厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）

分担研究報告書

DNA メチル化の分子機構の解析およびがんにおいて不活化される新規遺伝子の同定

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研究要旨

本研究では、ゲノムワイドなメチル化解析を行い、がんにおける DNA メチル化の役割を明らかにすることを目的とする。本年度は大腸前がん病変におけるメチル化異常の網羅的解析を行い、臨床病理像との相関を検証した結果、CIMP 陽性大腸腺腫に特徴的な形態（ピットパターン）を明らかにした。これまでゲノム網羅的手法で同定したがん関連遺伝子に関しては、大腸がん、胃がん、膀胱がんにおいて、それぞれ、内視鏡的に採取した剥離液や尿より DNA メチル化を検出する系を確立し、がんの浸潤度予測や再発リスク予測、術後のモニタリングへの応用が可能か検証を行っている。

Ａ．研究目的

本研究では、DNA メチル化の網羅的解析により発がんに関与する新規遺伝子を同定し、がん化における役割を明らかにすることを目的とする。H22 年度までの研究で、大腸がんにおける遺伝子変異と DNA メチル化異常の関連、microRNA（以下 miRNA）遺伝子のエピジェネティックな異常、乳がんにおけるエピジェネティックな標的の同定を行ってきた。本年度は、同定したメチル化異常のがん診断への応用を検討した。さらに大腸前がん病変のメチル化解析および膀胱がんの miRNA 遺伝子の解析を行った。

Ｂ．研究方法

網羅的 DNA メチル化解析を Methylated CpG island amplification microarray (MCAM)法によって行った。DNA メチル化は Methylation-specific PCR (MSP) 法、bisulfite sequencing 法および bisulfite pyrosequencing 法により検討した。遺伝子および microRNA 発現を microarray 法、RT-PCR 法および real-time PCR 法により解析した。

（倫理面への配慮）

平成 17 年厚生労働省告示第 255 号「臨床研究に関する倫理指針」に従い、倫理面に充分配慮して研究を進める。手術材料の残余の組織などの研究利用につき、患者に説明し文書で同意を得、連結可能匿名化して解析を行い、患者のプライバシーを遵守し、札幌医科大学の倫理委員会の承認を得て使用する。

Ｃ．研究結果

(1) 大腸粘膜剥離液中のメチル化

これまで我々は miR-34b/c、miR-1-1、SFRP1、DKK2 の大腸がんにおける高頻度なメチル化を明らかにしてきた。今回我々はこれらのメチル化が、大腸腫瘍の粘膜剥離液からも検出できることを明らかにした。剥離液は内視鏡下に腫瘍を生理食塩水で洗浄することで回収した。

剥離液中の遺伝子メチル化を、浸潤性大腸がんと非浸潤大腸腫瘍の間で比較した結果、浸潤性大腸がん由来の剥離液からより高レベルな miR-34b/c および SFRP1 のメチル化が検出された($P < 0.001$)。ROC 解析の結果、剥離液中の miR-34b/c のメチル化レベルは浸潤がんと最も強く相関した(AUC = 0.796)。これらの結果より、大腸粘膜剥離液中のメチル化検出による大腸がん浸潤予測システムが構築できることを示した。

(2) 大腸腫瘍の形態とメチル化異常の関連

大腸がんをはじめ様々な臓器のがんで CpG island methylator phenotype (CIMP)が見られることが知られているが、CIMP と臨床像との関連は未だ不明な点が多い。大腸がん発生初期におけるメチル化を解析する目的で 122 例の大腸腺腫のメチル化を解析し、内視鏡像および臨床病理像との関連を詳細に検討した。大腸腫瘍では、拡大内視鏡によるピットパターン分類が悪性度診断に有用であることが知られている。今回我々は、通常の Type II ピットに類似しながらも、円形に開いた形状のピットパターンが、BRAF 変異陽性かつ CIMP 陽性の鋸歯状腺腫にきわめて特異的に見られることを見いだし、これを Type II-Open ピットと命名した。この知見は、分子異常と腫瘍の形態の興味深い関連を明らかにするとともに、CIMP 陽性大腸がんの前癌病変を効率的に同定しうる診断法につながると期待される。

(3) 大腸前がん病変の網羅的メチル化解析

一般にメチル化異常は発がんの早期に発生するとされているが、その詳細は不明な点が多い。大腸発

がんにおける CIMP の意義を明らかにするため、16 例の大腸腺腫、14 例の粘膜内癌、28 例の大腸がん組織を対象に網羅的メチル化解析を行った。その結果、大腸腺腫の一群はすでに CIMP 陽性を獲得していることを明らかにした。また大腸発がん早期にメチル化する遺伝子として KCNV1、IGF2BP1 などを新たに同定した。今回の解析から、大腸発がんの早期の段階で多数の遺伝子にメチル化異常が発生していることが明らかとなった。これらのメチル化を便や大腸洗浄液から検出することで大腸がんの早期診断が可能であるか、検証を続けている。

(4) 膀胱がんの miRNA 遺伝子メチル化

膀胱がんでは多くの遺伝子が異常メチル化を受けること、さらに尿中からのメチル化検出による膀胱がん診断が期待できることが報告されている。しかし膀胱がんにおける miRNA 遺伝子のエピジェネティックな異常についての知見はわずかである。我々は膀胱がん細胞株を DNA メチル化阻害剤 5-aza-dC および HDAC 阻害剤 4-phenylbutyric acid で処理し、発現上昇する miRNA をマイクロアレイ解析した。その結果、miR-137, miR-124, miR-9 が膀胱がんにおいて高頻度にメチル化していることを明らかにした。これらの miRNA 遺伝子のメチル化は膀胱がん患者の尿中より容易に検出可能であり、手術切除後の尿中ではメチル化レベルが顕著に減少した。miRNA 遺伝子のメチル化が膀胱がんの尿中診断に応用しうるか、検証を続けている。

