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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

金井弥栄、新井恵吏、長塩亮「肝細胞癌のリスク評価方法」特願2011-06695 (2011年1月出願)

2. 実用新案登録

該当なし。

3. その他

該当なし。

厚生労働科学研究補助金（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

研究分担者　豊田　実　札幌医科大学生化学講座　教授

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、癌における DNA メチル化の役割を明らかにすることを目的とする。本年度は次世代シークエンサーを用いて ChIP シークエンス法による遺伝子転写開始点の網羅的解析、ゲノムキャプチャー法を用いた１塩基レベルでの DNA メチル化解析を行った。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、がんにおける 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。H21 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、mir-34b/c 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、ChIP シークエンス法による遺伝子転写開始点の網羅的解析、ゲノムキャプチャー法を用いた次世代シークエンサーによる網羅的 Bisulfite sequencing 法を行った。

B．研究方法

DNA メチル化に関しては、Bisulfite sequencing 法および Bisulfite-pyrosequence 法により、遺伝子発現に関しては、Microarray 法、RT-PCR 法および real-time PCR 法により解析した。ヒストン修飾に関しては、クロマチン免疫沈降(ChIP)後、次世代シークエンサーにより解析した。

（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

H21 年度までの研究で、ゲノムワイドな転写開始点のマッピングを行うため、ヒストン H3 リジン 4 トリメチル化(H3K4me3)を認識する抗体で免疫沈降後、次世代シークエンサーを用いて転写開始点の網羅的解析を行った。その結果、DNMT1 および DNMT3B をノックアウトした細胞（DKO）において、親株の HCT116 細胞と比べ、25,000 程度の H3K4me3 の新規のピークを認めた。しかし、データの出力が登録した遺伝子ごとのタグ数で表示されるのみで、生物学的意義の解析は困難であった。本年度は、独自にビューアの作成を行い、ヒストン修飾と遺伝子の関連を可視化することを可能にし、特に microRNA に関してより詳細な解析を行った。その結果、ゲノムの 174 カ所の領域に 233 の microRNA 前駆体に HCT116 細胞では認められず、DKO 細胞特異的な H3K4me3 のピークを認めた。これらのピークの中には、従来のプロモーターアレイでは同定出来なかった、新たな DNA メチル化の標的 microRNA が存在した。そのうち、47 個の microRNA が DKO 細胞で発現上昇していた。22 個が microRNA 前駆体の第一エクソン 5 kb 以内に CpG アイランドを有し、全てが DNA メチル化により遺伝子サイレンシングされていた。

同定されたマイクロ RNA の一つである、miR 1-1 を大腸がん細胞において、バイサルファイトパイロシークエンス法にて解析したところ、高いレベルでメチル化を示し、DNMT1 および DNMT3B をノックアウトした DKO 細胞ではメチル化レベルが低下し、発現が回復していた。また、大腸がん組織でプロモーター部位の DNA メチル化解析を実施すると、76%の大腸がんでメチル化されていたが、正常大腸組織ではメチル化レベルは非常に低く、便や血清からの大腸がんのスクリーニング有用である可能性が示唆された。マイクロ RNA、miR1-1 を、大腸がん細胞株に強制的に発現させ、Gene Ontology 解析を行うと、特に有意に発現が低下した遺伝子は、細胞の接着や細胞運動に関与する遺伝子であった。実際、miR1-1 を過剰発現させた細胞では、細胞運動が遅く、遊走も阻害されていた。miR1-1 が抑制されることが、がん細胞の

転移や浸潤を引き起こしている可能性が示唆された。

従来、DNA メチル化感受性制限酵素による処理や抗メチルシトシン抗体で免疫沈降した DNA をプロモーターオリゴアレイで解析してきたが、解析可能な部位が、プローブの位置に制約を受けることや、シグナルの特異性、定量性に問題があった。1 塩基レベルでの DNA メチル化解析には、バイサルファイトシークエンス解析が必要であるが、ゲノム網羅的な解析は容易ではなかった。最近、特定のゲノムの領域を RNA オリゴヌクレオチドでキャプチャーが開発された。われわれは、これまで困難とされてきた SOLiD を用いた方法で、遺伝子発現に重要な CpG アイランドの解析を試みた。当解析では無作為に選んだ CpG アイランド 1,976 個(1.5Mb)と、メチル化されている CpG アイランド 100 個(0.15Mb)の標的配列に、完全メチル化された配列とメチル化されていない配列に対するベイト(3.3Mb)を作って、ゲノムキャプチャーを行い、バイサルファイト法でシークエンスした。この解析で、選択した 2,076 遺伝子中 2,030 遺伝子を解析できた。また、164,027 個の CpG 配列のうち、110,654 個を解析することができた。1,849 個(89%)の遺伝子で50回以上カバーレージで解析可能であった。以上の結果より、ゲノムキャプチャーを用いたバイサルファイトシークエンス法は、1 塩基レベルでの DNA メチル化解析に有用な方法と考えられた。

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、胃がん膵胆がんにおいて、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。また、消化管間質腫瘍(GIST)において、LINE1 の低メチル化が予後因子となりうることを明らかにした。

D．考察

従来、DNA メチル化の標的遺伝子の同定は、がん細胞株を DNA メチル化阻害剤 5-aza-dC などで処理し、発現アレイ法を用いて、同定する方法を取ってきた。本研究では、転写開始点の指標となる H3K4me3 をゲノム網羅的にマッピングを行い、より効率よくエピジェネティックにサイレンシングされる microRNA を多数同定出来た。また、ゲノムキャプチャーを用いたバイサルファイトシークエンスは、1 塩基レベルでの DNA メチル化解析に有用な方法と考えられた。

E．結論

次世代シークエンサーを用いた ChIP シークエンス法により、遺伝子転写開始点の網羅的解析、ゲノムキャプチャー法を用いた 1 塩基レベルでの DNA メチル化解析を行った。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、がんの新しいバイオマーカーとして有用である可能性が示唆された。

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

豊田 実、山本英一郎、神前正幸、鈴木 拓、山野泰穂. Method for detection of diseases using colonic mucosa. 出願先：PCT/JP2010/064715、平成 22 年 8 月 30 日出願.

