の探索と機能解析	3,510,000 円
2015－2018 年度科学研究費補助金（基盤研究A）：15H04928	大島正伸	源　利成， ほか	大腸がん自然転移・再発モデルの開発による悪性化進展機構の研究	41,000,000 円
2015－2017 年度科学研究費補助金（基盤研究C）：15K09051	島崎猛夫	石垣靖人 源　利成	抗がん剤による膵がん細胞の浸潤形質獲得の分子機構の解明とがん治療への応用	4,810,000 円
2014－2015 年度科学研究費補助金（研究活動ｽﾀｰﾄ支援）（25670572）	Ilya V. Pyko		Development of combined cellular and molecular target-directed therapies for glioblastoma	2,300,000 円
2014－2016 年度科学研究費補助金（基盤研究B）（25670572）	中田光俊	源　利成 （連携）	ドラッグリポジショニングによる悪性グリオーマに対する新規化学療法の基盤構築	12,160,000 円
2013－2015 年度科学研究費補助金（若手研究B）（25860233）	堂本貴寛		エネルギー代謝特性に基づく消化器がん病態解明と制御への応用	3,770,000 円
2015 年度金沢大学がん研究所共同研究（一般）	小坂健夫	源　利成， ほか	大腸がんにおける β-カテニン/Tcf の転写標的 CRD-BP の分子病理学的特性と病態の解明	700,000 円
2015 年度金沢大学がん研究所共同研究（特定）	島崎猛夫	源　利成， ほか	GSK3β を標的とする新規核酸医薬の機能解析	500,000 円
2015 年度金沢大学がん研究所共同研究（一般）	松田陽子	源　利成， ほか	膵幹細胞の加齢と発癌機序の解明	500,000 円
2015 年度金沢大学がん研究所共同研究（特定）	吉村健太郎	源　利成， ほか	大気圧イオン化法-質量分析を用いた大腸がん診断システムの確立および病態解明	500,000 円

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
2015 年度金沢大学がん研究所共同研究（特定）	山本憲男	源 利成，ほか	軟部肉腫の GSK3β を標的とする新規治療法の開発と分子メカニズム	200,000 円
2015 年度金沢大学がん研究所共同研究（一般）	宮下知治	源 利成，ほか	GSK3β 阻害による食道発癌の予防とその機序の解明	200,000 円
2015 年度金沢大学がん研究所共同研究（一般）	Richard Wong	源 利成，ほか	大腸癌進行に関与する核膜孔複合体蛋白の同定および機能解析	200,000 円
2015 年度金沢大学がん研究所共同研究（一般）	古田拓也	源 利成，ほか	既存薬転用による膠芽腫に対する GSK3β 標的療法の開発	200,000 円
2015 年度金沢大学がん研究所共同研究（一般）	Xi Cheng	源 利成，ほか	The relationship between GSK3β expression and chemoresistance in ovarian clear cell carcinoma	200,000 円
奨学寄附金	源 利成	2015 年 3 月	予防医学関連財団（I）	800,000 円
奨学寄附金	源 利成	2015 年 3 月	予防医学関連財団（I）	400,000 円
			期間の総額	86,190,000 円

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・英文総説，著書

なし

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・その他（講演，社会・地域貢献を含む）

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Ⅲ. 研究成果による知的財産権の出願状況

1. 特許出願

該当なし

Ⅳ. 新聞，報道など

該当なし

【附記1】 COSMO SCIENCE I NEWS

No. 5

COSMO SCIENCE I NEWS

石川県立金沢泉丘高等学校 SSH 推進室

第５回コスモサイエンスⅠ　　平成 27 年１月 23 日（金）

講義①：「がんのエネルギー代謝」

講　師：金沢大学がん進展制御研究所

　　教授　源　利成　先生

講義②：「においの受容とノーベル賞」

講　師：金沢医科大学

　　教授　三輪　高喜　先生

実　習：救急処置（クリニカルシミュレーションセンター）

場　所：金沢医科大学

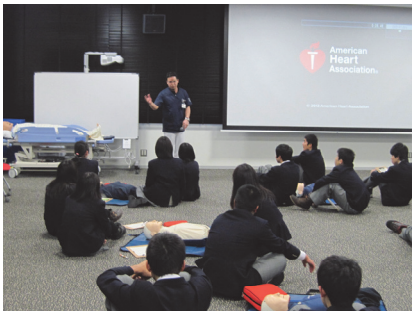
問　最新の医療技術を行うことは医学の進歩のために不可欠なことであるが、科学技術者としてあなたが身につけておかなくてはならない素養および必要であると考えるところをまとめなさい。

私が心得ておくべきだと考える素養は異なる視点をも持つということである。何事に対しても「それは当たり前ではない」「ではなぜそうなのか」と固定観念を捨てて疑問を持つことが新たな発見へとつながると考える。

固定観念、と言っても普段自分の中にあると認識しにくいが、場合によっては大きな壁となる。歴史で見ても、大勢の学者達が新しい学説を発表する度に、一つの考えに縛られた人々によって批判を浴びてきた。遺伝学者のメンデルもその一人だ。生前にその偉大な発見は認められず、証明されたのは随分あとだったという。科学におけるある進歩が遅れたということだ。この場合、メンデルに対してまわりの人々が固定観念にとらわれたわけだが、技術者、研究者自身もとらわれてはいけない。

現在、様々なことが証明され「当たり前」があふれているが、もう一つの視点から考え、自身に問いかけていけば小さな一歩につながるだろう。

（８番　藤尾）



私たちが科学技術者として世の中で活躍していくためには、技術や知識は欠かせない。だが、技術者は技術や知識だけではなく心の部分、つまり、倫理観も持ち合わせるべきではないだろうか。

現在の日本社会では、家電製品や交通機関、医療機器などにおける様々な科学技術が存在し、「便利」な生活が普及している。だが、人々はこの「便利さ」をさらに向上させようと新たな科学技術を求める。ここで考えるべきことは、この無限の「便利さ」の追求が社会にどのような影響を与えるかということである。福島第一原発問題は原子力、注射器による病気の感染は医療という科学技術によるものである。

他人が被害を受けるような科学技術は世に広めない。「便利さ」の中に生きる私たちには、技術よりも先に、一個人として持つべき倫理観が問われているのではないだろうか。

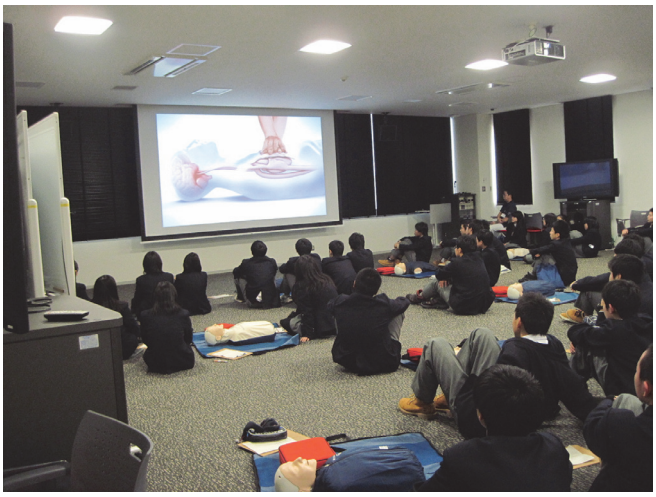
（２３番　鹿渡）

科学技術者ならば、探求心が必要である。なぜこのようになるのか、という謎から科学の発展につながることは今までに多々ある。

その中で、普段コスモサイエンスでやっている「質問」という行為は探求心そのものである。それは、今まで理解できていないことや知らないことが何なのかと考えることである。そして、それが先人によって解明・発明されていなければ、新たな研究のテーマと成り得る可能性が高いのだ。

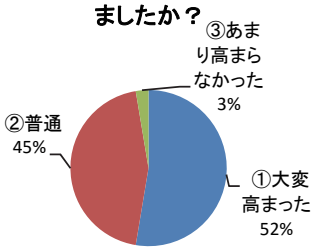
探求心とは、そのものの本質を理解しようとし、その中で出てきた矛盾に興味をもつことだ。しかし、世の中に矛盾は起こらない。何かの原因から結果は発生する。このコスモサイエンスを通じて、素朴な疑問を持つこと、そしてその疑問を発生させるための幅広い知見を得て、科学者の一員として参加できるようになることが今からの目標である。

（３６番　松井）

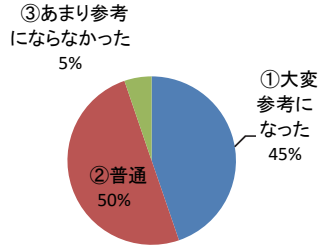


Result of a questionnaire

科学に対する興味・関心が高まりましたか？



将来の参考になりましたか？



【附記2】 第 121 回日本消化器病学会北陸支部例会，第 31 回教育講演会，第 10 回専門医セミナー

日本消化器病学会北陸支部

第 34 回　教 育 講 演 会
第121回　支　部　例　会
第 10 回　専門医セミナー

プログラム・抄録集

教 育 講 演 会
日　時：2015（平成27）年11月 8 日（日） 午前9時～12時
会　場：石川県地場産業振興センター新館　金沢市鞍月2－1
1 階コンベンションホール

北陸支部例会
日　時：2015（平成27）年11月 8 日（日） 午後2時5分～
会　場：第一会場　1階コンベンションホール
第二会場　5階第12研修室

専門医セミナー
日　時：2015（平成27）年11月 8 日（日） 午後1時～2時
会　場：第一会場　1 階コンベンションホール

当番会長　金沢大学がん進展制御研究所　源　　利成

JSGE
1898

The Hokuriku Branch of
the Japanese Society of Gastroenterology

第34回教育講演会

日　時：2015(平成27)年11月 8 日(日)　午前 9 時～12時

会　場：石川県地場産業振興センター新館
1 階コンベンションホール

1．9：00～ 9：40 「早期胃癌の内視鏡診断と治療の最前線」
座長：安本　和生　先生（金沢医科大学　腫瘍内科学）
講師：伊藤　　透　先生（金沢医科大学　消化器内視鏡学）

2．9：40～10：20 「高度進行大腸がんへの取り組み」
座長：中村　慶史　先生（金沢大学附属病院　胃腸外科）
講師：伴登　宏行　先生（石川県立中央病院　消化器外科）

3．10：20～11：10 「肝細胞がんの発がんと治療に関する最新の知見」
座長：高村　博之　先生（金沢大学附属病院　肝胆膵・移植再生外科）
講師：田中　真二　先生（東京医科歯科大学　分子腫瘍医学分野）

4．11：10～12：00 「職業胆管がん：印刷業者の胆管がん多発事例」
座長：原田　憲一　先生（金沢大学　医薬保健研究域医学系　形態機能病理学）
講師：久保　正二　先生（大阪市立大学大学院　肝胆膵外科学）

◎ 教育講演会の参加で専門医更新単位は 9 点取得できます。
◎ 参加費1,000円（当日会場入口の受付でお支払い下さい）

1．「早期胃癌の内視鏡診断と治療の最前線」

9：00～9：40

金沢医科大学　消化器内視鏡学　伊　藤　　透

昭和35年の国民健康保険が開始された当初より、当時開発して間もない胃カメラは日本国民の死亡原因として多かった胃癌の撲滅を目的に保険収載された。そのお蔭で、胃カメラは爆発的に売れ始めた。当時はバリウム透視による診断が全盛であったため医学書においては透視診断が優位であった。しかし、最初は方向性の自由にならない胃カメラの写真診断からファイバースコープで直接視ることに発展し、それに診断のための組織を採取する機能が付与された。診断学が格段進歩した。更にファイバースコープの機能が充実し治療用のスコープが開発された。ファイバースコープは電子スコープの開発により、解像能の向上と伴に処置機能も向上した。

日本の内視鏡診断学は、透視診断が基盤となっておりファイバースコープの時代から近年まで大きく変化していなかった。拡大内視鏡の開発により、早期食道癌、早期大腸癌においては質的診断が色素を併用した拡大内視鏡所見により、病理診断に匹敵するまでに発展した。更に内視鏡のNBI（Narrow Banding Image）、BLI（Blue Laser Image）モードの開発によってより効率的な質的、深達度診断が可能となった。しかし、早期胃癌においては、質的診断についてはかなり病理診断に肉薄していると思われるが、H. pylori感染等による胃炎の影響により深達度診断は依然として日常臨床に大きく貢献できる程に熟成しているとは言い難い状況である。

本講演では、現在日常診療で用いられている早期胃癌の内視鏡診断のデバイス、診断学とESDが全盛となっている内視鏡治療について述べ、外科的治療との間をなす腹腔鏡下内視鏡的全層切除についても言及する。

NBI＋拡大内視鏡診断では、田尻らが最初に報告して以来、多数の分類が報告されているが、いずれも分類が複雑で田尻論文を超える内容は見当たらない。内視鏡治療か外科的治療かの判断が求められる早期胃癌のうちSM胃癌の深達度診断においては、未だNBI＋拡大内視鏡所見で確定的な所見の観察は困難であると言わざるを得ない。すなわち深部SM浸潤にみられると言われるinterrupted signは、SM浸潤部は病変の厚みや、易出血性などのため観察が困難なことが多い。加えて術前診断を切除後の病変で再検討することが困難である。今後の検討課題であると思われる。

治療については、分化型腺癌で粘膜内早期胃癌の正確な深達度診断がなされれば、線維化の強い症例などを除けばほとんど部位がESD可能となっており、患者の機能温存に寄与すること大であると思われる。但し、本邦の報告では不適切切除、すなわち粘膜内浸潤の診断にてESD施行例の11～13%がSM胃癌であったとのことで深達度診断の難しさを物語っている。低分化腺癌においては、適応拡大が進められている現状であるが、20㎜以内の大きさで、切除後の標本で、粘膜内浸潤で完全切除、リンパ管、脈管侵襲陰性が確認されば経過観察可能であろう。

一方、早期胃癌全体の約30%を占めるSM胃癌においては、現在の標準的な治療は外科的手術である。しかし、全体で20%程度のリンパ節転移しかないと言われておりこれを術中に確認できれば、腫瘍の局所切除を行い機能温存が可能となる。国内の幾つか大学病院がリーダーとなり、幾つかに方法で腹腔鏡下センチネルリンパ節生検を行い、リンパ節転移陰性であれば、内視鏡的に全層切除（EFTR）を行う治療が進められている。

H. pylori除菌時代を迎えた早期胃癌の内視鏡診断・治療は機器の開発と伴に更に発展するものと思われる。

伊藤　　透（いとう　とおる）

略　歴：

1982年（S 57）3 月	金沢医科大学医学部卒
1986年（S 61）3 月	金沢医科大学大学院医学研究科　修了
1986年（S 61）4 月	金沢大学がん研究所附属病院　外科
11月	同　助手
1988年（S 63）5 月	国立がんセンター放射線科（内地留学）
1988年（S 63）10月	金沢大学がん研究所附属病院　外科
1990年（H 2）1 月	国立病院機構石川病院　外科医長
1992年（H 4）4 月	金沢大学がん研究所附属病院　外科
1994年（H 6）4 月	金沢医科大学　消化器内科　助手
1995年（H 7）4 月	同　講師
	金沢医科大学病院　内視鏡センター副部長
2001年（H13）7 月	金沢医科大学　総合診療科　講師
2001年（H13）8 月	金沢医科大学　総合診療科　助教授
2004年（H16）4 月	金沢医科大学　総合内科学（総合診療科）　助教授（改組のため）
2007年（H19）4 月	金沢医科大学病院　内視鏡科　科長・准教授
2008年（H20）4 月	金沢医科大学病院　内視鏡センター　部長
2008年（H20）10月	金沢医科大学病院　内視鏡科　臨床教授
2012年（H24）12月	金沢医科大学　消化器内視鏡学　臨床教授
2013年（H25）6 月	金沢医科大学　消化器内視鏡学　教授（講座主任）

専門分野：

消化器内視鏡

所属学会：

日本消化器内視鏡学会（専門医、指導医）・社団評議員
日本消化器病学会（専門医、指導医）・支部評議員
日本外科学会（認定医）
日本消化器外科学会（認定医）
日本内科学会（認定医）
日本東洋医学会
日本胃癌学会

委員（査読含）：

日本消化器内視鏡学会和文誌編集委員会査読委員（平成12年 4 月～）
エンドスコピックフォーラム編集監事（平成13年 1 月～）
日本消化器内視鏡学会雑誌英文誌（平成18年 4 月～）
日本消化器内視鏡学会技師制度技師会委員（平成24年 7 月～）
日本消化器内視鏡学会医療安全委員会委員（平成26年 7 月～）
日本消化器内視鏡学会卒後教育委員会委員（平成26年 7 月～）
一般社団法人 日本消化器関連学会機構社員（平成27年 1 月～）
一般社団法人 日本消化器関連学会機構総務企画委員会委員（平成27年 1 月～）

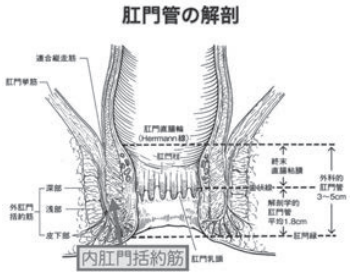
２．「高度進行大腸がんへの取り組み」

9 ：40～10：20

石川県立中央病院　消化器外科　伴　登　宏　行

下部直腸は狭い骨盤内に存在し、周囲に排尿、生殖に関係した重要臓器がある。そのため手術は難しいが、逆にやり甲斐のあるところである。今回、われわれの下部直腸がんに対する取り組みを紹介する。

１．肛門温存について



まず問題になるのは肛門の温存である。図は肛門管の解剖を示す。1980年代後半からは器械吻合などの導入で、肛門挙筋付着部上縁(Herrmann線)での吻合が可能となった。さらにSchiessel Rらにより内肛門括約筋も切除し、さらに低位での切除が提唱された。

１）括約筋間切除

内肛門括約筋は直腸の内輪状筋が肥厚したもので、平滑筋である。安静時に肛門を締める働きをしている。当科でもこの術式の導入により直腸切断術は年間１～２例に減った。しかし、術後の便失禁は必発である。若年者では１～２年の経過で排便機能は改善し、日常生活で支障を来すことは少ないが、高齢者では回復は難しく、吻合は行わず、永久的人工肛門を造設している。それでも肛門挙筋を切除しないため、術後の会陰創の合併症がなく、有用な方法である。

２）経肛門的内視鏡下手術

直径４㎝の円筒形の器具を肛門に挿入し、内視鏡下に鉗子で腫瘍を局所切除する手技である。従来、早期がんに行われてきたが、直腸壁を全層切除できるため、当科では高齢で、ADLの低下した患者さんを対象に積極的に施行している。現在、局所切除後に、術後化学放射線療法を行う臨床試験が進行中である。

２．局所進行直腸がんの治療

１）術前化学放射線療法

日本では直腸がんに対する治療は手術が第一選択であるが、欧米では術前化学放射線治療(CRT)が標準である。これにより、約20%の症例で、病理学的完全奏功が得られる。欧米では臨床的完全奏功例に対し、手術をしないで(non-operative management)、経過を見る(Watch & Wait)試みがされている。腫瘍の縮小効果は確実だが、術後に縫合不全の治癒の遅延、口側結腸の狭窄と認め、当科では標準治療になっていない。

２）術前化学療法

CRTは局所再発を抑えるが、遠隔転移は減少せず、生存率は向上しないとされている。一方、抗がん剤治療の進歩により、術前化学療法のみを行う試みがされている。当科ではオキサリプラチンを用いている。腫瘍縮小効果はCRTに劣るものの、術後合併症の増加はなく、今後期待できると思われる。

３）側方骨盤リンパ節郭清

術前放射線治療により、側方骨盤リンパ節郭清が省略できるという報告がある。しかし、外科医としては効果が同じであれば、手術で治療したいと考えている。外科医が手術で頑張れば、患者さんが術前に治療を受ける費用、手間、危険が減るのである。

伴登　宏行（ばんどう　ひろゆき）

略　歴：

1960年２月	金沢市生まれ
1978年３月	石川県立金沢泉丘高等学校　卒業
1979年４月	金沢大学医学部　入学
1985年３月	同上　卒業
〃　４月	同上　第１外科（現　心肺・総合外科）　入局
1985年５月	石川県立中央病院　小児外科　研修医
〃　８月	金沢大学医学部附属病院　研修医
1986年４月	金沢大学大学院医学研究科　入学
1986年５月	富山県　氷見市民病院　外科医員
1987年５月	富山県　厚生連高岡病院　外科医員
1988年５月	富山県　北陸中央病院　外科医員
1989年11月	石川県　公立穴水総合病院　外科医長
1990年５月	富山県　市立砺波総合病院　外科医員
〃　９月	金沢大学大学院医学研究科　卒業
1992年５月	石川県立中央病院　一般消化器外科医長
1995年４月	富山県　市立砺波総合病院　外科医長
2003年４月	石川県立中央病院　一般消化器外科診療部長

資格等：

日本外科学会指導医、日本消化器外科学会指導医、日本消化器内視鏡学会指導医、日本消化器病学会専門医、日本内視鏡外科学会技術認定医

３．「肝細胞がんの発がんと治療に関する最新の知見」

10：20～11：10

東京医科歯科大学医学部　分子腫瘍医学　田　中　真　二

肝細胞がん（肝がん）の多くは慢性肝疾患を背景として発生し、異形成結節、早期肝がんの段階を経て、高分化型から中・低分化型へと進行する、いわゆる多段階発がんのプロセスを辿る。近年、肝がん臨床検体を用いたゲノム全体の解析が進み、その背景に内在する様々な異常分子群が明らかになってきた。

発がんの遺伝的素因を網羅的に解析する手法として、ゲノムワイド関連解析（genome-wide association study；GWAS）が活用されている。GWASとはゲノム上の個人差を示す一塩基多型（single nucleotide polymorphism；SNP）をタグ・マーカーとして利用し、特定の個人集団の遺伝的背景を網羅的に炙り出すものであり、B型肝炎ウイルスやC型肝炎ウイルスによる慢性肝炎から肝発がんへの遺伝的危険因子として、腫瘍免疫（抗原提示、インターフェロン、NK活性など）やアポトーシス、代謝などに関わるSNPが同定されている。

さらに次世代シーケンサーの開発によって、全ゲノム・全エキソン解析が進められ、遺伝子変異の全容が明らかになりつつある。肝がんのゲノム全体では１万以上の遺伝子変異が認められるが、実際に病態に関与するドライバー変異はその一部であり、多くはパッセンジャー変異と考えられている。最も頻度の高いドライバー変異はテロメラーゼ逆転写酵素（telomerase reverse transcriptase；TERT）のプロモーターであり、そのホットスポット変異C228T/C250Tは肝がんの約60％に認められ、TERT高発現を伴っている。肝硬変の再生結節や異形成結節においても、25％にTERTプロモーター変異を検出しており、前がん病変でも既にゲノム異常が始まっていることを示す所見として特記すべきである。

アミノ酸変異を来すドライバー遺伝子は、肝がんでは少なくとも３つのファミリーに集約される。その１つはp53（*TP53*）であり、30％以上の肝がんに変異を認める代表的ながん抑制遺伝子である。もう１つのドライバーはβ-catenin（*CTNNB1*）であり、Axinなど制御分子を含めたWntシグナル伝達ファミリー全体では20～30％に変異が報告されている。そして第３のファミリーとして最近注目されているのが、SWI/SNFクロマチン・リモデリング複合体であり、全体として約20％に変異を認める。ヒストンをメチル化するMLLファミリーなどを含めるとクロマチン制御因子の遺伝子変異は肝がんの約50％にも昇り、合成致死作用を応用した治療開発が期待されている。

またゲノムワイドな遺伝子発現解析の結果、非がん部の発現遺伝子プロファイルが肝発がんや再発の強力な予測バイオマーカーとなることが判り、宿主反応の重要性に注目が集まっている。がん幹細胞の宿主ニッチ相互作用や免疫チェックポイント阻害剤ニボルマブの高い治療効果などの報告が相次ぎ、肝がん宿主反応のコントロールを標的とした新規治療法開発に拍車が掛かっている。

田中　真二（たなか　しんじ）

学歴・職歴：

1988年	九州大学医学部卒業、第二外科入局
	九州大学医学部附属病院、
	広島赤十字・原爆病院外科にて研修
1990-93年	九州大学医学系研究科
	大学院（ウイルス学）学位取得（医学博士）
1993-94年	九州大学医学部　助手（ウイルス学）
1994-96年	ハーバード大学医学部
	マサチューセッツ総合病院　研究員
1996-99年	九州大学　生体防御医学研究所　助手（腫瘍外科）
1999-2004年	九州大学医学部　助手（第二外科）
2004-05年	九州がんセンター　消化器外科
2005-06年	東京医科歯科大学　助手（肝胆膵外科）
2006-09年	東京医科歯科大学　特任准教授（肝胆膵外科／情報医科学センター）
2009-14年	東京医科歯科大学　准教授（肝胆膵・総合外科）
2014年	東京医科歯科大学　教授（分子腫瘍医学）
	現在にいたる

学　会：

日本外科学会（指導医、専門医、認定医）、日本消化器外科学会（評議員、指導医、専門医、認定医）、日本肝胆膵外科学会（評議員、高度技能指導医）、日本肝臓学会（評議員、専門医）、日本消化器病学会（支部評議員、専門医、学会誌編集委員）、日本癌学会（評議員）、日本肝癌研究会（幹事）、日本がん分子標的治療学会（評議員）、日本肝がん分子標的治療研究会（世話人）、米国癌学会、米国外科学会正会員（FACS）など

受賞歴：

1996年	アメリカ肝臓病学会 Young Investigator Award
1997年	日本医師会医学研究奨励賞
1999年	日本癌学会奨励賞
2004年	ISCRT Subsidy Award for Cancer Research
2006年	日本学術振興会賞
2007年	IRPC“Gold Medal”, Eminent Scientist of the Year
2012年	日本癌学会学術賞JCA-Mauvernay Award
2013年	日本消化器外科学会賞（第一回学術部門）JSGS Science of the Year 2013

４．「職業性胆管癌：印刷事業場での胆管がん多発事例」

11：10～12：00

大阪市立大学大学院　肝胆膵外科学　久　保　正　二

大阪の印刷事業場オフセット校正印刷部門の元および現従業員に胆管癌が多発している事例を踏まえて、2013年３月に厚生労働省から⑴胆管癌は、ジクロロメタン（dichloromethane；DCM）または1，2 - ジクロロプロパン（1，2 -dichloropropane；DCP）に長期間、高濃度曝露することにより発症し得ると医学的に推定でき、⑵本件事業場で発生した胆管癌は、DCPに長期間、高濃度曝露したことが原因で発症した蓋然性が極めて高いことが報告された。この結果、印刷労働者にみられた胆管癌が、新たな職業癌として認識されることとなった。

106名の同部門従業員のうち、18名（17例が労災認定、１例が労災申請中）に胆管癌が発症した。2015年３月の時点での標準化罹患比は1132.5と高値であった。職業性胆管癌17例全例がDCPに、11例がDCMに曝露していた。17例の胆管癌患者は診断時25歳から45歳と若年であった。塩素系有機溶剤の使用期間は、６年１ヶ月から16年１ヶ月であった。胆管癌診断のきっかけは腹痛や黄疸などの症状、検診時の臨床検査値異常や肝腫瘤の指摘などであった。胆管癌診断時、ASTやALT高値例が多く、γ -GTPは全例で上昇していた。画像診断上、腫瘤像、胆管内の乳頭状病変や隆起性病変、胆管の狭窄像や閉塞像、主腫瘍による末梢側胆管の拡張像に加えて、主腫瘍と無関係な限局性肝内胆管拡張像がみられた症例があった。

主腫瘍は腫瘤形成性胆管癌や浸潤性intraductal papillary neoplasm of the bile ductを示す胆管内発育型胆管癌あるいは乳頭状肝外胆管癌であった。さらに胆管癌の前癌病変と考えられているIPNBやBiliary intraepithelial neoplasia（BilIN）- ２/３病変が主腫瘍以外の広範囲の胆管に確認された。また、胆管付属腺を含めた広範囲の胆管に炎症性細胞浸潤を伴う胆管硬化像、胆管消失を伴う胆管障害像や増殖性病変がみられた。さらに、広範囲の胆管がγ -H２AH陽性であったことから、広範囲の胆管にDNA傷害が起こっていると考えられた。これらの所見から、職業性胆管癌症例では、DNA傷害を伴う慢性胆管傷害から胆管の増殖性変化、BilINやIPNBなどの前癌病変・早期癌を経て浸潤性胆管癌に至る発癌メカニズムが推定された。

現在、全国で36例が職業性胆管癌と認定されている。一方、これらの報告を受けて、2015年にInternational Agency for Research on Cancerでは、DCPがgroup １（carcinogenic to humans）に、DCMがgroup ２A（probably carcinogenic to humans）に改訂された。

久保　正二（くぼ　しょうじ）

略　歴：

昭和56年３月　大阪市立大学医学部　卒業
昭和56年６月　大阪市立大学医学部附属病院
　臨床研修医（第２外科）
昭和62年３月　大阪市立大学大学院医学研究科　修了
昭和63年４月　英国ケンブリッジ大学　外科（移植外科）　留学
平成元年10月　大阪市立大学医学部　第２外科　助手
平成４年３月～９月
　米国テキサス大学　MD Anderson Cancer Center　外科　留学
平成８年10月　大阪市立大学医学部　第２外科　講師
平成12年４月　大学院重点化に伴い大阪市立大学大学院医学研究科消化器外科学　講師
平成17年１月　大阪市立大学大学院医学研究科　肝胆膵外科学　助教授（准教授）
平成23年４月　神戸大学　客員教授
平成24年４月　大阪市立大学大学院医学研究科　肝胆膵外科学　病院教授
平成27年２月　職業性胆管癌臨床・解析センター　センター長

学会等：

日本外科学会代議員、日本消化器外科学会評議員、日本肝臓学会評議員、日本消化器病学会財団評議員、日本臨床外科学会評議員、日本肝胆膵外科学会評議員、日本移植学会評議員、日本化学療法学会評議員、日本腹部救急医学会評議員、日本消化器癌発生学会評議員、日本外科感染症学会理事、日本肝癌研究会幹事、日本肝移植研究会世話人、肝臓内視鏡外科研究会世話人、膵臓内視鏡外科研究会世話人、Microwave Surgery研究会世話人

日本肝臓学会　肝癌診療ガイドライン改訂委員会委員、専門医試験問題作成委員
日本肝癌研究会「原発性肝癌取扱い規約」規約委員、肝癌治療効果判定基準作成委員会委員
日本消化器外科学会　学術委員会委員、評議員選出小委員会委員
日本外科感染症学会・外科周術期感染管理医認定制度委員会担当理事、
医の質・安全委員会委員
日本肝胆膵外科学会　利益相反委員会委員
日本移植学会　臓器移植後の妊娠・出産に関するガイドライン作成委員会委員
日本化学療法学会　術後感染予防抗菌薬適正使用ガイドライン作成委員会委員

編集委員：

日本外科感染症学会会誌編集委員会委員、日本外科系連合学会誌編集委員会委員

資格等：

日本外科学会指導医、日本消化器外科学会指導医、日本消化器病学会指導医、日本肝臓学会指導医、日本肝胆膵外科学会高度技能指導医、日本がん治療認定医機構暫定教育医、Infection Control Doctor、日本化学療法学会抗菌化学療法認定医、日本胆道学会指導医、日本外科感染症学会外科周術期感染管理教育医、日本移植学会移植認定医

第10回専門医セミナー

会　場：石川県地場産業振興センター新館
　　1階コンベンションホール（第一会場）

日　時：2015（平成27）年11月8日㈰　13：00～14：00

「症例から学ぶ膵疾患」

司　会：元雄　良治　先生（金沢医科大学　腫瘍内科学）

講　師：大坪公士郎　先生（金沢大学附属病院　がん高度先進治療センター）

講　師：北川　裕久　先生（富山市立富山市民病院　外科）

（支部例会に参加の先生方はどなたでも参加できます）

専門医セミナーの参加で、専門医更新単位3点を取得できます。

「慢性膵炎における内視鏡診断と治療 ～早期慢性膵炎に対するEUS診断を中心に～」

金沢大学附属病院　がん高度先進治療センター　大　坪　公士郎

慢性膵炎は膵臓の内部に不規則な線維化、細胞浸潤、実質の脱落、肉芽組織などの慢性変化が生じ、進行すると膵外分泌・内分泌機能の低下を伴う病態である。成因はアルコール性と非アルコール性に大別され、後者には特発性、遺伝性、家族性などが挙げられる。

慢性膵炎に対する内視鏡治療は主膵管狭窄や膵石による膵炎症状がある症例に適応とされる。膵管狭窄に対しては、内視鏡的バルーン拡張術、内視鏡的経鼻膵管ドレナージ(ENPD)、膵管ステント留置などが行われる。膵石に対しては、ESWLの他に内視鏡的膵管口切開(EPST)が行われる。また、膵仮性嚢胞に対するドレナージに関しては、まずERPにて主膵管と嚢胞との交通の有無を評価し、交通がある場合には経乳頭的アプローチにてENPDや膵管ステント留置、交通がない場合、あるいは経乳頭的アプローチが困難な場合にはEUS下ドレナージを行う。

2009年に慢性膵炎臨床診断基準が改訂されたが、慢性膵炎に対してより早期に介入する目的で新たに早期慢性膵炎の疾患概念が提唱された。早期慢性膵炎は、①反復する上腹部痛、②血中または尿中膵酵素値の異常、③膵外分泌障害、④1日80g以上の持続する飲酒歴のいずれか2項目以上と早期慢性膵炎の画像所見が認められる場合に診断される(①または②の1項目のみ有し、早期慢性膵炎の画像所見を示すものは早期慢性膵炎の疑い)。早期慢性膵炎の画像所見は、EUSにて⑴蜂巣状分葉エコー、⑵不連続な分葉エコー、⑶点状高エコー、⑷索状高エコー、⑸嚢胞、⑹分枝膵管拡張、⑺膵管辺縁高エコーの7項目のうち、⑴～⑷のいずれかを含む2項目以上、あるいはERCP像で3本以上の分枝膵管に不規則な拡張が認められる場合とされる。当科では現在まで非アルコール性早期慢性膵炎4例、その疑い9例、計13例においてEUSを中心とした診断および経過観察を行ってきた。男女比は2：11と女性に多く、平均年齢50.5歳であり、20～30代が5名(38%)含まれていた。症状は腹痛以外に背部痛を10例(77%)に認めた。EUS所見は、索状高エコー13例(100%)、膵管辺縁高エコー11例(85%)、点状高エコー8例(62%)、不連続な分葉エコー7例(54%)、蜂巣様分葉エコー1例(8%)に認めた。なお、CTでは13例いずれにも異常を認めず、MRIを施行した6例にも異常はみられなかった。治療に関しては、メシル酸カモスタット、消化酵素薬にてほとんどの症例で症状の改善を認めたが、消化酵素薬では高力価のパンクレリパーゼが効果的であった。若年の女性で背部痛を認める症例、あるいはUS, CT, 上部消化管内視鏡検査にて異常がなく機能性胃腸症と診断されている症例の中には早期慢性膵炎症例が含まれている可能性があり、その診断にはEUSが有用と考えられる。

「次期膵癌取扱い規約（第７版）からみた膵癌の診断と治療の方向性」

富山市立富山市民病院　外科　北　川　裕　久

膵癌は罹患者数と死亡者数がほぼ同数と非常に予後が悪く、最新の統計では癌死亡者数は肝癌を抜いて第４位となった。男性では54人、女性では64人に１人の割合で膵癌によって死亡する計算であるという。この膵癌死亡者数を減少させるためには、集学的な診断・治療によって治癒症例を積み上げていくしかない。

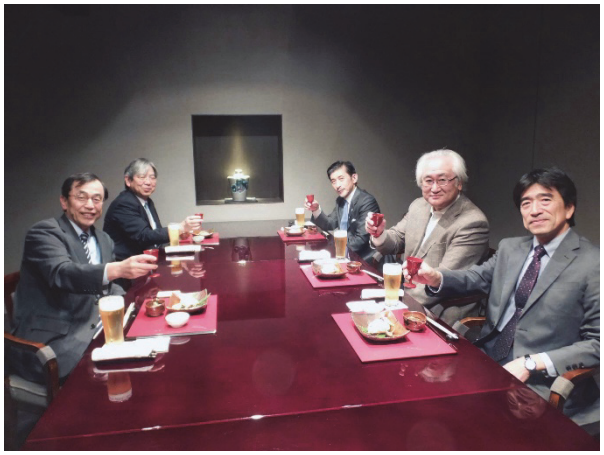
現行の膵癌取り扱い規約は第６版であるが、2013年より伊佐地秀司委員長（三重大学教授）を中心に私を含め20余名が改訂に携わり、第７版が2016年春に市販される予定である。第７版での大きな改訂点は、新たに切除可能性分類、術前治療の評価、リンパ節転移の程度分類、術前画像評価法などが加わり、進行度（Stage）分類はよりインターナショナルに互換性が保たれるようUICC分類に近い形となる。また、局所腫瘍遺残度（R）もR1は「病理組織学的検索で、切除断端から１㎜以内に遺残腫瘍を認める」となる予定である。

すなわち、術前に膵癌進展範囲を画像から正確に評価し、切除可能（Resectable）例では局所再発のないR0切除を行うべきという姿勢が根幹をなしている。そのために、膵癌の進展範囲をミリ単位でどのように読影するかについて、MDCTの撮像法から読影に至るまでの解説が新たに設けられており、外科手術ではこのポイントを確実に理解し、症例ごとに切除範囲をテーラーメードに設定する柔軟性が求められるようになる。一方、切除可能境界（Borderline resectable）例は標準的手術ではR1切除となる可能性が高いもので、何らかの術前治療や合併切除が必要であることを示し、術前治療の組織学的評価についても統一を図るべく、Evans分類、CAP分類などを基盤として曖昧な部分を明確にできるように工夫している。これによって、より効果的な術前治療は何なのかを共通の尺度のもと比較検討することが期待される。また、切除不能（Unresectable）例でも、近年放射線・化学療法などが奏功すれば切除したとの報告が散見されるが、この組織学的評価が有用となる。

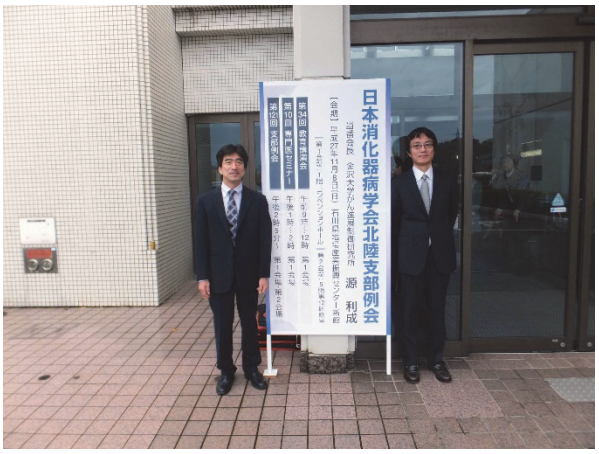
このように、次期新規約では、膵癌の診断・治療は多科にわたる連携を元に進めることが前提となっているが、特に内科、放射線科、病理と外科との連携は大切である。多科が連携して膵癌の診断・治療チームを作り、一例一例を詳細に検討してその知見をフィードバックすることを繰り返し、チーム内で情報や知見を共有することが、膵癌による死亡者数を減少させるために最も効果的と考えられる。我々は、これを実行することでR0切除率80～90％以上、R0症例の５年生存率45％前後という成績を得ることができた（金沢大学附属病院在任中の成績）。

診断学、化学・放射線療法の進歩とともに外科手術の機会が増加していくことは他臓器癌でも経験されてきたが、今後膵癌に対しても更に外科手術の要望が高まると思われる。次期新規約では、「どれだけ安全に切除できるのか」が問われた時代から、「治癒を目標に切除できるか」が問われる時代に変わることを願いつつ改訂に臨んでいる。

支部例会記録(1)



前日の講師懇談会(於:金沢茶屋)



会場：石川県地場産業振興センター新館

受付け準備

支部例会スタッフ

受付け

第1会場:司会席

支部例会記録(2)

伊藤 透先生（講師）：金沢医科大学教授

教育講演会場：手前が伴登宏行先生（講師）

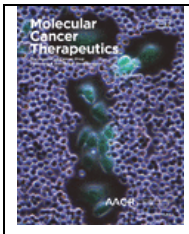
田中真二先生（講師）：東京医科歯科大学教授

久保正二先生（講師）：大阪市立大学教授

専門医セミナー司会：元雄良治教授（金沢医科大学）とご一緒に

支部例会反省会

【附記3】 論文発表（抜粋）



Chikano Y, Domoto T, Furuta T, Sabit H, Kitano-Tamura A, Pyko IV, Takino T, Sai Y, Hayashi Y, Sato H, Miyamoto KI, Nakada M, Minamoto T. Glycogen synthase kinase 3β sustains invasion of glioblastoma via the focal adhesion kinase, Rac1 and c-Jun N-terminal kinase-mediated pathway. *Mol Cancer Ther* 14 (2): 564-74, 2015. doi: 10.1158/1535-7163. MCT-14-0479.

The failure of current treatment options for glioblastoma stems from their inability to control tumor cell proliferation and invasion. Biologically targeted therapies offer great hope and one promising target is glycogen synthase kinase-3β (GSK3β), implicated in various diseases, including cancer. We previously reported that inhibition of GSK3β compromises the survival and proliferation of glioblastoma cells, induces their apoptosis, and sensitizes them to temozolomide and radiation. Here, we explore whether GSK3β also contributes to the highly invasive nature of glioblastoma. The effects of GSK3β inhibition on migration and invasion of glioblastoma cells were examined by wound-healing and Transwell assays, as well as in a mouse model of glioblastoma. We also investigated changes in cellular microarchitectures, cytoskeletal components, and proteins responsible for cell motility and invasion. Inhibition of GSK3β attenuated the migration and invasion of glioblastoma cells in vitro and that of tumor cells in a mouse model of glioblastoma. These effects were associated with suppression of the molecular axis involving focal adhesion kinase, guanine nucleotide exchange factors/Rac1 and c-Jun N-terminal kinase. Changes in cellular phenotypes responsible for cell motility and invasion were also observed, including decreased formation of lamellipodia and invadopodium-like microstructures and alterations in the subcellular localization, and activity of Rac1 and F-actin. These changes coincided with decreased expression of matrix metalloproteinases. Our results confirm the potential of GSK3β as an attractive therapeutic target against glioblastoma invasion, thus highlighting a second role in this tumor type in addition to its involvement in chemo- and radioresistance.

Kurklu B, Whitehead RH, Ong EK, Minamoto T, Fox JG, Mann JR, Judd LM, Giraud AS, Menheniott TR. Lineage-specific RUNX3 hypomethylation marks the pre-neoplastic immune component of gastric cancer. *Oncogene* 34 (22): 2856-66, 2015. doi:10.1038/onc.2014.233.

Runt domain transcription factor 3 (RUNX3) is widely regarded as a tumour-suppressor gene inactivated by DNA hypermethylation of its canonical CpG (cytidine-phosphate-guanidine) island (CGI) promoter in gastric cancer (GC). Absence of RUNX3 expression from normal gastric epithelial cells (GECs), the progenitors to GC, coupled with frequent RUNX3 overexpression in GC progression, challenge this longstanding paradigm. However, epigenetic models to better describe RUNX3 deregulation in GC have not emerged. Here, we identify lineage-specific DNA methylation at an alternate, non-CGI promoter (P1) as a new mechanism of RUNX3 epigenetic control. In normal GECs, P1 was hypermethylated and repressed, whereas in immune lineages P1 was hypomethylated and widely expressed. In human GC development, we detected aberrant P1 hypomethylation signatures associated with the early inflammatory, preneoplastic and tumour stages. Aberrant P1 hypomethylation was fully recapitulated in mouse models of gastric inflammation and tumorigenesis. Cell sorting showed that P1 hypomethylation reflects altered cell-type composition of the gastric epithelium/tumour microenvironment caused by immune cell recruitment, not methylation loss. Finally, via long-term culture of gastric tumour epithelium, we revealed that de novo methylation of the RUNX3 canonical CGI promoter is a bystander effect of oncogenic immortalization and not likely causal in GC pathogenesis as previously argued. We propose a new model of RUNX3 epigenetic control in cancer, based on immune-specific, non-CGI promoter hypomethylation. This novel epigenetic signature may have utility in early detection of GC and possibly other epithelial cancers with premalignant immune involvement.

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Cancer Biology and Signal Transduction

Molecular Cancer Therapeutics

Glycogen Synthase Kinase 3β Sustains Invasion of Glioblastoma via the Focal Adhesion Kinase, Rac1, and c-Jun N-Terminal Kinase-Mediated Pathway

Yuri Chikano[1,2], Takahiro Domoto[1], Takuya Furuta[3], Hemragul Sabit[3], Ayako Kitano-Tamura[1,2], Ilya V. Pyko[1,3], Takahisa Takino[4], Yoshimichi Sai[2], Yutaka Hayashi[3], Hiroshi Sato[4], Ken-ichi Miyamoto[2], Mitsutoshi Nakada[3], and Toshinari Minamoto[1]

Abstract

The failure of current treatment options for glioblastoma stems from their inability to control tumor cell proliferation and invasion. Biologically targeted therapies offer great hope and one promising target is glycogen synthase kinase-3β (GSK3β), implicated in various diseases, including cancer. We previously reported that inhibition of GSK3β compromises the survival and proliferation of glioblastoma cells, induces their apoptosis, and sensitizes them to temozolomide and radiation. Here, we explore whether GSK3β also contributes to the highly invasive nature of glioblastoma. The effects of GSK3β inhibition on migration and invasion of glioblastoma cells were examined by wound-healing and Transwell assays, as well as in a mouse model of glioblastoma. We also investigated changes in cellular microarchitectures, cytoskeletal components, and proteins responsible for cell motility and invasion. Inhibition of GSK3β attenuated the migration and invasion of glioblastoma cells *in vitro* and that of tumor cells in a mouse model of glioblastoma. These effects were associated with suppression of the molecular axis involving focal adhesion kinase, guanine nucleotide exchange factors/Rac1 and c-Jun N-terminal kinase. Changes in cellular phenotypes responsible for cell motility and invasion were also observed, including decreased formation of lamellipodia and invadopodium-like microstructures and alterations in the subcellular localization, and activity of Rac1 and F-actin. These changes coincided with decreased expression of matrix metalloproteinases. Our results confirm the potential of GSK3β as an attractive therapeutic target against glioblastoma invasion, thus highlighting a second role in this tumor type in addition to its involvement in chemo- and radioresistance. *Mol Cancer Ther; 14(2); 564–74. ©2014 AACR.*

Introduction

Glioblastoma is the most frequent and lethal type of malignant primary brain tumor (1). The mainstay of current treatment for patients with glioblastoma is maximal tumor-reduction surgery where this is safe, followed by chemoradiation therapy (1, 2). The pathologic triad of this disease includes the highly proliferative capacity of the tumor cells, their borderless invasive ability, and tumor neoangiogenesis (1). The invasive nature of glioblastomas prevents complete surgical removal and renders them resistant to temozolomide-based chemotherapy, radiation, and a combination of both (2). Consequently, the median overall survival of patients following diagnosis is just 15 months and has not improved significantly over the past 30 years (1, 3). Recent clinical trials using pharmacologic inhibitors of epidermal growth factor receptor (EGFR) and HER2 and therapeutic antibodies against EGFR failed to show significant clinical benefit (4). This is probably due to extensive heterogeneity at the cellular and molecular levels and the complex interplay between different oncogenic signaling pathways in tumor cells (5). Clinical trials using the antiangiogenic agent bevacizumab, a monoclonal antibody to vascular endothelial growth factor, demonstrated some improvement in progression-free survival in newly diagnosed and recurrent glioblastomas, but failed to show an overall survival benefit (4). An undesired consequence of this therapy is the enhancement of tumor cell invasion in the resulting hypoxic tumor environment due to a metabolic shift toward glycolysis and upregulation of other proangiogenic factors (6, 7). The effective targeting of biologic mechanisms that facilitate tumor cell invasion will be crucial for the development of more successful treatment strategies (8).

Glycogen synthase kinase-3β (GSK3β) is a serine/threonine protein kinase that has emerged as a key enzyme in a number of chronic progressive diseases, including diabetes mellitus, neurodegenerative disorders, and cancer (9, 10). We and others have demonstrated that inhibition of GSK3β attenuates the survival and proliferation of glioblastoma cells by modulating specific

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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molecular pathways (11, 12), thus sensitizing them to chemotherapeutic agents and radiation (12, 13). It has also been reported that GSK3β inhibition with lithium and indirubins reduces the invasive potential of glioblastoma cells (14, 15). However, a recent study indicated both pro- and anti-invasive roles for GSK3β depending on its subcellular localization in glioblastoma (16). Despite the increasing number of studies showing its participation in cell polarity and motility (17), relatively little is known about a putative role for GSK3β in the migration, invasion, and metastasis of tumor cells. Here, we demonstrate that inhibition of GSK3β attenuates the invasion of glioblastoma cells via effects on the proinvasive cellular microarchitectures and on a pivotal pathway involving focal adhesion kinase (FAK), Rac1, and c-Jun N-terminal kinase (JNK).