D．考察

これまで発現マイクロアレイおよびゲノムワイドなヒストン修飾解析を通じて、多数の遺伝子のメチル化異常を同定した。これらのメチル化は、大腸粘膜剥離液や尿など非侵襲的に得られる検体からも検出可能であった。SFRP や miR-34b/c 遺伝子は、大腸がん組織では浸潤の有無に関わらず高頻度にメチル化しているが、粘膜剥離液では浸潤がんの方がより高レベルなメチル化を示した。これは細胞の剥離しやすさと剥離後のアポトーシス抵抗性が寄与しているものと推察された。また大腸発がん早期にメチル化する遺伝子、および膀胱がんでメチル化する miRNA 遺伝子のメチル化については、腫瘍マーカーとしての性能を今後さらに検証していきたい。

E．結論

大腸がん早期に発生するメチル化異常を明らかにし、大腸腺腫において CIMP 陽性とピットパターンの興味深い相関を見いだした。また、大腸粘膜剥離液に含まれるメチル化は大腸がん浸潤予測マーカーとして有用であることを明らかにした。さらに膀胱がん診断マーカー候補として期待できる miRNA 遺伝子メチル化を同定した。

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G．知的財産権の出願・登録状況（予定を含む）

1. 特許取得

清水 崇、鈴木 拓、豊田 実、塚本泰司. 膀胱癌細胞の検出方法、膀胱癌細胞の検出方法に用いるプライマー及び膀胱癌マーカー. 特願2011-203705、平成23年9月16日出願.

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2. 実用新案登録

該当無し

3. その他

該当無し

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）

分担研究報告書

胃癌におけるエピジェネティック異常に基づいた高精度がん化予測診断

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研究要旨

内視鏡診断技術の発達により Stage I 胃癌症例が 50%以上を占めるわが国において、内視鏡的治療をはじめとした低侵襲胃がん治療の開発がめざましい。一方で、今後わが国のハイリスク残胃症例に対する適切な検査系の確立が必須であるものの未だ十分ではない。現在、残胃に対して内視鏡医による経験に基づいた診断、スポット生検による病理診断のみである。我々は、残胃を低侵襲かつ効率的よく診断する方法を開発した。すなわち胃洗浄廃液による遺伝子診断である。胃の発癌機構にエピジェネティックな異常が大きく関与することを臨床診断へ応用し、網羅的メチル化解析等により選出した候補遺伝子（MINT25, Sox17, miR34b/c, ACMG1）を分子マーカーとし、胃洗浄廃液により回収された胃粘膜細胞由来の gDNA を用いて癌存在診断、予測診断ができる可能性を予備試験により確認し、前向き多施設共同試験を実施中にある。

A．研究目的

通常内視鏡検査・治療時に発生する胃洗浄廃液から gDNA を抽出、網羅的メチル化解析により得た候補遺伝子（MINT25, Sox17, miR34b/c, ACMG1）を分子マーカーとし、前向き多施設共同試験を行うためのシステムを構築する。

B．研究方法

１．予備試験の実施：EMR: Endoscopic mucosal resection/ ESD: Endoscopic submucosal dissection 治療の適応となる 40 症例の治療前後（治療直前および、治療 1 週間後の内視鏡観察時）から採取した洗浄廃液を用い、4 つの候補遺伝子を用い、DNA メチル化解析を定量性に優れた Bisufite-Pyrosequence 法にて行う。

２．胃洗浄廃液を効率よく回収することが可能な専用採取管の設計および制作を行う。また、将来的な臨床検査系への応用を見据えた、検体搬送および、効率的な DNA 抽出系の検討を行い、既存の受諾サービスで対応が可能であるかを検討する。

３．平成 23 年 4 月キックオフを目標とした前向き多施設共同試験遂行のための参加施設登録および臨床試験デザイン作成、検体搬送、遺伝子検査系に関する一連のシステムを構築する。

（倫理面への配慮）

研究に必要な検体は通常破棄される胃洗浄廃液であり、大学施設生命倫理委員会への承諾を行った後、患者様への十分なインフォームドコンセントのもと同意を得た症例にのみ実施されるものである。また、試料については、連結可能匿名化を行い、医療情報管理を厳重に行うこととする。

C．研究結果

１．本年度までに、40 症例による予備試験登録を終了し、4 候補遺伝子（MINT25, Sox17, miR34b/c, ACMG1）を用いた DNA メチル化定量解析を行った。結果、症例の約 8 割が治療前において高メチル化を示し、治療後にそのメチル化レベルに有意な低下を示した。また、内視鏡治療後の病理組織学的な検討において切除断端陽性となった症例では、治療後も依然としてメチル化レベルが低下せず完全切除できていないことを予測することが出来る可能性が示唆された。

２．胃洗浄廃液を効率よく回収すべく、250mL 専用採取管を作成し、医療用としての使用認可を得た。

３．北海道大学光学診療部を中心とした多施設共同試験グループ（SGIST: Sapporo GI Study Team）による採択を受け、聖マリアンナ医科大学消化器・肝臓内科、筑波大学消化器内科、札幌厚生病院消化器科、札幌医療センター斗南病院消化器病センター、

札幌北楡病院消化器科、手稲渓仁会病院消化器病センター、恵佑会札幌病院消化器内科、小樽腋済会病院消化器科、小樽協会病院消化器内科、済生会横浜市東部病院消化器科が加わり、早期胃がんに対する内視鏡治療症例300症例を対象に治療前後、1年～5年後まで洗浄廃液を回収し、再発予測診断プログラムの構築を目的として前向き試験を開始した。現在1年目経過中である。

D．考察

通常の内視鏡検査時に破棄している胃洗浄廃液を用い、エピジェネティックな異常を診断に応用することが有用であることが強く示唆され、さらに候補遺伝子（MINT25, Sox17, miR34b/c, ACMG1）を用いて前向き多施設臨床試験を行うことで、臨床応用へ向けた大きな一歩となる可能性が考えられた。

