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創薬基盤推進研究事業：生物資源・創薬モデル動物

がんの高度専門医療施設において研究用に提供される試料及び情報を統合したバイオバンク構築と、その実証的活用に基づくがんの分子解析に関する研究
(H19-生物資源-一般-009)

平成19年度～21年度　総合研究報告書

研究代表者　金井　弥栄

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(H19-生物資源-一般-009)

研究代表者　金井　弥栄 (国立がんセンター研究所病理部長)

研究要旨

本研究は、オーム解析に耐える質と量を備え、疾患や病態の多様性に応じて十分数が確保され、説明と同意に基づく倫理性が担保され、質の高い標準化された臨床情報が付随している豊富な臨床試料よりなるバイオバンクを国立がんセンターに実証的に構築し、がん研究の推進に資することを目的とする。具体的には、がん診療連携拠点病院等全国約500施設に既に無償配布している院内がん登録アプリケーションHos-CanR 2.5に機能を付加する形で、カタログシステムHos-CanR 2.5バイオバンク版の開発・改良を進めた。要請のあったがん診療連携拠点病院へHos-CanR 2.5バイオバンク版を無償供与し、研究資源の標準化に貢献している。国立がんセンター内のバイオバンクにおいては、平成19年度に、バイオバンク事務室を開設し、標準作業手順書を策定した。平成19年度に598症例・2827バイアル、平成20年度に362症例・1359バイアル、平成21年度に946症例・4265バイアルの病理組織試料を新規に受け入れる一方で、国立がんセンター倫理審査委員会の承認を得た研究 (外部研究機関との共同研究を含む)のために平成19年度に687症例・866バイアル、平成20年度に669症例・1225バイアル、平成21年度に946症例・4265バイアルの病理組織試料を払い出し、10564症例分・41945バイアルを現有して適切に保管している。保管試料が最新のオーム解析に利用しうる質を保持していることを示すため、バンク試料を用いた分子解析を実際に行った。研究代表者は生物資源研究合同班会議に出席し、厚生科学行政上の要請の把握に努めた。本バイオバンクはノイズとバイアスが制御された十分な検出力を持つ研究資源となり、診断・予防・治療の革新を目指したがん研究に寄与すると期待される。

研究分担者氏名・所属研究機関名及び所属研究機関における職名

金井弥栄・国立がんセンター研究所 (部長)

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A．研究目的

国立がんセンターで生み出される質の高い診療情報・病理情報が付随した豊富な臨床試料を、研究資源バンクとして基盤整備する。バイオバンク試料を用いてオーム解析等を行い、バンクの活用ががんの予防・診断・治療の標的候補同定に有益であることを実証する。倫理面にも充分留意したバンク構築のノウハウを、全国のがん診療連携拠点病院等に提供し、我が国におけるがんバンクの標準化・ネットワーク化に資することを目指す。

B．研究方法

病理組織試料ならびに血清試料の管理情報と院内がん登録情報を匿名化の上統合し、研究目的での使用に提供者の同意が得られていることを確認できる、カタログシステムの開発を進める。

バイオバンク事務室を開設し、実務担当者を雇用し、標準作業手順書を策定して教育する。国立がんセンターに蓄積され、病理組織・血清・院内がん登録情報としてそれぞれ病理部門・臨床検査部門・がん対策情報センターに別々に集積・保管されてきた研究資源を、バイオバンクに移管する。新規の病理組織試料・血清試料を受け入れ、カタログシステムに登録する。バンク保管試料を、倫理委員会の承認を得て研究を行う国立がんセンターの研究者(外部研究機関との共同研究を含む)に払い出し、データベースを更新する。現有試料は、核酸・蛋白等の変性を防ぎ直ちに研究の用に供せる質を保持すべく適切な環境下に保管する。

保管試料が最新のゲノム・トランスクリプトーム・プロテオーム解析等に利用可能なことを示すため、研究代表者は倫理委員会の承認を得てバンク試料の一部を用いて分子解析を行う。学術的な成果を公表し、バイオバンク活用ががんの診断・予防・治療の革新を目指した研究に資することを実証する。

(倫理面への配慮)

バンク保管試料は、平成19年8月16日改正文部科学省・厚生労働省「疫学研究に関する倫理指針」に従い、国立がんセンター倫理委員会に研究の承認を得て行われる研究にのみ払い出した。実証的分子解析は、「疫学研究に関する倫理指針」に従い、国立がんセンター倫理委員会に研究の承認を得 (課題番号16-33 「ヒト多段階発がん過程におけるDNA メチル化の変化に関する研究」 研究代表者: 金井弥栄)、倫理面に充分配慮して研究を進めた。全ての分子病理学的解析は、連結可能匿名化し、患者の個人情報保護に充分配慮して進めた。すなわち、個人識別番号と匿名化番号の対応表は、研究所内におかれた匿名管理者によって終始厳重に管理され、診療情報と同時に閲覧されることはなかった。実験室においては、終始患者個人を特定することなく研究を進めた。