Materials and Methods

Cell lines

The human glioblastoma cell lines U87 and U251 were obtained from the American Type Culture Collection, whereas A172 and T98G were obtained from the Human Science Research Resource Bank (Osaka, Japan) and Biomedical Cell Resource Center, Tohoku University Geriatric Medicine Research Institute (Sendai, Japan) in 2009. These cell lines were characterized by the respective resource bank/institute by short tandem repeat profile analysis, and passaged in our laboratory for fewer than 6 months after resuscitation. They were maintained at 37°C with 5% CO_2 in high-glucose Dulbecco's modified Eagle medium (U87, U251, A172) and RPMI-1640 (T98G; Sigma-Aldrich). All medium was supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin-G and 100 μg/mL streptomycin; Gibco).

Western blotting

Protein was extracted from cultured cells and mouse brain tissues using lysis buffer (CelLytic-MT; Sigma-Aldrich) containing a mixture of protease and phosphatase inhibitors (Sigma-Aldrich). A 30-μg aliquot of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western immunoblotting for the proteins of interest. Electroblotted membranes (Amersham) were blocked with 5% bovine serum albumin before detection of phosphorylated protein fractions. The source and working dilutions of the primary antibodies are listed in Supplementary Table S1. Signals were developed using enhanced chemiluminescence (ECL; Amersham). The amount of protein extract in each sample was monitored by the expression of β-actin. Immunoblot signals were measured using the CS analyzer (version 2.0; ATTO).

Assays for cell migration and invasion

Glioblastoma cell migration and invasion were examined by wound-healing assay and Transwell assays, respectively. Confluent tumor cells grown in the presence of dimethyl sulfoxide (DMSO; Sigma-Aldrich) or the GSK3β inhibitor AR-A014418 (Calbiochem) dissolved in DMSO at the indicated concentrations were scratched with a 20-μL micropipette tip to create a cell-free zone (wound). For each condition studied, the gap distance between the wound edges was measured at three fixed reference points for 12 to 48 hours using a phase-contrast microscope (Axiovert 40 CFL; Zeiss). Cell migration at each time point was calculated as the mean distance of the gap measured at the three reference points. This was compared between cells treated with DMSO and AR-A014418. AR-A014418 does not inhibit the activity of 26 closely related kinases and is therefore considered highly specific for GSK3β (18). Because no subsequent information is available for the effect of AR-A014418 on activity of kinases other than those reported previously (18), the effects of GSK3β RNA interference (RNAi) on cell migration and invasion were examined for cells transfected with either nonspecific small interfering RNA (siRNA; Stealth RNAi Negative Control Low GC Duplex; Invitrogen) or GSK3β-specific siRNA (GSK3β Validated Stealth RNAi; Invitrogen). The effect of RNAi on GSK3β expression was determined by Western blotting using an antibody against both GSK3α and GSK3β (Supplementary Table S1). The specificity of GSK3β-specific siRNA was confirmed in our previous studies (12, 19).

The Transwell assays used a 24-well double-chamber system (BD BioCoat Matrigel Incubation Chamber; BD Bioscience) to examine cell migration and invasion by applying cells to uncoated and Matrigel-coated upper chambers, respectively. Cells were suspended in serum-free medium containing DMSO or AR-A014418 at the indicated concentrations and applied to the upper chamber. The paired lower chamber was filled with medium containing 10% FBS (as a chemoattracting agent) and DMSO or AR-A014418 at the indicated concentrations. The cells transfected with nonspecific or GSK3β-specific siRNA were also subjected for the Transwell assay. Cells were allowed to migrate or invade the Matrigel toward the lower side of the upper chamber. After 22 hours of incubation, cells on the upper side of the chamber were removed with a cotton-swab. Cells on the lower side of the chamber were fixed and stained with the Diff-Quick Kit (Symex). In each assay, the total number of cells per high-power microscopic field on the lower side of the uncoated or Matrigel-coated chamber was counted and scored for migrating or invading cells, respectively. The mean number of cells in five high-power microscopic fields was calculated.

Cell morphology and immunofluorescence cytochemistry

Glioblastoma cells grown to 50%–60% confluence on a cover slip were treated with either DMSO or AR-A014418 at the indicated concentrations for 24 hours and then observed under a phase-contrast microscope. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X (Sigma-Aldrich) for immunofluorescence and fluorescence staining. They were incubated with mouse monoclonal antibody to Rac1 (BD Bioscience; diluted 1:200) at 4°C overnight and then with Alexa Flour 488–labeled anti-mouse IgG (Invitrogen; diluted 1:1,000) at room temperature for 40 minutes in the dark. After washing off excess antibody, cells were stained for filamentous (F-) actin with Alexa Flour 546–labeled phalloidin (Invitrogen; diluted 1:40) for 20 minutes at room temperature. Following Rac1 and F-actin staining, cell nuclei were counterstained with Hoechst 33342 (Molecular Probes) for 20 minutes at room temperature. The cells were observed by fluorescence microscopy (Keyence) for expression and subcellular localization of Rac1 and F-actin.

To quantify effects of GSK3β inhibition on lamellipodia formation, lamellipodia-positive cells were scored for the same cells treated with DMSO and 25 μmol/L AR-A014418 and for those transfected with nonspecific and GSK3β-specific siRNA. The cells were stained for F-actin and observed under phase-contrast and fluorescence microscopy, respectively. In each assay, the mean percentage of lamellipodia-positive cells in five microscopic fields was calculated with standard deviations.

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Rac1 activity

Protein was extracted from cells treated with DMSO or AR-A014418 for 24 hours with 25 mmol/L Tris–HCl buffer (pH 7.5) containing 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1% NP-40, 1 mmol/L dithiothreitol, and 5% glycerol. Active Rac1 was isolated from the protein sample by the pull-down method using GST-human Pak1-PBD (Thermo) and resins (Glutathione Sepharose 4 Fast Flow; GE Healthcare) according to the manufacturers' instructions. The fraction of Rac1 bound to guanosine triphosphate (GTP; Rac1-GTP, an active form) was eluted from the resins and detected by Western blot analysis using rabbit polyclonal antibody to Rac1 (diluted 1:1,000; Thermo). Separately, whole cellular protein was probed for total Rac1 using the same antibody. The relative level of Rac1-GTP was quantified by densitometry and normalized to that of total Rac1 in the same cells.

Expression and secretion of matrix metalloproteinases

Expression of matrix metalloproteinase 2 (MMP-2) and membrane type 1 (MT1)-MMP mRNA in the cells was examined by quantitative reverse transcription-PCR (qRT-PCR) as described in our previous report (12) using SYBR Premix Ex Taq II (TaKaRa-Bio) and specific primers for the amplification of MMP-2, MT1-MMP, and β-actin (Supplementary Table S2).

MMP-2 expression was analyzed by gelatin zymography (20). Glioblastoma cells were seeded on 12-well plates for 48 hours and then treated with DMSO or AR-A014418 (10, 25 μmol/L) for 24 hours in serum-free medium. Conditioned medium or treated cells were incubated with SDS sample buffer for 30 minutes at 37°C. Samples were separated on 10% SDS-PAGE containing 0.005% Alexa Fluor 680–labelled gelatin. After electrophoresis, gels were washed in 2.5% Triton X-100 for 2 hours at room temperature to remove SDS and incubated in substrate buffer overnight at 37°C. The gel was scanned using the LI-COR Odyssey IR imaging system.

Expression of guanine nucleotide exchange factors and integrins

Guanine nucleotide exchange factors (GEF) shown to activate Rac1 and promote migration and invasion of glioblastoma include ELMO1, Dock180, Trio, Ect2, Vav3, and SWAP-70 (21). Among integrin family, integrins α2, α3, α5, αV, β1, β3, and β8 are associated with glioma invasion (22). Changes in the mRNA expression of these GEFs and integrins in glioblastoma cells were examined after treatment with AR-A014418 by qRT-PCR (12) using specific primers for these GEFs, integrins, and β-actin (Supplementary Table S2). The expression of GEFs proteins was analyzed by Western blotting with the respective specific antibodies (Supplementary Table S1) and compared between cells treated with DMSO and AR-A014418.

Invadopodia formation

The ability of glioblastoma cells to invade three-dimensional extracellular matrix (ECM) was assessed by observing the formation of invasive "feet" (invadopodia; ref. 23). Glioblastoma cells were cultured on cover slips coated with either poly-L-lysine (Sigma-Aldrich) or type I collagen gel (Nitta Gelatin). The cells were fixed, serially stained for F-actin, cortactin, and nuclei, and observed by fluorescence microscopy as described above to evaluate invadopodia. To examine the effect of GSK3β inhibition on the formation of invadopodia, cells seeded on glass-bottom dishes coated with Oregon Green (OG; Invitrogen)–labeled gelatin were treated with DMSO or 25 μmol/L AR-A014418 for 12 hours. After staining for F-actin, cortactin, and nuclei, the cells were observed by confocal laser-scanning microscopy (LSM5 EXCITER; Carl Zeiss). Area of degradation of OG-labeled gelatin mediated by invadopodia was measured by using the Image J Software.

Animal study, immunohistochemical and biochemical analysis

We generated a mouse brain tumor model of human glioblastoma by retrovirus-mediated introduction of mutant K-ras gene ($K\text{-}ras^{G12V}$) into neurospheres derived from the brain of a $p16^{Ink4A-/-}/p19^{Arf-/-}$ mouse. These were then transplanted into the brains of wild-type mice, as described in our previous study (24). Brain tumors developed in this mouse model as early as 20 days after transplantation, with most mice dying within 40 days. Histologically, the tumors showed dense palisades of tumor cells around areas of necrosis, the presence of bizarre giant cells and the proliferation of microvasculature, all of which are characteristic of human glioblastoma (1). The 12 mice were treated by intraperitoneal injection of either DMSO ($n = 6$) or AR-A014418 (2 mg/kg body weight; $n = 6$) three times a week, as described earlier (19, 25). All mice were euthanized at the end of 2 weeks of treatment. At autopsy, the brain (with tumor) and vital organs (lungs, liver, pancreas, and kidneys) were assessed histologically and biochemically. All animal experiments followed the Guidelines for the Care and Use of Laboratory Animals at Kanazawa University that covers the national guideline (26).

For histologic and immunohistochemical examinations, mice brains were fixed in 4% paraformaldehyde. Before fixation, the parts of fresh specimens were immediately frozen and stored at −80°C until use. Tissue sections were stained with hematoxylin and eosin and immunostained with the following primary antibodies: anti-nestin (BD Biosciences), anti-glycogen synthase (GS) and the fraction phosphorylated at the seine (S) 641 residue (pGS^{S641}; Cell Signaling). The staining signals were visualized with peroxidase-conjugated secondary antibody (Dako) followed by the avidin–biotin complex method (12). The sections were counterstained with hematoxylin. Images were acquired with a BZ-X700 microscope (Keyence) and digitally processed with the Keyence Analysis Software. To evaluate the irregular-shaped tumor size, we calculated the surface included by the contour of the region of interest in coronal section showing the maximal area of each tumor. Nestin-positive tumor cell clusters were scored to evaluate the degree of invasion as described previously (27). Level of pGS^{S641} in each tumor was analyzed by Western blotting as described above. Tumor size, degree of invasion, and pGS^{S641} levels were quantitatively compared between the mice treated with DMSO and AR-A014418, respectively.

Statistical analysis

Statistical significance was determined using the Student *t* test for comparison of two groups and analysis of variance (ANOVA) followed by Bonferroni/Dunn *post hoc* test for comparison of three groups in wound-healing and Transwell assays. A P value of < 0.05 was considered as statistically significant.

Results

Effect of GSK3β inhibition on tumor cell migration and invasion

In the wound-healing assay, treatment with 5 to 25 μmol/L AR-A014418 reduced the migration of all glioblastoma cells in

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response to mechanical stimulation (Fig. 1A). The selective effect of AR-A014418 on cell migration is supported by our previous observations that the same concentrations of inhibitor did not affect cell survival after 24 hours of treatment, although 25 μmol/L AR-A014418 slightly attenuated cell survival at 48 hours (12). The use of RNAi for GSK3β produced the same result in this assay as AR-A014418. The Transwell assay showed that AR-A014418 and GSK3β-RNAi inhibited the migration of glioblastoma cells and their invasion of ECM constituents (Fig. 1B and C; Supplementary Fig. S1).

Changes in cell migration phenotypes following GSK3β inhibition

Among the different cellular microarchitectures involved in cell motility and invasion, we focused here on lamellipodia (28) because these structures were frequently observed in glioblastoma cells growing in nonconfluent conditions (Fig. 2, Supplementary Fig. S2). Rac1, a member of the Rho-GTPase family, is involved in the formation of lamellipodia and membrane ruffling, thus promoting cell migration (21, 29). Immunofluorescence staining of glioblastoma cells showed preferential colocalization of Rac1

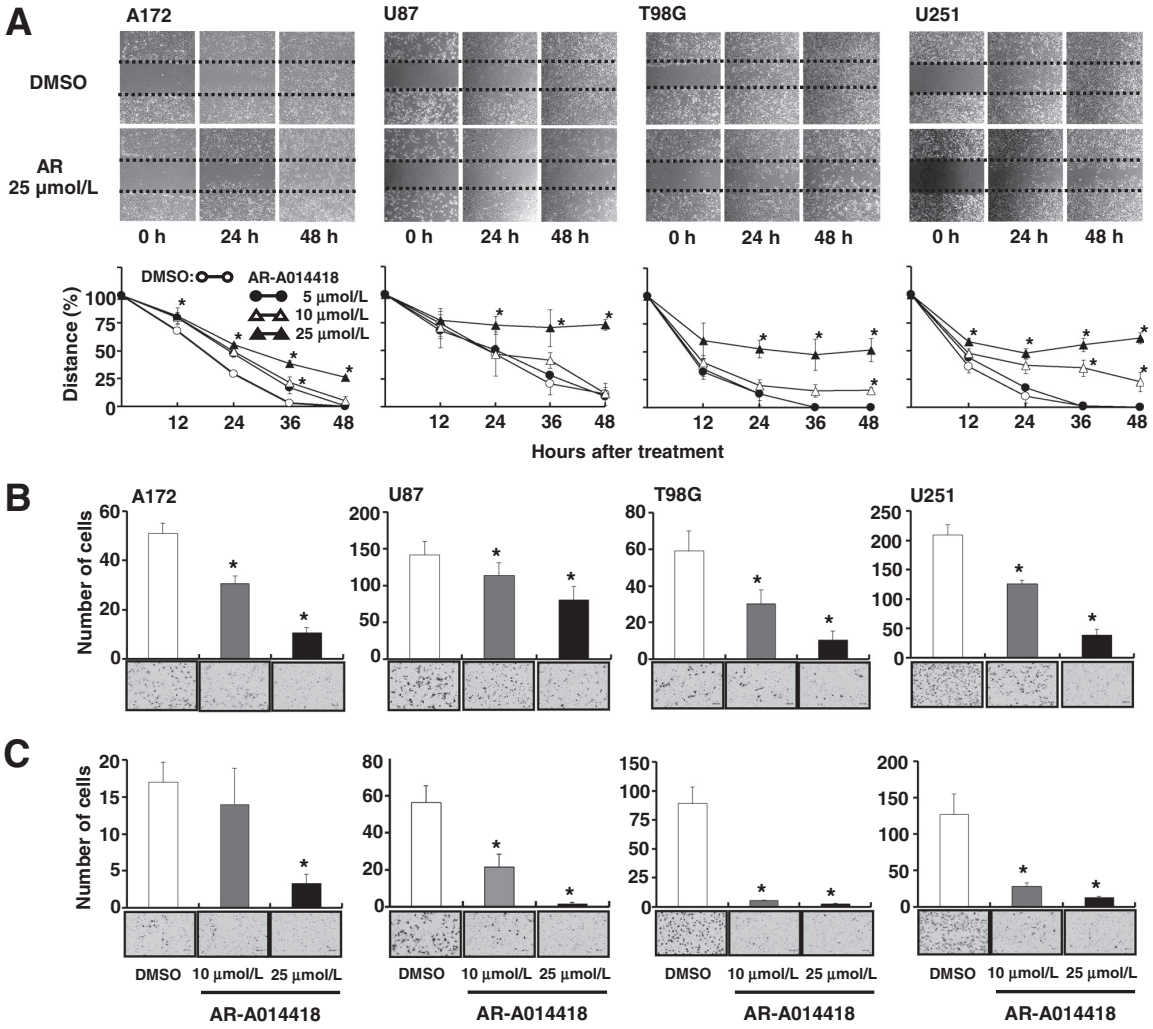

Figure 1.
Effect of GSK3β inhibition on the migration and invasion of glioblastoma cells under mechanical and chemoattractant stimuli. A, top, representative time course of glioblastoma cell migration in the wound-healing assay in the presence of DMSO or AR-A014418 (AR). The assay was performed by scratching confluent cells followed by serial observation of the same reference points at 24 and 48 hours under a phase-contrast microscope. Bottom, the relative widths of wounds at the indicated time after treatment with DMSO or AR-A014418 were measured and expressed as a percentage of the initial gap width at time zero. Values shown are the means ± SD of three separate reference points of observation. B and C, effects of GSK3β inhibition on three-dimensional migration and invasion of glioblastoma cells. Migrating cells through a Transwell chamber (B) and invading cells through a Matrigel-coated Transwell chamber (C) were scored for cells treated with DMSO or AR-A014418 for 22 hours. In each assay, the mean number of cells in five high-power microscopic fields was calculated with SDs. Representative photomicroscopic findings from the assay are shown below each column. *, $P < 0.05$, statistically significant difference between cells treated with DMSO and AR-A014418.

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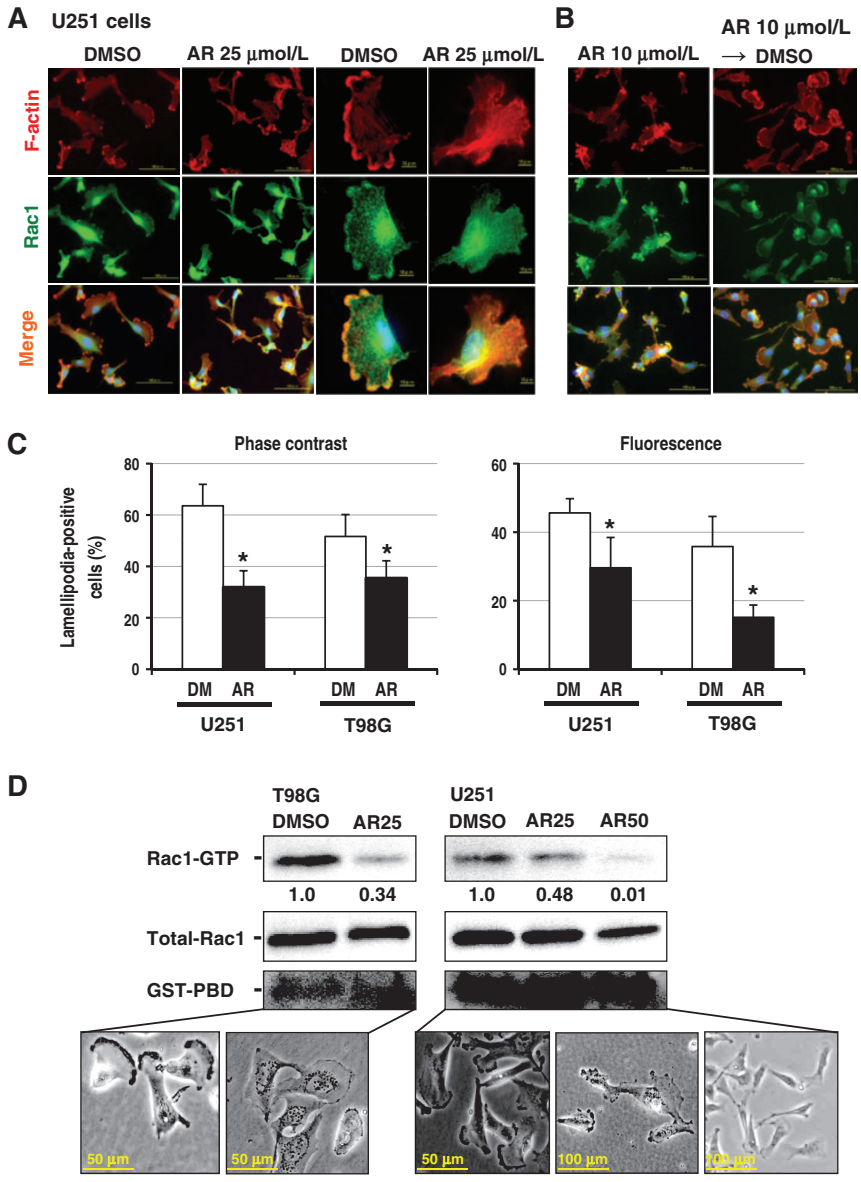

Figure 2.
Effect of GSK3β inhibition on the lamellipodia formation and the subcellular localization and activity of Rac1 in glioblastoma cells. A, U251 cells were treated with either DMSO or AR-A014418 (AR) for 24 hours and processed for immunofluorescence staining of F-actin (red fluorescence) and Rac1 (green fluorescence). Cell nuclei were counterstained with Hoechst 33342. Merged images are also shown. Scale bars in the left 6 panels represent 100 μm and in the right 6 panels represent 10 μm. B, the effect of GSK3β inhibition on cell morphology and motility was reversible. U251 cells were treated with AR-A014418 (AR) for 24 hours, then washed by PBS, and immediately replaced with medium containing DMSO alone (AR 10 μmol/L→DMSO) and cultured for 24 hours. Cells were then processed for immunofluorescence staining of F-actin and Rac1. Cell nuclei were counterstained with Hoechst 33342. Merged images are also shown. Scale bars, 100 μm. C, effect of DMSO (DM) and AR-A014418 (AR; 25 μmol/L) on the incidence of lamellipodia-positive glioblastoma cells scored under phase-contrast and fluorescence (F-actin) microscopy, respectively. *, $P < 0.05$. D, GSK3β inhibition decreases Rac1 activity in glioblastoma cells. The cells were treated with DMSO or AR-A014418 (AR; 25 and 50 μmol/L) for 24 hours. Active Rac1 (Rac1-GTP) was isolated by pull-down assay and detected by Western blotting (top). Whole-cell lysates were probed for total-Rac1 and GST-PBD (middle and bottom). The value below each lane shows the relative amount of Rac1-GTP quantified by densitometry and normalized to that of total Rac1 in the same cells. The panels in the bottom showed phase-contrast microscopic findings of T98G and U251 cells treated with DMSO and AR-A014418, respectively.

with actin filaments at the site of lamellipodia. Both proteins are involved in the organization of lamella structure. Treatment of glioblastoma cells with AR-A014418 inhibited lamellipodia formation and the colocalization of Rac1 and F-actin, and the effect was reversible (Fig. 2A and B; Supplementary Fig. S2A and S2B). Treatment with AR-A014418 and GSK3β-specific siRNA significantly decreased the incidence of lamellipodia-positive glioblastoma cells (Fig. 2C, Supplementary Fig. S2C). Associated with these changes, the pull-down assay showed decreased Rac1-GTP (active fraction) in cells treated with AR-A014418 (Fig. 2D).

Rac1 cycles between the active GTP-bound and inactive guanosine diphosphate (GDP)–bound forms (29). Of the three classes of protein that regulate Rho-GTPase activity, GEFs activate GTPase and participate in tumor cell migration and invasion (30). Among the GEFs previously shown to promote glioblastoma invasion via the activation of Rac1 (21), four (Dock180, SWAP-70, ELMO1, Trio) were constitutively expressed but this could be reduced following treatment with AR-A014418. Inhibition of GSK3β also decreased Vav3 expression in T98G and U87 cells (Fig. 3, Supplementary Fig. S3A). Thus, decreased Rac1 activity following

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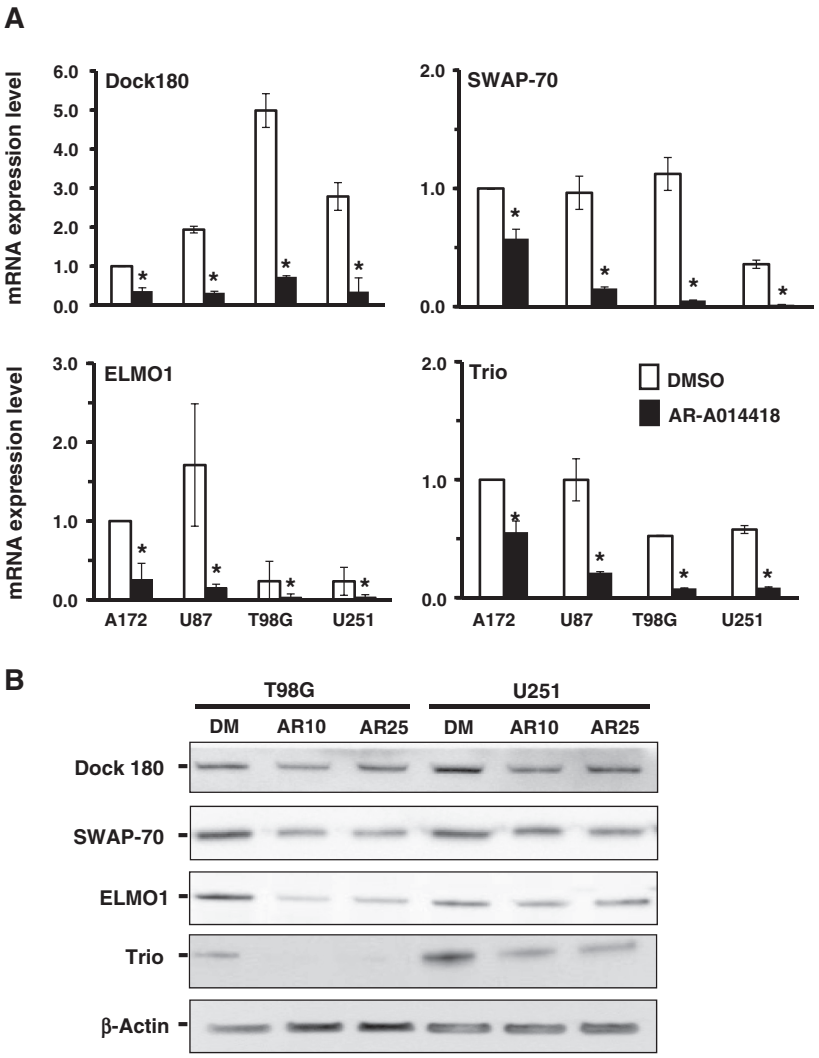

Figure 3.
Effects of GSK3β inhibition on the expression of GEFs (Dock180, SWAP-70, ELMO1, Trio) mRNA (A) and proteins (B) were examined by qRT-PCR and Western blotting in glioblastoma cells treated with DMSO (DM) or 25 μmol/L AR-A014418 (AR) for 24 hours. Relative mRNA expression levels of GEF genes (target mRNA: β-actin mRNA ratios) in the cells were calculated on the basis that the mRNA level in A172 cells treated with DMSO was 1.0. Columns, mean values from three experiments; bars, SD. *, $P < 0.05$, statistically significant difference between cells treated with DMSO and AR-A014418.

inhibition of GSK3β is partly due to the downregulation of specific GEFs.

Changes in the invasive phenotype following GSK3β inhibition

The ECM degrading enzymes MMP-2, MMP-9, and MT1-MMP play a major role in glioblastoma invasion (31) and Rac1 has been shown to enhance their expression and/or activity (29). Treatment of glioblastoma cells with AR-A014418 decreased the expression of mRNAs for MMP-2 and MT1-MMP and inhibited the secretion of MMP-2 in the glioblastoma (Fig. 4A and B) other than A172 cells (data not shown). Together with the changes in Rac1 activity (Fig. 2D), these findings indicate a mechanistic link between GSK3β and Rac1 in the dysregulation of MMP expression and secretion in glioblastoma cells.

It has been reported that FAK is regulated by GSK3β and interacts with Rac1 to promote cell motility and tumor cell invasion (32). Phosphorylation of the tyrosine (Y) 397 and Y861 residues of FAK is crucial for its kinase activity and its signal transduction to Rac1, respectively (33). In addition, the enhancement of cell motility and invasion mediated by the FAK–Rac1 axis is associated with JNK activation through phosphorylation of its threonine (T) 183 and/or Y185 residues (34). The phosphorylated fractions of FAK (p-FAK^{Y397}, p-FAK^{Y861}) and JNK (p-JNK$^{T183/Y185}$) in all glioblastoma cells tested were reduced following treatment with 25 μmol/L AR-A014418 (Fig. 4C and D). Together, these results suggest a pivotal role for the GSK3β-induced molecular axis mediated by FAK, Rac1, and JNK in sustaining glioblastoma cell invasion (Supplementary Figure S4). It is known that integrin-mediated signaling plays a pivotal role in activating FAK phosphorylation and invasion of tumor cells (33). Of integrin subunits associated with glioblastoma invasion (22), GSK3β inhibition decreased expression of integrin β8 in three of four cell lines tested (Supplementary Fig. S3B), suggesting an involvement of integrin β8-induced

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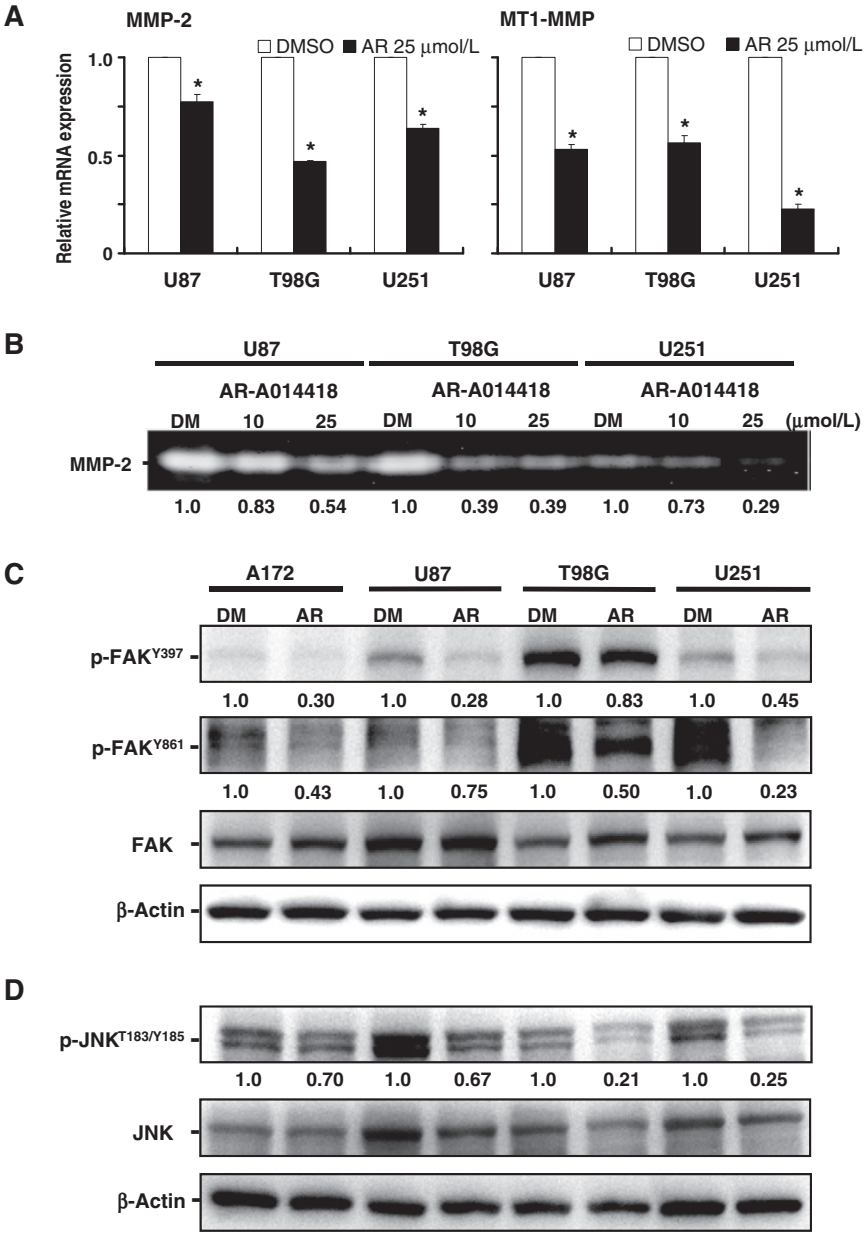

Figure 4.
Effect of GSK3β inhibition on the expression of MMP-2 and MT1-MMP (A), the secretion of MMP-2 (B), and the expression and phosphorylation of FAK (C) and JNK (D) in glioblastoma cells. A, the expression of mRNA for MMP-2 and MT1-MMP was measured by qRT-PCR in glioblastoma cells after 24 hours of treatment with DMSO or AR-A014418 (AR). Relative levels of mRNA expression shown are the means ± SDs from four separate experiments. *, $P < 0.05$, statistically significant difference between cells treated with DMSO and AR-A014418. B, glioblastoma cells were grown in regular medium for 24 hours and then in serum-free medium for 2 hours. Both media contained either DMSO (DM) or AR-A014418. The conditioned media were collected from the respective cell cultures and analyzed for MMP-2 by gelatin zymography. The value shown below each lane is the relative amount of MMP-2 quantified by densitometry and normalized to the same cells treated with DMSO. C and D, Western blotting analyses comparing the levels of phosphorylation for FAK (p-FAK^{Y397}, p-FAK^{Y861}) and JNK (p-JNK$^{T183/Y185}$) between glioblastoma cells treated with DMSO (DM) or with 25 μmol/L AR-A014418 (AR) for 24 hours. The value below each lane shows the relative level of p-FAK^{Y397}, p-FAK^{Y861}, or p-JNK$^{T183/Y185}$ quantified by densitometry and normalized to total FAK or JNK in the same cells.

pathway in GSK3β-mediated FAK phosphorylation and invasion of glioblastoma.

Although lamellipodia are important for cell motility, the cell surface microstructure known as invadopodium is critical for stromal degradation and invasion in several tumor types, including glioblastoma. MT1-MMP is one of the key molecules in the formation of invadopodia and is highly active in invasive tumor cells (23, 31, 35). Consistent with previous studies (reviewed in 35), glioblastoma cells in the type I collagen gel formed innumerable invadopodium-like microstructures where F-actin and cortactin colocalized (Fig. 5A). Treatment of cells with 25 μmol/L AR-A014418 decreased the number of invadopodium-like microstructures and inhibited the degradation of OG-labeled gelatin mediated by invadopodia (Fig. 5B, Supplementary Fig. S5). This is in line with the negative effects of GSK3β inhibition on FAK, Rac1, and MMPs described above.

Effect of GSK3β inhibition on glioblastoma cell invasion in an animal model

As described in Materials and Methods, the histology of tumors from our animal model showed several features that are characteristic of human glioblastoma (1). Inhibition of GSK3β by

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treatment with AR-A014418 was confirmed by the decreased level of phosphorylated GS observed in the tumor cells (Supplementary Fig. S6). Tumors were smaller in mice treated with AR-A014418 compared with those treated with DMSO but no significant difference was found between the two groups (Fig. 6). The number of diffusely infiltrating tumor cells that stained positive for nestin was significantly decreased (Fig. 6B). This resulted in a clearly demarcated border between the tumor and adjacent normal brain tissues in mice treated with AR-A014418 (Fig. 6A).

Similar to our previous studies (19, 25), no detrimental effects were observed in mice treated with GSK3β inhibitor. There was no significant difference in body weight between groups treated with DMSO and AR-A014418. At necropsy, gross observation and histologic examination revealed no pathologic findings, primary cancers, or metastatic tumors in the major vital organs, including the lungs, liver, gastrointestinal tract, pancreas, and kidneys (data not shown).

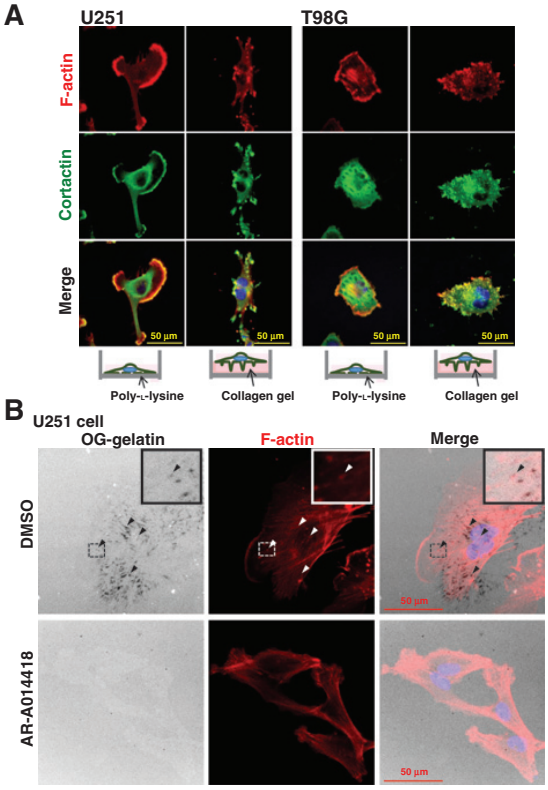

Figure 5.
Effect of GSK3β inhibition on extracellular matrix degradation by invadopodia formation. A, fluorescence and immunofluorescence microscopic findings of glioblastoma cells cultured for 12 hours on poly-L-lysine–coated (left 3 panels) or collagen gel-coated (right 3 panels) cover slips and stained for F-actin (red), cortactin (green), and cell nuclei (blue). B, U251 cells seeded on OG-gelatin (white)–coated glass-bottom culture dishes were treated with DMSO or AR-A014418 for 12 hours. The cells were fluorescence-stained for F-actin and nuclei (Hoechst 33342) and observed under confocal laser-scanning microscopy. The magnified image from the area within the square is shown in the right corner of each upper panel. Arrow heads, cellular structures responsible for degradation of OG-gelatin and accumulation of F-actin, corresponding to invadopodia.

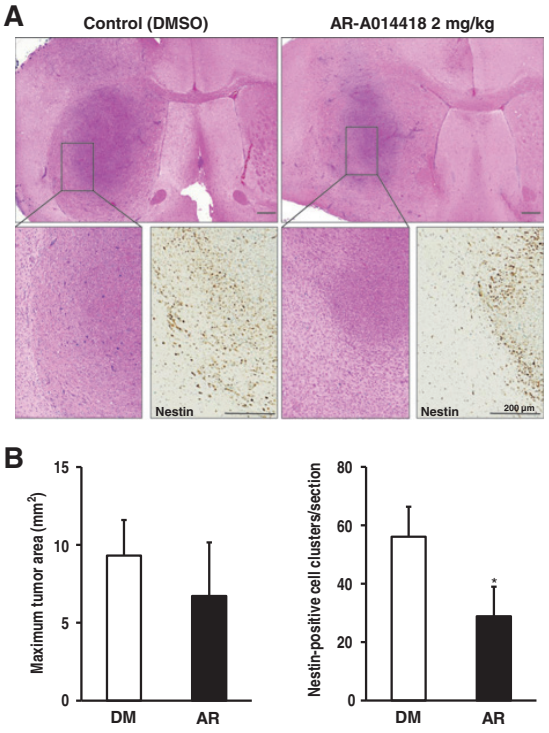

Figure 6.
Effects of GSK3β inhibition on the glioblastoma animal model. A, representative histologic and immunohistochemical sections of brain tumors treated with DMSO or AR-A014418. After 2 weeks of treatment, the tumor bulk was smaller in mice treated with AR-A014418 compared with those treated with DMSO. Tumor cells (positive for nestin) invaded adjacent normal brain tissue in control mice, whereas mice treated with AR-A014418 showed a well-demarcated border between the tumor and normal brain tissue. Scale bars, 200 μm. B, effects of DMSO (DM) and AR-A014418 (AR) on the maximum tumor area (mm^2) and the number of nestin-positive cell clusters. *, $P < 0.05$.

Discussion

The highly invasive nature of tumor cells is one of the most challenging hallmarks that prevents cure of refractory cancers, including glioblastoma. In the present study, we have shown that inhibition of GSK3β attenuates the migration and invasion of glioblastoma cells *in vitro*, as well as the invasion of malignant glioma cells in an animal model that recapitulates human glioblastoma (24). These effects were associated with alterations in molecular pathways mediated by FAK, GEFs/Rac1, and JNK. In addition, changes were observed in the cellular microstructures of lamellipodia and invadopodia, which coordinate to influence the motility and invasion of glioblastoma cells, respectively. We have previously reported that deregulated GSK3β is important for the survival and proliferation of glioblastoma cells and confers them with resistance to chemotherapeutic agents and radiation (12, 13). Taken together, these results establish GSK3β as a therapeutic target with multiple functional roles in glioblastoma.

An earlier study of glioblastoma cells showed an inverse association between cell migration and transcriptional

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coactivation of β-catenin following treatment with indirubin or lithium chloride (15). However, no data were presented to suggest how activated β-catenin might inhibit cell migration and the results are inconsistent with other studies demonstrating a role for the oncogenic Wnt/β-catenin pathway in promoting tumor cell stemness, proliferation, and invasion of glioblastoma cells (36–38). In our previous study, the fraction of β-catenin phosphorylated at its GSK3β phospho-acceptor sites (S33, 37 and/or T41 residues) was frequently detected in clinical samples of glioblastoma at elevated levels compared with adjacent, normal brain tissue (12). Furthermore, we did not find constitutive activation of β-catenin in glioblastoma cells following GSK3β inhibition. The role of β-catenin–mediated signaling in glioblastoma cell motility and invasion therefore requires further investigation.

The proinvasive phenotype of cancer cells includes an epithelial–mesenchymal transition (EMT) state (39) and distinct cellular microstructures such as lamellipodia and invadopodia (23, 28, 35) responsible for cell migration and invasion. In contrast with its role in cell migration (17), GSK3β was shown to inhibit EMT in normal cells by phosphorylating and stabilizing snail, a repressor of E-cadherin transcription (40). This suggests that EMT may not be involved in the mechanism by which GSK3β inhibition suppresses the migration and invasion of glioblastoma cells. As previously reported (28, 35), we observed the formation of lamellipodia and invadopodia in glioblastoma cells grown in nonconfluent conditions and on gelatin-coated slides, respectively. Treatment of cells with GSK3β inhibitor decreased the formation of these cellular microstructures and altered the subcellular colocalization of the cytoskeletal molecules F-actin and Rac1, resulting in their redistribution throughout the cytoplasm. Coinciding with these structural and functional changes in the cell, the inhibition of GSK3β reduced the phosphorylation of FAK and JNK. This in turn reduced their activity as well as that of active Rac1, the expression of GEFs responsible for Rac1 activation (22) and the expression and secretion of MMP-2 and MT1-MMP. Previous studies have shown these molecules (FAK, JNK, GEFs/Rac-1, MMPs other than GSK3β) interact to facilitate cell motility (32–34) and are involved in glioblastoma cell migration and invasion (22, 31, 41, 42). With this in consideration, our findings indicate that a GSK3β-mediated pathway involving FAK, JNK, GEFs/Rac-1 and MT1-MMP/MMP-2 plays a major role in promoting glioblastoma invasion (Supplementary Fig. S4) and suggest a novel therapeutic approach to control the invasive nature of this lethal disease.

It is becoming increasingly recognized that the invasive behavior of tumor cells and their resistance to therapy may not be separate properties and could in fact be interconnected processes (43). This notion is particularly relevant for glioblastoma in which a strong association is seen between invasive and therapy-resistant phenotypes (44, 45), leading to dismal survival outcomes (1–4). These two major cancer hallmarks share distinctive molecular pathways with several signaling hubs that include Rac1, FAK, and JNK (reviewed in 43). Rac1 has been reported to sustain stemness and the invasive ability of glioma stem-like cells, thus rendering them resistant to radiotherapy (46). It is also implicated in the radiation-induced enhancement of the invasive potential of primary glioblastoma cells (47). Similar to observations made with gene-manipulated fibroblasts (34), Rac1 has been shown to mediate phorbol 12-myristate 13-acetate–induced migration of glioblastoma cells via phosphorylation and activation of JNK and its translocation to paxillin-containing focal complexes (42). A previous study also showed that Rac1-mediated enhancement of glioma cell invasion in response to radiation was associated with activation of JNK and p38 MAP kinases (48). Moreover, an inhibitor of FAK autophosphorylation (Y15) reduces the viability of glioblastoma cells, induces their apoptosis, inhibits their invasion, and synergizes with temozolomide to increase survival in a murine glioma model (41). We previously reported that glioblastoma cells depend on deregulated GSK3β to survive, proliferate, and resist chemotherapy and radiation via the inactivation of p53- and/or Rb-mediated pathways (12). We have also demonstrated that a specific GSK3β inhibitor (AR-A014418; ref. 18) synergizes with temozolomide against glioblastoma cells by silencing O^6-methylguanine DNA methyltransferase expression via c-Myc–mediated promoter methylation (13). GSK3β is, therefore, heavily implicated in the two major pathologic characteristics of glioblastoma cells, invasive activity and therapy resistance, through pivotal oncogenic pathways.

We are currently undertaking a phase I/II clinical trial of recurrent glioblastoma patients treated with temozolomide in combination with drugs known to inhibit GSK3β activity and which are already in clinical use (UMIN:00005111; ref. 49). Our preliminary results show that the repurposed drugs inhibit GSK3β activity in the tumor cells, enhance the therapeutic effect of temozolomide and reduce invasion by the residual tumors, resulting in significantly longer patient survival times compared with patients treated with temozolomide alone (Nakada and colleagues; unpublished data). There is increasing evidence to show that GSK3β has neurodegenerative effects and that its inhibition has neuroprotective consequences (9). This has been observed for example with cranial irradiation-induced neurocognitive dysfunction (50). Therefore, the inhibition of GSK3β can provide dual benefits for the treatment of patients with glioblastoma by first reducing tumor invasion and therapy resistance, and second by protecting the host brain tissue from injury.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Sato, K. Miyamoto, M. Nakada, T. Minamoto
Development of methodology: T. Domoto, H. Sato, M. Nakada
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Chikano, T. Domoto, T. Furuta, H. Sabit, A. Kitano-Tamura, I.V. Pyko, T. Takino
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Chikano, T. Domoto, H. Sabit, I.V. Pyko, Y. Hayashi, M. Nakada, T. Minamoto
Writing, review, and/or revision of the manuscript: Y. Chikano, T. Domoto, T. Furuta, H. Sato, M. Nakada, T. Minamoto
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Kitano-Tamura, Y. Hayashi
Study supervision: Y. Sai, K. Miyamoto, T. Minamoto

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ORIGINAL ARTICLE

Lineage-specific *RUNX3* hypomethylation marks the preneoplastic immune component of gastric cancer

B Kurklu[1,2], RH Whitehead[2], EK Ong[1], T Minamoto[3], JG Fox[4], JR Mann[1,5], LM Judd[1,2], AS Giraud[1,2,6] and TR Menheniott[1,2,6]

Runt domain transcription factor 3 (*RUNX3*) is widely regarded as a tumour-suppressor gene inactivated by DNA hypermethylation of its canonical CpG (cytidine-phosphate-guanidine) island (CGI) promoter in gastric cancer (GC). Absence of *RUNX3* expression from normal gastric epithelial cells (GECs), the progenitors to GC, coupled with frequent *RUNX3* overexpression in GC progression, challenge this longstanding paradigm. However, epigenetic models to better describe *RUNX3* deregulation in GC have not emerged. Here, we identify lineage-specific DNA methylation at an alternate, non-CGI promoter (P1) as a new mechanism of *RUNX3* epigenetic control. In normal GECs, P1 was hypermethylated and repressed, whereas in immune lineages P1 was hypomethylated and widely expressed. In human GC development, we detected aberrant P1 hypomethylation signatures associated with the early inflammatory, preneoplastic and tumour stages. Aberrant P1 hypomethylation was fully recapitulated in mouse models of gastric inflammation and tumorigenesis. Cell sorting showed that P1 hypomethylation reflects altered cell-type composition of the gastric epithelium/tumour microenvironment caused by immune cell recruitment, not methylation loss. Finally, via long-term culture of gastric tumour epithelium, we revealed that *de novo* methylation of the *RUNX3* canonical CGI promoter is a bystander effect of oncogenic immortalization and not likely causal in GC pathogenesis as previously argued. We propose a new model of *RUNX3* epigenetic control in cancer, based on immune-specific, non-CGI promoter hypomethylation. This novel epigenetic signature may have utility in early detection of GC and possibly other epithelial cancers with premalignant immune involvement.

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INTRODUCTION

Gastric cancer (GC) has the second highest rate of cancer-related mortality worldwide, accounting for >700 000 deaths annually.[1] Late diagnosis is a major challenge to GC management, with disease presentation typical at advanced stages when treatment is ineffective and prognosis is poor.[2] Chronic inflammation after infection with *Helicobacter pylori* is a primary risk factor for the most common or 'intestinal-type' GC,[3] but definitive mechanisms remain elusive. GC is believed to be of epithelial origin, deriving from gastric epithelial cells (GECs) or their progenitors.[4,5] Elucidation of molecular events underlying the inflammation-related preneoplastic transformation of GECs[3] will be critical for the advancement of GC management, allowing earlier disease detection and improved survival.