E．結論

胃洗浄液を用いたエピジェネティック診断は、今までにない視点からの診断法であるだけでなく、通常廃棄される廃液を利用する、侵襲度の非常に低い新たな検査法として非常に有望であり、前向き多施設臨床試験（1年目）症例エントリー中にある。

F．研究発表

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G．知的財産権の出願・登録状況（予定を含む）

1. 特許取得

該当無し

2. 実用新案登録

該当無し

3. その他

該当無し

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）

分担研究報告書

がん細胞 DNA メチル化異常の起源解明

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研究要旨

本研究では iPS 細胞(iPSC)作製技術を応用することで、がん細胞のエピゲノム異常の起源および意義を明らかにすることを目的としている。昨年度までの研究において、家族性大腸腺腫症のモデルである Apc Min マウスの大腸腫瘍細胞にレトロウイルスを用いて山中 4 因子を強制発現させることで、iPSC に類似した細胞(T-iPSC 様細胞)を樹立した。しかしながら、T-iPSC 様細胞には完全に初期化された細胞のマーカーである *Nanog* の発現が確認されず、腫瘍細胞の完全な初期化はなし得なかった。

本年度は、ドキシサイクリン投与により山中 4 因子が誘導可能である「初期化可能マウス」を用いて *Apc* Min マウス大腸腫瘍細胞の完全な初期化を目指した。初期化可能マウスに発生した大腸腫瘍細胞にドキシサイクリンを用いて初期化因子を発現させることで、ES 細胞並みに *Nanog* 遺伝子を発現する細胞を得ることが出来た(reprogrammed tumor cells; RT 細胞)。分化誘導培地を用いた培養にて、RT 細胞では、分化に重要な転写因子の発現増加が観察され、細胞分化の誘導が可能であることが示唆された。我々が作製した初期化可能マウスを用いたリプログラミング／再分化の系は、腫瘍細胞におけるエピゲノム異常の起源、意義を解析するために有用なツールとなることが示唆された。

A．研究目的

DNA メチル化異常に代表されるエピゲノム異常は多くのがんに観察され、発がんに促進的な役割を果たしていることが明らかとなっている。しかし、がん細胞におけるエピゲノム異常の原因や発がんにおける意義については、未だ不明な点が多く、より効果的な治療方法の開発には、がん細胞におけるエピゲノム異常の起源、役割を解明する必要がある。

iPS 細胞(iPSC)作製に必要な初期化因子の発現は、エピゲノム状態の改変を誘導し、多能性幹細胞樹立に至る。本研究では iPSC 作製技術をエピゲノム変化を誘導するツールとして捉え、腫瘍細胞のゲノム異常をそのままに、エピジェネティック修飾状態に強制的な変化を誘導することで、がん細胞のエピゲノム異常の起源および意義を明らかにすることを目的とした。

昨年度までの研究により、家族性大腸腺腫症のモデルマウスである *Apc* Min/+マウス大腸腫瘍細胞から iPS 細胞様の T-iPSC 様細胞を樹立できることが明らかとなったものの、T-iPSC 様細胞には、*Nanog* 遺伝子など完全な初期化マーカーは発現しておらず、不完全な初期化細胞と考えられた。本研究では、まずはマウス大腸腫瘍細胞の完全な初期化を目的とした。

B．研究方法

ドキシサイクリンによる遺伝子発現系を利用して山中因子を発現コントロールできるマウスシステムを用いた。*Rosa26* プロモーター制御下に *M2rtTA* を発現し、*Col1a1* 遺伝子座において tet オペレーター下に 2A polypeptide で連結させた山中 4 因子を有する「初期化可能マウス」を作製し、研究に用いた。初期化可能マウスを *Apc* Min マウスに交配し、強力な大腸発がんプロモーターである dextran sodium sulfate (DSS)を投与することで大腸腫瘍誘発を行った。7 週齢マウスに DSS (2%)を 1 週間飲水投与し、投与終了後 4 週後に屠殺し、大腸を摘出し、ドキシサイクリンにより初期化因子誘導可能な大腸腫瘍を得た。試験管内でドキシサイクリンを添加することで、大腸腫瘍細胞に初期化因子を発現させ、完全な初期化を試みた。樹立した初期化細胞が、腫瘍細胞由来であることを確認するために、*Apc* 遺伝子の LOH を、PCR-RFLP 法にて検索した。

樹立された細胞の完全な初期化の評価は、*Nanog* 遺伝子の real-time PCR による発現解析にて行った。また初期化細胞の分化能の検討は、マウス初期胚へのマイクロインジェクションにより検討した。

（倫理面への配慮）

全ての動物実験は、動物実験実施機関（京都大学iPS細胞研究所）の動物実験委員会の承認を得た。動物愛護の精神に配慮し、3Rに努めて実験を施行した。

C．研究結果

初期化可能マウスに発生した大腸腫瘍細胞に初期化因子を誘導することで、形態が多能性幹細胞に類似した細胞株を複数得ることが出来た。*Apc* 遺伝子のLOH解析により、得られた細胞株でLOHが確認され、実際に腫瘍細胞由来であることが確認された。多能性幹細胞に類似した細胞株は、高いアルカリフォスファターゼ活性を持ち、多能性幹細胞に類似した性質を有することが分かった。さらに、完全初期化のマーカーとして使用される *Nanog* 遺伝子の発現が確認され、その発現レベルもES　細胞とほぼ同等であることが分かった。腫瘍細胞の完全な初期化が可能であることが示唆された。

次に我々は樹立したreprogrammed tumor cells (RT細胞)の分化能について検討した。昨年度までにレトロウイルスで作製したiPSC様細胞の解析から、大腸腫瘍由来のiPSC様細胞は胎盤組織へ分化する可能性が示唆されていた。今回樹立されたRT細胞においても、胎盤分化能を持つのかを検討した。分化誘導培養条件で培養するとRT細胞では胎盤分化に重要な転写因子である *Cdx2* 遺伝子の発現が上昇ことを確認した。さらに、大腸腫瘍由来のRT細胞をマウス初期胚にマイクロインジェクションすることで、大腸腫瘍由来RT細胞が胎盤組織へと分化することが確認された。胎盤組織に分化した大腸腫瘍細胞由来RT細胞は、組織学的に周囲の胎盤組織と区別不可能であり、非腫瘍性胎盤組織に分化したことが示唆された。

