金沢大学がん進展制御研究所
腫瘍制御（旧：遺伝子診断）研究分野

研究のあゆみと業績 2022

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Annual Report 2022

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

2022 年 12 月
December 2022

まえがき

本誌の編集は今回が 21 回目です（研究のあゆみと業績 2001 年と 2002 年は合冊のため）。この間に研究分野開設 10 年（Decennial Report 2001–2010: Downstream, Present & Upstream）、15 年（Quinquennial Report ）と 20 年（Vicennial Report 2016–2020）の記念誌を作りましたので、この手の編集は 24 回におよびます。外科と腫瘍学の教えを受けた磨伊正義先生（故人）が生前、心配なさっていたとはいえ、弱小体制ながらなんとか 22 年間は持ちこたえたようです。そして、23 年に向かおうとしています。惰性で日記のようになってきても、もうしばらく編集を続けます。

今春、もう当たらないと思っていた研究費2件が採択され、ほっとしました（第8頁）。ところがこれと前後して3月 16 日に、病理学とその後の生き方を絶えず指導してくださった中西功夫先生（本学旧第一病理学：名誉教授）が他界されました。初期の本誌を届けに伺ったときには、「源さんの文章は硬くてまわりくどくていかん。もう少し、柔らかく書けばいい。それでも、よくやっている」と云われました。今でも昨日のことのように思い出されます。この後は、研究室独自の成果が芳しくなく、淡々と日々を過ごしていましたところ、9月 19 日の朝、共同研究者の石渡俊行先生（東京都健康長寿医療センター研究所）から、松田陽子先生（香川大学腫瘍病理学：教授）の訃報を伝えるメールが届きました。2019年2月の教授内定のささやかなお祝いなどこれまでの交流を考えると、かけがえのない仲間を失ったとても悲しい知らせでした。8月初旬に私が推薦した第31回日本消化器癌発生学会：田原榮一賞に内定した直後だったのです。それでも、ご逝去後の11月に田原賞を受賞され、遺作となった共同研究論文が12月下旬にJ Natl Cancer Instに採択されたこと（第9頁：研究業績を参照）は、私にとってはなによりの供養になったと思い、ご冥福を祈るしだいです。私と松田先生との交流の経緯については、研究のあゆみと業績 2021年にも掲載した当研究所ニュースレター（抜粋）を再掲します【附記2：第13–15頁】。

2019年2月27日 駅前居酒屋 狼煙（金沢駅前）

私自身は2021年末に、日本消化器内視鏡学会から第31回（2022年度）北陸支部セミナーを担当するように指名されました。そしてこの一年間、多くの旧知や新たにできた仲間の皆さんに助けられて、年明けの開催の目途がたちました。2008年には消化器・腫瘍外科から身を引いたものの、消化器内視鏡専門医、指導医としてなんとか持ちこたえている自身に課せられた任務と受けとめ、できるかぎりの役目をはたすよう努めます。

この年末が過ぎて来春には、大学での最後の年度を迎えます。年明けから1年3か月のあいだ、何をしようか思案することにします。それでは、これからもどうぞよろしくお願いします。

2022年12月 年末

源 利成

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【目次】

【研究メンバー】(2022 年 12 月現在)

教授	源　利成	金沢大学附属病院がんセンター(併任)
助教	堂本貴寛(たかひろ)	
博士研究員	上原将大(まさひろ)	
	Diliraba Bolidong(ディリレバ ボリドン)	～2022 年 3 月
大学院	竹中　哲(さとし)	大学院医学系研究科肝胆膵・移植外科学
(博士課程)	太田亮介	大学院医薬保健学総合研究科 腫瘍制御学(～3 月)
研究生	小竹優範(まさのり)	厚生連高岡病院 外科(～12 月)
研究支援員	浅香敦子	研究支援推進員
研究協力員	旭井亮一(あさい)	(株)凸版印刷研究所
	川島篤弘	独立行政法人国立病院機構金沢医療センター 臨床検査科
	藤沢弘範(ひろのり)	独立行政法人国立病院機構金沢医療センター 脳神経外科
	横井健二	米国 ヒューストンメソジスト研究所ナノ医療部門
	島崎猛夫(しまさき)	金沢医科大学総合医学研究所, 消化器内科
	東　朋美(ひがし)	金沢大学医薬保健研究域医学系 環境分子応答学(衛生学)
	笠島里美(かさしま)	金沢大学医薬保健研究域保健学系 検査技術科学
	宮下勝吉(かつよし)	福井県立病院 脳神経外科
	中島日出夫(なかじま)	上尾中央総合病院 腫瘍内科
	下崎真吾	医療法人社団 下崎整形外科医院
	澤田　武	名古屋市立大学消化器・内分泌内科学
	太田亮介	金沢大学大学院医薬保健学総合研究科 腫瘍制御(～3 月)
共同研究者	塚(づか)　正彦	金沢大学医薬保健研究域医学系 法・社会医学(法医学)

【共同研究者】（2022 年現在で共同研究が稼動および予定しているもの．敬称略）

教授	中田光俊（なかだ）	金沢大学医薬保健研究域医学系 脳機能制御学/脳神経外科学
教授	土屋弘行	金沢大学医薬保健研究域医学系 機能再建学／整形外科学
特任教授	山本憲男	金沢大学医薬保健研究域医学系 機能再建学／整形外科学
教授	竹村博文	金沢大学医薬保健研究域医学系 心血管外科学
教授	稲木紀幸	金沢大学医薬保健研究域医学系 消化管外科学
准教授	木下　淳	金沢大学医薬保健研究域医学系 消化管外科学
助教	中村慶史（けいし）	金沢大学医薬保健研究域医学系 消化管外科学
教授	八木真太郎	金沢大学医薬保健研究域医学系 肝胆膵・移植外科学
講師	牧野　勇	金沢大学医薬保健研究域医学系 肝胆膵・移植外科学
教授	Richard Wong	金沢大学理工学研究域自然システム学系／ナノ生命科学研究所
准教授	宮下知治	金沢医科大学 一般・消化器外科学／腫瘍外科学
教授	曽我朋義	慶應義塾大学 先端生命科学研究所
教授	竹田　扇（せん）	帝京大学医学部 解剖学
学部内准教授	吉村健太郎	山梨大学大学院総合研究部医学域 総合医科学センター 分子生物学研究室
研究部長	石渡俊行（いしわた）	東京都健康長寿医療センター 老年病理学，高齢者がん研究
教授	松田陽子（故人）	香川大学医学部病理病態・生体防御講座 腫瘍病理学（～9 月）
准教授	水津　太（すいつ　ふとし）	香川大学医学部病理病態・生体防御講座 腫瘍病理学
教授	松下一之	千葉大学医学研究院　分子病態解析学，附属病院検査部
教授	佐々木泰史（やすし）	札幌医科大学 教養教育研究部門 医療人育成センター
准教授	久保田英嗣（えいじ）	名古屋市立大学消化器・内分泌内科学
助教	古田拓也	久留米大学医学部 病理学
研究員	小泉恵太	上尾中央総合病院
	紙（かみ） 健次郎	（株）ヒューマンメタボロームテクノロジーズ
教授	Ze’ev Ronai	Sanford Burnham Prebys Medical Discovery Institute, La Jolla, USA
教授	Andy Giraud	豪州オーストラリア王立小児病院
	Louise M. Judd	豪州オーストラリア王立小児病院
	Trevelyan R. Menheniott	豪州オーストラリア王立小児病院
	Phil Sutton	豪州オーストラリア王立小児病院
准教授	Serge Y. Fuchs	ペンシルヴェニア大学 生物学

【2022 年のあゆみとできごと】

2022 年 3 月 31 日
【附記3】
・太田亮介君が大学院を修了し、医学博士の学位を取得。
課題：Integrated genetic and epigenetic analysis of cancer-related genes in non-ampullary duodenal adenomas and intramucosal adeno-carcinomas. *J Pathol* 252 (3): 330-42, 2020.

2022 年 3 月 31 日
・博士研究員：Dilireba Bolidong さんが退職。

2022 年 4 月 1 日
・科学研究費2件が採択された。
2022 年－2024 年度 基盤研究（B） 課題番号 22H03144
源　利成（代表），宮下知治（分担）
課題：治療耐性膵がんの悪性形質を繋ぐ分子経路の解明と耐性制御法開発への応用
2022 年－2024 年度 基盤研究（C） 課題番号 22K07227
堂本貴寛（代表）
課題：抗がん剤耐性獲得膵がん細胞における悪性形質連関の解明と治療法開発

2022 年 6 月 11 日
【附記1】
・源 利成が金沢大学公開講座「がん医療の最前線」で講演した。
課題：大腸がんのしくみと医療を一緒に学びましょう

2022 年 9 月 4 日
・源 利成が第 38 回日本臨床細胞学会北陸連合会学術集会で講演した。
課題：膵がん難治性の細胞特性 ― その仕組みと私たちの取り組み ―

2022 年 9 月 17 日
【附記2】
・共同研究者：松田陽子さん（香川大学医学部病理病態・生体防御講座 腫瘍病理学 教授）がご逝去された（享年 49 歳）。

2022 年 12 月 31 日
【附記3】
・研究生：小竹優範君（厚生連高岡病院 外科）が研究期間を終え、論文を提出して医学博士の学位を取得した。
課題：LOH of the thymidylate synthase locus in combination with genotype has prognostic and predictive significance in colorectal cancer. *Mol Clin Oncol* 15: 235, 2021.

博士研究員：Dilireba Bolidong さん退職のときのささやかな食事会
（2022 年 3 月 24 日：金沢まいもん寿司 白山インター店）

【研究分野と活動の概要】

当研究分野は 1998 年に遺伝子診断の旧称で開設され、その後 24 年間にわたって、消化器がんを中心にがんの多様な生物病態と腫瘍外科学的特性について、基礎と臨床を関係づけるかたちの研究を指向している。そして、その成果を難治がんや希少がんの病態解明と制御に応用するために、学内外のグループと共同研究を進める。

Division Summary

The mission of our division centers on laboratory and clinical research to develop the novel strategies and modalities for diagnosis and treatment of the gastrointestinal (GI), refractory and rare cancer types including glioblastoma, bone and soft tissue sarcomas and pancreatic neuroendocrine neoplasms. Research projects are based on biological characteristics of individual tumor types that are relevant to their invasive and metastatic potential, resistance to therapy, recurrence and outcome of patients. Our current efforts are focused on (1) research and development of the cancer therapy by targeting aberrant glycogen synthase kinase (GSK)3β; (2) understanding of malignant phenotypes of cancer by investigating pathological metabolic properties (eg., aerobic glycolysis, tumor-promoting autophagy); and (3) biological basis of GI and refractory cancer, all for clinical translation. We have been also establishing the tissue material resources of human stomach and colorectal cancer for our own projects described above as well as for studies collaborating with our institutional and many other research groups. During an immediate couple of years, we have obtained the preliminary results indicating the putative roles of tumor GSK3β as a molecular node that is intersected by the pathways responsible for tumor invasion and resistance to therapy, thus enforcing its potential as an emerging cancer therapeutic target. We are extending this project toward investigation of the putative roles for GSK3β in promoting pancreatic neuroendocrine neoplasms as well as in interconnecting malignant phenotypes in pancreatic cancer with acquired chemoresistance. Following the project on GSK3β, we have recently identified a number of molecules potentially responsible for acquired resistance to gemcitabine in pancreatic cancer by comparing the cDNA microarray-based gene expression between gemcitabine-sensitive BxPC-3 cells and BxPC-3-derived clones that acquired stepwise resistance to gemcitabine. In addition to these projects, we have participated in the collaborative studies showing the association of hTERT phosphorylation and longer telomere length in cancer cells with worth prognosis in various cancer types.

＜2022 年の研究成果，進捗状況及び今後の計画＞

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

Wnt 経路抑制因子と認識されている GSK3β が固有の分子経路を介して、がんの悪性形質を

推進することを系統的に示してきた。そして、GSK3β 阻害のがん治療効果を細胞レベルと担がん動物で実証した。また、学内外の外科系グループと連携し、膵がん、膠芽腫や骨軟部肉腫などの難治，希少がんで高活性を示す GSK3β が，腫瘍浸潤性と治療（抗がん剤，放射線）不応性の悪性形質を連結することを見出した。一連の研究をもとに、GSK3β 阻害薬品の転用と抗がん剤を併用するがん治療法を開発し、再発膠芽腫（附属病院脳神経外科）と進行膵がん（金沢医科大学病院）を対象とする医師主導臨床研究によりその安全性と抗腫瘍効果を検証した。ついで、食道扁平上皮がんと抗がん剤耐性獲得膵がんに対する GSK3β 阻害の治療効果とメカニズムを明らかにした。2022 年には GSK3β 阻害によるがん治療の概念実証のため、膵内分泌腫瘍（PNET）の共同研究に加えて、米国で臨床試験中の GSK3β 阻害剤 9-ING-41 を開発した Actuate 社と共同で治療耐性膵がんの前臨床試験を計画した。

2. 膵がんの抗がん剤耐性獲得に伴う悪性形質の解析研究

膵がんで汎用されているゲムシタビン（GEM）に対する耐性獲得は、膵がん治療の未解明医療ニーズである。そこで、GEM 感受性膵がん BxPC-3 細胞から段階的に GEM 耐性を獲得させた 3 種のクローン細胞株（Anticancer Drug 2015;26:90, 北里大から供与）を対象に、GEM 耐性獲得の生物学的特性を検討した。その結果、耐性獲得に伴い腫瘍浸潤とがん幹細胞形質が増強した。また、最耐性細胞株をマウス膵内に移植すると、高度の局所浸潤と肝や腹膜転移をきたし、GEM 耐性を獲得した膵がん患者の病態を模倣する所見を呈した。さらに、GSK3β がこれらの悪性形質のハブとして作用することを見出した（論文作成中）。ついで、耐性細胞株の遺伝子発現解析により、ある種の cyclin D 亜型をはじめ複数の新たな耐性獲得に関わる候補責任分子群を見出した。現在、個々の分子について解析を進めている。

3. ヒト消化管がん組織バンクを中心とする大腸がんの分子病理学的研究

消化管がん研究や臨床研究の基礎資源として 2008 年から本事業を開始し、2010 年にこの事業を当研究所ヒトがん組織バンクに継承し現在に至っている。この組織資源の共同利用促進のために、日本医療研究開発機構ゲノム医療支援サイト（http://www.biobank.amed.go.jp/biobank/index. html）に情報公開している。帝京大学：竹田 扇らが山梨大学で開発した大気圧イオン化法－質量分析を用いて、大腸がん質量分析診断法開発の共同研究を継続している。大腸組織の質量分析パターンをもとに特有の統計解析と機械学習を組合わせて非がん/がんの判別（診断）アルゴリズムを構築し、90%以上の感度と特異度による判別を可能にした（論文作成予定）。現在、山梨大学：吉村らと島津製作所基盤技術研究所との共同で、大腸がんの質量分析－内視鏡診断法の内視鏡デバイス開発を進めている。組織バンク検体を共用して、名古屋市立大学（十二指腸腺腫，早期がん）や香川大学（がんテロメア解析）と共同研究を継続している。そして、香川大学：松田（故人）が主導する多施設共同研究で、hTERT の第 249 スレオニンのリン酸化と、がん細胞とがん随伴線維芽細胞におけるテロメア長が主要がん種の悪性度と相関し、患者生存期間と逆相関することを明らかにした。

