12.3 プライバシーの保護と患者識別

登録患者の氏名は参加施設からJGOGデータセンターに知らされることはない。患者の同定や照会は、登録時に発行される症例登録番号、患者イニシャル、生年月日、カルテ番号を用いて行われ、患者名など第3者が当該施設の職員やデータベースの不正アクセスを介さずに直接患者を識別できる情報がJGOGデータセンターのデータベースに登録されることはない。イニシャル、カルテ番号を開示できない場合、本試験へ参加するためには原則として以下の条件を満たすこととする。

- 参加施設の常設機関として設けられた組織(治験管理室、臨床試験管理室等)で患者識別コードの発行がなされ、真のカルテ番号との対応表が管理されること。
  (施設研究責任医師や施設コーディネーターなど個人や当該診療科教室／医局などによる管理ではなく、施設内の常設の組織での管理が望ましい。)
- 患者識別コードと真のカルテ番号の対応表の保管期間は当該施設のカルテの保管期間より短くないこと。
- JGOG の施設監査が行われる場合、患者識別コードと真のカルテ番号の対応表の管理体制の監査を受け入れること。
- イニシャルをマスクする場合は、ダミーであることが判別できるように一律「X.X.」と記載すること。

12.4 試験実施計画書の遵守

本試験に参加する研究者は、患者の安全と人権を損なわない限りにおいて本試験実施計画書を遵守する。

12.5 施設の倫理審査委員会(機関審査委員会)の承認

本試験への参加に際しては、本試験実施計画書および患者への説明文書・同意書が各施設の倫理審査委員会(機関審査委員会)(IRB : Institutional Review Board)で承認されなければならない。

IRB承認が得られた場合、各施設の施設研究責任医師、担当医師あるいは施設コーディネーターはIRB承認文書のコピーをJGOG事務局へFAX送付する。IRB承認原本は各施設が保管、コピーはJGOG事務局及びJGOGデータセンターが保管する。

IRB 承認文書送付先(JGOG 事務局)
特定非営利活動法人　婦人科悪性腫瘍化学療法研究機構　事務局
住所：〒162-0825　東京都新宿区神楽坂 6-22 小松ビル 4 階

FAX : 03-5206-1983　　TEL : 03-5206-1982

12.6 IRB 承認の年次更新

本試験実施計画書および患者への説明文書の各施設倫理審査委員会もしくは IRB の審査承認年次更新の有無は各参加施設の規定に従う。

12.7 試験実施計画書変更等の取り扱い

安全性情報あるいはその他の理由により臨床試験審査委員会承認後に試験実施計画書の変更が必要となった場合、その内容により改正・改訂と区別して取り扱う。また、試験実施計画書の変更に該当しない補足説明の追加を覚え書き/メモランダムとして別に定める。その定義・取り扱いは下記の通りとする。

1) 改正(amendment)

① 定義

試験に参加する患者の危険(risk)を増大させる可能性のある、もしくは試験の primary endpoint に関連する試験実施計画書の部分的変更。

② 変更手順

- 試験実施計画書改正が必要と判断した当該試験の研究代表者・研究事務局は、子宮体がん委員会および JGOG データセンターと協議し、試験実施計画書改正を発案するか否かを決定する。発案する場合、研究代表者・研究事務局は変更試験実施計画書を作成する。
- 子宮体がん委員会委員長は運営委員会へ変更試験実施計画書の審査を依頼し、承認を得る。
- 研究代表者・研究事務局は JGOG 事務局を通して、臨床試験審査委員会へ変更試験実施計画書の審査を依頼し、承認を得る。
- 変更試験実施計画書の承認が得られた場合、JGOG 事務局は当該試験の子宮体がん委員会へ報告し、子宮体がん委員会委員長は運営委員会へ報告する。

③ 会員への連絡と変更試験実施計画書の送付

- JGOG 事務局は、試験実施計画書が改正された旨とその内容をホームページへ掲載すると共に JGOG 会員へ一斉メールを送信する。
- JGOG 事務局はカバーページに試験実施計画書改正日(臨床試験審査委員会の承認日)を記載した変更試験実施計画書を当該試験参加施設へ送付する。

④ 施設 IRB の承認

各施設 IRB の審査・承認を要する

2) 改訂(revision)

① 定義

試験に参加する患者の危険(risk)を増大させる可能性がなく、かつ試験の primary endpoint にも関連しない試験実施計画書の部分的変更。

② 変更手順

- 試験実施計画書改訂が必要と判断した当該試験の研究代表者・研究事務局は、子宮体がん委員会および JGOG データセンターと協議し、試験実施計画書改訂を発案するか否かを決定する。発案する場合、研究代表者・研究事務局は変更試験実施計画書を作成する。
- 研究代表者・研究事務局は JGOG 事務局を通して、試験実施計画書改訂を臨床試験審査委員長に報告する。
- 子宮体がん委員会委員長は試験実施計画書改訂を運営委員会へ報告する。

③ 会員への連絡と変更試験実施計画書の送付

- JGOG 事務局は、試験実施計画書が改訂された旨とその内容をホームページへ掲載すると共に JGOG 会員へ一斉メールを送信する。
- JGOG 事務局はカバーページに試験実施計画書改訂日(臨床試験審査委員会への報告日)を記載した変更試験実施計画書を当該試験参加施設へ送付する。

④ 施設 IRB の承認
施設 IRB の審査・承認は各施設の取り決めに従う。

3) メモランダム/覚え書き(memorandum)
① 定義
試験実施計画書内容の変更ではなく、文面の解釈上のばらつきを減らしたり、特に注意を喚起する等の目的で試験の関係者に配布される試験実施計画書の補足説明。
② 作成手順
・ メモランダム/覚え書きが必要と判断した当該試験の研究代表者・研究事務局は、子宮体がん委員会および JGOG データセンターと協議し、メモランダム/覚え書きを作成するか否かを決定する。作成する場合、研究代表者・研究事務局がこれを作成する。
・ 研究代表者・研究事務局は JGOG 事務局にメモランダム/覚え書きの作成を報告する。
③ 会員への連絡とメモランダム/覚え書きの送付
・ JGOG 事務局は、メモランダム/覚え書きが作成された旨とその内容をホームページへ掲載すると共に JGOG 会員へ一斉メールを送信する。
・ JGOG 事務局は作成されたメモランダム/覚え書きを当該試験参加施設へ送付する。
④ 施設 IRB の承認
施設 IRB の審査・承認は各施設の取り決めに従う。

12.8 試験実施計画書改正/改訂時の施設 IRB 承認

試験中に臨床試験審査委員会の承認を得て本研究実施計画書もしくは患者への説明文書の改正がなされた場合は、改正された研究実施計画書および説明文書が各施設の倫理審査委員会(もしくは IRB)で承認されなければならない。内容変更が改正ではなく改訂の場合に、各施設の倫理審査委員会(もしくは IRB)の審査承認を要するか否かは各施設の取り決めに従う。

改正に対する IRB 承認が得られた場合、各施設の施設研究責任医師、担当医師あるいは施設コーディネーターは IRB 承認文書のコピーを JGOG 事務局へ FAX 送付する(FAX : 03-5206-1983)。IRB 承認文書原本は担当医師あるいは施設コーディネーターが保管、コピーは JGOG 事務局および JGOG データセンターが保管する。

12.9 試験実施計画書作成記録

2006 年 3 月 31 日　実施計画書　提案
2006 年 8 月 5 日　実施計画書　初版作成　Version 1.0
2006 年 8 月 31 日　JGOG 臨床試験審査委員会　承認　Version 1.1

13. モニタリング、監査および報告方法

13.1 定期モニタリング

試験が安全に、かつ試験実施計画書に従って実施されているかを確認する目的で定期モニタリングが行われる。定期モニタリングは原則として年 2 回行われる。収集された「症例報告書」(別紙 9)等の記入データに基づき JGOG データセンターで作成されるモニタリングレポートは、必要な手続き、評価を経て確定され当該試験の参加施設へ伝達されるが、その手順を以下のように定める。