Aberrant DNA methylation is one of the earliest molecular alterations in cancer and has been linked to GC pathogenesis.[6,7] Hypermethylated sequences become hypomethylated on a global scale. Conversely, unmethylated CpG (cytidine-phosphate-guanidine) island (CGI) promoters, including those of some tumour-suppressor genes (TSGs), become hypermethylated leading to their repression.[8] Hypermethylation of TSGs has been traditionally viewed as a persuasive mechanism of cancer pathogenesis, as well as providing a target for cancer detection.[9] Nonetheless, recent evidence that *de novo* hypermethylation in cancer mostly affects CGI promoters already repressed in normal tissues argues that methylation is not always required for their repression and therefore is not necessarily a driver of cancer.[10] Cell-type composition of the tumour microenvironment has gained attention as an alternative influence on cancer methylation profiles.[11] In this context, CpG-depleted or 'non-CGI' promoters, which show considerable variation in lineage-specific methylation, may offer complementary clinical utility to CGI promoters.[12,13] Indeed, the prognostic value of methylation signatures corresponding to non-neoplastic tumour lineages, such as cancer-associated fibroblasts or cytolytic T-lymphocytes, has recently been demonstrated.[14,15]

Runt domain transcription factor 3 (RUNX3) belongs to the family of conserved 'runt-domain' transcription factors that have diverse roles in hematopoiesis, neurogenesis and skeletal development.[16–18] *RUNX3* transcription initiates from two *cis*-regulatory regions designated the P1 (distal) and P2 (proximal) promoters. Expressed predominantly in hematopoietic lineages, RUNX3 regulates several aspects of immune function, including T-cell differentiation,[19,20] dendritic cell (DC) maturation[21] and natural killer (NK) cell activation.[22,23] In contrast to these definitive immune roles, the *RUNX3* locus (located on human chromosome 1p36.1) has been controversially linked to a TSG function in GC. In 2002, Li *et al.*[24] proposed that *RUNX3* inactivation via hypermethylation of a large CGI overlapping its P2 promoter is a pivotal event in GC pathogenesis. Subsequent studies have confirmed the association of *RUNX3* P2 hypermethylation with GC incidence (reviewed in Fan *et al.*[25] and Subramaniam *et al.*[26]).

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However, fresh evidence that *RUNX3* is never expressed in the normal GEC from which GC originates[27] casts doubt not only on its proposed TSG function, but also on the role of P2 methylation in RUNX3 silencing and its associated utility as a functional marker of GC. Limitations of this widely disseminated epigenetic model[24] are further compounded by paradoxical observations of *RUNX3* overexpression in GC and other cancers[28–30] where P2 is reportedly hypermethylated.[31] Although *RUNX3* continues to feature in the literature, models to better explain its deregulation in cancer have not emerged. With its TSG function now disputed,[27] unravelling the inconsistencies of *RUNX3* epigenetic control in GC will lead to an improved understanding of its broader role in cancer biology.

In focussing exclusively on the mechanistic importance of P2 methylation, researchers have overlooked a role for the alternate 'non-CGI' *RUNX3* P1 promoter. Here we address this gap in understanding, showing that lineage-specific P1 methylation constitutes a novel and unexpected mechanism of *RUNX3* epigenetic control in GC. We find that, in normal GEC, P1 is strongly hypermethylated and repressed. By striking contrast, in immune lineages, P1 is hypomethylated and widely expressed. Through studies of human and mouse GC progression, we uncover a preneoplastic P1 hypomethylation signature reflecting altered cell-type composition of the gastric epithelium/tumour microenvironment via immune cell infiltration. Finally, we reveal that *de novo* methylation of the P2 CGI promoter in GEC, argued previously as a driver of GC pathogenesis, arises as a bystander effect of oncogenic immortalization and is unlikely to exert any significant impact on GC progression. These results delineate a novel, more parsimonious model of *RUNX3* epigenetic control with emphasis on lineage-specific P1 methylation as a marker of preneoplastic tissue remodelling. The strong translational relevance of our findings to GC, and potential application to other epithelial cancers of inflammatory provenance, is discussed.

RESULTS

RUNX3 P1 hypomethylation correlates with human GC progression

To date, all *RUNX3* methylation studies have targeted the CGI overlapping the P2 promoter, but none have interrogated P1 methylation in GC (Figure 1a). The P1 sequence falls short of the minimum CpG density criteria to qualify as a CGI (CpG observed/expected ratio >0.6; >200 bp[32]) and is therefore 'non-CGI'-associated. In contrast to CGI promoters that generally resist methylation, non-CGI promoters are very likely to be regulated by methylation if CpG dinucleotides are present.[12,33] Indeed, this is the case for the human and mouse P1 sequences (Supplementary Figure S1). We therefore investigated *RUNX3* P1 expression and methylation in human gastric epithelial tissue collected from individuals displaying early-, intermediate- or late-stage intestinal-type GC development: *H. pylori*-infected/gastritis,[34] intestinal metaplasia (IM) and GC, respectively,[6] together with normal (disease-free) controls (Figure 1b). Quantitative reverse transcriptase–PCR (QRT–PCR) revealed increased total *RUNX3* mRNA expression in *H. pylori*-infected (4.05 ± 0.60-fold; $P < 0.001$), IM (8.47 ± 3.04-fold; $P < 0.01$) and GC (2.54 ± 0.73-fold; $P < 0.01$) tissues relative to normal controls (Figure 1c). Specific measurement of P1 mRNA similarly revealed increased expression in *H. pylori*-infected (8.77 ± 1.74-fold; $P < 0.001$) but not in IM or GC tissues. The high GC content of the P2 transcript 5′ leader exon precluded its direct interrogation by QRT–PCR. These results show increased *RUNX3* P1 expression, particularly during preneoplastic stages of GC. We next examined *RUNX3* P1 methylation levels using Sequenom EpiTYPER assays. EpiTYPER quantifies the ratio of methylated to unmethylated cytosines at individual CpG dinucleotides at specific loci.[35] Although P1 hypermethylation predominated in normal gastric epithelial tissues, a P1 hypomethylation signature was significantly associated with *H. pylori*-positive, IM and GC tissues. Consistent with its CGI status, P2 remained unmethylated in *H. pylori*-infected and IM preneoplastic tissues, showing modestly increased methylation only in a subset of tumours but not exceeding 15% (that is, methylation ratio 0.15) of strands in any individual tumour (Figure 1d). Hierarchical clustering showed a clear association of P1 hypomethylation with early inflammatory, preneoplastic and tumour stages of GC. Conversely, P2 was less vulnerable to epigenetic perturbation, showing weak hypermethylation in a subset of tumours (Figure 1e). *RUNX3* P1 and P2 are thus oppositely methylated in normal gastric tissue, respectively showing uniform early loss and variable late gain of methylation in GC progression. These results identify *RUNX3* P1 hypomethylation as a novel epigenetic signature with potential utility in GC risk prediction.

Conserved *Runx3* P1 hypomethylation following *H. pylori* infection, genetic induction of gastric inflammation or tumorigenesis in mice

Human and mouse *RUNX3/Runx3* are highly conserved with respect to their genomic organization, dual promoter structure (Figure 2a) and tissue expression profile. Therefore, mouse genetic and infection models recapitulating *H. pylori*-related preneoplastic and tumorigenic stages of human GC progression (Figure 2b), offered the most stringent approach to pinpoint the origin and significance of *RUNX3* P1 hypomethylation *in vivo*. Accordingly, we first determined *Runx3* transcription and methylation in stomachs of C57BL6 (wild type; WT) mice infected with mouse-adapted *H. pylori* SS1 for either 3- or 12 months. QRT–PCR showed that P1 and P2 transcripts were progressively upregulated in 3-month (4.10 ± 1.08; $P = 0.036$ and 12.58 ± 3.00; $P = 0.008$) and 12-month (8.22 ± 1.43-fold; $P = 0.005$ and 66.59 ± 12.28-fold; $P < 0.001$) infected mice compared with uninfected littermate controls (Figure 2c). Similar to our observations in *H. pylori*-infected humans, aberrant P1 hypomethylation was evident in *H. pylori*-infected mice, showing progression from a moderate to a strong signature in 3- and 12-month infected mice, respectively. Conversely, P2 methylation was unaltered with *H. pylori* infection (Figures 2d and e). To discern effects of *H. pylori*-dependent inflammation versus bacterial presence on P1 hypomethylation, we utilized transgenic mice with stomach-specific overexpression of the pro-inflammatory cytokine, gmcsf, (*gmcsf*^{Tg}). *Gmcsf*^{Tg} mice develop spontaneous gastric inflammation independently of *H. pylori* infection.[36] *Runx3* P1 transcripts were upregulated (2.58 ± 0.31-fold; $P < 0.01$) in *gmcsf*^{Tg} compared with WT stomachs (Figure 2c) and correlated with P1 hypomethylation (Figures 2d and e). P2 was not differentially expressed or methylated (Figures 2c and e). Therefore P1 hypomethylation correlates with gastric inflammation, not with *H. pylori*'s presence *per se*. To determine whether P1 hypomethylation persists later in GC progression, we utilized the *gp130*$^{F/F}$ GC model.[37] *Gp130*$^{F/F}$ mice develop tumours of the distal stomach with similar histopathology to human GC. Though epithelial in origin, *gp130*$^{F/F}$ tumours are strongly infiltrated by cells of the innate and adaptive immune system. Increased P1 transcription (9 ± 1.46-fold; $P < 0.05$) (Figure 2b) and P1 hypomethylation were clearly correlated in *gp130*$^{F/F}$ tumours (Figures 2c and d), further supporting a link with gastric inflammation. Conversely, P2 transcription was increased (23.19 ± 6.10-fold; $P < 0.01$) independently of its methylation status (Figures 2b and d). These results show that aberrant *RUNX3* P1 hypomethylation is a conserved, inflammation-associated process correlating with the preneoplastic and tumorigenic stages of GC.

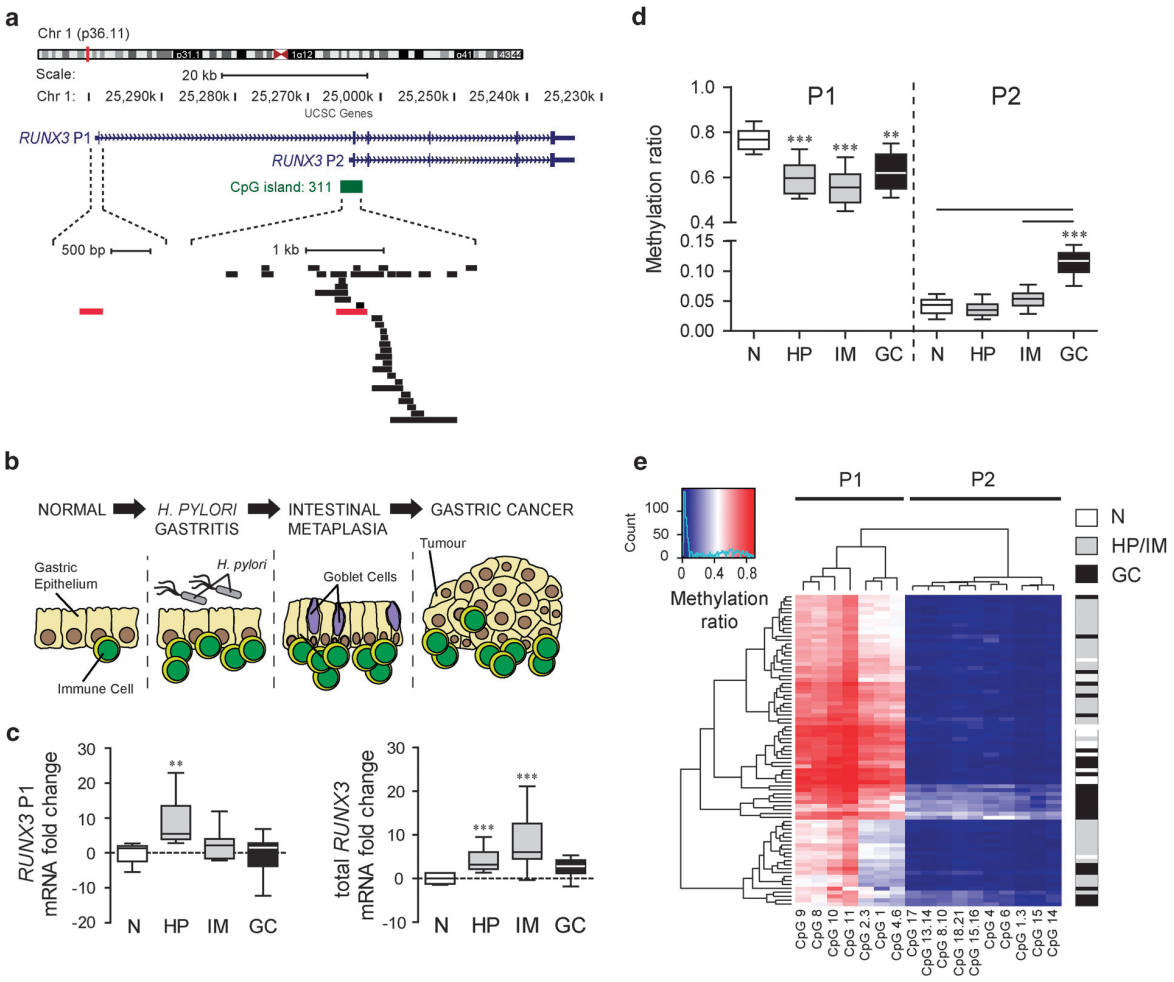

Figure 1. Epigenetic regulation of *RUNX3* P1 in human GC. (**a**) Mapping of methylation amplicons within human RUNX3. Genome browser output for human *RUNX3* on chromosome 1p36 (GRCh37 Hg19; http://genome.ucsc.edu/) shows relative locations of P1 and P2 promoter regions and intron/exon structures of the derived transcripts. Browser tracks show locations of *RUNX3* methylation amplicons (from previously published studies, black bars; from current study, red bars) aligned with the human genome using the 'Blast Like Alignment Tool' (BLAT). (**b**) Schematic showing progressive alteration in cell-type composition of the gastric epithelium during human GC progression. (**c**) QRT–PCR analysis of *RUNX3* mRNA expression in human GC progression. Box plots show mRNA fold change relative to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) internal reference gene expression for normal (N; $n=6$), *H. pylori*-infected (HP; $n=16$), preneoplastic adjacent to tumour with intestinal metaplasia (IM; $n=28$) and gastric cancer (GC; $n=28$) mucosal tissues. (**d**) EpiTYPER analysis of *RUNX3* P1 and P2 methylation in tissue samples analysed in panel (**b**). Box plots show combined CpG methylation values for P1 and P2, respectively. (**e**) Heatmap showing two-way hierarchical clustering of methylation data presented in panel (**c**). CpGs are shown on the horizontal axis; tissue samples are shown on the vertical axis. Tissue identities are indicated to the right of the heatmap. Asterisks show statistical significance: $^{**}P<0.01$; $^{***}P<0.001$.

RUNX3 localizes to infiltrating immune cells and not to epithelial cells in gastric preneoplasia and cancer

RUNX3 was localized in *H. pylori*-infected ($n=16$) and uninfected ($n=6$) human gastric tissues by immunohistochemistry. RUNX3-specific staining was detected in nuclei of immune cells infiltrating the lamina propria of *H. pylori*-infected but was not detected in uninfected tissues (Figure 3a). RUNX3 was not detected in GECs of infected or uninfected individuals. We similarly analysed the stomachs from $gmcsf^{Tg}$ and $gp130^{F/F}$ mice. In $gmcsf^{Tg}$ mice, RUNX3-specific staining localized to the nuclei of immune cells infiltrating the gastric epithelium but was not detected in GECs (Figure 3b). Similarly, in $gp130^{F/F}$ mice, RUNX3 was detected in immune cells infiltrating lamina propria of antral tumours and in submucosal lymphoid pockets but was not detected in GECs (Figure 3c). Earlier work showed absence of RUNX3 in normal mouse intestinal epithelium;[27] however, our results make the novel and critical distinction of showing absence of RUNX3 in normal, preneoplastic and tumour gastric epithelium in both humans as well as in mice. Therefore, *RUNX3* overexpression in gastric preneoplasia and cancer is likely dependent on immune cell recruitment.

Differential *Runx3* P1 methylation in GECs and immune lineages

Runx3 is known to be highly expressed in immune lineages (Supplementary Figure S2). Localization of gastric RUNX3 to infiltrating immune cells suggested that altered P1 methylation could similarly reflect immune cell recruitment. To address this, we isolated immune lineages known to have high Runx3 expression: NK1.1+CD3− NK cells, CD8+ T-cells, CD11c+ DCs, or low/absent Runx3 expression: CD11b+ macrophages, Gr1+ neutrophils, and CD45R+ B-cells from the spleens of WT mice by fluorescence-activated cell sorting (FACS) (Figure 4a;

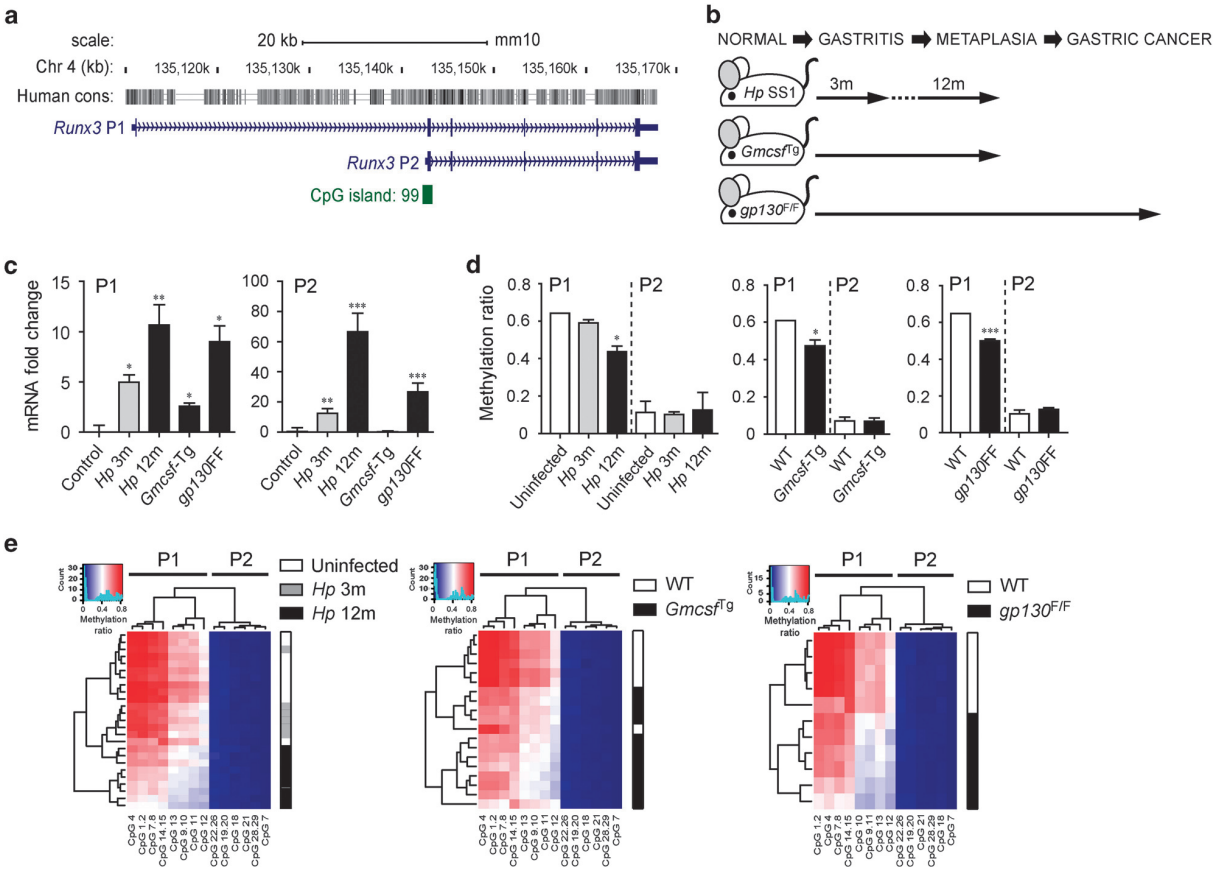

Figure 2. Conserved *Runx3* P1 hypomethylation in mouse gastric inflammation and tumorigenesis. (**a**) Genome browser output for the mouse *Runx3* locus on chromosome 4qD3 (GRCm38/mm10; *http://genome.ucsc.edu/*) shows relative locations of P1 and P2 promoters and intron/exon structure of their transcripts. (**b**) Schematic showing the mouse genetic and infection models used to recapitulate key stages of human GC progression. (**c**) QRT–PCR analysis of *Runx3* P1 and P2 transcripts in stomach tissues collected from C57BL6 (WT) mice infected with *H. pylori* SS1 for 3 months (*Hp* 3m) and 12 months (*Hp* 12m) in $Gmcsf^{Tg}$ and $gp130^{F/F}$ mice. Histograms show mRNA fold change relative to uninfected or WT controls. (**d**) EpiTYPER quantitative DNA methylation analysis of *Runx3* P1 and P2 promoters of gastric tissues analysed for mRNA in panel (**b**). Histograms show the combined CpG methylation levels for P1 and P2, respectively. Error bars ± s.e.m. Asterisks show statistical significance: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (**e**) Heatmaps show two-way hierarchical clustering of P1 and P2 individual CpG methylation values for data shown in panel (**d**).

Supplementary Figure S3) and characterized their *Runx3* mRNA and methylation profiles relative to primary GECs. *Runx3* P1 transcripts were detected in all immune cell types except B-cells, being most abundant in NK cells, CD8+ T-cells and DCs. P2 transcripts were abundant only in NK cells, showing modest levels in other lineages and were absent in GECs (Figure 4b). Strikingly, P1 was hypomethylated in all immune cell types irrespective of their *Runx3* expression level. This suggests that P1 hypomethylation is permissive but not sufficient for transcription. Conversely, P1 was hypermethylated in primary GECs and mouse embryonic fibroblasts, which lack *Runx3* expression (Figure 4c). By reference to the public domain transcriptome data, mast cells (MCs) were noted for abundant *Runx3* mRNA expression (Supplementary Figure S2) but have otherwise not been formally described to express *Runx3*. Analysis of spleen-derived, c-kit/CD117+FcεR1+ MCs (Figure 4a) confirmed *Runx3* P1 hypomethylation and transcription at similar levels to CD8+ T-cells (Figures 4b and c).

Our results showing universal P1 hypomethylation in immune cells suggested that its specific epigenetic state might originate before, or during, hematopoiesis. To address this question, we tracked P1 methylation levels during MC differentiation from mouse bone marrow stem cells (BMSCs) cultured with interleukin (IL)-3. Correct MC differentiation was verified by the acquisition of a c-kit/CD117+FcεR1α+ surface phenotype (Figure 4d) and induction of *carboxypeptidase* (*Cpa3*) mRNA (Supplementary Figure S3). Bone marrow-derived MCs showed strong induction of *Runx3* P1 mRNA relative to BMSCs showing only marginal expression, while P1 was equivalently hypomethylated in BMSCs and differentiated MCs (Figures 4e and f). Similar results were obtained in bone marrow-derived DCs (B Kurklu, unpublished data). These results argue that immune-specific P1 hypomethylation is inherited from BMSCs, further suggesting the existence of a developmental mechanism that protects P1 against *de novo* methylation in certain contexts.

Immune cell recruitment accounts for *Runx3* P1 hypomethylation in gastric epithelial tumours

We next examined P1 methylation in GECs and immune cells isolated directly from $gp130^{F/F}$ gastric tumours. Dissected tumours were non-enzymatically disaggregated, and the following cell types were recovered by FACS: e-cadherin+ GECs, CD8+ T cells, and CD11c+ DCs (Figure 5a). EpiTYPER analysis of these tumour cell fractions revealed that P1 hypomethylated alleles were enriched in CD8+ T-cells and CD11c+ DCs. Conversely, P1 hypermethylated alleles were enriched in e-cadherin+ GECs. P2 was unmethylated in tumour-derived immune cells and GECs

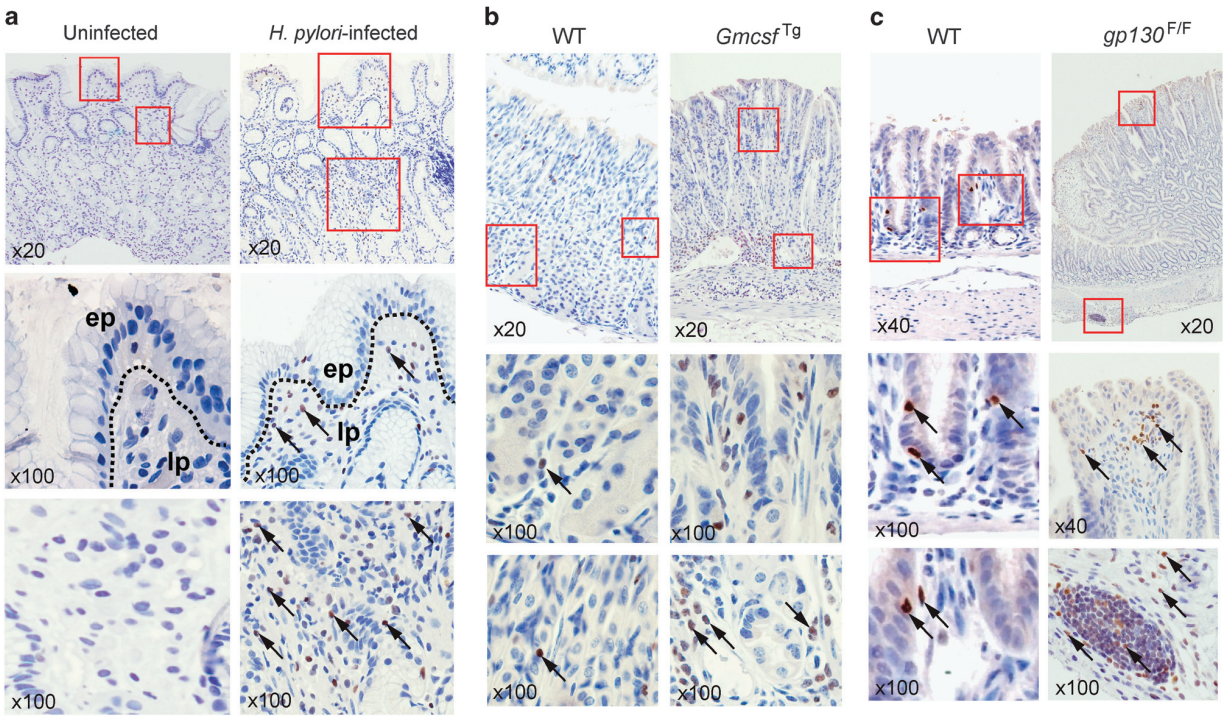

Figure 3. Immunolocalization of RUNX3 in human and murine gastric preneoplasia and tumorigenesis. (**a**) RUNX3 immunohistochemistry in human gastric mucosal tissue collected from *H. pylori*-infected and uninfected individuals. (**b**) Runx3 immunohistochemistry in $Gmcsf^{Tg}$ gastric fundus. (**c**) Runx3 immunohistochemistry in $gp130^{F/F}$ gastric antral tumours. Tissue sections were counterstained with hematoxylin (blue staining). Magnifications are indicated (×20, ×40, ×100). Arrows indicate highly discrete Runx3 nuclear staining. lp, lamina propria; ep, epithelium.

(Figures 5b and c). That is, hypomethylated P1 alleles reside predominantly within the immune, but not the epithelial component of gastric tumours, supporting immune cell recruitment as a key mechanism underlying a *RUNX3* P1 hypomethylation signature in GC progression.

Runx3 P2 hypermethylation triggered by immortalization of GECs

RUNX3 P2 hypermethylation clearly has no direct role in *RUNX3* repression or as an autonomous driver of GC; however, its significance remains unexplained. Cancer-related *de novo* methylation targets repressed CGI promoters,[10,38] and this global deregulation of methylation may arise as an effect of cellular immortalization.[38] Immortalized cell lines often show more extensive CGI hypermethylation than corresponding primary tumours.[39] Accordingly, we compared P2 methylation levels in immortal human GC cell lines and primary GC tissues. Five out of the six cell lines showed near complete (>90%) P2 hypermethylation (Figure 6a) contrasting with much lower levels (<15%) in primary GC tumour tissues. The single exception was the slow growing NCI-N87 line, which showed only marginally increased P2 methylation levels. P1 methylation levels were similar in both normal, tumour and cell lines. These results reveal a specific correlation between hypermethylation of the P2 CGI and GEC immortalization. To explore this concept, we next quantitated P2 methylation levels in mouse GECs both before and after their immortalization *in vitro*. Immortalization, defined as the inappropriate acquisition of indefinite proliferation, was induced by serial passage of cultured primary GECs isolated from $gp130^{F/F}$ gastric tumours (Figure 6b). We cloned five GEC lines showing key features of an immortalized phenotype, including a high level of aneuploidy and the capacity to proliferate indefinitely in culture. Additionally, two of the lines displayed anchorage-independent growth in soft agar, yet all of the lines retained expression of key epithelial marker proteins, including cytokeratin (Krt)8 (Figures 6c and d; Supplementary Table S1). Though derived from primary GECs lacking P2 methylation, four of the five GEC lines had acquired high-level P2 hypermethylation after immortalization (Figure 6e). P2 methylation levels were the highest in the fastest growing lines (clones1, 3 and 5), whereas only minor gains in methylation were observed in the slowest growing line (clone 2). Conversely, P1 methylation in the immortal GECs was unperturbed relative to normal GECs (Figure 6e), consistent with evidence that *de novo* methylation in epithelial carcinogenesis may specifically target repressed CGI promoters. Therefore, P2 hypermethylation can be induced during GEC immortalization, likely in concert with *de novo* hypermethylation of other CGI promoters on a global scale. The over-representation of P2 hypermethylation among GC cell lines argues that CGI hypermethylated GEC clones may have a growth advantage, resulting in their preferential expansion in culture.

DISCUSSION

This work is the first to reveal a role for *RUNX3* P1 promoter methylation in regulating lineage-specific *RUNX3* transcription in the stomach. We specifically showed that P1 methylation is established differently in GEC and immune cells and that increased recruitment of the latter underlies aberrant P1 hypomethylation in GC progression. Finally, we showed that *de novo* methylation of the P2 CGI promoter in GEC/tumour cells, argued previously as a driver of GC progression, likely arises as a bystander effect of oncogenic immortalization and is unlikely to have a causal role in the disease. We surmise that an *in vitro* growth advantage, associated with global CGI promoter hypermethylation, leads to an over-representation of hypermethylated

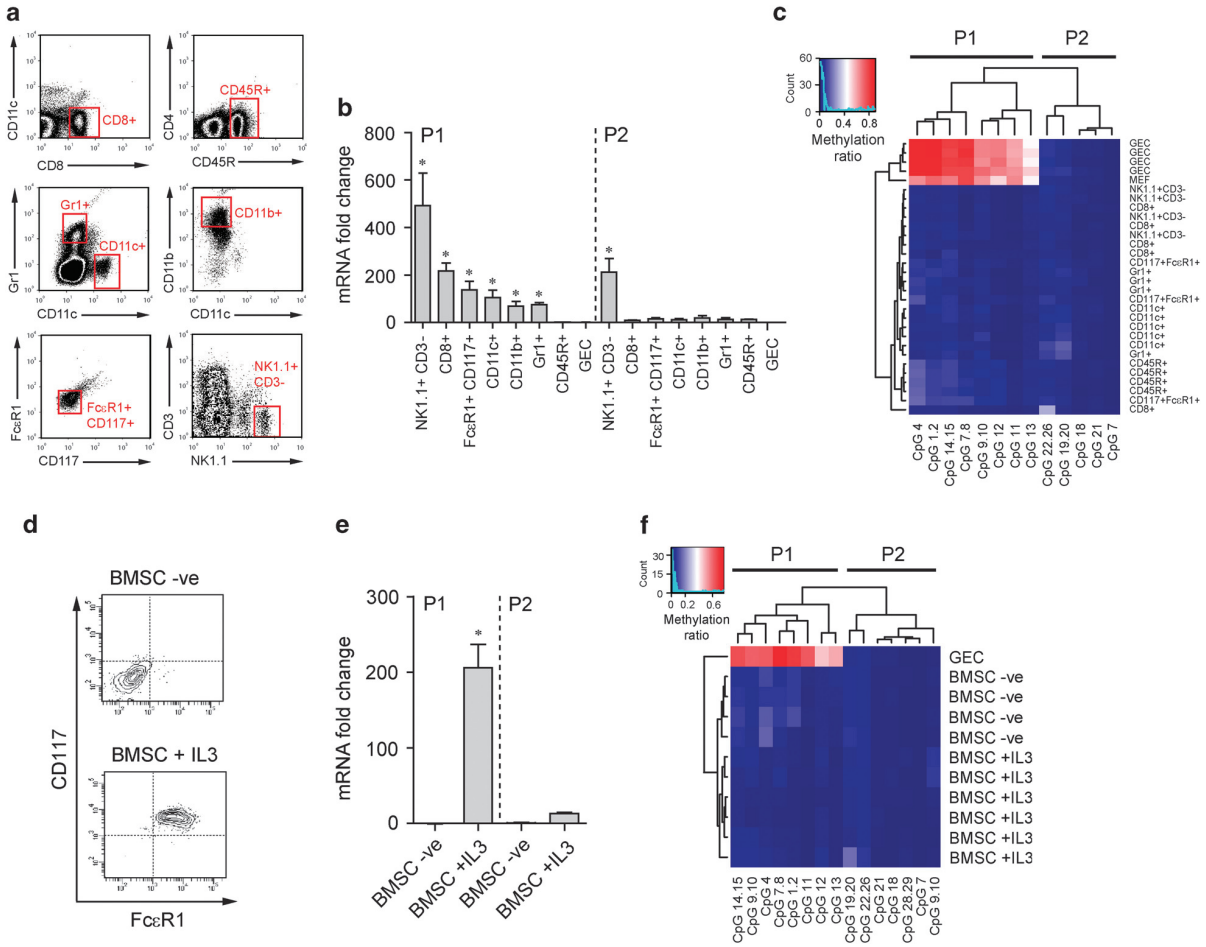

Figure 4. Differential *Runx3* P1 methylation in GEC and hematopoietic lineages. (**a**) Isolation of immune cell populations from splenocytes by FACS: NK1.1+ CD3− NK cells; CD3+CD8+ T-cells; CD117+ FcεR1a+ MCs; CD11c+ DCs; CD11b+ macrophages; Gr1+ neutrophils; and CD45R+ B-cells. (**b**) QRT–PCR analysis of *Runx3* P1 and P2 transcripts in FACS-sorted immune cell types. Histograms show mRNA fold changes relative to levels in primary GEC. (**c**) Quantitative DNA methylation analysis of Runx3 P1 and P2 promoters in FACS-sorted immune cell types, primary GECs and primary mouse embryonic fibroblasts. Heatmap showing two-way hierarchical clustering of P1 and P2 methylation data. (**d**) *In vitro* generation of bone marrow-derived MCs. Flow cytometric analysis of murine bone marrow stem cells (BMSC) cultured with IL-3 (BMSC+IL3) or untreated (BMSC −ve). MC differentiation is shown by the acquisition of a CD117+ FcεR1+ double-positive phenotype (one representative experiment of four replicates is shown). (**e**) QRT–PCR analysis of *Runx3* P1 and P2 mRNA in BMSC+IL3 and BMSC control cultures. Error bars ± s.e.m. Asterisks show statistical significance: $*P < 0.05$. (**f**) Heatmap showing EpiTYPER quantitative methylation analysis of Runx3 P1 and P2 promoters corresponding to the samples analysed in panel (**e**).

P2 alleles in immortal GC cell lines. Our novel findings are summarized in Figure 7.

More than a decade has passed since Li *et al.*[24] initially described *RUNX3* as a TSG repressed by P2 hypermethylation in GC. Then recognized as a significant advance, the findings engendered a long-standing paradigm of gastric tumorigenesis via TSG epigenetic loss. Frequent P2 hypermethylation in GC and other cancers remains undoubted, having been verified by hundreds of subsequent studies seeking to replicate and extend the pioneering work of Li *et al.* (reviewed in Fan *et al.*[25] and Subramaniam *et al.*[26]). However, the demonstrable absence of RUNX3 from normal GECs, the progenitors to GC, casts doubt upon its much vaunted TSG function.[27] By inference, the existing model of P2 hypermethylation as a driver of GC is also inadequate given that *RUNX3* is repressed by default in normal GECs and is often overexpressed in GC and other cancers.

We propose an alternative model in which differential methylation of P1 dictates lineage-specific *RUNX3* expression. This novel modality was evident from observations in normal GEC and mesenchymal (fibroblast) lineages, where P1 was hypermethylated and repressed. By contrast, in immune lineages P1 lacked methylation and was widely transcribed. The fact that not all immune cell types have Runx3 expression, despite their universal P1 hypomethylation, argues that *trans*-factors are also required for full activation of transcription. This mode of differential methylation was highly significant in the context of human and mouse GC progression. P1 hypomethylation (and increased transcription) signatures reflected evolving cell-type composition of the preneoplastic epithelium and/or tumour microenvironment due to immune cell recruitment. Moreover, localization of RUNX3 to immune cells infiltrating the preneoplastic and tumour epithelium as shown here excludes the possibility that preneoplastic epithelial lesions, such as atrophic gastritis or mucous cell metaplasia, may have influenced P1 transcription and/or methylation. In breast cancer, DNA methylation profiles reflecting the tumour immune component have been used to great effect for clinical prediction.[14] Presence of T-cell 'methylation biomarkers' in breast tumours correlated with better prognosis, suggesting a link with anti-tumour immunity. We postulate that *RUNX3* P1 hypomethylation, as a marker of cytotoxic lymphocytes,

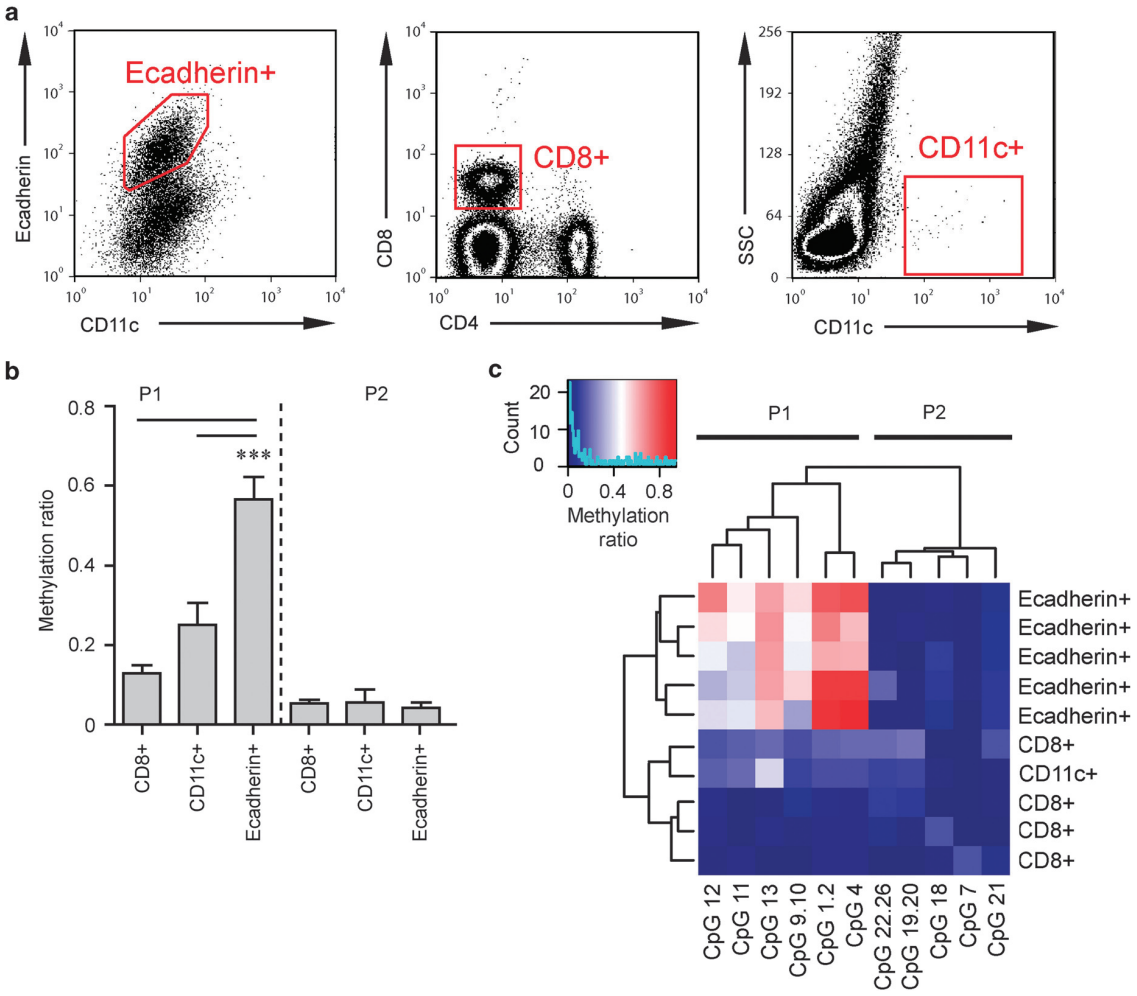

Figure 5. *Runx3* P1 methylation in gastric tumour lineages. (**a**) Isolation of immune cells and GECs from *gp130*$^{F/F}$ gastric tumour tissue by FACS: CD8+ T-cells; CD11c+ DCs; and e-cadherin+ GECs. Shown is one representative example of five replicate experiments. (**b**) EpiTYPER quantitative DNA methylation analysis of *Runx3* P1 and P2 promoters of isolated cell types shown in panel (**a**). Histograms show the combined CpG methylation levels for P1 and P2, respectively. Error bars ± s.e.m. Asterisks show statistical significance: ***$P < 0.001$. (**c**) Heatmap showing two-way hierarchical clustering of P1 and P2 individual CpG methylation data represented in panel (**b**).

may have similar prognostic value in GC, a postulate supported by the fact that cytolytic properties of CD8+ T-cells and NK cells are *Runx3*-dependent.[23]

A second major conclusion here is that P2 hypermethylation is a phenotypic feature of transformed, or immortal, GECs and has no causal role in *RUNX3* repression or GC pathogenesis. Our findings are reminiscent of recent work in hTERT-immortalized fibroblasts showing progressive accumulation of P2 hypermethylation as a function of increasing generations in culture, without affecting transcription.[40] However, our findings make the key distinction of showing evolution of immortal P2 hypermethylated clones from primary GECs ostensibly lacking P2 methylation. Whether P2 hypermethylation can arise *de novo* in culture, or amplifies clonally from rare aberrant cells in primary tumours, remains to be elucidated. Resonant with our findings, repressed CGI promoters are more vulnerable to *de novo* methylation in cancer than active CGI promoters[10,38] based on polycomb-mediated premarking by repressive histone (H)3-lysine (K)27 tri-methylation.[41] Together with evidence that the polycomb repressor complex 2 can promote *RUNX3* P2 repression in GEC,[42] these studies illustrate how a program of DNA methylation-independent repression might promote *de novo* P2 hypermethylation in immortal GECs. Nonetheless, it is increasingly apparent that CGI hypermethylation is less frequent in cancer than initially hypothesized.[43] We indeed observed lower P2 methylation frequencies here using quantitative EpiTYPER analysis than reported by earlier studies using non-quantitative methylation-specific PCR (MSP).[25,26] The propensity of non-quantitative MSP to overestimate low methylation levels[44] may explain this discrepancy. In agreement, recent work found that P2 methylation levels of $< 10\%$ can be reported as 'hypermethylated' by MSP.[45]

With its TSG function now in doubt,[27] alternative roles for *RUNX3* in the stomach must be considered. It has not escaped our notice that gastric epithelial hyperplasia in *Runx3*$^{-/-}$ mice may arise by a non-autonomous mechanism. This phenotype was attributed to *Runx3* deficiency in CD8+ cytolytic T-cells and consequent impairment of anti-tumour immunity.[46] An immune-specific anti-tumour role was similarly implicated by spontaneous colitis and tumour growth in mice with lymphocyte-specific *Runx3* deficiency.[47] It may be significant that *Runx3* expression is particularly abundant in immune lineages with known roles in anti-tumour immunity. *Runx3* is essential for the cytolytic functions of CD8+ T-cells and NK cells,[19,23] but if either of these

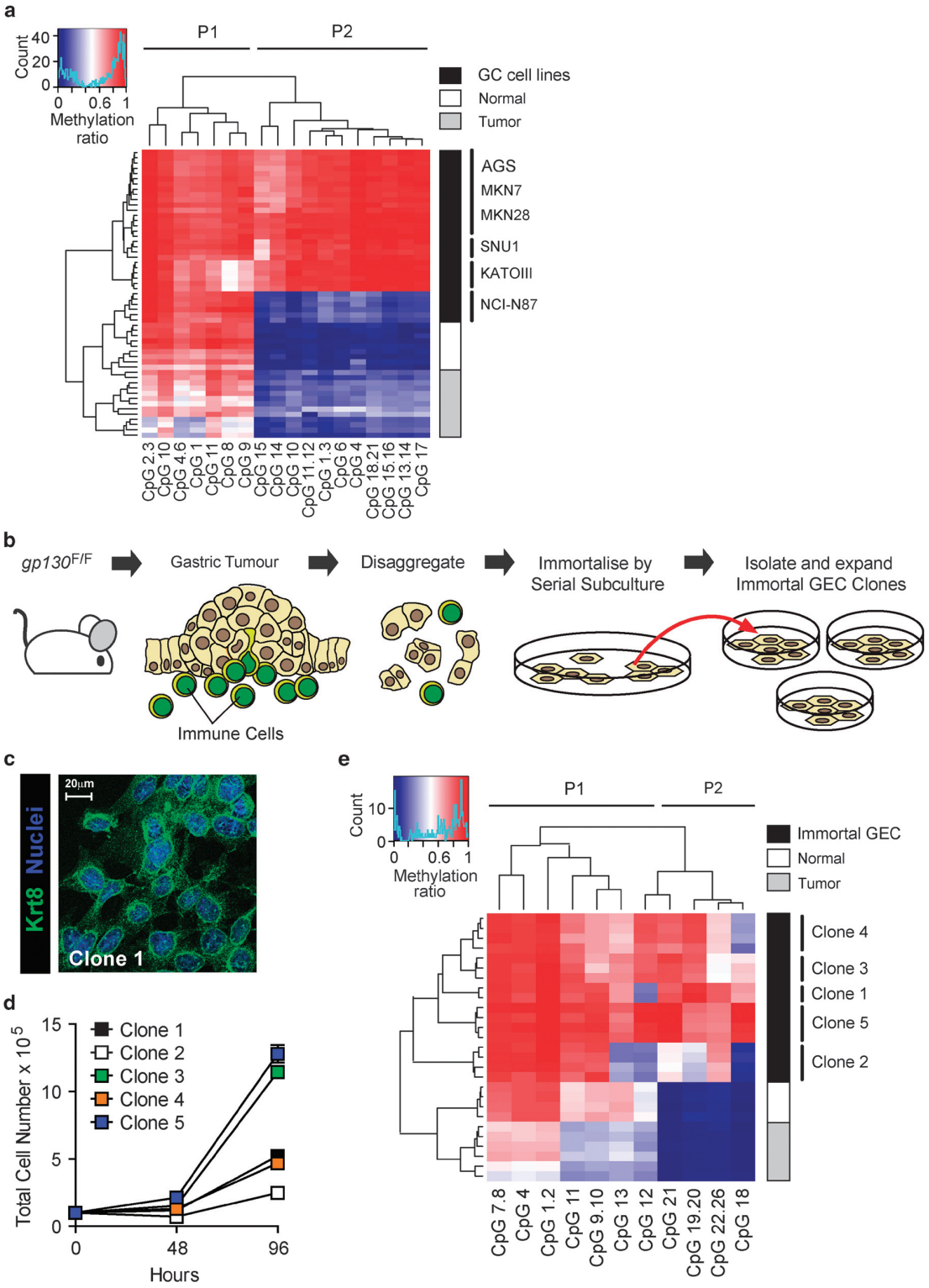

Figure 6. *RUNX3* P2 methylation is triggered by immortalization of human and mouse GECs. (**a**) EpiTYPER analysis of *RUNX3* P2 (and P1) methylation levels in human GC cell lines, primary GC tumours and normal gastric epithelial tissues. Heatmap shows two-way hierarchical clustering of individual CpG methylation values against cell line or tissue type. (**b**) Flow diagram showing the derivation of mouse immortal GEC lines from $gp130^{F/F}$ primary gastric epithelial tumours. (**c**) Immunofluorescent detection of cytokeratin (Krt)8 in immortal GEC lines. Representative staining for one of the lines (clone 1) is shown. (**d**) Growth curves of mouse immortal GEC lines in monolayer culture. Viable cell count data are shown as the mean of six replicate wells for each clone. (**e**) EpiTYPER analysis of *Runx3* P1 and P2 methylation in immortal mouse GEC, primary $gp130^{F/F}$ tumours and WT gastric tissue. Heatmaps show two-way hierarchical clustering of individual CpG methylation values for P1 and P2.