【研究費】（2022 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
2022年－2024年度 科学研究費補助金（基盤研究Ｂ）（22H03144）	源　利成	宮下知治	治療耐性膵がんの悪性形質を繋ぐ分子経路の解明と耐性制御法開発への応用	直接経費 13,400,000 円 間接経費 4,020,000 円
2022年－2024年度 科学研究費補助金（基盤研究Ｃ）（22K07227）	堂本貴寛		抗がん剤耐性獲得膵がん細胞における悪性形質連関の解明と治療法開発	直接経費 3,100,000 円 間接経費 930,000 円
2020年－2022年度 科学研究費補助金（基盤研究Ｃ）（20K09100）	宮下知治	太田哲生，源　利成，ほか	GSK3βを基軸とした食道発癌機構の解明と新規治療戦略の開発	直接経費 3,200,000 円 間接経費 960,000 円
2022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	源　利成	質量分析と機械学習を用いたハイスループット大腸がん診断システムの構築	350,000 円
2022年度金沢大学がん進展制御研究所共同研究（若手）	水津　太	源　利成	テロメアを介したオートファジー制御と発癌機構の解明	350,000 円
2022年度金沢大学がん進展制御研究所共同研究	宮下知治	源　利成	膵癌微小環境内の腫瘍関連線維芽細胞の改変に着目した新規治療法の開発	350,000 円
2022年度金沢大学がん進展制御研究所共同研究（一般）	佐々木泰史	澤田　武，源　利成，ほか	非乳頭部十二指腸腫瘍におけるERBB受容体ファミリーの解析と治療標的の探索（一般）	300,000 円
2022年度金沢大学がん進展制御研究所共同研究（若手）	北村浩一	松下一之，源　利成，ほか	消化器・難治がんのリボソーム生合成の新規メカニズム解明と診断，治療法への応用（若手）	350,000 円
2022年度金沢大学がん進展制御研究所共同研究（一般）	久保田英嗣	澤田　武，源　利成，ほか	大腸癌における循環腫瘍DNAを用いた抗EGFR抗体薬耐性の検出と病状モニタリングの確立（一般）	300,000 円
2022年度金沢大学がん進展制御研究所共同研究（一般）	石渡俊行	源　利成，ほか	スフェア形成法を用いた膵癌幹細胞に有効な薬剤の探索	300,000 円
			期間の総額（間接経費を含む）	27,910,000 円

【研究業績】

Ⅰ. 論文発表 [Impact factor 2021]

・英文総説, 著書

なし

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Ⅱ. 学会発表

・国際学会

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（金），鳴門市.

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5. Hiroki Sato, Katsuya Sakai, Ryu Imamura, Toshinari Minamoto, Kunio Matsumoto. Metastatic platform formation triggered by distal HGF conversion. 佐藤拓輝，酒井克也，今村 龍，源 利成，松本邦夫. HGF 活性の遠隔制御によるがん転移ニッチ形成. 第 81 回日本癌学会学術総会，2022 年 9 月 29 日（木）-10 月 01 日（土），パシフィコ横浜，横浜市.

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9. 橋本明史，宮下知治，島崎猛夫，源 利成，高村博之. バレット食道から発癌過程での微小環境の変化と GSK3β 阻害による抑制効果. 第 33 回日本消化器癌発生学会総会，ワークショップ 1（食道），2022 年 11 月 11 日（金），12 日（土），一橋大学一橋講堂，東京.

・司会・座長など

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04-1 辻尾 元，丸尾晃司，山本百合恵，瀬良知央，杉本敦史，笠島裕明，三木友一朗，吉井真美，田村達郎，豊川貴弘，田中浩明，六車一哉，大平雅一，八代正和. クラスター分析による胃癌リンパ節転移および予後に関与する蛋白および遺伝子の解析.

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04-3 菅 元泰，千葉哲博，紺野 亮，中川 良，大山 広，大野 泉，日下部裕子，高橋幸治，永嶌裕樹，三浦義史，大内麻愉，川島祐介，小原 収，加藤直也. 高精度質量分析を用いた胆汁プロテオーム解析の胆道癌の新規バイオマーカー探索における有用性の検討.

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04-6 山道 岳, 加藤大悟, 明庭昇平, 岡 利樹, 奥田洋平, 植村俊彦, 山本顕生, 冨山栄輔, 石津谷祐, 山本致之, 波多野浩士, 河嶋厚成, 植村元秀, 野々村祝夫. 前立腺癌における GDF15 プロペプチドの骨転移診断マーカーとしての有用性.

・その他（講演, 社会・地域貢献を含む）

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Ⅲ. 学会開催

該当なし

IV. 究成果による知的財産権の出願状況

該当なし

V. 新聞, 報道など

該当なし

【附記1】 金沢大公開講座（抜粋）

2208 | 医療・健康

がん医療の最前線

講座概要

がんは、我が国の死因第一位の疾患で、生涯のうちに約２人に１人が罹患すると推計されるなど国民の生命及び健康にとって重大な問題となっています。近年の医学の進歩によって、がんの原因となる遺伝子異常や悪性化を引き起こす仕組みが次々と明らかにされてきました。その研究成果は、新しいがんの診断や治療に結びつき、多くのがん患者にとって福音となっています。

この講座では、金沢大学がん進展制御研究所と文部科学省がん専門医療人材（がんプロフェッショナル）養成プラン事業（北信がんプロ）が共同で、最新のがん医療に関する情報を、一般の方にわかりやすく紹介し、がんの知識を深めて頂く機会を提供いたします。本講座が、早期発見、早期治療、早期社会復帰ができる社会の実現の一助になればと思います。

講　師	松本　邦夫 （がん進展制御研究所　所長）
日　時	６月 11 日（土）～６月 25 日（土） 全３回　いずれも 10:30 ～ 12:00
会　場	サテライト・プラザ
対　象	一般
定　員	20 名
受 講 料	2,250 円
申込期限	６月３日（金）

受講スタンプ欄

印

●プログラム

6/11（土）	大腸がんのしくみと医療をいっしょに学びましょう（仮） 源　利成（がん進展制御研究所　教授）
6/18（土）	乳がん　～乳がん治療の過去・現在・未来～ 石川　聡子（附属病院乳腺外科　助教）
6/25（土）	がんゲノム医療の最前線　～肺がん治療から発展したがんゲノム医療～ 竹内　伸司（附属病院がんセンター　講師）

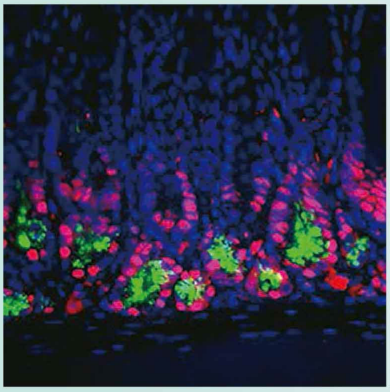
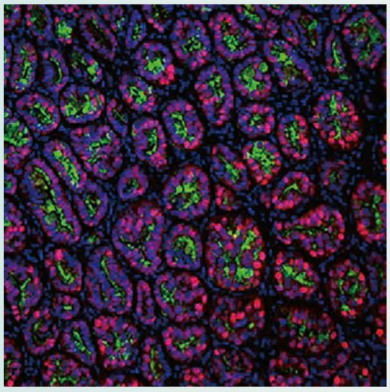
14

【附記2】 金沢大がん研 News Letter（抜粋）：共同研究者紹介

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

News Letter

Vol.15 October 2021


Contents

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

※金沢大学がん進展制御研究所共同利用・共同研究拠点の承諾を得て転載。

共同研究者の紹介　松田教授と源教授は2021年度

教　授　松田　陽子
MATSUDA　YOKO

香川大学医学部
腫瘍病理学

共同研究からはじまった
源先生との出会い

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

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

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

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

2019年2月金沢にて

採択課題で共同研究をすすめています。

松田陽子さんとの８年間

教　授　源　利成
MINAMOTO TOSHINARI

金沢大学がん進展制御研究所
腫瘍制御研究分野

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

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

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

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

【附記3】 学位論文：2022 年 3 月および 12 月修了

Ota R, Sawada T, Tsuyama S, Yao T, Sasaki Y, Suzuki H, Kaizaki Y, Hasatani K, Yamamoto E, Nakanishi H, Inagaki S, Tsuji S, Yoshida N, Doyama H, Kasashima S, Kubota E, Kataoka H, Tokino T, Minamoto T. Integrated genetic and epigenetic analysis of cancer-related genes in non-ampullary duodenal adenomas and intramucosal adenocarcinomas. *J Pathol* 252 (3): 330-42, 2020. doi: 10.1002/path.5529.

The molecular and clinical characteristics of non-ampullary duodenal adenomas and intramucosal adenocarcinomas are not fully understood because they are rare. To clarify these characteristics, we performed genetic and epigenetic analysis of cancer-related genes in these lesions. One hundred and seven non-ampullary duodenal adenomas and intramucosal adenocarcinomas, including 100 small intestinal-type tumors (90 adenomas and 10 intramucosal adenocarcinomas) and 7 gastric-type tumors (2 pyloric gland adenomas and 5 intramucosal adenocarcinomas), were investigated. Using bisulfite pyrosequencing, we assessed the methylation status of CpG island methylator phenotype (CIMP) markers and MLH1. Then using next-generation sequencing, we performed targeted exome sequence analysis within 75 cancer-related genes in 102 lesions. There were significant differences in the clinicopathological and molecular variables between small intestinal- and gastric-type tumors, which suggests the presence of at least two separate carcinogenic pathways in non-ampullary duodenal adenocarcinomas. The prevalence of CIMP-positive lesions was higher in intramucosal adenocarcinomas than in adenomas. Thus, concurrent hypermethylation of multiple CpG islands is likely associated with development of non-ampullary duodenal intramucosal adenocarcinomas. Mutation analysis showed that APC was the most frequently mutated gene in these lesions (56/102; 55%), followed by KRAS (13/102; 13%), LRP1B (10/102; 10%), GNAS (8/102; 8%), ERBB3 (7/102; 7%), and RNF43 (6/102; 6%). Additionally, the high prevalence of diffuse or focal nuclear β-catenin accumulation (87/102; 85%) as well as mutations of WNT pathway components (60/102; 59%) indicates the importance of WNT signaling to the initiation of duodenal adenomas. The higher than previously reported frequency of APC gene mutations in small bowel adenocarcinomas as well as the difference in the APC mutation distributions between small intestinal-type adenomas and intramucosal adenocarcinomas may indicate that the adenoma-carcinoma sequence has only limited involvement in duodenal carcinogenesis.

Kotake M, Bando H, Kaneko M, Takemura H, Minamoto T, Kawakami K. LOH of the thymidylate synthase locus in combination with genotype has prognostic and predictive significance in colorectal cancer. *Mol Clin Oncol* 15 (5): 235, 2021. doi: 10.3892/mco.2021.2398.

The aim of the current study was to investigate the prognostic and predictive significance of polymorphisms in the thymidylate synthase (TS) gene, alongside the loss of heterozygocity (LOH) at this gene locus in patients with colorectal cancer. Genotyping was carried out for a variable number tandem repeat (VNTR) polymorphism in the TS 5'-untranslated region, a G/C single nucleotide polymorphism (SNP) located within this VNTR, and for TS LOH status in 246 colorectal cancer and paired normal DNA samples. The results were analyzed in relation to clinicopathological features, including the prognostic and predictive significance of TS genotype in patients who underwent curative surgery. Complete VNTR, SNP and LOH information for TS was obtained in 226 cases. No significant associations were observed between normal tissue TS genotype status and clinicopathological features. LOH of TS was observed in 58% of tumor samples and was associated with poor prognosis independently of clinical stage. Cases exhibiting TS LOH were classified into the three groups of 2R/loss, 3G/loss and 3C/loss. Patients with 3C/loss genotype status had poor outcomes when treated by surgery alone, but their survival was similar to patients with other genotypes following Fluorouracil (5-FU)-based adjuvant chemotherapy. The results suggested that LOH of the TS locus may be a significant prognostic factor in colorectal cancer, with the genotype of the residual allele also demonstrating an influence on prognosis. In conclusion, LOH status should be considered when TS genotype is explored as a potential prognostic and predictive marker for 5-FU-based adjuvant chemotherapy in colorectal cancer.

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ORIGINAL PAPER

Integrated genetic and epigenetic analysis of cancer-related genes in non-ampullary duodenal adenomas and intramucosal adenocarcinomas

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Abstract

The molecular and clinical characteristics of non-ampullary duodenal adenomas and intramucosal adenocarcinomas are not fully understood because they are rare. To clarify these characteristics, we performed genetic and epigenetic analysis of cancer-related genes in these lesions. One hundred and seven non-ampullary duodenal adenomas and intramucosal adenocarcinomas, including 100 small intestinal-type tumors (90 adenomas and 10 intramucosal adenocarcinomas) and 7 gastric-type tumors (2 pyloric gland adenomas and 5 intramucosal adenocarcinomas), were investigated. Using bisulfite pyrosequencing, we assessed the methylation status of CpG island methylator phenotype (CIMP) markers and *MLH1*. Then using next-generation sequencing, we performed targeted exome sequence analysis within 75 cancer-related genes in 102 lesions. There were significant differences in the clinicopathological and molecular variables between small intestinal- and gastric-type tumors, which suggests the presence of at least two separate carcinogenic pathways in non-ampullary duodenal adenocarcinomas. The prevalence of CIMP-positive lesions was higher in intramucosal adenocarcinomas than in adenomas. Thus, concurrent hypermethylation of multiple CpG islands is likely associated with development of non-ampullary duodenal intramucosal adenocarcinomas. Mutation analysis showed that *APC* was the most frequently mutated gene in these lesions (56/102; 55%), followed by *KRAS* (13/102; 13%), *LRP1B* (10/102; 10%), *GNAS* (8/102; 8%), *ERBB3* (7/102; 7%), and *RNF43* (6/102; 6%). Additionally, the high prevalence of diffuse or focal nuclear β-catenin accumulation (87/102; 85%) as well as mutations of WNT pathway components (60/102; 59%) indicates the importance of WNT signaling to the initiation of duodenal adenomas. The higher than previously reported frequency of *APC* gene mutations in small bowel adenocarcinomas as well as the difference in the *APC* mutation distributions between small intestinal-type adenomas and intramucosal adenocarcinomas may indicate that the adenoma–carcinoma sequence has only limited involvement in duodenal carcinogenesis.

Keywords: non-ampullary duodenal adenoma; small bowel adenocarcinoma; methylation; CpG island methylator phenotype; mutation

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No conflicts of interest were declared.