13.1.1 モニタリングの手順

1) JGOG データセンターは、当該試験の実施計画書に定められた頻度で収集された「症例報告書」(別紙 9)等の記入データに基づき定期モニタリングレポートを作成し、モニタリング委員会へ報告する。
2) モニタリング委員会のメンバーは、子宮体がん委員会のメンバー、研究代表者・研究事務局で構成される。モニタリング委員会はモニタリング委員長を子宮体がん委員会委員長を除いた子宮体がん委員会のメンバーから選出する。
3) 当該試験のモニタリング委員会委員長は、子宮体がん委員会の際に、定期モニタリングレポートにつき検討を加え、定期モニタリングレポートの内容を確定する。
   当該試験のモニタリング委員会委員長は、効果・安全性評価委員会に JGOG 事務局を通して定期モニタリングレポートを提出する。効果・安全性評価委員会はモニタリングレポートに対して意見・質問がある場合には、2 週間以内に、JGOG 事務局を通じて報告する。
4) JGOG 事務局は、効果・安全性評価委員会の承認後、定期モニタリングレポートを JGOG ホームページへ掲載する。

13.1.2 項目

1) 症例集積達成状況：登録症例数－累積/期間別、全施設/施設別
2) 適格性：不適格例/不適格の可能性のある症例：群/施設
3) 試験実施計画書治療中/治療終了の別、中止/終了理由：群/施設
4) 治療前背景因子：群
5) 重篤な有害事象：群/施設
6) 有害反応/有害事象：群
7) 試験実施計画書逸脱(逸脱の可能性のある症例を含む)：群/施設
8) 外科的切除術の内容：群/施設
9) 生存期間：全登録例
10) その他、試験の進捗や安全性に関する問題点

13.2 試験実施計画書逸脱・違反

薬剤投与、臨床検査や毒性・有効性の評価等が試験実施計画書の規定に従って行われなかったものを試験実施計画書逸脱とする。

モニタリングに際しては、原則として予め JGOG データセンターと研究代表者/研究事務局間で取り決めた一定の許容範囲を超える逸脱が「逸脱の可能性のある患者」としてモニタリングレポートに列記され、研究事務局および研究グループの検討を経て、以下のいずれかに分類される。

1) 違反(violation)
   原則として以下の複数項目に該当する試験実施計画書規定からの逸脱を「違反」とする。
   ① 試験のエンドポイントの評価に影響を及ぼす
   ② 施設研究責任医師／施設分担医師／施設に原因がある
   ③ 故意もしくは系統的
   ④ 危険もしくは逸脱の程度が著しい
   「違反」は論文公表する際に患者ごとの内容を記載する。
   <違反の例>
   ・ 試験実施計画書治療中に他の抗がん剤や併用禁止治療を併用(薬剤、放射線照射等)

・ 複数の患者で継続的に治療レジメン中にある薬剤を使用しなかった
・ 大幅な過量投与

2) 逸脱(deviation)
1)の違反にも、3)の許容範囲にも該当しない逸脱
特定の逸脱が多く見られた場合は論文公表の際に記載する。

3) 許容範囲(acceptable deviation)
研究グループ、もしくは研究代表者/研究事務局とJGOGデータセンター間で、事前にもしくは事後的に設けた許容範囲内の逸脱はモニタリングレポートに掲載しない。

13.3 施設訪問監査

監査委員会が指名する監査担当者が本試験参加施設を訪問し、施設IRB承認文書の確認、患者同意文書の確認、症例報告書記入データとカルテとの照合(原資料の直接閲覧)等を監査委員会の定める監査マニュアルに従って行う。なお、各施設の監査結果は、当該施設の施設研究責任医師、監査委員会にのみ報告され、それ以外に公表する場合には施設名は伏せられるものとする。

14. 研究結果の発表

研究代表者は、研究の進捗状況および研究結果等について厚生労働省研究事業研究発表会ならびに報告書にて報告することができる。

研究結果は、主たる公表論文、学会発表は最終解析終了後に効果･安全性評価委員会の承認を経て、専門誌または英文誌に投稿する。

14.1 論文発表に関するauthorship等に関する取り決め

原則として論文発表に関するauthorshipは以下の通りとする。

試験結果の公表論文の 1st author は症例登録数が最も多かった施設の実務担当者もしくは代表者(該当施設内の筆頭者の選択は、その施設の判断による。ただし研究に最も直接実務に貢献した者を原則とする)とする。ただしその authorship を取得するかどうかは当該施設の選択による。2nd author は研究代表者(当該試験実施計画書の発案者：study chair)、3rd author はJGOGデータセンターの統計担当(公表のための解析を行った時点での担当者1名)とする。それ以外は、論文の投稿規定による制限に従って、登録数の多い順に選び共著者とする。症例登録数が最も多かった施設の代表者が1st authorを辞退した場合は、1st authorは研究代表者、2nd authorは症例登録数が最も多かった施設の代表者となる。

プロトコール委員会のメンバーにおいては、その試験実施計画書の完成への過程、研究結果の解析など、研究の作成、運営に対する貢献度を考慮して author とする。すべての共著者は投稿前に論文内容を review し、発表内容に合意した者のみとする。JGOG データセンターの担当データマネージャーに対する謝辞を acknowledgement で述べる。

学会発表は複数回に及ぶ可能性があり、1.症例登録数が最も多かった施設の代表者、2.研究代表者、3.以下、登録数が多い順の施設代表者の優先順位で発表する権利を与える。

15. 研究組織

本臨床試験は厚生労働科学研究費補助金がん臨床研究事業「子宮体がんに対する標準的化学療法の確立に関する研究」の一環として行われる厚生労働省研究班(主任研究者：青木大輔　分担研究者：勝俣範之、木口一成、寒河江悟、櫻木範明、進　伸幸、竹内正弘、星合　昊、深澤一雄、八重樫伸生)から提案された試験実施計画書を基に、婦人科悪性腫瘍化学療法研究機構(Japanese Gynecologic Oncology Group(JGOG)理事長：野田起一郎)の臨床研究として行うものとする。

15.1 研究組織

婦人科悪性腫瘍化学療法研究機構(JGOG)
子宮体がん委員会　　委員長　　八重樫伸生
東北大学医学部　産婦人科
〒980-8574　宮城県仙台市青葉区星陵町 1-1
TEL：022-717-7251　　FAX：022-717-7258

15.2 研究代表者

慶應義塾大学医学部　産婦人科　　青木　大輔
〒160-8582　東京都新宿区信濃町 35
TEL：03-3353-1211　　FAX：03-3226-1667

15.3 研究事務局

慶應義塾大学医学部　産婦人科　　進　　伸幸、野村　弘行
〒160-8582　東京都新宿区信濃町 35
TEL：03-3353-1211(内線：63960)　　FAX：03-3226-1667
e-mail：jgog2043@jgog.gr.jp

15.4 JGOG 事務局

特定非営利活動法人　婦人科悪性腫瘍化学療法研究機構　　高橋　幸男
〒162-0825　東京都新宿区神楽坂 6-22 小松ビル 4 階
TEL：03-5206-1982　　FAX：03-5206-1983
e-mail：info@jgog.gr.jp

15.5 JGOG 登録センター／JGOG データセンター

社団法人北里研究所　臨床薬理研究所　臨床試験コーディネーティング部門
〒108-8642　東京都港区白金 5-9-1
TEL：03-5791-6398　　FAX：03-5791-6399
e-mail：jgog-dc@kitasato-ctcc.jp