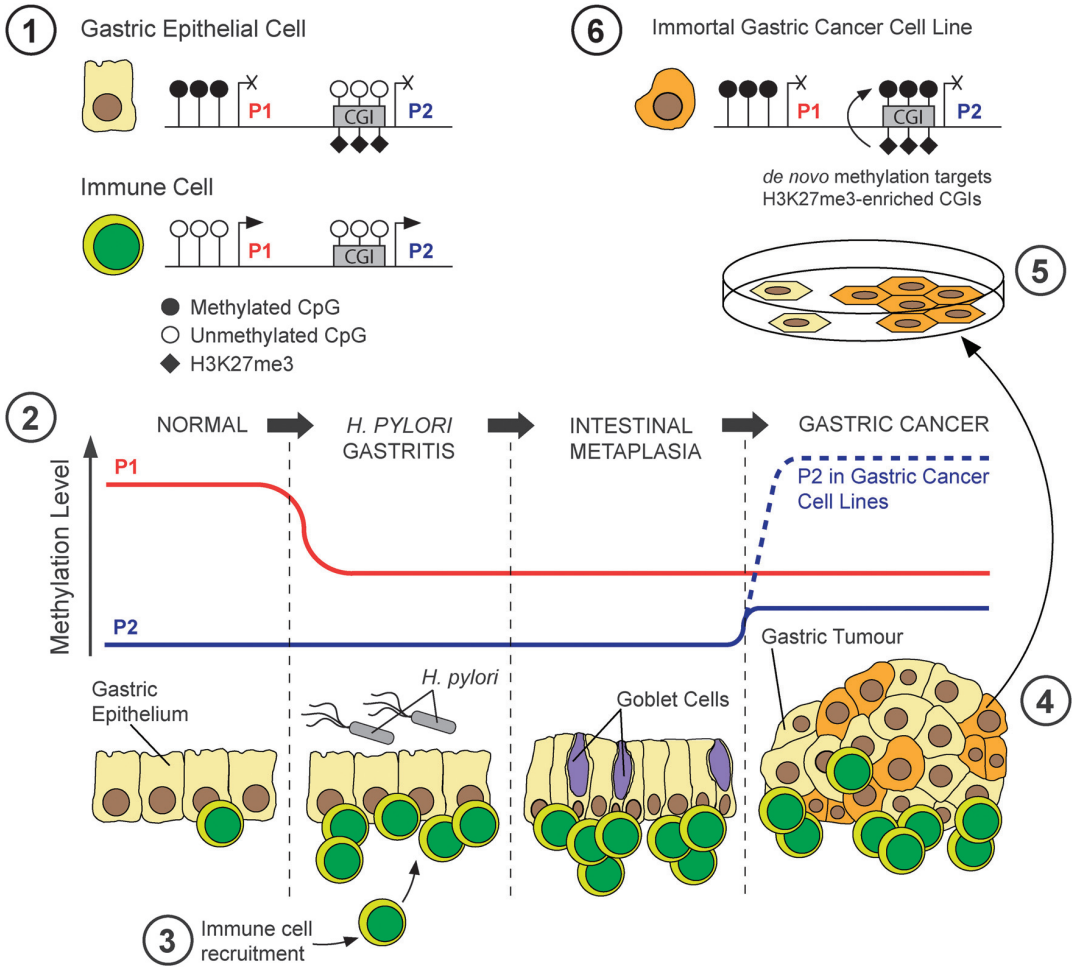

Figure 7. Schematic showing effects of cell-type composition and tumour clonality on *RUNX3* promoter methylation in GC progression and cell line establishment. In GECs, P1 is hypermethylated and repressed. In immune cells, P1 is hypomethylated and expressed (1). These lineage-specific differences in P1 methylation underlie P1 hypomethylation in GC progression (2). Immune cell recruitment triggered *H. pylori* infection (3), leads to altered cell-type composition of the gastric epithelium leading to decreased P1 methylation. Conversely, P2 lacks methylation in both GECs and immune cells, thus its methylation level is unaffected by altered tissue cellularity. In gastric tumours, P2 becomes aberrantly hypermethylated (in concert with other CGI promoters on a global scale) in a subset of highly immortalized/transformed GECs (orange shading), leading to a modest increase in P2 methylation in tumour tissue (4). P2 hypermethylated clones have an *in vitro* growth advantage over non-hypermethylated clones, allowing their preferential expansion during cell line establishment from primary tumour tissue (5), leading to a significant over-representation of P2 hypermethylated clones among GC cell lines (6).

modalities might extend to restrain gastric tumour growth remains to be proven.

Here we have proposed a new model of *RUNX3* epigenetic control based on lineage-specific hypomethylation of its (non-CGI) P1 promoter in immune cells. Furthermore, aberrant *RUNX3* P1 hypomethylation derives from the mobile immune component of the preneoplastic and tumour epithelium during GC development. The significance of this new data lies in the potential clinical utility of P1 methylation in identifying 'at-risk' individuals during the preneoplastic stages of GC, before the emergence of malignant adenocarcinoma. P1 methylation may therefore offer superior clinical potential to P2 methylation, which appears later in GC progression and, reportedly, has no predictive value in staging, prognosis, recurrence or survival.[25]

MATERIALS AND METHODS

Human tissues

H. pylori-infected and uninfected human gastric epithelial tissues, GCs and preneoplastic adjacent to cancer tissues with IM were obtained endoscopically as described.[6] Ethics approvals were obtained from the Royal Melbourne Hospital Human Research Ethics Committee (approval number 2004.176) and the Kanazawa University Ethics Committee for Human Genome Research (approval number 174.2008). Written informed consent was obtained for all study participants.

Mice

H+/K+ATPase-gmcsf transgenic mice[36] were maintained on a Balb/C genetic background. *Gp130*$^{F/F}$ co-receptor knock-in mice[37] were maintained on a C57BL6/J genetic background. WT littermate controls were used in all the experiments. Mice were housed under specific pathogen-free conditions. Experiments were approved by the Murdoch Children's Research Institute Animal Ethics Committee (approval numbers A693 and A713). WT (C57BL6) mice were infected with the *H. pylori* Sydney strain (SS) 1 as described.[6]

Mammalian cell culture

Human GC cell lines AGS, MKN7, MKN28, NCI-SNU1, KATO III and NCI-N87 were cultured in RPMI 1640 Glutamax medium supplemented with 10% fetal bovine serum (FBS); 2 mM non-essential amino acids; 50 IU penicillin;

50 ug/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified incubator with 5% CO_2/air. To derive mouse primary GECs, stomachs from 3-week-old mice were chopped into ~1-mm^3 pieces and digested with 2 mg/ml collagenase A (Roche, Mannheim, Germany) at 37 °C for 1 h. Digested tissue was resuspended in Dulbecco's modified Eagle's medium Glutamax medium supplemented with 20% FBS, 2 mM non-essential amino acids; 50 IU penicillin; 50 ug/ml streptomycin (Invitrogen), disaggregated by repeated pipetting, seeded into 24-well plates and incubated at 37 °C with 5% CO_2/air for 48 h to allow for culture maturation. To derive immortal mouse GEC lines, freshly dissected *gp130*$^{F/F}$ gastric tumours were surface sterilized by incubation in 0.04% sodium hypochlorite in phosphate-buffered saline (PBS) for 20 min at room temperature, rinsed in PBS, chopped into ~1-mm^3 pieces, resuspended in sterile PBS and allowed to settle for 1 min. Supernatants were aspirated, and tissue was collected by centrifugation, resuspended in 1 mg/ml collagenase and 1 IU/ml neutral protease (Sigma, St Louis, MO, USA) in PBS and digested at 37 °C for 60 min. Digested tissue was collected by centrifugation, resuspended in growth medium[48] seeded onto collagen-coated 24-well culture plates and incubated undisturbed for 7 days. Rapidly expanding epithelial clones were isolated from contaminating non-epithelial cells by limiting dilution.

Generation of bone marrow-derived cultured MCs

Bone marrow stem cells were extracted from femurs of 6–8-week-old C57BL6 mice and cultured in Dulbecco's modified Eagle's medium Glutamax medium, supplemented with 10% FBS, 50 IU penicillin, 50 ug/ml streptomycin and IL-3 for 6 weeks as described.[49] IL-3-enriched medium was sourced from murine WEHI-3 myelomonocytic leukaemia cultures and added to growth medium at 20%v/v.[50] MC differentiation was verified by cell surface staining with CD117-FITC (fluorescein isothiocyanate; 1:300) and FcεR1α-PE (1:300; BD Biosciences, San Jose, CA, USA). Stained cells were analysed on a LSR-II flow cytometer using the FacsDiva software (BD Biosciences).

Immunohistochemistry and immunofluorescence

Immunohistochemistry with peroxidase detection was done essentially as described.[51] A well-characterized rabbit polyclonal anti-Runx3 antiserum (poly-G) was used at a dilution of 1:1000.[52] Bound immunocomplexes were detected using Vectastain ABC reagents (Vector Laboratories, Burlingame, CA, USA), and staining was visualized by incubation in 3, 3′-diaminobenzidine tetrahydrochloride buffer (Sigma). Immunofluorescence in cultured cells was performed as described.[51] A FITC-conjugated rat polyclonal anti-mouse cytokeratin (Krt)8 antibody (Sigma) was used at a dilution of 1:100.

FACS

Splenocytes were prepared from 12-week mice, erythrocytes were removed by incubation in lysis buffer (1:9 v/v 0.17M Tris: 0.16 M ammonium chloride) for 5 min and enriched splenocytes were resuspended in 2% FBS and 1 mM EDTA in Hank's Balanced Salt Solution. For isolation of tumour lineages, dissected *gp130*$^{F/F}$ gastric tumours were chopped into ~3–4 mm^3 pieces and disaggregated non-enzymatically by incubation in dissociation buffer (5% FBS, 1 mM dithiothreitol, 1 mM EDTA in PBS) for 1 h at 37 °C with agitation. Digested tissue pieces were passed through a 70-μm strainer, and the cells were resuspended in 2% FBS and 2 mM EDTA in Hank's Balanced Salt Solution. Splenocytes and gastric tumour cells were stained with CD11c-APC (1:500), CD8α-APC-Cy7 (1:500), CD45R/B221-FITC (1:500), CD11b-PE (1:500), Gr-1 (Ly6-G/C)-PerCP-Cy5.5 (1:300) (all from BD Biosciences) and NK1.1-brilliant violet 421 (1:300) E-cadherin-PE (1:300) (from BioLegend, San Diego, CA, USA). Cells were sorted (at low pressure with a 100-μm nozzle) on a MoFlo sorter (Beckman-Coulter, Brea, CA, USA). Cells were not cultured in the period between isolation and sorting.

Gene expression and DNA methylation analysis

QRT–PCR was performed as described.[53] Primer sequences (Supplementary Table S2) were designed using the primer3 tool (http://frodo.wi.mit.edu/primer3/). Relative gene expression was normalized to the reference genes *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase; human) or *Rpl32* (mouse) using $-2\Delta\Delta Ct = \Delta Ct$ sample $- \Delta Ct$ calibrator. Quantitative DNA methylation analysis was performed by EpiTYPER (Sequenom, San Diego, CA, USA) as described.[6] Primer sequences for methylation amplicons (Supplementary Table S3) were selected using EpiDesigner (http://www.epidesigner.com). Data cleaning and hierarchical clustering were performed in R script using the gplots package (http://www.r-project.org/).

Statistical analysis

Data were analysed with GraphPad Prism V5.1 software (GraphPad Software, La Jolla, CA, USA). Data were expressed as mean ± s.e.m. Statistical analysis was performed by one-way analysis of variance and the appropriate parametric or nonparametric post test. *P*-values of $\leqslant 0.05$ were considered statistically significant.

ABBREVIATIONS

CGI, CpG island; CpG, cytidine-phosphate-guanidine; DC, dendritic cell; GC, gastric cancer; GEC, gastric epithelial cell; hTERT, human telomerase reverse transcriptase; IM, intestinal metaplasia; MC, mast cell; MSP, methylation-specific PCR; NK, natural killer; RUNX, runt-domain family transcription factor; TSG, tumour-suppressor gene.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

TRM, ASG, LMJ and JRM conceived, designed and led the study. BK, TRM, EK and RHW performed the experiments. BK and TRM analysed the data. TM and JGF contributed reagents and materials. TRM and BK wrote the manuscript. TRM, ASG, LMJ and JRM edited and revised the manuscript. All authors approved the final version of the manuscript before submission

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

【附記4】 研究のあゆみと業績 2011 年－2015 年

Quinquennial Report 2011－2015

研究のあゆみと業績　2011－2015

猿山灯台（輪島市門前町） 2015 年 4 月 4 日撮影（源）

【この５年間を返りみて，その先へ】

表題の「Quinquennial」は聞き慣れない言葉であり，舌を噛みそうになります．辞書では「５年目ごとの」，「５年間の」，あるいは「５年続く」，などを意味するようです．

2011 年に「Deccenial Report 2001～2010」を編集してから5年が経過しました．2016 年６月 30 日には，この研究室が開設されて満 15 年を迎えます．自身のことでは大学医学部を卒業して 33 年間のうちの 15 年を占めます．表紙の米印（＊）は 15 個目を数えました．気の遠くなるような長さでもあり，見かたを変えればときの流れのはやさを実感させられる年月でもあります．ありきたりにいえば，責任ある立場であること以外はとりたてて感慨のようなものはなく，これから 16 年目への通過点ととらえています．中途半端な区切りでもあり，その意味合いからの「Quinquennial Report」です．

それでも，この研究室とわが身が，この５年間にどれほどのことが出来たかを振り返るきっかけになります．これまでに編集した「あゆみと業績」と同様に，ちまたの年報や業績集などにありがちな国内外の世情や時事論評のたぐいに関する内容は一切なく，私たちの活動の変遷を淡々と綴ることが本誌の趣旨であり，逆につまらない点でもあると思っています．そして，本誌をお読みくださる皆さまと仕事仲間が，この報告をどのように批評，批判してくださるのかなぁと思いめぐらせるのが楽しみです．どうぞ，ご感想やご批評をお寄せください．

本誌の表紙，裏表紙と「Quinquennial Repor 2011～2015」の表題に載せてあるのは「猿山（さるやま）灯台」と猿山岬にひっそりと生息している「雪割草（ゆきわりそう）」です．海抜 200 メートル以上の断崖が続く猿山岬は能登半島の北西端にある秘境で，その突端に白亜の猿山灯台が立っています．この灯台は近海を航行するすべての船舶の”seamark（海標）”であり，春先には「雪割草」が訪れる多くの人たちの目を和ませてくれます．私の幼少時から年長の子供たちの遠足の目的地であり，半日かけて山道を辿り，この灯台に着いたときの歓びと達成感はいまも残っています．私たちと共同研究者の皆さんの最終目的ではなくても，少しでも先を目指す共通の”landmark（道標）”のような存在として連想しています．

それではどうぞ「Quinquennial Repor 2011～2015」をご覧ください．

2016 年6月　源 利成

雪割草（ゆきわりそう：輪島市門前町猿山岬）2015 年 4 月 4 日撮影（源）

【研究スタッフと在籍者】（1998 年～2015 年7月現在）

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教 授	源 利成	1998 年 4 月～2001 年 6 月　主任助教授 2001 年 7 月～現在　教授 2006 年 4 月－2009 年 3 月　分子標的がん医療研究開発センター長
准教授	川上和之	2006 年 4 月～2013 年 3 月（東京女子医科大学に転出）　*2006 年 3 月まで:医学系研究科心肺病態制御学 学内講師
助教	堂本貴寛	2013 年 11 月～テニュア
博士研究員	Abbas Shakoori	2005 年 4 月～2006 年 4 月 (米国 NIH に異動)
	Mingji Jin (金 明姫)	2008 年 4 月～2010 年 2 月
	廣瀬まゆみ	2010 年 4 月～2014 年 3 月
	堂本貴寛	2012 年 4 月～2013 年 11 月
	Ilya V. Pyko	2013 年 12 月～現在
	堂口裕士	2014 年 4 月～2014 年 11 月
	石井浩子	2015 年 6 月～現在
日本学術振興会	Andrei V. Ougolkov	2002 年 9 月～2004 年 4 月 (Mayo Clinc に異動)
特別研究員	Bin Zhang（張 濱）	2003 年 9 月～2005 年 4 月 (Minnesota 大に異動)
大学院医学系	平野晃一	1998 年 4 月～2001 年 3 月（課程修了）
研究科	Andrei V. Ougolkov	1998 年 4 月～2002 年 3 月（医学博士）【国費】
	Bin Zhang（張 濱）	1999 年 4 月～2003 年 6 月（医学博士）
	Abbas Shakoori	2003 年 4 月～2005 年 3 月（医学博士）【大使館推薦】
	辻端亜紀彦	2004 年 3 月～2005 年 3 月（退学）
	Zhiwei Yu (于 志偉)	2004 年 4 月～2006 年 3 月（除籍）【国費】
	宮下勝吉	2005 年 6 月～2008 年 3 月（医学博士）【脳神経外科学】
	Wei Mai（麦 威）	2006 年 4 月～2009 年 3 月（医学博士）
	斎藤健一郎	2007 年 11 月～2010 年 9 月（医学博士）【心肺病態制御学／心肺総合外科学】
	松之木愛香	2008 年 7 月～現在 【心肺病態制御学／心肺総合外科学】
	Liming Wang (王 利明)	2009 年 10 月～2010 年 10 月（退学）【国費】
	金子真美	2010 年 4 月～現在 【心肺総合外科学／先進総合外科学】

	氏 名	在籍期間, 備考
	富田泰斗（やすと）	2011 年 7 月～現在 【金沢医科大学一般･消化器外科学】
	北村祥貴（ひろたか）	2011 年 8 月～現在 【心肺総合外科学／先進総合外科学】
	下崎真吾	2011 年 11 月～現在 【整形外科学】
	阿部健作	2015 年 1 月～現在 【整形外科学】
	竹中 哲（さとし）	2015 年 5 月～現在 【消化器･腫瘍･再生外科学】
大学院修士課程	引地哲郎	2002 年 7 月～2003 年 3 月 （保健学科課程修了）
	玉野裕子	2002 年 4 月～2004 年 3 月 （保健学科課程修了）
	北野綾子	2008 年 4 月～2010 年 3 月 （薬学系課程修了）
	近野祐里（ゆうり）	2009 年 4 月～2011 年 3 月 （薬学系課程修了）
	伊藤有美	2012 年 4 月～2014 年 3 月 （医学修士課程修了）
	佐々木規雄	2014 年 4 月～現在 （保健学系修士課程）
	上原将大（まさひろ）	2015 年 4 月～現在 （医学系修士課程）
研究員	小竹優範（まさのり）	2009 年 12 月～現在 （厚生連高岡病院外科）
研究協力員	北川治恵	金沢工業大学 2001 年 5 月～2004 年 3 月
	Andrei V. Ougolkov	2002 年 4 月～2002 年 8 月
	旭井亮一（あさい）	株式会社 凸版印刷 2002 年 9 月～現在
	小田惠夫（よしお）	株式会社アルプ 病理研究所 2004 年 4 月～2009 年 4 月
	川島篤弘	独立行政法人国立病院機構金沢医療センター 研究検査科 2004 年 4 月～現在
	川上和之	金沢大学大学院医学系研究科心肺病態制御学／心肺総合外科 2005 年 4 月～2006 年 3 月
	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 2005 年 4 月～現在
	横井健二	金沢大学大学院医学系研究科心肺病態制御学／心肺総合外科；現 米国メソジスト病院研究所 2007 年 4 月～現在
	島崎猛夫（しまさき）	金沢医科大学総合医学研究所, 同消化器内科学 2007 年 4 月～現在

	氏　名	在籍期間, 備考
	東（ひがし） 朋美	金沢大学医学系研究科環境分子応答学／衛生学 2007 年 4 月～現在
	笠島（かさしま）里美	独立行政法人国立病院機構 金沢医療センター 研究検査科　2007 年 4 月～現在
	宮下勝吉（かつよし）	金沢大学大学院医学系研究科 脳機能制御学／脳神経外科学　2009 年 4 月～現在
	中島（なかじま）日出男	医療法人社団 愛友会 上尾中央総合病院 腫瘍内科　2012 年 6 月～現在
卒業研究生（保健学類）	樺木（かばき）陽子	2011 年 10 月～2012 年 8 月
	辰巳暁哉（あきや）	2011 年 10 月～2012 年 8 月
	佐々木則雄	2012 年 10 月～2013 年 8 月
	柴田莉穂（りほ）	2012 年 10 月～2013 年 8 月
	森脇美優（みゆう）	2013 年 10 月～2014 年 8 月
	米村圭祐	2013 年 10 月～2014 年 8 月
	藤井瑞希（みずき）	2014 年 10 月～2015 年 8 月
	丸山幸奈（ゆきな）	2014 年 10 月～2015 年 8 月
教務補佐員	Wei Mai（麦 威）	2005 年 4 月～2007 年 3 月（現 中国：ハルビン医科大学）
技術･技能補佐員	Andrei V. Ougolkov	2002 年 4 月～2002 年 8 月
	Bin Zhang（張 濱）	2003 年 5 月～2003 年 8 月（現：ミネソタ大学）
	Mingji Jin（金明姫）	2007 年 6 月～2008 年 3 月
	Wei Mai（麦 威）	2007 年 4 月～2009 年 3 月
研究支援推進員	金田（清水）敦子	1998 年 4 月～2003 年 5 月
	北村（横村）律子	1998 年 4 月～2007 年 3 月
	寺島昌代	2004 年 4 月～2007 年 3 月
	浅香敦子	2008 年 4 月～現在
	枡井亜希子	2009 年 11 月～2013 年 3 月（がん組織バンク）
	中みぎわ	2011 年 4 月～2012 年 9 月（がん組織バンク）
	阿部尚子	2013 年 4 月～現在（がん組織バンク）

【共同研究者】

研究室開設以来，研究指導，共同研究や研究協力などをいただいた学内外の方々のリストです．５０音順で，敬称と期間は略しました．研究協力員は含まれていません．

氏名	所属機関
石垣靖人	金沢医科大学総合医学研究所
石渡俊行	東京都長寿医療センター研究所
伊藤 透	金沢医科大学病院内視鏡科
江角浩安	国立がん研究センター研究所
大井章史	金沢大学大学院医学系研究科分子細胞病理学
大島正伸	金沢大学がん研究所腫瘍遺伝学
太田哲生	金沢大学大学院医学系研究科がん局所制御学／消化器外科学
大野博司	理化学研究所免疫アレルギー科学総合研究センター(RCAI)
小田 誠	金沢大学大学院医学系研究科心肺病態制御学／心肺総合外科
表 和彦	金沢医科大学消化器外科治療学
勝田省吾	金沢医科大学
河原 栄	金沢大学医学部保健学科
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小坂健夫	金沢医科大学一般・消化器外科学
京 哲	金沢大学大学院医学系研究科分子移植学／産科婦人科学
崔 吉道	金沢大学附属病院薬剤部
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澤田 武	金沢大学
須田貴司	金沢大学がん研究所免疫炎症制御学
清尾康志	東京工業大学
曽我朋義	慶應義塾大学先端生命科学研究所
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古田拓也	金沢大学医学系研究科脳機能制御学／脳神経外科学
磨伊正義（故）	金沢大学がん研究所腫瘍外科（当時）
松田陽子	東京都長寿医療センター病理診断科
三村美津子	神戸大学大学院消化器内科
宮下知治	金沢大学消化器・腫瘍・再生外科学
宮本謙一	金沢大学附属病院薬剤部
三輪真嗣	金沢大学医学系研究科機能再建学／整形外科学
元雄良治	金沢医科大学腫瘍内科学
山下 要	金沢大学がん研究所腫瘍外科
山本聡子	金沢医科大学総合医学研究所
山本憲男	金沢大学医学系研究科機能再建学／整形外科学
吉田 優	神戸大学大学院消化器内科
吉村健太郎	山梨大学解剖学講座
渡邊 剛	金沢大学大学院医学系研究科心肺病態制御学／心肺総合外科
渡辺弥壽夫	金沢工業大学情報工学科
Daniel D. Billadeau	米国メイヨクリニック発生腫瘍研究分野
Peter V. Danenberg	米国南カリフォルニア大学生化学
Serge Y. Fuchs	米国ペンシルヴェニア大学生物学
Andrew S. Giraud	豪州オーストラリア王立小児病院
Francesco Graziano	豪州ウルビーノ病院臨床腫瘍科
Barry Iacopetta	豪州西オーストラリア大学腫瘍学
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
Ze’ev Ronai	米国バーナム研究所
Vladimir S. Spiegelman	米国ウィスコンシン大学皮膚科学
Timothy C. Wang	米国コロンビア大学イルヴィングがん研究センター
Richard Wong	金沢大学大学院自然システム系

【研究費・外部資金】

2011 年以降の受入れの概要

区 分	種 類		件 数	研究経費
代 表	科学研究費		9	９３，８１０千円
	その他		1	５００千円
		小 計	10	９４，３１０千円
分 担 *	科学研究費		6	７２，９２０千円
	その他		25	１９，７００千円
		小 計	31	９２，６２０千円
代表＋分担		中 計	41	１８６，９３０千円
	奨学寄附金		14	７，２７０千円
		総 計	55	１９４，２００千円

* 連携を含む

2011 年以降の受入れ状況

[1] 2015－2017 年度 科学研究費補助金（基盤研究B）：課題番号 15H04928
源 利成（代表），宮下知治（分担），太田哲生，曽我朋義，清尾康志（連携）
課題：GSK3β 経路を標的とする大腸がんの病態解明と治療法開発の基盤形成
研究経費：14,240,000 円

[2] 2015－2016 年度 科学研究費補助金（挑戦的萌芽）：課題番号 15K15493
源 利成（代表），Wong Richard，石垣靖人，太田哲生，宮下知治（連携）
課題：大腸がんにおける β-カテニン核移送に作用する核膜孔複合体因子の探索と機能解析
研究経費：3,510,000 円

[3] 2015－2018 年度 科学研究費補助金（基盤研究A）：課題番号 15H04928
大島正伸（代表），ほか（分担），源 利成（連携）
課題：大腸がん自然転移・再発モデルの開発による悪性化進展機構の研究
研究経費：41,000,000 円

[4] 2015－2017 年度 科学研究費補助金（基盤研究C）：課題番号 15K09051
島崎猛夫（代表），石垣靖人（分担），源 利成（連携）
課題：抗がん剤による膵がん細胞の浸潤形質獲得の分子機構の解明とがん治療への応用
研究経費：4,810,000 円

[5] 2015 年度 金沢大学がん進展制御研究所共同研究（一般）
小坂健夫（代表），源 利成，ほか（分担）
課題：大腸がんにおける β-カテニン/Tcf の転写標的 CRD-BP の分子病理学的特性と病態の解明
研究経費：700,000 円

[6] 2015 年度 金沢大学がん進展制御研究所共同研究（一般）
島崎猛夫（代表），源 利成，ほか（分担）
課題：GSK3β を標的とする新規核酸医薬の機能解析
研究経費：500,000 円

[7] 2015 年度 金沢大学がん進展制御研究所共同研究（一般）
松田陽子（代表），源 利成，ほか（分担）

課題：膵幹細胞の加齢と発癌機序の解明
研究経費：500,000 円

[8] 2015 年度 金沢大学がん進展制御研究所共同研究（一般）
吉村健太郎（代表），源 利成，ほか（分担）
課題：大気圧イオン化法-質量分析を用いた大腸がん診断システムの確立および病態解明
研究経費：500,000 円

[9] 2015 年度 金沢大学がん進展制御研究所共同研究（一般）
山本憲男（代表），源 利成，ほか（分担）
課題：軟部肉腫の GSK3β を標的とする新規治療法の開発と分子メカニズム
研究経費：200,000 円

[10] 2015 年度 金沢大学がん進展制御研究所共同研究（一般）
Richard Wong（代表），源 利成，ほか（分担）
課題：大腸癌進行に関与する核膜孔複合体蛋白の同定および機能解析
研究経費：200,000 円

[11] 2015 年度 金沢大学がん進展制御研究所共同研究（一般）
宮下知治（代表），源 利成，ほか（分担）
課題：GSK3β 阻害による食道発癌の予防とその機序の解明
研究経費：200,000 円

[12] 2015 年度 金沢大学がん進展制御研究所共同研究（一般）
古田拓也（代表），源 利成，ほか（分担）
課題：既存薬転用による膠芽腫に対する GSK3β 標的療法の開発
研究経費：200,000 円

[13] 2015 年度 金沢大学がん進展制御研究所共同研究（一般）
Xi Cheng（代表），源 利成，ほか（分担）
課題：The relationship between GSK3β expression and chemoresistance in ovarian clear cell carcinoma
研究経費：200,000 円

[14] 2014－2016 年度 科学研究費補助金 (基盤研究B)：課題番号 26293322
中田光俊（代表），源 利成（連携）
課題：ドラッグリポジショニングによる悪性グリオーマに対する新規化学療法の基盤構築
研究経費：12,160,000 円

[15] 2014－2015 年度 科学研究費補助金（研究活動スタート支援）：課題番号 26893096
ピコ イリア（Ilya V. Pyko：代表）
課題：Development of combined cellular and molecular target-directed therapies for glioblastoma
研究経費：2,300,000 円

[16] 2014 年度 金沢大学がん進展制御研究所共同研究（一般）
小坂健夫（代表），源 利成，ほか（分担）
課題：大腸がんにおける Wnt 経路標的分子 CRD-BP の分子病理学的特性と病態の解明
研究経費：700,000 円

[17] 2014 年度 金沢大学がん進展制御研究所共同研究（一般）
島崎猛夫 (代表), 源 利成, ほか（分担）
課題：膵がんエキソソームと GSK3β の交絡的病理作用の解明とがん治療薬スクリーニングへの応用
研究経費：500,000 円

[18] 2014 年度 金沢大学がん進展制御研究所共同研究（一般）
松田陽子 (代表), 源 利成, ほか（分担）
課題：Nestin のリン酸化制御による, 膵癌分子標的治療の開発
研究経費：500,000 円

[19] 2014 年度 金沢大学がん進展制御研究所共同研究（一般）
吉村健太郎 (代表), 源 利成, ほか（分担）
課題：質量分析型迅速がん診断システムを用いた大腸がんの新規診断法の開発と発がんメカニズムの解明
研究経費：500,000 円

[20] 2014 年度 金沢大学がん進展制御研究所共同研究（一般）
山本憲男 (代表), 源 利成, ほか（分担）
課題：骨肉腫の GSK3β を標的とする新規治療法の開発と分子メカニズム
研究経費：200,000 円

[21] 2014 年度 金沢大学がん進展制御研究所共同研究（一般）
宮下知治 (代表), 源 利成, ほか（分担）
課題：GSK3β 阻害による食道発癌の予防とその機序の解明
研究経費：200,000 円

[22] 2013－2014 年度 科学研究費補助金（挑戦的萌芽）：課題番号 25670572
源 利成（代表), 杉山直幸（連携）
課題：蛋白質リン酸化特性の網羅的解析による大腸がんの病態解明と制御への応用
研究経費：3,770,000 円

[23] 2013－2015 年度 科学研究費補助金（若手研究B）：課題番号 25860233
堂本貴寛（代表）
課題：エネルギー代謝特性に基づく消化器がん病態解明と制御への応用
研究経費：3,770,000 円

[24] 2013 年度 金沢大学がん進展制御研究所共同研究（特定）
島崎猛夫（代表), 源 利成, ほか（分担）
課題：GSK3β 阻害による膵がん治療法の分子基盤の解明と臨床試験への応用
研究経費：1,250,000 円

[25] 2013 年度 金沢大学がん進展制御研究所共同研究（一般）
小坂健夫（代表), 源 利成, ほか（分担）
課題：新規の Wnt 経路分子を中心とする大腸がんの分子病理学的特性と病態の解明
研究経費：700,000 円

[26] 2013 年度 金沢大学がん進展制御研究所共同研究（一般）
松田陽子（代表), 源 利成, ほか（分担）
課題：Nestin の制御による膵癌幹細胞および腫瘍新生血管に対する新たな治療戦略

研究経費：500,000 円

[27] 2013 年度 金沢大学がん進展制御研究所共同研究（一般）
太田哲生（代表），源 利成，ほか（分担）
課題：GSK3β 阻害による食道発癌の予防とその機序の解明
研究経費：200,000 円

[28] 2012－2013 年度 金沢大学重点研究経費（拠点形成型）
松本邦夫（代表），源 利成，ほか（分担）
課題：アカデミアがん創薬拠点形成のための人材と知の集約・循環プログラム
研究経費：4,000,000 円

[29] 2012－2013 年度 金沢大学重点研究経費（拠点形成型）
Richard Wong（代表），源 利成，ほか（分担）
課題：細胞核輸送分子機構の機能的および構造的動態の解析
研究経費：4,000,000 円

[30] 2012 年度 金沢大学がん進展制御研究所共同研究（一般）
元雄良治（代表），源 利成，ほか（分担）
課題：GSK3β 阻害による新規膵がん化学療法の開発と臨床試験
研究経費：500,000 円

[31] 2012 年度 金沢大学がん進展制御研究所共同研究（一般）
小坂健夫（代表），源 利成，川上和之，ほか（分担）
課題：大腸がんの分子病理学的特性の解析と診断，治療のための分子指標の解明
研究経費：750,000 円

[32] 2011－2013 年度 科学研究費補助金（基盤研究 B）：課題番号 23390321
川上和之（代表），源 利成（分担），曽我朋義（連携）
課題：ゲノムの低メチル化とレトロポゾンの活性化を特徴とする大腸がんの診断・治療開発
研究経費：16,340,000 円

[33] 2011－2013 年度 科学研究費補助金（基盤研究 C）：課題番号 23591955
廣瀬まゆみ（代表），源 利成，川上和之（分担）
課題：GSK3β 阻害による消化器がん治療法の開発と分子機構の解明
研究経費：4,550,000 円

[34] 2011－2012 年度 科学研究費補助金（挑戦的萌芽研究）：課題番号 23659643
源 利成（代表），川上和之（分担），曽我朋義（連携）
課題：がん特異的ネルギー代謝を標的とする消化器がん治療法の開発
研究経費：3,340,000 円

[35] 2011－2013 年度 科学研究費補助金（基盤研究 C）：課題番号
中田光俊（代表），源 利成（連携）
課題：悪性グリオーマの浸潤シグナルを狙った分子標的療法の確立
研究経費：4,550,000 円

[36] 2011－2013 年度 科学研究費補助金（基盤研究 C）：課題番号 23590898
中島日出夫（代表），源 利成，ほか（分担）
課題：がん温熱療法の新規分子マーカー候補 FAM107 ファミリー蛋白質の発現・機能解析

研究経費: 5,200,000 円

[37] 2011－2013 年度 科学研究費補助金（基盤研究 C）: 課題番号 23591016
島崎猛夫（代表）, 源 利成, ほか（分担）
課題: 化学療法により誘発される EMT 誘導因子の同定とその制御による膵がん治療法の開発
研究経費: 5,200,000 円

[38] 2011 年度 金沢大学がん進展制御研究所共同研究（一般） 整理番号: 12
元雄良治（代表）, 源 利成, ほか（分担）
課題: GSK3β 阻害による新規膵がん化学療法の開発と臨床試験
研究経費: 1,000,000 円

[39] 2011 年度 金沢大学がん進展制御研究所共同研究（一般） 整理番号: 12
小坂健夫（代表）, 源 利成, 川上和之, ほか（分担）
課題: 大腸がん個別化医療のためのバイオマーカー探索
研究経費: 1,000,000 円

[40] 2011 年度 財団法人金沢総合技術研究センター研究助成金
源 利成, 川上和之, ほか
課題: がん細胞の代謝特性を標的とするがん治療法の開発
研究経費: 500,000 円

[41] 2010－2012 年度 科学研究費補助金（基盤研究 A）: 課題番号 20890086
源 利成（代表）, 川上和之, 太田哲生（分担）, 大野博司（連携）
課題: 基幹的細胞調節経路の異常に起因する消化器がんの病態解明とがん制御への応用
研究経費: 41,990,000 円

【知的財産権の出願】

日本国特許出願　2013 年 4 月 25 日
出願番号：特願 2013-093072
発明者：島崎猛夫，中田光俊，源 利成
出願者：学校法人金沢医科大学，国立大学法人金沢大学
名称：膵臓癌治療剤

【5年間（2011 年～2015 年）のあゆみ】

あゆみ 2011 年

2011 年3月 11 日に発生した東日本大震災は国家を揺るがす災害となりました．これに比べれば小規模ではありましたが，2007 年3月 26 日の能登半島地震で郷里が被災した自身（研究のあゆみと業績 2007 年を参照）は日々，被災された皆さまのご冥福を祈り，お見舞いを申し上げずにはいられません．おかげさまで昨夏，能登半島の震災は復旧宣言がなされ，郷里では記念行事が執り行なわれました．

2011 年7月には，私たちの研究分野の開設からちょうど10年を迎えました．当初は金沢大学がん研究所附属病院（金沢市米泉キャンパス）の一隅で専用スペースはなく，専任スタッフは私一人いう状況でした．とても心細いなかで，10年ももつとは想像もしていませんでした．少しずつではありますが思いを共有する仲間が増え，研究所や大学のご支援により今まで継続できたものと，ありがたく思っています．7月2日には，これまでに導いていただいた先生方や仲間の皆さんと一緒に，金沢市しいのき迎賓館で記念発表会を企画しました．そして，例年7月の親睦会を少し大きくして“七夕の会スペシャル”を開き，皆でお祝いしました．年齢の上下や立場に関係なく，「仲間はいいいなぁ」と実感したひとときでした．

前後しますが，11年目の出発となる今春，たくさんの科学研究費をいただきました．研究代表，分担，連携を合わせて新規に6課題が採択されました．私たちのところに研究費がどれくらい入るかということではなく，これでまた一緒に研究が続けられるという嬉しさを仲間の皆さんと分かち合っています．自身はいろいろありますが，もうしばらく今の仕事を続けてみるつもりです．

もう一つ，皆さまにお知らせしたいことがあります．11 月 26 日に佐賀で開催された第 22 回日本消化器癌発生学会の理事会と評議委員会で，私たちの研究分野が第 24 回日本消化器癌発生学会総会を担当することが決まりました．身に余る責務です．弱小な私たちに総会開催を託してくださった前原喜彦理事長（九州大学消化器総合外科学 教授）の心意気に応えられるようにつとめます．開催は 2013 年9月5－6日の予定です．金沢で磨伊正義先生（故人）が第4回総会をご担当されてから 20 年目にあたります．私と磨伊先生は歳がちょうど 20 違うので，これもご縁かなと思います．

私たちの取組みが少し評価されるようになってきたのか，2011 年は外科系大学院博士課程や卒業研究生などが来てくれるようになりました．ここにお知らせしたいくつかのことは私たちにとってはとても励みとなります．10年前を振り返ることなく，いつも前をみて，がんの診療や研究に繋がるよう，地道に仕事をすすめたいと思います．10年を期にやめようと思っていた紙媒体の本報告書をもうしばらく続けることにしました．電子媒体では伝わらない，私たちの思いとあゆみをご覧ください．また，皆様の忌憚のないご意見やご批評とともに，ご助言をいただければ幸いです．これからもご指導とご支援をよろしくお願いします．

2012 年1月

源　利成

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2011 年のできごと

2011 年 1 月 19 日　・源 利成が「がんにおける質の高い看護師育成研修会」で講演（本学附属病院） 課題：発がん学，がん医科学とがん医療―消化器がんを中心に―

2011 年 1 月 23 日　・源 利成が北陸がんプロ市民公開講座（金沢市赤羽ホール）で講演
課題：難治がんに対する新たながん治療薬開発への道
1 月 24 日と 2 月 27 日の北國新聞日刊に掲載

2011 年 3 月 11 日　・東日本大震災発生

2011 年 3 月 31 日　・近野祐里君が薬学系修士課程を修了
課題：神経膠芽腫におよぼす GSK3β の病的作用の解明と臨床応用の可能性

2011 年 5 月 14 日　・太田孝仁先生（金沢大がん研外科同門）：久藤病院院長就任祝賀会

2011 年 7 月 01 日　・金沢医科大学大学院消化器外科学：富田泰斗君が研究に参画（研究指導）
課題：大腸がんの生物学的特性の解明と診断，治療への応用

2011 年 7 月 02 日　・腫瘍制御研究分野開設 10 周年記念 研究発表会：しいの木迎賓館（金沢）
七夕の会スペシャル：ホテル日航金沢**【写真】**

2011 年 7 月 27 日　・共同研究員：小竹優範君（石川県立中央病院消化器外科）を代表とする研究チームが北國がん基金研究活動助成を受賞
受賞者：小竹優範，伴登宏行（石川県立中央病院消化器外科）
西村元一（金沢赤十字病院外科），川上和之，源 利成
課題：大腸がん組織検体資源化によるがん生物学的特性の大規模解析とがん医療への展開
報道：7 月 28 日の北國新聞日刊に掲載

2011 年 8 月 19 日　・金沢医科大学病理学教室：上田善道教授 就任祝賀会　ホテル日航金沢

2011 年 10 月 1 日　・本学保健学類の樺木陽子さんと辰巳暁哉君が卒業研修を開始

2011 年 11 月 1 日　・金沢大学大学院医学系研究科 整形外科学：下崎真吾君が研究に参画
課題：GSK3β を標的とする骨・軟部悪性腫瘍の病態解明と治療法の開発

2011 年 11 月 25 日　・第 22 回日本消化器癌発生学会総会の理事会と評議員会において，源 利成が同学会の第 24 回総会長に選出された．同会は 2013 年 9 月 5-6 日に石川県立音楽堂で開催予定

2011 年 11 月 26 日　・金沢大学がん研究所外科同門会総会：ホテル日航金沢

2011 年 12 月 16 日　・石川県立金沢泉丘高等学校 SSH (Super Science High School)プログラムで講義
三輪高喜（金沢医科大学耳鼻咽喉科頭頸部外科学）：鼻は何故あるのか？
源 利成：がんの科学と医療

2011 年 12 月 16 日　・腫瘍制御・金沢医科大学腫瘍内科合同忘年会：とと屋 金沢駅前店

七夕の会 2015（10 周年会）
当時の研究メンバー
2011 年 7 月 2 日
ホテル日航金沢

あゆみ 2012 年

昨年，遅れに遅れた研究室開設 10 周年記念誌「金沢大学がん研究所腫瘍制御研究分野：研究のあゆみと業績 2001－2010 年」を“Downstream, Present & Upstream”と称して発刊しました．厚くもなく薄くもなく微妙なできあがりでした．それでも，それはとりもなおさず当研究室が 10 年経過した証であり，通過点でもあります．10 年を区切りにやめようと思っていた紙媒体の本報告書を昨年も発行し，皆さまに紹介しました．電子媒体ではうまくあらわせない私たちの生身の思いとあゆみをお伝えしたく，今年も編集しました．

私の敬愛するお一人の山本悦秀先生は本学をご退任されたあと，東京で歯科クリニックを開院され，地域医療に尽くされています．いつものように気楽な気持ちで，本誌と 10 周年記念誌を差し上げましたところ，つぎのようなご感想と励ましのおことばを頂戴しました．以下は原文：

源 教授： 拝復、貴研究分野の 10 周年記念誌、他を御恵贈いただき誠にありがとうございました。質量共に立派な業績の敬意を表します。よく頑張られたと思います。
おそらく殆どおひとりで、この記念誌を作られたのではないでしょうか。実は不肖・私の退職記念誌も依頼原稿以外は全て自分でやりました。・・・（中略）・・・でしたが、何か突き動かすものがありました。私の恩師は「書き記すことの大切さ」を教室員に説き続けてこられました。すなわち、私の退職記念誌のあとがきにも書きましたが、業績集については、「教室の運営費が国の税金で賄われている以上、教授が在任中に何をしたかを記録として残しておくことは国民に対する義務である」といった類のことを我々弟子達は聞いて育ってきました。従って、先生のこういった研究者としての姿勢には大変、共感するところがあります。先生のさらなる御活躍を東京の地より祈念しております。ちなみに私の・・・（中略）・・・経過しています。（中略）・・・ることに感謝の日々です。【2012 年9月 11 日】

たしかに毎年，本誌の編集には余分のエネルギーを費やしてしまいます．それでも，小さいながらも研究室を担当する者の責務のようにも感じます．このようなお便りを頂いたあとですので，今回はとくに，皆さまが「研究のあゆみと業績 2012 年」をどのように受けとめてくださるのかなぁ・・と想像しながら，お届けすることにします．皆さまの忌憚のないご意見，ご批評とご助言をいただければ幸いです．

今年の 11 月に当研究所外部評価が実施されました**【附記1】**．前回（2008 年 11 月 27 日；研究の歩みと業績 2008 参照）から早や5年が経過し，この間にどれほどのことができたかは自分でも評価は困難です．それでも，上西紀夫（かみにし みちお）先生と勝田省吾先生のお二方はとても暖かくご評価くださいました．ありがたいことです．研究の質量は軽視できませんが，やはり大切なのは師，先輩，同僚や仲間であることをあらためて感じています．一昨年の春に発生した東日本大震災といまも続く余震は国家を揺るがす災害となったものの，災い転じて「ひとの絆」の大切さを私たちに問い続けています．これとは次元が違いますが，研究室でも皆が楽しく過ごせるよう心がけたいと思います．

来年の初秋に第 24 回日本消化器癌発生学会総会を担当します．身に余る責務です．準備は大変ですが，おかげさまで助けてくれる仲間がたくさんいます．感謝の気持ちで一杯です．日常の診療や研究のアクティビティを落とすことなく努めたいと思います．これからもご指導とご支援をお願いします．

2012 年12 月

源 利成

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2012 年のできごと

2012 年 1 月 18 日 ・源 利成：富山大学和漢医薬学総合研究所ジョイントセミナーで講演
課題：がんを含む慢性進行性疾患の創薬標的 GSK3β

・源 利成：「がんにおける質の高い看護師育成研修会」で講演（本学附属病院） 課題：がん医科学とがん医療－消化器がんを中心に－

2012 年 1 月 21 日 ・源 利成：第 5 回金沢脳腫瘍セミナー（ホテル日航金沢）で講演
課題：大腸がん研究から開発した新しいがん治療法
－分子基盤と膠芽腫治療への橋渡し－

2012 年 1 月 27 日 ・源 利成：金沢医科大学大学院第 29 回医学研究セミナーで講演
課題：がん細胞の代謝特性と治療

2012 年 4 月 01 日 ・堂本貴寛君が博士研究員に採用され，研究開始

・伊藤有美さんが大学院医学系研究科修士課程に入学

2012 年 6 月 10 日 ・宮下勝吉君（研究協力員；本学脳神経外科学）がご結婚：横浜元町で挙式

2012 年 6 月 16 日 ・源 利成：金沢大学公開講座：がん研究の最前線で講演
課題：難治がんの新しい治療法－膵がんと脳腫瘍への取り組み－

2012 年 7 月 14 日 ・金沢医科大学腫瘍内科学との合同親睦会（七夕の会）：べに屋（金沢駅前）

2012 年 8 月 02 日 ・金沢大学保健学類卒業研究発表会：樺木陽子さん，辰巳暁哉君
課題：異なる検出法による大腸癌の K-*ras* と B-*raf* 遺伝子変異検出の比較解析

2012 年 9 月 21 日 ・2011 年度北国がん基金助成の成果が北國新聞日刊に掲載
受賞者：小竹優範，伴登宏行（石川県立中央病院消化器外科）
西村元一（金沢赤十字病院外科），川上和之，源 利成
北國がん基金 27 日に助成金贈呈式．解明，治療，確かな歩み．昨年の助成対象者の成果：組織のデータを蓄積

2012 年 9 月 30 日 ・中みぎわさんが退職（技能補佐員：ヒトがん組織バンク担当）

2012 年 10 月 01 日 ・本学保健学類の佐々木則雄君と柴田莉穂さんが卒業研修を開始

2012 年 10 月 10 日 ・源 利成が日本消化器病学会評議員に内定

2012 年 10 月 16 日 ・佐々木琢磨先生（当研究所名誉教授）ご逝去

2012 年 10 月 18 日 ・源 利成：「がんにおける質の高い看護師育成研修会」で講演（本学附属病院） 課題：がん医科学とがん医療－消化器がんを中心に－

2012 年 11 月 01 日 ・金沢大学がん進展制御研究所外部評価委員会が開催
まえがき 当研究分野は上西紀夫氏（かみにし みちお；日本消化器内視鏡学会理事長，公立昭和病院 院長）と勝田省吾氏（金沢医科大学 学長）の評価を受けた．

2012 年 11 月 22 日 ・源 利成：STOP 学術講演会（福井パレスホテル）で講演
課題：大腸がん研究から発見したがん治療標的－金沢発膠芽腫治療法への展開－

2012 年 11 月 23 日 ・金沢大学がん研究所外科同門会総会・懇親会：ホテル日航金沢

2012 年 12 月 21 日 ・腫瘍制御・金沢医科大学腫瘍内科合同忘年会：もんぜん（金沢駅前）

七夕の会 2012 年 7 月 14 日
べに屋 金沢駅前

あゆみ 2013 年

2013 年を振り返ってみると，その多くの期間を学会の開催準備に追われていたように感じます．また，人との別れや出会い，そして対人関係の難しさも考えさせられる 1 年でした．

