⑨婦人科癌化学療法クリニカルパス

はじめに

クリニカルパスは，医療の質を確保しつつ効率的に医療を提供するために，達成目標を立ててだれがいつどのような医療やケアを行うかを日程表にまとめたものである。クリニカルパスを導入することにより，平均在院日数の短縮，EBMに基づいた医療の標準化，チーム医療の活性化，安全性の向上，患者満足度の向上が期待される。本稿では，婦人科癌において主に用いられる化学療法のレジメンについて，クリニカルパスの実例をあげながら考察する。

クリニカルパスの実際

1．AP療法

AP療法（ドキソルビシン＋シスプラチン）は子宮体癌Ⅲ・Ⅳ期で用いられる標準療法の一つである[1]。ドキソルビシン60 mg/m²，シスプラチン50 mg/m²を3週ごとに投与するレジメンである。表1にクリニカルパスの例を示す。

クリニカルパス作成において重要となるポイントは，シスプラチンによる腎毒性である[2]。輸液による尿量確保が腎毒性予防において最も重要である。輸液指示，尿量測定指示，体重測定指示（体内水分保持の指標として）を行う。

シスプラチン投与前，投与後に十分な生理食塩水の輸液を行い，十分な尿量（明確な基準はないが，100 mL/hr以上の尿量を確保するように努める）を確保する。もし患者が十分な水分摂取ができ，十分な尿量が得られている場合は，必ずしも点滴静注を行う必要はない。もし基準の尿量が得られない，もしくは体重増加をきたした場合は，利尿薬を投与する。また，シスプラチン投与により低マグネシウム血症が起こり，加えて低マグネシウム血症がシスプラチンによる腎毒性を増悪させるため[3]，輸液にマグネシウム製剤を加えることも重要である。当院では入院で化学療法を行っているが，day 2以降に飲水を取れれば輸液投与は不要であることから，外来投与が可能なレジメンである。

2．TC療法

TC療法（パクリタキセル＋カルボプラチン）は卵巣癌で用いられる標準療法である[4]。パクリタキセル175 mg/m²（3時間で静注），カルボプラチンAUC 6を3週ごとに投与するレジメンである。当院では初回から外来投与で行っているが，今回は入院での投与を想定し，表2にクリニカルパスの例を示す。

クリニカルパス作成において重要となるポイントは，パクリタキセルによる過敏性反応である。パクリタキセルの溶解剤であるpolyoxethylated castor oil vehicle（クレモフォールEL）による反応と考えられている。初期の臨床試験ではパクリタキセルによる過敏性反応が高頻度に起こったため，アレルギー予防のためにパクリタキセル投与の12時間前，6時間前にデキサメタゾン20 mgを経口投与していた。ところが近年，short premedicationといわれる，パクリタキセル投与の30分前にリン酸デキサメタゾン20 mg，ジフェンヒドラミン50 mg，シメチジン300 mgもしくはラニチジン50 mgを静注することにより，過敏性反応の発現を予防することができることが示された[5]。過敏

表 1　AP 療法クリニカルパスの一例

クリニカルパス名　AP療法（子宮体癌）　コースII

患者氏名　様　ID　担当医：

担当看護師：　区域看護

適応基準		退院基準
1）子宮体癌Ⅲ期術後または再発 2）75歳以下 3）PSが0から3	4）好中球1,000/μL 5）血小板10万以上 6）クレアチニン2.0 mg/dL 未満 7）ALT/AST 100未満 8）合併症を有する場合、経口薬投与で症状が安定している	day 4にコップ1杯の水を飲むことができる