JGOG 登録センター／JGOG データセンター代表者
社団法人北里研究所　臨床薬理研究所　所長　　竹内　正弘

15.6 統計解析

高橋　史朗
北里大学大学院薬学研究科　臨床統計部門
〒108-8642　東京都港区白金 5-9-1
TEL : 03-5791-6322　　FAX : 03-3444-2546
e-mail : takahashifu@pharm.kitasato-u.ac.jp

15.7 モニタリング委員会【→別添 1 参照】

15.8 監査委員会(JGOG)【→別添 1 参照】

15.9 臨床試験審査委員会(JGOG)【→別添 1 参照】

15.10 効果･安全性評価委員会(JGOG)【→別添 1 参照】

15.11 プロトコール委員会【→別添 1 参照】

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Relationship of the aberrant DNA hypermethylation of cancer-related genes with carcinogenesis of endometrial cancer

KOUJI BANNO*, MEGUMI YANOKURA*, NOBUYUKI SUSUMU, MAKIKO KAWAGUCHI, NOBUMARU HIRAO, AKIRA HIRASAWA, KATSUMI TSUKAZAKI and DAISUKE AOKI

Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo, Japan

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Abstract. Epigenetic abnormalities including the aberrant DNA hypermethylation of the promoter CpG islands play a key role in the mechanism of gene inactivation in cell carcinogenesis. To identify the genes associated with aberrant DNA hypermethylation in endometrial carcinogenesis, we studied the hypermethylation of the promoter regions of five genes: *hMLH1*, *APC*, *E-cadherin*, *RAR-ß* and *p16*. The frequencies of aberrant hypermethylation were 40.4% (21/52) in *hMLH1*, 22% (11/50) in *APC*, 14% (7/50) in *E-cadherin*, and 2.3% (1/44) in *RAR-ß* in endometrial cancer specimens. No aberrant DNA methylation was found in *p16*. In atypical endometrial hyperplasia, the frequencies of aberrant methylation were 14.3% (2/14) in *hMLH1* and 7.3% (1/14) in *APC*, whereas normal endometrial cells showed no aberrant hypermethylation of any of the five genes. The high frequencies of the aberrant DNA hypermethylation of *hMLH1*, *APC* and *E-cadherin* suggest that the methylation of the DNA mismatch repair and Wnt signal-related genes may be associated with endometrial carcinogenesis.

Introduction

The relationship of cellular oncogenic transformation with aberrant DNA hypermethylation in promoter regions (i.e., epigenetic changes) is an area of growing interest. Genes ranging from tumor suppressors to DNA mismatch repair and cell cycle-related genes are known to be inactivated by aberrant DNA methylation in cancer. The DNA mismatch repair genes human MutL homolog-1 (*hMLH1*) and human MutS homolog (*hMSH2*) function in the repair of base-pair mismatches that occur in gene amplification during cell division. The characteristic seen in cancer cells when the DNA mismatch repair system breaks down is referred to as microsatellite instability (MSI). Microsatellites are repeated DNA sequences of ~1 to 5 bases, and DNA replication errors occur frequently at these sites upon the inactivation of the DNA mismatch repair genes. MSI is detected in ~40% of patients with endometrial cancer (1,2), suggesting that mutations of the DNA mismatch repair genes are associated with endometrial carcinogenesis. Therefore, in this study we examined the aberrant DNA methylation of *hMLH1*, a leading candidate in the DNA mismatch repair gene group regarding the production of MSI.

The *ß-catenin* gene codes for a cell adhesion molecule that plays a key role in the Wnt signaling pathway and is generally localized in the cell membrane, where it binds to *E-cadherin*, an adhesion molecule. Free *ß-catenin* forms a complex with *adenomatous polyposis coli* (*APC*) and axin is phosphorylated by GSK-3ß and degraded via the proteosome pathway. The mutation of the *ß-catenin* gene increases the level of undegraded *ß-catenin* in the cells and causes the transition of *ß-catenin* into the nucleus, which induces the activation of the Wnt signaling pathway and enhances the transcriptional activity of target genes including *cyclin D*, leading to cell cycle aberrations. The activation of the Wnt signaling pathway is also thought to be important in endometrial carcinogenesis (3), and therefore *E-cadherin* and *APC*, which are components of the Wnt signaling pathway, are also candidate genes for aberrant DNA methylation in endometrial cancer.

p16 is a tumor suppressor gene that codes for a protein that binds to CDK4 and CDK6 and inhibits the phosphorylation of the RB/E2F complex by the CDK-Cyclin D. *p16*-knockout mice develop multiple cancers in different organs, and therefore *p16* inactivation is thought to play an important role in cell carcinogenesis. The frequencies of *p16* mutation and deletion in endometrial cancer are only 5-6% and 3%, respectively (4,5), but reduced protein levels have been found in 19% of cases (5), and this may be associated with aberrant DNA methylation.

Type I endometrial cancer is also estrogen-dependent; estrogen increases the risk of endometrial cancer through a mechanism that has yet to be fully explained. Estrogen acts in a receptor-specific manner as a molecular switch to regulate transcription factor function. Estrogen receptors have highly differentiated structures, and aberrant methylation of the *estrogen receptor* (*ER*) gene in endometrial cancer has

Correspondence to: Dr Kouji Banno, Department of Obstetrics and Gynecology, Keio University School of Medicine, Shinanomachi 35, Shinjuku-ku, Tokyo 160-8582, Japan
E-mail: kbanno@sc.itc.keio.ac.jp

*Contributed equally

Key words: endometrial cancer, DNA hypermethylation, human MutL homolog-1, *E-cadherin*, *adenomatous polyposis coli*, *retinoic acid receptor-ß*

Table I. Primer sequences used in MSP analysis and RT-PCR.

Gene name	PCR analysis	Sense	Antisense	Size (bp)	Ann[a] temp (°C)
hMLH1	Methylated	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	112	60
	Unmethylated	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124	60
APC	Methylated	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	100	68
	Unmethylated	GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	110	67
RAR-ß	Methylated	GGTTAGTAGTTCGGGTAGGGTTTATC	CCGAATCCTACCCCGACG	235	59
	Unmethylated	TTAGTAGTTTGGGTAGGGTTTATT	CCAAATCCTACCCCAACA	233	59
p16	Methylated	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	67
	Unmethylated	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	66

[a]Annealing temperature.

been reported (6). The *ER* protein shares a common fold with glucocorticoid and retinoic acid receptors (these receptors all belong to the nuclear receptor superfamily), but the frequency of the aberrant methylation of the *retinoic acid receptor-ß* (*RAR-ß*) gene in endometrial cancer has not been determined. However, studies of cancers in other organs (7,8) suggest a relationship between those cancers and the aberrant DNA methylation of *RAR-ß*.

To identify genes associated with aberrant DNA methylation in endometrial carcinogenesis, we studied the aberrant DNA methylation of the promoter regions of five genes (*hMLH1*, *APC*, *E-cadherin*, *RAR-ß* and *p16*) that show high frequencies of aberrant DNA methylation in different cancers and may be important in endometrial carcinogenesis.

Materials and methods

Clinical specimens. The subjects were 93 patients who gave informed consent to the collection of endometrial specimens (27 normal endometria, 14 atypical endometrial hyperplasia, and 52 endometrial cancers). The cells obtained from the tissue specimens were examined by liquid-based cytology using the ThinPrep system (Cytyc Corporation, Boxborough, MA, USA) with preservation fluid (PreservCyt Solution, Cytyc Corporation) (9). A pathological diagnosis of the endometrial tissue was consistent with the cytology results for all the 93 subjects. Of the 27 patients with a normal endometrium, 16 were in the secretory phase and 11 were in the proliferative phase. Of the 52 patients with endometrial cancer, 44 had ovarian endometrioid adenocarcinoma (G1, 24; G2, 10; G3, 10) and 8 had adenosquamous carcinoma. The grade of histological differentiation (G1 to G3) and the cancer stage at surgery were determined based on the Guidelines for Endometrial Cancer published by the Japan Society of Obstetrics and Gynecology.