C．研究結果

研究協力者等とともに、バイオバンクカタログシステムHos-CanR 2.5バイオバンク版の開発を進めた。

本システムは、がん診療連携拠点病院等全国約500施設に既に無償配布している院内がん登録アプリケーションHos-CanR 2.5に、機能を付加する形で開発した。試験ユーザーである国立がんセンターの研究者に対し、Hos-CanR 2.5バイオバンク版の機能性について評価を求め、改良を続けている。要請のあったがん診療連携拠点病院へ、Hos-CanR 2.5バイオバンク版を無償供与し、具体的な試料管理方法等についても助言した。これを基に、一部のがん診療連携拠点病院においては、施設内バイオバンクが実稼働している。研究代表者は生物資源研究合同班会議に出席し、厚生科学行政上の要請の把握に努めた。

セキュリティー対策と液体窒素タンク・冷凍冷蔵庫等の設備を備えたバイオバンク事務室を開設し、臨床検査技師の国家資格を有する管理実務担当者を雇用して教育した。バイオバンク事務室運用規程ならびに標準作業手順書を策定した。研究協力者等とともに、バイオバンク事務室の運営にあたった。平成19年度に598症例・2827バイアル、平成20年度に362症例・1359バイアル、平成21年度に946症例・4265バイアルの病理組織試料を新規に受け入れる一方で、国立がんセンター倫理審査委員会の承認を得た研究(外部研究機関との共同研究を含む)のために平成19年度に687症例・866バイアル、平成20年度に669症例・1225バイアル、平成21年度に946症例・4265バイアルの病理組織試料を払い出し、データベースを更新した。血清試料の受け入れには、研究協力者古田耕があたった。平成22年3月31日現在でバイオバンクが現有する病理組織試料は10564症例分・41945バイアルで、核酸・蛋白等の変性を防ぎ直ちに研究の用に供せる質を保持すべく適切な環境下に保管している。

研究代表者等は、倫理委員会の承認を得ておこなう諸臓器がんの多段階発生過程におけるDNAメチル化異常の網羅的解析に、バンク試料の一部を供した。バイオバンク保管病理組織試料から抽出した核酸検体が、BACアレイを基盤とするメチル化CpGアイランド増幅 (BAMCA)法に供するに充分な質を保持していることを確認した。網羅的解析結果を検証するために、バンク試料由来のゲノムDNAは、DNAメチル化率を精密に定量することができるパイロシークエンス法等にも供した。

例えば、BAMCA法で、前がん状態にあると考えられる慢性肝炎ないし肝硬変症を呈する非がん肝組織において既に、多数のBACにおけるDNAメチル化減弱・亢進を認めた。DNAメチル化減弱・亢進を示すBACクローンは、肝細胞がん組織でさらに有意に増加していた。正常肝組織と前がん状態にある肝組織を区別し得るDNAメチル化減弱・亢進を示し、かつその変化が肝細胞がんに進展するまで受け継がれるBACクローンを抽出し得た。肝細胞がんの分化度・門脈侵襲の有無・肝内転移の有無と有意に相関するDNAメチル化減弱・亢進を示す、BACクローンを抽出し得た。

さらに、BAMCA法で肝発がんリスクを反映するDNAメチル化の変化を示すことが判明した25BAC領域上の、203 *Xma* I/*Sma* I 認識部位におけるDNAメチル化状態を、パイロシークエンス法で定量的に再評価した。学習コホートの正常肝組織と肝細胞がん症例より得られた非がん肝組織の間で有意にDNAメチル化率が異なる30領域を抽出した。抽出した30領域において学習コホートの肝細胞がん症例より得られた非がん肝組織を正常肝組織から区別するためのカットオフ値を設定することで、学習コホートの肝細胞がん症例より得られた非がん肝組織を感度・特異度とも100%で発がん高リスク状態にあると診断できた。同指標を用いると検証コホートにおいても感度・特異度とも100%で肝細胞がん症例より得られた非がん肝組織を発がん高リスク状態にあると診断できた。バイオバンク試料におけるDNAメチル化解析で、感度・特異度に優れた発がんリスク指標を開発することができた。

尿路上皮がん多段階発生の諸過程に対応する組織標本におけるBAMCA法では、DNAメチル化減弱・亢進を示したBACクローン数は、正常尿路上皮に比して、尿中の発がん物質に暴露されて前がん状態にある可能性のある尿路上皮がん症例より得られた非がん尿路上皮において既に有意に増加しており、尿路上皮がん組織で更に有意に増加していた。尿路上皮がん症例より得られた非がん尿路上皮を、正常尿路上皮から、充分な感度と特異度を持って区別し得る、BACクローンを83個同定した。83クローンを組み合わせ、学習コホートの尿路上皮がん症例より得られた非がん尿路上皮を、感度・特異度とも100%で前がん状態にあると判断し得る発がんリスク評価指標を設定した。同様に、尿路上皮がんの転移・再発予測指標ならびに、腎盂尿管がん術後の膀胱における異時性尿路上皮がん発生リスク指標を獲得した。