Introduction

Small intestinal cancers are rare. They comprise only 2–3% of the total annual cancer incidence in the digestive system [1,2], and a recent epidemiological study in the Unites States of America showed that the age-adjusted incidence rate (IR) for small intestinal cancers is only 2.10/100 000 person-years [3]. These small intestinal cancers include neuroendocrine cancers (IR = 0.83), carcinomas (IR = 0.66), sarcomas (IR = 0.20), and lymphomas (IR = 0.38), with small bowel adenocarcinomas (SBAs) accounting for approximately 69% of the carcinomas. The incidence of carcinomas is most prominent in the duodenum, and duodenal carcinomas have increased more markedly than other small intestinal cancers []. Duodenal adenomas are also uncommon lesions, with a reported prevalence of less than 0.1–0.3% in patients undergoing upper gastrointestinal endoscopy [4,5]. Nonetheless, with the advent of surveillance endoscopy and improvements in endoscopic imaging, these lesions are now being detected incidentally [6]. Moreover, these lesions are thought to progress into duodenal adenocarcinomas (DAs), via the adenoma–carcinoma sequence [7–9], a common pathway for colorectal cancer (CRC) development [10]. However, their clinicopathological characteristics and natural course have not been investigated in detail, due to their rarity. Because SBAs have a significantly poorer prognosis than CRCs [11], early detection and treatment are crucial. In particular, preoperative diagnosis to distinguish lesions that should be followed up from those that require treatment is an important problem [6], and molecular characterization of premalignant duodenal lesions is essential to address this issue.

Concurrent methylation of multiple CpG islands (CGIs) was first characterized as CpG island methylator phenotype (CIMP) in CRC by Toyota *et al* [12]. Since then, CIMP has been reported in various types of tumors [13]. Multiple studies also reported the presence of CIMP in SBAs, including DAs [14,15], and the methylation profiles of DAs reportedly differ from biliary and ampullary carcinomas [14]. Moreover, CIMP positivity, albeit without *MLH1* methylation, is reportedly associated with a poor prognosis in DA patients [15]. An earlier study analyzed DNA methylation in ampullary and non-ampullary duodenal adenomas, but that study did not analyze these tumor types separately [16]. Thus, epigenetic alterations in early non-ampullary duodenal lesions remain to be elucidated.

By contrast, genetic analyses of SBAs, including non-ampullary DAs, have been performed by multiple groups [17–23]. Schrock *et al* [20] analyzed mutations of cancer-related genes in 317 SBA samples and showed that the genetic signatures of SBAs were distinct from those in CRCs or gastric cancers. In addition, exome sequencing revealed potential driver genes, dysregulated oncogenic pathways, and targetable mutations in SBAs [18,19,21,22]. Still, the genetic alterations in early duodenal lesions are not fully understood, with only one earlier study analyzing mutations of 50 cancer-related genes in a limited number of samples [24].

To gain further insight into duodenal carcinogenesis, we analyzed the methylation and mutation status of a large number of non-ampullary duodenal adenomas and intramucosal adenocarcinoma samples, and assessed their clinicopathological significance. We also evaluated the involvement of the WNT signaling pathway, which is reportedly dysregulated in non-ampullary duodenal adenomas and adenocarcinomas [25–27].

Materials and methods

Patients and tissue samples

Specimens of non-ampullary duodenal adenomas and intramucosal adenocarcinomas (n = 107) were obtained from 107 Japanese patients who underwent endoscopic mucosal resection or endoscopic submucosal dissection at Ishikawa Prefectural Central Hospital or Fukui Prefectural Hospital between 2008 and 2019. Normal samples were also obtained from normal-appearing adjacent mucosa in 15 patients with small intestinal-type duodenal adenomas included in the present study. Patients with hereditary cancer syndrome such as familial adenomatous polyposis (FAP) or Peutz–Jeghers syndrome, as well as those with inflammatory bowel diseases, such as Crohn's disease, were excluded. Information on their BMI and smoking status at the time of treatment was also obtained. Approval of this study was obtained from the Institutional Review Board of Ishikawa Prefectural Central Hospital and Fukui Prefectural Hospital, Kanazawa University, Juntendo University, and Sapporo Medical University.

Endoscopic analysis

High-resolution magnifying endoscopes (GIF-H290Z; Olympus, Tokyo, Japan) were used for all upper gastrointestinal endoscopic analyses. The morphology of duodenal lesions was determined according to the Paris classification [28]. All lesions detected during esophagogastroduodenoscopy were observed at high magnification using narrow band imaging, after which samples were treated by endoscopic resection for histological analysis. Locations within the duodenum were subcategorized into bulb, descending part, and transverse part. Ninety-five small intestinal-type tumors, of which detailed information about location was available, were also subcategorized into tumors within the proximal part (from bulb to periampulla) and those within the distal part (from periampulla to transverse part), as described previously [26].

Histological analysis

Histological studies were first carried out at Ishikawa Prefectural Central Hospital and Fukui Prefectural Hospital. The histological findings for all specimens were

then re-reviewed by two independent pathologists (ST and TY) blinded to the clinical and molecular information. The presence of small intestinal phenotypes was determined based on the presence of a brush border, goblet cells, and Paneth cells in H&E-stained specimens. Immunohistochemical staining for CD10, MUC2, MUC5AC, and MUC6 was performed as described previously [29]. Small intestinal-type tumors were defined by CD10 and MUC2 staining. The presence of gastric-type differentiation was defined by MUC5AC and MUC6 staining. Tumors with the small intestinal phenotype were classified as small intestinal-type low-grade adenomas (SLAs), small intestinal-type high-grade adenomas (SHAs), or small intestinal-type intramucosal adenocarcinomas (SCAs) according to the World Health Organization (WHO) tumor classification system (supplementary material, Figure S1) [8]. Tumors with the gastric phenotype were classified as pyloric gland adenomas (PGAs) or gastric-type intramucosal adenocarcinomas (GCAs). SLAs and PGAs were classified as category 3 tumors, while SHAs, SCAs, and GCAs were classified as category 4 tumors according to the revised Vienna classification of gastrointestinal epithelial neoplasia [30]. SCAs correspond to non-invasive carcinomas (category 4.2), suspicious for invasive carcinomas (category 4.3), or intramucosal carcinomas (category 4.4). The clinicopathological features of the lesions are summarized in Table 1.

DNA isolation

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

DNA methylation analysis

DNA methylation was analyzed using bisulfite pyrosequencing as described previously [31]. A cut-off value of 15% was used to define genes as methylation-positive. We used five CIMP markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*) proposed by Weisenberger *et al* [32]. Tumors were defined as CIMP-positive if two or more loci showed methylation and as CIMP-high (CIMP-H) if three or more loci showed methylation, as previously reported [16]. In addition, methylation of the *MLH1* gene was investigated. Primer sequences are shown in supplementary material, Table S1.

Targeted amplicon sequencing analysis

A customized panel, encompassing all exons for 75 cancer-related genes including those frequently mutated in SBAs [17–21,33,34], was created using the Ion AmpliSeq Designer (Thermo Fisher Scientific) (supplementary material, Table S2). Genes whose mutations had been reported in duodenal adenomas were also included [24]. The assay design consisted of 3663 amplicons with an average length of 112 bp, covering 95.5% of the 366 kb target sequence. Library preparation and sequencing with an Ion Proton sequencer were performed as described previously [35,36]. The templates were sequenced after emulsion PCR with 12–16 samples per Ion PI chip using an Ion PI HI-Q Chef kit (Thermo Fisher Scientific).

Identification of somatic mutations and copy number variations

Somatic mutations and copy number variations (CNVs) were detected as described previously [35]. Human genome build 19 (hg19) was used as a reference. Signal processing, base-calling, mapping to the hg19 reference, alignment, and further quality filtering were performed using Torrent Suite version 5.0 (Thermo Fisher Scientific). Somatic mutations, including point mutations, insertions, and deletions, were detected using Ion Reporter Software 5.0 (Thermo Fisher Scientific). Because matched normal controls were not available, we utilized Ion Reporter software tumor–normal workflow using Demo AmpliSeq Exome control as the normal control for excluding common single nucleotide polymorphisms (SNPs). A sequencing coverage of 20x and a minimum variant frequency of 5% of the total number of distinct tags were used as cut-offs. The pathogenic status of the variant was stated if it was a missense variant with less than 0.1% global minor allele frequency in the dbSNP and/or the variant was suggested as being pathogenic in the ClinVar, COSMIC, SIFT, or PolyPhen-2 databases. Variants with allele frequencies between 0.4 and 0.6 or greater than 0.9 were considered germline variants unless listed as a pathogenic variant. Integrative Genomics Viewer (IGV) software (http://software.broadinstitute.org/software/igv/) was used to filter out possible strand-specific errors, such as a mutation that was identified in the forward or reverse DNA strand but not in both strands. CNVs were also detected using Ion Reporter Software with an algorithm based on the Hidden Markov Model. Recurrent genomic regions with CNVs were detected using copy numbers greater than 3 and less than 1 for gains and losses, respectively.

Immunohistochemistry

Immunohistochemical studies of β-catenin expression were performed in 102 samples, as described previously [37]. A mouse anti-β-catenin monoclonal antibody (1:1000 dilution, Clone 14; BD Biosciences, San Jose, CA, USA) was used. Expression of β-catenin was evaluated semi-quantitatively in tumor cells with β-catenin-positive nuclei, as reported previously [38]: negative, 0–9%; focal, 10–49%; and diffuse, > 50%. All slides were evaluated by two pathologists (ST and TY) blinded to the clinical and molecular data. In addition, immunohistochemical studies of two mismatch repair (MMR) proteins, MLH1 (1:1000 dilution, Ab92312; Abcam, Cambridge, UK) and MSH2 (1:1000 dilution, Ab

Table 1. Clinicopathological features of non-ampullary duodenal lesions.

Histology Vienna classification	Total	SLA Category 3	SHA Category 4 Small intestinal type	SCA	PGA Category 3	GCA Category 4 Gastric type	*P* value (Small intestinal type versus gastric type)
Patients' characteristics (*n*)	107	32	58	10	2	5	
Age (years, mean ± SD)	64 ± 10	64 ± 9	63 ± 10	66 ± 12	69 ± 3	78 ± 4	0.0022
Gender							
Male, *n* (%)	80 (75%)	24 (75%)	43 (74%)	8 (80%)	1 (50%)	4 (80%)	> 0.9999
Female, *n* (%)	27 (25%)	8 (25%)	15 (26%)	2 (20%)	1 (50%)	1 (20%)	
BMI (kg/m^2, mean ± SD)	22.9 ± 3.4	23.2 ± 3.5	22.4 ± 3.5	24.4 ± 2.4	22.2 ± 1.1	23.7 ± 3.2	0.79
Smoking status							
Current smoker, *n* (%)	29 (27%)	7 (22%)	20 (34%)	0 (0%)	0 (0%)	2 (40%)	> 0.9999
Former/non-smoker, *n* (%)	78 (73%)	25 (78%)	38 (66%)	10 (100%)	2 (100%)	3 (60%)	
Lesion characteristics							
Location in duodenum							
Bulb, *n* (%)	17 (16%)	4 (13%)	6 (10%)	1 (10%)	2 (100%)	4 (80%)	< 0.0001
Descending part, *n* (%)	86 (80%)	27 (84%)	49 (85%)	9 (90%)	0 (0%)	1 (20%)	(Bulb versus non-bulb)
Transverse part, *n* (%)	3 (3%)	1 (3%)	2 (3%)	0 (0%)	0 (0%)	0 (0%)	
Others, *n* (%)	1 (1%)	0 (0%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)	
Lesion size (mm, mean ± SD)	13 ± 8	11 ± 6	14 ± 8	19 ± 10	10 ± 0	18 ± 5	0.41
Morphology							
Protruding (0-Ip, 0-Is, 0-Isp), *n* (%)	16 (15%)	4 (13%)	4 (7%)	3 (30%)	1 (50%)	4 (80%)	0.0002
Mixed (0-I + IIa), *n* (%)	5 (5%)	0 (0%)	3 (5%)	1 (10%)	1 (50%)	0 (0%)	(Protruding or mixed versus nonprotruding)
Nonprotruding and nonexcavated (0-IIa, 0-IIc, 0-IIa + IIc), *n* (%)	85 (79%)	28 (87%)	50 (86%)	6 (60%)	0 (10%)	1 (20%)	
N/A, *n* (%)	1 (1%)	0 (0%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)	

GCA, gastric-type intramucosal adenocarcinoma; N/A, not available; PGA, pyloric gland adenoma; SCA, small intestinal-type intramucosal adenocarcinoma; SHA, small intestinal-type high-grade adenoma; SLA, small intestinal-type low-grade adenoma.

227 841; Abcam), was performed in 107 samples to assess microsatellite instability (MSI) status. To evaluate expression, lymphocytes in adjacent normal tissue were used as an internal positive control. When nuclear staining was identified in epithelial cells, the lesion was defined as positive for MMR proteins. All slides were evaluated by a pathologist (SK) blinded to the clinical and molecular data.

Statistical analysis

Continuous data were analyzed using *t*-tests (for two groups) or ANOVA with a *post hoc* Tukey's HSD test (for more than two groups). Comparison of categorical data between two or more groups was performed using the Fisher's exact test or chi-squared test. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

Results

Clinicopathological characteristics of non-ampullary duodenal lesions

The clinicopathological and molecular characteristics of 107 non-ampullary duodenal lesions analyzed in this study are summarized in Table 1. The average age at endoscopic treatment was 64 years. The male-to-female ratio was approximately 3:1, which is consistent with previous studies from Japan [9,26,39]. When compared between cases with small intestinal-type (SLA, SHA, and SCA) and gastric-type (PGA and GCA) tumors, age at treatment was significantly higher in those with gastric-type tumors (*t*-test, $p = 0.0022$). In addition, tumors with the gastric phenotype were more prevalent in the duodenal bulb (6/7, 86%) than were those with the small intestinal phenotype (11/100, 11%) (Fisher's exact test, $p < 0.0001$). On endoscopic observation, most tumors with gastric differentiation were protruding lesions or mixed lesions that included a protruding portion (6/7, 86%), and differed significantly from tumors with small intestinal differentiation, most of which were non-protruding and non-excavated lesions (84/100, 84%) (Fisher's exact test, $p = 0.0002$, Table 1). We also compared the clinicopathological characteristics of small intestinal-type tumors based on the proximal or distal locations (supplementary material, Table S3). Significant differences were found in the tumor histological types (SLA, SHA or SCA, chi-squared test, $p = 0.017$) and categories (3 or 4, Fisher's exact test, $p = 0.0075$) between tumors in the proximal and distal duodenum. In addition, the prevalence of nonprotruding and nonexcavated type tumors was significantly higher in the distal part (Fisher's exact test, $p = 0.037$).