D．考察

本研究の最終目的であるエピジェネティック修飾異常の起源および意義解明には、腫瘍細胞の完全な初期化が必須である。しかしながら、腫瘍細胞の完全な初期化は困難であることが示唆されている。本研究により、初期化可能マウスを用いることで、少なくとも一部の腫瘍細胞に完全な初期化が誘導できることが分かった。今後は樹立された *Nanog* 陽性RT細胞を用いて、エピゲノム解析を進める予定である。

レトロウイルスにより作製されたiPSC様細胞では、レトロウイルス由来の外来遺伝子のリークが観察されていた。外来遺伝子の発現により、エピジェネティクス修飾状態の変化が誘導される可能性があり、本研究の目的達成のための障壁となることが予想されていた。本年度の研究により、外来遺伝子の発現が見られない初期化細胞株が樹立できたことから、今後のエピゲノム解析に有用なツールとなることが期待される。

大腸腫瘍由来のRT細胞から非腫瘍性胎盤組織への分化誘導は、腫瘍発生に十分な遺伝子変異を有する細胞であってもエピジェネティック修飾状態次第で非腫瘍性細胞に変化することを示唆する。エピゲノム制御を標的としたがん治療の妥当性を示す結果と考えられた。

E．結論

大腸腫瘍細胞から *Nanog* 陽性かつ外来遺伝子リークのない初期化細胞が樹立できた。腫瘍細胞の完全な初期化が可能であることが示唆された。腫瘍細胞のリプログラミング／再分化の系は、がん細胞におけるエピゲノム異常の起源、および意義を解明するために有用なモデルと考えられる。

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G．知的財産権の出願・登録状況（予定を含む）

1. 特許取得

該当無し

2. 実用新案登録

該当無し

3. その他

該当無し

別紙４

研究成果の刊行に関する一覧表

書籍

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Kanai Y and Arai E.	DNA methylation alterations in human cancers.	Tollefsbol T.	Epigenetics in Human Disease	Elsevier			in press

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Chapter 10

Field Cancerization in Gastric Cancer

Toshikazu Ushijima and Takeichi Yoshida

Abstract

Frequent occurrence of multiple gastric cancers became clear as their endoscopic resection became popular. As an important mechanism, accumulation of aberrant DNA methylation of various genes, both driver and passenger genes, in "normal-appearing" gastric mucosae has been revealed.

Aberrant DNA methylation is induced by inflammation caused by *Helicobacter pylori* infection, a major causative agent of gastric cancers, and consists of permanent and temporary components that will remain and disappear, respectively, after discontinuance of *Helicobacter pylori* infection. Permanent methylation is almost absent in gastric mucosae of healthy individuals, and is present at low levels in gastric mucosae of patients with a single gastric cancer and at high levels in those of patients with multiple gastric cancers.

The presence of microsatellite instability in gastric cancers, mostly due to *MLH1* methylation, is also known to be associated with multiple gastric cancers. Accumulation of aberrant DNA methylation in gastric mucosae constitutes the major mechanism of field defect for gastric cancers.

10.1. Introduction

Gastric cancer is a major cause of cancer deaths world-wide [1]. Its incidence has markedly declined in the last century in the US and Europe [2], as shown by its crude mortality rate in Caucasian males at 33/100,000 in the early 20th century and at 5 in the late 20th century. However, gastric cancer incidence is still high in many Asian countries and Russia [1]. Histologically, gastric cancers are classified into intestinal and diffuse types, established by Lauren, in Western countries, and these two types largely correspond to differentiated and undifferentiated types in Japanese classification [3].

Early gastric cancers used to be treated by gastrectomy (total and partial gastrectomy), but now early intestinal-type gastric cancers are treated by endoscopic resection (ER),

including endoscopic mucosal resection and endoscopic submucosal dissection [4,5]. ER conserves a much larger part of gastric mucosae than partial gastrectomy, and brought a dramatic improvement of quality of life after treatment. At the same time, it became clear that metachronous gastric cancers occur in 8.5-14.0 % of patients after ER [6,7], which was much higher than the incidence after partial gastrectomy (1.8 – 2.4 %) [8,9]. Also, patients with multiple gastric cancers are known to have a higher risk of developing another gastric cancer than patients with a single gastric cancer [10,11]. The very high incidence of metachronous gastric cancers and high risk of gastric cancer patients with multiple gastric cancers strongly indicate that "field cancerization" or "field defect" is involved in gastric carcinogenesis. As its molecular basis, accumulation of aberrant DNA methylation in "normal-appearing" gastric mucosae is now recognized to be deeply involved [12,13]. The aberrant DNA methylation is induced by *H. pylori* infection, a major etiologic factor for gastric cancers [14], mainly through inflammation [12,15]. In this chapter, we will describe both epigenetic and genetic field defects, placing emphasis on the epigenetic field defect.

10.2. Conventional Changes Indicative of the Presence of Field Defect

The presence of individuals with high risk of gastric cancers has been known for a long time, and suggested that a field defect for gastric cancers is present. Conventionally, the presence of gastric atrophy and/or intestinal metaplasia in the stomach of gastric cancer patients has been well known [16]. Recently, much effort has been made to develop serum and other molecular markers to detect individuals with high risk of gastric cancers.

10.2.1. Histological Changes Associated with Increased Risk

Gastric atrophy is characterized by loss of gastric glandular cells, and appearance of fibrous tissue. Intestinal metaplasia is characterized by the appearance of intestinal-type epithelia in the stomach, and is considered as an abnormal differentiation. Gastric atrophy and intestinal metaplasia are produced as a result of chronic inflammation due to *H. pylori* infection. A prospective study involving 5,373 subjects for more than 10 years revealed that subjects with moderate atrophy at the baseline had a hazard ratio of 2.22 to develop gastric cancers [17].