夏休み明けの９月５日，６日に第 24 回日本消化器癌発生学会総会を担当しました【附記２，４】．1992 年当時，私が金沢大学がん研究所外科（磨伊正義教授）から国立がんセンター研究所生化学部（江角浩安部長）に出向した年の９月に磨伊先生が第４回研究会を開催されてから 20 年後の節目にあたります．このことをご存じだったかどうかは伺っていませんが，前原喜彦理事長（九州大学消化器・総合外科学：教授）のご配慮をありがたく思っています．それまでは地方会でさえまともに担当したことのない私に，はたしてこの総会をうまくできるかは不安でした．それでも，前日の荒天を撞いて全国から多くの皆さまがご参加くださり，温かいご評価をいただきました．一般公開プログラムも好評でした【附記３】．亡き磨伊先生は心の支えであり，江角先生の応援も心強く思いました．学会事務局幹事の清水伸行先生（前 東京大学消化管外科学）は学会 News Letter (2013, No.1)で次のように述べられています・・・第４回総会を磨伊先生，第 14 回総会を三輪晃一先生（金沢大学がん局所制御学）が主催してくださいましたので，金沢での本会開催は３回目になります．これまで同一都市での開催は東京の３回が最高で（中略），これに肩を並べました・・・．私たち金沢の消化器がん研究のレベルも肩を並べられるようになることを願っています．この総会を支えてくれた教室関連のスタッフと，本学や金沢医科大学で消化器がん研究を志している仲間に改めて感謝します．

なお，総会記録【附記2-4】は日本消化器癌発生学会本部事務局と第 24 回総会事務局の了解を得て転載しました．

このような慌しいなか，春には川上准教授が退職し，年末に助教を補充していただきました．一部の研究員や研究生とは思いがけずも齟齬が生じることもありました．必ずしも性善説ではうまくいかない人間関係があることがわかり，よい経験になりました．晩秋には悲しい別れがありました．本学の脳神経外科学教授：濵田潤一郎先生が急逝されたことです．私たちのこれまでの大腸がん研究をもとにした膠芽腫の新しい治療法の開発と，drug repositioning（既存薬転用）による膠芽腫治療の臨床研究（UMIN: 000005111）を先導してくださっている矢先のことでした．これだけではなく，医師，研究者ひいては人間としての生き方についても薫陶を受けました．慎んでご冥福を祈ります．先の総会開催やこれらのできごとの所為にはできませんが，研究活動が低下したことを自省し，前向きに受け止めています．

幸いなことに，この年には本学消化器外科学，整形外科学や脳神経外科学などから新たに共同研究を提案いただき，また少しずつ研究に対してモチベーションが回復してきたように感じています．私たちの研究はとても小さな課題ですが，2012 年の年報をお送りしたところ，九州大学消化器・総合外科学の調 憲先生から次のようなお便りを頂戴しました・・・九大二外科の先々代の教授でいらっしゃいます井口 潔先生のお言葉に「小さくとも，ささやかでも，珠玉のごときものにつくりあげたい．」という文章があります．私も大好きなのですが，先生の GSK3β のお仕事を拝見し，思い出しました・・・というものです．とても暖かいご感想で，元気づけられました．皆さまにはこれからもご指導とご支援をお願いします．

2013 年12 月

源　利成

2013 年のできごと

2013 年 1 月 11 日　・源 利成：石川県立金沢泉丘高等学校ｽｰﾊﾟｰｻｲｴﾝｽﾊｲｽｸｰﾙで模擬講義
課題：研究の質と評価：独創性と国際性　－みんなで考えてみよう－
会場：金沢医科大学医学部

2013 年 1 月 18 日　・源 利成：日本癌学会主催：がんと代謝シンポジウム 2013 で講演
課題：がんの代謝と治療標的 GSK3β の接点

2013 年 3 月 21 日　・第 99 回日本消化器病学会総会（鹿児島市）で研究協力員：島崎猛夫君が第 26 回日本消化器病学会奨励賞を受賞
受賞課題：Shimasaki T, Minamoto T, et al. Glycogen synthase kinase 3β inhibition sensitizes pancreatic cancer cells to gemcitabine. *J Gastroenterol* 47(3): 321-33, 2012.**【研究のあゆみと業績 2012 に転載】**

2013 年 3 月 31 日　・川上和之 准教授：辞職　・枡井亜希子さん（研究支援推進員）が退職

2013 年 4 月 01 日　・阿部尚子さんが研究支援推進員に採用（組織バンク担当）

2013 年 7 月 01 日　・金沢大学保健学類卒業研究発表会：柴田莉穂さん，佐々木規雄君
課題：大腸がんの血清 p53 抗体値と腫瘍における p53 発現や臨床病理学的因子の比較解析

2013 年 7 月 12 日　・金沢医科大学腫瘍内科学との合同親睦会（七夕の会）：水魚（金沢駅前）

2013 年 9 月 04 日　・日本消化器癌発生学会委員会（石川県立音楽堂 楽屋）
同役員懇談会（ホテル日航 金沢）

2013 年 9 月 05 日 ～ 9 月 06 日 **【附記２，４】**　・第 24 回日本消化器癌発生学会総会を開催
会長：源　利成
会場：石川県立音楽堂 邦楽ホール，交流ホール

2013 年 9 月 05 日 **【附記３】**　・日本消化器癌発生学会主催：一般公開プログラムを第 24 回総会と併催
時局講演：福島原発事故と県民の健康管理
講　師：神谷研二先生　広島大学 副学長（復興支援・被ばく医療担当）
広島大学原爆放射斜線医科学研究所 所長
福島県立医科大学 副学長（非常勤）
育成講演：ありがとう　高校野球の一期一会に感謝します
講　師：山下智茂先生　学校法人 稲置学園　　理事
星稜高等学校野球部　名誉監督

2013 年 10 月 01 日　・本学保健学類の森脇美優さんと米村圭祐君が卒業研修を開始

2013 年 10 月 07 日　・源 利成が 2013 年度石川県看護師資質向上研修会【がん看護】で講演
会場：金沢大学附属病院宝ホール
課題：がん医科学とがん医療　－消化器がんを中心に－

2013 年 10 月 28 日　・濵田潤一郎先生（金沢大学脳神経外科学 教授）ご逝去

2013 年 11 月 14 日 ～11 月 15 日　・源 利成が第 7 回国際消化器発癌会議で講演
課題：Targeting GSK3β in gastrointestinal and refractory cancer
会場：University of Pennsylvania, Philadelphia, PA
・源 利成が国際消化器発癌会議 理事に承認された

2013 年 11 月 23 日　・金沢大学がん研究所外科同門会総会・懇親会：ホテル日航金沢

2013 年 12 月 01 日　・堂本貴寛が助教（テニュアトラック）に採用
・Ilya V. Pyko 君が博士研究員に採用

2013 年 12 月 12 日　・源 利成が順天堂大学腫瘍病理学特別セミナーで講演
課題：大腸がん研究から同定した治療標的－難治がん制御への応用－

2013 年 12 月 12 日　・源 利成が順天堂大学基礎研究医養成プログラム M1「医学研究入門Ⅱ」セミナーで講演
課題：研究力と臨床力　－研究と臨床は表裏一体－

2013 年 12 月 21 日　・腫瘍制御・金沢医科大学腫瘍内科合同忘年会：くろ屋（ 金沢駅前）

あゆみ 2014 年

とても慌ただしく過ごした昨年とはうって変わり，年末まで（下記）はわりと落ちついた１年になったように感じます．一方，研究が格段に進んだわけでもなく，昨年までの惰性できているように思われる年でした．昨秋に全国的な学術集会（第 24 回日本消化器癌発生学会総会）を担当したことはとても有意義で，今までにない経験となりました．確かに，開催準備のため日常業務や研究の活力が停滞したことは否めません．それでも，開催のプロセスはそれに代えがたい財産となり，モチベーションを高めてくれました．個人的にはもともと付き合いの狭い私の交流範囲を少し広げてくれたみたいです．

教室自体はこのようにメリハリのない年になりそうなところ，私たちの研究仲間の躍進が私たちを活気づけてくれました．まず，2014 年４月に共同研究者のお一人で，石垣靖人（やすひと）先生が金沢医科大学総合医学研究所生命科学研究領域の教授にご就任されました．石垣先生はいまも，私たちの基礎研究をご指導，支援してくださっています．晩秋には，同じく大切な研究仲間の中田光俊先生が本学脳神経外科学の教授に内定したとの朗報がもたらされました．中田先生は私たちの主要な研究課題について膠芽腫を中心に病態生物学研究を推進してくださり，その成果を応用して再発膠芽腫治療の医師主導型臨床研究（UMIN000005111）を実施されているところです．さらに，両先生のお祝いをかねた研究仲間（七夕の会）の忘年会の２日前になって，私たちに懇意にしてくださっている塚（づか） 正彦先生が本学法・社会医学の教授に医学系で内定したとの連絡が内々に伝えられました．塚先生は私が大学院で在籍した本学第一病理学（現：分子細胞病理学）教室の同門であり，ご出身の輪島市は私の郷里：門前町と近隣ということもあり，自分のことのように嬉しく思いました．石垣先生，中田先生，塚先生の前途をお祝いできることになった忘年会はささやかながらとても華やかで楽しい会になったことはいうまでもありません．ここまでお読みになられた皆さまには是非，第７頁の記念撮影をご覧ください．

塚先生の教授候補の内々定の翌々日，私の大学院在籍時の恩師：中西功夫先生（本学名誉教授）から思いがけずもつぎのような電子メールをいただきました．“源 先生： このたびは、脳外科で中田先生、法医で塚先生とたてつづけに、先生の研究チームの人材が教授に押されており、心からお祝い申しあげます。−中略−。では、よろしく。中西功夫拝”．簡略なお便りでしたが，その中身は静かに私たちの研究グループを評価してくださるものであり，私自身にはとても暖かいご感想で，元気をいただきました．そして，単に研究成果や名声にとらわれることなく，かけがえのない仲間をこれからも大切にしていこうという気持ちを新たにしてくださいました．皆さまにはこれからもご指導とご支援をお願いします．

2014 年12 月年末

源 利成

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2014 年のできごと

2014 年 1 月 11 日　・源　利成: 石川県立金沢泉丘高等学校ｽｰﾊﾟｰｻｲｴﾝｽﾊｲｽｸｰﾙで模擬講義
課題: がんのエネルギー代謝　ーがん細胞は甘いものが大好きー
会場: 金沢大学医学類 医学図書館十全記念スタジオ

2014 年 3 月 21 日　・伊藤　透先生（金沢大学がん研究所外科同門）
金沢医科大学消化器内視鏡学 教授就任祝賀会　ホテル日航金沢

2014 年 3 月 31 日　・廣瀬まゆみさん: 退職

2014 年 4 月　・石垣靖人先生が金沢医科大学総合医学研究所 生命科学研究領域 教授に就任
・佐々木規雄君（保健学系修士課程）が研究を開始

2014 年 6 月 28 日　・源　利成: 金沢大学公開講座「がん研究の最前線」で講演
金沢大学サテライトプラザ西町
課題: 大腸がん研究からみつけた新しいがん治療法

2014 年 7 月 11 日　・金沢医科大学腫瘍内科学との合同親睦会（七夕の会）
金沢はこまち: 花より魚（金沢武蔵辻）

2014 年 7 月 27 日　・磨伊正義先生: 七回忌法要
金沢ニューグランドホテル

2014 年 7 月 31 日　・金沢大学保健学類 卒業研究発表会: 森脇美優（みゆう）さん, 米村圭祐君
課題: 大腸がんにおける GSK3β 制御性 micro-RNA (miR) の発現とがん病態との関連解析

2014 年 10 月 01 日　・本学保健学類の藤井瑞希（みずき）さん, 丸山幸奈（ゆきな）さんが卒業研修を開始

2014 年 10 月 10 日　・源 利成が 2014 年度石川県看護師資質向上研修会【がん看護】で講演
会場: 金沢大学附属病院宝ホール
課題: がんの研究と医療・看護の資質向上

2014 年 11 月 05 日　・中田光俊先生が医学系で本学脳神経外科学 教授候補に選出

2014 年 11 月 16 日　・第 119 回日本消化器病学会北陸支部例会
支部評議員会で, 源　利成が第 121 回支部例会会長に承認された

2014 年 11 月 23 日　・金沢大学がん研究所外科同門会総会・懇親会: ホテル日航金沢

2014 年 12 月　・中田光俊先生が本学脳神経外科学教授に内定

2014 年 12 月 03 日　・塚　正彦先生が医学系で本学法・社会医学 教授候補に選出

2014 年 12 月 05 日　・腫瘍制御・金沢医科大学腫瘍内科合同忘年会: 和台（ 金沢駅前）
石垣靖人先生, 中田光俊先生, 塚　正彦先生のお祝いの会

【研究業績】

（註）論文は英文のみ．[IF:]は 2014 年現在のインパクトファクター

Ⅰ．原著論文

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Ⅲ. 学会発表

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【講演など】

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【報道など】

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【受賞】

なし

治療法開発の最前線

北陸がんプロ養成プログラム

ロボット手術、免疫療法紹介

北陸がんプロフェッショナル養成プログラムの市民公開講座「みんなで拓(ひら)こう新しいがん治療への道～最近注目の高度・先進治療および臨床試験・治験～」（北國新聞社主催）は23日、金沢市の北國新聞交流ホールで開かれた。金大と金沢先進医学センターの研究者ら5人が講演し、がん克服に向けた治療法開発の最前線を紹介した。

がん治療法を紹介した講座＝北國新聞交流ホール

北國新聞交流ホール 市民講座に5氏

「前立腺がんに対する新たな集学的治療」と題して話した金大附属病院泌尿器科の並木幹夫教授は、2009（平成21）年に同病院に導入されたロボット手術や、ホルモン剤と放射線を併用した臨床試験を紹介した。「早期発見できれば外科手術、放射線、薬剤を組み合わせた集学的治療で治せる」として、採血検査を呼び掛けた。

金沢先進医学センターの和田道彦個別化医療センター長は、同センターで臨床研究が進む腫瘍免疫療法の仕組みを説明した。自分の細胞を用いる同療法は副作用が少なく、「ほかの治療法との併用で大きな相乗効果が期待できる」と話した。

金大がん研究所腫瘍制御研究分野の源利成教授は、腫瘍増殖を促す物質にのみ働き掛ける脳腫瘍の「分子標的療法」開発の取り組みを紹介した。治療が難しいすい臓などのがん治療に道を開く可能性を示唆した。

金大附属病院放射線科の香田渉助教は、肝臓や腎臓、肺のがんにラジオ波を流して焼き殺す「ラジオ波焼灼(しゃく)術」の特徴を説明し、手術に匹敵する生存率が得られ、体への負担も小さい、再発しても再治療できるなどの長所を強調した。

同病院臨床試験管理センターの松嶋由紀子薬剤主任は、臨床試験と治験が新治療法開発に果たす役割や手続きについて解説した。

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北陸がんプロフェッショナル養成プログラム市民公開講座

みんなで拓こう 新しいがん治療への道

～最近注目の高度・先進治療および臨床試験・治験～

北陸がんプロフェッショナル養成プログラムの市民公開講座「みんなで拓こう新しいがん治療への道～最近注目の高度・先進治療および臨床試験・治験～」はこのほど、北國新聞交流ホールで開かれました。金沢大学と金沢先進医学センターの研究者ら5人が、がん治療法開発の最前線を紹介しました。並木幹夫（北陸がんプロ統括コーディネーター）、元雄良治（金沢医科大病院集学的がん治療センター長）の両氏が座長を務めました。

［主催］北陸がんプロフェッショナル養成プログラム（金沢大学、富山大学、福井大学、金沢医科大学、石川県立看護大学）
NPO法人がんプロフェッショナル認定機構、中外製薬㈱、北國新聞社、金沢ケーブルテレビネット㈱
［後援］石川県医師会、金沢市医師会、テレビ金沢

がん治療の新たな腫瘍免疫療法

和田道彦氏（医療法人社団金沢先進医学センター 個別化医療センター長）

相乗効果、再発予防に注目

金沢先進医学センターは昨年7月開設され、私どもの個別化医療センターは、実質的に10月から診療を開始、免疫療法に取り組んでいます。

免疫の仕組みについてはこの20年の間に徐々に解明され、第4世代といわれる免疫療法へと進展しています。免疫で主に活躍するのは、白血球の中の樹状細胞とT細胞です。樹状細胞は、がん細胞を摂取し「がんの目印」を探し出す。そして、がんだけがつくるたんぱく質の一部（がん特異抗原）を切り出す。これがペプチドです。がん攻撃の司令官である樹状細胞は、リンパ節でT細胞にその情報を伝えます。T細胞は兵士となってがん細胞に立ち向かいます。

免疫の仕組みを利用した療法の利点は、自分の細胞を使うことで重篤な副作用が少ないことです。最近のトピックスとしては、がんペプチドワクチン療法と、樹状細胞ワクチン療法があります。前者は創薬として取り組まれています。がんに特異的なペプチドを人工的に合成して注射する。後者は取り出した樹状細胞に自分のがんをすりつぶして混ぜたり、人工ペプチドを投与し再び体内に戻す治療法です。考え方は違いますが患者さんの体内での仕組みは同じといえます。外科、化学、放射線療法との相乗効果、術後の再発予防効果に最近注目が集まっています。

前立腺がんに対する新しい集学的治療

並木幹夫氏（金沢大学附属病院泌尿器科教授）

ホルモン療法指針の確立へ

前立腺がんの早期発見と適切治療をアピールするブルークローバーキャンペーンのキャラクターを務めた間寛平さんが、アースマラソンのためよくよく検査したら、前立腺がんだったことがわかり大きな話題となりました。間さんは「早く見つければそれだけ治療法も多い」と述べておりましたが、この点が実はとても大事なことなのです。PSA検査という血液検査で、簡便に、概ね発見できるので、乳がんのピンクリボンキャンペーンに負けずに普及に努めたい。

早期の限局性前立腺がんは全摘すれば10年生存率が100％という成績ですが、それでも医療現場では、放射線療法とともにホルモン療法がよく行われています。良好な効果があるから使っているのに欧米のデータでは推奨レベルに達していない。性機能の低下などの副作用、欧米人との感受性の違いもあると思いますが、このレベルを上げることはできないか。そのためにホルモン療法の有効性に関する観察研究を進めています。105の施設で1200人の患者さんの登録があり、今後10年をかけてわが国独自の指針を確立したいと思っています。

また、当院では2年前から高度医療としてロボット手術を行っています。ロボットが自動的に行うのではなく、拡大した立体画像を見ながらの遠隔手術のことです。鉗子など手術器具を自在に動かせ、細かい所に手が届くのです。出血量が圧倒的に少ないことなどメリットはあり、実績を積み上げていきたい。

新たな局所治療としてのラジオ波焼灼術

香田 渉氏（金沢大学附属病院放射線科 助教）

体にやさしい根治療法

ラジオ波焼灼術（RFA）は、電極針をCT、超音波などの画像を見ながら経皮的に腫瘍に刺し、高熱で凝固壊死させる局所治療です。治療可能な腫瘍の大きさは3センチぐらいまでで、適切な症例選択が必要ですが、低侵襲で根治性が高く、再発時に再治療が可能などのメリットがあります。欧米では1990年代より臨床応用され、肝がんのほか、腎がん、肺がんなどに対しても行われており、術後の腎不全や呼吸不全を回避できる体にやさしい根治的療法といわれています。

わが国では1999年に本格導入され、2004年から肝悪性腫瘍についてのみ保険適応となりました。腎、肺がんなどに対するRFAは保険適応が無く自由診療となります。さまざまな報告が上がっており、腎がんの場合、3センチ以下、外方突出型の腫瘍では外科手術に匹敵する治療成績が報告されています。また、肺がんも原発性非小細胞がん、大腸がんの肺転移などに良い結果が報告されています。骨腫瘍については転移性骨腫瘍の痛みを和らげるためにRFAが実施されることがあります。いずれの腫瘍でも再発時など繰り返し実施できるのも強みといえます。これらの腫瘍に対するRFAは先進医療としても取り組まれており、低侵襲治療の可能性は広がっています。

難治がんに対する新たながん治療薬開発への道

源 利成氏（金沢大学がん研究所腫瘍制御研究分野 教授）

悪玉酵素をたたく新治療法

難治がんには、進行の早いスキルス胃がん、浸潤、転移しやすく発見した時はすでに手遅れの膵臓がんなどがあります。そして、手術すら難しい、最も悪性のがんが脳に発生する「神経膠芽腫」です。周囲の脳に染み込むように急速に広がるため、手術で腫瘍を取りきれず再発することから、この30年間、脳神経外科医らの苦闘にもかかわらず、予後はほとんど改善されていません。新たな治療法を求め、金沢大学附属病院脳神経外科との共同研究をもとに2年前から臨床試験を実施しています。

がんの増殖を促す酵素「GSK3β」の悪玉的働きを、大腸がんで解明しました。この酵素を阻害すると、がん細胞の増殖や浸潤が抑制されることに着目、これまで統合失調症や胃潰瘍などの治療に使われていた4種類の薬剤がGSK3βの働きを阻害することを利用し、脳腫瘍の治療に転用しました。臨床試験の結果、抗がん剤のみの治療を受けた患者さんの平均生存期間が再発後23週なのに対し、新たな治療法では28～73週となりました。2月から金沢医科大学病院のチームがやはり悪性度の高い膵臓がんに対しても臨床試験を開始しました。ゼロから新薬を創り出すのは莫大な労力、時間と費用がかかるので、なんとしても眼の前の患者さんを救いたいという思いで研究開発を進めています。

新たながん治療が生まれるまで ～臨床試験・治験って何？～

松嶋由紀子氏（金沢大学附属病院臨床試験管理センター副センター長）

厳格なルールで実施

臨床試験とは、患者さんにご協力頂いて「新しい治療法の候補」の有効性や安全性を調べる試験のことをいい、臨床試験のうち、薬や機器の候補を薬と認めてもらうために実施するものを治験といいます。

臨床試験は、ルールを守って実施されます。ヒトを対象とする医学研究の倫理原則は「ヘルシンキ宣言」（1964年）にうたわれています。研究に参加される方の生命・健康・プライバシー及び尊厳を守ることは、医学研究に携わる医師の責務であることなどが盛り込まれています。参加するかどうかは、試験のメリット、デメリットを十分理解した上で、自分自身で判断してください。もちろん参加しないことによって不利益が生じることはありません。

当院で抗がん剤治験対象になっているのは、肝細胞がん、肺がん、白血病、悪性リンパ腫、多発性骨髄腫、骨・軟部腫瘍、乳がん、前立腺がんです。参加については主治医とよく相談していただきたい。

研究助成など11件

北國がん基金 9月21日に贈呈式

財団法人北國がん研究振興財団の理事会は27日、金沢市の北國新聞会館で開かれ、第25回北國がん基金の助成対象として、研究活動助成部門に10件、特別表彰部門に1件の計11件総額1060万円を決めた。贈呈式は9月21日に同市の北國新聞交流ホールで行われる。

第25回北國がん基金の助成には、県内の大学、病院、関係団体から22件の推薦、応募があり、選考委員会と理事会で内容を審議した。

理事会は飛田秀一理事長らが出席して開かれ、事業計画として、11月に県産業展示館で開催される「シニアライフフェア2011」のブース出展や、9月に金沢市内で実施される「がん征圧月間行事」に協賛することを決めた。公益財団法人への移行認定を目指すことも了承された。

新たに選任された役員は次の各氏。

▽相談役　山野之義（金沢市長）▽理事　竹鼻巖（金沢医科大理事長）

研究活動助成部門

小田　誠氏（金大心肺・総合外科／呼吸器外科／病院臨床教授）
松本　勲氏（同病院臨床准教授）
滝沢　昌也氏（同助教）
早稲田　龍一氏（同大地域医療心肺・総合外科／呼吸器外科／特任助教）
【肺がんに対する肋弓下経横隔膜アプローチを用いた新たな低侵襲手術の開発に関する研究―肺部分切除術～遠隔操作用ロボット支援下肺葉切除術まで】＝200万円▽肺がん治療で、横隔膜側からのアプローチを用いた胸腔鏡補助下手術の有用性や問題点を検討する。

京　哲氏（金大医薬保健研究域医学系分子移植学講師）
高倉　正博氏（同大附属病院産科婦人科助教）
【血中循環がん細胞の検出技術の確立とがん診断への応用】＝100万円▽がん細胞でのみ増殖するウイルスに蛍光遺伝子を組み込み、がん細胞を光らせて判別。血中循環がん細胞を検出する新技術を確立し、がん診断への応用を目指す。

小出　寛氏（金大医薬保健研究域医学系再生分子医学研究分野准教授）
【胚性幹細胞に発現している遺伝子群からの新規がん遺伝子の単離】＝100万円▽胚性幹細胞（ES細胞）の遺伝子の中から、がん細胞の増殖に関与する新たながん遺伝子を特定する。

小竹　優範氏（県立中央病院消化器外科医長）
伴登　宏行氏（同科長）
西村　元一氏（金沢赤十字病院外科／副院長）
川上　和之氏（金大がん進展制御研究所腫瘍制御研究分野准教授）
源　利成氏（同教授）
【大腸がん組織検体資源化によるがん生物学的特性の大規模解析とがん医療への展開】＝100万円▽患者から提供を受けた大腸がんの組織検体を多角的に解析し、臨床情報や遺伝子や分子の情報などと合わせてデータベース化する。大腸がんの研究や医療の進展に役立てる。

笹川　寿之氏（金沢医科大産科婦人科学准教授）
【子宮頸がんに対する新しい免疫療法の開発とその効果発現機序の解明】＝100万円▽ヒトパピローマウイルス感染が原因とされる子宮頸がんについて、免疫療法などの有効性を検証する。

高橋　智聡氏（金大がん進展制御研究所教授）
【がん細胞特異的な脂質代謝異常の分子機構と治療標的としての可能性】＝100万円▽がん細胞に特徴的な脂質代謝異常の仕組みを解明し、これを制御することによるがん治療が可能かどうかを検討する。

溝上　敦氏（金大附属病院泌尿器科講師）
【再燃時における前立腺がんの増殖・アンドロゲン感受性変化に影響を与える遺伝子の同定】＝100万円▽進行性前立腺がんでは、いったんホルモン療法を行うと数年後には効かなくなることが多い。ホルモン療法の効果を延長させることを目指し、前立腺がんの増殖にかかわる遺伝子の中で、アンドロゲン応答性にも影響する遺伝子を特定する。

渡邉　直人氏（金沢医科大放射線医学特任教授）
道合　万里子氏（同助教）
【がんに対する放射線内部照射治療におけるリンパ球の放射線組織障害に関する検討】＝100万円▽がんの放射線内部照射治療について、リンパ球を用いて組織障害がどの程度出現するかを基礎的に解明する。

和田　道彦氏（金沢先進医学センター個別化医療センター長）
金子　周一氏（金大大学院医学系研究科恒常性制御学教授）
【悪性腫瘍に対する免疫細胞療法の効果の前向きコホート研究】＝100万円▽金沢先進医学センターと金大が連携し、がんと闘う免疫細胞を培養して体内に戻す「免疫細胞療法」を受けている患者の治療効果を評価する。

特別表彰部門

赤い靴ひもの会　＝10万円▽がん体験者による白山登山を企画、実施し、新聞報道などを通して県民にがん早期発見の重要性を啓発している。

山田　圭輔氏
金田　礼三氏
丸谷　晃子氏
原　祐輔氏（金大附属病院緩和ケアチーム）
【医学生に対するスピリチュアルケア教育プログラムの作成】＝50万円▽がん患者の精神的苦痛を理解し共感できる医師の育成を目指し、金大の医学生を対象に、作成したプログラムに基づいて緩和医療臨床実習を行う。実習後、学生の態度の変化なども評価する。

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2012年（平成24年）9月21日 （金曜日） 北 國 新 聞

北國がん基金

27日に助成金贈呈式

北國新聞交流ホール

財団法人北國がん研究振興財団の第26回北國がん基金助成金贈呈式は27日午後1時半から、金沢市の北國新聞交流ホールで行われる。研究活動助成部門の10件、啓発活動助成部門の2件に助成金が贈られ、1団体が特別表彰される。贈呈式を前に、昨年の助成対象者の取り組みを紹介する。

当日、贈呈式会場で開かれる北國がん基金市民講座では、金大附属病院集中治療部の谷口巧部長が「がん患者の集中治療管理」と題して講演する。入場無料。問い合わせは北國がん研究振興財団（平日午前10時～午後6時）＝076（260）3402＝まで。

研究活動

肺手術の痛みを軽減

小田 誠氏 金大心肺・総合外科病院臨床教授
松本 勲氏 同病院臨床准教授
滝沢 昌也氏 同助教
早稲田龍一氏 金大地域医療心肺・総合外科特任助教

組織のデータを蓄積

小竹 優範氏 県立中央病院消化器外科医長
伴登 宏行氏 同科長
西村 元一氏 金沢赤十字病院副院長
川上 和之氏 金大がん進展制御研究所腫瘍制御研究分野准教授
源 利成氏 同教授

【大腸がん組織検体資源化によるがん生物学的特性の大規模解析とがん医療への展開】県立中央病院、金大附属病院、金沢赤十字病院、金沢医科大病院が連携し、患者から提供を受けた大腸がんの組織を収集・解析しデータベース化している。

臨床情報や遺伝子情報などと合わせ、現在約850例のデータを蓄積。個別化医療の実現を目指し、がん関連遺伝子や分子異常を探す研究に活用する。

【学位（博士・修士）関連論文】

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3. **Ilya V. Pyko**: 大学院医学系研究科 脳機能制御学（脳神経外科学）

Pyko IV, Nakada M, Sabit H, Lei T, Furuyama N, Hayashi Y, Kawakami K, Minamoto T, Fedlau AS, Hamada JI. Glycogen synthase kinase 3β inhibition sensitizes human glioblastoma cells to temozolomide by affecting O^6-methylguanine DNA methyltransferase promoter methylation via c-Myc signaling. ***Carcinogenesis*** 34 (10): 2206-17, 2013. doi: 10.1093/carcin/bgt182. Epub 2013 May 28.

4. **近野祐里**: 大学院薬学系研究科修士課程 【本誌 32－42 頁に掲載】

Chikano Y, Domoto T, Furuta T, Sabit H, Kitano-Tamura A, Pyko IV, Takino T, Sai Y, Hayashi Y, Sato H, Miyamoto KI, Nakada M, Minamoto T. Glycogen synthase kinase 3β sustains invasion of glioblastoma via the focal adhesion kinase, Rac1 and c-Jun N-terminal kinase-mediated pathway. ***Mol Cancer Ther*** 14 (2): 564-74, 2015. [IF: 5.683]

Matsunoki A, Kawakami K, Kotake M, Kaneko M, Kitamura H, Ooi A, Watanabe G, Minamoto T. LINE-1 methylation shows little intra-patient heterogeneity in primary and synchronous metastatic colorectal cancer. *BMC Cancer* 12: 574, 2012.

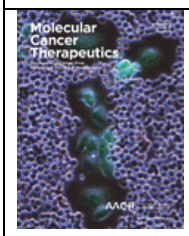
BACKGROUND: Long interspersed nucleotide element 1 (LINE-1) hypomethylation is suggested to play a role in the progression of colorectal cancer (CRC). To assess intra-patient heterogeneity of LINE-1 methylation in CRC and to understand its biological relevance in invasion and metastasis, we evaluated the LINE-1 methylation at multiple tumor sites. In addition, the influence of stromal cell content on the measurement of LINE-1 methylation in tumor tissue was analyzed. **METHODS:** Formalin-fixed paraffin-embedded primary tumor tissue was obtained from 48 CRC patients. Matched adjacent normal colon tissue, lymph node metastases and distant metastases were obtained from 12, 18 and 7 of these patients, respectively. Three different areas were microdissected from each primary tumor and included the tumor center and invasive front. Normal mucosal and stromal cells were also microdissected for comparison with the tumor cells. The microdissected samples were compared in LINE-1 methylation level measured by multicolor MethyLight assay. The assay results were also compared between microdissected and macrodissected tissue samples. **RESULTS:** LINE-1 methylation within primary tumors showed no significant intra-tumoral heterogeneity, with the tumor center and invasive front showing identical methylation levels. Moreover, no difference in LINE-1 methylation was observed between the primary tumor and lymph node and distant metastases from the same patient. Tumor cells showed significantly less LINE-1 methylation compared to adjacent stromal and normal mucosal epithelial cells. Consequently, LINE-1 methylation was significantly lower in microdissected samples compared to macrodissected samples. A trend for less LINE-1 methylation was also observed in more advanced stages of CRC. **CONCLUSIONS:** LINE-1 methylation shows little intra-patient tumor heterogeneity, indicating the suitability of its use for molecular diagnosis in CRC. The methylation is relatively stable during CRC progression, leading us to propose a new concept for the association between LINE-1 methylation and disease stage.

Kitano A, Shimasaki T, Chikano Y, Nakada M, Hirose M, Higashi T, Ishigaki Y, Endo Y, Takino T, Sato H, Sai Y, Miyamoto KI, Motoo Y, Kawakami K, Minamoto T. Aberrant glycogen synthase kinase 3β is involved in pancreatic cancer cell invasion and resistance to therapy. *PLoS One* 8 (2): e55289, 2013.

BACKGROUND AND PURPOSE: The major obstacles to treatment of pancreatic cancer are the highly invasive capacity and resistance to chemo- and radiotherapy. Glycogen synthase kinase 3β (GSK3β) regulates multiple cellular pathways and is implicated in various diseases including cancer. Here we investigate a pathological role for GSK3β in the invasive and treatment resistant phenotype of pancreatic cancer. **METHODS:** Pancreatic cancer cells were examined for GSK3β expression, phosphorylation and activity using Western blotting and in vitro kinase assay. The effects of GSK3β inhibition on cancer cell survival, proliferation, invasive ability and susceptibility to gemcitabine and radiation were examined following treatment with a pharmacological inhibitor or by RNA interference. Effects of GSK3β inhibition on cancer cell xenografts were also examined. **RESULTS:** Pancreatic cancer cells showed higher expression and activity of GSK3β than non-neoplastic cells, which were associated with changes in its differential phosphorylation. Inhibition of GSK3β significantly reduced the proliferation and survival of cancer cells, sensitized them to gemcitabine and ionizing radiation, and attenuated their migration and invasion. These effects were associated with decreases in cyclin D1 expression and Rb phosphorylation. Inhibition of GSK3β also altered the subcellular localization of Rac1 and F-actin and the cellular microarchitecture, including lamellipodia. Coincident with these changes were the reduced secretion of matrix metalloproteinase-2 (MMP-2) and decreased phosphorylation of focal adhesion kinase (FAK). The effects of GSK3β inhibition on tumor invasion, susceptibility to gemcitabine, MMP-2 expression and FAK phosphorylation were observed in tumor xenografts. **CONCLUSION:** The targeting of GSK3β represents an effective strategy to overcome the dual challenges of invasiveness and treatment resistance in pancreatic cancer.

Pyko IV, Nakada M, Sabit H, Lei T, Furuyama N, Hayashi Y, Kawakami K, Minamoto T, Fedlau AS, Hamada JI. Glycogen synthase kinase 3β inhibition sensitizes human glioblastoma cells to temozolomide by affecting O^6-methylguanine DNA methyl-transferase promoter methylation via c-Myc signaling. *Carcinogenesis* 34 (10): 2206-17, 2013.

Glycogen synthase kinase 3β (GSK3β) is a serine/threonine protein kinase involved in human cancers including glioblastoma. We have previously demonstrated that GSK3β inhibition enhances temozolomide effect in glioma cells. In this report, we investigated the molecular mechanisms of sensitization of glioblastoma cells to temozolomide by GSK3β inhibition, focusing on O(6)-methylguanine DNA methyltransferase (MGMT) gene silencing. Glioblastoma tissues from patients treated with the GSK3β-inhibiting drugs were subjected to immunohistochemistry and methylation-specific PCR assay. Human glioblastoma cell lines T98G, U138, U251 and U87 were treated with a small-molecule GSK3β inhibitor, AR-A014418 or GSK3β-specific small interfering RNA. The combined effect of temozolomide and AR-A014418 on cell proliferation was determined by AlamarBlue assay and an isobologram method. MGMT promoter methylation was estimated by methylation-specific PCR and MethyLight assay. MGMT gene expression was evaluated by real-time quantitative reverse transcriptase-PCR. c-Myc and DNA (cytosine-5)-methyltransferase 3A binding to the MGMT promoter was estimated by chromatin immunoprecipitation assay. GSK3β inhibition decreased phosphorylation of glycogen synthase and reduced MGMT expression and increased MGMT promoter methylation in clinical tumors. In glioblastoma cell lines, GSK3β inhibition decreased cell viability, enhanced temozolomide effect and downregulated MGMT expression with relevant changes in the methylation levels of the MGMT promoter. Here, we showed for the first time that c-Myc binds to the MGMT promoter with consequent recruitment of DNA (cytosine-5)-methyltransferase 3A, regulating the levels of MGMT promoter methylation. The results of this study suggest that GSK3β inhibition enhances temozolomide effect by silencing MGMT expression via c-Myc-mediated promoter methylation.



Chikano Y, Domoto T, Furuta T, Sabit H, Kitano-Tamura A, Pyko IV, Takino T, Sai Y, Hayashi Y, Sato H, Miyamoto KI, Nakada M, Minamoto T. Glycogen synthase kinase 3β sustains invasion of glioblastoma via the focal adhesion kinase, Rac1 and c-Jun N-terminal kinase-mediated pathway. *Mol Cancer Ther* 14 (2): 564-74, 2015. doi: 10.1158/1535-7163. MCT-14-0479.

The failure of current treatment options for glioblastoma stems from their inability to control tumor cell proliferation and invasion. Biologically targeted therapies offer great hope and one promising target is glycogen synthase kinase-3β (GSK3β), implicated in various diseases, including cancer. We previously reported that inhibition of GSK3β compromises the survival and proliferation of glioblastoma cells, induces their apoptosis, and sensitizes them to temozolomide and radiation. Here, we explore whether GSK3β also contributes to the highly invasive nature of glioblastoma. The effects of GSK3β inhibition on migration and invasion of glioblastoma cells were examined by wound-healing and Transwell assays, as well as in a mouse model of glioblastoma. We also investigated changes in cellular microarchitectures, cytoskeletal components, and proteins responsible for cell motility and invasion. Inhibition of GSK3β attenuated the migration and invasion of glioblastoma cells in vitro and that of tumor cells in a mouse model of glioblastoma. These effects were associated with suppression of the molecular axis involving focal adhesion kinase, guanine nucleotide exchange factors/Rac1 and c-Jun N-terminal kinase. Changes in cellular phenotypes responsible for cell motility and invasion were also observed, including decreased formation of lamellipodia and invadopodium-like microstructures and alterations in the subcellular localization, and activity of Rac1 and F-actin. These changes coincided with decreased expression of matrix metalloproteinases. Our results confirm the potential of GSK3β as an attractive therapeutic target against glioblastoma invasion, thus highlighting a second role in this tumor type in addition to its involvement in chemo- and radioresistance.

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LINE-1 methylation shows little intra-patient heterogeneity in primary and synchronous metastatic colorectal cancer

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Abstract

Background: Long interspersed nucleotide element 1 (LINE-1) hypomethylation is suggested to play a role in the progression of colorectal cancer (CRC). To assess intra-patient heterogeneity of LINE-1 methylation in CRC and to understand its biological relevance in invasion and metastasis, we evaluated the LINE-1 methylation at multiple tumor sites. In addition, the influence of stromal cell content on the measurement of LINE-1 methylation in tumor tissue was analyzed.

Methods: Formalin-fixed paraffin-embedded primary tumor tissue was obtained from 48 CRC patients. Matched adjacent normal colon tissue, lymph node metastases and distant metastases were obtained from 12, 18 and 7 of these patients, respectively. Three different areas were microdissected from each primary tumor and included the tumor center and invasive front. Normal mucosal and stromal cells were also microdissected for comparison with the tumor cells. The microdissected samples were compared in LINE-1 methylation level measured by multicolor MethyLight assay. The assay results were also compared between microdissected and macrodissected tissue samples.

Results: LINE-1 methylation within primary tumors showed no significant intra-tumoral heterogeneity, with the tumor center and invasive front showing identical methylation levels. Moreover, no difference in LINE-1 methylation was observed between the primary tumor and lymph node and distant metastases from the same patient. Tumor cells showed significantly less LINE-1 methylation compared to adjacent stromal and normal mucosal epithelial cells. Consequently, LINE-1 methylation was significantly lower in microdissected samples compared to macrodissected samples. A trend for less LINE-1 methylation was also observed in more advanced stages of CRC.

Conclusions: LINE-1 methylation shows little intra-patient tumor heterogeneity, indicating the suitability of its use for molecular diagnosis in CRC. The methylation is relatively stable during CRC progression, leading us to propose a new concept for the association between LINE-1 methylation and disease stage.

Keywords: LINE-1, DNA methylation, Colorectal cancer, Laser microdissection

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Background

Global DNA hypomethylation is frequently observed in various malignancies including colorectal cancer (CRC) [1,2], where it is thought to play a pivotal role in carcinogenesis [3]. One of the possible mechanisms for the involvement of DNA hypomethylation in cancer development is through the activation of long interspersed nucleotide element-1 (LINE-1) and genomic instability [4,5]. LINE-1 is a non-long-terminal-repeat class of retroposon. It is the most successfully integrated mobile element and accounts for about 18% of the human genome [6]. LINE-1 has the potential to transpose in the human genome, thus creating new genetic sequences that are one of the driving forces of human evolution [7,8]. Although the majority of LINE-1-derived elements in the human genome no longer have the ability to transpose due to mutations and deletions in their sequence, approximately 100 full-length copies of LINE-1 retain this ability [9,10]. Global DNA hypomethylation is also accompanied by hypomethylation of LINE-1 promoter [11], suggested to result in aberrant expression and active transposition of this sequence. The hypomethylation and/or transposition of LINE-1 elements during carcinogenesis have been suggested to alter the transcriptome [12] and to play a role in the acquisition of multiple cancer phenotypes including invasion and metastasis.

Consistent with the suggested link between LINE-1 hypomethylation and carcinogenesis, previous studies reported that LINE-1 methylation levels are lower in more advanced stages of CRC, leading to the concept of a progressive loss of genomic methylation during CRC development [13]. The inverse association between LINE-1 methylation and CRC stage suggested that LINE-1 hypomethylation was causally involved in the acquisition of invasive and metastatic phenotypes since these are critical factors in TNM staging. If this was true, the LINE-1 methylation level could be expected to differ between the tumor center and invasive front, and between primary and metastatic tumor tissue from the same patient. However, previous studies have only measured LINE-1 methylation in the primary tumor and it is still unknown whether this differs from metastatic lesions.

Aside from its biological relevance, the possible heterogeneity of LINE-1 methylation is a potential problem for any clinical application using the methylation status. Molecular analyses of tumor samples are generally performed using biopsy specimens obtained prior to surgery, or using the surgically resected tissue. Because tissue from metastatic deposits is not easily accessible, results obtained from analysis of the primary tumor are used in clinical decision making. The strategies based on molecular analysis assume the marker shows no significant intra-patient heterogeneity. However, this issue is rarely investigated for candidate prognostic and predictive markers.

LINE-1 methylation is a promising prognostic factor in CRC [14] and may also be a predictive marker for the response to fluoropyrimidines in microsatellite stable and CpG island methylator phenotype-negative CRC [15]. For LINE-1 methylation to be used in personalized medicine, the intra-patient heterogeneity of this molecular marker first needs to be established. Furthermore, the impact of contamination of tumor tissue with stromal cells on the analysis of LINE-1 methylation also needs to be investigated. A previous study showed that stromal cells such as fibroblasts and infiltrating lymphocytes have significantly higher LINE-1 methylation levels compared to the adjacent tumor cells [13]. The degree of contamination of tumor specimens with stromal cells could therefore compromise the accuracy of LINE-1 methylation assays. Laser-capture microdissection (LCM) can reduce this interference by allowing the exclusive collection of tumor cells. However, LCM is labor intensive and time consuming compared to the use of whole tumor tissue for molecular diagnosis. Comparison of the results for LINE-1 methylation levels in microdissected tumors compared to whole tumors should resolve whether contamination with stromal cells has a significant influence on the measurement of this marker.

The issues of intra-patient heterogeneity and contamination with stromal cells were addressed in this study to increase our understanding of LINE-1 methylation in cancer biology, as well as for possible future clinical applications of this marker in CRC. Using LCM, CRC samples were collected from multiple sites including the center and invasive front of primary tumors, as well as from lymph node and distant metastases. LINE-1 methylation levels were found to show little intra-patient heterogeneity, indicating this marker is suitable for clinical applications. Stromal cells significantly influenced the measurement of LINE-1 methylation in tumors, demonstrating the need for LCM. Finally, from a biological perspective the prevailing view of a progressive loss of genomic methylation during CRC development was not supported in this study. Instead, we propose another mechanism to explain the link between LINE-1 methylation and disease stage in CRC.

Materials and methods

Patients and tissue samples

Formalin-fixed paraffin-embedded (FFPE) CRC tissue specimens were obtained from 48 patients who underwent surgery at Kanazawa University Hospital. The patients comprised 26 males and 22 females and ranged in age from 37–91 years (mean 68.8 years). In addition to the primary CRC tissue, FFPE specimens of matched adjacent normal colon tissue, lymph node metastases

and distant metastases were available for 12, 18 and 7 of these patients, respectively. Tumor stage was defined according to the International Union Against Cancer (UICC) TNM system. Approval for this project was obtained from the Kanazawa University Medical Ethics Committee.

Tissue collection by LCM and macrodissection

All tissue samples were reviewed for quality and tumor content, followed by histological diagnosis with hematoxylin-eosin staining. Ten μm thickness sections were placed on a special foil on a glass slide, deparaffinized with xylene and then hydrated and stained with hematoxylin. Cells were collected using the Leica AS LMD system (Leica mycrosystems, Wetzlar, Germany) and captured into a microcentrifuge tube. To verify the accuracy of capture, images of tissue sections taken before and after microdissection were recorded. Representative images were presented in Additional file 1: Figure S1. Cell populations from three different areas were collected and included the tumor center and invasive front for all 48 primary tumors. For 12 patients, matched epithelial cells from normal mucosa and stromal cells surrounding the tumor cells were also isolated. In 21 cases, metastatic tumor cells from lymph nodes and/or distant metastases were collected. In addition to the microdissected tissue samples, a scalpel was used to collect macrodissected tumor tissue for assessment of the influence of stromal cell contamination and for analysis of microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) status.

DNA isolation and bisulfite treatment

DNA was extracted from tissue samples using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) and treated with bisulfite using the EpiTect bisulfite Kit (Qiagen) according to the manufacturer's protocols.

Multicolor MethyLight assay

LINE-1 methylation was measured using the MethyLight assay [16] and the use of two different probes for unmethylated- and methylated-LINE-1 sequences. These were labelled with FAM and Yakima yellow, respectively. Primers used for PCR (forward, GGGAGTGTTAGA TAGTGGG; reverse, AAACTCCCTAACCCCTTA) contained no CpG sites and amplifed the bisulfite-converted LINE-1 sequence independently of its methylation status. The two probes were synthesized by Nippon EGT (Toyama, Japan) and consisted of FAM-CCTACTT CAACTCACACACAATAC-Eclipse Dark Quencher and Yakima yellow-CCTACTTCGACTCGCGCACGATAC-Eclipse Dark Quencher. Locked nucleic acid was used for the underlined nucleotides in order to match the melting temperature between the probes. A standard sample was created by ligating unmethylated- and methylated-LINE-1 sequences, and cloning into a plasmid (Additional file 2: Figure S2). Real-time detection was performed simultaneously with the standard sample that was equivalent to 50% methylated LINE-1 sequence. The percentage of methylated LINE-1 was calculated using the formula: 100 × methylated reaction / (unmethylated reaction + methylated reaction).

Analysis of MSI and CIMP

DNA isolated from macrodissected tissue was used for the analysis of MSI and CIMP as described previously [15]. MSI status was determined using 3 mononucleotide repeat markers (BAT26, NR21 and NR27) and CIMP by the methylation status of 5 genes (CACNA1G, IGF2, NEUROG1, RUNX3 and SOCS1).

Statistical analysis

Paired t-test was used for the comparison between the LINE-1 methylation levels in different samples. The Mann–Whitney U test or the Kruskal-Wallis test was used to compare LINE-1 methylation levels between two or three clinicopathological variables, respectively. All statistical analyses were carried out using the R software package version 2.7.2 [17].

Results

Development and validation of a multicolor MethyLight assay

In a previous study we developed a quantitative methylation-specific PCR (qMSP) assay to measure LINE-1 methylation [18]. This assay used methylation-specific primers and SYBR green and was initially employed here to analyze the microdissected samples. However, the results obtained with this method were not reproducible due to the low threshold cycle (data not shown). We therefore developed a new multicolor MethyLight assay to measure LINE-1 methylation with high sensitivity and reproducibility. To validate this method, samples with 10% increments in LINE-1 methylation level were prepared by mixing varying ratios of plasmids cloned with unmethylated- or methylated-LINE-1 sequence. Using the multicolor MethyLight assay, a linear increase in LINE-1 methylation was observed in the prepared samples (Additional file 3: Figure S3). To evaluate inter-assay variation, DNA from four CRC cell lines with different LINE-1 methylation levels was analyzed in four independent assays. The inter-assay variation was within an acceptable range (Additional file 4: Table S1). These results demonstrate that the newly established assay was accurate and reproducible across a range of LINE-1 methylation levels.