膵がん組織を、非膵がん症例より得られた正常膵組織ならびに膵がん症例より得られた非がん膵組織から区別するのに有用な、12BACクローンを抽出した。12BACクローンを組み合わせた膵がんの存在診断指標により、検証コホート中の膵がん検体を、感度・特異度とも100%でがんであると診断できた。次に、学習コホートの早期再発群を長期無再発群から区別するのに有用な、11BACクローンを抽出した。11BACクローンを組み合わせた予後予測指標を設定した。検証コホート症例における11BACクローンのDNAメチル化状態は、無再発生存率・全生存率と有意に相関した。多変量解析で、我々の指標は、切除断端の状態やリンパ節転移の有無とは独立した予後予測因子であることが分かった。バイオバンク試料において解析し得たDNAメチル化プロファイルに基づいて、膵がんの新規の存在診断法・予後予測法を実用化させうると期待された。

バイオバンク試料を用いたそのほかの分子病理学研究の成果は、G．研究発表の項に記した。

D．考察

ゲノム規模のDNAメチル化異常は、諸臓器における前がん状態からがんの悪性進展に至るまで、多段階発がん過程に寄与する可能性があると考えられた。バンク試料を用いた実証的分子解析の結果から、バンク保管組織標本から抽出した核酸検体が、最新のオーム解析に耐える質を保持していることが分かった。バイオバンク保管試料を用いた解析が、発がんリスク診断・がんの個性診断ツールの開発の基盤となる可能性が示された。

平成21年度においては、新規受け入れ・払い出し試料数ともに、平成19年・平成20年に比して増加している。管理アプリケーションの充実等により悉皆的収集が徹底し、バイオバンク室の整備で研究に適した試料の検索等が容易になり、体細胞研究が促進されたためと考えている。今後長期にわたって試料を研究に用いることができるよう、バイオバンク試料に関するコンタクトパーソンや研究組織の原則・コンタクトパーソンが稀少例であるから払い出しが不適切と判断した場合の調停委員会のありかた等を定めた、「バイオバンク運用ポリシー」の策定に向けて、バイオバンク関係者・ユーザーと協議をおこなっている。

E．結論

本研究で開発したカタログシステムHos-CanR 2.5バイオバンク版の検証と改善・必要な機能の追加を、試験ユーザーである国立がんセンターの研究者の評価を反映させつつ、随時継続して行う必要がある。本研究期間中に、がん診療連携拠点病院にノウハウを提供することで、各施設におけるバイオバンク事業立ち上げの支援に貢献できた事例があった。今後、がん診療連携拠点病院に配布したカタログシステムHos-CanR 2.5を仲介として、わが国における研究資源のネットワーク化が進むことが期待される。今後もバイオバンク試料を用いた疾患オーム研究が広く発

展し、がんの診断・予防・治療の革新に資する研究成果が継続して挙げられることを期待する。特に、「バイオバンク運用ポリシー」に関する充分な議論を経て、産学官の外部機関の研究者からの求めに応じ、倫理委員会の承認を得たバイオバンク試料を用いた共同研究が推進されることが望まれる。
バイオバンク試料を用いたがん研究の重要性についての認識がさらに浸透し、悉皆的試料確保が継続するとともに、構築してきたバイオバンクが本研究終了後も散逸することなく、恒久的研究基盤として長く継承されることが望まれる。

F．健康危険情報

該当なし

G．研究発表

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H．知的財産権の出願・登録状況

該当なし

別添4

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state

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Alterations of DNA methylation are one of the most consistent epigenetic changes in human cancers. Human cancers generally show global DNA hypomethylation accompanied by region-specific hypermethylation. Alterations of DNA methylation may result in chromosomal instability as a result of changes in chromatin structure. DNA hypermethylation of CpG islands silences various tumor-related genes. Alterations of DNA methylation are frequently observed in cancers associated with chronic inflammation and/or persistent infection with viruses or other pathogenic microorganisms, such as hepatitis B or C viruses, Epstein–Barr virus, human papillomavirus and *Helicobacter pylori*, or with cigarette smoking. Accumulating evidence suggests that alterations of DNA methylation are involved even in the early and precancerous stages. On the other hand, in patients with cancers, aberrant DNA methylation is significantly associated with poorer tumor differentiation, tumor aggressiveness and poor prognosis. Precancerous conditions showing alterations of DNA methylation may progress rapidly and generate more malignant cancers. DNA methyltransferase (DNMT) 1 over-expression is not a secondary result of increased cell proliferative activity but is significantly correlated with the CpG island methylator phenotype, which is defined as frequent DNA hypermethylation of C-type CpG islands that are usually methylated in a cancer-specific (not age-dependent) manner. Splicing alteration of DNMT3b may result in chromosomal instability through DNA hypomethylation of pericentromeric satellite regions. Alteration of DNA methylation may become an indicator for carcinogenetic risk estimation and early diagnosis of cancers and a biological predictor of poor prognosis in patients with cancers. Correction of DNA methylation status may offer a new strategy for prevention and therapy of cancers.