Methylation analysis of CIMP markers and *MLH1*

Among non-ampullary duodenal lesions, we assessed the methylation status of cancer-associated genes in 107 lesions and assessed the CIMP and CIMP-H status

in 106 lesions. Twenty-five lesions (25/106, 24%) were defined as CIMP-positive, and seven lesions (7/106, 7%) were defined as CIMP-H (Figure 1 and Table 2). The prevalence of CIMP-positive lesions was significantly associated with male gender (Fisher's exact test, $p = 0.033$), older age (cut-off value 75 years, Fisher's exact test, $p = 0.043$), and larger tumor size (cut-off value 15 mm, Fisher's exact test, $p = 0.032$) (Table 3). By contrast, BMI, smoking status, and the location and endoscopic morphology of the lesions were not associated with CIMP positivity. When samples were divided into adenomas (SLA, SHA, and PGA) and intramucosal adenocarcinomas (SCA and GCA), the prevalence of CIMP-positive lesions was higher in intramucosal adenocarcinomas (6/15, 40%) than in adenomas (19/91, 21%), although there were no statistical significant differences (Fisher's exact test, $p = 0.18$, Table 3). When only small intestinal-type tumors were analyzed, CIMP positivity was not associated with tumor histological type (SLA, SHA, and SCA). Moreover, the prevalence of CIMP-positive lesions was not associated with either histological classification (small intestinal type or gastric type) or Vienna classification (category 3 or 4). Although two *MLH1* methylation-positive lesions were detected among the SHA samples, methylation levels were below 20% (15.8% and 16.5%) in these lesions. In addition, there was no association between *MLH1* methylation status and CIMP or CIMP-H positivity (Figure 1). Although there

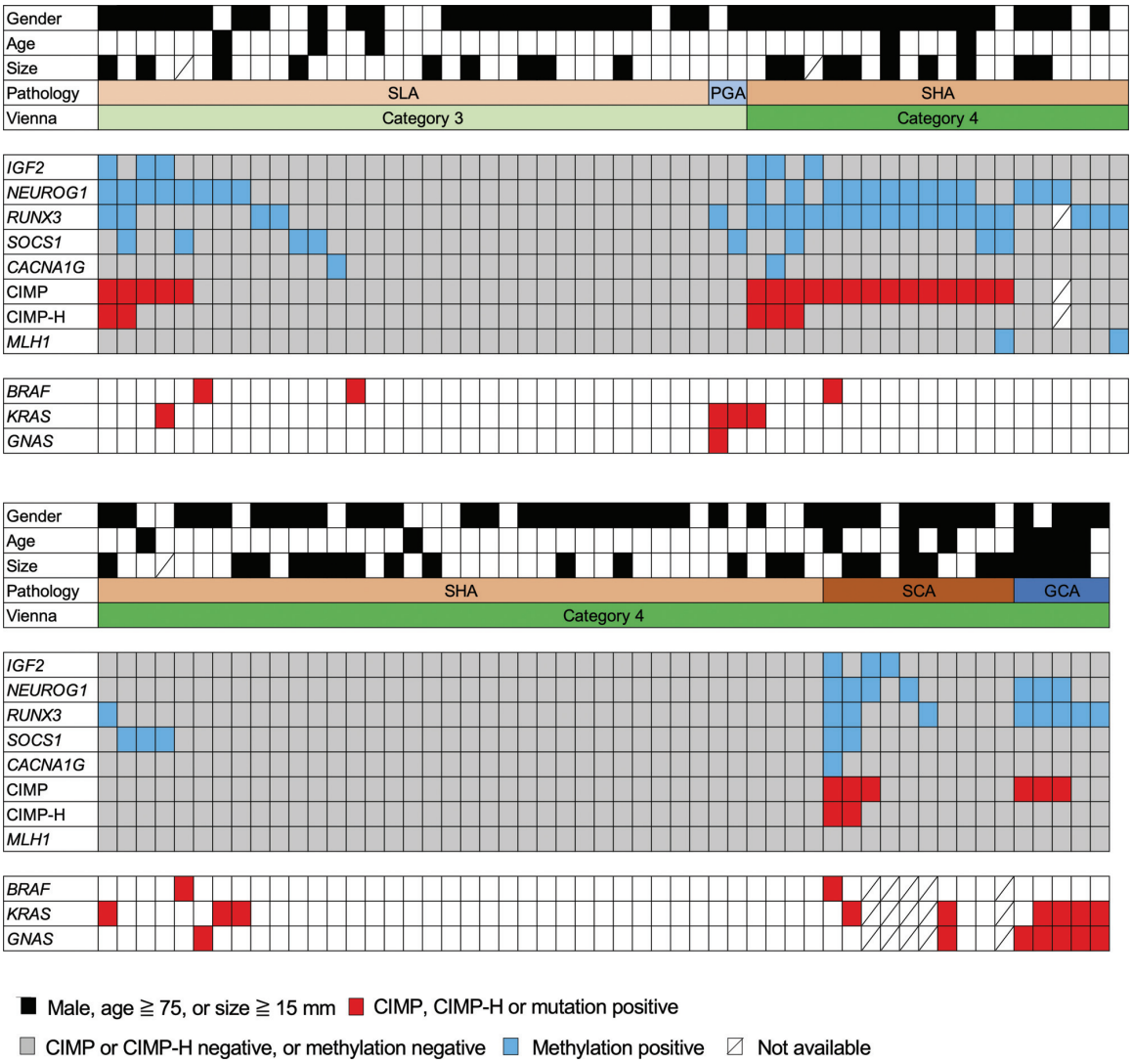

Figure 1. Methylation and mutation profiles in non-ampullary duodenal adenomas and intramucosal adenocarcinomas. Summarized results for CIMP marker methylation, CIMP status, *MLH1* methylation, and *BRAF/KRAS/GNAS* mutations in tumors with the indicated histological types are shown. CIMP, CpG island methylator phenotype; GCA, gastric-type intramucosal adenocarcinoma; PGA, pyloric gland adenoma; SCA, small intestinal-type intramucosal adenocarcinoma; SHA, small intestinal-type high-grade adenoma; SLA, small intestinal-type low-grade adenoma.

Table 2. Molecular characteristics and β-catenin expression of the respective histological types of non-ampullary duodenal lesions.

Histology	Total	SLA	SHA	SCA	PGA	GCA	*P* value
		Small intestinal type			Gastric type		(small intestinal type versus gastric type)
Gene mutation (*n*)	102	32	58	5	2	5	
APC mutation, *n* (%)	56 (55%)	17 (53%)	34 (59%)	3 (60%)	1 (50%)	1 (20%)	0.24
BRAF mutation, *n* (%)	5 (5%)	2 (6%)	2 (3%)	1 (20%)	0 (0%)	0 (0%)	> 0.9999
KRAS mutation, *n* (%)	13 (13%)	1 (3%)	4 (7%)	2 (40%)	2 (100%)	4 (80%)	< 0.0001
GNAS mutation, *n* (%)	8 (8%)	0 (0%)	1 (2%)	1 (20%)	1 (50%)	5 (100%)	< 0.0001
Epigenetic alteration (*n*)	107	32	58	10	2	5	
CIMP*, *n* (%)	25 (24%)	5 (16%)	14 (24%)	3 (30%)	0 (0%)	3 (60%)	0.36
CIMP-high*, *n* (%)	7 (7%)	2 (6%)	3 (5%)	2 (20%)	0 (0%)	0 (0%)	> 0.9999
MLH1 methylation, *n* (%)	2 (2%)	0 (0%)	2 (3%)	0 (0%)	0 (0%)	0 (0%)	> 0.9999
β-catenin expression (*n*)	102	32	58	5	2	5	
Negative, *n* (%)	15 (15%)	3 (10%)	5 (8%)	1 (20%)	1 (50%)	5 (100%)	< 0.0001
Focal, *n* (%)	33 (32%)	11 (34%)	19 (33%)	2 (40%)	1 (50%)	0 (0%)	(focal or diffuse versus negative)
Diffuse, *n* (%)	54 (53%)	18 (56%)	34 (59%)	2 (40%)	0 (0%)	0 (0%)	

CIMP, CpG island methylator phenotype; GCA, gastric-type intramucosal adenocarcinoma; PGA, pyloric gland adenoma; SCA, small intestinal-type intramucosal adenocarcinoma; SHA, small intestinal-type high-grade adenoma; SLA, small intestinal-type low-grade adenoma.
β-catenin expression was categorized as negative (0–9%), focal (10–49%), and diffuse (> 50%).
*CIMP status and CIMP-high status were analyzed in 106 lesions.

Table 3. Relationship between CIMP status and clinicopathological characteristics of non-ampullary duodenal lesions.

Characteristics	Total	CIMP positive	CIMP negative	*P value*
Patients (*n*)	106	25	81	
Age (years, mean ± SD)	64 ± 10	68 ± 9	63 ± 10	0.026
< 75, *n* (%)	91 (86%)	18 (72%)	73 (90%)	0.043
≥ 75, *n* (%)	15 (14%)	7 (28%)	8 (10%)	
Gender				
Male, *n* (%)	79 (75%)	23 (92%)	56 (69%)	0.033
Female, *n* (%)	27 (25%)	2 (8%)	25 (31%)	
BMI (kg/m^2, mean ± SD)	22.9 ± 3.4	23.4 ± 3.4	22.7 ± 3.4	0.39
Smoking status				
Current smoker, *n* (%)	29 (27%)	4 (16%)	25 (31%)	0.20
Former/non-smoker, *n* (%)	77 (73%)	21 (84%)	56 (69%)	
Location				
Bulb, *n* (%)	16 (15%)	5 (20%)	11 (14%)	0.52
Descending – transverse part, *n* (%)	89 (84%)	20 (80%)	69 (85%)	(Bulb versus non-bulb)
Other, *n* (%)	1 (1%)	0 (0%)	1 (1%)	
Size (mm, mean ± SD)	13 ± 8	17 ± 10	12 ± 7	0.032
< 15, *n* (%)	61 (57%)	9 (36%)	52 (64%)	0.032
≥ 15, *n* (%)	42 (40%)	14 (56%)	28 (35%)	
N/A, *n* (%)	3 (3%)	2 (8%)	1 (1%)	
Morphology				
Protruding, *n* (%)	16 (15%)	4 (16%)	12 (15%)	0.78
Mixed, *n* (%)	5 (5%)	0 (0%)	5 (6%)	(Protruding or mixed versus nonprotruding)
Nonprotruding and nonexcavated, *n* (%)	84 (79%)	21 (84%)	63 (78%)	
N/A, *n* (%)	1 (1%)	0 (0%)	1 (1%)	
Histology				
SLA, *n* (%)	32 (30%)	5 (20%)	27 (34%)	0.51*
SHA, *n* (%)	57 (54%)	14 (56%)	43 (53%)	
SCA, *n* (%)	10 (9%)	3 (12%)	7 (9%)	
PGA, *n* (%)	2 (2%)	0 (0%)	2 (2%)	
GCA, *n* (%)	5 (5%)	3 (12%)	2 (2%)	
Small intestinal type, *n* (%)	99 (93%)	22 (88%)	77 (95%)	0.35
Gastric type, *n* (%)	7 (7%)	3 (12%)	4 (5%)	
Adenoma, *n* (%)	91 (86%)	19 (76%)	72 (89%)	0.18
Adenocarcinoma, *n* (%)	15 (14%)	6 (24%)	9 (11%)	
Vienna classification				
Category 3, *n* (%)	34 (32%)	5 (20%)	29 (36%)	0.14
Category 4, *n* (%)	72 (68%)	20 (80%)	52 (64%)	

CIMP, CpG island methylator phenotype; GCA, gastric-type intramucosal adenocarcinoma; N/A, not available; PGA, pyloric gland adenoma; SCA, small intestinal-type intramucosal adenocarcinoma; SHA, small intestinal-type high-grade adenoma; SLA, small intestinal-type low-grade adenoma.
*Analyzed with SLA, SHA, and SCA.

was no relationship between CIMP-H status and age, gender, smoking status, or disease location, CIMP-H positivity was associated with BMI (*t*-test, $p = 0.022$) and lesion size (*t*-test, $p = 0.012$) (supplementary material, Table S4). In addition, the prevalence of CIMP-H tumors did not significantly differ among histological subtypes or between small intestinal-type and gastric-type tumors.

Targeted amplicon sequencing of non-ampullary duodenal lesions

We performed targeted sequencing of all exons in 75 cancer-related genes frequently mutated in SBAs, including DAs, as well as duodenal adenomas. A sequencing overview, including reads, coverage, and uniformity of the read coverage distribution, is shown in supplementary material, Table S5. Each FFPE sample underwent an average of 8.5 million sequencing reads after quality filtering. A mean coverage depth of 2461.0 reads (737.0–10 950.0) per base was observed. All single nucleotide variations and insertions/deletions detected through bioinformatics analysis underwent visual inspection using the Integrative Genomics Viewer for confirmation. We identified a mean of 1.9 somatic nonsynonymous mutations (range 0–8) per sample (supplementary material, Table S6). At least one somatic nonsynonymous mutation was observed in 45 of the 75 genes. The ten most commonly mutated genes in non-ampullary duodenal lesions are illustrated in Figure 2. *APC* was the most frequently mutated gene in these lesions (56 of 102 samples; 55%), followed by *KRAS* (13/102; 13%), *LRP1B* (10/102; 10%), *GNAS* (8/102; 8%), *ERBB3* (7/102; 7%), and *RNF43* (6/102; 6%).

For *APC*, a single mutation per sample was detected in 48 subjects, and two different mutations per sample were detected in eight subjects, resulting in a total of 64 mutations in this study. Most *APC* mutations were nonsense (43 mutations) or frameshift (16 mutations), though a number of missense mutations (five mutations) were also detected. The mutation distribution within *APC* was visualized using MutationMapper in cBioPortal (https://www.cbioportal.org) [40,41] with several modifications (Figure 3A). Most of the deleterious mutations were distributed between codons 700 and 1200 or between codons 1400 and 1600. Although a prior study suggested that T1556fs is a mutation hotspot within *APC* in duodenal adenomas [24], we most frequently found a mutation at R1450X (in ten samples), which is similar to CRC [42] but different from ampullary carcinoma [34] (Figure 3B). Although the frequencies of mutations within mutation cluster regions (codons 700–1200 and 1400–1600) among the total mutations detected in each histological type were similar between SLAs and SHAs, the mutation frequency in small intestinal-type adenomas (SLAs + SHAs) (86%; 51/59 mutations) within these regions was higher than in SCAs (33%; 1/3 mutations) (Fisher's exact test, $p = 0.065$). Overall, mutations within WNT signaling pathway components were detected in 59% (60/102) of the samples (Figure 4).

There were significant differences in the prevalence of *KRAS* mutations among the histological subtypes of small intestinal-type tumors: SLA (1/32; 3%), SHA (4/58; 7%), and SCA (2/5; 40%) (chi-squared test, $p = 0.013$), though there was no significant difference in the prevalence of *APC* mutations among histological subtypes (Table 2). *TP53* mutations were detected in five samples (5%), and samples harboring a mutation within at least one gene among *APC*, *KRAS*, and *TP53* were observed in 62 patients (61%) (Figure 2). All eight detected *GNAS* mutations were at codon 201, and six of those are associated with *KRAS* mutations. When compared between small intestinal-type tumors and gastric-type tumors, *GNAS* and *KRAS* mutations were significantly associated with gastric-type tumors (Table 2).