10.2.2. Serum and Molecular Markers for Increased Risk

Most serum markers to detect individuals at high risk for gastric cancers are related to atrophy of gastric mucosae [18]. Among these, the pepsinogen concentrations are most widely used. Pepsinogen I is produced in chief and mucous neck cells, and Pepsinogen II is produced in not only chief and mucous neck cells but also in cardiac, pyloric, and duodenal Brunner gland cells. Since gastric atrophy advances from the pyloric glands towards the cardiac glands, the level of pepsinogen I and the ratio of pepsinogen I/II decrease with the

advancement [19]. If individuals are classified according to *H. pylori* infection status and the presence of atrophy, gastric cancer risk increases in the order of Group A (*H. pylori*-negative, atrophy-negative), Group B (*H. pylori*-positive, atrophy-negative), Group C (*H. pylori*-positive, atrophy-positive), and then Group D (*H. pylori*-negative, atrophy-positive) [20]. Groups B, C, and D have hazard ratios of 3.0, 3.7, and 32 compared with Group A [20,21].

Molecular risk markers that can be assessed in gastric biopsy specimens are still analyzed for research purpose, and their clinical usefulness has not been established. For example, expression of brain-type glycogen phosphorylase in non-cancerous gastric mucosae has been reported to be useful to predict occurrence of another gastric cancer [22]. *CDX2* is known as a master regulator of the intestinal phenotype, and is expressed in intestinal metaplasia. However, its expression has been shown to progressively decrease from intestinal metaplasia, dysplasia, and then cancers [23].

10.3. Epigenetic Field for Cancerization

Epigenetic mechanisms are deeply involved in gastric cancers because tumor-suppressor genes that can be inactivated by genetic or epigenetic mechanisms are more frequently inactivated by epigenetic mechanisms in gastric cancers [24]. Now, the deep involvement is underlain by a mechanism, induction of aberrant DNA methylation by *H. pylori* infection in gastric mucosae and formation of "epigenetic field defect".

10.3.1. Epigenetic Alterations

Epigenetic modifications are characterized by their inheritance upon somatic cell division, and represented by DNA methylation and histone modifications. They control development, cellular differentiation, and reprogramming by establishing gene usage patterns. DNA methylation of a promoter CpG island (CGI) is known to cause silencing of its downstream gene (figure 10-1) [25].

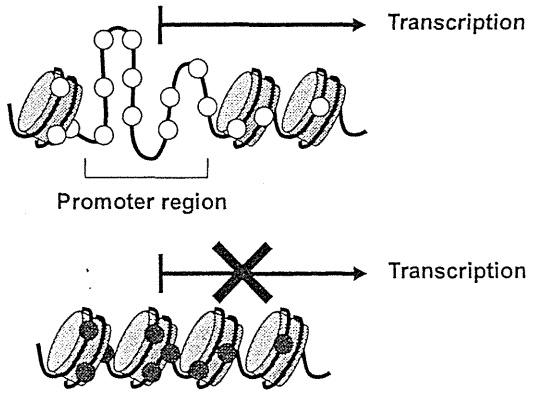

Figure 10.1. Silencing effect of DNA methylation of a CGI in promoter region. Most CGIs in gene promoter regions lack DNA methylation, and no nucleosome is formed. Therefore, transcription factors and RNA polymerase can have access to DNA, and the gene is transcribed. In contrast, if the CGI is methylated, a nucleosome is formed. Therefore, transcription machinery does not have access to DNA, and the gene is silenced.

When a promoter CGIs of a tumor-suppressor gene is aberrantly methylated, it leads to inactivation of the gene and can be causally involved in cancer development [26,27,28]. Therefore, aberrant methylation of promoter CGIs is considered to be equivalent to point mutations and chromosomal deletions.

Recent genome-wide studies on aberrant DNA methylation in cancers revealed that a large number of genes are methylated in their promoter CGIs in a single cancer cell [29,30,31]. Methylation of some genes, such as *CDKN2A*, *CDH1*, *MLH1*, *RUNX3*, *LOX*, and *MiR-124a*, is considered to be causally involved in cancer development [24,32,33], and these genes are designated as drivers. Methylation of other genes, such as *HAND1*, *FLNc*, and *THBD*, are unlikely to be involved in cancer development considering their low expression in normal gastric mucosae and known functions [12], and these genes are designated as passengers. The distinction between the drivers and passengers is just as in mutations.

10.3.2. Epigenetic Field for Cancerization, or Epigenetic Field Defect

Aberrant methylation of some genes, especially that of passenger genes, is accumulated in a large fraction of epithelial cells of the stomach, reaching up to several tens % [12,13,15]. The accumulation levels of methylation of specific passenger genes correlate with those of methylation of driver genes, and can be quantified more precisely because their methylation levels are high (figure 10-2).

Healthy people without *H. pylori* infection have very low methylation levels in their gastric mucosae, but gastric cancer patients without *H. pylori* infection have 5- to 300-fold higher methylation levels in their non-cancerous gastric mucosae (not in cancer tissues) (figure 10-2) [12].