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、腹腔鏡下手術や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、残胃に対するフォローは内視鏡医による経験に基づいた診断および、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た。

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、Bisulfite-Pyroseqeunce にて行った。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、通常内視鏡検査時の洗浄行為に使用する洗浄液量に合わせて 250mL 採取管を特注することとした。採取管には 2 つの専用連結管を連結させ、すべての内視鏡装置の吸引口にあうようコネクタを設計しなおした。さらに洗浄廃液採取量の視認性を挙げるべく、PET 素材を採用、遠心機への対応、遠心力への耐性にも考慮した形状を設計した。現在、金型を作成中にある。

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が加わり、早期胃がん（腺腫も含む）に対する内視鏡治療症例300症例を対象に治療前後および、1年おきの胃洗浄廃液を5年後まで回収し、再発予測診断プログラムの構築をエンドポイントにおいて前向き試験を行う。

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の結果が大いに期待される。

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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

Date of Filing: 15.05.07
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Designated States: AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC MT NL PL PT RO SE SK TR

2. 実用新案登録

該当無し。

3. その他

該当無し。

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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

近年 DNA 脱メチル化剤などエピジェネティック修飾異常を標的とした治療への応用が注目されている。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のために、がん細胞におけるエピゲノム異常の起源、役割を解明することが求められている。本研究では iPS 細胞(iPSC)作製技術を用いることにより、腫瘍細胞のリプログラミング／再分化におけるエピジェネティック修飾状態を解析することで、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。

家族性大腸腺腫症のモデルマウスである Apc Min マウス大腸腫瘍に山中 4 因子を強制発現させることで、形態的に iPSC に類似した細胞(T-iPSC 様細胞)を樹立した。T-iPSC 様細胞を分化誘導培地にて培養すると、一部は Cdx2 陽性細胞へと分化した。また、T-iPSC 様細胞を分化誘導条件下にて持続的に培養すると、細胞増殖能が徐々に亢進し、免疫不全マウス皮下での造腫瘍能を獲得した。現在、得られたそれぞれの細胞の DNA メチル化状態を解析し、いかに腫瘍細胞の DNA メチル化が変動するのか検討している。この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。

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、がん抑制遺伝子のサイレンシングなどを介して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。

4 種類の転写因子を強制発現させることで、体細胞が ES 細胞とほぼ同等の細胞に変化する、つまり induced pluripotent stem 細胞(iPSC)が樹立できることが明らかとなった。本研究では iPSC 作製技術を用いることにより、腫瘍細胞の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。

B．研究方法

腫瘍モデルとして、家族性大腸腺腫症のモデルマウスである Apc Min/+マウス大腸発がんモデルを用いた。Apc Min/+マウスに、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、大腸を摘出した。大腸腫瘍を初代培養し、レトロウイルスにより Oct3/4, Sox2, Klf4, Myc を導入した。樹立した iPSC 様細胞から DNA を抽出後、腫瘍細胞由来であるかを確認するために、Apc 遺伝子の LOH を、PCR-RFLP 法にて検索し、Apc LOH を有する T-iPSC 様細胞を同定した。

T-iPSC 様細胞は、多能性幹細胞の培養条件下にて維持した。Leukemia Inhibitory Factor (LIF)および feeder 細胞の非存在下にて T-iPSC 様細胞の分化誘導を試みた。

DNA メチル化状態の検索は、Apc Min/+マウス大腸腫瘍特異的に見られる遺伝子に対して、バイサルファイトシークエンス法により検索した。

免疫不全マウス皮下での腫瘍形成能を検討するために、未分化培養条件、および分化誘導条件における T-iPSC 様細胞 5×10^6 個をヌードマウスの皮下に移植した。皮下腫瘤は摘出後、4%PFA にて 24 時間固定後、パラフィン包埋し組織切片を作製した。組織切片は HE 染色および免疫染色にて検索した。

（倫理面への配慮）

全ての動物実験は、動物実験実施機関（京都大学および岐阜大学）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て実験を施行した。

C．研究結果

T-iPSC 様細胞では多能性幹細胞で発現が高い *Nanog* 発現は確認されなかったが、初代 iPSC のマーカーの一つである *Fbxo15* の発現が高いことが明らかとなった。また、T-iPSC 様細胞は、高いアルカリフォスファターゼ活性を持ち、形態的な類似性に加えて、多能性幹細胞に類似した性質を有することが分かった。マウス大腸腫瘍にて異常な高 DNA メチル化状態が見られる遺伝子において、DNA メチル化状態を検索すると、T-iPSC 様細胞では、高 DNA メチル化状態が見られないことが分かった。一方で、大腸腫瘍由来 iPSC 様細胞を、分化誘導培地にて培養すると、*Fbxo15* の発現が低下し、細胞形態が上皮様に変化した。分化様細胞では、分化マーカーの一つである Cdx2 の発現が見られ、実際に分化の誘導が示唆された。この大腸腫瘍由来 iPSC 様細胞の培養を継続すると、細胞の継代数とともに、徐々に細胞増殖活性の亢進が見られた。さらに継代を続けた大腸腫瘍由来 iPSC 様細胞は、ヌードマウス皮下における造腫瘍能を獲得することを見いだした。ヌードマウス皮下腫瘍は組織学的に、一部で上皮様の形態を示す未分化様細胞の増生から構成されており、大腸腫瘍とは異なる組織像を呈した。

D．考察

マウス大腸腫瘍細胞に初期化因子を強制発現させることで樹立された T-iPSC 様細胞は、形態的な類似性のみならず、多能性幹細胞関連遺伝子の発現などにも類似性が確認され、部分的にリプログラミングされた細胞であることが示唆された。実際に大腸腫瘍特異的に見られた異常 DNA メチル化状態が、T-iPSC 様細胞では確認されず、腫瘍細胞のエピジェネティック修飾状態に変化を誘導できたと考えられる。さらに T-iPSC 様細胞を分化培地にて培養することで、Cdx2 陽性細胞へと分化誘導できることが明らかとなった。興味深いことに、T-iPSC 様細胞は分化状態で継代を繰り返すことで、細胞増殖能の亢進、造腫瘍能の獲得が見られた。近年、培養細胞における異常な DNA メチル化修飾獲得が着目されている。T-iPSC 様細胞の持続培養過程において新たな遺伝子変異を獲得していないと仮定すると、T-iPSC 様細胞における性質変化は、DNA メチル化をはじめとするエピジェネティック修飾状態の変化に起因する可能性がある。従って、T-iPSC 様細胞の持続培養におけるエピジェネティック修飾変化を解析することで、造腫瘍能獲得に関わるエピジェネティック修飾変化を同定できる可能性があると考えられる。また、持続培養後に形成された腫瘍は、大腸腫瘍とは組織像が異なっており、共通した遺伝子変異を有する異なる腫瘍を形成できた可能性がある。現在、これらの細胞を用いて、DNA メチル化を中心とした網羅的なエピゲノム解析を行っている。