Introduction

In the earlier days of cancer research, stepwise and orderly progression of genetic alterations causing activation of oncogenes and inactivation of tumor suppressor genes was considered to be the molecular framework responsible for multistage carcinogenesis in humans. However, genetic events alone may not explain the entire process of carcinogenesis: only a few genetic alterations are known to be responsible, especially in the earlier, precancerous stages. Moreover, microscopic observation of cancers frequently reveals histological heterogeneity (e.g. well, moderately or poorly differentiated carcinoma components are simultaneously observed even in tissue sections from any single patient), reflecting complexity of the biological characteristics of tumors. In addition to genetic events, epigenetic events such as alterations of DNA methylation, which can be reversible and underlie the histological heterogeneity of cancers, are another leading player in multistage carcinogenesis.

DNA methylation, a covalent chemical modification resulting in addition of a methyl (CH3) group at the carbon 5 position of the cytosine ring in CpG dinucleotides, plays important roles in chromatin structure modulation, transcriptional regulation and genomic stability, and is essential for the development of mammals (1). The C-terminal catalytic domain of DNA methyltransferases (DNMTs), the major and best known of which is DNMT1, transfers methyl groups from *S*-adenosylmethionine to cytosines (2). DNMT1's preference for hemimethylated over unmethylated substrates *in vitro* and its targeting of replication foci are believed to allow copying of the methylation pattern of the parental strand to the newly synthesized daughter DNA strand (3). Thus, DNMT1 has been recognized as the 'maintenance' DNMT. DNMT1 can interact with the DNMT1-associated protein 1, histone deacetylase 1 and 2 and Rb and can repress gene transcription (4–6). Since DNMT1−/− embryonic stem cells are able to methylate viral DNA *de novo* (7), independently encoded DNMTs have been sought. Among the subsequently identified DNMTs 2 (8), 3a and 3b (9), DNMT activity of DNMT2 has never been demonstrated (10), whereas DNMT3a and DNMT3b do show *de novo* DNA methylation activity *in vitro* (11).

In comparison with normal cells, human cancer cells show a drastic change in DNA methylation status, generally exhibiting global DNA hypomethylation as well as accompanying region-specific hypermethylation (12,13). As 5-methylcytosine is deaminated to thymine, DNA hypermethylation facilitates gene mutation in human cancers. DNA methylation normally promotes a highly condensed chromatin structure through recruitment of DNA-organizing proteins, and DNA hypomethylation in cancer cells causes chromatin decondensation and chromosomal rearrangements that may result in chromosomal instability. Moreover, DNA hypermethylation of CpG islands near gene-regulatory regions silences specific genes, in cooperation with histone modification, including tumor suppressor genes (14).

Association of DNA methylation alterations with both the precancerous stage and malignant progression

In general, candidate tumor suppressor genes, which are located in the commonly deleted chromosomal regions revealed by genomic structural analysis in human cancers, are frequently silenced by alternative two-hit mechanisms consisting of loss of heterozygosity (LOH) and DNA hypermethylation, rather than gene mutation. Thus, DNA hypermethylation of some chromosomal loci is frequently associated with LOH at the same chromosomal loci in human cancers. LOH on chromosome 16 has been frequently detected by classical restriction fragment length polymorphism analysis using Southern blotting in hepatocellular carcinomas (HCCs) which are poorly differentiated, large in size and associated with metastasis (15). Therefore, LOH on chromosome 16 seems to be a late event during multistage hepatocarcinogenesis. At the time of these discoveries, only a few molecular events in the earlier stage of hepatocarcinogenesis were known. Therefore, we first examined DNA methylation status on chromosome 16 in surgically resected tissue specimens.

Classical Southern blotting showed that the digestion patterns obtained using HpaII, a DNA methylation-sensitive restriction enzyme, were similar to those obtained using MspI, a DNA methylation non-sensitive restriction enzyme, at the D16S32 (16 pter to p13), TAT (16q22.2) and D16S7 (16q24.3) loci in normal liver tissue obtained from patients with liver metastases from primary colon cancer, indicating that genomic DNA is normally unmethylated in these regions. Surprisingly, DNA hypermethylation at the D16S32, TAT and D16S7 loci, compared with normal liver tissues, was frequently

Abbreviations: CIMP, CpG island methylator phenotype; DNMT, DNA methyltransferase; HCC, hepatocellular carcinoma; LOH, loss of heterozygosity; MBD, methyl-CpG-binding protein; mRNA, messenger RNA; PanIN, pancreatic intra-epithelial neoplasia; PCR, polymerase chain reaction; PCNA, proliferating cell nuclear antigen; RCC, renal cell carcinoma; STAT, signal transducer and activator of transcription; TCC, transitional cell carcinoma.

detected even in non-cancerous liver tissues showing chronic hepatitis or liver cirrhosis, which are widely considered to be precancerous conditions, indicating that alterations of DNA methylation are a very early event during multistage hepatocarcinogenesis (16). This was one of the earliest reports of alterations of DNA methylation in the precancerous stage.