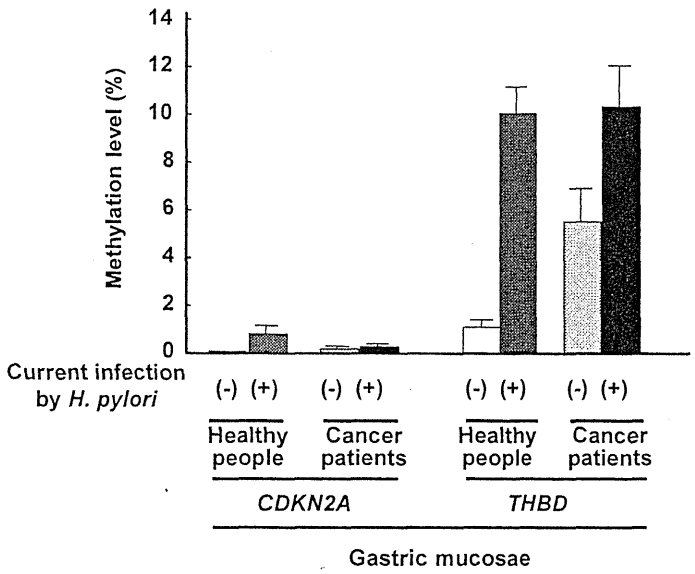

Figure 10.2. DNA methylation levels of *CDKN2A* and *THBD* in gastric mucosae of healthy people and cancer patients with and without *H. pylori* infection. Modified from Maekita et al [12]. Among healthy people, methylation levels were high in individuals with *H. pylori* infection, suggesting that *H. pylori* infection induce aberrant DNA methylation. Among individuals without *H. pylori* infection, gastric cancer patients showed higher methylation levels than healthy people, indicating that accumulation of aberrant DNA methylation is involved in formation of field defect.

Further, among individuals without *H. pylori* infection, patients with multiple gastric cancers have higher methylation levels in their non-cancerous gastric mucosae than those with a single gastric cancer, showing methylation levels in gastric mucosae are correlated with gastric cancer risk [13]. This shows that accumulation of aberrant DNA methylation of various genes in non-cancerous gastric mucosae forms an epigenetic field for cancerization, or epigenetic field defect.

10.3.3. Temporal Profile of Formation of Epigenetic Field Defect

Individuals with *H. pylori* infection have a very high methylation level irrespective of their cancer status and cancer risk. Since the vast majority of gastric cancer patients without current *H. pylori* infection are known to have had this infection in their past [14], the very high methylation level in individuals with *H. pylori* infection is expected to decrease when *H. pylori* infection discontinues. Temporal analysis of methylation levels in individuals who underwent eradication therapy for *H. pylori* confirmed that methylation levels decrease after eradication [34,35]. In other words, a methylation level in gastric mucosae is composed of two components – a temporary component that disappears when *H. pylori* infection discontinues and a permanent component that persists even after *H. pylori* infection discontinues.

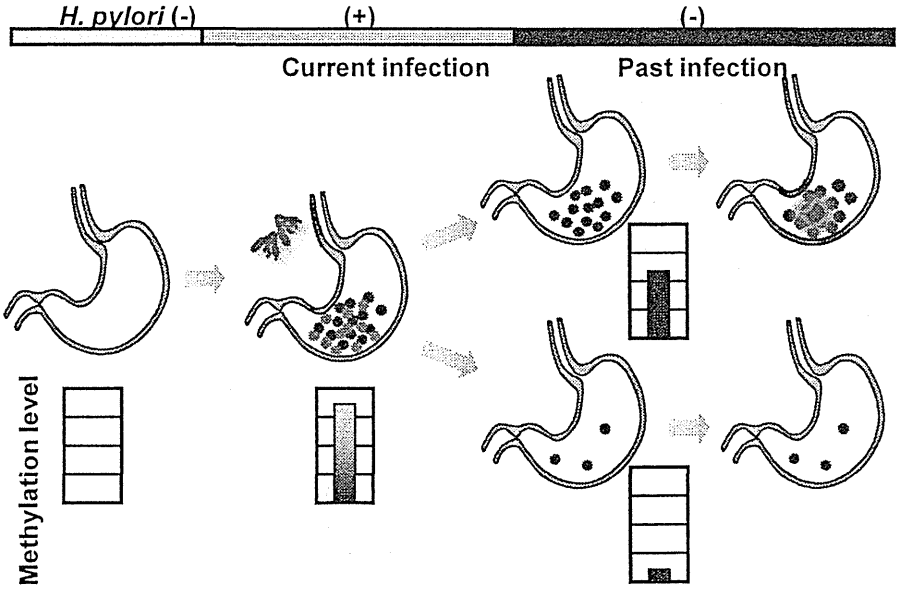

Figure 10.3. Induction of temporary and permanent components of methylation by *H. pylori* infection, and correlation between the permanent component and gastric cancer risk. Almost no methylation is present in gastric mucosae of individuals without any *H. pylori* infection. In gastric mucosae of individuals with *H. pylori* infection, very high levels of aberrant DNA methylation is induced, consisting of temporary and permanent components. When *H. pylori* infection discontinues by eradication or progression of atrophy, the temporary component, which is likely to be methylation in progenitor or differentiated cells, will disappear. In contrast, the permanent component, which is likely to be methylation in stem cells, will remain, and its level correlates with gastric cancer risk.

Based on these findings, a methylation level in gastric mucosae in one's life can be inferred to be very low before *H. pylori* infection takes place, to be very high while *H. pylori* infection is present, and to decrease when *H. pylori* infection discontinues (figure 10-3). If

one's methylation level decreases to a low level, this indicates that his gastric mucosa has limited epigenetic damage in stem cells and has a low risk for gastric cancers. If one's methylation level shows little decrease, this indicates that his gastric mucosa has already accumulated a lot of epigenetic damage in stem cells and has a high risk for gastric cancers.

10.3.4. Mechanisms of Methylation Induction by *H. Pylori* Infection

The observations in humans described above strongly indicate that *H. pylori* infection induces aberrant methylation in gastric mucosae, but lack demonstration of a causal relationship.

Now, the causal role of *H. pylori* infection in methylation induction has been demonstrated by use of Mongolian gerbils. Infection of gerbils with *H. pylori* induced aberrant methylation in gastric mucosae while little methylation was induced in non-infected age-matched gerbils [15].

Mechanisms how *H. pylori* infection induces aberrant DNA methylation were also unclear in humans. *H. pylori* has endogenous methyltransferases and the type IV secretion system that allows its endogenous proteins to infect epithelial cells [36,37], and there was a possibility that bacterial methyltransferase was directly involved in methylation induction.