E．結論

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）

該当無し

別紙４

研究成果の刊行に関する一覧表

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Epigenomic Analysis in Toxicology

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1 INTRODUCTION

The epigenome, the totality of epigenetic modifications in a cell, plays a fundamental role in development, differentiation, and reprogramming (Law and Jacobsen, 2010). Like the genome and unlike transcriptome and proteome in a cell, the epigenome is replicated upon somatic DNA replication (Ushijima *et al.*, 2003; Laird *et al.*, 2004; Riggs and Xiong, 2004; Margueron and Reinberg, 2010). At the same time, unlike the genome, the epigenome undergoes dynamic changes during development, differentiation, and reprogramming (Bird, 2007; Cedar and Bergman, 2009). In other words, the epigenome is established as a consequence of interactions between the genome and environmental input (Gan *et al.*, 2007), and serves as a cellular memory once established.

From a toxicological viewpoint, agents that induce aberrations in the epigenome are of serious concern. Once an aberrant epigenome is established by some factors, the aberrant epigenome is inherited at somatic cell divisions even if the aberrant status is hazardous to the cell or host. It is well established now that aberration of the epigenome can be causally involved in cancer development and progression (Jones and Baylin, 2007), and it is expected that aberration of the epigenome could be involved in a broader range of acquired disorders (Jones *et al.*, 2008; Robertson, 2005). This chapter will introduce what is the epigenome, how it is altered in cancers and other disorders, what induces epigenetic alterations, and essential techniques for epigenome analysis.

2 EPIGENETIC MODIFICATIONS AND EPIGENOME

Epigenetic modifications include DNA methylation and histone modifications. DNA methylation is well known for its high fidelity at somatic cell replication (Ushijima *et al.*, 2003; Laird *et al.*, 2004; Riggs and Xiong, 2004), and thus is considered as the central player in maintenance of long-term cellular memory in mammalian cells. Histone modifications are more diverse, and individual modifications seem to have their own roles and fidelity in somatic cell replication.

2.1 DNA Methylation

DNA methylation in epigenetics refers to physiological methylation at the 5 position of cytosines at some CpG sites (Figure 1a). This methylation is different from pathological DNA methylations at O^6 and *N*7 positions of guanines, which are abnormal adducts produced by alkylating agents and important in the field of toxicology. DNA methylation at CpG sites is characterized by its inheritance upon somatic cell division, and critical roles in regulation of gene transcription.

2.1.1 Maintenance of DNA Methylation Statuses

When a CpG site is methylated, cytosines on both strands are methylated (Figure 1b). At DNA

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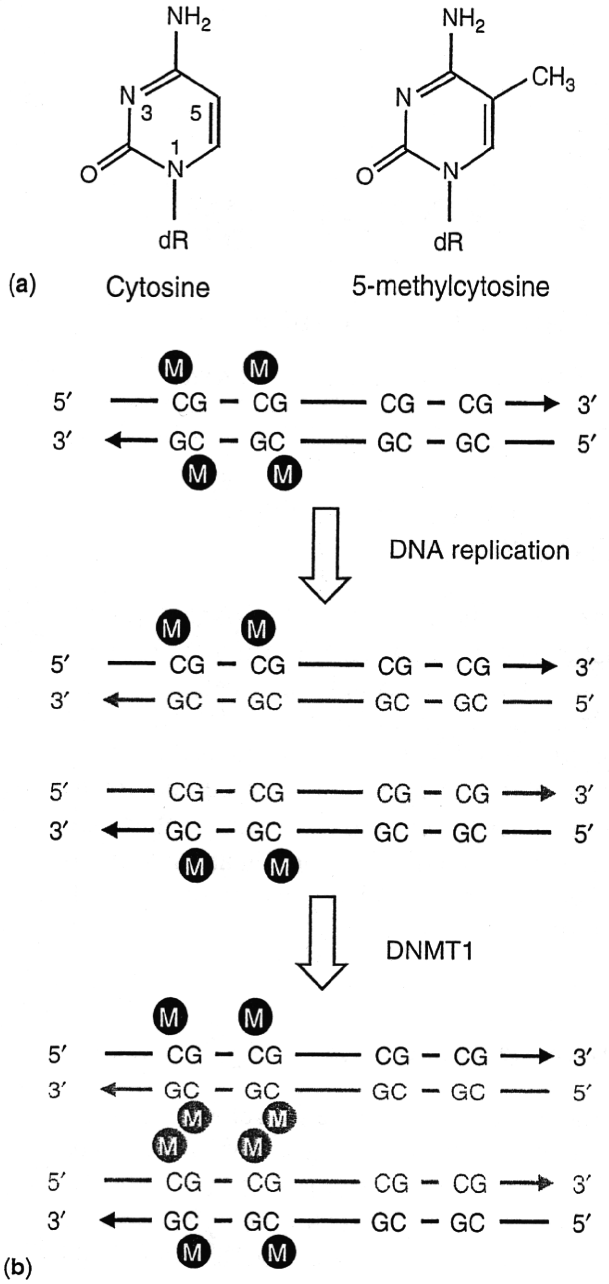

Figure 1. Characteristics of DNA methylation: (a) structure of 5-methylcytosine; (b) maintenance of DNA methylation at somatic cell replication. DNMT1 restores fully methylated statuses by methylating hemi-methylated CpG sites at DNA replication. Methylated or unmethylated statuses are inherited with high fidelity.

replication, cytosines in a newly synthesized DNA strand do not contain methyl groups, and hemi-methylated CpG sites are temporarily formed. However, a maintenance methylase, DNA methyltransferase 1 (DNMT1), associated with a replication fork (Hermann, Goyal and Jeltsch, 2004), restores those hemi-methylated CpG sites into fully methylated CpG sites. DNMT1 has much lower activity on unmethylated CpG sites, and unmethylated CpG sites are kept unmethylated. Therefore, DNA methylation patterns are replicated at somatic DNA replication with a high fidelity (~99.9%), especially in CpG islands (Ushijima *et al.*, 2003; Laird *et al.*, 2004; Riggs and Xiong, 2004).