Since the molecular weight of HpaII-digested DNA fragments in HCCs was higher than that in precancerous conditions and the intensity of larger sized bands was increased in HCCs in comparison with precancerous conditions, the numbers of methylated CpG dinucleotides and cells showing DNA hypermethylation may increase progressively as precancerous conditions develop into HCCs. The incidence of DNA hypermethylation at any of the D16S32, TAT or D16S7 loci in progressed HCCs was significantly higher than that in early HCCs and significantly correlated with higher histological grade (16). DNA hypermethylation at any of the D16S32, TAT or D16S7 loci was detected more frequently in HCCs showing associated involvement of the portal vein and intrahepatic metastasis than in HCCs without these features (16). The presence of DNA hypermethylation in both precancerous conditions and progressed HCCs suggests that precancerous conditions with aberrant DNA methylation might generate HCCs rapidly and that the HCCs thus generated might already be at a progressed stage when diagnosed.

Silencing of tumor suppressor genes by DNA hypermethylation

The *E-cadherin* gene is located on 16q22.1 near to the above-mentioned hot spots of both DNA hypermethylation and LOH in HCCs. E-cadherin acts as a Ca^{2+}-dependent cell–cell adhesion molecule in the adherens junctions of epithelial cells (17). Interactions between E-cadherin and cytoskeletal actin proteins through α- and β-catenins confer stability on the adherens junctions. Cell–cell adhesion determines cell polarity and participates in histogenesis. The mutual adhesiveness of cancer cells is significantly weaker than that of normal cells, and this allows cancer cells to disobey the social order, resulting in destruction of histological architecture, which is a morphological hallmark of malignant tumors (18). In signet-ring cell carcinoma of the stomach (19) and lobular carcinoma of the breast (20), in which cancer cells completely lose their mutual adhesiveness even in the *in situ* carcinoma stage, the *E-cadherin* gene is silenced by a two-hit mechanism comprising LOH and gene mutation. A large kindred study of early-onset, diffuse-type stomach cancers in New Zealand revealed a germ line mutation (21), indicating that the *E-cadherin* gene actually satisfies the criteria for a tumor suppressor gene.

On the other hand, suppression of E-cadherin activity is believed to trigger the release of cancer cells from primary cancer nests, resulting in cancer invasion and metastasis. In fact, non-invasive epithelial cells acquired the ability to invade into collagen gels upon addition of antibodies against E-cadherin (22) or plasmids encoding E-cadherin-specific anti-sense RNA (23). Generally, E-cadherin expression is reduced in poorly differentiated cancers that have lost their cell–cell adhesion and show a strong invasive tendency (18). Significant correlations between reduced E-cadherin expression and poor prognosis have been reported in patients with cancers (18). In order to clarify the mechanism responsible for regulation of E-cadherin expression in cancers, we cloned the promoter region of the human *E-cadherin* gene and demonstrated that it showed DNA methylation in human cancer cell lines lacking E-cadherin expression (24). We also observed induction of E-cadherin expression after treatment with the DNMT inhibitor 5-azacytidine in such cell lines (24). Thus, following the *RB* and *VHL* genes, the *E-cadherin* gene became the third example of a tumor suppressor gene that is silenced by DNA hypermethylation.

When assessed by Southern blotting analysis, DNA hypermethylation around the promoter region of the *E-cadherin* gene was detected in 46% of examined non-cancerous liver tissues showing chronic hepatitis or cirrhosis and in 67% of examined HCCs (25). Immunohistochemical examination revealed that hepatocytes in normal liver tissues showed strong E-cadherin immunoreactivity at their cell–cell borders. We found a significant correlation between DNA hypermethylation around the promoter region and reduced E-cadherin expression in primary HCCs (25). This was the first demonstration of a significant correlation between DNA hypermethylation and reduced expression in clinical tissue samples. Heterogeneous E-cadherin expression in non-cancerous liver tissues showing chronic hepatitis or cirrhosis, which is associated with small focal areas of hepatocytes showing only slight E-cadherin immunoreactivity and is not observed in normal liver tissues, might be due, at least partly, to DNA hypermethylation (25). DNA hypermethylation around the promoter region, which increases during progression from precancerous conditions to HCCs, may participate in hepatocarcinogenesis through reduction of E-cadherin expression, resulting in loss of intercellular adhesiveness and destruction of tissue morphology.

DNA hypermethylation of NotI sites at the D17S5 locus has been detected in various human cancers (26). The *hypermethylated-in-cancer-1* gene at this locus (17q13.3) was the first tumor suppressor gene to be identified in commonly methylated chromosomal loci in human cancers (27); human cancer cells transfected with the *hypermethylated-in-cancer-1* gene grew slowly (27) and mice with germ line disruption of one allele of *Hic1* developed different spontaneous malignant tumors (28). DNA methylation at the D17S5 locus was never detected in normal liver tissues but was detected in 44% of examined non-cancerous liver tissues showing chronic hepatitis or cirrhosis and in 90% of examined HCCs (29). In almost all the paired samples showing DNA hypermethylation, the molecular weight of NotI-digested DNA fragments in HCCs was higher than that in precancerous conditions and the intensity of the larger sized bands was higher in HCCs than in precancerous conditions, indicating that the degree of DNA methylation seems to further increase during progression from a precancerous condition to an HCC (29). The level of hypermethylated-in-cancer-1 messenger RNA (mRNA) expression in non-cancerous liver tissues showing chronic hepatitis or cirrhosis was significantly lower than that in normal liver tissues and was further decreased in HCCs (29).