DNA extraction and methylation-specific PCR (MSP) analysis. DNA was extracted from 93 endometrial specimens using liquid-based cytology with a Get Pure DNA kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Distilled water was added to 1 µg of the extracted DNA up to a volume of 50 µl, 5.5 µl 3 N NaOH solution was added, and, after mixing, the solution was incubated at 37°C for 15 min. Following this, 520 µl 3 M sodium bisulfite (Sigma, St. Louis, MO, USA), which was prepared at pH 5.5 with 30 µl 10 mM hydroquinone (Sigma) and 10 N NaOH, was added to the solution. After mixing in an upturned position to prevent vaporization, the solution was overlaid with mineral oil and incubated at 50°C overnight. Next, 1 ml clean-up resin (Promega Corporation, Madison, WI, USA) was added to the lower layer, and the resulting solution was mixed in an upturned position and then injected into a column. After rinsing with 2 ml 80% isopropanol, the column was centrifuged at 15,000 rpm for 3 min to remove the isopropanol completely, after which 50 µl distilled water (70°C) was added directly to the column, and the column was centrifuged at 15,000 rpm for 2 min to extract the DNA adsorbed in the column. Then, 5.5 µl 2 N NaOH was added to the resulting DNA solution, and, after mixing, the solution was incubated at 37°C for 20 min, after which 66 µl 5 N ammonium acetate solution and 243 µl 95% ethanol were added, and the solution was incubated at 80°C for 1 h and centrifuged at 15,000 rpm for 30 min to precipitate the DNA. Approximately 50 µl of the supernatant was left in the tube, and the rest of the supernatant was collected, mixed with 1 ml 70% ethanol, and then centrifuged at 15,000 rpm for 30 min to rinse the DNA. The precipitated DNA was air-dried and dissolved in 20 µl distilled water; 2 µl of this solution was used as the MSP template solution. AmpliTaq Gold & 10X PCR buffer/$MgCl_2$ with dNTP (Applied Biosystems, Foster City, CA, USA) was used in the PCR analysis, and the DNA was analyzed using a GeneAmp PCR 9700 system (Applied Biosystems). A CpG WIZ *E-cadherin* amplification kit (Chemicon, Temecula, CA, USA) was used as the MSP for the *E-cadherin* gene. The PCR conditions and primer sequences for the other genes are shown in Table I.

Immunohistochemical analysis of endometrial cancer tissues. Twenty surgical endometrial specimens from 52 patients were examined using liquid-based cytology. Formalin-fixed,

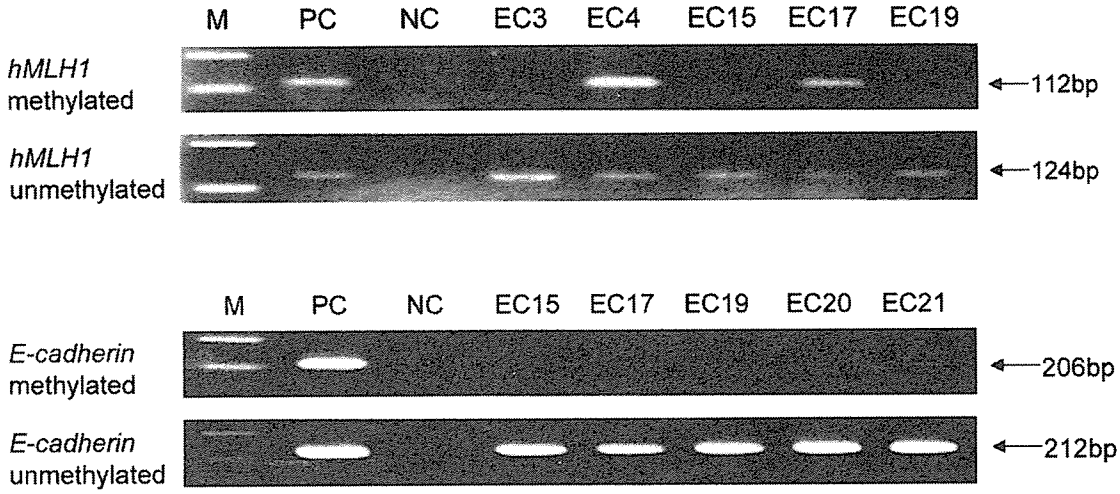

Figure 1. MSP analysis of the *hMLH1* and *E-cadherin* genes in endometrial cancer specimens. MSP analysis was conducted using DNA extracted from endometrial cancer specimens. The results for *hMLH1* and *E-cadherin* are shown in the upper and lower panels, respectively. For *hMLH1* the aberrant methylation band is shown in lanes EC4 and EC17, and for *E-cadherin* this band is shown in lanes EC20 and EC21. M, marker; PC, positive control; NC, negative control; EC, endometrial cancer.

paraffin-embedded specimens were prepared and the slices were stained in a silane-coated slide using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). After deparaffinizing, the slides were heated in 10 mM citric acid buffer solution (pH 7.0) at 120°C for 10 min in an autoclave for antigen retrieval. After allowing the slides to cool to room temperature, intrinsic peroxidase activity was eliminated by treating the slides with 3% H_2O_2 in phosphate-buffered saline (PBS) for 5 min. The slides were rinsed twice with PBS and blocked with normal goat serum, and then rinsed twice again with PBS and diluted with 1% bovine serum albumin (BSA) in PBS and incubated with the primary antibody at 4°C overnight. The primary antibodies were the 50-fold diluted anti-*hMLH1* antibody (BD Bioscience Pharmingen, San Diego, CA, USA) and the 500-fold diluted anti-*E-cadherin* antibody (Takara, Tokyo, Japan). After rinsing three times with PBS, the slides were incubated with the secondary antibody (biotin-labeled anti-mouse IgG) at room temperature for 30 min. After rinsing three more times with PBS, the slides were incubated with ABC (avidin-biotin peroxidase) complex at room temperature for 30 min. After further rinsing three times with PBS, the slides were treated with 0.2 mg/ml diaminobenzidine (DAB) for about 5 min for coloring. After rinsing twice with PBS, the slides were treated with hematoxylin solution for nuclear staining, and then dehydrated and observed microscopically. For judging the immunohistochemical staining intensity of the *hMLH1* protein, the nuclei of endometrial stromal cells were used as an internal control; if the nuclei of the tumor cells containing the protein showed a stronger staining intensity than the control nuclei, the specimen was considered positive, whereas a specimen was considered negative if the tumor cell nuclei showed a lower staining intensity than the control nuclei (10). Regarding the *E-cadherin* staining, the protein is localized in the cell membrane in normal epithelial cells, and the immunohistochemical analysis was conducted in accordance with the criteria of Wu *et al*: Specimens with ≥25% of the tumor cells that stained for *E-cadherin* in the cell membrane were considered positive, and specimens with <25% of the tumor cells giving this staining result were considered negative (11).

Statistical analysis. The correlation of the aberrant DNA methylation of the *hMLH1*, *APC* and *E-cadherin* genes with the clinicopathological factors, grade of histological differentiation and cancer stage at surgery was analyzed using Mann-Whitney tests. The correlation of the aberrant DNA methylation of each of these genes with the patients' age was also examined, after confirming that the groups of patients with and without aberrant methylation showed a normal age distribution based on a normal distribution test. An F test was used to confirm that the population variances of the two independent groups were equal to each other, and then the differences in the population means were examined using the Student's t-test. The correlation of the aberrant DNA methylation level of *hMLH1* with those of *APC*, *E-cadherin* and *RAR-β*, respectively, was calculated using Fisher's exact test, and the correlations of the aberrant DNA methylation of *hMLH1* and *E-cadherin* with the immunohistochemical staining data were also examined using Fisher's exact test.