DNA methyltransferases are essential machineries to establish and maintain DNA methylation. As mentioned above, DNMT1 has the major role in maintaining DNA methylation upon DNA replication, and homozygous knockout of *Dnmt1* is lethal in mid-gestation (Li *et al.*, 1992). In contrast, two *de novo* methylases, DNMT3A and DNMT3B, are involved in establishment of genome-wide DNA methylation patterns (Okano, Xie and Li, 1998; Hermann, Goyal and Jeltsch, 2004). While Dnmt3a cannot methylate nucleosomal DNA, Dnmt3b can (Takeshima *et al.*, 2006). Homozygous knockout of *Dnmt3a* causes lethality after birth (Okano *et al.*, 1999), and Dnmt3a is essential in establishment of genomic imprinting (Kaneda *et al.*, 2004). Homozygous knockout of *Dnmt3b* causes lethality before birth, and germline mutations of *DNMT3B* cause a recessive inherited disorder, ICF syndrome, in humans (Okano *et al.*, 1999).

2.1.2 *Gene Silencing Caused By DNA Methylation of Promoter CpG Islands*

DNA methylation of a CpG island in a gene promoter region has been known to be consistently associated with transcriptional repression of its downstream gene (Baylin and Ohm, 2006; Ushijima, 2005). This was further supported by recent genome-wide analyses of DNA methylation and gene expression (Weber *et al.*, 2007; Rauch *et al.*, 2009; Yamashita *et al.*, 2009). As a mechanism how DNA methylation of a CpG island in a promoter region causes silencing of its downstream gene, the role of nucleosome formation, as discussed below, is currently believed to be important (Li *et al.*, 2007), in addition to induction of inactive histone modifications and inhibition of binding of methylation-sensitive transcription factors.

2.1.3 *Gene Body Methylation and Increased Transcription*

Recent genome-wide analyses also showed that methylation of CpG islands in gene bodies is often associated with increased gene transcription (Hellman and Chess, 2007; Rauch *et al.*, 2009; Yamashita *et al.*, 2009). Since the association is much weaker than that between methylation of promoter CpG islands and gene repression, the association observed in gene bodies is considered to have no direct cause-consequence relationship.

2.2 Nucleosomes and Histone Modifications

DNA in the nucleus is not naked, and forms nucleosome structures along with core histones. The critical importance of histone modifications and nucleosomes in transcriptional regulation is now recognized.

2.2.1 *Nucleosome and Nucleosome-Free Region in Promoters*

A core nucleosome is made of 146 ± 2 base-pair stretches of DNA around the histone octamer for 1.65 turns in a left-handed superhelix (Figure 2) (Luger *et al.*, 1997). A linker is made of a short stretch of DNA and linker histone H1, and connects two nucleosomes. Thus one nucleosome contains a core and a linker, and is approximately 200 base-pairs long. The histone octamer consists of two of each of four core histone proteins, H2A, H2B, H3, and H4. It is known that DNA in nucleosomes is resistant to micrococcal nuclease and *Sss*I methylase activity, which is experimentally important.

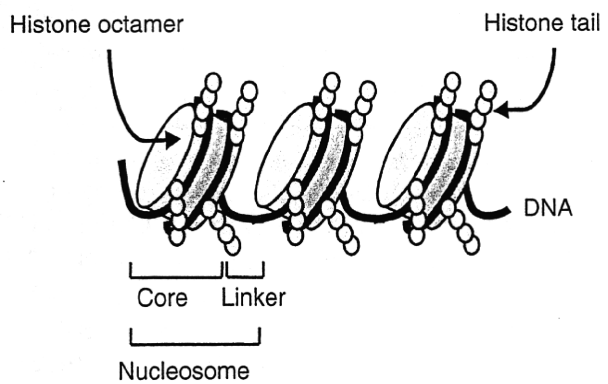

Figure 2. Structure of nucleosome. DNA wraps around the histone octamer, forming a core nucleosome. Core nucleosomes are connected by a linker, and the core and linker forms a nucleosome. Histone tails protrude from the histone octamer.

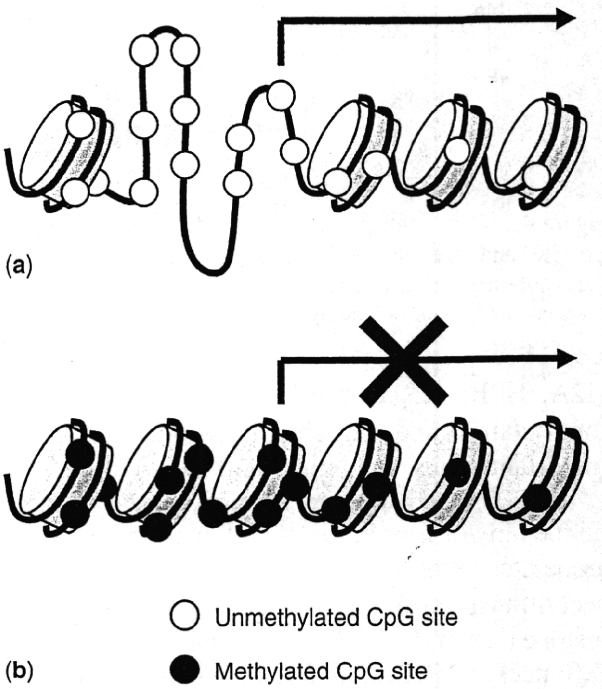

Figure 3. Nucleosome-free region (NFR) and its role in transcription: (a) an approximately 200 bp region upstream of a transcription start site (TSS) lack a nucleosome, and is designated as a NFR. RNA polymerase II and other transcription factors are considered to bind to the NFR; (b) if a NFR in a promoter CpG island is methylated, a nucleosome is formed, and transcription from the NFR is markedly impaired.

It is now known that an approximately 200 bp region just upstream of a transcription start site (TSS) lacks a nucleosome, forming a nucleosome-free region (NFR) (Figure 3a) (Lee *et al.*, 2004; Li *et al.*, 2007; Ozsolak *et al.*, 2007). When a NFR of a CpG-rich promoter is unmethylated, no nucleosomes are formed there, and transcription can be initiated. In contrast, if a NFR is methylated, a nucleosome is formed in the region, and transcription is markedly impaired (gene silencing by promoter methylation) (Figure 3b) (Lin *et al.*, 2007).