It is now recognized that numerous tumor-related genes, such as *p16, hMLH1, BRCA1, MGMT, GSTP1, TIMP-3 and DAPK-1*, are silenced by regional DNA hypermethylation around their promoter regions in human cancers (14). Several techniques, such as restriction landmark genomic scanning (30), methylation-sensitive representational difference analysis (31) and methylated CpG islands amplification (32), have been developed for cloning genes that are differentially methylated between cancer cells and normal cells (33), and the list of tumor-related genes silenced by DNA hypermethylation is being expanded (14).

Alterations of DNA methylation precede chromosomal instability during multistage carcinogenesis

The hot spot for DNA hypermethylation in HCCs corresponds to a previously reported hot spot of LOH on chromosome 16. It remains to be examined whether alterations of DNA methylation might predispose the locus to allelic loss or whether common or different causes facilitate both alterations of DNA methylation and LOH at certain loci. However, it is at least clear that DNA hypermethylation precedes LOH at the same chromosomal loci during hepatocarcinogenesis: even classical Southern blotting has detected DNA hypermethylation in bulk non-cancerous liver tissues showing chronic hepatitis or cirrhosis, in which LOH has never been detected using the same method (16).

Recently, microdissection techniques and polymerase chain reaction (PCR) using microsatellite markers have been developed for detecting LOH in small numbers of cells from paraffin-embedded tissues. LOH has been reported even in microdissected specimens from non-cancerous lesions, e.g. hyperplastic or dysplastic lesions accompanying non-small cell lung cancers (34) and proliferative lesions adjacent to breast cancers (35). In order to re-examine whether aberrant DNA methylation precedes chromosomal instability during hepatocarcinogenesis, we obtained 308 microdissected specimens and examined LOH and microsatellite instability by PCR using 39 microsatellite markers and DNA methylation status of eight C-type CpG islands that are known to be methylated in a cancer-specific, but not age-dependent, manner, as shown in Table I, by methylation-specific PCR and combined bisulfite restriction enzyme analysis.

Table I. DNA methylation status on C-type CpG islands in tissue samples of various organs

Tissue samples	The incidence of DNA methylation[a] (%)								References
	CpG islands								
	p16	*hMLH1*	*THBS-1*	MINT1	MINT2	MINT12	MINT25	MINT31	
Normal liver tissues	0	0	0	0	0	0	0	0	(36)
Non-cancerous liver tissues obtained from patients with HCCs	15	0	0	10	73	23	0	0	
HCCs	70	0	0	45	85	45	8	65	
Non-cancerous stomach mucosae obtained from patients with stomach cancers	17	14	2	22	1	6	ND	0	(37)
Stomach cancers	22	17	24	37	25	17	ND	10	
Colorectal cancers	23	27	9	23	20	23	20	10	(38)
Normal renal tissues	11	0	0	0	0	11	22	0	(39)
Non-tumorous renal tissues obtained from patients with renal tumors	62	12	29	2	5	10	24	0	
Renal tumors	73	12	43	17	8	17	35	5	
Normal urothelia	0	ND	ND	ND	56	0	25	45	(40)
Urinary bladder cancers	21	ND	ND	ND	76	30	35	79	

MINT, methylated in tumor; ND, not done.
[a]Analyzed by methylation-specific PCR or combined bisulfite restriction enzyme analysis.

In non-cancerous liver tissues showing chronic hepatitis, LOH for at least one marker was found in 20% of informative microdissected specimens, and LOH in at least one microdissected specimen was found in 45% of informative cases (36). In non-cancerous liver tissues showing cirrhosis, LOH for at least one marker was found in 15% of informative microdissected specimens, and LOH in at least one microdissected specimen was found in 40% of informative cases (36). LOH was never detected in normal liver tissues obtained from patients with liver metastases from primary colon cancer and in non-cancerous liver tissue showing no remarkable histological findings from patients with HCCs. Although no degree of DNA methylation of any of the examined CpG islands was ever detected in normal liver tissues obtained from patients with liver metastases from primary colon cancer, DNA hypermethylation was found on at least one CpG island even in 58% of examined microdissected specimens of non-cancerous liver tissue showing no remarkable histological features obtained from patients with HCCs, in which LOH was never detected (36). Thus, aberrant DNA methylation is an earlier event preceding chromosomal instability during hepatocarcinogenesis, even when examined using microdissection techniques (41). The low incidence of microsatellite instability in Japanese patients (42) was compatible with absence of silencing of the *hMLH1* gene by DNA hypermethylation during hepatocarcinogenesis (36).