		入院	day 1	day 2	day 3	day 4
年月日						
達成目標			嘔気・嘔吐がGrade 1以下である 尿量が1,500 mL以上確保されている	嘔気・嘔吐がGrade 1以下である 尿量が1,500 mL以上確保されている 水分摂れていれば下記点滴不要、退院可	嘔気・嘔吐がGrade 1以下である 水分摂れていれば下記点滴不要、退院可	コップ1杯の水を飲むことができる
指示	薬剤		9：00 ①生食 1,000 mL 3時間 12：00 ②生食50 mL リン酸デキサメタゾン20 mg グラニセトロン1A（1 mg/3 mg）15分 12：15 ③アドリアマイシン mg 生食50 mL 15分 12：30 ④シスプラチン mg 生食250 mL 60分 13：30 ⑤生食500 mL 60分 15：00 ⑥D-マンニトール300 mL 15分 15：30 ⑦生食(00 mL+硫酸マグネシウム液1A(00分 18：30 終了	9：00 ①生食50 mL リン酸デキサメタゾン16 mg 15分 9：15 ②生食 1,500 mL +硫酸マグネシウム液 1A 6時間	9：00 ①生食50 mL リン酸デキサメタゾン16mg 15分 9：15 ②生食1,500 mL +硫酸マグネシウム液1A 6時間	
		悪心・嘔吐時（1から④の順番に） ①メトクロプラミド 5mg 1錠（1日5回まで、経口不能時②へ） ②ドンペリドン坐剤60 mg 1個 ③生食 50 mL+メトクロプラミド 10 mg 1A/15分（1日4回まで） ④アルプラゾラム0.4 mg 1錠（1日3回まで）		便秘時 酸化マグネシウム（カマ、マグミット）、センノシド、ピコスルファートナトリウム内服可 GE、レシカルボン坐剤使用可 （各薬剤の投与量、タイミングは調節可） 発熱時（38.0℃以上） ①アセトアミノフェン（200）2錠内服	不眠時 ゾピクロン 7.5 mg 1錠 （追加1錠まで可） 退院時の発熱時処方（38.0℃以上） ①アセトアミノフェン（200）6錠分3内服 ②塩酸シプロフロキサシン（200）6錠分3内服 7日間継続	
	尿量・体重測定指示	1日1回測定（入院時）	1 尿量測定：9時から19時 19時尿量1,000 mL以下のとき 2 体重測定：10時、19時 19時体重がday 1(10時)の体重より2 kg以上増加したとき 1あるいは2の場合、フロセミド10 mgワンショット静注	尿量測定（1日量のみ）1,500 mL以下のとき フロセミド 10 mg ワンショット静注	尿量測定（1日量のみ）1,500 mL以下のとき フロセミド10 mg ワンショット静注	
	その他指示	有・無	有・無	有・無	有・無	有・無
処置		尿測準備	サーフロー挿入 ヘパロック	ヘパロック （点滴ない場合、サーフロー抜去）	終了後サーフロー抜去	
検査		血算・生化				
活動/安静度		制限なし	制限なし	制限なし	制限なし	
栄養		栄養指導（入院時） NST 依頼票提出		栄養指導（初回のみ）		
排泄			尿測	尿測	尿測	
清潔		入浴可	入浴可	入浴可	入浴可	
指導・説明		入院治療計画書 入院オリエンテーション（看護師） 治療オリエンテーション（医師）	シスプラチン終了時までに飲水1,000 mLを終了しておく		退院時オリエンテーション 退院時処方確認 退院療養計画書	
観察	体重	kg	10時 kg 19時 kg	19時 kg		
	尿量		10時～19時 mL 1日尿量 mL	10時～19時 mL 1日尿量 mL	1日尿量 mL	
	体温（℃）					
	脈拍（回/分）					
	血圧（mmHg）					
	食事摂取量					
	排便					
	悪心					
	嘔吐					
	食欲不振					
	便秘					
	倦怠感					
	血管外漏出					
特記記録						
予測される看護問題						
バリアンス						
担当看護師名						

* grade は NCI CTC AE ver 3.0にて評価すること。
国立がんセンター中央病院　16A 病棟　2007年3月改訂

表 2　TC 療法クリニカルパスの一例

医療者用

クリニカルパス名：TC 療法（一泊詳細）　（　　　）コース目
患者ID：　　　　患者氏名：　　　　担当医：　　　　担当看護師：
在院日数：2日

適応基準　1）根治不可能　2）卵巣癌術後　3）卵巣癌再発後　4）婦人科系悪性腫瘍　5）泌尿器系悪性腫瘍　6）呼吸器系悪性腫瘍　7）好中球数≧1,500/mm³　8）血小板数≧75,000/mm³　9）GOT/GPT 100 IU/ml 未満　10）PS 0－3