However, such a direct role was excluded since suppression of inflammation without decreasing number of *H. pylori* in the stomach markedly suppressed methylation induction [15]. It is still unknown what component of inflammation induced by *H. pylori* is responsible for methylation induction.

10.3.5. Gene Specificity in Methylation Induction

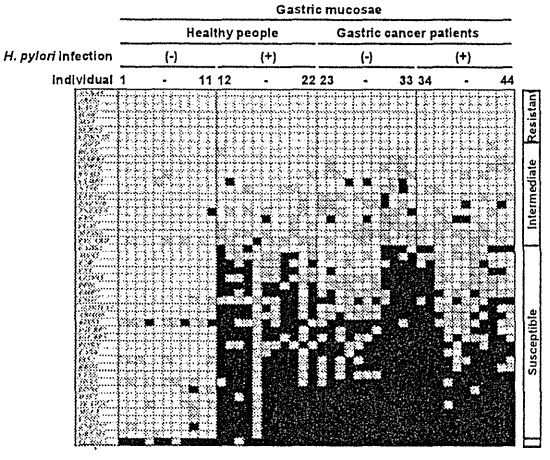

Figure 10.4. The presence of target gene specificity in methylation induction. Modified from Nakajima et al [38]. The presence of aberrant DNA methylation was analyzed by a high-sensitivity method, methylation-specific PCR, in gastric mucosae of healthy people and gastric cancer patients with and without *H. pylori* infection. Filled box, high levels of methylation detected; hatched box, low levels of methylation detected; and open box, no methylation detected. Some genes were easily methylated by *H. pylori* infection, and their methylation persisted in gastric cancer patients. The other genes were resistant to methylation induction.

Both driver and passenger genes are methylated in gastric mucosae, but there is a gene specificity in methylation induction [38]. A panel of genes was analyzed for methylation induction in gastric mucosae of individuals with and without *H. pylori* infection, and it was found that only a fraction of the genes were methylated in individuals with *H. pylori* infection (figure 10-4).

Similar target gene specificity has been found in esophageal mucosae of tobacco smokers [39]. Target genes for methylation induction are known to be determined by low transcription, the presence of a specific histone modification (trimethylation of lysine 27 of histone H3), and the lack of stalled RNA polymerase II [40,41].

10.4. Genetic Changes in Gastric and Other Tissues Associated with Gastric Cancers

Germline mutations and genetic polymorphisms are known to be associated with increased inborn risk of gastric cancers, and thus can be considered to be involved in the formation of "genetic field defect" of gastric cancers. Germline mutations have high penetrance and odds ratios, but are very rare. In contrast, genetic polymorphisms are commonly observed in general populations, but their effects on gastric cancer susceptibility are very weak.

10.4.1. Germline Mutations Associated with Gastric Cancers

Germline mutations of *CDH1* (E-cadherin) were first found in a large family from New Zealand in which diffuse-type gastric cancers took place at an early age [42,43]. *CDH1* germline mutations are very rare, but have been found in other areas in the world [44]. An individual with a *CDH1* germline mutation starts to accumulate a number of small foci of signet ring cells, most of which have methylation of the wild-type allele, and a small fraction of the foci develop into diffuse type cancers [45].

Since the penetrance of *CDH1* germline mutations is very high, prophylactic gastrectomy is a treatment option [42].

In families with hereditary nonpolyposis colorectal cancer (HNPCC), caused by germline mutations of mismatch repair genes, such as *MLH1*, *MSH2*, and *MSH6*, there used to be cases of gastric cancers. The gastric manifestation used to be common in older generations of HNPCC families [46], and is common in Asian populations who have high incidence of gastric cancers[47]. Patients with familial adenomatous polyposis, which is caused by *APC* germline mutations, often present gastric polyps, and also have increased risk for gastric cancers [48].

10.4.2. Genetic Polymorphisms Associated with Gastric Cancer

Genes whose genetic polymorphisms are most widely analyzed are pro-inflammatory cytokine, IL1b, and its receptor antagonist, *IL1RN*. It was initially reported that a single nucleotide polymorphism (SNP) in the *IL1b* promoter was associated with approximately 10-

fold higher risk of gastric atrophy and 2- to 3-fold higher risk of gastric cancers [49]. A SNP in *IL1RN* was also associated with increased gastric cancer risk. Many studies followed this initial study, and a meta-analysis reports that overall gastric cancer risk associated with *IL1b* and *IL1RN* are 1.26 and 1.20 folds, respectively [50].

It is noteworthy that *IL1b* is one of the candidate cytokines involved in methylation induction [15], and that frequent methylation in gastric cancers (CGI methylator phenotype; CIMP) was associated with a SNP in *IL1b* [51]. Taken together, the SNPs in cytokines can be involved in the susceptibility in methylation induction, and thus in gastric cancer susceptibility.

A SNP in the first exon of *PSCA* was identified by a large-scale genome-wide association study, and was associated with 1.62-fold increased risk of diffuse-type gastric cancers [52]. A meta-analysis showed that a SNP in *EGFR* is associated with 1.54-fold increased risk of gastric cancers [53].

Another meta-analysis supported that there is 1.42-fold increased risk for a SNP in folate metabolizing enzyme, *MTHFR* [54]. SNPs in *IL8*, *IL10*, and *TP53* might be risk factors for gastric cancer, but definitive conclusions cannot be made [55,56,57].