2.2.2 *Histone Modifications and Their Roles in Transcription Regulation*

All core histones are composed of a histone fold domain and a structurally undefined tail region (Zheng and Hayes, 2003). Tail regions of histones

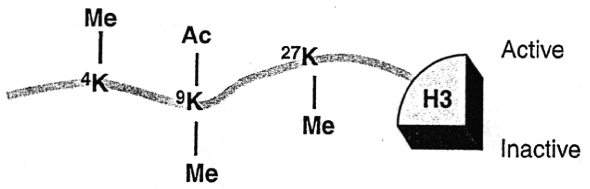

Figure 4. Representative histone modifications. Methylation of H3K4 and acetylation of H3K9 are associated with increased gene transcription, and methylation of H3K9 and H3K27 are associated with gene silencing.

H2A, H2B, H3, and H4 protrude from the histone octamer, and their chemical modifications play important roles in gene regulation (Ruthenburg *et al.*, 2007). Histone acetylation can be observed on the tails of four kinds of histones, and is usually associated with active gene transcription. Histone acetyltransferases (HATs) acetylate histones, and histone deacetylases (HDACs) deacetylate histones (Minucci and Pelicci, 2006). Four classes of HDACs are known, and HDAC1, HDAC2, and HDAC4 are considered to be good targets of HDAC inhibitor drugs.

In addition to histone acetylation, histone methylation at specific lysine and arginine residues is now known to have specific meanings (Figure 4) (Ruthenburg *et al.*, 2007). Especially, methylation of lysine 4, 9, and 27 of histone H3 (H3K4, H3K9, and H3K27, respectively) is associated with active or inactive gene transcription (Barski *et al.*, 2007). At transcription start sites, trimethylation of H3K4 (H3K4me3) is strongly associated with active transcription, and H3K27me3 is associated with silencing of a group of genes (Barski *et al.*, 2007; Kondo *et al.*, 2008). In transcribed regions, H3K4me1, H3K4me2, and H3K4me3 are associated with active transcription, H3K27me2 and H3K27me3 are associated with inactive transcription, and H3K9me2 and H3K9me3 are weakly associated with inactive transcription. The methylation statuses of histones are finely regulated by histone methyltransferases (Kouzarides, 2007; Hublitz, Albert and Peters, 2009) and demethylases (Shi, 2007; Klose and Zhang, 2007).

2.3 Interplay Between DNA Methylation and Histone Modifications

DNA methylation and histone modifications are often dependent upon each other. For example, DNA methylation is recognized by multiple proteins, such as MeCP2 and MBDs, and these proteins recruit histone deacetylases (Richards and Elgin, 2002) and a histone methyltransferase, SUV39H1 (Fujita *et al.*, 2003), which is known to be involved in formation of a heterochromatin structure (Stewart, Li and Wong, 2005). Deacetylated histones are known to be positively charged and to associate tightly with DNA, inhibiting accession of transcription complexes to DNA. On the other hand, H3K9me3 is recognized by heterochromatin protein 1 (HP1), and HP1 recruits DNMT3A and DNMT3B (Fuks, 2005). It helps that inactive histone modification is re-enforced by DNA methylation.

3 EPIGENOME ALTERATIONS IN CANCERS AND OTHER DISORDERS

Epigenomes of normal cells are precisely established and maintained according to developmental stages (Meissner *et al.*, 2008; Rauch *et al.*, 2009). The vast majority of CpG islands are kept unmethylated, and repetitive sequences, which consist of more than 40% of the genome (Lander *et al.*, 2001), are heavily methylated. In cancer cells, an altered epigenome, characterized by "global hypomethylation and regional hypermethylation", is observed (Figure 5).

3.1 Global Hypomethylation

Global hypomethylation, defined as a decrease in 5-methylcytosine content in the genome, is proposed to be present in almost all types of cancer cells (Feinberg and Tycko, 2004). Global hypomethylation is closely associated with hypomethylation of repetitive sequences (Feinberg and Tycko, 2004; Kaneda *et al.*, 2004), but can involve demethylation of normally methylated CpG islands. Demethylation of normally methylated promoter CpG islands leads to aberrant transcription of cancer-testis antigen genes, such as melanoma antigen genes (*MAGEs*) (de Smet *et al.*, 1999), and potentially oncogenes. Also, hypomethylation of a differentially methylated region (DMR) of *IGF2*, known as loss of imprinting, can lead to increased expression and tumor development (Cui *et al.*, 2002). A mouse strain with global hypomethylation demonstrated increased rates of chromosomal loss

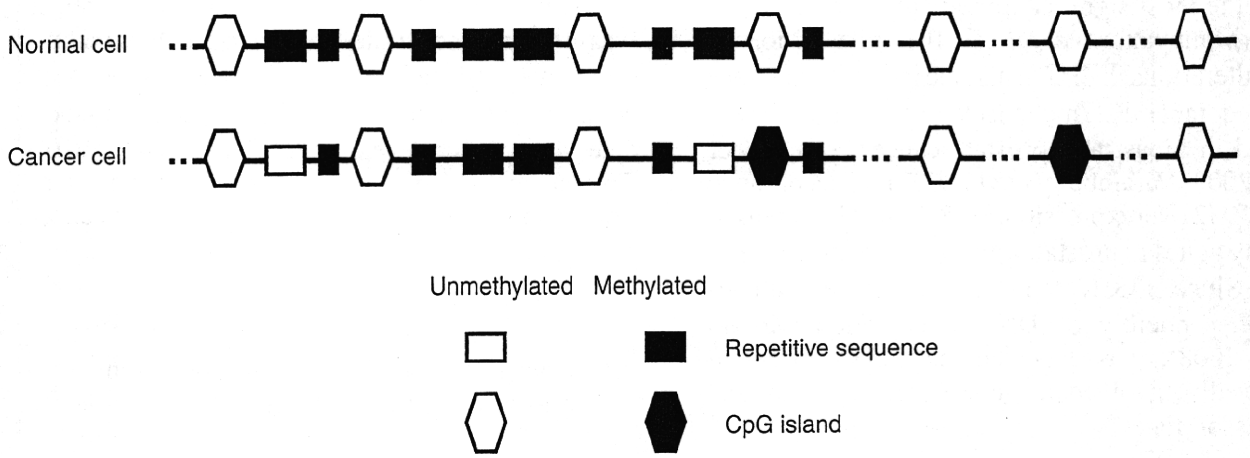

Figure 5. Epigenomic alterations in cancers. Normally methylated repetitive sequences are hypomethylated, and a fraction of normally unmethylated CpG islands are methylated.