退院基準：コップ1杯の水を摂取可能

年月日		（　/　）	（　/　）	（　/　）	（　/　）	（　/　）	（　/　）
病日		入院前	Day 1（入院日）	Day 2（退院日）	Day 3	Day 4	Day 5
達成目標			悪心・嘔吐が grade 1 以下である。コップ1杯の水を摂取可能である				
指示	薬剤		1 生食50 mL リン酸デキサメタゾン20 mg ラニチジン　50 mg/2 mL　15分 2 生食50 mL クロルフェニラミンマレイン酸塩　10 mg　15分 3 5%ブドウ糖　250 mL パクリタキセル＿＿＿mg　180分 4 生食50 mL グラニセトロン　1 mg・3 mg　15分 5 生食250 mL カルボプラチン＿＿＿mg　60分 6 生食50 mL　15分	デキサメタゾン0.5 mg 16錠分2（朝・昼食後） ラニチジン150 mg錠 2錠分2（朝食後・眠前） 38℃発熱時 塩酸シプロフロキサシン（200）6錠分3で7日間内服開始 アセトアミノフェン（200）1回2錠1日3回まで	デキサメタゾン0.5 mg 12錠分2 （朝・昼食後） ラニチジン 150 mg 2錠分2 （朝食後・眠前）	デキサメタゾン0.5 mg 8錠分2 （朝・昼食後） ラニチジン 150 mg 2錠分2 （朝食後・眠前）	デキサメタゾン0.5 mg 4錠分2 （朝・昼食後） ラニチジン 150 mg 2錠分2 （朝食後・眠前）
			不眠時：ゾピクロン7.5 mg 1錠（追加1錠まで可） 発熱（38℃以上）時：1）アセトアミノフェン（200mg）2錠内服 2）アセトアミノフェン内服で解熱なければ Drcall 悪心・嘔吐時：1）メトクロプラミド5 mg 1錠内服（1日5回まで、経口不能時2へ） 2）生食50 mL・メトクロプラミド10 mg 1A/15分（1日4回まで） 3）アルプラゾラム0.4 mg（1日3回まで） 便秘時：酸化マグネシウム（マグミット）、センノシド、ピコスルファートナトリウム当日排便なければ内服可。 GE、レシカルボン坐薬使用可				
	その他		有・無	有・無			
処置			サーフロー挿入、終了時サーフロー抜針。 （1コース目のみヘパロック） パクリタキセル専用ルートを使用 1コース目のみ ECG モニター装着（パクリタキセル中のみ） （アラーム設定：不整脈　HR60未満160以上 Drcall） バイタル測定パクリタキセル前・中・後				
検査		採血 CT（1・5・7コース目の前）					
活動・安静度			制限なし	制限なし			
栄養			常食（本人の希望にて変更可）				
排泄			尿測不要	尿測不要			
清潔			入浴可	入浴可			
指導・説明			入院診療計画書 入院オリエンテーション（看護師） 治療オリエンテーション（医師） 退院診療計画書 退院オリエンテーション 1）退院処方 2）次回入院日 3）次回外来日 4）CT、採血の有無	塩酸シプロフロキサシンを3日間内服しても解熱しない場合、悪寒がある、呼吸苦がある場合は病院に連絡するよう説明			

観察		入院前	タキソール投与前	投与中	投与後		Day 2		Day 3	Day 4	Day 5
	体温（℃）										
	脈拍（回/分）										
	血圧（mmHg）										
	悪心										
	嘔吐										
	静脈炎										
	アレルギー反応										
経過記録											
予測される看護問題											
バリアンス											
担当看護師サイン											

国立がんセンター中央病院

NCI-CTC AE ver.3.0による観察項目

有害事象	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
悪心	なし	摂食習慣に影響のない食欲低下	顕著な体重減少、脱水または栄養失調を伴わない経口摂取量の減少；<24時間の静脈内輸液を要する	カロリーや水分の経口摂取が不十分；≧24時間の静脈内輸液/経管栄養/TPNを要する	生命を脅かす
嘔吐	なし	24時間に1エピソードの嘔吐	24時間に2－5エピソードの嘔吐；<24時間の静脈内輸液を要する	24時間に≧6エピソードの嘔吐；≧24時間の静脈内輸液またはTPNを要する	生命を脅かす
静脈炎	なし		あり		
アレルギー反応	なし	一過性の潮紅あるいは皮疹；<38℃の薬剤熱	皮疹；潮紅；蕁麻疹；呼吸困難；≧38℃の薬剤熱	蕁麻疹の有無によらず症状のある気管支痙攣；非経口的治療を要する；アレルギーによる浮腫/血管性浮腫；血圧低下	アナフィラキシー