Etiologic backgrounds of carcinogenesis and regional DNA hypermethylation

Alterations of DNA methylation are frequently associated with carcinogenesis related to chronic inflammation and/or persistent infection with viruses or other pathogenic microorganisms, such as chronic hepatitis associated with hepatitis B virus or hepatitis C virus infection. As mentioned above, alterations of DNA methylation occur even in non-cancerous liver tissues showing no remarkable histological findings obtained from patients with HCCs, i.e. even before inflammation has become histologically obvious. This phenomenon might be at least partly attributable to hepatitis viral infection. Hepatitis B virus DNA is integrated into the cellular genome, and the integrated viral DNA is known to alter the DNA methylation status in several adjacent cellular genes and DNA segments (43). Epstein–Barr virus infection in stomach cancers is significantly associated with marked accumulation of DNA hypermethylation of C-type CpG islands (37). Induction of latent membrane protein 1 of Epstein–Barr virus has been reported to induce DNMT1 over-expression in cultured cancer cells (44). *Helicobacter pylori* infection, another etiologic factor that is believed to be involved in stomach carcinogenesis, has also been reported to strongly promote regional DNA hypermethylation (45), although the molecular mechanisms by which *H.pylori* infection alters DNA methylation are still unclear and warrant further investigation. Cervical intra-epithelial neoplasia is a precursor lesion for squamous cell carcinoma of the uterine cervix closely associated with human papillomavirus infection. DNMT1 protein expression is increased even in low-grade cervical intra-epithelial neoplasias compared with normal squamous epithelium and further increased in higher-grade cervical intra-epithelial neoplasias and squamous cell carcinomas of the uterine cervix (46). Human papillomavirus-16 E7 protein has been reported to associate directly with DNMT1 and stimulate the methyltransferase activity of DNMT1 *in vitro* (47), and accumulation of DNA hypermethylation on tumor-related genes has also been observed during cervical carcinogenesis (48).

In the same way that HCCs are preceded by chronic hepatitis, ductal carcinomas frequently emerge in pancreases damaged by chronic pancreatitis. Therefore, at least a proportion of peripheral pancreatic duct epithelia with an inflammatory background may be at the precancerous stage. When the DNA methylation status of the *p14, p15, p16, p73, APC, hMLH1, MGMT, BRCA1, GSTP1, TIMP-3, CDH1 and DAPK-1* tumor-related genes was examined, the incidence of DNA hypermethylation of at least one of the genes and the average number of methylated genes were significantly higher in microdissected specimens of peripheral pancreatic duct epithelia with an inflammatory background and in another precancerous lesion, pancreatic intra-epithelial neoplasia (PanIN), compared with that in peripheral pancreatic duct epithelia without an inflammatory background, and was further increased in ductal carcinomas (Figure 1; 50). The *BRCA1, APC, p16 and TIMP-3* genes are frequently methylated in ductal carcinomas of the pancreas (50). With respect to inflammation-related carcinogenesis, cytokine interleukin-6 treatment has been reported to induce DNMT1 over-expression in cultured cells (51), though the significance of cytokine signaling in alterations of DNA methylation has never been confirmed in chronic pancreatitis *in vivo*.

Cigarette smoking is another background factor associated with alterations of DNA methylation during multistage carcinogenesis. DNA hypermethylation at the D17S5 locus was observed in 31% of examined non-cancerous lung tissues, which may contain progenitor cells for cancers, obtained from patients with non-small cell lung cancers and in 33% of corresponding non-small cell lung cancers (52). The incidence of DNA hypermethylation at the D17S5 locus was significantly associated with poorer differentiation of lung adenocarcinomas (52). The incidence of DNA hypermethylation in both non-cancerous lung tissues and non-small cell lung cancers of patients