(Chen *et al.*, 1998) and increased incidences of lymphomas, colonic microadenomas and liver tumors (Chen *et al.*, 1998; Eden *et al.*, 2003; Yamada *et al.*, 2005). At the same time, global hypomethylation led to suppression of macroscopic tumors of the intestine (Laird *et al.*, 1995; Yamada *et al.*, 2005).

3.2 Regional Hypermethylation – Aberrant Methylation of CpG Islands

"Regional hypermethylation" denotes methylation of CpG islands that are normally unmethylated. If such methylation is induced in the promoter CpG island of a tumor-suppressor gene, the gene is permanently silenced, and the silencing can be causally involved in cancer development and progression (Baylin and Ohm, 2006; Jones and Baylin, 2007). Now, many tumor-suppressor genes involved in various cellular processes, such as cell cycle regulation (*CDKN2A*), WNT signalling (*SFRP* family and *CDH1*), and DNA repair (*MLH1* and *MGMT*), are known to be inactivated by promoter methylation (Baylin and Ohm, 2006). In some cancer types, such as gastric cancers, tumor-suppressor genes are inactivated more frequently by promoter methylation than by mutations (Ushijima and Sasako, 2004). Importantly, CpG islands aberrantly methylated in cancers are not limited to those in promoter regions, and can be present in CpG islands in gene bodies. Methylation of such regions is often associated with increased gene expression (Ushijima, 2005; Rauch *et al.*, 2009; Yamashita *et al.*, 2009).

3.3 Driver Methylation and Passenger Methylation

Now it is known that several hundred to one thousand promoter CpG islands are methylated in cancer cells (Rauch *et al.*, 2009; Yamashita *et al.*, 2009). Most of the genes methylated in cancers have no or only low expression, have H3K27me3 modification, and lack stalled RNA polymerase II in normal counterpart cells (Takeshima and Ushijima, 2010; Takeshima *et al.*, 2009). Therefore, it is considered that most of the promoter CpG islands aberrantly methylated in cancers are not causally involved in carcinogenesis, but methylated in association with it. As mutations are classified as driver and passenger mutations, methylation causally involved in carcinogenesis is designated as "driver methylation", and methylation that simply accompanies the process is designated as "passenger methylation".

3.4 Aberrant Histone Modifications

Histone modifications are also known to be altered in cancers. The global decrease in acetylation of lysine 16 and trimethylation of lysine 20 of histone H4 is known as a hallmark of cancer cells (Fraga *et al.*, 2005a). A global decrease in H3K4me1, H3K9me2, and H3K9me3 and acetylation of histone H3 and H4 are reported in prostate cancer cells (Ellinger *et al.*, 2010; Seligson *et al.*, 2009). A decrease in H3K4me2, H3K9me2, and acetylation of H3K18 is present in pancreatic cancers,

and is the most significant predictor of overall survival (Manuyakorn *et al.*, 2010). In addition to these alterations, EZH2, a histone methyltransferase involved in H3K27me3, is known to be overexpressed in breast and prostate cancer cells (Kleer *et al.*, 2003; Varambally *et al.*, 2002). In accordance with EZH2 overexpression, H3K27me3 is increased in many genes in prostate cancer cells (Kondo *et al.*, 2008). Since H3K27me3 is involved in gene silencing independently of DNA methylation (Kondo *et al.*, 2008), it is expected that H3K27me3 can be causally involved in gene silencing of tumor-suppressor genes.

3.5 Epigenetic Field for Cancerization

Aberrant DNA methylation is present in non-cancerous tissues of cancer patients, forming an epigenetic field for cancerization (epigenetic field defect) (Figure 6) (Ushijima, 2007). High levels of methylation of specific CpG islands, if appropriately selected, are observed in non-cancerous tissues of cancer patients, but not in the corresponding tissues of age-matched individuals (Maekita *et al.*, 2006). The methylation level is correlated with risk of cancer development (Nakajima *et al.*, 2006), and the accumulation can be considered to be associated with cancer development. In an animal model, it was clearly demonstrated that aberrant DNA methylation was induced as a result of exposure to an environmental factor, and accumulation is associated with cancer development (Niwa *et al.*, 2010). Epigenetic field defects are now attracting attention as a target for cancer risk diagnosis and cancer prevention.

3.6 Comparison Between Point Mutations and Aberrant DNA Methylation

Aberrant DNA methylation of promoter CpG islands, especially in NFRs, is now accepted as an equivalent of inactivating mutations, such as inactivating point mutations and chromosomal losses. However, when compared with point mutations, sharp contrasts have been clarified (Table 1) (Ushijima and Asada, 2010). The number of alterations in a cancer is estimated to be approximately 80 for mutations and several hundred to 1,000 for methylation (Gao *et al.*, 2008; Hayashi *et al.*, 2007; Keshet *et al.*, 2006; Rauch *et al.*, 2008; Wood *et al.*, 2007; Yamashita *et al.*, 2009). The fraction of cells with alterations in non-cancerous (thus polyclonal) tissues is very small for mutations (usually at 1×10^{-5}/cell) and can be large for methylation

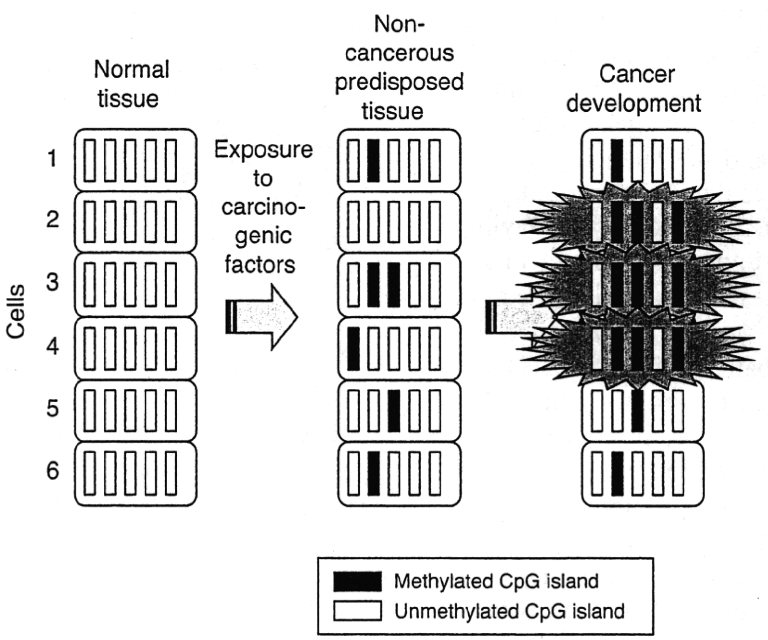

Figure 6. Epigenetic field for cancerization. By exposure to carcinogenic factors, methylation of various, but specific genes, involving both passenger and driver genes, is induced in normal appearing tissues. However, the accumulation level is correlated with cancer risk, and the status is designated as an epigenetic field for cancerization or epigenetic field defect.