Results

Aberrant DNA methylation of cancer-related genes in endometrial specimens. Fig. 1 shows partial results of the MSP analysis of the endometrial cancer cells obtained using liquid-based cytology. A band due to the aberrant methylation of the *hMLH1* gene was present in samples EC4 and EC17 (size, 112 bp), and bands due to the aberrant methylation of *E-cadherin* were found in samples EC20 and EC21 (206 bp). MSP analysis of the endometrial cancer specimens indicated that the frequencies of the aberrant methylation of the promoter regions were 40.4% (21/52) for *hMLH1*, 22% (11/50) for *APC*, 14% (7/50) for *E-cadherin*, and 2.3% (1/44) for *RAR-β*. No aberrant methylation was found in the promoter region of the

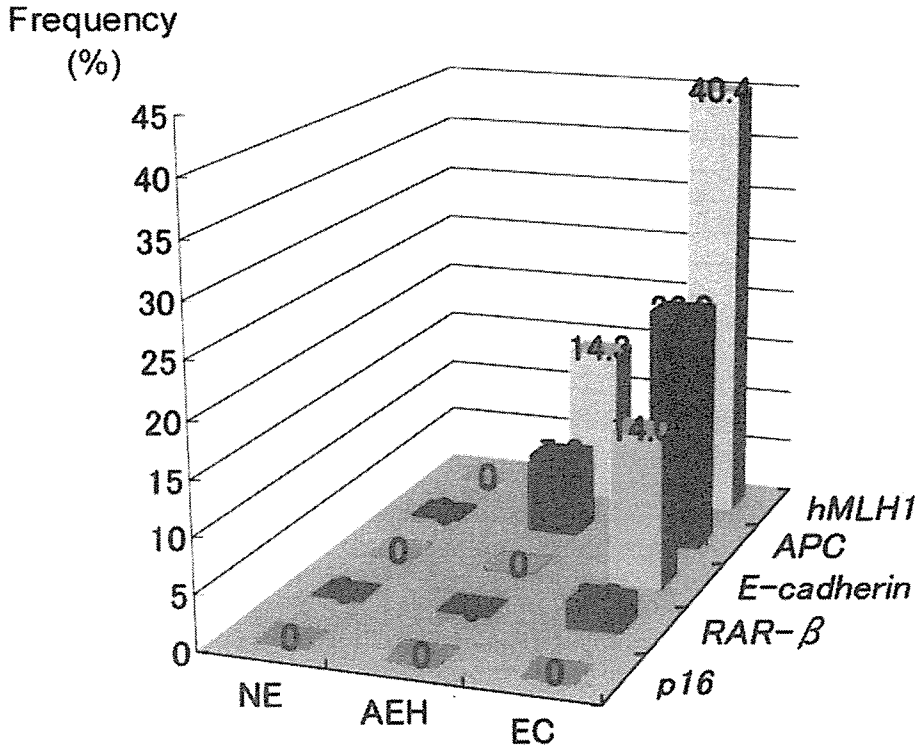

Figure 2. Frequencies of aberrant methylation of cancer-related genes in specimens from normal endometria, atypical endometrial hyperplasia and endometrial cancer. In the endometrial cancer specimens, *hMLH1* exhibited the highest frequency of aberrant methylation, followed by *APC* and *E-cadherin*. Aberrant methylation of *hMLH1* was also found in atypical endometrial hyperplasia, whereas normal endometrial cells showed no aberrant methylation of the five genes. NE, normal endometrium; AEH, atypical endometrial hyperplasia; EC, endometrial cancer.

Case	Age	Tissue type	Cancer stage	Differentiation grade	*hMLH1*	*E-cad*	*APC*	*RAR-β*	*p16*
EC1	52	endometrioid adenocarcinoma	Ⅰb	G3	■				
EC2	50	endometrioid adenocarcinoma	Ⅰa	G1					
EC3	51	endometrioid adenocarcinoma	Ⅲc	G1					
EC4	54	adenosquamous carcinoma	Ⅲc	G3	■				
EC5	51	endometrioid adenocarcinoma	Ⅰa	G1		■			
EC6	61	endometrioid adenocarcinoma	Ⅰb	G1	■				
EC7	70	endometrioid adenocarcinoma	Ⅲc	G2					
EC8	61	endometrioid adenocarcinoma	Ⅱb	G1		■			
EC9	62	adenosquamous carcinoma	Ⅲa	G2					
EC10	40	endometrioid adenocarcinoma	Ⅱa	G1					
EC11	59	endometrioid adenocarcinoma	Ⅱa	G3					
EC12	57	endometrioid adenocarcinoma	Ⅰb	G3		■	■		
EC13	80	endometrioid adenocarcinoma	Ⅲc	G3		■			
EC14	54	adenosquamous carcinoma	Ⅰb	G1					
EC15	53	endometrioid adenocarcinoma	Ⅰb	G3			■		
EC16	42	endometrioid adenocarcinoma	Ⅱb	G1	■		■		
EC17	71	endometrioid adenocarcinoma	Ⅲc	G3	■		■		
EC18	60	endometrioid adenocarcinoma	Ⅰb	G1		■			
EC19	57	endometrioid adenocarcinoma	Ⅲa	G2					
EC20	71	endometrioid adenocarcinoma	Ⅱa	G1		■			
EC21	37	endometrioid adenocarcinoma	Ⅱa	G2	■	■			
EC22	47	endometrioid adenocarcinoma	Ⅲb	G1	■				
EC23	67	endometrioid adenocarcinoma	Ⅰc	G2					
EC24	53	endometrioid adenocarcinoma	Ⅰa	G1	■				
EC25	69	endometrioid adenocarcinoma	Ⅲc	G2	■				
EC26	55	endometrioid adenocarcinoma	Ⅲc	G2	■		■		
EC27	54	endometrioid adenocarcinoma	Ⅰa	G1					
EC28	63	endometrioid adenocarcinoma	Ⅰa	G1	■		■		
EC29	41	endometrioid adenocarcinoma	Ⅰb	G1	■				

Case	Age	Tissue type	Cancer stage	Differentiation grade	*hMLH1*	*E-cad*	*APC*	*RAR-β*	*p16*
EC30	62	adenosquamous carcinoma	Ⅰb	G1	■		■		
EC31	58	endometrioid adenocarcinoma	Ⅰb	G2	■		■		
EC32	66	endometrioid adenocarcinoma	Ⅲc	G3					
EC33	71	endometrioid adenocarcinoma	Ⅰb	G2					
EC34	53	adenosquamous carcinoma	Ⅰb	G3					
EC35	50	endometrioid adenocarcinoma	Ⅲa	G3					
EC36	42	adenosquamous carcinoma	Ⅲc	G3					
EC37	55	endometrioid adenocarcinoma	Ⅰc	G3				■	
EC38	34	adenosquamous carcinoma	Ⅲc	G1					
EC39	61	endometrioid adenocarcinoma	Ⅰc	G1					
EC40	61	endometrioid adenocarcinoma	Ⅰc	G1			■		
EC41	61	endometrioid adenocarcinoma	Ⅰb	G1	■				
EC42	59	endometrioid adenocarcinoma	Ⅰb	G1	■				
EC43	55	adenosquamous carcinoma	Ⅳb	G2	■				
EC44	54	endometrioid adenocarcinoma	Ⅱa	G1	■				
EC45	57	endometrioid adenocarcinoma	Ⅱa	G1	■		■		
EC46	56	endometrioid adenocarcinoma	Ⅰb	G2					
EC47	78	endometrioid adenocarcinoma	Ⅰb	G3			■		
EC48	65	endometrioid adenocarcinoma	Ⅰb	G2	■				
EC49	37	endometrioid adenocarcinoma	Ⅰa	G1	■				
EC50	36	endometrioid adenocarcinoma	Ⅰa	G1					
EC51	26	endometrioid adenocarcinoma	Ⅰa	G1	■				
EC52	18	endometrioid adenocarcinoma	Ⅰa	G1					

■ Methylated □ Unmethylated ▨ Not done

Figure 3. Aberrant methylation of the promoter regions of cancer-related genes in endometrial cancer. G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; EC, endometrial cancer; *E-cad*, *E-cadherin*.

p16 gene. In the atypical endometrial hyperplasia samples, the frequencies of the aberrant methylation of the promoter regions were 14.3% (2/14) for *hMLH1* and 7.3% (1/14) for *APC*. Normal endometrial cells in the proliferative and secretory phases showed no aberrant methylation of the promoter regions of the five examined genes (Figs. 2-4).