LINE-1 methylation level shows little intra-patient heterogeneity

Tumor samples microdissected from three different areas of each primary tumor including the center and invasive front were evaluated in duplicate by the multicolor MethyLight assay. Samples with a low threshold cycle value (<32) showed inconsistent results for duplicate assays and were thus deemed as "not available" (NA). All the results of the assay are shown in Additional file 5: Table S2. The measurement of LINE-1 methylation was successful in 133/144 (92.4%) of the microdissected samples from the primary tumors, despite the small amounts of DNA collected by LCM. The LINE-1 methylation level showed little intra-tumor heterogeneity, with all values from the same tumor being within 10% difference. This level of difference is not strong considering the distribution of the LINE-1 methylation (range of 27.2 to 88.3%, Additional file 5: Table S2) and up to 4.9% of the inter-assay variation (Additional file 4: Table S1). No significant difference was observed in LINE-1 methylation between the central area and invasive front from the same tumor (Figure 1A).

To investigate for possible differences in LINE-1 methylation between matched primary and metastatic tumor tissues, tumor cells collected by LCM from lymph node (18 cases) and distant metastases (7 cases) were also evaluated. Distant metastases from liver (5 samples), peritoneum (4 samples) and ovarium (1 sample) were available for the analysis. The mean LINE-1 methylation value observed in multiple areas of the primary tumor was identical to that found in metastatic lymph nodes (Figure 1B). No significant difference in the methylation between primary tumor and distant metastases was observed (Table 1). These results demonstrate there is little intra-patient heterogeneity for LINE-1 methylation and that it remains relatively stable during CRC progression.

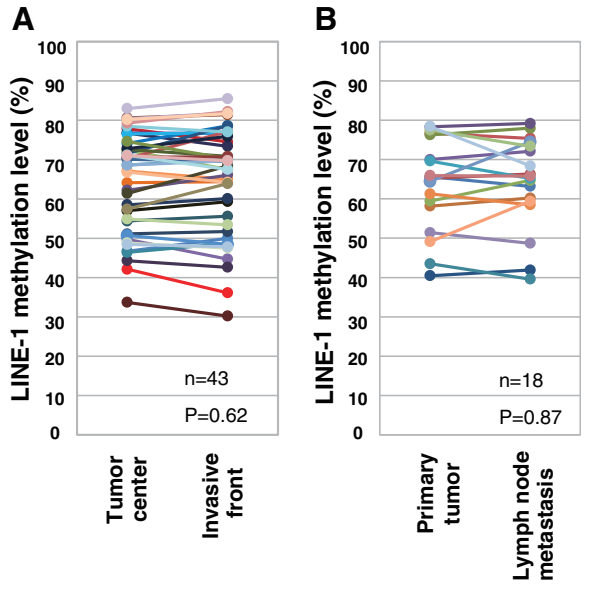

Figure 1 LINE-1 methylation at multiple tumor sites. LINE-1 methylation level was compared between cancer cells from the center and invasive front of the tumor **(A)**, and between the primary tumor and lymph node metastasis **(B)**.

Contamination of tumor sample with stromal cells significantly influences the measurement of LINE-1 methylation

Cancer cells and surrounding stromal cells and normal mucosal cells were obtained from 12 primary tumors and matched adjacent colonic mucosa using LCM. Consistent with a previous report [13], LINE-1 methylation level was significantly lower in tumor cells compared to stromal and adjacent normal cells (Figure 2A). This finding suggests that a high content of stromal cells could influence the accuracy of LINE-1 methylation measurement in tumor samples. To investigate this, we compared microdissected and macrodissected tumor samples, with the latter having moderate-heavy contamination of stromal cells. LINE-1 methylation was significantly lower in the microdissected samples compared to the macrodissected samples ($P<0.0001$, Figure 2B), with the former also showing more cases with low levels of methylation (Figure 2C).

Associations between LINE-1 methylation and clinicopathological characteristics

Amongst the 48 primary CRC, 3 were CIMP-positive/microsatellite stable, 1 was CIMP-positive/MSI and 1 was CIMP-negative/MSI. These phenotypes show high LINE-1 methylation [19,20] and distinct clinicopathological features [21,22] and were therefore excluded from the analysis. Table 2 summarizes the associations between LINE-1 methylation in primary tumors and the clinicopathological characteristics of CIMP-negative/microsatellite stable

Table 1 LINE-1 methylation level in the primary tumor and in synchronous metastases of CRC

LINE-1 methylation level (%)					
Patient ID	Primary	Metastatic sites			
		Lymph node	Liver	Peritoneum	Ovarium
12	73.4	NA	78.0	NA	NA
18	43.5	39.6	38.7	NA	NA
27	48.2	NA	NA	40.6	NA
29	59.5	64.7	66.9	65.2	NA
30	70.0	72.1	77.7	70.8	67.5
36	77.5	73.4	NA	74.6	NA
40	70.0	NA	66.6	NA	NA

The LINE-1 methylation level at the primary tumor site is the mean value of assay results from multiple areas including the tumor center and invasion front.
NA, samples were not available or no metastasis was found at the indicated site.

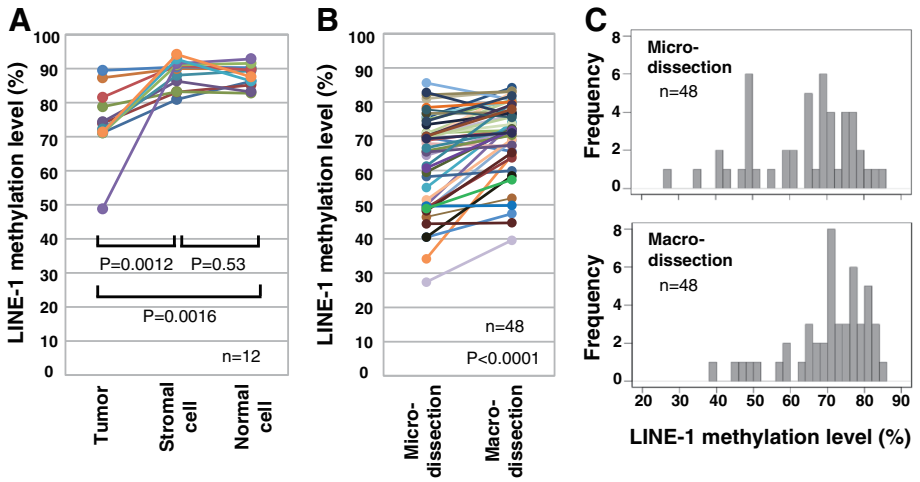

Figure 2 Influence of stromal cell contamination of the tumor sample on results from the LINE-1 methylation assay. (A) LINE-1 methylation levels in tumor, stromal and normal cells. **(B)** LINE-1 methylation levels in microdissected tumor cells and in macrodissected tumor tissues. **(C)** Distribution of LINE-1 methylation levels in microdissected tumor cells (upper panel) and in macrodissected tumor tissues (lower panel).

Table 2 Associations between LINE-1 methylation in primary CRC and clinicopathological features

		n	LINE-1 methylation	p-value
Age	>70	22	69.5 (62.1 – 70.7)	0.17
	≤70	21	59.5 (48.4 – 73.4)	
Gender	male	25	64.4 (48.9 – 70.4)	0.49
	female	18	68.3 (52.3 – 74.3)	
Site	proximal	19	65.3 (53.2 – 69.8)	0.77
	distal	24	69.3 (49.0 – 73.8)	
Histology	well	16	68.6 (49.0 – 72.1)	0.98
	moderately	27	65.7 (54.8 – 71.9)	
T factor	1	1	76.3	0.55
	2	7	67.3 (49.3 – 70.7)	
	3	26	67.3 (49.3 – 70.7)	
	4	9	58.2 (48.2 – 69.3)	
N factor	0	20	69.5 (49.7 – 75.0)	0.67
	1	16	59.4 (48.9 – 74.1)	
	2	7	65.7 (62.9 – 68.0)	
M factor	0	34	67.3 (50.3 – 73.8)	0.49
	1	9	60.7 (48.2 – 70.0)	
Stage	I	4	72.3 (69.5 – 75.0)	0.47
	II	15	69.3 (49.4 – 73.8)	
	III	15	64.4 (50.5 – 68.1)	
	IV	9	60.7 (48.2 – 70.0)	

n, number of patients.
LINE-1 methylation levels are shown as the median (25^{th} percentile – 75^{th} percentile).
Histology of adenocarcinoma was sub-classified into well- and moderately-differentiated adenocarcinoma according to their grading.
TNM factors and stage were defined according to the International Union Against Cancer (UICC) TNM system.

CRC. No significant associations were found, although this could be due to the small sample size. Consistent with a previous report [13], LINE-1 methylation level was lower in the CRC with advanced stage.

Discussion

LINE-1 hypomethylation has been observed in various malignancies and suggested to play a role in carcinogenesis. Although an association between LINE-1 methylation level and disease stage has been reported in leukemia [23], colorectal [13] and lung cancers [24], the underlying mechanism for this association is unknown. Sunami et al. proposed the concept of a progressive loss of genomic methylation during CRC development [13], as illustrated in Figure 3A. This concept involves that acquisition of metastatic potential in CRC is accompanied by decrease of LINE-1 methylation. From this, it could be expected that LINE-1 methylation level would be lower in metastatic CRC tissue compared to the primary tumor of the same patient if metastasis occurs by a clone of cancer cell with lower LINE-1 methylation. However, our results showed almost identical LINE-1 methylation levels between primary and metastatic sites (both lymph node and distant). Our observation may represent that LINE-1 methylation level is identical between primary tumor cells and metastatic clone, and cannot exclude the concept illustrated in Figure 3A. Nevertheless, it might be unlikely that the LINE-1 methylation is progressively decreased with same grade even in these different cells that reside in different environment including primary, lymph node and distant metastatic sites.

We propose another explanation for the association of LINE-1 methylation with disease stage in Figure 3B. This concept is consistent with the observation that LINE-1

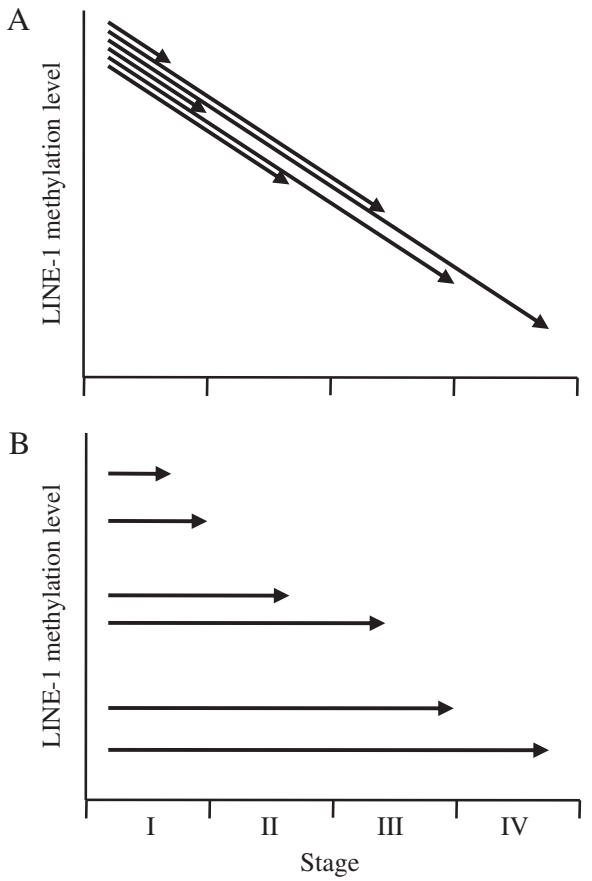

Figure 3 Possible models to explain the association between LINE-1 hypomethylation and CRC progression. (A) In the prevailing model, LINE-1 methylation decreases progressively during CRC progression. **(B)** In an alternate model, LINE-1 methylation is stable during CRC progression and the time of diagnosis may be the critical factor that determines the observed association between methylation level and disease stage. In both models, the solid line shows a change of LINE-1 methylation during CRC progression and the arrowhead indicates the time of diagnosis, surgery and sample collection.

methylation is relatively stable during disease progression and takes into account the time of diagnosis. Cancers with low LINE-1 methylation levels can progress rapidly because they have unstable genomes, more frequent transposition and may be difficult to diagnose early. Such a case is represented by the long arrow in Figure 3B. If the CRC arises within a polyp that has high LINE-1 methylation, such as a serrated adenoma/polyp [25,26], this type of cancer is more likely to be detected early by colonoscopy. In contrast, if the CRC arises within a flat or depressed type of tumor with low LINE-1 methylation, it will be much harder to detect by colonoscopy. Further investigations into LINE-1 methylation and cancer progression are required to test this concept. Possible associations between LINE-1 methylation and the morphology of early stage CRC should also be the subjects of further studies.

LINE-1 methylation is an ideal molecular marker because it exhibits little intra-tumoral heterogeneity. Molecular analysis is usually performed on a small portion of the surgical specimen of primary tumor. In the neoadjuvant setting, tumor tissue obtained by preoperative endoscopic biopsy is used instead of the surgical sample. Intra-tumoral genetic heterogeneity for K-ras and p53 gene mutations has been demonstrated in CRC tissue [27,28]. DNA methylation may show greater heterogeneity than gene mutations because it is a reversible modification. Tumor heterogeneity in samples used for molecular analysis can give rise to false positives and negatives that lead to diagnostic and therapeutic failure. Only one study has so far addressed the issue of intra-tumoral heterogeneity for LINE-1 methylation [13]. This work reported no significant difference in methylation between samples from the luminal surface and those from the most invasive primary tumor site, which is consistent with our data showing no significant difference in methylation between the central area and invasive front from the same tumor. However, there was a weak difference of less than 10% in the LINE-1 methylation level between three analyzed areas, suggesting that some CRCs might have weak heterogeneity in the LINE-1 methylation level. Although further study is required to demonstrate how stable the LINE-1 methylation is in a primary tumor, sampling errors due to strong intra-tumoral heterogeneity are not the case for this molecular marker.

Because the present work found that LINE-1 methylation level was identical between matching primary and metastatic tumor tissues, the molecular analysis of this marker can be performed using tissue samples from either of these sites. Secondary tumor tissues are not readily accessible and hence the molecular features of a metastatic tumor must sometimes be evaluated using the primary tumor, although the reverse is sometimes also the case. The LINE-1 methylation level of an individual CRC patient can therefore be represented by a single sample from any site. One limitation of our work is that cases of metachronous metastases were not included in the CRC study cohort. Considering the long natural history of CRC development, the primary tumor and synchronous metastases evaluated here were from the same time period and hence this could account for the identical levels of LINE-1 methylation. Lower LINE-1 methylation due to clonal evolution might be observed in metachronous metastases. Furthermore, discordance in the LINE-1 methylation level between primary tumor and metachronous metastases might arise in recurrent CRC following chemotherapy. Molecular analysis of relapsed cancer is generally performed on the primary

tumor tissue because of the difficulty of access to recurrent cancer sites. Future studies should compare primary tumors and metachronous metastases from individual patients.

In order to use LINE-1 methylation for routine clinical application, LCM is necessary to avoid erroneous results caused by stromal cell contamination. The present study is the first to compare the results for LINE-1 methylation between samples prepared with and without microdissection. LINE-1 methylation level was significantly lower in microdissected compared to macrodissected samples (Figure 2B), demonstrating that measurement of this marker may be inaccurate in tumor samples that have not undergone LCM enrichment. Continuous variables are often stratified into a small number of groups. Absolute assay values are therefore not critical provided their distribution shows a similar pattern between micro- and macro-dissected samples and patients are stratified according to this pattern. However, we observed a considerable alteration in the distribution of LINE-1 methylation values due to contamination with stromal cells (Figure 2C), making it more difficult to achieve accurate stratification of patients. We therefore recommend LCM of tumor samples in order to achieve accurate quantification of LINE-1 methylation.

The multicolor MethyLight assay developed in this study allows the analysis of LINE-1 methylation even in the small amounts of DNA obtained from microdissected samples. Although LINE-1 consists of many numbers of variants [9,29], our MethyLight assay only measures the one of variants, L1.2 (M80343). This major variant of LINE-1 sequence presents in human genome with about 16000 copies compared to single copy gene of ACTB, which is estimated by qPCR and ddCt method (data not shown). The high copy number suggests that the L1.2 sequence can be used to estimate global methylation status. In addition, there was significant relationship between the LINE-1 methylation level measured by this MethyLight assay and those measured by previously developed qMSP assay [18] (Additional file 6: Figure S4). The latter assay was not compatible to microdissected samples but used in our previous studies to demonstrate clinical significance of LINE-1 methylation [15,24]. Together, the new MethyLight assay could be used in broader clinical appreciation than the qMSP assay and in further validation studies of clinical significance of the LINE-1 methylation.

Conclusions

We have demonstrated that LINE-1 methylation level shows little intra-patient heterogeneity, thus making it suitable for possible clinical applications in CRC. LINE-1 methylation appears to be relatively stable during CRC progression. We propose a new concept to explain the relation between LINE-1 methylation and disease stage. Further studies are required to establish whether LINE-1 methylation could be used in personalized medicine for CRC and to better understand its role in cancer progression and metastasis.

Additional files

Additional file 1: Figure S1. Representative images of tissue sections taken before and after LCM.

Additional file 2: Figure S2. Methods for the synthesis of assay standards.

Additional file 3: Figure S3. Accuracy of newly developed LINE-1 MethyLight assay.

Additional file 4: Table S1. Results of four independent assays for LINE-1 methylation.

Additional file 5: Table S2. LINE-1 methylation levels in primary tumor of CRC patients. Samples are obtained from tumor center, invasive front and other area.

Additional file 6: Figure S4. Relationship between the LINE-1 methylation level measured by MethyLight assay and those measured by qMSP assay.

Competing interests

There are no conflicts of interest with this article.

Authors' contributions

AM and KK formulated the design and concept of the study, carried out the methylation assay, and drafted the manuscript. MK, MK and HK participated in DNA preparation, CIMP and MSI analysis. AM and AO carried out preparation of FFPE sections and LCM. GW and TM collected clinical samples and helped to draft the manuscript. All authors read and approved the final manuscript.

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Aberrant Glycogen Synthase Kinase 3β Is Involved in Pancreatic Cancer Cell Invasion and Resistance to Therapy

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Abstract

Background and Purpose: The major obstacles to treatment of pancreatic cancer are the highly invasive capacity and resistance to chemo- and radiotherapy. Glycogen synthase kinase 3β (GSK3β) regulates multiple cellular pathways and is implicated in various diseases including cancer. Here we investigate a pathological role for GSK3β in the invasive and treatment resistant phenotype of pancreatic cancer.

Methods: Pancreatic cancer cells were examined for GSK3β expression, phosphorylation and activity using Western blotting and *in vitro* kinase assay. The effects of GSK3β inhibition on cancer cell survival, proliferation, invasive ability and susceptibility to gemcitabine and radiation were examined following treatment with a pharmacological inhibitor or by RNA interference. Effects of GSK3β inhibition on cancer cell xenografts were also examined.

Results: Pancreatic cancer cells showed higher expression and activity of GSK3β than non-neoplastic cells, which were associated with changes in its differential phosphorylation. Inhibition of GSK3β significantly reduced the proliferation and survival of cancer cells, sensitized them to gemcitabine and ionizing radiation, and attenuated their migration and invasion. These effects were associated with decreases in cyclin D1 expression and Rb phosphorylation. Inhibition of GSK3β also altered the subcellular localization of Rac1 and F-actin and the cellular microarchitecture, including lamellipodia. Coincident with these changes were the reduced secretion of matrix metalloproteinase-2 (MMP-2) and decreased phosphorylation of focal adhesion kinase (FAK). The effects of GSK3β inhibition on tumor invasion, susceptibility to gemcitabine, MMP-2 expression and FAK phosphorylation were observed in tumor xenografts.

Conclusion: The targeting of GSK3β represents an effective strategy to overcome the dual challenges of invasiveness and treatment resistance in pancreatic cancer.

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Introduction

Pancreatic cancer is a major health problem due to an overall 5-year survival rate of less than 10% [1]. It is characterized by the highly proliferative and invasive capacity of the tumor cells and a strong predisposition for metastasis [2–4]. The aggressive nature of pancreatic cancer hampers early diagnosis and curative surgical intervention and renders it resistant to chemotherapy and radiation [3,4]. The widely used therapy is infusional gemcitabine, although fewer than 20% of patients respond to this treatment [3,4]. Novel therapeutic strategies that enhance the effects of gemcitabine and attenuate the invasive properties of pancreatic cancer cells are needed. Molecular target-directed therapy has emerged and includes targeting of the growth factor receptors, angiogenic factor/receptor and matrix metalloproteinases, since these are aberrantly expressed in pancreatic cancer [2–4]. Several clinical trials of pancreatic cancer have already targeted these growth factors, either as monotherapy or in combination with

gemcitabine, but most have shown little or no therapeutic benefit [5]. Identification of novel molecular targets that could enhance the therapeutic effects of gemcitabine and radiation is therefore a high priority [6].

Glycogen synthase kinase 3β (GSK3β) is a serine/threonine protein kinase that regulates multiple signaling pathways [7]. Based on its known functions and involvement in primary pathologies, GSK3β has been implicated as a therapeutic target for glucose intolerance, neurodegenerative disorders and inflammation [8]. We previously demonstrated that deregulated expression, activity and phosphorylation of GSK3β are distinct features of gastrointestinal cancers and glioblastoma and that GSK3β sustains the survival and proliferation of these tumor cells. A role for aberrant GSK3β in these tumor types is supported by the observation that pharmacological inhibition of its activity reduces the survival and proliferation of cancer cells and predisposes them to apoptosis *in vitro* and in tumor xenografts [9–12]. Although its role in cancer is still debated [13], the overall results so far indicate that aberrant expression and activity of GSK3β is a common and fundamental characteristic in a broad spectrum of cancers (reviewed in [14]).

Based on earlier studies that demonstrated involvement of GSK3β in NF-κB-mediated cell survival [15], GSK3β was found to support the survival of pancreatic cancer cells via this pathway [16,17]. Although GSK3β is a key regulator of cell polarization and migration during physiological processes such as tissue development and wound healing [18], very little is known about its role in the migration and invasion of cancer cells. Here we investigated the potential involvement of GSK3β in the invasive nature of pancreatic cancer and its resistance to gemcitabine and ionizing radiation, the two major obstacles to more effective treatment.

Materials and Methods

Ethics Statement

Written informed consent was obtained from all patients with pancreatic cancer before surgery. This study was approved by the Medical Ethics Committee of Kanazawa University.

Animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals in Kanazawa Medical University, and in accordance with national guidelines for animal use in research in Japan (http://www.lifescience.mext.go.jp/policies/pdf/an_material011.pdf). The protocol was approved by the Committee on Animal Experiments of Kanazawa Medical University.

Cell Lines and Tissue Specimens

Human embryonic kidney cells (HEK-293) and pancreatic cancer cells (PANC-1, MIA PaCa-2, BxPC-3, Capan-1) were obtained from the American Type Culture Collection (ATCC). These cell lines were characterized by DNA profiling in ATCC, and passaged for fewer than 6 month after resuscitation. They were maintained at 37°C with 5% CO_2 in DMEM (HEK-293, PANC-1, MIA PaCa-2, Capan-1) and RPMI 1640 (BxPC-3) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin G and streptomycin) (GIBCO). Cells were harvested during the exponential growth phase for extraction of RNA and protein.

This study included 15 patients with pancreatic cancer who underwent surgery at the Department of Surgical Oncology, Kanazawa University Hospital (Table S1). The surgical specimens were fixed in neutral-buffered 10% formalin, embedded in paraffin and processed for histopathologic diagnosis and immunohistochemical examinations.

Western Blotting

Protein was extracted from cultured cells using lysis buffer (CelLytic-MT, Sigma-Aldrich) containing a mixture of protease and phosphatase inhibitors (Sigma-Aldrich). Concentrations of protein extracts were measured by Coomassie Protein Assay Reagents (Pierce). A 30 μg aliquot of protein was separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting for the proteins of interest and their phosphorylation [9,11,12] using the respective primary antibodies (Table S2). Electroblotted membranes (Amersham) were blocked with 5% bovine serum albumin prior to detection of phosphorylated protein fractions. The amount of protein in each sample was monitored by the expression of β-actin. Signals were developed using enhanced chemiluminescence (ECL).

In vitro Kinase Assay for GSK3β Activity

A nonradioisotopic *in vitro* kinase assay (NRIKA) [19] was used to detect the activity of GSK3β derived from cells. The NRIKA uses a sequential combination of immunoprecipitations to isolate GSK3β in cellular protein samples, an *in vitro* kinase reaction that uses recombinant human β-catenin protein (substrate) and non-radioisotopic adenosine triphosphate (ATP), followed by immunoblotting to detect the β-catenin phosporylated at serine (S) 33, S37 and/or threonine (T) 41 residues (p-β-catenin$^{S33/37/T41}$) [19]. As a negative control, the mouse monoclonal antibody to GSK3β was replaced by an equal amount of non-immune mouse IgG (Sigma-Aldrich) in the immunoprecipitation step. GSK3β activity is demonstrated by the presence of p-β-catenin$^{S33/37/T41}$ in the test reaction and by its absence in the negative control reaction. The amount of immunoprecipitated GSK3β and the presence of recombinant β-catenin protein in the kinase reaction were monitored by immunoblotting.

Immunohistochemistry

Expression and/or phosphorylation of GSK3β, matrix metalloproteinase (MMP)-2 and focal adhesion kinase (FAK) in tumor and adjacent non-neoplastic tissues of pancreatic cancer patients were examined by the avidin-biotin-peroxidase complex method [11,12]. Following deparaffinization, microwave antigen unmasking and blocking of non-specific immunoreactions, paraffin sections were incubated with antibody to GSK3β, tyrosine (Y) 216-phosphorylated GSK3β (p-GSK3β^{Y216}), MMP-2, FAK, Y397-phosphorylated or Y861-phosphorylated FAK (p-FAK^{Y397}, p-FAK^{Y861}) and p-β-catenin$^{S33/37/T41}$, respectively. Sources and working dilutions of these antibodies were shown in Table S2. Sections were then incubated with biotinylated goat anti-rabbit IgG or horse anti-mouse IgG (diluted 1:200; Vector). Immunoreactivity was detected using the ABComplex/HRP kit (DakoCytomation). For the negative control, primary antibodies were replaced by non-immune rabbit or mouse IgG (DakoCytomation). Overexpression or higher phosphorylation of the respective molecules in tumors was defined as stronger expression or phosphorylation of either protein in the tumor cells compared to non-neoplastic pancreatic ducts in the same patient.

Effects of GSK3β Inhibition on Cell Survival and Proliferation

Cells seeded in 96-well plates were treated with dimethyl sulfoxide (DMSO; Sigma-Aldrich) or a GSK3β inhibitor (AR-

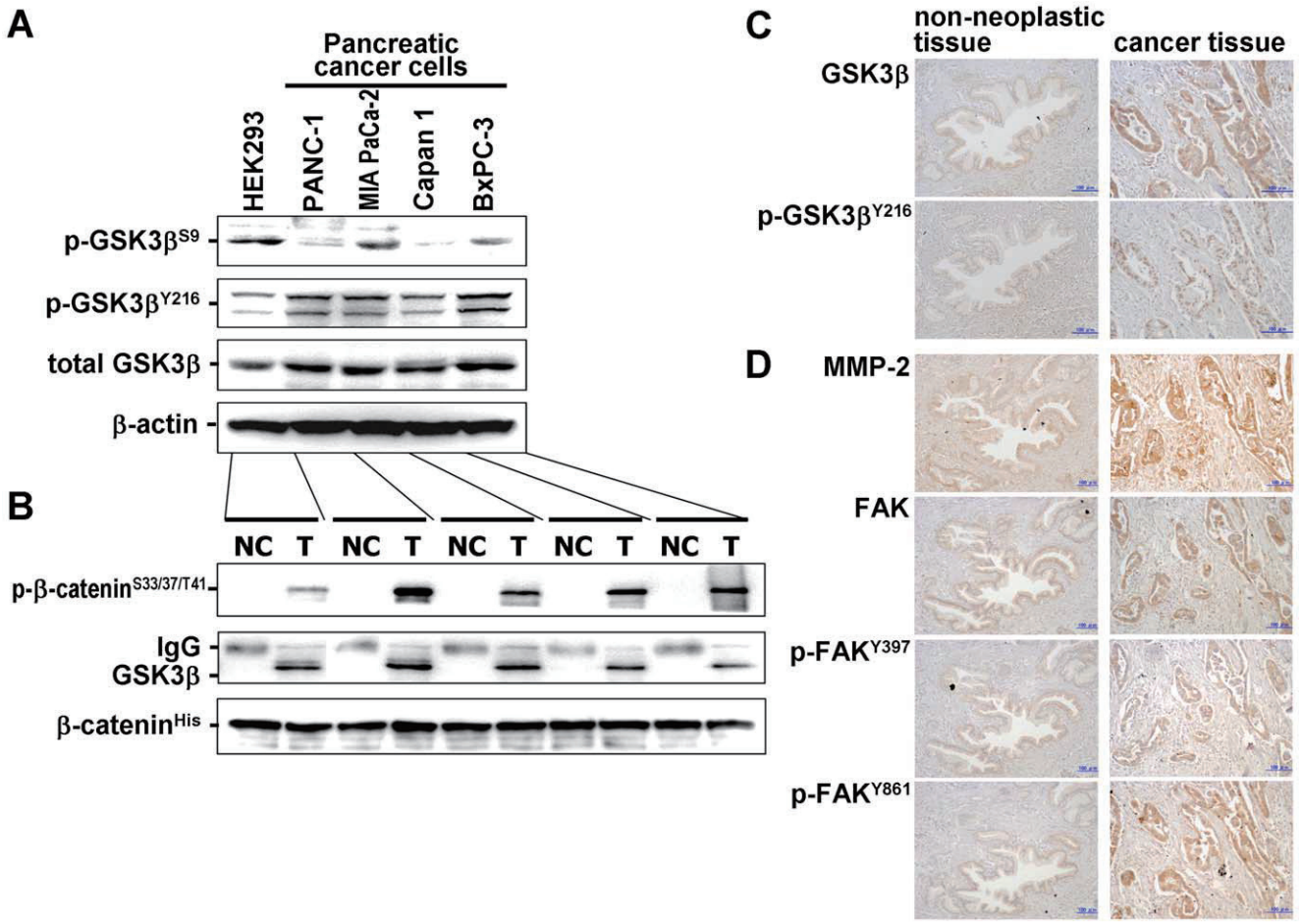

Figure 1. Expression, phosphorylation and activity of GSK3β in pancreatic cancer cells and primary pancreatic cancers. (A) Protein extract from each cell line was analyzed by Western immunoblotting for the expression of GSK3β and its phosphorylation (p-GSK3β^{S9}, p-GSK3β^{Y216}). β-actin expression was monitored as a loading control. (B) GSK3β activity was detected by NRIKA [19] in the respective cells. As described in Materials and Methods, GSK3β activity is demonstrated by the presence of p-β-catenin$^{S33/37/T41}$ in the test reaction (T) and by its absence in the negative control reaction (NC). The amount of immunoprecipitated GSK3β and the presence of substrate (β-catenin) in the kinase reaction were monitored by immunoblotting. (C, D) Serial paraffin sections of a primary pancreatic cancer and its adjacent non-neoplastic tissue (patient No. 5 in Table S1) were immunostained for GSK3β and p-GSK3β^{Y216} (C), and for MMP-2, FAK, p-FAK^{Y397} and p-FAK^{Y861} (D). The scale bar in each panel indicates 100-μm in length.
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A014418; Calbiochem) dissolved in DMSO at the indicated final concentrations in the medium. Notably, AR-A014418 does not inhibit the activity of 26 closely related kinases and is considered highly specific for GSK3β [20]. At designated time points, the relative numbers of viable and proliferating cells were determined by using WST-8 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay kit (Cell counting kit-8; Wako) and Cell Proliferation ELISA BrdU Kit (Roche), respectively.

Small interfering RNA (siRNA) specific to human GSK3β (GSK3β Validated Stealth RNAi) and negative control siRNA (Stealth RNAi Negative Control Low GC duplex) were purchased from Invitrogen. The GSK3β-specific siRNA targets the sequence 5′-GCUCCAGAUCAUGAGAAAGCUAGAU-3′. Cells were transfected with 10 nM of either siRNA using Lipofectamine RNAiMAX (Invitrogen). The effect of RNA interference on GSK3β expression was determined by Western blotting using an antibody against both GSK3α and β (Table S2). The specificity of GSK3β-specific siRNA was confirmed in our previous studies [11,12]. To examine the effect of GSK3β RNA interference on cell survival and proliferation, cells seeded into 96-well plates were transfected with 10 nM of control or GSK3β-specific siRNA. At 72 hrs after transfection, the relative numbers of viable and proliferating cells were determined by the methods described above.

Effects of GSK3β Inhibition on the Susceptibility of Cancer Cells to Gemcitabine and to Ionizing Radiation

Cancer cells seeded into 96-well plates ($3–6\times10^4$ cells per well) were treated with escalating concentrations of either gemcitabine (Eli Lily) or AR-A014418. Following treatment for 72 hrs, the relative number of viable cells was measured by WST-8 assay to determine IC_{50} (50% cell survival inhibitory concentration) for gemcitabine and AR-A014418 and to generate isobolograms. Cells were then treated with a dose of gemcitabine close to IC_{50} in the presence of DMSO or a low dose (5 or 10 μM) of AR-A014418. The isobologram method [21] was used to determine whether the effect of GSK3β inhibitor on pancreatic cancer cell susceptibility to gemcitabine was additive, synergistic or antagonistic.

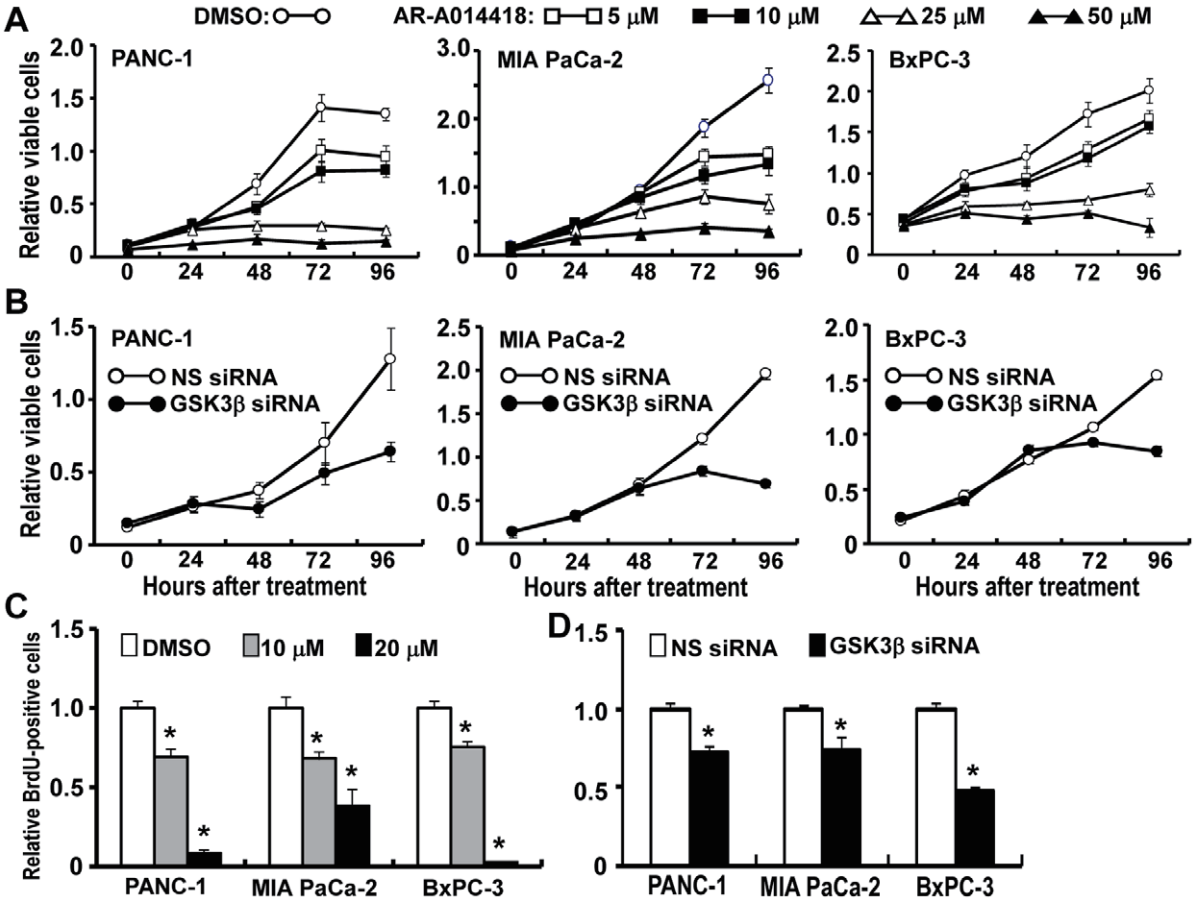

Figure 2. Effects of GSK3β inhibition on the survival and proliferation of pancreatic cancer cells. (A) Relative numbers of viable cells at the designated time points were measured by WST-8 assay for the respective cells in the presence of DMSO or AR-A014418 at the indicated concentrations. (B) Relative numbers of viable cells were measured for the respective cells after transfection of non-specific (NS) or GSK3β-specific siRNA. (C, D) The relative number of proliferating cells was determined by measuring the amount of BrdU incorporation. Proliferating cells were scored at 48 hrs after treatment with DMSO or AR-A014418 (10 μM, 20 μM) (C), or after transfection with non-specific (NS) or GSK3β-specific siRNA (D). Values shown in (A–D) are the means ± SD of five separate experiments. *$p<0.05$, statistically significant difference between cells treated with DMSO or AR-A014418 and between cells treated with non-specific and GSK3β-specific siRNA.
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The effect of GSK3β inhibitor on pancreatic cancer cell susceptibility to ionizing radiation was examined by colony-forming cell survival assay. In each well of 6-well plates, 1,000 pancreatic cancer cells were seeded and treated sequentially with either DMSO or a low dose (5 or 10 μM) of AR-A014418 for 24 hrs and with ionizing radiation at doses of 0, 4 and 8 Gy. At 6 days after irradiation, the total number of colonies stained with 0.1% crystal violet (Wako) was scored in each well. The mean number of colonies in three separate experiments was calculated with standard deviations.

Assays for Cell Migration and Invasion

Cancer cell migration and invasion were examined by monolayer-based wound-healing assay and transwell assay. Confluent monolayers of pancreatic cancer cells in the presence of DMSO or AR-A014418 at a dose of 5 or 10 μM were scratched with a 20 μL-micropipette tip to create a cell-free zone (wound). In each condition, the gap distance between the wound edges was monitored at three fixed reference points for 6 to 24 hrs by a CCD camera (Axiocam MRm, Zeiss) connected to a phase-contrast microscope (Axiovert 40 CFL, Zeiss). Cell migration at each time point was calculated as the mean distance of wound measured at the three points and compared between cells treated with DMSO and AR-A014418.

Cell migration and invasion were examined by the transwell assays using uncoated and matrigel-coated 24-well double-chamber system (BD BioCoat^{TM} Matrigel^{TM} Incubation Chamber, BD Bioscience). Cancer cells were suspended in serum-free medium containing DMSO or AR-A014418 (5 or 10 μM), and 2×10^4 cells were applied to the upper chamber pairing with the lower chamber filled with medium containing 10% FBS (as a chemo-attractant) and DMSO or AR-A014418 (5 or 10 μM). At 22 hrs after allowing cells to migrate and invade, cells on the lower side of the chamber were fixed and stained with Diff-Quick Kit (Symex). In each assay, the total number of cells per high-power microscopic field on the lower side of the uncoated or matrigel-coated chamber was counted and scored for migrating or invading cells. The mean number of cells in five high-power microscopic fields was calculated with standard deviations.

Cell Morphology and Immunofluorescence Cytochemistry

Cancer cells grown on a cover slip were treated with either DMSO or AR-A014418 (5 or 10 μM) for 12 hrs and then

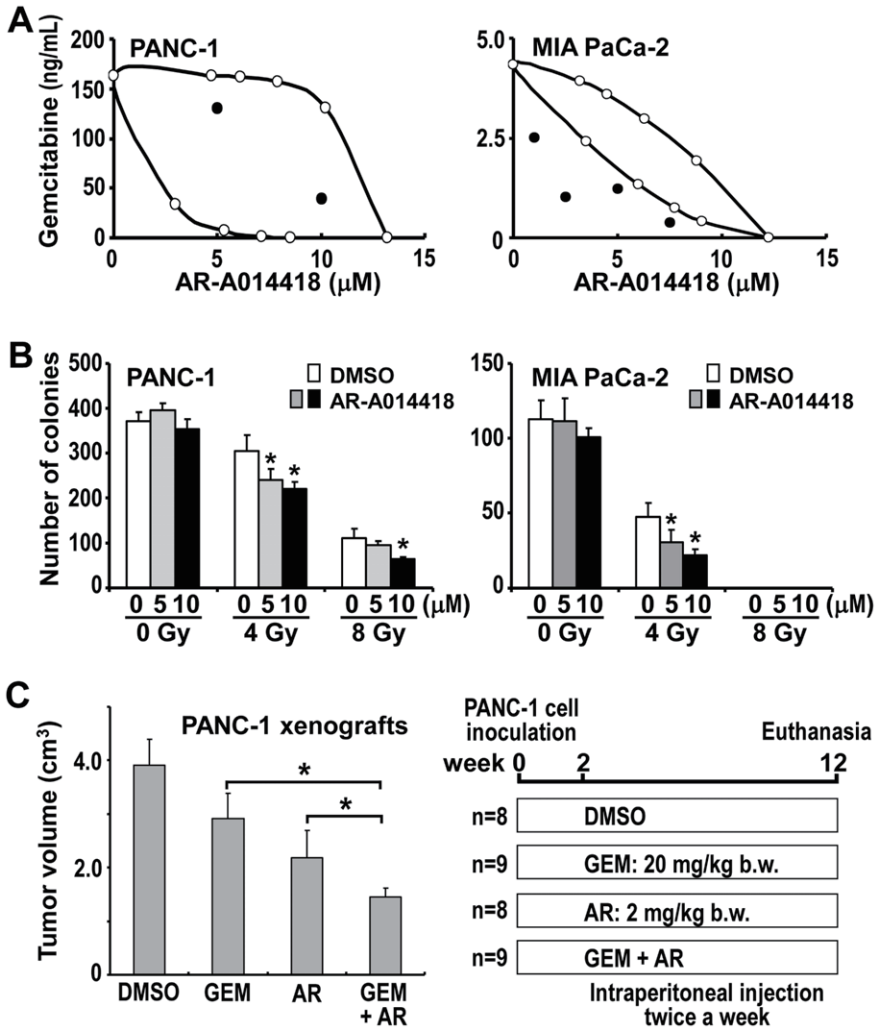

Figure 3. Combined effect of gemcitabine or ionizong radiation and GSK3β inhibitor against cancer cells and xenografts. (A) The influence of AR-A014418 on the effect of gemcitabine was analyzed using the isobologram [21] by plotting the IC_{50} of the combination therapy (Fig. S2, Table S4). (B) The combined effect of ionizing radiation and AR-A014418 was tested in PANC-1 and MIA PaCa-2 cells by colony formation assay. *$p<0.05$, statistically significant difference between cells treated with DMSO or AR-A014418. (C) The combined effect of gemcitabine and AR-A014418 was tested in PANC-1 xenografts. Athymic mice with PANC-1 xenograft were assigned to four groups for treatment with intraperitoneal injection (twice a week) of DMSO (control; 8 mice), gemcitabine (GEM; 20 mg/kg body weight; 9 mice) and AR-A014418 (AR; 2 mg/kg body weight; 8 mice), alone or in combination (GEM+AR; 9 mice). At the time after treatment for 10 weeks, 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) [10,12]. The mean tumor volume was compared between the 4 groups. *$p<0.05$, statistically significant difference between data.
doi:10.1371/journal.pone.0055289.g003

scratched as described above. At 12 hrs after scratching, the cells along the wound edges were observed by phase-contrast microscopy. These cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X (Sigma-Aldrich). The cells were incubated serially with mouse monoclonal antibody to Rac1 (BD Bioscience; diluted 1:200) at 4°C overnight and with Alexa Flour® 488-labeled anti-mouse IgG (Invitrogen; diluted 1:1,000) at room temperature for 40 min in the dark. After washing off excess antibody, the cells were stained for filamentous (F-) actin with Alexa Flour® 546-labeled phalloidin (Invitrogen; diluted 1:40) for 20 min. Then, cell nuclei were counterstained with Hoechst 33342 (Molecular Probes) and observed by fluorescence microscopy (Keyence) for expression and subcellular localization of Rac1 and F-actin.

Rac1 Activity

Protein was extracted from cells treated with DMSO or 10 μM AR-A014418 for 24 hrs in 25 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM dithiothreitol and 5% glycerol. Active Rac1 was isolated from the protein sample by the pull-down method using GST-human Pak1-PBD (Thermo) and resins (Glutathione Sepharose 4 Fast Flow; GE Healthcare). The fraction of Rac1 bound to guanosine triphosphate (GTP) (Rac1-GTP, an active form) was eluted from the resins and detected by Western blot analysis using rabbit polyclonal antibody to Rac1 (diluted 1:1,000; Thermo). Separately, whole cellular protein was probed for total Rac1 using the same antibody.

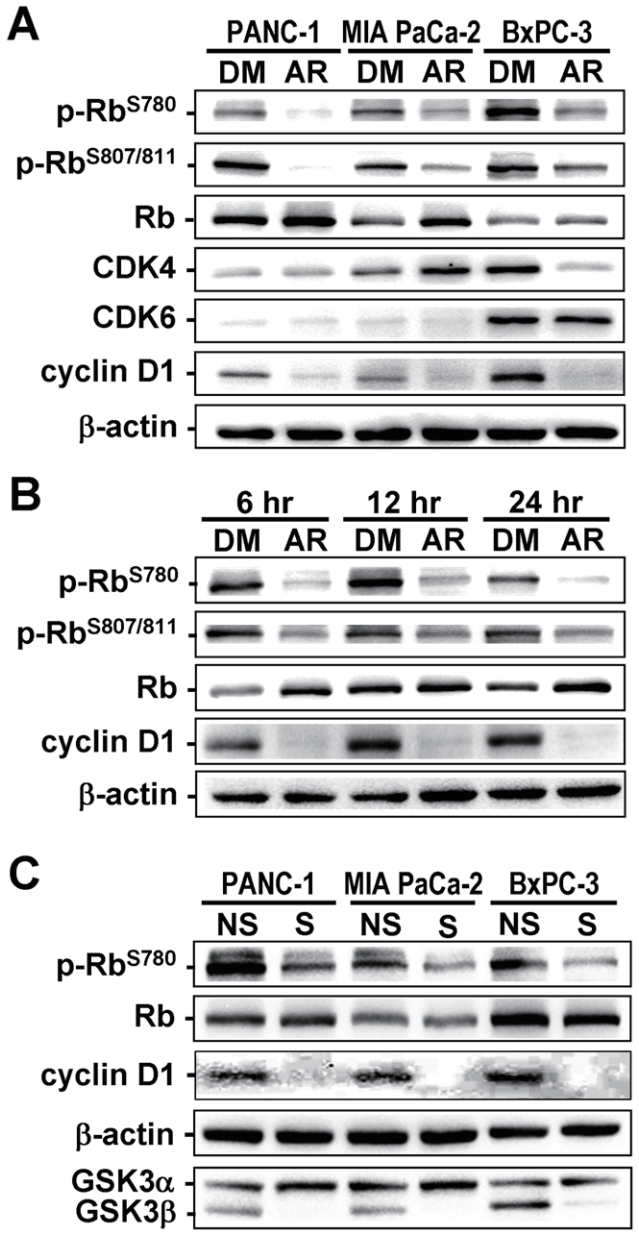

Figure 4. Changes in expression and phosphorylation of the proteins in cancer cells following GSK3β inhibition. (A) Immunoblotting analysis compares the expression of Rb, CDK4, CDK6 and cyclin D1, and the phosphorylation of Rb at S780 and S807/811 residues (p-Rb^{S780}, p-Rb$^{S807/811}$) between cells treated with DMSO (DM) or 10 μM AR-A014418 (AR) for 24 hrs. (B) Changes in levels of p-Rb^{S780} and p-Rb$^{S807/811}$ and expression of Rb and cyclin D1 were examined in MIA PaCa-2 cells at the indicated time points after treatment with 10 μM AR-A014418. (C) Expression of Rb, cyclin D1, GSK3α and GSK3β proteins and levels of Rb phosphorylation (p-Rb^{S780}) were examined and compared between the same pancreatic cancer cells transfected with non-specific siRNA (NS) or GSK3β-specific siRNA (S) (10 nM each). (A–C) β-actin expression was monitored as a loading control.
doi:10.1371/journal.pone.0055289.g004

Expression and Secretion of Matrix Metalloproteinase-2 (MMP-2)

Expression of MMP-2 mRNA was examined by quantitative reverse transcription-PCR (qRT-PCR). Total RNA was isolated from cells using ISOGEN (Wako). Complementary DNA (cDNA) was generated using a Reverse Transcription Kit (Promega). qRT-PCR was performed using SYBR Premix Ex Taq^{TM}II (Takara Bio) with the respective sets of sense and antisense primers for amplification of MMP-2 and β-actin (Table S2) [11].

MMP-2 expression was analyzed by gelatin zymography [22]. Cancer cells were seeded on 12-well plates for 48 hrs and then treated with DMSO or AR-A01418 (10 or 25 μM) for 24 hrs in serum-free medium. Conditioned medium or treated cells were incubated with SDS sample buffer for 30 min at 37°C. Samples were separated on 10% SDS-PAGE containing 0.005% Alexa Fluor 680-labelled gelatin. After electrophoresis, gels were washed in 2.5% Triton X-100 for 2 hrs and then incubated in substrate buffer overnight at 37°C. The gel was scanned by the LI-COR Odyssey IR imaging system (Lincoln).