Table 1. Comparison between aberrant DNA methylation and point mutations.

	Point mutation	DNA methylation	References
Number of alterations per cancer cell	~80	Several hundred to 1,000	(Gao *et al.*, 2008; Hayashi *et al.*, 2007; Keshet *et al.*, 2006; Rauch *et al.*, 2008; Wood *et al.*, 2007; Yamashita *et al.*, 2009)
Frequency of alterations of a specific gene in non-cancerous tissues	10^{-5}/cell up to 10^{-3}/cell	0.1 to several % up to several 10%	(Maekita *et al.*, 2006; Nagao *et al.*, 2001)
Target gene	Random	Specific	(Costello *et al.*, 2000; Keshet *et al.*, 2006; Loeb, 2001; Wood *et al.*, 2007)
Reversibility	Irreversible	Reversible	(Gan *et al.*, 2007; Issa and Kantarjian, 2009; Jones and Taylor, 1980; Loeb, 2001; Meissner *et al.*, 2008; Wood *et al.*, 2007)

Modified from (Ushijima and Asada, 2010).

(up to several 10% of cells) (Maekita *et al.*, 2006; Nagao *et al.*, 2001). Regarding target genes, mutations are induced mostly in random genes, but methylation is induced in specific genes depending on tissues and inducers (Costello *et al.*, 2000; Keshet *et al.*, 2006; Loeb, 2001; Wood *et al.*, 2007; Nakajima *et al.*, 2009; Oka *et al.*, 2009). Although mutations are essentially irreversible, methylation is potentially reversible, and is now used as a therapeutic target (Gan *et al.*, 2007; Issa and Kantarjian, 2009; Jones and Taylor, 1980; Loeb, 2001; Meissner *et al.*, 2008; Wood *et al.*, 2007). As discussed in Section 4, inducers of aberrant DNA methylation are markedly different from those of mutations.

3.7 Possible Involvement of Epigenomic Alterations in Acquired Disorders Other Than Cancers

Epigenomic alterations are known to be responsible for some inborn disorders other than cancers, such as Rett syndrome (inborn mutations of *MeCP2*), ICF syndrome (inborn mutations of *DNMT3B*), and Beckwith-Wiedemann syndrome (imprinting disorder). From toxicological viewpoints, involvement of epigenomic alterations in acquired human disorders other than cancers is of great interest. As described above, aberrant methylation of specific genes can be present in up to several 10% of cells in non-cancerous tissues, different from mutations. Even if one of 10^5 cells in a tissue had lost expression of specific genes by mutations, it does not harm the function of the tissue. However, it is well expected that, if 10% of cells in a tissue had lost expression of specific genes by methylation, it could harm the function of the tissue.

Epigenomic differences become larger as monozygotic twins grow older, and this could explain different disease susceptibility between twins (Fraga *et al.*, 2005b). Monozygotic twins with and without multiple sclerosis had exactly the same genome and transcriptome, but a slightly different epigenome (Baranzini *et al.*, 2010). Glucocorticoid receptor is reported to be aberrantly methylated in the hippocampus of suicide victims (McGowan *et al.*, 2009). Activating epigenetic changes are induced in the nuclear factor kappaB (NF-κB) subunit *p65* gene in aortic endothelial cells after transient high glucose, and the epigenetic changes and altered gene expression persists during subsequent normoglycemia (El-Osta *et al.*, 2008). Involvement of epigenetic alterations in autoimmune disorders and atopic disorders is also proposed (Maciejewska Rodrigues *et al.*, 2009; van Panhuys, Le Gros and McConnell, 2008). The mechanistic basis and evidence in human and animal studies strongly indicate that epigenomic alterations are involved in common acquired human disorders.

4 INDUCERS OF EPIGENETIC ALTERATIONS

Epigenetic alterations play a major role in cancer development, as described, and possibly in other disorders (Robertson, 2005; Ushijima and

Asada, 2010). Nevertheless, only limited information is available on the factors that induce epigenetic alterations, including aging, inflammation, virus infection, one carbon metabolism, and chemicals (Ushijima and Okochi-Takada, 2005). These inducers are also in a sharp contrast with those of mutations, such as mutagenic chemicals, radiation, and ultraviolet light. Little information is available on how epigenetic alterations are induced.

4.1 Interpretation of Changes in Epigenetic Modifications

Epigenetic modifications can regulate gene transcription, but can be regulated by it at the same time. Therefore, we have to be cautious in interpreting the meaning of changes of epigenetic modifications. An agent may target epigenetic modifications first, and the epigenetic changes can then lead to permanent changes in gene expression (left panel in Figure 7). This change of epigenetic modifications corresponds to mutations, and can be designated as epigenetic alterations. Inducers of epigenetic modifications are of great concern from a toxicological viewpoint.

At the same time, an agent may induce gene expression changes first, and then the expression changes can lead to changes in epigenetic modifications, such as histone acetylation statuses (right panel in Figure 7). Such changes in epigenetic modifications might be inherited upon cell division, or might not be inherited. It is often observed that DNA methylation of a CpG island in an exon is induced when expression of the gene is reduced, or that DNA methylation of the CpG island is reduced when its expression is induced (see sections 2.1.2 and 2.1.3).

Even limited to regions within a promoter CpG island, methylation outside a NFR is often observed while the NFR is kept unmethylated (Graff *et al.*, 1997; Issa *et al.*, 2001; Abe *et al.*, 2002). A gene is usually kept to be transcribed even if regions outside the NFRs are methylated (Ushijima, 2005). This shows that methylation outside NFRs is relatively easily induced, but does not cause gene silencing.