Case	Age	Tissue type	*hMLH1*	*E-cad*	*APC*	*RAR-β*	*p16*
NE1	37	sec					
NE2	43	sec					
NE3	51	sec					
NE4	35	sec					
NE5	39	sec					
NE6	41	sec					
NE7	47	sec					
NE8	40	sec					
NE9	36	sec					
NE10	49	sec					
NE11	51	sec					
NE12	52	sec					
NE13	44	sec					
NE14	47	sec					
NE15	23	sec					
NE16	34	sec					
NE17	37	pro					
NE18	37	pro					
NE19	51	pro					
NE20	49	pro					
NE21	43	pro					
NE22	36	pro					
NE23	43	pro					
NE24	42	pro					
NE25	27	pro					
NE26	44	pro					
NE27	32	pro					

Case	Age	*hMLH1*	*E-cad*	*APC*	*RAR-β*	*p16*
AEH1	34					
AEH2	30					
AEH3	32					
AEH4	35					
AEH5	35					
AEH6	46					
AEH7	41					
AEH8	33					
AEH9	41					
AEH10	50					
AEH11	45					
AEH12	47					
AEH13	45					
AEH14	33					

Methylated Unmethylated

Not done

Figure 4. Aberrant methylation of the promoter regions of cancer-related genes in atypical endometrial hyperplasia. AEH, atypical endometrial hyperplasia; *E-cad*, *E-cadherin*.

Immunohistochemical analysis of hMLH1 and E-cadherin protein expression. The relationship of the aberrant DNA methylation of the promoter regions of the *hMLH1* and *E-cadherin* genes with protein expression was determined immunohistochemically. Of the 20 surgical specimens of endometrial cancer showing aberrant methylation, most showed negative protein staining (*hMLH1*, $p<0.01$; *E-cadherin*, $p<0.05$) (Fig. 5) (Table II).

Correlation of aberrant DNA methylation of cancer-related genes with clinicopathological factors. The correlations of the aberrant DNA methylation of the promoter regions of *hMLH1*, *APC* and *E-cadherin* with the clinicopathological factors were examined in endometrial cancer patients. For the *hMLH1*, APC and *E-cadherin* genes, no correlation was found between aberrant methylation and the grade of histological differentiation or with cancer stage at surgery. Aberrant DNA methylation is generally thought to increase with age, but no significant difference was found in the mean age between patients with and without aberrant methylation of *hMLH1*, *APC* and *E-cadherin*, respectively. Therefore, these data do not indicate that aberrant methylation occurs more frequently in elderly patients with endometrial cancer (Table IV).

The relationship of the aberrant methylation of the promoter region of *hMLH1*, which showed the highest frequency in the endometrial cancer samples, was also examined with that of other genes, but no correlation was found with the methylation of *APC*, *E-cadherin* or *RAR-β*.

Discussion

Of the five endometrial cancer-related genes examined, the aberrant methylation of *hMLH1*, a DNA mismatch repair gene, was found most frequently (40.4%). The frequencies of the aberrant methylation of *hMLH1* have been reported as 14% to 26% in gastric cancer (12,13) and 7% to 32% in lung cancer (14,15); therefore, the frequency of the aberrant methylation of this gene in endometrial cancer is higher than in other cancers. After *hMLH1*, the second most likely genes to show aberrant methylation were *APC* and *E-cadherin*, which are Wnt-related genes. Collectively, these data suggest that abnormal DNA mismatch repair and aberrant Wnt signaling are associated with endometrial carcinogenesis. However, patients with an aberrant methylation of *hMLH1* rarely corresponded to those with an aberrant methylation of *E-cadherin*, and therefore carcinogenesis due to the aberrant methylation of these respective genes may occur through independent mechanisms.

The aberrant methylation of *hMLH1* may cause a reduced protein expression that leads to abnormal DNA mismatch repair and MSI. However, although MSI has been found in ~40% of patients with endometrial cancer, the *hMLH1*

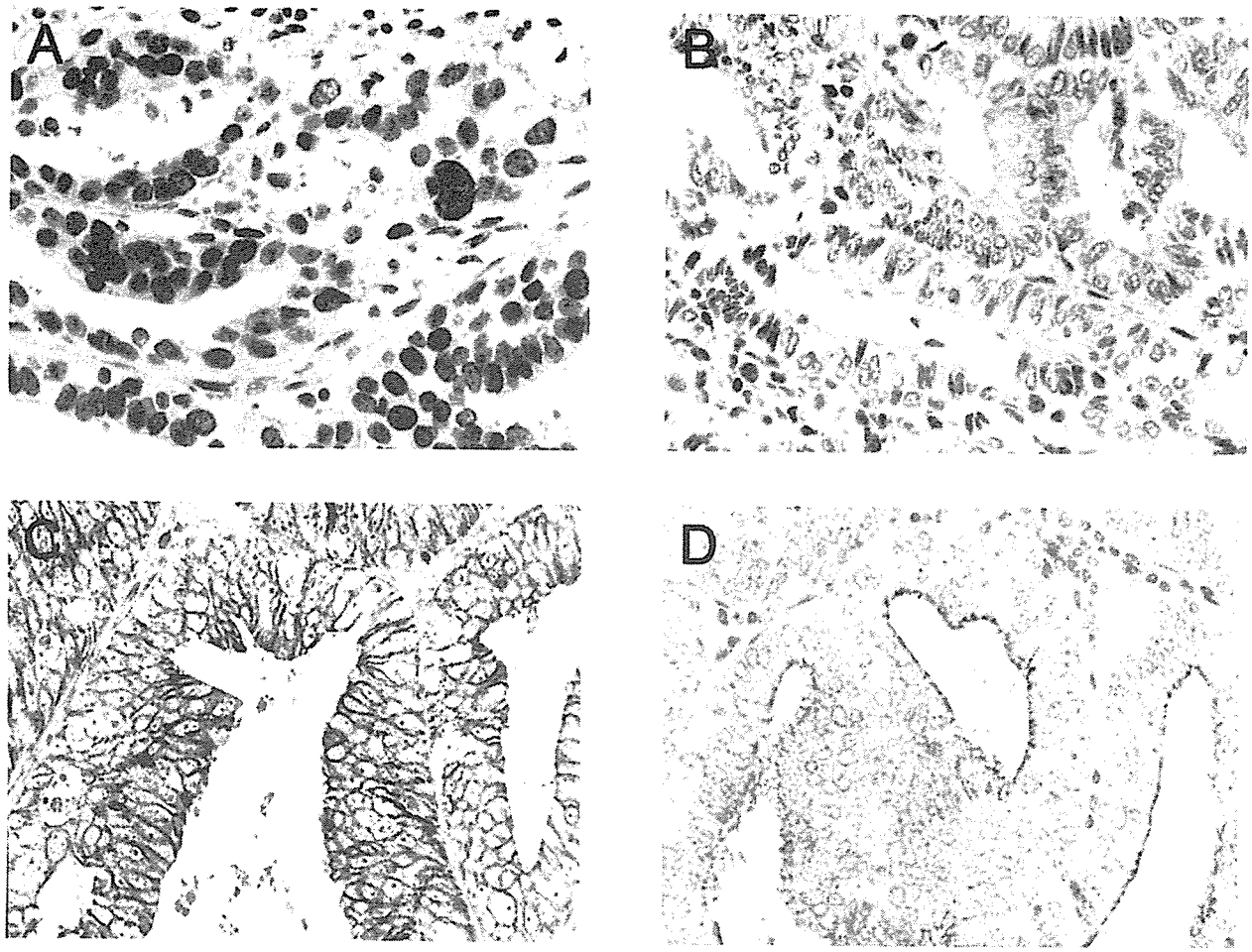

Figure 5. (A and B) Immunohistochemical analysis of the endometrial cancer specimens using the anti-*hMLH1* antibody. (A) In patients with an unmethylated *hMLH1* gene (EC23), the nuclei of the cancer cells were strongly stained. (B) In patients with aberrant methylation of *hMLH1* (EC31), the cell nuclei were less strongly stained. (C and D) Immunohistochemical analysis of the endometrial cancer specimens using the anti-*E-cadherin* antibody. (C) In patients with unmethylated *E-cadherin* (EC29), the cell membranes of the cancer cells were strongly stained. (D) In patients with aberrant methylation of *E-cadherin* (EC8), the cell membranes were less strongly stained. EC, endometrial cancer.