ERBB2 mutations, which have been frequently reported in SBAs, were also detected in four of the 102 samples (4%). The frequency of lesions harboring at least one mutation in an ERBB receptor family member (*ERBB2*, *ERBB3*, or *ERBB4*) was 12% (12/102) (Figure 4). *BRAF* mutation was detected in five samples (5%), which is comparable to earlier studies reporting frequencies of 6–11% in SBA samples [17,20,21]. *BRAF* V600E mutation was detected in only one SCA sample with CIMP and CIMP-H (Figure 1), which is consistent with an earlier report that only about 10% of *BRAF*-mutated SBAs harbor V600E mutations [20].

Targeted amplicon sequencing detects CNVs

We also detected CNVs in segments of the genome that could be duplicated or deleted from the sequencing data (Figure 2 and supplementary material, Table S7). Based on copy number gains in all samples, the most frequently affected genes that were considered oncogenic were *EPHA6* (43/102; 42%), followed by *KRAS* (30/102; 29%), *ERBB4* (25/102; 25%), *BRAF* (12/102; 15%), and *GNAS* (13/102; 13%). On the other hand, the most frequently affected tumor suppressive genes by copy number losses were *MIB2* (41/102; 40%), *CDKN2A* (37/102; 36%), *TP53* (10/102; 10%), and *ARID1B* (10/102; 10%). The distributions of CNVs were not different among tumor histological subtypes. As for ERBB family genes, copy number gains were observed in one case each for *ERBB2* and *ERBB3*, while frequent copy number gains (25/102, 25%) were detected in *ERBB4* (Figure 4). Lastly, the frequency of lesions harboring at least one mutation or copy number gain in an ERBB family member was 34% (35/102).

Immunohistochemistry

Of the 102 non-ampullary duodenal lesions analyzed, 33 (32%) showed focal nuclear expression and 54 (53%) showed diffuse nuclear expression, whereas 15 (15%) showed no nuclear expression of β-catenin (Table 2, Figure 4, and supplementary material, Figure S2). The prevalence of diffuse and focal nuclear β-catenin accumulation in duodenal adenomas (SLA,

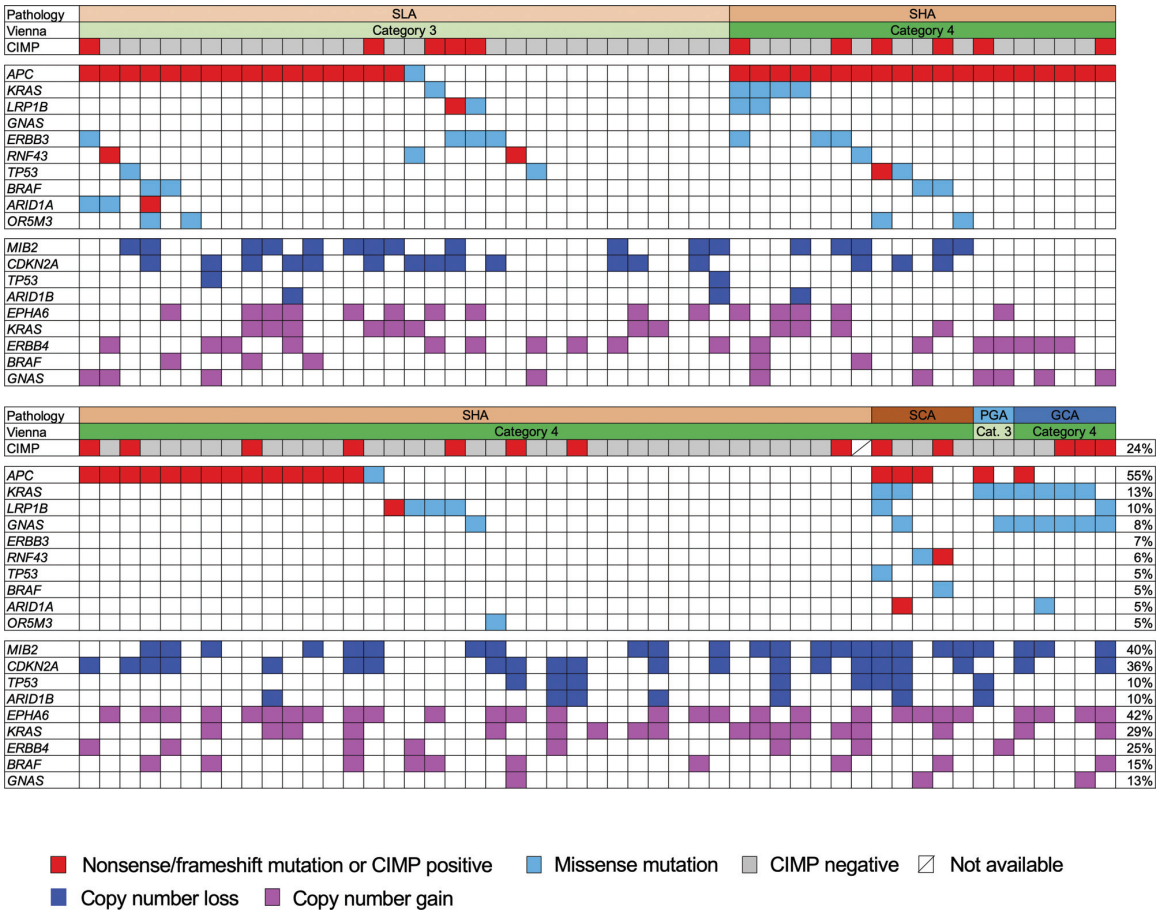

Figure 2. Mutation and CNV profiles in non-ampullary duodenal adenomas and intramucosal adenocarcinomas. Upper panels show histological types, Vienna classification, and CIMP status. Middle panels show summarized results for targeted sequencing of cancer-related genes in tumors with the indicated histological types. Lower panels show frequently detected CNVs. Frequencies of CIMP status as well as mutations and CNVs in respective genes are shown on the right. CIMP, CpG island methylator phenotype; GCA, gastric-type intramucosal adenocarcinoma; PGA, pyloric gland adenoma; SCA, small intestinal-type intramucosal adenocarcinoma; SHA, small intestinal-type high-grade adenoma; SLA, small intestinal-type low-grade adenoma.

SHA, and PGA) (90%; 83/92) was consistent with earlier reports of nuclear β-catenin accumulation in 64–84% of non-ampullary adenomas [25–27]. Lesions showing nuclear β-catenin accumulation (diffuse or focal) were not associated with *APC* mutation-positive tumors or tumors with mutations of WNT signaling components. The prevalence of nuclear β-catenin accumulation was significantly higher in small intestinal-type tumors (86/95, 91%) than in gastric-type tumors (1/7, 14%) (Fisher's exact test, $p < 0.0001$, Table 2). However, when we focused on tumors with the small intestinal phenotype, the prevalence of nuclear β-catenin accumulation did not significantly differ among SLAs, SHAs, and SCAs. We found no significant differences between β-catenin expression or molecular variables and the locations (proximal and distal) of the 90 small intestinal-type tumors (supplementary material, Table S3).

Among MMR proteins, expression of MLH1 and MSH2 was evaluated immunohistochemically across all non-ampullary duodenal lesions. All samples stained positively for MLH1, which was largely consistent with the methylation data. By contrast, loss of MSH2 expression was detected in one SCA sample (KT23) (supplementary material, Figure S3). That sample harbored the largest number of somatic mutations (eight) among the 75 genes investigated (supplementary material, Table S6). Six of the eight mutations were insertions of one base resulting in frameshift mutations, which supports the possibility of MSI.

Discussion

In the present study, we performed integrated genetic and epigenetic analyses of non-ampullary duodenal lesions. In addition to clinicopathological variables, there were significant differences in molecular variables between small intestinal-type and gastric-type tumors. High prevalences of *KRAS* and *GNAS* mutations in PGAs and GCAs were consistent with earlier

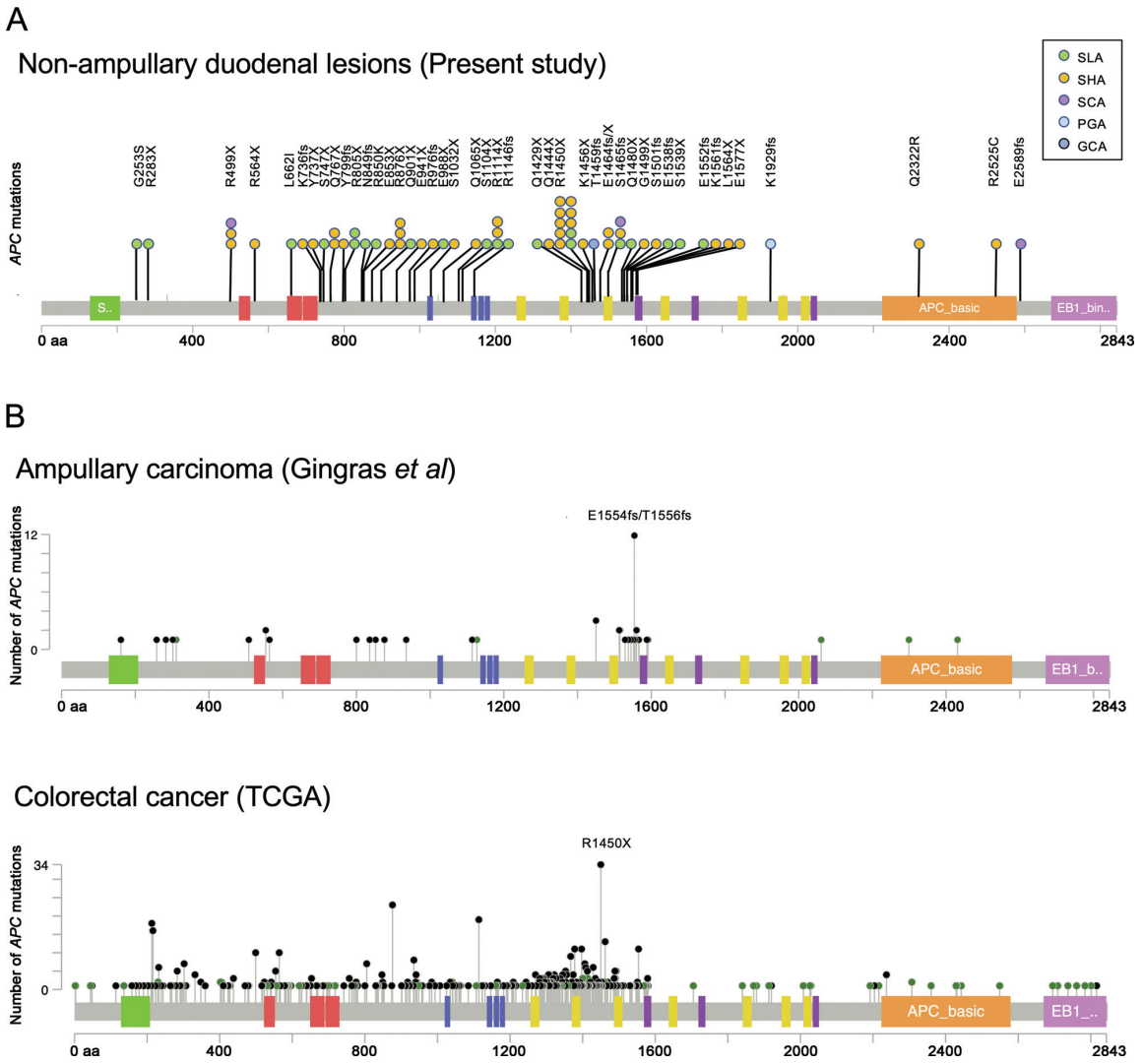

Figure 3. *APC* mutations detected in non-ampullary duodenal lesions. (A) Schematic representation of 64 *APC* mutations detected in the present study. Most of the somatic mutations detected in SLAs and SHAs were clustered within codons 700–1200 and 1400–1600, where hotspot mutation (R1450X) is located. Each circle represents an individual mutation in each patient. (B) Mutations previously reported in ampullary carcinomas [34] and colorectal cancers [42] are also indicated for comparison. GCA, gastric-type intramucosal adenocarcinoma; PGA, pyloric gland adenoma; SCA, small intestinal-type intramucosal adenocarcinoma; SHA, small intestinal-type high-grade adenoma; SLA, small intestinal-type low-grade adenoma.

reports [43,44]. The low prevalences of nuclear β-catenin expression in PGAs and GCAs, which is also consistent with an earlier study [26], may suggest that the WNT signaling pathway is less involved in development of non-ampullary duodenal tumors with the gastric phenotype. These results indicate that small intestinal-type and gastric-type tumors arise via separate carcinogenic pathways. When small intestinal-type tumors were divided based on whether they were located within the proximal or distal duodenum, SLAs and nonprotruding and nonexcavated type tumors were more prevalent in the distal part. However, there were no significant differences in the molecular characteristics including β-catenin expression between tumors in these two locations. Previous reports suggested that development of gastric-type tumors in the proximal duodenum is potentially associated with gastric acid and *H. pylori* infection, whereas small intestinal-type tumors in the distal duodenum may be associated with bile acids [26]. Further study will be necessary to clarify the pathological and molecular differences between tumors in the proximal and distal duodenum.