4.2 Aging

Issa *et al.* (1994) first reported that a *Not*I site in exon 1 of estrogen receptor (*ESR*) was methylated in normal colon mucosa in association with aging (Issa *et al.*, 1994). The age-dependent methylation

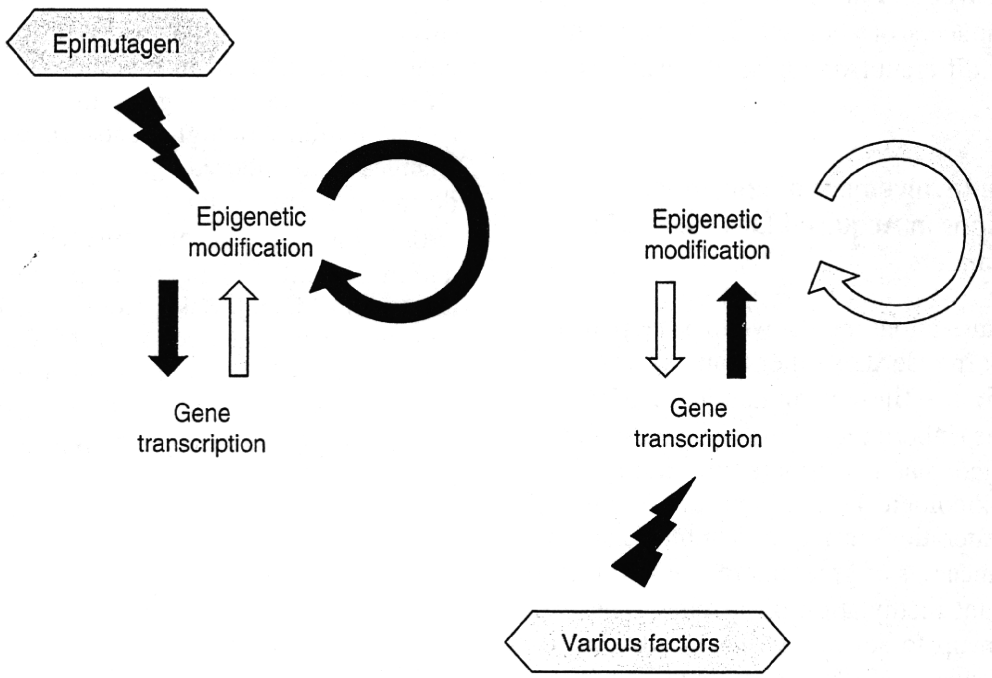

Figure 7. Direct and indirect effects on epigenetic modifications by exogenous factors. *bona fide* epimutagens (defined in Section 4.6) target epigenetic modifications first, and their alterations are inherited and lead to changes in gene transcription. On the other hand, many chemicals induce changes in gene transcription first, and the changes can be accompanied by changes in epigenetic modifications.

was later confirmed by many investigators using human and animal samples (Abe *et al.*, 2002; Waki *et al.*, 2003). It was later shown that age-dependent methylation takes place in specific CpG islands (type A CpG islands) (Ahuja *et al.*, 1998; Toyota *et al.*, 1999). However, it is often observed that, even within the same CpG island, only peripheral regions are methylated but its central regions, which correspond to NFRs in promoter CpG islands, are kept unmethylated (Graff *et al.*, 1997; Issa *et al.*, 2001; Abe *et al.*, 2002). This suggests that mechanisms for methylation induction are different between NFRs in central regions of CpG islands. As a mechanism of age-dependent methylation, an increase in the cumulative number of cell proliferations is considered to give a higher chance of induction of "aberrant" DNA methylation (Issa *et al.*, 2001; Issa *et al.*, 1994).

4.3 Chronic Inflammation

Among the poorly characterized inducers, the best-characterized inducer is possibly chronic inflammation. Aberrant DNA methylation is known to be present in colonic tissues with long-standing ulcerative colitis (Hsieh *et al.*, 1998; Issa *et al.*, 2001; Toyota *et al.*, 2002), in the liver with chronic hepatitis (Kondo *et al.*, 2000), and in gastric tissues exposed to *Helicobacter pylori* (*H. pylori*) infection (Maekita *et al.*, 2006; Park *et al.*, 2009). In addition to these associations, we recently demonstrated using an animal model that inflammation triggered by *H. pylori* infection, not *H. pylori* itself, is indeed the cause of methylation induction (Niwa *et al.*, 2010). Exact mechanisms of how chronic inflammation induces aberrant DNA methylation are still unknown, but expression levels of *Tnfa*, *Il1b*, *Cxcl2*, and *Nos2* are well correlated with methylation induction.

4.4 Viral Infection and Exogenous DNA

It was noted decades ago that viral DNA is methylated upon infection into mammalian cells (Doerfler *et al.*, 1995). It was shown that cells transgenic for an adenovirus type have methylation of not only the transfected viral DNA but also cellular DNA (Muller, Heller and Doerfler, 2001), and the presence of exogenous DNA was suggested to induce methylation of even endogenous genes. The Epstein-Bar (EB) virus infection is occasionally associated with human gastric cancers, and such cancers are known to have more methylated CGIs than gastric cancers without EB virus infection (Kang *et al.*, 2002; Chang *et al.*, 2006). As a potential mechanism, it was recently reported that DNMT1 is activated by EBV latent membraneprotein 2A (Hino *et al.*, 2009). Liver tissues infected by Hepatitis virus C have methylation of multiple genes (Nishida *et al.*, 2008). An adult T-cell leukemia virus was also shown to induce methylation of endogenous genes (Yasunaga *et al.*, 2004). All these indicate that viral infection and exogenous DNA are inducers of aberrant DNA methylation of endogenous genes.

4.5 Disturbances in One Carbon Metabolism

Disturbances in one carbon (methyl group) metabolism, due to deficiency of folate, vitamin B_{12}, or choline, can influence DNA methylation status by limiting availability of the methyl donor, *S*-adenosylmethionine (Poirier, 2002). In animal experiments, methyl supplementation in maternal diet during pregnancy affected methylation levels of a transposable element of offsprings, and the resultant phenotype persisted for a life time (Waterland and Jirtle, 2003). In human, malnutrition during intrauterine and neonatal periods is known to be associated with the development of obesity, type 2 diabetes, and other related co-morbidities (Kalhan, 2009). This strongly indicates that disturbances of one carbon metabolism can induce changes in DNA methylation, which predispose individuals to disease conditions.

4.6 Chemicals

Some chemicals are considered to induce epigenetic alterations, and are designated as "epimutagens" (Holliday, 1991; MacPhee, 1998; Holliday and Ho, 2002). One of the most well characterized epimutagens is a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), which is widely used in laboratories and has now been approved as a therapeutic drug for myelodysplastic syndrome (Jones, 1985; Issa *et al.*, 2005; Issa and Kantarjian, 2009). 5-Aza-dC is incorporated into DNA strands and