Table II. Relationship of the aberrant DNA methylation of the *hMLH1* and *E-cadherin* genes with reduced protein expression.

	Expressed	Not expressed	
hMLH1			
methylated	3	9	p<0.01
unmethylated	8	0	
E-cadherin			
methylated	2	2	p<0.05
unmethylated	15	1	

mutation frequency in MSI-positive endometrial cancer patients is extremely low (16,17), suggesting that MSI may occur due to the aberrant methylation of the promoter regions, and not due to the *hMLH1* mutation. Furthermore, the aberrant methylation of *hMLH1* has been found in atypical endometrial hyperplasia, but is not observed in the normal endometrium;

Table III. Correlation of the aberrant DNA methylation of cancer-related genes with the grade of histological differentiation and clinical stage at surgery.

	G1	G2	G3	Stage			
				I	II	III	IV
hMLH1							
M	12	6	3	11	4	5	1
U	15	6	10	19	4	8	0
E-cadherin							
M	4	1	2	3	3	1	0
U	22	11	10	24	5	13	1
APC							
M	5	2	4	7	2	2	0
U	21	10	8	20	6	12	1

G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; M, methylated; U, unmethylated.

Table IV. Correlation of the aberrant DNA methylation of cancer-related genes with the mean onset age of endometrial cancer.

	hMLH1		*E-cadherin*		*APC*		*RAR-β*
Methylated	53.3±11.46	NS	59.6±13.78	NS	59.7±9.37	NS	55
Unmethylated	55.7±12.78		54.9± 10.84		54.3±11.57		56.26±9.76

NS, not significant.

therefore, aberrant methylation occurs in the early stage of carcinogenesis. Since such aberrant methylation can be detected in a small amount of cytological material by using minimally invasive procedures, the determination of the methylation levels of genes such as *hMLH1* is a potential supplementary diagnostic method for endometrial cancer.

An accumulation of *β-catenin* in the nucleus, which indicates aberrant Wnt-signaling, has been observed in 23.8% of patients with endometrial cancer (18), and is thought to be one of the causes of endometrial cancer. In contrast, the *β-catenin* mutation frequency is only 11%, significantly lower than the frequency of the accumulation of *β-catenin* in the nucleus (18). Furthermore, the accumulation of *β-catenin* in the nucleus has been observed in patients without *β-catenin* mutations; therefore, the transition and accumulation of *β-catenin* in the nucleus are dependent on a mechanism other than gene mutation. The aberrant methylation of the promoter region of *E-cadherin*, which codes for a scaffolding protein that binds to *β-catenin* and is present in the cell membrane as a cell adhesion molecule, was found in 14% of the patients with endometrial cancer in our patient population. Reduced levels of the *E-cadherin* protein were frequently observed in the patients with aberrant methylation of *E-cadherin*, suggesting that the inactivation of *E-cadherin* by aberrant methylation could be associated with changes in the localization of *β-catenin* in endometrial cancer. The aberrant methylation of *E-cadherin* has also been found in G3 adenocarcinoma (19), but no correlation with the localization of *β-catenin* has been investigated. The aberrant methylation of *E-cadherin* was not detected in the patients with atypical endometrial hyperplasia, which is considered pathologically to be Stage 0 endometrial cancer, but was found in the patients with Stage Ia or higher endometrial cancer. This suggests that the aberrant methylation of *E-cadherin* is not involved in early-stage carcinogenesis, in contrast to *hMLH1*.

Similar to the aberrant methylation of the *hMLH1* gene, the aberrant methylation of *APC*, a component of the Wnt signaling pathway, was observed in 7.3% of the patients with atypical endometrial hyperplasia and 22% of the patients with endometrial cancer; however, no reduction in the levels of the *APC* protein was observed. Therefore, our results indicate that there is no relationship between the inactivation of *APC* by aberrant methylation and the onset of endometrial cancer.

The frequencies of the aberrant methylation of *RAR-β* and *p16* were 2.3% and 0% in the endometrial cancer patients, respectively, which are significantly lower than those in cancers of other organs. These results suggest that the type and frequency of genes undergoing aberrant methylation in endometrial cancer are specific and differ from those in other cancers. The aberrant methylation of the promoter region of *p16* has been reported in 20% of non-Japanese patients with endometrial cancer (20); the difference between this result and our study suggests that the frequencies of aberrant DNA methylation in endometrial cancer may also vary between races. Furthermore, aging is generally thought to be an important factor for aberrant DNA methylation, but we found no tendency for increased aberrant methylation in elderly patients with endometrial cancer. The mechanism of the induction of aberrant DNA methylation may also differ widely between organs and tissues (21), and this may account for the differences in results between the studies.

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Relationship of aberrant DNA hypermethylation of *CHFR* with sensitivity to taxanes in endometrial cancer

MEGUMI YANOKURA*, KOUJI BANNO*, MAKIKO KAWAGUCHI, NOBUMARU HIRAO, AKIRA HIRASAWA, NOBUYUKI SUSUMU, KATSUMI TSUKAZAKI and DAISUKE AOKI

Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo, Japan

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Abstract. The relationship of aberrant DNA hypermethylation of cell cycle checkpoint genes with the sensitivity of cancer cells to anticancer drugs is a question of current interest. In this study, we investigated the relationship between aberrant hypermethylation of the *CHFR* (checkpoint with forkhead-associated and ring finger) mitotic checkpoint gene and sensitivity to taxanes in endometrial cancer. Methylation-specific PCR (MSP) indicated aberrant hypermethylation of *CHFR* in 12.0% (6/50) of endometrial cancer specimens, and suggested that aberrant hypermethylation is significantly more frequent in poorly differentiated adenocarcinoma (G3) ($p<0.05$). Of six culture cell lines, SNG-II and HEC108 cells showed aberrant hypermethylation and reduced expression of *CHFR*. These cells had high sensitivity to taxanes but became resistant after demethylation. Cancer specimens with aberrant hypermethylation of *CHFR* also exhibited high sensitivity to taxanes. To our knowledge, this study is the first to examine aberrant hypermethylation of *CHFR* in endometrial cancer, and our results suggest that the methylation status of *CHFR* may be a new molecular index that will allow design of personalized treatment in endometrial cancer. This may be particularly important in poorly differentiated adenocarcinoma (G3), which is known to have a poor prognosis.

Introduction

Recent studies have shown that aberrant DNA hypermethylation of cell cycle checkpoint genes in cancer cells has a major effect on specific anticancer drugs (1,2). The *CHFR* (checkpoint with forkhead-associated and ring finger) mitotic checkpoint gene is located in 12q24.33 and has the function of delaying chromatin condensation and progression to the mitotic phase (3). The CHFR protein has a forkhead-associated domain in the N-terminal region and a finger domain in the central region; these two domains act as a sensor for mitotic stress and therefore function as a cell cycle M phase checkpoint. Upon detection of mitotic stress in a cell, CHFR action causes arrest of the cell cycle in G2 phase to allow repair of damaged DNA (G2 arrest).