Tumor Xenograft Study

We prepared subcutaneous PANC-1 xenografts in mice as described [23]. These mice were assigned to 4 groups and treated with intraperitoneal injection (twice a week for 10 weeks) of DMSO (diluent), gemcitabine (20 mg/kg body weight) and AR-A014418 (2 mg/kg body weight, equivalent to 10 μM in culture medium as determined and optimized in our previous studies [10,12]) alone or in combination, respectively. All mice were terminated after treatment and the xenograft tumors were removed and processed for histological examination and immunohistochemistry for expression of MMP-2 and FAK and phosphorylation of FAK in tumor cells, as described above.

Statistical Analysis

Between-group statistical significance was determined using the Student *t* test. In tumor xenograft study, tumor volumes in each treatment group were expressed as means ± standard deviation (SD). The statistical significance of differences among the data was determined with Kruskal Wallis H-test followed by Mann-Whitney U-test with Bonferroni correction. A P value of <0.05 was considered statistically significant.

Results

Expression, Phosphorylation and Activity of GSK3β in Cancer Cells

Pancreatic cancer cells showed higher basal levels of GSK3β and the Y216-phosphorylated active form (p-GSK3β^{Y216}) and lower levels of the S9-phosphorylated inactive form (p-GSK3β^{S9}) compared to HEK 293 cells (Fig. 1A). Cancer cell-derived GSK3β was active for phosphorylation of its substrate, β-catenin (Fig. 1B). These results indicate that pancreatic cancer cells express active GSK3β that is not regulated by differential phosphorylation at S9 and Y216. Immunohistochemistry for the serial sections showed that GSK3β and p-GSK3β^{Y216} were diffusely expressed and colocalized in the tumor cells and overexpressed in the invasive tumor cells of 8/15 (53%) pancreatic cancer patients (Fig. 1C). Overexpression was more frequent in patients with T3/T4 primary tumor or with lymph node and distant metastasis at the time of surgery (Table S1).

Effects of GSK3β Inhibitor on Cell Survival and Proliferation

We investigated whether GSK3β contributes to cancer cell survival and proliferation. The levels of glycogen synthase (GS) phosphorylated at Y641 (p-GS^{S641}) and p-β-catenin$^{S33/37/T41}$ decreased in cancer cells following treatment with AR-A014418 (Fig. S1A, B), indicating its activity against GSK3β in cancer cells.

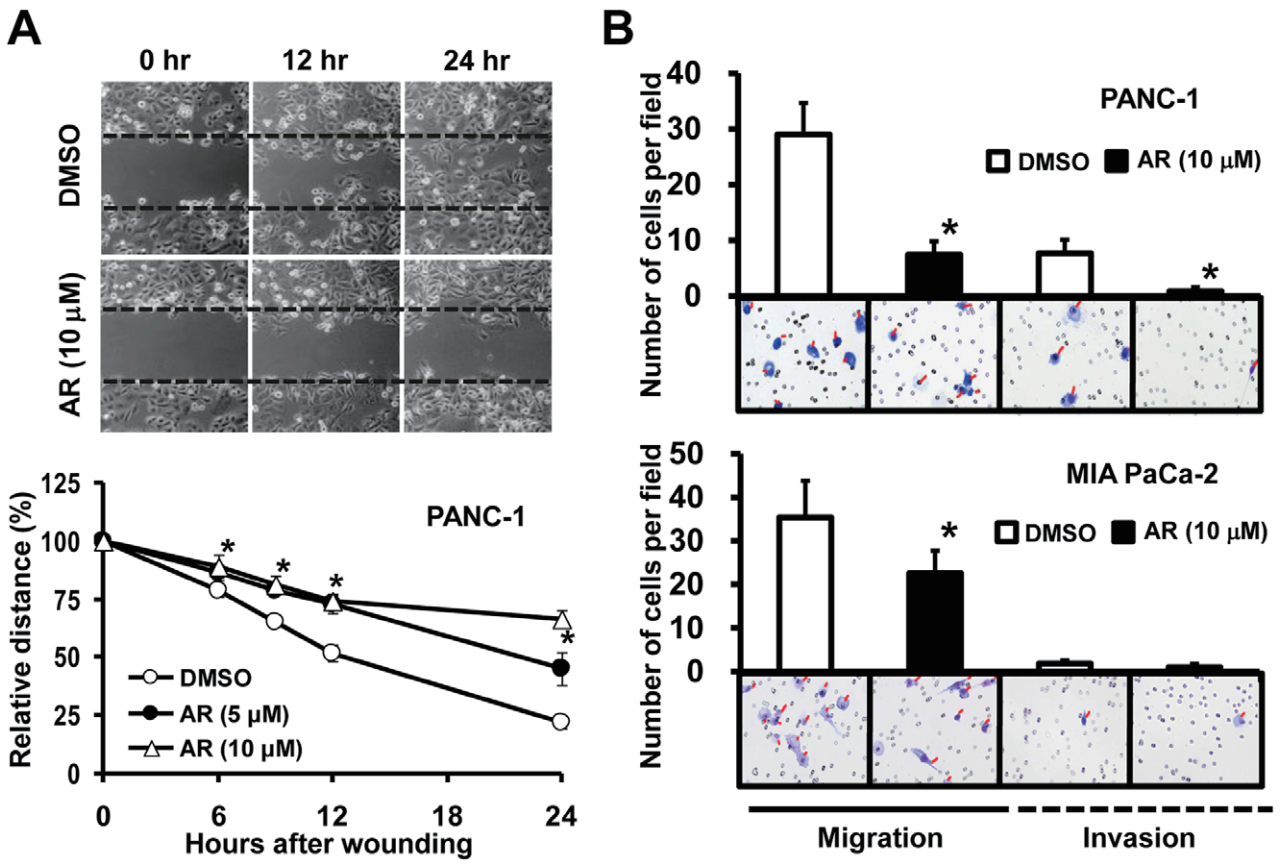

Figure 5. Effects of GSK3β inhibition on the migration and invasion of pancreatic cancer cells. (A) Upper panels show the time course for PANC-1 cell migration in a wound-healing assay in the presence of DMSO or AR-A014418 (AR). The lower panel shows the relative widths of wounds measured as a percentage of the initial gap at time zero. *$p<0.05$, statistically significant difference between cells treated with DMSO or AR-A014418. (B) Migrating cells through uncoated transwell and invading cells through matrigel-coated transwell were scored for PANC-1 and MIA PaCa-2 cells treated with DMSO or AR-A014418 (AR) for 22 hrs. Representative photomicroscopic findings in each assay are shown below the columns. *$p<0.05$, statistically significant difference between cells treated with DMSO or AR-A014418.
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GSK3β inhibition attenuated the survival and proliferation of cancer cells (Fig. 2A, C). Depletion of GSK3β by RNA interference attenuated cell viability and proliferation in all cancer cell lines (Fig. 2B, D). These results are consistent with the previous studies [16,17] suggesting that aberrant GSK3β impacts upon the survival and proliferation of pancreatic cancer cells.

Effects of GSK3β Inhibitor Combined with Gemcitabine or Radiation Against Cancer Cells

The above results led us to address whether GSK3β inhibition could enhance the effects of gemcitabine and ionizing radiation. High doses (25 or 50 μM) of AR-A014418 alone had a therapeutic effect against cancer cells (Fig. 2A, C). Therefore, the effects of relatively low doses (5 or 10 μM) were tested in combination with gemcitabine or ionizing radiation. First, we examined dose-dependent effects of AR-A014418 and gemcitabine on cancer cell survival and determined their IC_{50} values (Fig. 2A, Fig. S2). IC_{50} values for AR-A014418 were similar in PANC-1, MIA PaCa-2 and BxPC-3 cells, whereas those for gemcitabine varied (Table S3). We next examined the effect of AR-A014418 on the susceptibility of cancer cells to gemcitabine. When cells were treated with escalating doses (1 ng/mL to 10 μg/mL) of gemcitabine, combination with low dose AR-A014418 significantly reduced the IC_{50} of gemcitabine (Fig. S2, Table S4). Isobologram analysis [21] of the data revealed that low-dose AR-A014418 in combination with gemcitabine was additive against PANC-1 cells and synergistic against MIA PaCa-2 cells (Fig. 3A). We confirmed that the combined treatment with AR-A014418 significantly enhanced the effect of gemcitabine against cancer cell xenografts (Fig. 3C) in rodents with no detrimental effects by the reagent.

The effect of AR-A014418 combined with ionizing radiation was tested in cancer cells. In colony-forming cell survival assay, presence of 10 μM AR-A014418 significantly reduced viability of the cancer cells compared to treatment with ionizing radiation alone (Fig. 3B). Together, these results demonstrate that combined treatment with GSK3β inhibitor sensitizes cancer cells to gemcitabine and to ionizing radiation.

Molecular Alterations Associated with GSK3β Inhibition in Cancer Cells

To understand the mechanism that underlies involvement of GSK3β in cancer cell proliferation and resistance to therapy, we investigated the effect of GSK3β inhibition on expression and phosphorylation of proteins involved in cell cycle regulation and proliferation. Consistent with previous studies (reviewed in [2]), pancreatic cancer cells showed phosphorylation of Rb protein (p-Rb^{S780}, p-$Rb^{S807/811}$; Fig. 4), suggesting that binding to the E2F

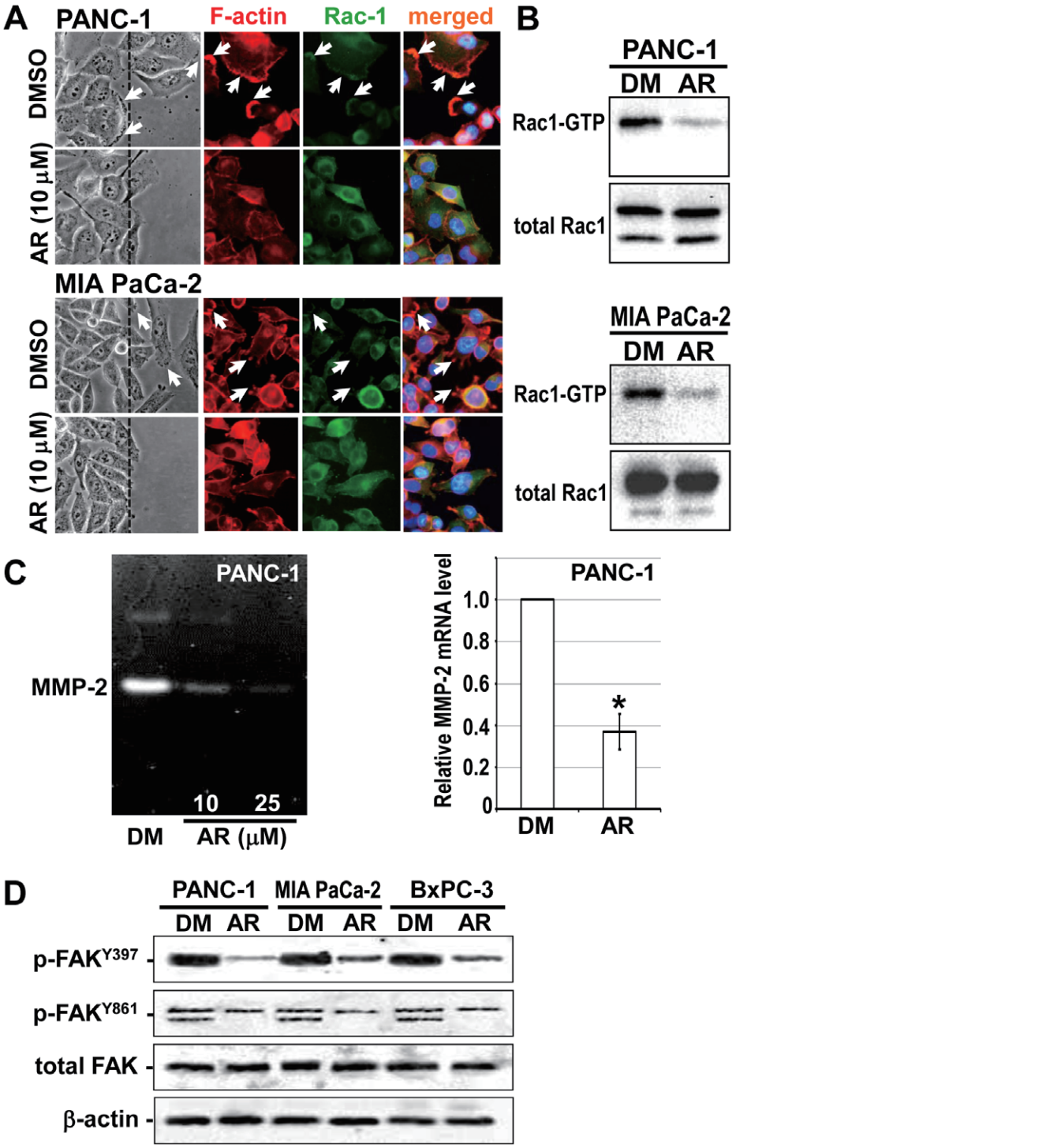

Figure 6. Changes in the invasive phenotype of pancreatic cancer cells following GSK3β inhibition. (A) Phase-contrast microscopic findings (left panels), expression and subcellular localization of F-actin and Rac-1 (middle panels) and their merged images (right panels) in cancer cells along the wound edge (dashed line) were observed in the wound-healing assay in the presence of DMSO or AR-A014418 (AR). Arrows indicate lamellipodia. (B) Changes in the levels of active (Rac1-GTP) and total Rac1 examined by pull-down assay and Western blotting between the cancer cells treated with DMSO (DM) or 10 μM AR-A014418 (AR) for 24 hrs. (C) Changes in the secretion and mRNA expression of MMP-2 examined by gelatin zymography (left panel) and qRT-PCR (right panel) between PANC-1 cells treated with DMSO (DM) or AR-A014418 (AR) for 24 hrs. Values for the relative levels of mRNA expression are shown as means ± SDs of four separate experiments. *$p<0.05$, statistically significant difference between cells treated with DMSO or AR-A014418. (D) Changes in the expression of FAK and its phosphorylation (p-FAK^{Y397}, p-FAK^{Y861}) examined by Western blotting in cancer cells following GSK3β inhibition. The cells in confluent monolayer were wounded multiple times and cultured in the presence of DMSO (DM) or 10 μM AR-A014418 (AR) for 24 hrs. β-actin expression was monitored as a loading control.
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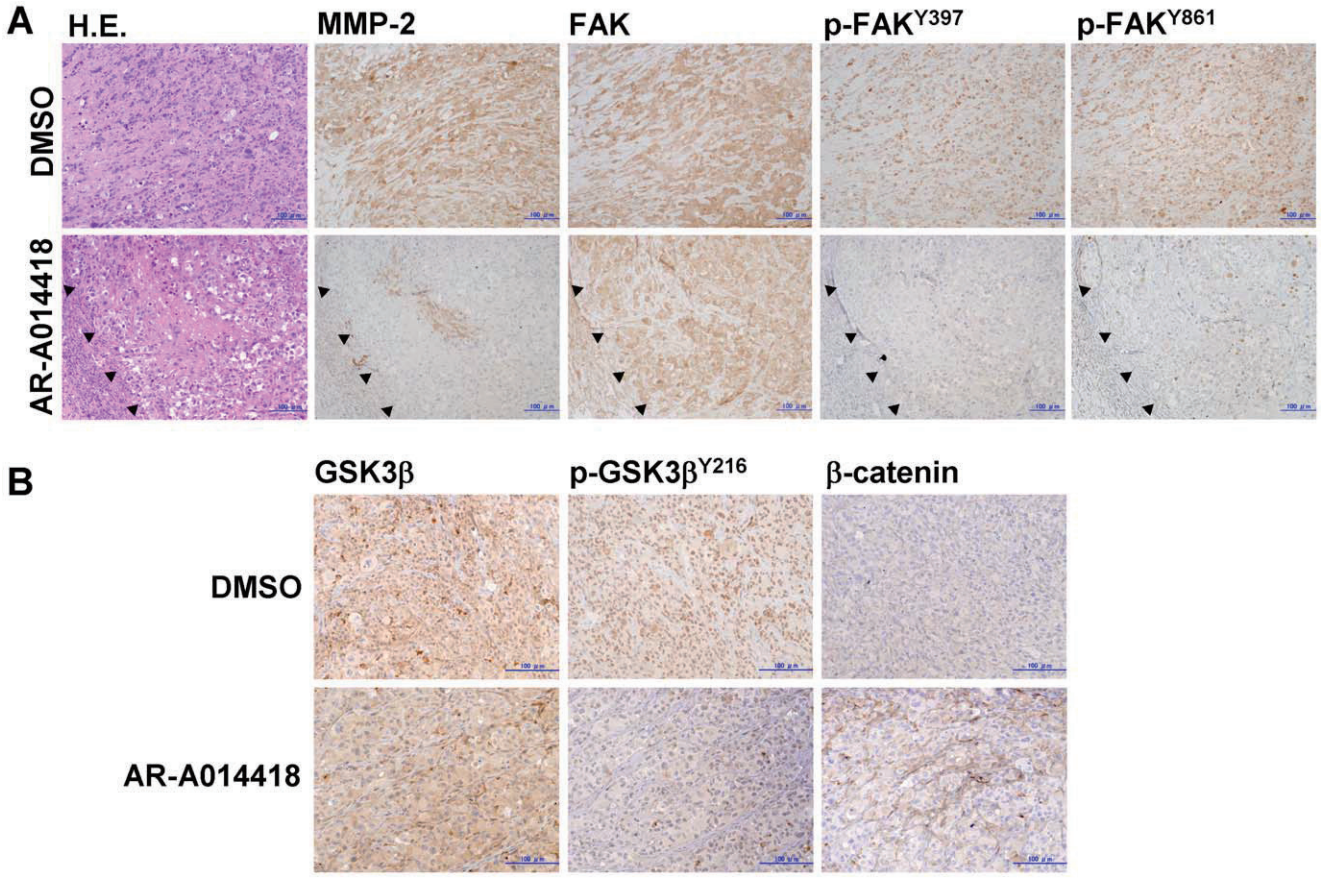

Figure 7. Changes in the invasive phenotype of pancreatic cancer cells in xenografts following GSK3β inhibition. (A) The left two panels showed represntative histological findings of the deeper part of PANC-1 xenografts in rodents after treatment with intraperitoneal injection (twice a week) of DMSO and AR-A014418, respectively, as shown in Fig. 3C. The serial sections of these tumors were immunostained for MMP-2, FAK, p-FAK^{Y397} and p-FAK^{Y861}. Closed triangles in the lower panels delineate an interface between the xenograft and host stromal tissue. (B) These tumors were immunostained for GSK3β, p-GSK3β^{Y216} and β-catenin. (A, B) The scale bar in each panel indicates 100-μm in length.
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transcription factor was impaired [24]. Treatment with either AR-A014418 or GSK3β-specific siRNA decreased the levels of Rb phosphorylation and cyclin D1 expression (Fig. 4), however no consistent changes were found for CDK4 or CDK6 expression.

Effects of GSK3β Inhibition on Cancer Cell Migration and Invasion

The wound-healing assay showed that migration of cancer cells was significantly reduced by treatment with 5 μM and 10 μM AR-A014418 (Fig. 5A, Fig. S3). Importantly, these concentrations were insufficient to inhibit cell proliferation 24 hrs after treatment (Fig. 2A). In the transwell assay, 5 μM and 10 μM AR-A014418 inhibited chemotactic migration of cancer cells and their invasion of extracellular matrix component (Fig. 5B).

Changes in the Invasive Phenotype of Cancer Cells Following GSK3β Inhibition

The above results led us to hypothesize that GSK3β has a role in cancer cell migration and invasion. This may be attributed to epithelial-mesenchymal transition (EMT), a phenotype responsible for cancer cell invasion and metastasis [25]. We investigated expression of the EMT-related molecules E-cadherin, N-cadherin and vimentin in cancer cells following treatment with AR-A04418 and GSK3β-specific siRNA. No consistent changes were observed in the levels of expression of these molecules (Fig. S1C, D), implying that EMT is unlikely to be the mechanism by which GSK3β inhibition attenuates cancer cell migration and invasion. A possible explanation may be that established cancer cell lines have already acquired the EMT phenotype.

We next focused on cellular microarchitecture and in particular on lamellipodia that plays an important role in cell migration during physiological processes and in cancer cell migration and invasion [26]. A member of the Rho-GTPase family, Rac1, participates in lamellipodia formation and in cancer progression [27]. Wound healing assay showed that migrating cancer cells form lamellipodia at the site of Rac1 localization, with actin filaments organizing the lamella structure. Treatment with AR-A014418 decreased lamellipodia formation in cancer cells at the wound edge and resulted in diffuse cytoplasmic distribution of Rac1 and F-actin (Fig. 6A). Concomitant with these changes, treatment with AR-A014418 decreased the Rac1-GTP (active form) in cancer cells stimulated to multiply by the regenerating wounds (Fig. 6B).

Rac1 was reported to increase the secretion and activity of MMP-2 in cancer cells [28]. Treatment with AR-A014418 inhibited MMP-2 secretion and decreased MMP-2 mRNA expression (Fig. 6C). These results indicate a mechanistic link between GSK3β and Rac1 in the regulation of MMP-2 expression and secretion. Previous studies have reported that FAK regulates

Rac1 [29]. We therefore investigated the effect of GSK3β inhibition on FAK activity in cancer cells responding to wound stimulation by determining the levels of p-FAK^{Y397} and p-FAK^{Y861} [29]. FAK phosphorylation was detected in cancer cells stimulated by multiple wounds. Treatment with 10 μM AR-A014418 reduced the levels of p-FAK^{Y397} and p-FAK^{Y861} (Fig. 6D), suggesting a pivotal role for the GSK3β/FAK/Rac1 pathway in promoting pancreatic cancer cell invasion.

Immunohistochemical examination of the primary pancreatic cancers showed higher expression of MMP-2 and FAK and tyrosine phosphorylation of FAK in tumor cells than non-neoplastic pancreatic ducts (Fig. 1D). In histological examination of the deep part of cancer cell xenografts, the tumor in mice treated with AR-A014418 showed less invasive than those in sham (DMSO)-treated mice (Fig. 7A, left panels). Immunohistochemistry showed decreases in MMP-2 expression and FAK phosphorylation in the tumors by treatment with AR-A014418 (Fig. 7A). Both groups of mice showed the similar levels of GSK3β expression in tumor xenografts (Fig. 7B, left panels). In AR-A014418-treated mice, Y216-phosphorylation of GSK3β appeared to decrease in the tumors, and membranous expression, but no nuclear accumulation of β-catenin was observed in tumor cells (Fig. 7B).

Discussion

Failure of treatments for pancreatic cancer is due to the high propensity of these tumors to invade surrounding tissues and because of their resistance to chemotherapy and radiation [3,4]. The present study confirmed previously reported roles for GSK3β in cancer cell survival and proliferation [16,17]. However, the novel findings of this study relate to the effects of GSK3β inhibition on the invasive ability and phenotype of pancreatic cancer cells and on their susceptibility to gemcitabine and radiation. The results provide a biological rationale for combinational treatment strategies that include targeting of GSK3β in order to control refractory pancreatic cancer.

The proinvasive phenotype of cancer cells includes EMT and increased cell motility [25,26]. Here, we found overexpression of GSK3β and its active form in the tumor cells in the invasive primary pancreatic cancers. Inhibition of GSK3β attenuated cancer cell migration and invasion in wound-healing and transwell assays. No consistent changes were observed for the expression of EMT-related molecules in cancer cells treated with GSK3β inhibitor, in contrast to a previous study that found GSK3β inhibits EMT by phosphorylating and destabilizing snail, a transcriptional repressor of E-cadherin [30]. Our study suggests that EMT may not be involved in the mechanism by which GSK3β inhibition attenuates cancer cell migration and invasion. In wound-healing assays, the migrating cancer cells formed lamellipodia where Rac1 and F-actin preferentially co-localized. Treatment with GSK3β inhibitor decreased lamellipodia formation and re-distributed Rac1 and F-actin throughout the cytoplasm. GSK3β inhibition decreased the active fractions of FAK and Rac1 and the expression and secretion of MMP-2. These findings indicate for the first time the existence of a key pathway involving GSK3β, FAK and Rac1 that plays a pivotal role in promoting pancreatic cancer invasion and could provide a novel target for inhibiting cancer cell invasion.

Rac1 cycles between active GTP-bound and inactive guanosine diphosphate (GDP)-bound forms. Rho-GTPase activity is regulated by three classes of proteins including guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and GDP dissociation inhibitors [27]. GEFs that activate GTPases participate in cancer progression and are considered as therapeutic targets [31]. Aberrant expression of Vav1, a Vav family member of GEFs, is frequently observed in pancreatic cancer and associated with active Rac1 and worse prognosis [32]. Within the cell migration machinery, the IQ motif-containing GAPs (IQGAPs) form scaffolds with Rac1 and Cdc42 (another member of Rho-GTPases) and bind to F-actin. In migrating cells, this molecular complex localizes to actin-rich cellular structures such as lamellipodia and controls F-actin and microtubule dynamics [33]. GSK3β regulates microtubule stability by phosphorylating microtubule-binding proteins, while Cdc42 and GSK3β interact spatially to dictate the polarity of migrating cells [34,35]. IQGAPs participate in cancer cell invasion and the overexpression of IQGAP1 is associated with unfavorable prognosis in various cancers [36,37]. A previous study indicated that Rac1 destabilizes E-cadherin-mediated cell adhesion in pancreatic cancer by interacting with IQGAP1, thereby promoting cancer cell migration [38]. Further work is required to clarify the putative roles for GSK3β in regulating cytoskeletal structure, cell polarity and motility and its promotion of cancer cell migration and invasion.

Resistance of cancer cells to chemotherapeutic agents, radiation and molecular target-directed agents is a critical determinant of outcome from recurrent and unresectable pancreatic cancer [3–5]. Many studies have investigated the mechanisms by which pancreatic cancer cells resist or acquire resistance to chemotherapy and radiation, focusing mainly on NF-κB [39]. GSK3β was reported to sustain pancreatic cancer cell survival by maintaining the transcriptional activity of NF-κB [16,17]. However, a recent study found that disruption of NF-κB activity through GSK3β inhibition did not sensitize cancer cells to gemcitabine [40]. Whereas these studies examined the activity of exogenous (transfected) NF-κB, we previously found no effect of GSK3β inhibition on endogenous NF-κB transcriptional activity in gastrointestinal cancers (including pancreatic cancer) and glioblastoma [11,12]. Therefore, a role for GSK3β in regulating NF-κB activity in cancer cells remains controversial.

In the present study, we tested various combinations and doses of gemcitabine and AR-A014418 on the survival of pancreatic cancer cells. AR-A014418 sensitized the cells to gemcitabine when the doses of both agents were individually optimized. Consistent with our preliminary study [23], the present study confirmed the effect of this drug combination in the cancer cell xenografts. Elucidation of the underlying biological mechanism is important to justify the combinational treatment of GSK3β inhibitor with gemcitabine. One possible mechanism is suggested by our observation that GSK3β inhibition decreased Rb phosphorylation, thus restoring its function against E2F. Ribonucleotide reductase (RR), thymidylate synthase (TS) and thymidine kinase (TK) are transcriptional targets for E2F and essential for DNA synthesis and replication [41]. It was reported that pancreatic cancers with increased RR expression are resistant to gemcitabine [42]. Therefore, restoration of Rb ability to bind to E2F following GSK3β inhibition may sensitizes cancer cells to gemcitabine by affecting RR expression. Inhibition of GSK3β also enhanced the effect of ionizing radiation against cancer cells. Decreases in E2F-dependent transcription of TS and TK may be responsible for this radio-sensitization effect via the impairment of radiation-induced DNA damage repair. In contrast to our result, a recent study reported that siRNA-mediated GSK3β silencing promotes pancreatic cancer cell survival following irradiation via stabilization and activation of β-catenin [43]. Clarification of the role of GSK3β in modulating the anti-tumor effects of radiation is therefore a priority for future investigations.

While the levels of GSK3β activity differ between the pancreatic cancer cells examined in this study (Fig. 1B), its inhibitor AR-A014418 has the similar IC_{50} values against them (Fig. 2A, Table S3). This is consistent with our previous studies showing the similar therapeutic effects of this inhibitor in its pharmacologic doses [20] against different gastrointestinal cancer cells [9,12] and glioblastoma cells [11]. Other studies also reported the similar result in pancreatic cancer cells [16,17]. These studies may suggest that survival of different cancer cells and/or their susceptibility to AR-A014418 might similarly depend on GSK3β activity in cells. This issue should be an important future task for cancer treatment targeting GSK3β.

Several studies have suggested opposite roles for GSK3β in the same cellular events mediated by the protooncoproteins and tumor suppressors between non-neoplastic and cancer cells [14]. Whereas GSK3β phosphorylates and stabilizes $p27^{Kip1}$ in normal cells [44], it down-regulates $p27^{Kip1}$ in leukemia cells and selectively maintains the survival and proliferation of these cells [45]. Despite its role in destabilizing cyclin D1 in physiological cells [7,8,13], inhibition of GSK3β decreases cyclin D1 expression in cancer cells [11,12,46]. Other studies reported various roles for GSK3β in regulating cell stemness. It was shown that GSK3β inhibition maintains the pluripotency of embryonic stem cells and the repopulation of hematopoietic stem cells through activation of the Wnt and hedgehog pathways, respectively [47,48]. Conversely, it was reported that GSK3β sustains tumor cell stemness in leukemia and glioblastoma [45,49]. Our present and previous studies showing the presence of both inactive (p-$GSK3\beta^{S9}$) and active (p-$GSK3\beta^{Y216}$) forms in non-neoplastic cells and tissues [9,12] suggest that the kinase activity is regulated by differential phosphorylation in these key residues depending on stimuli in cells of non-neoplastic origin. Although the underlying mechanisms are yet to be understood, differential roles for GSK3β in normal and neoplastic cells could be advantageous for cancer treatment strategies that target this kinase. Consistently, the animal studies showed little detrimental effects of GSK3β inhibition on normal cells and vital organs [10,12,23], leading to promotion of future clinical application of cancer treatment targeting GSK3β.

Concerns regarding the therapeutic use of GSK3β inhibitors remain, because these may activate oncogenic (e.g., Wnt) signaling and thus promote cellular transformation [7,8,13]. Unlike this hypothesis, inhibition of GSK3β is not sufficient to stabilize β-catenin in normal cells and this seems to occur only when other transforming events (eg., adenomatous polyposis coli [APC] protein truncation) have already taken place [50]. In normal cells, the known function of GSK3β in mediating Wnt/β-catenin signaling depends on cell membrane-associated fraction of GSK3β that antagonizes the phosphorylation of β-catenin by cytoplasmic GSK3β, a key step initiating β-catenin degradation [51]. These paradoxical roles of GSK3β in cells partly supports the reports showing that GSK3β inhibition does not influence the survival or growth of normal cells, nor induce their transformation ([9,12,16,19]; reviewed in [14]). Consistently the above concerns have not deterred preclinical studies of GSK3β inhibitors for the treatment of many cancer types [14], or Phase II clinical trials for the treatment of neurological diseases [52]. These trials are rationally supported by the differential roles of GSK3β in cellular signaling events between normal and tumor cells and by the phosphorylation-dependent regulation of GSK3β activity that presumably protects normal cells from transformation by GSK3β inhibition [9–12,14]. Currently, two clinical trials are being undertaken to test whether the GSK3β inhibitor LY2090314 (Eli Lily) enhances the efficacy of established chemotherapeutic agents for advanced solid cancers (http://clinicaltrials.gov/ct2/show/study/NCT01287520) and leukemia (http://clinicaltrials.gov/ct2/show/study/NCT01214603).

Supporting Information

Figure S1 Effects of GSK3β inhibition on expression and phosphorylation of the proteins in pancreatic cancer cells. The levels of expression and phosphorylation of the indicated proteins were examined by Western blotting in pancreatic cancer cells after treatment with the respective agents. (A, B) Expression of GS and β-catenin and their phosphorylation (p-GS^{S641}, p-β-catenin $^{S33/37/T41}$) were examined and compared between the same pancreatic cancer cells treated with DMSO (DM) or 10 μM AR-A014418 (AR) for 6 hrs. (C) Expression of E-cadherin, N-cadherin and vimentin in pancreatic cancer cells treated with DMSO (DM) or 10 μM AR-A014418 (AR) for 6 hrs. (D) Expression of E-cadherin, N-cadherin, vimentin and GSK3α and GSK3β in pancreatic cancer cells transfected with non-specific siRNA (NS) or GSK3β-specific (S) siRNA (10 nM each). (A–D) The amount of protein extract in each sample was monitored by expression of β-actin.
(TIF)

Figure S2 Effects of gemciatbine and AR-A014418, alone or in combination, against pancreatic cancer cells. Inhibitory effects of gemcitabine, AR-A014418 and combinations of the two agents at different doses were examined on the survival of pancreatic cancer cells. PANC-1 (A) and MIA PaCa-2 (B) cells were treated with escalating doses of either gemcitabine, AR-A014418 or both agents in combination at the doses indicated. Relative (%) cell survival ratios for each cell line were examined by WST-8 assay at 48 hrs after treatment with the respective agent. IC_{50} of gemcitabine in the absence (+ DMSO) or presence of AR-A014418 (+ AR) at the indicated doses was determined and is shown in Table S4.
(TIF)

Figure S3 Effect of GSK3β inhibitor on pancreatic cancer cell migration. The time course for cell migration was minitored by monolayer-based wound healing assay for MIA PaCa-2 and BxPC-3 cells in the presence of DMSO or AR-A014418 (AR 5 μM, AR 10 μM). The relative widths of wounds were measured and expressed as a percentage of the initial gap at time zero. Values are means ± SD of three separate experiments. *$p<0.05$, statistically significant difference between cells treated with DMSO or AR-A014418.
(TIF)

Table S1 Clinical and pathologic characteristics of patients with pancreatic cancer.
(DOC)

Table S2 Primary antibodies used for Western blotting and Sequences of the primers used for RT-PCR amplification.
(DOC)

Table S3 Comparison of IC_{50} values of gemcitabine and a GSK3β inhibitor (AR-A014418) between pancreatic cancer cell lines (PANC-1, MIA PaCa-2 and BxPC-3).
(DOC)

Table S4 Changes in 50% cell survival inhibitory concentration (IC_{50}) of gemcitabine in combination with different doses of AR-A014418 in pancreatic cancer.
(DOC)

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Author Contributions

Conceived and designed the experiments: AK TS YM TM. Performed the experiments: AK TS YC MH HS. Analyzed the data: AK TS YC MN MH YI TT HS YM KK TM. Contributed reagents/materials/analysis tools: AK TS MN TH YE TT HS YS KK. Wrote the paper: TS MN KM YM KK TM.

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Glycogen synthase kinase 3β inhibition sensitizes human glioblastoma cells to temozolomide by affecting O^6-methylguanine DNA methyltransferase promoter methylation via c-Myc signaling

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Glycogen synthase kinase 3β (GSK3β) is a serine/threonine protein kinase involved in human cancers including glioblastoma. We have previously demonstrated that GSK3β inhibition enhances temozolomide effect in glioma cells. In this report, we investigated the molecular mechanisms of sensitization of glioblastoma cells to temozolomide by GSK3β inhibition, focusing on O^6-methylguanine DNA methyltransferase (MGMT) gene silencing. Glioblastoma tissues from patients treated with the GSK3β-inhibiting drugs were subjected to immunohistochemistry and methylation-specific PCR assay. Human glioblastoma cell lines T98G, U138, U251 and U87 were treated with a small-molecule GSK3β inhibitor, AR-A014418 or GSK3β-specific small interfering RNA. The combined effect of temozolomide and AR-A014418 on cell proliferation was determined by AlamarBlue assay and an isobologram method. MGMT promoter methylation was estimated by methylation-specific PCR and MethyLight assay. MGMT gene expression was evaluated by real-time quantitative reverse transcriptase-PCR. c-Myc and DNA (cytosine-5)-methyltransferase 3A binding to the MGMT promoter was estimated by chromatin immunoprecipitation assay. GSK3β inhibition decreased phosphorylation of glycogen synthase and reduced MGMT expression and increased MGMT promoter methylation in clinical tumors. In glioblastoma cell lines, GSK3β inhibition decreased cell viability, enhanced temozolomide effect and downregulated MGMT expression with relevant changes in the methylation levels of the MGMT promoter. Here, we showed for the first time that c-Myc binds to the MGMT promoter with consequent recruitment of DNA (cytosine-5)-methyltransferase 3A, regulating the levels of MGMT promoter methylation. The results of this study suggest that GSK3β inhibition enhances temozolomide effect by silencing MGMT expression via c-Myc-mediated promoter methylation.

Introduction

Glioblastoma (GBM) is the most frequent malignant tumor of the brain and is highly unresponsive to the currently available anticancer treatments. The proliferative and invasive activity of GBM (1) hinders curable surgical intervention and makes GBM highly resistant to radiation and chemotherapy (2) with median patient survival showing little improvement over the past 30 years (3). Consequently, there is an urgent need to develop new treatment modalities represented by molecular target-directed therapies (4,5).

O^6-methylguanine DNA methyltransferase (MGMT) promoter methylation is an independent favorable prognostic factor in patients with GBM (6). The levels of MGMT gene promoter methylation are associated with MGMT expression and are important in setting the transcriptional state of the MGMT promoter through changes in chromatin structure (7). Although the MGMT promoter is methylated only in 22% of GBM cases, methylation status of the promoter is implicated in chemosensitivity to temozolomide (TMZ) (6). Therefore, patients with a methylated MGMT promoter may benefit from TMZ therapy (6).

Glycogen synthase kinase 3β (GSK3β) is a multifunctional protein kinase that regulates various cellular pathways depending on its substrates for phosphorylation (8). We have shown that deregulated GSK3β expression and activity maintains survival and proliferation and that GSK3β-specific inhibitors suppress survival and proliferation and induce apoptosis in human GBM cells (9,10). We have also demonstrated that GSK3β inhibition significantly sensitized GBM cell lines to TMZ (10). Based on our research, we are conducting clinical research for the therapeutic effect of GSK3β inhibition in recurrent GBM.

Here, we investigated the molecular mechanisms underlying a combined effect of GSK3β inhibition with TMZ, focusing on MGMT gene silencing and its causal relationship with c-Myc signaling, which is known to be upregulated by GSK3β inhibition (11,12). The results suggest that GSK3β inhibition enhances TMZ effect by decreasing MGMT expression via its c-Myc-mediated promoter methylation.

Material and methods

Clinical tumor samples and histological examination

In our institute, clinical research entitled 'Chemotherapy for recurrent malignant glioma with combined usage of temozolomide and GSK3β-inhibiting drugs' is ongoing under the approval by the Medical Ethics Committee of Kanazawa University Hospital (UMIN Clinical Trial Registry: UMIN000005111). According to the institutional review board-approved protocol, fresh GBM tissues were obtained at surgery and/or autopsy. The histological diagnosis of tumor was determined according to the revised World Health Organization criteria (13). In seven patients registered for the trial, autopsy was available for three patients with obtained informed consent from their families and was performed 2 h after death. Patients 1 and 2 underwent administration of GSK3β-inhibiting drugs until death with last administration within 8 h antemortem. One patient stopped GSK3β-inhibiting drugs 3 months prior to death. Patients 1 and 3 died from cerebrospinal fluid dissemination of GBM. Histology did not differ between the primary tumor and the recurrent tumor obtained by autopsy. In patient 2, the primary brain stem tumor tissues obtained by needle biopsy were diagnosed with GBM. This patient died from enlargement of the brain stem lesion. The autopsy specimens showed the presence of GBM. All tumor samples obtained were fixed in neutral-buffered 10% formalin and were embedded in paraffin. The detailed information and outcome of this clinical research will be submitted elsewhere.

Immunohistochemistry

Immunohistochemistry was performed for the clinical tumor tissues as described previously (14). Briefly, formalin-fixed and paraffin-embedded tissue blocks were sectioned (6 μm thick) onto slides and then deparaffinized. Sections were immunostained using an Envision+ Kit (Dako, Tokyo, Japan) with anti-glycogen synthase polyclonal antibody, anti-phospho-glycogen synthase polyclonal antibody specific to serine (S) 641-phosphorylated glycogen synthase (p-GS^{S641}) and anti-MGMT antibody (Cell Signaling

Abbreviations: ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; DNMT3A, DNA (cytosine-5)-methyltransferase 3A; EGS, ethylene glycol bis(succinimidylsuccinate); GBM, glioblastoma; GS, glycogen synthase; GSK3β, glycogen synthase kinase 3β; MGMT, O^6-methylguanine DNA methyltransferase; MSP, methylation-specific PCR; p-GS^{S641}, serine (S) 641-phosphorylated glycogen synthase; qPCR, quantitative real-time PCR; QRT–PCR, quantitative reverse transcriptase–polymerase chain reaction; siRNA, small interfering RNA; TMZ, temozolomide.

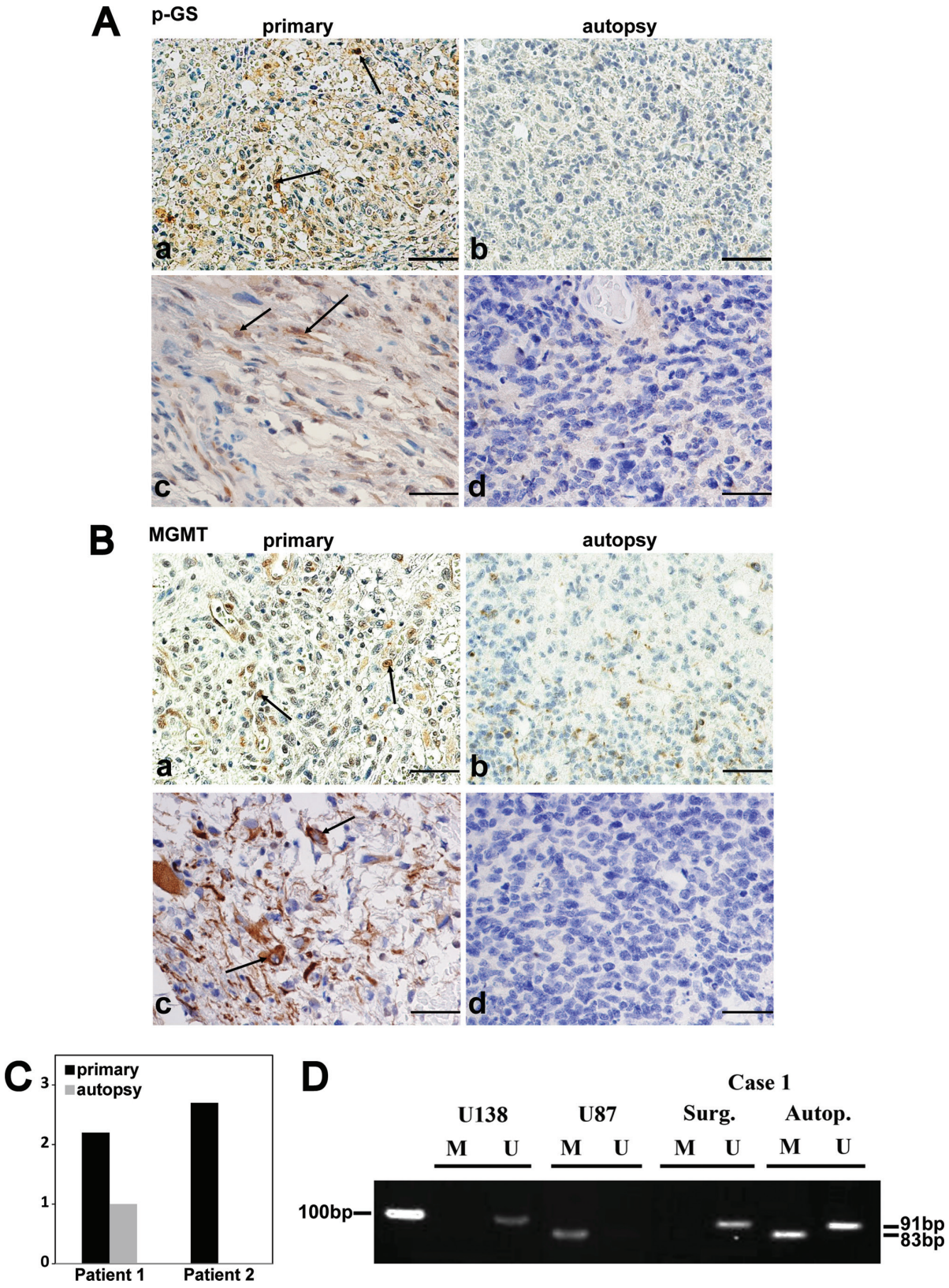

Fig. 1. (**A**) The immunohistochemical localization of phosphorylated GS in tissues. a and b, patient 1; c and d, patient 2; a and c, primary tumor; b and d, the GSK3β-inhibiting drug-treated recurrent tumor obtained at autopsy. Paraffin sections were immunostained with the antibody against p-GS^{S641}. Note that

Technology, Danvers, MA) using a 1:200 dilution. Sections were microwaved for 15 min in target retrieval solution (pH 9.0; Dako), blocked by incubation in 0.3% H_2O_2 solution in methanol for 20 min. Non-specific immunoreactions were blocked at room temperature for 30 min using a Protein-Block Kit (Dako). Slides were incubated with rabbit anti-p-GS^{S641} antibody, anti-glycogen synthase antibody or rabbit anti-MGMT antibody overnight at 4°C, and they were washed and secondary antibody was applied for 30 min. Sections were exposed to diaminobenzidine peroxidase substrate (Funakoshi) for 3–5 min and counterstained with Mayer's hematoxylin. Non-immune rabbit IgG (Sigma–Aldrich, St Louis, MO) was used as a negative control.

DNA extraction

After microscopic observation of hematoxylin and eosin-stained sections by a neuropathologist (H.S.), DNA was extracted from the tumor areas of formalin-fixed and paraffin-embedded tissue samples of patient 1 by using a QIAamp DNA Mini Kit™ (Qiagen, GmbH, Hilden, Germany). The amount of the sample obtained by needle biopsy in patient 2 was not enough for DNA extraction.

Cell cultures

Human GBM cell lines T98G, U138, U251 and U87 were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum both from GIBCO (Grand Island, NY) at 37°C and 5% CO_2.

Effects of GSK3β inhibitors on tumor cells

GBM cells were serum starved for 24 h and seeded in the cell proliferation assay format, as detailed elsewhere (15–17). Briefly, cells were seeded in 96-well plastic plates and treated with dimethyl sulfoxide (DMSO) as a vehicle control or with a small-molecule GSK3β inhibitor AR-A014418 (Sigma) dissolved in equivalent amount of DMSO at escalating final concentrations (5–80 μmol/l). Medium was replaced after 72 h of incubation in all treatment and control wells with the medium of the same formulation (containing the same final concentration of AR-A014418) (17). The proliferation assay was performed by adding AlamarBlue (AbD Serotec, Kidlington, UK) to the cells 144 h after cell seeding to assess proliferation according to the manufacturer's protocol. Absorbance values were measured by spectrophotometry using a Microplate Reader Model 550 (Bio-Rad, Hercules, CA) at 540 and 590 nm. The relative cell viability was determined by calculated values of the percent difference in reduction of AlamarBlue between treated and control cells as described in the manufacturer's protocol. The experiments were carried out in sextuplicates.

RNA interference

GBM cells seeded into 96-well plates were transfected with small interfering RNA (siRNA) specific to human GSK3β (target sequence, 5′-GCUCCAGAUCAUGAGAAAGCUAGAU-3′) proved to be effective in glioma cells in our previous study (10) or negative control siRNA (Stealth RNAi Negative Control Low GC duplex) both from Invitrogen (Grand Island, NY), or β-catenin-specific siRNA (Cell Signaling Technology). Lipofectamine RNAiMAX (Invitrogen) was used for transfection according to the manufacturer's protocol. The relative cell viability was determined 72 h after incubation by the AlamarBlue assay.

Western blot analysis

Western blot analysis was performed on polyvinylidene difluoride membrane (Invitrogen), according to the standard procedure as described previously (14,16). Rabbit monoclonal anti-c-Myc antibody (Epitomics, Burlingame, CA), mouse monoclonal anti-GSK3β antibody and mouse monoclonal anti-tyrosine 216-phosphorylated GSK3β antibody (BD Biosciences, San Jose, CA), anti-DNA (cytosine-5)-methyltransferase 3A antibody (Imgenex, San Diego, CA), anti-β-catenin antibody and anti-MGMT antibody (Cell Signaling Technology) were used at a dilution of 1:5000, 1:10 000, 1:5000, 1:1000, 1:5000 and 1:2500, respectively.