10.4.3. Somatic Genetic Changes Associated with Field Defect

Microsatellite instability (MSI) is caused by inactivation of mismatch repair genes, such as *MLH1* and *MSH2*. Especially, *MLH1* is known to be inactivated by its promoter methylation, and one of the important drivers involved in epigenetic field defect. By analysis of cancer tissues, cancers of patients with multiple gastric cancers have been reported to exhibit a higher incidence of MSI than cancers of patients with a single gastric cancer [58]. Since the major mechanism of *MLH1* inactivation is its promoter methylation [24], the presence of MSI in cancer tissues indicates that the patient has high levels of methylation and thus epigenetic field defect. It is difficult to analyze the presence of MSI in non-cancerous gastric mucosae since the fraction of cells with MSI is expected to be very small, and individual gastric glands with MSI are expected to have different types of microsatellite mutation. However, *MLH1* methylation can be detected using a sensitive method, methylation-specific PCR (MSP), and was shown to be present in non-cancerous gastric mucosae of gastric cancer patients with MSI [59].

Activation-induced cytidine deaminase (AID) is a member of the cytidine-deaminase family that acts as a DNA- and RNA-editing enzyme. Infection of gastric epithelial cells with *H. pylori* is known to induce aberrant expression of AID via the IkB kinase-dependent nuclear factor-kB activation pathway. Upregulation of AID is reported to lead to increased *TP53* mutations in gastric epithelial cells *in vitro*, and it seems to contribute to formation of field defect for gastric cancers [60].

Conclusion

Gastric cancer is closely associated with *H. pylori* infection. It induces aberrant methylation of various but specific genes in gastric mucosae mainly through inflammation, and produces an epigenetic field defect (figure 10-5).

Methylation levels of some genes are correlated with gastric cancer risk, and are promising cancer risk markers. *H. pylori* infection can also induce AID expression, which leads to induction of mutations in gastric epithelial cells. Germline mutations of *CDH1*, *MLH1*, and *APC* are involved in gastric cancer susceptibility, but all these are very rare. In contrast, SNPs of some cytokines, such as *IL1b* and *IL1RN*, are common but only weakly associated with gastric cancer risk. These could be involved in the differential responses to *H. pylori* infection and thus in how "efficiently" epigenetic field defect is formed.

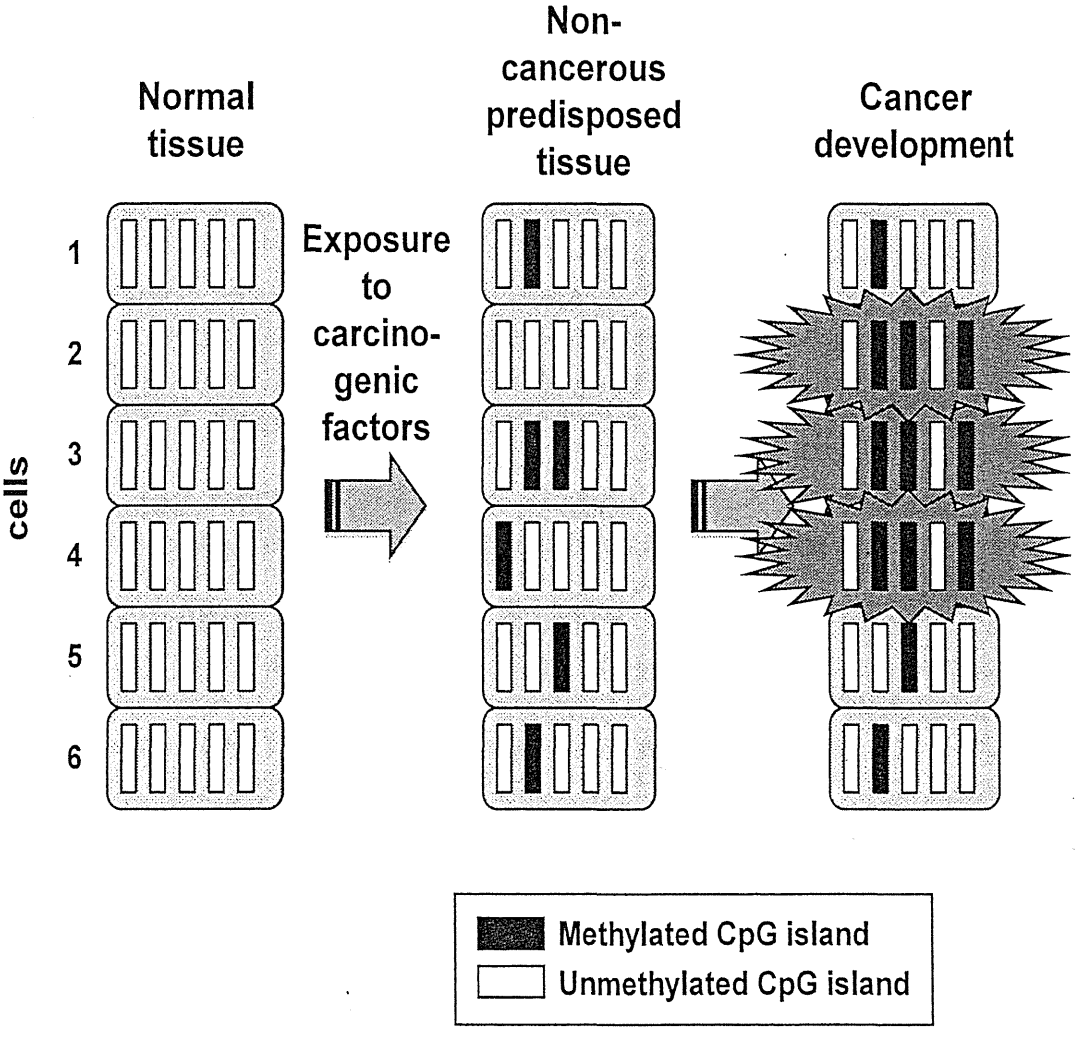

Figure 10.5. Formation of epigenetic field defect and cancer development. If a normal tissue is exposed to an inducer of aberrant DNA methylation, such as *H. pylori* infection, methylation of various but specific genes is induced (non-cancerous predisposed tissue). Both driver and passenger genes (genes A, B, and C) are methylated, but driver genes usually have very low methylation levels. If a predisposed cell harbors an additional hit (e. g. methylation of gene E), a cancer is considered to develop.

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