Taxanes are anticancer agents that act in M phase as microtubule depolymerization inhibitors. Upon administration of a taxane to cancer cells, those cells with normal *CHFR* develop G2 arrest and repair damaged DNA, thereby exhibiting resistance to taxanes. In contrast, cells with inactivated *CHFR* due to aberrant hypermethylation proceed with the cell cycle due to failed detection of damaged DNA and subsequently cannot go on to normal cell division, leading to mitotic catastrophe and cell death; i.e., these cells show high sensitivity to taxanes. Given this background, the methylation status of *CHFR* is likely to be a highly sensitive molecular index for taxane sensitivity of cancer cells.

A relationship between aberrant hypermethylation of *CHFR* and sensitivity to taxanes has been reported in colon and gastric cancer cells in culture (2,4), but not in endometrial cancer. Therefore, we investigated this relationship in endometrial cancer, with the goal of establishing a molecular index that might lead to personalized treatment strategies for endometrial cancer.

Materials and methods

Subjects and specimens for biopsy. The subjects were 69 patients who gave informed consent for collection of endometrial specimens (9 of normal endometrium, 10 of atypical endometrial hyperplasia and 50 of endometrial cancer). Cells obtained from the tissue specimens were examined by liquid-based cytology using the ThinPrep System (Cytyc Corp., Boxborough, MA) with preservation fluid (PreservCyt Solution, Cytyc Corp.) (5). A pathological diagnosis of the endometrial tissue was consistent with cytology results for all 69 subjects. Of the 9 patients with a normal endometrium, 5 were in the secretory phase and 4 were in the proliferative phase, and of the 50 patients with endometrial cancer, 42 had ovarian endometrioid adenocarcinoma (G1, 20; G2, 12; G3, 10) and 8 had adenosquamous carcinoma. The grade of histo-

Correspondence to: Dr Kouji Banno, Department of Obstetrics and Gynecology, Keio University School of Medicine, Shinanomachi 35, Shinjuku-ku, Tokyo 160-8582, Japan
E-mail: kbanno@sc.itc.keio.ac.jp

*Contributed equally

Key words: CHFR, endometrial cancer, DNA hypermethylation, taxane, chemosensitivity

Table I. Primer sequences used in MSP and RT-PCR analyses.

Gene name	PCR analysis	Sense	Antisense	Size (bp)	Annealing temperature (°C)
CHFR	Methylated	GTCGGGTCGGGGTTC	CCCAAAACTACGACGACG	150	60
	Unmethylated	ATATAATATGGTGTTGATT	TCAACTAATCCACAAAACA	206	53
CHFR	RT-PCR	TGGAACAGTGATTAACAAGC	AGGTATCTTTGGTCCCATGG	206	55
ß-actin	RT-PCR	TTATTTGAGCTTTGGTTCTG	CTCCTTAATGTCACGCACGATTTC	303	50

logical differentiation (G1-G3) and the cancer stage at surgery were determined based on the Guidelines for Endometrial Cancer published by the Japan Society of Obstetrics and Gynecology.

Culture cell lines. Six cell strains were used: HEC108 (a human endometrial cancer-derived culture cell line supplied by Dr Hiroyuki Kuramoto), HOOUA and HHUA (supplied by Dr Isamu Ishiwata) and SNG-II, HEC1B and KLE. KLE cells were cultured in a DMEM/F12 (1:1) medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Sanko Junyaku Co., Ltd., Tokyo, Japan), and the other cells were cultured in 10% FBS-supplemented F12 medium (Sigma, St. Louis, MO, USA). The cells were incubated in a 10-cm dish under 5% CO_2 at 37°C.

DNA extraction and methylation-specific PCR (MSP) analysis of CHFR. DNA was extracted from 69 endometrial specimens and 6 endometrial cancer-derived cell lines using liquid-based cytology with a GetPure DNA Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Distilled water was added to 1 μg of the extracted DNA up to a volume of 50 μl, 5.5 μl of 3 N NaOH solution was added, and after mixing the solution was incubated at 37°C for 15 min. Following this, 520 μl of 3 M sodium bisulfate (Sigma), which was prepared at pH 5.5 with 30 μl of 10 mM hydroquinone (Sigma) and 10 N NaOH, was added to the solution. After mixing in an upturned position to prevent vaporization, the solution was overlaid with mineral oil and incubated at 50°C overnight. Next, 1 ml of clean-up resin (Promega Corp., Madison, WI, USA) was added to the lower layer, and the resulting solution was mixed in an upturned position and then injected into a column. After rinsing with 2 μl of 80% isopropanol, the column was centrifuged at 15,000 rpm for 3 min to remove isopropanol completely, after which 50 μl of distilled water (70°C) was added directly to the column, and the column was centrifuged at 15,000 rpm for 2 min to extract DNA adsorbed on the column. Then, 5.5 μl of 2 N NaOH was added to the resulting DNA solution, and after mixing the solution was incubated at 37°C for 20 min, after which 66 μl of 5 N ammonium acetate and 243 μl of 95% ethanol were added. The solution was then incubated at -80°C for 1 h and centrifuged at 15,000 rpm for 30 min to precipitate DNA. Approximately, 50 μl of the supernatant was left in the tube, and the rest of the supernatant was collected, mixed with 1 ml of 70% ethanol, and then centrifuged at 15,000 rpm for 30 min to rinse the DNA. The precipitated DNA was air-dried and dissolved in 20 μl of distilled water; 2 μl of this solution was used as the MSP template solution. AmpliTaq Gold and 10X PCR buffer/$MgCl_2$ with dNTP (Applied Biosystems, Foster City, CA, USA) was used in the PCR analysis, and DNA was analyzed using a GeneAmp PCR System 9700 (Applied Biosystems). The PCR conditions for other genes and primer sequences are shown in Table I. DNA extracted from the culture cell lines was also used in MSP analysis of *CHFR*.

Statistical analysis. Correlations of aberrant DNA hypermethylation of *CHFR* with the grade of histological differentiation and the cancer stage at surgery were analyzed using the χ^2 test and Mann-Whitney test, respectively. Correlation of aberrant DNA hypermethylation of *CHFR* with patient age was also examined, after establishing that the groups of patients with and without aberrant hypermethylation had a normal age distribution. The Mann-Whitney test was used to examine whether the population medians of the two independent groups differed significantly.

RNA extraction and expression analysis of CHFR using RT-PCR. Total-RNA was extracted from 6 endometrial cancer-derived cell lines using a RNeasy mini-Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized with 1 μg of total-RNA using a SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). Synthesized 1st strand cDNA (1 μl) was used as a template solution in RT-PCR analysis of *CHFR* expression. AmpliTaq Gold and 10X PCR buffer/$MgCl_2$ with dNTP (Applied Biosystems) was used in the PCR analysis, and DNA was analyzed using a GeneAmp PCR System 9700 (Applied Biosystems). The PCR conditions and primer sequences are shown in Table I.

Demethylation. SNG-II cells, which are endometrial cancer-derived cells with aberrant hypermethylation of *CHFR*, were plated on a 10-cm dish at 10^6 cells/dish and incubated for 72 h. A demethylating agent, 5-aza-dC (Sigma), was then added until its final concentration in the culture medium was 1 μM. Forty-eight hours after the first addition 5-aza-dC was added again, and DNA and RNA were extracted 24 and 72 h after the second addition, respectively.

Cell cycle analysis using flow cytometry. SNG-II and KLE cells, which are both endometrial cancer-derived cell lines, were plated on a 10-cm dish at $5x10^5$ cell/dish and incubated