Effect of GSK3β inhibition on the sensitivity of GBM cells to TMZ

After serum starvation for 24 h, GBM cells were seeded in the cell proliferation assay format. The cells were treated with DMSO (vehicle control), with escalating concentrations (50, 100, 200 and 400 μmol/l) of TMZ (Sigma), AR-A014418 (5, 10 and 20 μmol/l) or the combinations of indicated concentrations of TMZ and AR-A014418 dissolved in equivalent amount of DMSO. After treatment for 144 h, cells were subjected to the AlamarBlue assay to determine the dose-dependent effect and IC50 of TMZ and AR-A014418 against GBM cells and to observe the influence of AR-A014418 (5, 10 or 20 μmol/l) on TMZ effect against GBM cells. The influence of GSK3β inhibition on TMZ effect against GBM cells was analyzed using classic isobologram method (18) and median dose-effect analysis (19,20). The IC_{50} values and combination indices of the combination therapy by various concentrations of TMZ and AR-A014418 were plotted to determine whether the effect of GSK3β inhibitor on GBM cell sensitivity to TMZ was additive, synergistic or antagonistic.

Methylation-specific PCR assay

Isolated effect of GSK3β inhibition and combined influence of GSK3β and c-Myc inhibition on methylation status of the MGMT promoter were evaluated by methylation-specific PCR (MSP) assay as described previously (21,22). T98G, U251 and U138 cells were serum starved for 24 h and then treated with DMSO (control) or AR-A014418 (5, 10 or 20 μmol/l) or combination of DMSO–AR-A014418 (5, 10 or 20 μmol/l) and c-Myc inhibitor 20 μmol/l (EMD Millipore, Billerica, MA) dissolved in equivalent amount of DMSO. After treatment for 72 h, genomic DNA was extracted from the cells using a QIAamp DNA Mini Kit (Qiagen). Sodium bisulfite conversion of 200 ng of the purified DNA was performed using an EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's protocol. MSP of bisulfate-converted DNA was carried out in a nested, two-stage PCR approach as described previously (22) using GeneAmp PCR System 2700 (Applied Biosystems, Grand Island, NY). U87 and U138 cell lines were used as methylated and unmethylated controls, respectively. Amplified PCR products were separated by 3% agarose gel electrophoresis and visualized with ethidium bromide.

MethyLight assay

T98G, U251 and U138 cells were serum starved for 24 h and then treated with DMSO (control) or escalating concentrations of AR-A014418 (5, 10 or 20 μmol/l) dissolved in equivalent amount of DMSO or combination of DMSO–AR-A014418 (5, 10 or 20 μmol/l) and c-Myc inhibitor 20 μmol/l. After treatment for 72 h, genomic DNA was extracted from the cells using a QIAamp DNA Mini Kit and subjected to bisulfite conversion using an EpiTect Bisulfite Kit followed by quantitative real-time PCR (qPCR) of bisulfite-converted DNA using PCR premix TaKaRa ExTaq (TaKaRa, Otsu, Japan) with primers and probe specific to methylated fraction of the MGMT promoter. Probe and forward primer sequences were taken from other study (23): probe, 6FAM-CCTTACCTCTAAATACCAACCCCAAACCCG-BHQ-1; forward primer, 5′-CTAACGTATAACGAAAATCGTAACAACC-3′; reverse primer designed by authors, 5′-AGTATGGAAGGGTAGGAAGAATTCG-3′. Alu was utilized as a calibrator (24) with following probe and primers: probe, 6FAM-CCTACCTTAACCTCCC-BHQ-1; forward primer, 5′-GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA-3′; reverse primer, 5′-ATTAACTAAACTAATCTTAAACTCCTAACCTCA-3′. The qPCR was performed using LightCycler 1.5 (Roche Diagnostics GmbH, Mannheim, Germany) with protocol consisting of an initial denaturation step at 95°C for 30 s followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. LightCycler Software 4.1 was used to analyze the qPCR data. Threshold cycle values (Ct) were determined and relative methylation of the MGMT promoter was calculated using the ΔC_T method described by the manufacturer using Alu as a calibrator gene.

Real-time quantitative reverse transcriptase–polymerase chain reaction

T98G, U251 and U138 cells were serum starved for 24 h and then treated with DMSO (control) or escalating concentrations of AR-A014418 (5, 10 or 20 μmol/l) dissolved in equivalent amount of DMSO. After treatment for 72 h, total RNA was isolated from the cells using a GenElute Mammalian Total RNA Miniprep Kit (Sigma). Complementary DNA was synthesized from total RNA using a High

←
phosphorylation of GS is found in the cells of primary tumors (arrows), whereas faint staining is observed in the recurrent tumors obtained at autopsy. Cell nuclei were counterstained with hematoxylin. Scale bars, 50 μm. (**B**) The immunohistochemical expression of MGMT protein in the tumor tissues. a and b, patient 1; c and d, patient 2; a and c, primary tumor; b and d, the GSK3β-inhibiting drug-treated recurrent tumor obtained at autopsy. Cells with nuclear staining were considered as positive for MGMT expression (arrows). Cell nuclei were counterstained with hematoxylin. Scale bars, 50 μm. (**C**) Staining intensity of glioma cells was scored individually for MGMT antibody in each specimen and was classified as no (0), weak (1), moderate (2) or strong staining (3); mean staining intensity was calculated separately for primary specimen and autopsy specimen in patients 1 and 2. (**D**) MSP assay for methylation status of the MGMT promoter in GBM cells and the primary and recurrent tumors of the patients. PCR products in the M lanes and U lanes indicate methylated and unmethylated status of the MGMT promoter, respectively. U138, U138 GBM cell line as an unmethylated control; U87, U87 GBM cell line as a methylated control. The representative case (patient 1) is shown. Surg., primary tumors at surgery; Autop., recurrent tumor obtained at autopsy following the treatment with the GSK3β-inhibiting drugs.

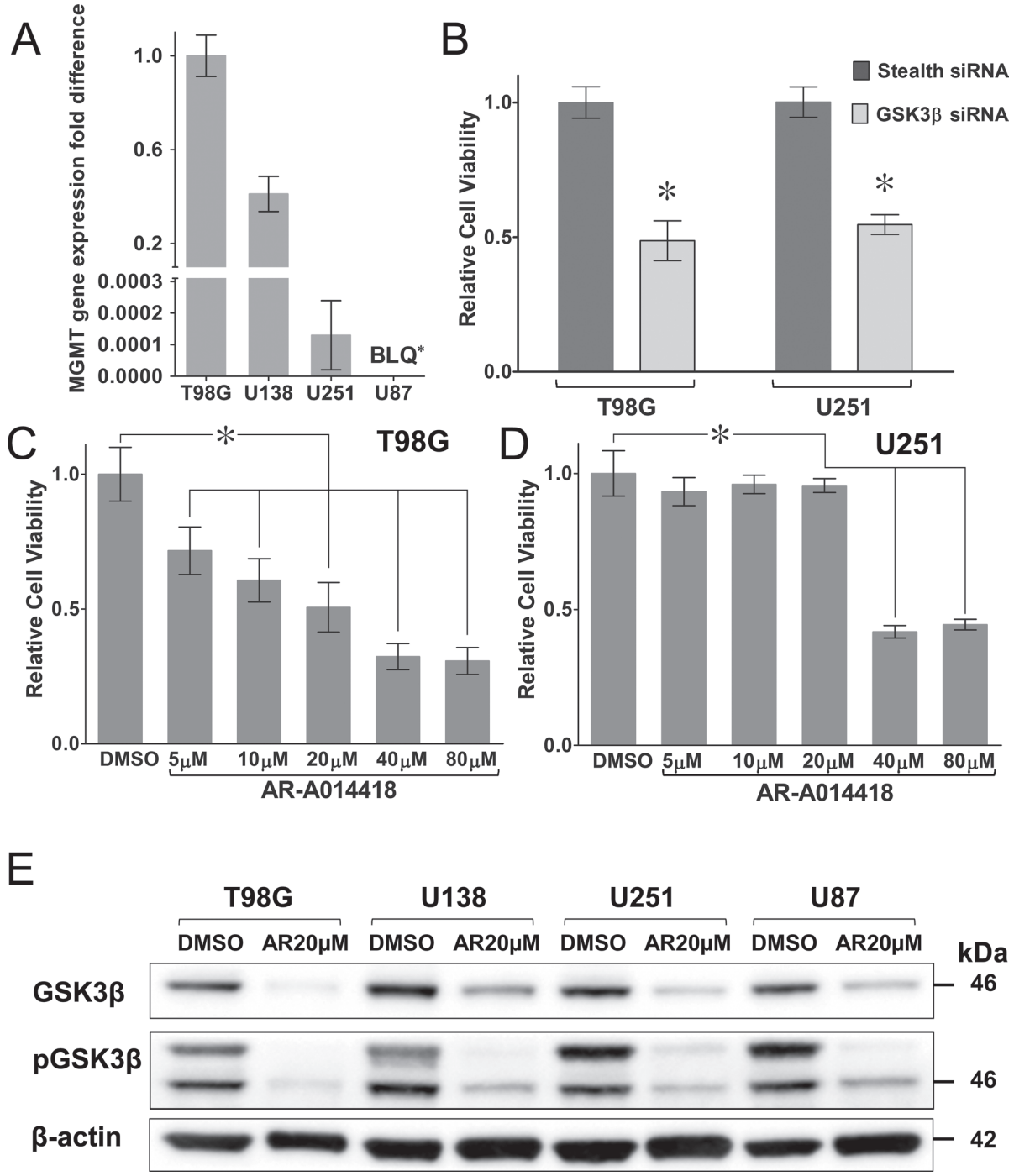

Fig. 2. (**A**) A comparison of the levels of MGMT gene expression in T98G, U138, U251 and U87 GBM cells by QRT–PCR with primers specific to MGMT. The relative value of MGMT messenger RNA expression in T98G was scored as 1.0. *BLQ, below the limit of quantitation; bars show standard deviations in the data. The figure shows the data from three independent experiments. (**B**) The effect of GSK3β–RNA interference on GBM cell survival. Values of relative cell viability were measured by the AlamarBlue assay and compared between T98G and U251 cells transfected with GSK3β-specific and non-specific siRNA (10 nmol/l each), respectively, for 72 h. The relative viability of the cells treated with non-specific siRNA was scored as 1.0. *$P = 0.002$; Mann–Whitney *U*-test, bars show standard deviation. The figure shows the data from experiments carried out in sextuplicates. (**C** and **D**) The relative cell viability of T98G and U251 cells was measured by the AlamarBlue assay and compared between cells treated with DMSO (control) or AR-A014418 (5, 10, 20, 40 or 80 μmol/l) for 144 h. The relative viability of the cells treated with DMSO was scored as 1.0. *$P < 0.05$; Mann–Whitney *U*-test, bars show standard deviation. The figure shows the data from experiments carried out in sextuplicates. (**E**) The effect of GSK3β inhibitor (AR-A014418) on the expression of total and tyrosine 216-phosphorylated GSK3β. Equal amounts of whole-cell lysates from T98G, U138, U251 and U87 cells treated with DMSO or 20 μmol/l AR-A014418, respectively, for 72 h were analyzed by western blot of total and phosphorylated GSK3β proteins, with β-actin used as loading control. AR, AR-A014418. The figure shows the representative data from three independent western blots.

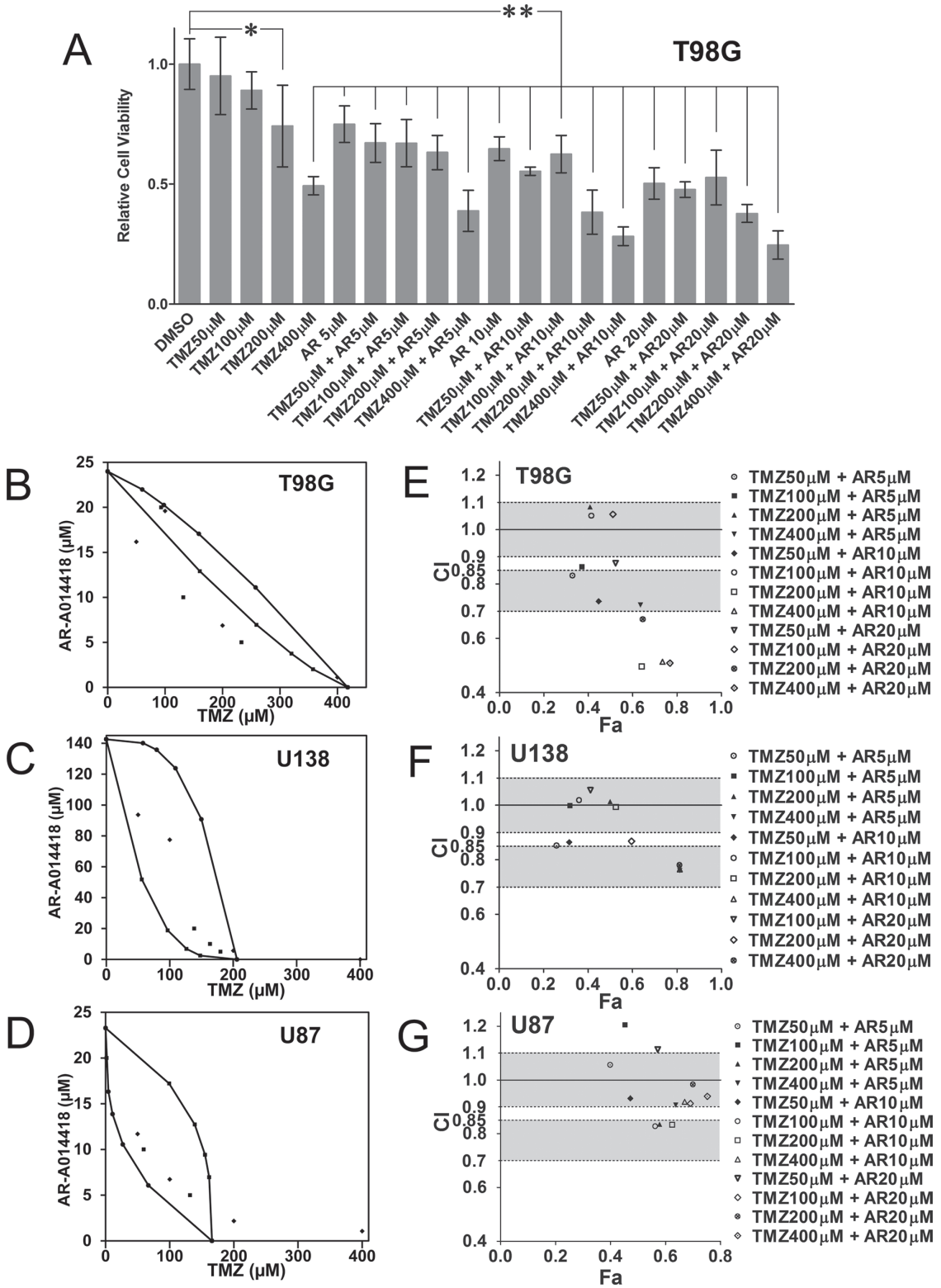

Fig. 3. The combined effect of TMZ and a GSK3β inhibitor (AR-A014418) on GBM cells. (**A**) The relative cell viability of T98G cells after treatment for 144 h with TMZ (50, 100, 200 and 400 μmol/l) or AR-A014418 (5, 10 and 20 μmol/l), alone or in different combinations shown below the panel, measured by the AlamarBlue assay. The relative viability of the cells treated with DMSO alone was scored as 1.0. **P* < 0.05, ***P* < 0.01, AR, AR-A014418; Mann–Whitney *U*-test, compared with DMSO-treated cells; bars show standard deviation. (**B**–**G**) A comparison of combined effect of TMZ (50, 100, 200 and 400 μmol/l) and AR-A014418 (5, 10 and 20 μmol/l) on T98G, U138 and U87 cells. (B–D) Classic isobologram method. The IC_{50} values of the combination therapy by TMZ and AR-A014418 determined by the AlamarBlue assay were plotted (TMZ IC_{50}, closed square; AR-A014418 IC_{50}, closed diamond).

Capacity complementary DNA Reverse Transcription Kit (Applied Biosystems). Quantitative reverse transcriptase–polymerase chain reaction (QRT–PCR) was performed using a LightCycler FastStart DNA MasterPlus SYBRGreen kit (Roche Diagnostics GmbH) with the respective sense and antisense primers for MGMT and glyceraldehyde 3-phosphate dehydrogenase (all from Sigma) that span exon–exon junctions preventing amplification of contaminating genomic DNA. QRT–PCR was carried out in a LightCycler 1.5 as described previously (25). QRT–PCR was done with the following set of primers for MGMT (NM_002412.3): forward primer, 5′-CCTGGCTGAATGCCTATTTC-3′ and reverse primer, 5′-TGTCTGGTGAACGACTCTTG-3′ (amplicon size: 100 bp); and for glyceraldehyde 3-phosphate dehydrogenase (NM_002046.3): forward primer, 5′-CTCCTCCTGTTCGACAGTCA-3′ and reverse primer, 5′-CCAATACGACCAAATCCGTTG-3′ (amplicon size: 112 bp). All primers other than the MGMT forward primer (26) were designed with Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The QRT–PCR reaction protocol consisted of an initial denaturation step at 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 5 s. LightCycler Software 4.1 was used to analyze the QRT–PCR data. Ct was determined and relative messenger RNA expression was calculated using the ΔC_T method described by the manufacturer using glyceraldehyde 3-phosphate dehydrogenase as a calibrator gene.

Chromatin immunoprecipitation assay

To assess c-Myc and DNA (cytosine-5)-methyltransferase 3A (DNMT3A) binding to the MGMT promoter chromatin immunoprecipitation (ChIP) assay was performed utilizing a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) as described previously (27). Briefly, two-step protein and chromatin fixation was performed with N-hydroxysuccinimide ester-ester cross-linking reagent ethylene glycol bis(succinimidylsuccinate) (EGS) (Pierce Biotechnology, Rockford, IL) and formaldehyde (Sigma). EGS stock solution was added to T98G and U138 cells to a final concentration of 2 mM and rapidly mixed. Cells were fixed with EGS for 45 min at room temperature. After EGS fixation, the cells were washed three times with phosphate-buffered saline. Ten milliliters of a freshly prepared 1% (v/v) solution of formaldehyde in phosphate-buffered saline, pH 8.0, were added, and the cells were incubated at room temperature for 15 min and processed according to the SimpleChIP Enzymatic Chromatin IP Kit protocol. We used anti-c-Myc (Cell Signaling Technology), anti-DNMT3A antibodies (Imgenex) and anti-histone H3 (D2B12) XP antibody (Cell Signaling Technology) as a positive control or anti-FLAG M2 antibody (Sigma) as an unrelated antibody as a negative control. Immunoprecipitated DNA was analyzed by qPCR using LightCycler 1.5 with a LightCycler FastStart DNA MasterPlus SYBRGreen kit and the primers for E-box localized in the human MGMT promoter (NT_008818.16 2498978-83). We designed these primers and tested their specificity with Primer-BLAST software. Sequences of the primers used are available on request. The LightCycler Software 4.1 was used to evaluate the qPCR data. Ct were determined and % input values were calculated.

Statistical analyses

Statistical analyses were done using Mann–Whitney *U*-test and Kruskal–Wallis one-way analysis of variance test. $P < 0.05$ was considered significant.

Results

Inhibition of glycogen synthase phosphorylation by GSK3β-inhibiting drugs

To assess the effect of the GSK3β-inhibiting drugs in the patients with recurrent GBM, immunohistochemistry was performed on tumor specimens. The primary tumor and the recurrent tumor tissues in all patients were positive for glycogen synthase (GS) (Supplementary Figure S1, available at *Carcinogenesis* Online). Phosphorylation of GS reflects GSK3β activity since GS is the primary substrate for GSK3β (8). Immunohistochemistry demonstrated that the cytoplasm of tumor cells was strongly positive for p-GS^{S641} in patients 1 and 2 (Figure 1A, a and c). However, staining was weak in the tumors obtained by autopsy (Figure 1A, b and d). In contrast, p-GS^{S641} was preserved in autopsy specimen in patient 3 (Supplementary Figure S2, available at *Carcinogenesis* Online). This result suggests that the GSK3β-inhibiting drugs function in the tumor cells of the patients.

Changes in the expression and promoter methylation of MGMT in the tumors

The level of MGMT expression was compared by immunohistochemistry of the primary tumor and the recurrent tumor tissues from the same patients treated with the GSK3β-inhibiting drugs (Figure 1B and C). Tumor cells were strongly positive for MGMT in primary tumors (Figure 1B, a and c), whereas weak expression was observed in the tumors obtained at autopsy in patients 1 and 2, respectively (Figure 1B, b and d). In contrast, MGMT was detected both in primary tumor and autopsy specimen in patient 3 (Supplementary Figure S3, available at *Carcinogenesis* Online). This suggests that MGMT was downregulated in response to the inhibition of GSK3β in the tumor tissues.

With the findings shown above, we hypothesized that treatment with the GSK3β-inhibiting drugs decreased MGMT expression in the tumors by epigenetic silencing of its gene. To address this, a MSP assay was performed to examine the methylation status of the MGMT promoter in the tumor tissues. Although the MGMT promoter was unmethylated in the primary tumors at surgery, it became methylated in the tumors obtained at autopsy in patient 1 (Figure 1D).

MGMT gene expression in glioma cells

We investigated the mechanism of MGMT silencing by GSK3β inhibition. Initially, we screened for the glioma cell lines T98G, U138, U251 and U87. Basal MGMT gene expression was determined by QRT–PCR. T98G, U138 and U251 cell lines expressed the MGMT gene transcripts, whereas U87 showed no detectable MGMT gene expression (Figure 2A).

GSK3β inhibition and glioma cell viability

To determine the function of GSK3β in the glioma cell lines, we used siRNA to specifically knockdown endogenous GSK3β. The specific reduction of GSK3β by siRNA was determined in our previous study (10). Depletion of GSK3β, confirmed by western blot analysis (Supplementary Figure S4, available at *Carcinogenesis* Online), significantly decreased cell viability in T98G and U251 cells (Figure 2B). To further assess the effect of inhibition of GSK3β activity, we treated the cells with dose-escalating AR-A014418. Cell viability was decreased by AR-A014418 treatment in a dose-dependent manner in T98G cells (Figure 2C). IC_{50} was ~20 μmol/l. Decreased viability by AR-014418 was also observed in U251 cells (Figure 2D). Consistently with previous study showing depletion of GSK3β by AR-A014418 treatment (28), depletion of total and tyrosine 216-phosphorylated GSK3β by AR-A014418 was observed in glioma cells by western blot analysis (Figure 2E). These data showed that GSK3β inhibition attenuates viability in T98G and U251 cells. According to the range of linear dose-dependent response in T98G cells, a 5–20 μmol/l dose range of AR-A014418 was used to assess combination treatment with TMZ.

Combined effect of GSK3β inhibitor and TMZ in GBM cells

To investigate whether GSK3β inhibition enhances TMZ effect, T98G, U138 and U87 cells were treated with different doses of TMZ or AR-A014418, alone or in combination. Compared with U138, U87 (Supplementary Figure S5, available at *Carcinogenesis* Online) and U251 as shown previously (10), T98G cells were much

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A 5 and 10 μmol/l of AR-A014418 and 50 and 200 μmol/l of TMZ appeared to be under the *envelope of additivity* (continuous line with closed square and continuous line with closed circle, respectively), showing synergistic action against T98G cells. IC_{50} values for combination treatment, which are inside the envelope of additivity, reveal the additive action of TMZ and AR-A014418 treatment against GBM cells. (E–G) Median dose-effect analysis. Dose-normalized combination indices (CI) of the combination therapy by TMZ and AR-A014418 were plotted. CI values for combination treatment within intervals 0.3–0.7, 0.7–0.85, 0.85–0.90 and 0.90–1.10 reveal synergism, moderate synergism, slight synergism and nearly additive action, respectively, of TMZ and AR-A014418 treatment against GBM cells. Fa, affected cell fraction. The figure shows the data from experiments carried out in sextuplicates.

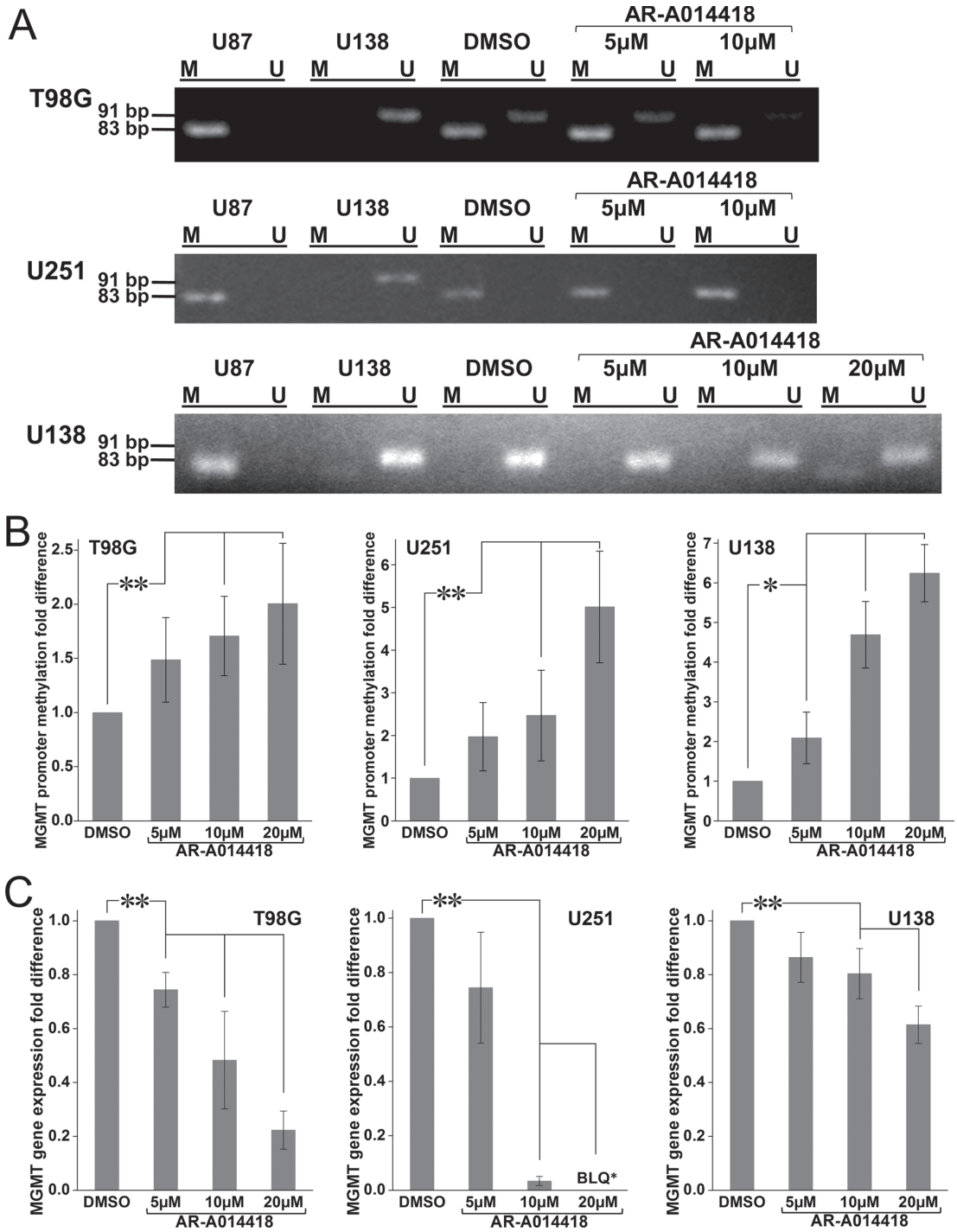

Fig. 4. (**A**) Changes in MGMT gene methylation status in GBM cells by GSK3β inhibition. The effect of GSK3β inhibition with 5, 10 or 20 μmol/l of AR-A014418 for 72 h on the methylation status of the MGMT promoter in T98G, U251 and 138 cells was observed by MSP assay. PCR products in the M lanes and U lanes indicate methylated and unmethylated status of the MGMT promoter, respectively. U87, U87 GBM cell line as a methylated control; U138, U138 GBM cell line as an unmethylated control; AR, AR-A014418. (**B**) The effect of GSK3β inhibition on methylation status of the MGMT promoter examined with MethyLight assay in T98G, U251 and U138 cells treated for 72 h with DMSO or AR-A014418 (5, 10 or 20 μmol/l). qPCR of bisulfite-converted DNA with the primers and probes specific to the methylated fraction of the MGMT promoter. The level of MGMT promoter methylation in the cells treated with DMSO was scored as 1.0. *$P < 0.05$, **$P < 0.01$, AR, AR-A014418; Mann–Whitney *U*-test, bars show standard deviation. The figure shows the data from three independent experiments. (**C**) QRT–PCR analysis of the effect of GSK3β inhibition on MGMT gene expression in T98G, U251 and U138 cells treated with DMSO or AR-A014418 (5, 10 or 20 μmol/l), respectively, for 72 h. The level of MGMT messenger RNA expression in the cells treated with DMSO was scored as 1.0. *BLQ, below the limit of quantitation. **$P < 0.01$; Mann–Whitney *U*-test, bars show standard deviation. The figure shows the data from three independent experiments.

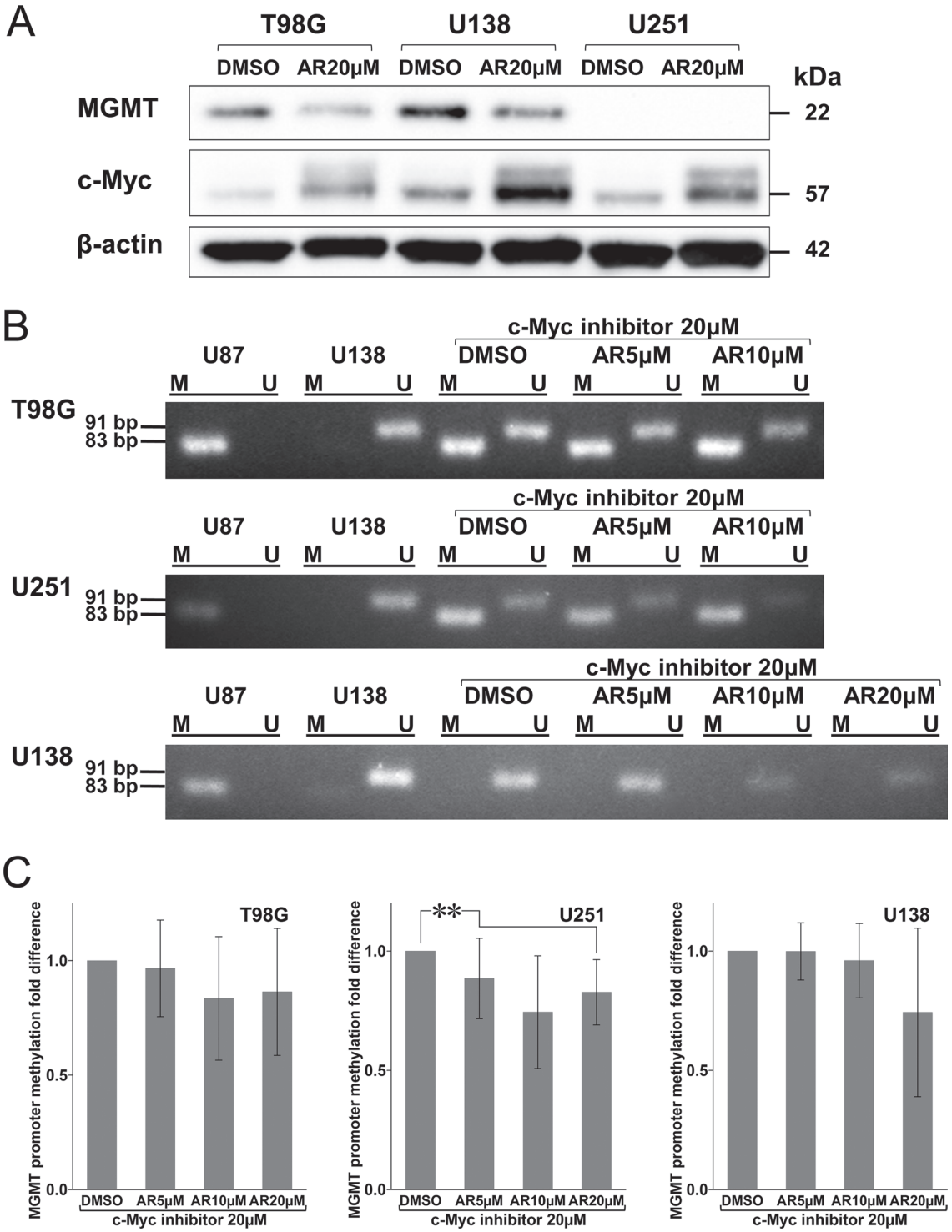

Fig. 5. (**A**) The effect of GSK3β inhibitor (AR-A014418) on the expression of MGMT and c-Myc. Equal amounts of whole-cell lysates from T98G, U138 and U251 cells treated with DMSO or 20 μmol/l AR-A014418, respectively, for 72h were analyzed by western blot of c-Myc and MGMT proteins, with β-actin used as loading control, AR, AR-A014418. The figure shows the representative data from three independent western blots. (**B**) Changes in MGMT gene methylation status in GBM cells by GSK3β and c-Myc inhibition. The effect of combination of DMSO–AR-A014418 (5, 10 or 20 μmol/l) and c-Myc inhibitor 20 μmol/l for 72h on the methylation status of the MGMT promoter in T98G, U251 and U138 cells was observed by MSP assay. PCR products in the M lanes and U lanes indicate methylated and unmethylated status of the MGMT promoter, respectively. U87, U87 GBM cell line as a methylated control; U138, U138 GBM cell line as an unmethylated control; AR, AR-A014418. (**C**) The effect of GSK3β and c-Myc inhibition on methylation status of the MGMT promoter examined with MethyLight assay in T98G, U251 and U138 cells treated for 72h with combination of DMSO–AR-A014418 (5, 10 or 20 μmol/l) and c-Myc inhibitor 20 μmol/l. qPCR of bisulfite-converted DNA with the primers and probes specific to the methylated fraction of the MGMT promoter. The level of MGMT promoter methylation in the cells treated with DMSO was scored as 1.0. $^{**}P < 0.01$, AR, AR-A014418; Mann–Whitney *U*-test, bars show standard deviation. The figure shows the data from three independent experiments.

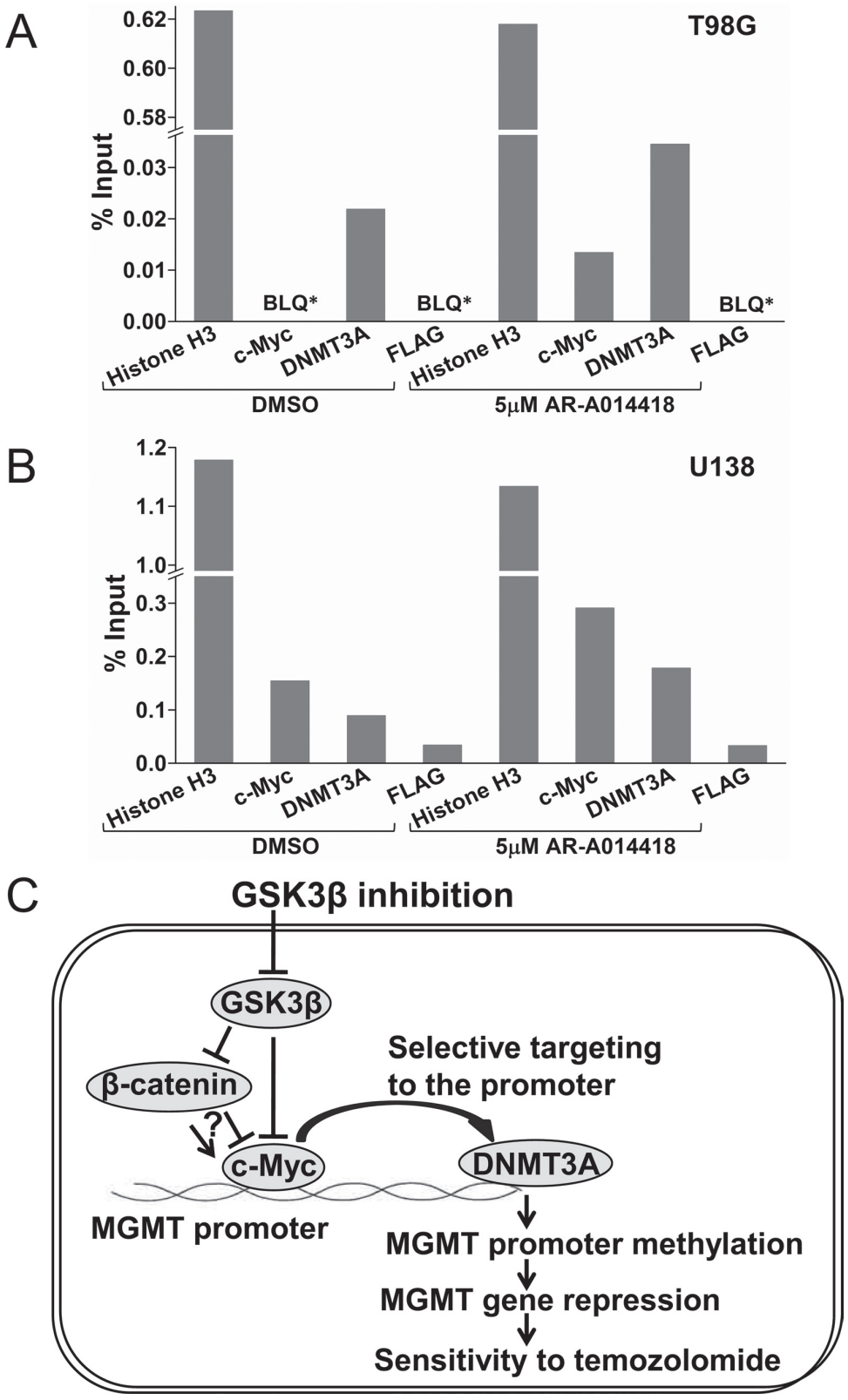

Fig. 6. Epigenetic silencing of MGMT expression by GSK3β inhibition. (**A** and **B**) Comparison by ChIP assay of the binding of histone H3, c-Myc, DNMT3A and FLAG to the E-box site in the MGMT promoter between the GBM cells treated with DMSO or with a GSK3β inhibitor (AR-A014418). T98G and U138 cells were treated for 72 h with DMSO or 5 μmol/l of AR-A014418. The amount of DNA coprecipitated with the antibody to each molecule was measured by

more chemoresistant to TMZ (Figure 3A), which is consistent with a higher basal level of MGMT expression (Figure 2A). The combination of low-dose AR-A014418 and TMZ significantly reduced cell viability compared with the treatment with TMZ or low-dose AR-A014418 alone (Figure 3A). Classic isobologram analysis and median dose-effect analysis were performed to evaluate whether low-dose AR-A014418 potentiates TMZ effect against GBM cells. The data obtained showed mainly synergistic antitumor effect of low-dose AR-A014418 (5, 10 and 20 μmol/l) in combination with TMZ in T98G cells (Figure 3B and E), whereas U138 demonstrating lower basal MGMT expression (Figure 2A) showed additive and moderate synergistic effect (Figure 3C and F), while mainly additive effect was noted in U87 (Figure 3D and G) and in U251 in our previous report (10) with undetectable and low MGMT expression, respectively (Figure 2A).

Effect of GSK3β inhibition on MGMT promoter methylation status and gene expression

To investigate the influence of GSK3β inhibition on MGMT promoter methylation, a MSP assay was performed in T98G, U251 and U138 cells expressing MGMT (Figure 2A). AR-A014418 treatment decreased or ablated unmethylated MGMT promoter in T98G, increased methylated MGMT promoter in U251 and decreased unmethylated MGMT promoter in U138 cells revealing methylated MGMT promoter at 20 μmol/l (Figure 4A). MSP assay data were confirmed by the MethyLight assay showing that GSK3β inhibition increased methylation of CpG islands in the MGMT promoter in T98G, U251 and U138 cells after treatment with AR-A014418 (Figure 4B). Consistent with the changes in methylation levels, GSK3β inhibition by AR-A014418 decreased the levels of MGMT gene expression in T98G, U138 and U251 cells as measured by QRT–PCR (Figure 4C). Consistently, MGMT protein expression was decreased by AR-A014418 treatment in T98G and U138, whereas MGMT was undetectable in U251 cells with low MGMT expression (Figures 2A and 5A).

Effect of GSK3β inhibition on c-Myc and DNMT3A binding to the MGMT promoter

To investigate the molecules that are responsible for the changes in MGMT promoter methylation status, we focused on c-Myc signaling. c-Myc is a good candidate since GSK3β inhibition increases c-Myc expression in U251 (12), T98G, U138 (Figure 5A and Supplementary Figure S6, available at *Carcinogenesis* Online) and U87 cells (I.V.Pyko, unpublished results), as shown by western blot analysis and QRT–PCR (I.V.Pyko, unpublished results).

To determine if the effect of GSK3β inhibition on MGMT promoter methylation is mediated by c-Myc signaling, we performed MSP assay and MethyLight assay in T98G, U251 and U138 cell lines treated with combination of DMSO–AR-A014418 and c-Myc inhibitor. As shown in Figure 5B, the c-Myc inhibitor caused increase/appearance of unmethylated MGMT promoter in T98G and U251 cells and ablated AR-A014418-induced MGMT promoter methylation (Figure 4A) in U138 cells in MSP assay, suggesting that c-Myc plays a role in MGMT promoter methylation in the GBM cells. Comparing with the data shown in Figure 4A, concomitant treatment with the c-Myc inhibitor and AR-A014418 maintained the fraction of unmethylated MGMT promoter in these cells. MethyLight assay demonstrated that c-Myc inhibitor abrogated the AR-A014418-induced increase of MGMT promoter methylation in all cells (Figure 5C). The results of MSP and MethyLight assays collectively suggest that c-Myc-mediated signaling is responsible for MGMT promoter methylation induced by the GSK3β inhibitor in the GBM cells.

c-Myc is a target for β-catenin-mediated gene transcription by T-cell factor/lymphoid-enhancer factor (29). We have shown previously that GSK3β inhibition increases β-catenin expression in T98G and U251 cells (10). To determine if c-Myc expression is increased via β-catenin signaling in the glioma cell lines, we used siRNA to specifically knockdown endogenous β-catenin. Depletion of β-catenin, confirmed by western blot analysis (Supplementary Figure S7, available at *Carcinogenesis* Online), resulted in decreased c-Myc expression in U138; however, in T98G and U251, β-catenin depletion increased c-Myc expression and produced no significant changes in U87 cells (Supplementary Figure S7, available at *Carcinogenesis* Online). It suggests that the regulation of c-Myc by β-catenin under GSK3β inhibition is cell dependent.

c-Myc is known to selectively target DNMT3A to the promoter of the gene, resulting in DNA methylation *de novo* and silencing of the targeted promoter (30). DNMT3A protein expression was not changed by GSK3β siRNA (Supplementary Figure S8, available at *Carcinogenesis* Online), suggesting that changes in DNMT3A expression are not involved in regulation of MGMT promoter methylation via DNMT3A in GBM cells.

We examined possible interaction between c-Myc and DNMT3A by the ChIP assay. We chose T98G and U138 cells since T98G is highly resistant to TMZ due to the high basal level of MGMT gene expression (Figure 2A), and the observed synergistic effect of combined treatment with AR-A014418 and TMZ against T98G and U138 cells (Figure 3B, C, E and F) was accompanied by a decrease in unmethylated MGMT promoter after AR-A014418 treatment (Figure 4A). In ChIP assay, c-Myc binding to the E-box transcription factor-binding site in the MGMT promoter was on detectable level in U138 and below the limit of quantitation in T98G in DMSO-treated control, whereas AR-A014418 treatment increased c-Myc binding to the same site in the MGMT promoter in both cell lines. This finding was accompanied by increased DNMT3A binding to the same region in the MGMT promoter in T98G and U138 cells (Figure 6A and B). These data suggest that, in response to GSK3β inhibition, c-Myc recruits DNMT3A to the MGMT promoter, which increases its methylation level in T98G and U138 cells.

Discussion

In our clinical research, the GSK3β-inhibiting drugs inhibited GS phosphorylation and decreased MGMT expression, a predictor for resistance to TMZ, in tumor cells in patients with recurrent GBM. Consistent with these findings, the status of the MGMT promoter changed from unmethylated to methylated after treatment with GSK3β-inhibiting drugs. These data suggest that GSK3β inhibition decreases MGMT production in GBM affecting the methylation status of the MGMT promoter. This observation in patient tumors was extrapolated by novel findings in this study, demonstrating that GSK3β inhibition in GBM cells induced c-Myc-dependent recruitment of DNMT3A, leading to MGMT promoter methylation and consequent silencing of MGMT expression (Figure 6A and B).

In our study, T98G and U138 cells appeared to be highly resistant to TMZ treatment, and this can be attributed to high MGMT gene expression level in these cell lines. It is consistent with previous reports asserting that T98G has the highest MGMT expression level among 10 GBM cell lines (7) and is extremely resistant to TMZ (31,32). Here, we showed that GSK3β inhibition enhanced TMZ effect in T98G cells to sensitivity levels seen in TMZ-sensitive cell lines (10). The synergistic effect observed for the combination treatment of GSK3β inhibitor with low-dose TMZ (50 μmol/l) may be considered in future clinical applications as it is within the TMZ therapeutic window for systemic administration (33,34).

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qPCR with a set of primers specific to the E-box transcription factor-binding site in the MGMT promoter. *BLQ, below the limit of quantitation. The figure shows the representative data from two independent immunoprecipitations. (**C**) Regulation of MGMT expression by GSK3β signaling. GSK3β inhibition results in c-Myc activation directly and via activation of β-catenin-mediated signaling, which consequently increases recruitment of DNMT3A by c-Myc to the MGMT promoter, thus increasing *de novo* DNA methylation in the MGMT promoter. The methylated status of the MGMT promoter increases the sensitivity of GBM to TMZ.

c-Myc is an important factor in cell proliferation that enables G_1/S cell cycle progression (35). Despite its role in cell proliferation, several studies show an essential role for c-Myc in apoptosis (36,37). A previous report demonstrated that c-Myc has a suppressive effect in human GBM cells, as c-Myc upregulation is associated with increased apoptosis (12). GSK3β has also been reported to phosphorylate (38) and destabilize c-Myc (11) and GSK3β inhibition in glioma cells along with the upregulation of Ser^{62} phosphorylation and downregulation of Thr^{58} phosphorylation of c-Myc, results in increased c-Myc expression (12). Taken together, c-Myc expression is increased in GBM cells by GSK3β inhibition via its effects on c-Myc phosphorylation and in some cell lines its effect on β-catenin mediated gene transcription.

Considering the role of c-Myc in apoptosis (12), additive effect of GSK3β inhibition and TMZ in U87 showing undetectable MGMT expression (Figure 3D and G) can be explained by c-Myc upregulation (I.V.Pyko, unpublished results). Moreover, TMZ induces G_2/M arrest decreasing its effect (39) by retention of proliferative capacity in U87 cells (40). Accordingly, our laboratory previously have shown cyclin D1 upregulation in U87 by GSK3β inhibition (10), suggesting that GSK3β inhibition can enhance TMZ effect in U87 cells reducing TMZ-induced G_2/M arrest.

It is known that c-Myc can silence gene expression by active recruitment of corepressor proteins and that it can target DNMT3A selectively to the promoter of the gene (30). However, there are no studies showing binding of c-Myc to the MGMT promoter (41), despite the presence of an E-box transcription factor-binding site in the MGMT promoter. In this study, we hypothesized that a high expression level of c-Myc in GBM cells induced by GSK3β inhibition induces c-Myc binding to the E-box, resulting in transcriptional repression via recruitment of DNMT3A.

Our ChIP assay demonstrated for the first time that c-Myc binds to the MGMT promoter in intact cells and that c-Myc and DNMT3A binding to the MGMT promoter increases after AR-A014418 treatment. The mediation of c-Myc for recruitment of DNMT3A to the MGMT promoter was confirmed by comparison of data from the conventional ChIP assay, which includes a one-step protein and chromatin fixation with formaldehyde (I.V.Pyko, unpublished results) and data from the ChIP assay with a two-step protein and chromatin fixation shown in this study. Conventional ChIP assay, which effectively detects direct protein–DNA binding, did not reveal detectable DNMT3A binding to the MGMT promoter. In contrast, ChIP assay with a two-step protein and chromatin fixation provides effective detection of both direct protein–DNA binding and indirect protein–protein–DNA binding (27). Consequently, our ChIP assay with a two-step protein and chromatin fixation showed detectable DNMT3A binding to the MGMT promoter with an increased binding level in GBM cells treated with AR-A014418. This was consistent with an increase in c-Myc binding after AR-A014418 treatment, suggesting recruitment of DNMT3A by c-Myc to the MGMT promoter (Figure 6A and B).

Based on our data, we speculate that GSK3β inhibition eliminates the suppressive effect of GSK3β on c-Myc expression by the effects on c-Myc phosphorylation, which subsequently increases recruitment of DNMT3A by c-Myc to the MGMT promoter in GBM cells. The interaction between c-Myc and DNMT3A would then activate DNA methyltransferase, which increases local *de novo* DNA methylation in the CpG islands of the MGMT promoter. MGMT promoter methylation silences MGMT gene expression, and this finally sensitizes GBM cells to TMZ (Figure 6C). The provided data will facilitate the development of an optimal combination of GSK3β inhibitor and alkylating agents for their use in the treatment of patients with GBM. Further *in vivo* research is needed to optimize combination regimens of TMZ and GSK3β inhibitor for the treatment of refractory GBM.

Supplementary material

Supplementary Figures S1–S8 can be found at http://carcin.oxfordjournals.org/

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