We also found that CIMP and CIMP-H frequencies are higher in intramucosal adenocarcinomas than in adenomas, indicating that concurrent methylation of CGIs is likely associated with malignant transformation of non-ampullary duodenal adenomas. In the clinical settings,

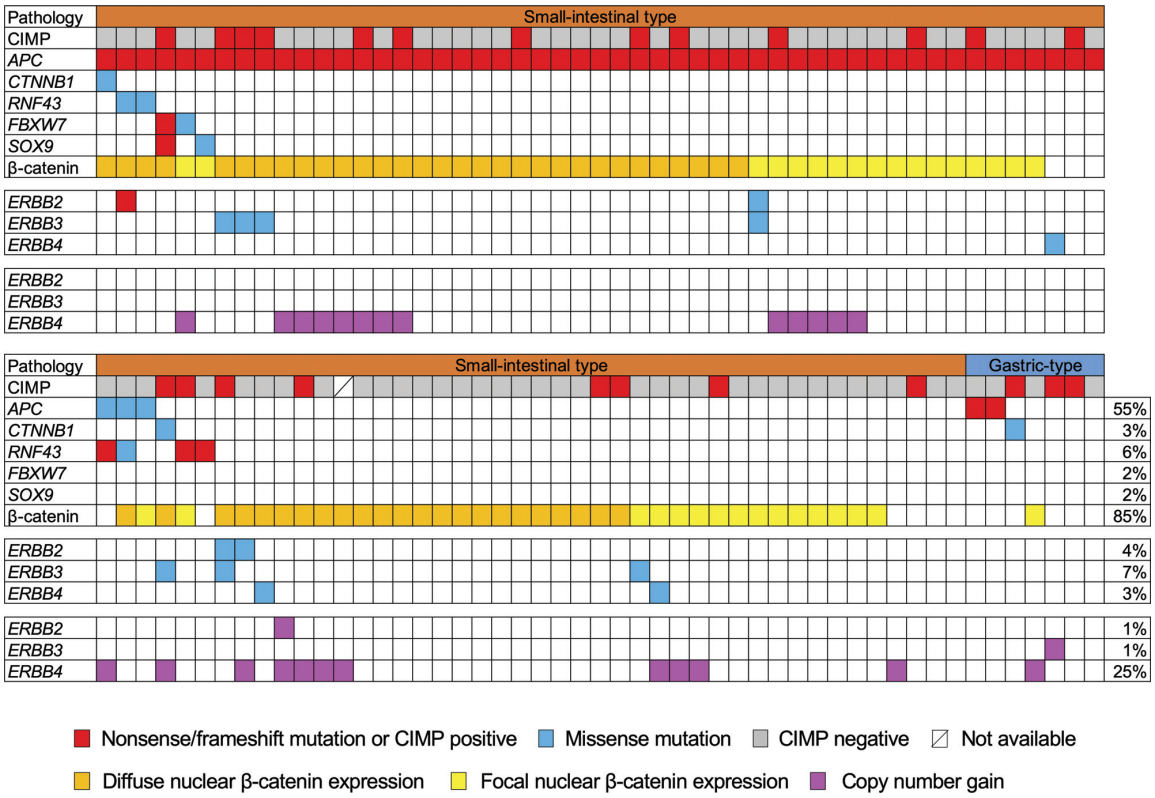

Figure 4. Mutation and copy number gains of genes related to the WNT signaling pathway and ERBB receptor family members in non-ampullary duodenal lesions. Pathological types (small intestinal or gastric type) and CIMP status, mutations in WNT signaling-associated genes, and β-catenin expression status are shown at the top. Mutations and copy number gains in ERBB receptor family members are shown in the middle panels and lower panels. Frequencies of mutations and copy number gains in respective genes as well as β-catenin expression positivity are shown on the right. CIMP, CpG island methylator phenotype.

tumors classified as category 3 according to the revised Vienna classification (SLAs and PGAs) can be monitored and followed up [30], while it is recommended that category 4 tumors (SHAs, SCAs, and GCAs) be treated, especially those that are 20 mm in diameter or larger, as these lesions have a high risk of progression to adenocarcinoma [9]. The fact that CIMP or CIMP-H positivity was associated with larger lesions in the present study may support this recommendation. At the same time, from the viewpoint of aberrant DNA methylation, SHAs could be followed up at a later time, especially if they are small in size. Despite recent advances in endoscopic technology, including magnifying endoscopy and image enhanced endoscopy, it remains difficult to distinguish between lesions that should be followed up or treated [6]. Pretreatment diagnosis by using biopsy is also difficult because the biopsy procedure itself may induce unintended fibrosis, possibly causing unsuccessful endoscopic resection. If specific endoscopic findings for lesions with concurrent methylation are determined, detailed endoscopic assessment could contribute to the prediction of premalignant lesions, as was previously reported for colorectal serrated lesions [45].

MLH1 methylation has been reported in 12% of non-ampullary adenomas [16] as well as in 14% [15] or 25% [14] of DAs. MSI has also been reported in 20% [15] or 33% [17] of DAs, where it is reportedly associated with *MLH1* methylation [15]. In the present study, only two *MLH1* methylation-positive lesions, with relatively low methylation levels, and no samples with loss of MLH1 immunoreactivity were detected. Additionally, there was only one sample with loss of MSH2 expression in possible association with MSI. These results suggest that acquisition of MSI in association with MMR deficiency, especially with *MLH1* methylation, is an infrequent event in early duodenal carcinogenesis.

Recent studies indicated that the genes recurrently mutated in SBAs are *TP53* (41–58%), *KRAS* (27–54%), and *APC* (11–27%) [17–22]. An earlier study analyzed 50 hotspot mutations in cancer-related genes in 19 patients with non-ampullary duodenal adenomas and adenocarcinomas, and found prevalent mutations in *KRAS* (63%), *APC* (47%), and *TP53* (37%) [24]. In the present study, we found mutations in *APC* (55%) and *KRAS* (13%); *TP53* mutations were observed in only 5% of the samples. These differences are likely due to the proportion of the adenocarcinomas or high-grade adenomas. In the present study, most *KRAS* mutations appeared to occur at the progression of intramucosal

adenocarcinomas. In addition, the lower prevalence of *TP53* mutations in early duodenal lesions than in SBAs suggests that most *TP53* mutations occur at a later stage of tumorigenesis. These results in SBAs appear to some degree consistent with the adenoma–carcinoma sequence previously seen in CRC [10]. We also detected a higher prevalence of gene mutations associated with the WNT signaling pathway (59%) and nuclear β-catenin accumulation (85%) in non-ampullary duodenal lesions. Together with earlier immunohistochemical and gene expression studies of duodenal adenomas [25–27], these results indicate the importance of the WNT signaling pathway to duodenal adenoma development.

Interestingly, we detected *APC* mutations (55%) more frequently in early non-ampullary duodenal lesions than did earlier studies in advanced lesions, which reported frequencies of 13–27% in SBAs or 8% in DAs [17,20–22]. Kojima *et al* separately analyzed multiple components in non-ampullary duodenal lesions that exhibited different histological grades, and observed *APC* mutations more frequently in the adenoma (58%) than in the adenocarcinoma (25%) components [24]. The earlier observation that abnormal nuclear localization of β-catenin is more frequent in non-ampullary duodenal adenomas than in adenocarcinomas (84% versus 33%) may support these results [26]. Although a number of studies have reported that gastric intramucosal neoplasias with the small intestinal phenotype also frequently show *APC* mutations, a few dysplasia/intramucosal neoplasias with *APC* mutation reportedly progress to gastric cancer [46]. Together with the different mutation distribution patterns between duodenal adenomas (SLAs + SHAs) and SCAs, the higher prevalence of *APC* mutations in duodenal adenomas than in SBAs or DAs may suggest that most duodenal adenomas, especially those with mutations within mutation cluster regions, also have low malignant potential and do not progress to DA. This suggests that the adenoma–carcinoma sequence has only limited impact on duodenal carcinogenesis. Targeted deep DNA sequencing with tumor variant allele frequency analysis in larger numbers of adenoma, intramucosal adenocarcinoma, and DA samples may confirm these findings and improve our understanding of the mechanisms underlying duodenal carcinogenesis, as has been described for gastric intramucosal neoplasias with the small intestinal phenotype [46].

ERBB2 mutations and amplifications have been detected in 12–23% of SBAs, which suggests that they are potential therapeutic targets [17,20–22]. *ERBB2* alterations are also significantly associated with a duodenal location when compared to other parts of the small intestine [17,20]. In the present study, *ERBB2* mutations were observed in only 4% of samples. Because a significant number of *ERBB2* mutations were observed in SBAs with MMR deficiency [17,21], most of these mutations may have been acquired after the cancer progression and/or acquisition of MSI. A recent study using SBA patient-derived cell lines demonstrated that small-molecule ERBB2 inhibitors have anti-cancer activity both *in vitro* and *in vivo* [22]. Such small-molecule ERBB2 inhibitors may be useful not only for the treatment of metastatic SBA but also for the chemoprevention of multiple non-ampullary duodenal adenoma in patients with FAP, if activating *ERBB2* mutations are detected.

As for CNVs, previous conventional and array comparative genomic hybridization studies reported that in SBAs, the regions most commonly exhibiting copy number gains were at chromosomes 5p, 7, 8q, 13, 16, and 20, while copy number losses were detected at chromosomes 4, 5q, 8p, 17p, and 18 [23]. In the present study, the frequent occurrence of copy number losses at the *TP53* locus (17p) as well as the gains at the *BRAF* (7q) and *GNAS* (20q) loci was consistent with those earlier results. Notably, frequent copy number gains at the *ERBB4* locus were observed in the present study. In ovarian serous carcinoma, immunohistochemical detection of high ERBB4 expression reportedly correlated with cisplatin resistance and a poorer prognosis [47]. Because recent studies suggest that somatic CNVs at oncogenic loci are not always associated with gene expression [42,48,49], further validation of the effect of CNVs through comparison with expression is needed before the utilization of CNVs as biomarkers.

This study has several limitations. One is the lack of MSI analysis other than the immunohistochemical comparison of MMR proteins. In addition, the sample sizes were small when the specimens were divided into intestinal- and gastric-type tumors or CIMP-H and non-CIMP-H tumors. Furthermore, analysis of corresponding normal samples for validation of somatic mutations was not performed. Although rigorous bioinformatics pipelines were used to discriminate somatic from germline mutations, the results of mutation analyses may still include germline mutations. However, we have several novel findings. First, molecular and clinicopathological characteristics differ among small intestinal-type tumors and gastric-type tumors, which is suggestive of separate carcinogenic pathways. Second, concurrent methylation of CGIs appears to be associated with the development of non-ampullary duodenal lesions. Third, prevalent WNT pathway gene mutations and positive staining for nuclear β-catenin indicate the involvement of WNT signaling in the development of duodenal tumors. Finally, we observed a higher frequency of *APC* mutations than previously reported in SBA patients as well as differences in the mutation distributions within *APC* between adenoma and intramucosal adenocarcinoma samples. This suggests that the adenoma–carcinoma sequence has only limited involvement in duodenal carcinogenesis.

At present, it remains difficult to predict the progression from adenoma to adenocarcinoma or to determine endoscopically which lesions can be followed and which must be treated. The differences between the pathogenesis of small intestinal-type tumors in the proximal duodenum and those in the distal part are also uncharacterized. Accumulation of data from

comprehensive genetic and epigenetic analyses and comparison with more detailed endoscopic and pathological findings will likely provide new insight into non-duodenal ampullary carcinogenesis as well as the endoscopic diagnosis of early non-ampullary duodenal lesions.

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Author contributions statement

RO and TS proposed the concept, contributed to the study design, performed statistical analysis, and wrote the manuscript. RO, TS, ST, YK, HM, KN, SK, TY and TM contributed to the collection and interpretation of pathological and immunohistochemical data. RO, TS, YS, HS, EY, EK, HK and TT contributed to the collection and interpretation of molecular data. RO, KH, HN, SI, ST, NY and HD contributed to the collection of clinical data. ST, YS, HS, TY and TM assisted with draft revision of the manuscript. All the authors approved the final draft submitted for publication.

Data availability statement

Access to the data from our study is available upon reasonable request.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Representative views of duodenal adenomas and intramucosal adenocarcinomas (H&E staining)

Figure S2. Representative views of H&E staining (left) and β-catenin nuclear expression (right)

Figure S3. Representative views of immunohistochemistry analysis of mismatch repair (MMR) proteins

Table S1. Primer sequences used in this study

Table S2. List of genes in the custom Ampliseq gene panel used in this study

Table S3. Comparison of clinicopathological and molecular characteristics between small intestinal-type non-ampullary duodenal lesions located in the proximal and distal parts of the duodenum

Table S4. Relationship between CIMP-high status and clinicopathological characteristics of non-ampullary duodenal lesions

Table S5. Summary of targeted amplicon sequencing data

Table S6. Somatic nonsynonymous mutations found in 102 non-ampullary duodenal lesions

Table S7. Copy number alterations found in 102 non-ampullary duodenal lesions

Thymidylate synthase locus LOH in combination with genotype has prognostic and predictive significance in colorectal cancer

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Abstract. The aim of the current study was to investigate the prognostic and predictive significance of polymorphisms in the thymidylate synthase (TS) gene, alongside the loss of heterozygocity (LOH) at this gene locus in patients with colorectal cancer. Genotyping was carried out for a variable number tandem repeat (VNTR) polymorphism in the TS 5'-untranslated region, a G/C single nucleotide polymorphism (SNP) located within this VNTR, and for TS LOH status in 246 colorectal cancer and paired normal DNA samples. The results were analyzed in relation to clinicopathological features, including the prognostic and predictive significance of TS genotype in patients who underwent curative surgery. Complete VNTR, SNP and LOH information for TS was obtained in 226 cases. No significant associations were observed between normal tissue TS genotype status and clinicopathological features. LOH of TS was observed in 58% of tumor samples and was associated with poor prognosis independently of clinical stage. Cases exhibiting TS LOH were classified into the three groups of 2R/loss, 3G/loss and 3C/loss. Patients with 3C/loss genotype status had poor outcomes when treated by surgery alone, but their survival was similar to patients with other genotypes following Fluorouracil (5-FU)-based adjuvant chemotherapy. The results suggested that LOH of the TS locus may be a significant prognostic factor in colorectal cancer, with the genotype of the residual allele also demonstrating an influence on prognosis. In conclusion, LOH status should be considered when TS genotype is explored as a potential prognostic and predictive marker for 5-FU-based adjuvant chemotherapy in colorectal cancer.

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Abbreviations: CRC, colorectal cancer; LOH, loss of heterozygosity; PE, primer extension; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; TS, thymidylate synthase; 5'-UTR, 5'-untranslated region; VNTR, variable number tandem repeat

Key words: 5-fluorouracil, colorectal cancer, genetic polymorphism, loss of heterozygocity, thymidylate synthase

Introdution

Colorectal cancer (CRC) is one of the most common malignancies worldwide. Although most localized cases of the disease are treated surgically, a considerable number of patients experience disease recurrence. Adjuvant chemotherapy after curative surgery has been shown to reduce the recurrence rate and therefore all CRC patients with clinical stage III disease are recommended to receive adjuvant chemotherapy (1), even though only a proportion of these derive a benefit. Patients with clinical stage II disease are mostly treated with surgery alone, however some may benefit from adjuvant therapy because of a high risk of recurrence (2,3). To maximize the efficacy of adjuvant chemotherapy, accurate predictive markers are needed to select patients who will benefit most from treatment.

5-FU-based chemotherapy is the current standard of care for adjuvant therapy following surgical treatment of CRC (4). Thymidylate synthase (TS) is a target enzyme for 5-FU (5), leading to extensive studies of TS mRNA expression (6), TS protein expression (7,8) and TS gene polymorphisms (9,10) as potential predictive factors for the efficacy of 5-FU-based adjuvant chemotherapy. Currently however, no information regarding TS status is recommended for routine clinical use in the selection of patients to receive 5-FU-based chemotherapy (11).

TS shows unique genetic variants comprising a variable number of tandem repeat (VNTR) and a single nucleotide polymorphism (SNP) in its 5' untranslated region (5'UTR) (12-14). These may be predictive markers for 5-FU efficacy and for adverse events from this treatment. We previously reported that VNTR and SNP can give rise to four TS allele types: 2G, 2C, 3G and 3C. These may affect the translational activity of TS mRNA, thus influencing TS protein expression and therefore constitute a marker for the efficacy of 5-FU-based adjuvant chemotherapy (14-16). In addition to these four allele types, other rarer alleles comprising more than three repeats and novel SNPs in the 2R allele have also been reported (17), thus giving rise to a larger number of allele types. Furthermore, we observed that frequent loss of heterozygosity (LOH) of the TS locus in tumors can affect the genotype, thereby indirectly influencing the TS expression level in tumors (18,19). This potential change in genotype status due to LOH should be considered in studies of the TS genotype as a predictive marker. The status of VNTR, SNPs in both 2R and 3R, and LOH must all be evaluated before TS genotype information can be introduced into the clinical setting.