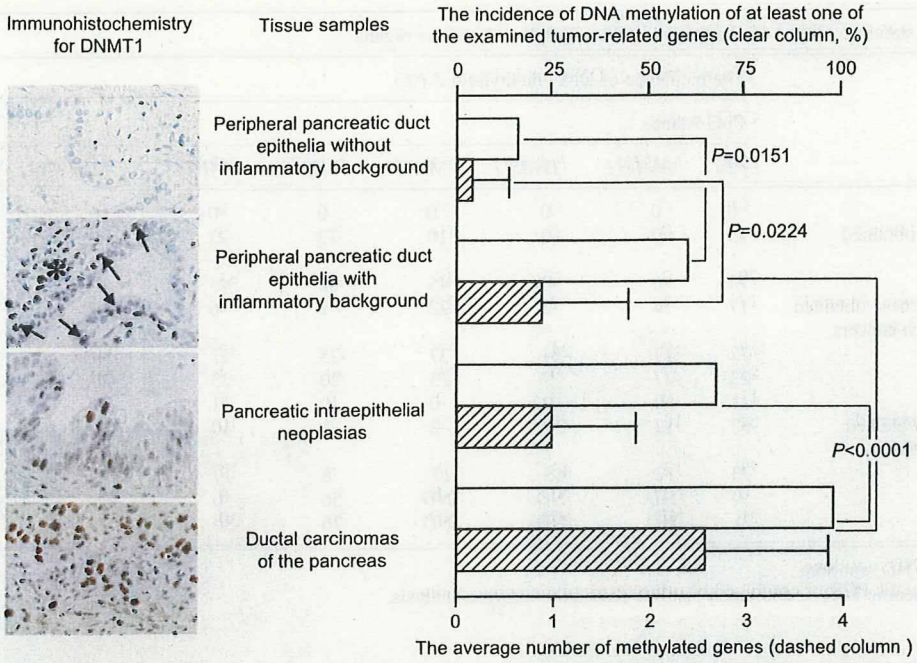

Fig. 1. DNMT1 protein expression and DNA methylation status of CpG islands in tumor-related genes during multistage carcinogenesis of the pancreas. Immunohistochemical examination for DNMT1 was performed in peripheral pancreatic duct epithelia without an inflammatory background, peripheral pancreatic duct epithelia with an inflammatory background (arrows), PanINs and ductal carcinomas (49). Infiltrating lymphocytes (asterisk) were used as an internal positive control for DNMT1 immunoreactivity. Tissue samples were microdissected from surgically resected materials, embedded in agarose beads and subjected to methylation-specific PCR to evaluate the DNA methylation status of the *p14, p15, p16, p73, APC, hMLH1, MGMT, BRCA1, GSTP1, TIMP-3, CDH1 and DAPK-1* genes (50). The incidence of DNMT1 nuclear immunoreactivity, the incidence of DNA methylation of at least one of the 12 genes and the average number of methylated genes increased progressively during multistage carcinogenesis of the pancreas. The average number of methylated genes in ductal carcinomas was significantly correlated with DNMT1 protein expression level ($P = 0.0093$).

who were current smokers was significantly higher than in patients who had never smoked (52). The incidence of DNA hypermethylation in non-cancerous lung tissues obtained from patients with non-small cell lung cancers was significantly correlated with the extent of pulmonary anthracosis, as an index for the cumulative effects of smoking (Figure 2; K. Eguchi, Y. Kanai, K. Kobayashi and S. Hirohashi, unpublished data). Cigarette smoking may participate in alteration of DNA methylation during the development of non-small cell lung cancers. The molecular mechanisms by which carcinogens related to cigarette smoking affect DNA methylation status are still unclear and warrant further investigation.

The incidence of DNA hypermethylation at multiple C-type CpG islands in non-cancerous tissues and cancers from various organs is summarized in Table I. For example, the methylated in tumor-25 clone is methylated in normal renal tissues obtained from patients without renal cancers as frequently as in non-cancerous renal tissues showing no marked histological findings obtained from patients with renal cancers or in renal cancers (39), although it is never methylated in normal liver tissues. DNA methylation profiles of normal tissues tend to be organ specific. Moreover, hot spots of DNA hypermethylation vary among cancers arising in different organs and may reflect the influence of various carcinogenetic factors. The molecular mechanisms responsible for determination of target genes of the CpG island methylator phenotype (CIMP), defined by frequent DNA hypermethylation of C-type CpG islands (53), should be further clarified.

Alterations of DNA methylation are a hallmark of precancerous conditions even in histologically normal tissues

Alterations of DNA methylation are considered to participate in the precancerous stage in various organs, in association with obvious etiological factors, e.g. chronic inflammation, persistent infection with viruses or other pathogenic microorganisms, as mentioned above. Unlike cancers derived from such organs, precancerous conditions in the kidney have been rarely described: pathologists hardly ever observe histological changes in non-cancerous renal tissues obtained from patients with renal cancers. Surprisingly, even in non-cancerous renal tissues showing no marked histological findings obtained from patients with renal cancers, the average number of methylated CpG islands was significantly higher than that in normal renal tissues obtained from patients without renal cancers, regardless of patient age and smoking history (39). The average number of methylated CpG islands was even higher in renal cancers. From the viewpoint of alterations of DNA methylation, the presence of precancerous conditions can be recognized even in the kidney. In other words, regional DNA hypermethylation participates in the early and precancerous stage of multistage renal carcinogenesis. More surprisingly, the average number of methylated CpG islands in non-cancerous renal tissues showing no marked histological change obtained from patients with conventional renal cell carcinomas (RCCs) was significantly correlated with a higher histological grade of corresponding RCCs developing in individual patients (Figure 3; 39), indicating that precancerous conditions showing accumulation of DNA methylation may generate more malignant RCCs.

Regional DNA hypermethylation has a prognostic impact on patients with cancers

Accumulation of DNA methylation at CpG islands in conventional RCCs is significantly correlated with higher histological grade, an infiltrating growth pattern and vascular involvement (39), suggesting that regional DNA hypermethylation is continuously involved in multistage renal carcinogenesis from precancerous conditions to malignant progression. The recurrence-free survival rate of patients with RCCs showing accumulated DNA methylation of CpG islands was significantly lower than that of patients with RCCs not showing this feature (39).

The incidence of increased DNMT1 protein expression in HCCs is significantly correlated with poorer tumor differentiation and portal