In this study, we analyzed the TS VNTR, the SNPs in both the 2R and 3R alleles, as well as the LOH status of the TS locus in order to explore their potential significance as prognostic and predictive markers of 5-FU-based adjuvant chemotherapy in CRC.

Materials and methods

Patient cohort and DNA isolation. Matched tumor and normal tissue samples were obtained following surgical resection for primary colorectal adenocarcinoma in 246 patients. The patients were all Japanese and comprised 146 males and 100 females, ranging in age from 33 to 93 years (mean age 66.0 years). The resected tissues were fixed in formalin and embedded in paraffin followed by H&E staining and histological diagnosis. Tumor tissue was dissected manually from 10 μm sections of formalin-fixed, paraffin-embeded tissue blocks. After deparaffinization using xylene and ethanol, genomic DNA was isolated using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) following the protocol provided by the manufacturer. Approval for this project was obtained from the Kanazawa University Genome/Gene Analysis Research Ethics Committee.

Genotyping of TS VNTR and SNP. TS genotypes for the VNTR and the SNP in the 3R allele were determined by PCR and PCR-restriction fragment length polymorphism (RFLP) using the forward primer TS25: 5'-AGGCGCGCG GAAGGGGTCCT-3' and reverse primer TS18: 5'-TCCGAG CCGGCCACAGGCAT-3' as described previously (14) with a modification of PCR conditions. PCR with the genomic DNA template was performed in reaction mixtures containing 1X TaKaRa HS Taq buffer (TaKaRa Bio, Otsu, Japan), 200 μM deoxyribonucleoside triphosphates, 500 nM of each primer, 0.5 unit of TaKaRa HS Taq DNA polymerase (TaKaRa Bio) and 100 ng of genomic DNA. The cycling conditions were: one cycle at 95˚C for 3 min, 35 cycles at 98˚C for 10 sec and 68˚C for 60 sec, with a final extension at 72˚C for 5 min. Aliquots of amplified fragments were separated on 3% agarose gels to determine the TS VNTR genotype.

Samples showing the 2R/3R or 3R/3R genotypes were analyzed further for the G/C polymorphism in the 3R allele by using the RFLP method. *Hae*III digestion of the 3R fragment produced 66-, 37-, 28- and 10-bp bands for the 3G allele, and 94-, 37- and 10-bp bands for the 3C allele after separation on 3% agarose gels. For samples with 2R/2R or 2R/3R genotypes, the G/C polymorphism in the 2R allele was determined by PCR-PERFLP method, consisting of PCR followed by primer extension (PE) and RFLP analysis with *Hae*III digestion. The PCR reaction was performed using reverse primer TS21: 5'-CAGCTCCGAGCCGGCCACAG-3' instead of TS18. Five microliters of PCR product was mixed with extension primer TS105: 5'-TCCGAGCCAGCCACAGGCAT-3' labeled with fluorescein 5'-isothiocyanate to a total volume of 7.5 μl. The mixture was denatured for 5 min at 98˚C, annealed for 10 min at room temperature and then combined with 2.5 μl of PE reaction mixture containing 0.5 unit of Vent (exo-) DNA polymerase (New England BioLabs, Ipswich, MA), 200 μM deoxyribonucleoside triphosphates, 1X ThermoPol Reaction Buffer provided by the manufacturer, followed by incubation for 10 min at 72˚C. The product of primer extension was digested with *Hae*III, separated on 3% agarose gels and visualized with the Typhoon fluoroimager. The 2G allele produced a 48 bp fragment and the 2C allele a 76 bp fragment with the fluorescein 5'-isothiocyanate label. The TS genotype was thus classified into 2G/2G, 2G/2C, 2C/2C, 2G/3G, 2G/3C, 2C/3G, 2C/3C, 3G/3G, 3G/3C, or 3C/3C by comprehensive genotyping of the VNTR and SNP in the TS 5'UTR. Analyses were performed at least twice to confirm the genotype.

LOH analysis. LOH of the TS locus was determined in three distinct ways depending on the TS genotype observed in the normal tissue. The G/C SNP in the 2R allele was not taken into consideration for LOH analyses. Samples that were 2R/3G or 2R/3C were analyzed by PCR followed by separation on Spreadex gel (Elchrom Scientific, Cham, Switzerland). Samples that were 2R/2R, 3G/3G or 3C/3C were evaluated for LOH using the microsatellite marker D18S59, as described previously (18). Samples that were 3G/3C were analyzed using the PCR-PERFLP method, as described above for the SNP genotyping method with the 2R allele. PCR-PERFLP avoids interference due to heteroduplex formation, thereby allowing the exact allele ratio to be determined. The 3G allele produced a 76 bp fragment and the 3C allele a 104 bp fragment with PCR-PERFLP, as visualized by Typhoon fluoroimaging. The image was analyzed using ImageQuant software and the relative ratio between 3G and 3C alleles in tumor DNA was normalized using the ratio measured in the corresponding normal tissue DNA sample. LOH was defined as either the complete absence of one allele, or a decrease in intensity of one allele by at least 50%. LOH of 18q was analyzed using the microsatellite markers D18S58, D18S61 and D18S64. Forward primers were labeled with fluorescein 5'-isothiocyanate and the same method as for microsatellite marker D18S59 was used.

Statistical analysis. Relationships between variables were analyzed by Chi-square analysis or the Scheffe post-hoc test used following ANOVA. The cumulative survival rate was

estimated using the Kaplan-Meier method and statistical significance was assessed by the log-rank test. Cox regression modelling was used for multivariate analysis. P-values less than 0.05 were considered significant.

Results

Genotype analysis in normal tissue samples. We previously investigated and compared the TS genotypes between matched normal and tumor tissues from 151 patients with colorectal cancer. The results suggest that frequent LOH of the TS locus detected in the tumors affect the functional TS genotyping (18). Therefore, in the present study, TS genotyping was carried out on DNA from normal tissue rather than from tumor samples in order to avoid possible artifacts from LOH. The VNTR genotype distribution amongst the 246 cases was: 2R/2R (n=9), 2R/3R (n=70), 3R/3R (n=162) and 3R/5R (n=5). Because the 5R allele is rare and analysis of the G/C SNP in the repeat component is difficult, the 5 cases with 3R/5R genotype were excluded. The remaining 241 cases of 2R/2R, 2R/3R and 3R/3R VNTR genotype cases were then screened for the G/C SNP in both the 2R and 3R alleles (Fig. 1). The combined VNTR and SNP genotype frequencies were: 2G/2C (n=1), 2C/2C (n=8), 2G/3G (n=3), 2G/3C (n=3), 2C/3G (n=25), 2C/3C (n=39), 3G/3G (n=48), 3G/3C (n=81) and 3C/3C (n=33). The previously reported 2R allele with a G→C SNP located in the first tandem repeat (17) was not found in our subjects. This should have resulted in a 121 bp fragment following PCR-PERFLP and gel electrophoresis (Fig. 1B). The 2G allele was quite rare in our population and showed no significant associations with any clinicopathological variable or with clinical course (data not shown). Therefore, SNP information for the 2R allele was not considered in further analysis. The distribution of clinicopathological features according to TS VNTR/SNP genotype are shown in Table I. As reported previously (14), the 3G allele was less frequent in females (P=0.04, chi-square test) (male G=115, female G=62, male C=79, female C=68). No significant associations were apparent between the normal tissue TS genotype and any other clinicopathological feature.

LOH and the residual TS allele in colorectal cancer. The appropriate method for LOH analysis was selected according to the genotype found in the normal tissue, as shown in Fig. 2. The genotype frequency according to LOH status is also shown in Fig. 2. A novel method for LOH analysis of the 3G/3C genotype was employed in this study and involved the use of PCR-PERFLP to avoid interference with heteroduplex product (Fig. 3). Fifteen cases could not be evaluated for LOH status because both the TS genotype and the D18S59 microsatellite marker were homozygous. Therefore, complete TS VNTR/SNP genotype information together with tumor LOH status was available for 226 patients. The frequencies were: 2R/2R (n=3), 2R/3G (n=11), 2R/3C (n=20), 3G/3G (n=13), 3G/3C (n=34), 3C/3C (n=14), 2R/loss (n=26), 3G/loss (n=58) and 3C/loss (n=47). The overall frequency of LOH for the TS locus was 58.0% (131/226). Lossed alleles were evenly distributed between 2R (n=23), 3G (n=58) and 3C (n=50). Associations between LOH status and clinicopathological features are shown in Table II. The absence of LOH for TS was significantly associated with proximal tumor location and with mucinous histology. No other statistically significant associations were observed.

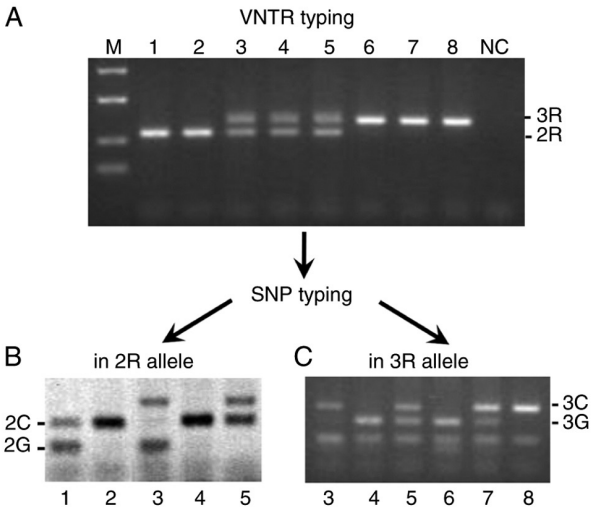

Figure 1. TS VNTR and SNP analysis. (A) VNTR analysis using PCR amplification and separation of products on a 3% agarose gel. (B) SNP analysis in the 2R allele using PERFLP followed by separation on a 3% agarose gel and scanning with a fluoroimager. (C) SNP analysis in the 3R allele by RFLP and separation on a 3% agarose gel. The DNA fragments stained using ethidium bromide are displayed as white pixels on a black background, whereas those labeled by fluoresein are displayed as black pixels on a white background. The numbers on these panels indicate the same samples being analyzed. The genotypes are as follows: 1, 2G/2C; 2, 2C/2C; 3, 2G/3C; 4, 2C/3G; 5, 2C/3C; 6, 3G/3G; 7, 3G/3C; and 8, 3C/3C. M indicates the size marker (50, 100, 200 and 300 bp) and NC indicates that there was no template control. TS, thymidylate synthase gene; VNTR, variable number tandem repeat; SNP, single nucleotide polymorphisml RFLP, restriction fragment length polymorphism.

Patient prognosis and TS LOH status. A number of studies have reported that LOH at a given gene locus is associated with poor prognosis. We therefore analyzed the prognostic significance of TS LOH status before examining the prognostic role of the TS genotype. This was performed for 153 patients with clinical stage II or III disease who underwent curative surgery and where clinical information including long term follow-up and use of adjuvant therapy was available. Of these patients, 90 (59%) showed TS LOH and had significantly shorter survival (P=0.0005) compared to patients with no LOH (n=63; Fig. 4). We also compared the frequency of LOH between the tumors at clinical stage II and III by Chi-square test. We found no statistical difference in frequency of LOH between the two groups of tumors (P=0.22). Multivariate analysis with the parameters listed in Table III demonstrated that TS LOH status, receipt of adjuvant chemotherapy and clinical stage were independent prognostic factors in this patient cohort.

TS LOH is often accompanied by 18q LOH. TS is located on 18p11.32. Earlier studies reported that 18q LOH was associated with poor prognosis in CRC (20,21). Thus, we analyzed the relation between TS LOH and 18q LOH in 41 randomly selected samples comprising 15 cases with no TS LOH and 26 cases with TS LOH. Three microsatellite markers (D18S58, D18S61, D18S64) located at 18q22.3, 18q22.2 and 18q21.32, respectively, were used to determine 18q LOH. Fig. 5 shows the TS LOH

Table I. Thymidylate synthase genotype and clinicopathological features.

	2R/3R			3R/3R		
Parameter	2R/3R	2R/3G	2R/3C	3G/3G	3G/3C	3C/3C
Total	9	28	42	48	81	33
Sex						
Male	5	18	23	37	41	19
Female	4	10	19	11	40	14
Age (years)						
Mean	63.3	70.4	63.7	66.3	65.5	66.3
SD	15.8	11.1	11.2	13.2	11.1	13.5
Stage						
I	1	4	6	3	7	3
II	5	5	17	18	32	10
III	3	13	17	14	27	13
IV	0	6	2	13	15	7
Tumor site						
Proximal	2	9	12	19	32	9
Distal	7	19	30	29	49	24
Pathology						
Tub1	7	8	17	21	39	13
Tub2	1	17	21	23	36	16
Muc	1	0	1	2	1	2
Por	0	3	3	2	5	2

Muc, mucinous adenocarcinoma; Por, poorly differentiated adenocarcinoma; Tub1, well differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma.

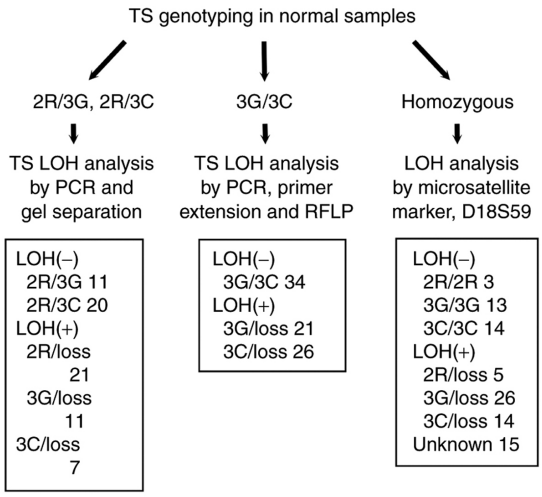

Figure 2. Flow chart of LOH analysis and the frequency of TS genotypes. The LOH status of tumors was analyzed according to the TS genotype in normal sample. The TS genotype frequency in relation to LOH status is shown in the box. LOH, loss of heterozygocity; TS, thymidylate synthase gene; RFLP, restriction fragment length polymorphism.

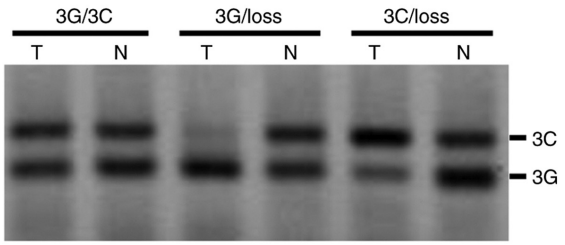

Figure 3. Representative cases of LOH analysis in 3G/3C genotype samples. The PCR-primer extention restriction fragment length polymorphism method detailed in the materials and methods was used to avoid interference by the heteroduplex product. The image of DNA fragments labeled by fluoresein are displayed as black pixels on a white background. Thymidylate synthase genotype and LOH status is indicated on the top of matched T and N lanes. LOH, loss of heterozygocity; T, tumor; N, normal.

status as well as that of each 18q microsatellite marker. Whenever LOH was observed at the TS gene locus, it was also consistently present at other 18q loci. On the other hand, three tumors (from patients no. 4, 13 and 16 shown in Fig. 5) showed LOH at one or more 18q microsatellite markers in the absence of LOH at TS. In these tumors, LOH was not observed for all three markers, indicating the chromosomal loss occurred in a relatively small area of 18q. These results suggest that LOH at TS and 18q are simultaneous events in most CRC, although a few tumors have small areas of LOH at 18q without allelic loss at the TS locus.

TS genotype as a prognostic and predictive factor in tumors with LOH. Since TS LOH was a strong prognostic factor (Fig. 4)

Table II. Loss of heterozygosity of the thymidylate synthase locus and clinicopathological features.

Parameter	No LOH	LOH	P-value
Total	95	131	
Sex			0.63
Male	55	80	
Female	40	51	
Age (years)			0.16
Mean	64.6	66.9	
SD	12.9	11.3	
Stage			0.61
I	11	12	
II	30	52	
III	39	46	
IV	15	21	
Tumor site			<0.0001
Proximal	48	30	
Distal	47	101	
Pathology			0.038
Tub1	42	55	
Tub2	39	69	
Muc	6	1	
Por	8	6	

Muc, mucinous adenocarcinoma; Por, poorly differentiated adenocarcinoma; SD, standard deviation; Tub1, well differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma.

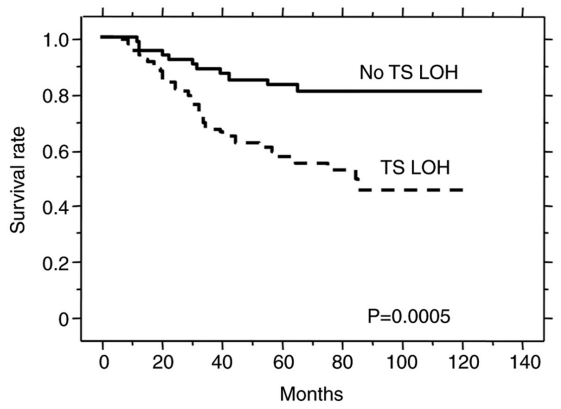

Figure 4. Overall survival of patients with curatively resected colorectal cancer according to their TS LOH status. TS, thymidylate synthase gene; LOH, loss of heterozygocity.

and also influences the TS genotype observed in tumors, we explored the role of TS genotype separately in patient groups stratified according to their LOH status. In patients without TS LOH (n=63), the tumor genotype is identical to that found in normal tissue. These patients were classified into 6 groups: 2R/2R (n=2), 2R/3G (n=6), 2R/3C (n=13), 3G/3G (n=10), 3G/3C (n=23) and 3C/3C (n=9). The relatively small number of cases for each genotype prevented analysis of the prognostic value of these groups. When the genotypes were grouped into L-type (2R/2R, 2R/3C, 3C/3C; n=24) and H-type (2R/3G, 3G/3C, 3G/3G; n=39) according to criteria from our previous report (14), no prognostic significance was observed in the overall patient group, in patients treated by surgery alone, or in patients treated with adjuvant chemotherapy (data not shown).

In patients with TS LOH (n=90), the tumor TS genotypes were: 2R/loss (n=20), 3G/loss (n=39) and 3C/loss (n=31). No prognostic significance was observed for these genotypes in the overall group of patients with TS LOH (Fig. 6A) or in patients who received adjuvant chemotherapy (Fig. 6C). However, the 3C/loss genotype was associated with significantly shorter survival in patients treated by surgery alone (Fig. 6B). These results suggest that patients with the 3C/loss genotype have poor prognosis when treated by surgery alone, but may benefit from chemotherapy as observed by the similar survival rate to patients with other genotypes. Indeed, patients with the 3C/loss genotype who received adjuvant chemotherapy survived significantly longer than those treated by surgery alone (Fig. 6D). Thus, the 3C/loss genotype appears to be a prognostic marker for poor outcome, as well as a predictive marker for good response to 5-FU-based chemotherapy.

Table III. Multivariate analysis of prognostic factors.

Variable	Hazard ratio	95% CI	P-value
TS LOH			
Yes vs. no	3.01	1.36-6.64	0.0065
Adjuvant chemotherapy			
Yes vs. no	0.53	0.28-0.98	0.045
Sex			
Male vs. female	1.22	0.66-2.26	0.53
Age			
≥66 vs. <66	1.15	0.62-2.16	0.66
Stage			
II vs. III	0.53	0.29-0.96	0.039
Tumor site			
Proximal vs. distal	0.63	0.28-1.43	0.27
Pathology			
Tub2 vs. Tub1	1.27	0.368-2.38	0.45
Muc vs. Tub1	2.42	0.50-11.7	0.27
Por vs. Tub1	0.53	0.068-4.21	0.55

CI, confidence interval; LOH, loss of heterozygosity; M, mucinous adenocarcinoma; Por, poorly differentiated adenocarcinoma; TS, thymidylate synthase; Tub1, well differentiated tubular adenocarcinoma; Tub2, moderately differentiated tubular adenocarcinoma.

Discussion

In this report we genotyped TS for VNTR status and for SNPs located within the 2R and 3R alleles. We have evaluated the TS locus for LOH in CRC. In agreement with our previous observations, LOH was quite frequent regardless of the TS

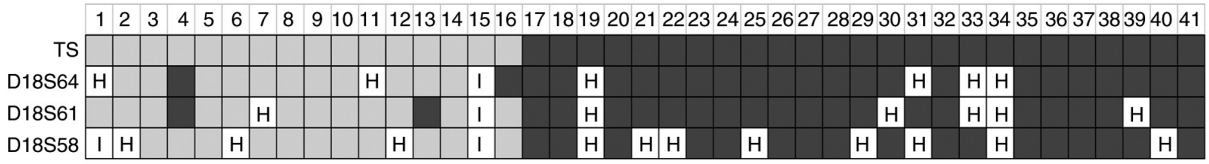

Figure 5. LOH status of the TS locus and of 18q in 41 randomly selected tumor samples. White squares indicate that the LOH status could not be determined either due to microsatellite instability (I) or homozygosity (H). Light grey squares indicate no LOH and dark grey squares indicate LOH. LOH, loss of heterozygocity; TS, thymidylate synthase gene.

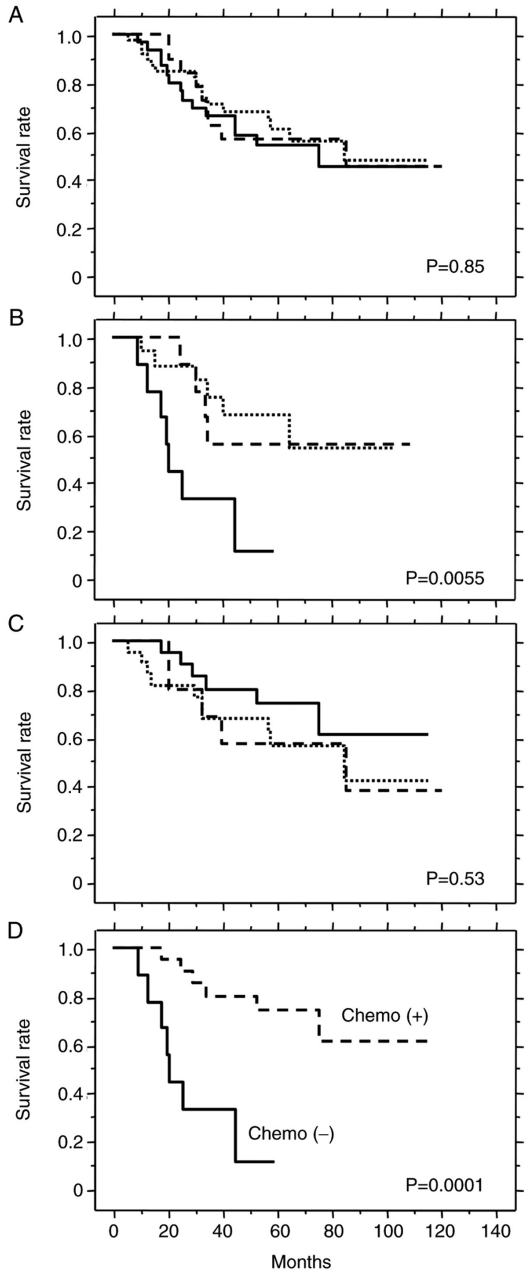

Figure 6. Survival analysis of patients with colorectal cancer exhibiting TS LOH. The survival rates of patients with 2R/loss (broken line), 3G/loss (dotted line) and 3C/loss (continuous line) genotype were compared using the Kaplan-Meier method in (A) all patients with TS LOH, (B) patients treated with surgery alone and (C) patients who received adjuvant chemotherapy. (D) Survival of patients with the 3C/loss genotype who did (broken line) or did not (continous line) receive 5-FU-based adjuvant chemotherapy. TS, thymidylate synthase gene; LOH, loss of heterozygocity; 5-FU, Fluorouracil.

genotype (18,19). TS LOH was a prognostic factor for poor survival (Fig. 4), independently of clinical stage and other clinicopathological features. Because of the high frequency of TS LOH (58%) and also its significant prognostic impact, the TS genotype cannot be combined with LOH status to give one simple prognostic indicator similar for example to the '3G-containing type' we used previously (14). The 3G/3G and 3G/loss genotypes are identical in that both have only the 3G allele, however their prognostic significance is quite different due to the presence of LOH in the latter. Important prognostic information derived from the LOH status would be lost if the 3G/3G and 3G/loss genotypes were combined into a simple '3G type'.

In exploring the predictive value of a given factor for adjuvant chemotherapy, it is also important to consider its prognostic value in the absence of such treatment. The TS LOH status is essential for the correct use of TS genotype as a prognostic and predictive factor in 5-FU-based adjuvant chemotherapy. The different number of TS genotypes in tumor DNA is another reason to stratify patients according to their TS LOH status. In cases with no LOH, 6 major TS genotypes are observed (2R/2R, 2R/3G, 2R/3C, 3G/3G, 3G/3C, 3C/3C) whereas three groups are seen in cases with LOH (2R/loss, 3G/loss, 3C/loss). Future investigations into the role of TS genotype in the clinical setting will require large patient cohorts so that the LOH status of TS can also be taken into account.

In the current study we classified patients according to their TS LOH status and then followed by investigating the prognostic and predictive significance of TS genotype. In cases with no LOH, the overall patient group showed relatively good prognosis (Fig. 4) and there were 6 major genotype groups, making it difficult to obtain statistically meaningful results because of the relatively small patient numbers. Moreover, no prognostic or predictive significance was observed when these 6 genotypes were classified into just two groups (H and L) according to our previous results (14). This indicates that TS genotype is not a useful marker in patients without TS LOH, although study of a larger number of patients is required to confirm this observation.

Cases with TS LOH showed poor prognosis (Fig. 4). These were further classified into three simple TS genotype groups (2R/loss, 3G/loss, 3C/loss) in order to explore their prognostic and predictive values (Fig. 6). The 3C/loss genotype was a marker for poor prognosis in patients treated by surgery alone (Fig. 6B). Furthermore, the 3C/loss genotype also predicted good response to 5-FU-based adjuvant chemotherapy (Fig. 6D). Despite the relatively small number of patients (n=31), this result reached a high level of statistical significance (P=0.0001). Using an *in vitro* reporter assay, we previously showed the 3C allele was associated with lower translational activity compared

to the 3G allele (14). Low expression of TS mRNA (6) and of the TS protein (8) in CRC have both been associated with good response to 5-FU-based chemotherapy. The current result showing the TS 3C/loss genotype is a marker for good response to 5-FU-based adjuvant chemotherapy (Fig. 6D) is therefore consistent with our previous *in vitro* observations and with the results of Salonga *et al* (6) and Soong *et al* (8). Although we cannot explain why this genotype was associated with poor prognosis (Fig. 6B), the result concurs with a previous study showing that low TS expression is a marker of worse prognosis in patients treated by surgery alone (8).

Due to the retrospective nature of this study and the potential for biases, further analyses are required to validate the results, particularly for the TS genotype groups in cases with LOH. The 2G allele was rare and no 2R allele with the G→C SNP in the first tandem repeat was found in our patient cohort, suggesting that SNP typing of the 2R allele can be omitted in further studies of the Japanese population. However, there is considerable ethnic variation in the allele frequency for 2R and the incidence is higher in Western populations (22). Therefore, additional analysis of the SNP in the 2R allele may be required for Caucasian populations.

The LOH status of TS was closely associated with 18q LOH status, with the latter being reported previously as a prognostic factor in CRC (23,24). The mechanism by which 18q LOH is linked to poor prognosis of CRC patients is not known, although the loss of several tumor suppressor genes in this region including *DCC*, *SMAD4* and *SMAD22* has been implicated. The current study sheds light on loss of the whole of chromosome 18 as a prognostic factor. LOH of 18p and 18q should be analyzed simultaneously to investigate whether the minimally lost regions on 18q or the whole allelic loss of chromosome 18 have stronger prognostic significance.

TS LOH is a significant prognostic factor in CRC. Furthermore, the 3C/loss genotype appears to be prognostic in patients treated by surgery alone and predictive in patients who receive 5-FU-based adjuvant chemotherapy. Since TS LOH status influences the TS genotype of tumors and also has a significant prognostic role, TS LOH should be incorporated into all future studies of TS genotype, particularly in relation to its predictive value. Stratification of CRC patients into subgroups defined by TS LOH status is therefore essential in obtaining clear evidence for a clinical role of the TS genotype.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MKo and KK conceived and designed the current study. MKo, HB, MKa and KK collected clinical samples. MKo, HB, MKa, HT and KK performed the experiments and analyzed the data. MKo, TM and KK drafted the manuscript. MKo and TM confirmed the authenticity of all the raw data. All authors read and approved the final version of manuscript.

Ethics approval and consent to participate

The current study was performed in accordance with the Declaration of Helsinki. Since tissues used in this study were obtained from the patients diagnosed between 1999 and 2010, written informed consent was available for most but not all patients. However in accordance with Japanese ethical guidelines and law, the study protocol was reviewed and approved by the Kanazawa University Human Genome/Gene Analysis Research Ethics Committee (approval nos. 181 and 264). Following instruction by the Ethics Committee at approval, all patients were publicly provided with an opportunity to opt-out from registration to the current study. None declined. All samples were anonymized before analysis was performed to guarantee the protection of privacy.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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