乳腺腫瘍内科（16A病棟）　2007年3月改訂

性反応への対応として，パクリタキセル投与前にshort-premedicationを行い，バイタル測定を行う。初回投与では投与中に心電図モニターを装着する。もし過敏性反応が起こった場合はバリアンスとなり，過敏性反応に対する治療を行うとともに，症例に応じてパクリタキセルの投与方法の変更，投与中止を行う。

3．悪心嘔吐に対する対策

化学療法に伴う副作用として，いずれのレジメンにおいても問題となるのは，悪心嘔吐である。ASCO（American Society of Clinical Oncology）ガイドライン，MASCC（Multinational Association of Supportive Care in Cancer）のコンセンサス会議により抗がん剤の催吐性リスク分類が提唱されており[6,7]，それによるとシスプラチンは高リスク，ドキソルビシン，カルボプラチンは中間リスクに分類されており，十分な悪心嘔吐対策が必要である。

化学療法投与開始から24時間以内の急性嘔吐に対しては，5-HT$_3$受容体拮抗薬と副腎皮質ステロイドの併用が勧められる[7]。ASCOガイドラインではaprepitantを含めた3剤併用を勧めているが，aprepitantは日本ではまだ承認されていない。上記薬剤で無効であれば，メトクロプロミド，アルプラゾラムの追加を行う。化学療法投与開始から24時間以降の遅発性嘔吐に対しては副腎皮質ステロイドの投与が確立しており，それにて効果がなければメトクロプロミド，アルプラゾラムの追加投与を行う。5-HT$_3$受容体拮抗薬の有効性は定かでなく，投与は勧められない。処方例を以下に示す。詳しくはASCOガイドラインを参照されたい。

＜シスプラチン（high emetic risk）投与時＞

day 1　・カイトリル®（グラニセトロン）1 mg静注もしくは2 mg経口
・デカドロン®4 mg（aprepitant併用時は16 mg）静注もしくは経口

day 2-3　・デカドロン®4 mg（aprepitant併用時は10 mg）静注または経口

※デカドロン®4 mg中にはデキサメタゾンとして3.3 mgしか含有していないことに注意

悪心時　・プリンペラン®（メトクロプラミド）20 mg静注または経口
・ソラナックス®（アルプラゾラム）0.4 mg静注または経口

おわりに

婦人科癌で代表的に用いられるレジメンのクリニカルパスの例を示し，重要な事項についてそれぞれ説明した。各病院の実情に合わせ，かつエビデンスに基づいたクリニカルパスを作成し発展させていくことにより，より一層適正で良質な医療が提供されるものと思われる。

（原野　謙一，勝俣　範之）

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Analysis of a correlation between the *BRAF* V600E mutation and abnormal DNA mismatch repair in patients with sporadic endometrial cancer

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Abstract. Point mutations of *KRAS* and *BRAF* genes are thought to be important in carcinogenesis of colon cancer. In particular, gene instability caused by decreased expression of the *hMLH1* gene, a DNA mismatch repair (MMR) gene, may be linked to the activating *BRAF* V600E point mutation in sporadic colon cancer. However, a consensus has not been established regarding the correlation between point mutations of *KRAS* or *BRAF* and carcinogenesis in patients with endometrial cancer, which is closely related to colon cancer. Therefore, we analyzed aberrant hypermethylation of the *hMLH1* gene, microsatellite instability (MSI), and point mutations of *KRAS* and *BRAF* in 44 samples of sporadic endometrial cancer, with the aim of examining the mechanism of carcinogenesis in patients with endometrial cancer. Aberrant *hMLH1* hypermethylation was found in 17 of the 44 cases (38.6%) and showed a significant positive correlation with MSI (p=0.02). This suggests that an abnormal MMR mechanism plays an important role in carcinogenesis of sporadic endometrial cancer. Point mutation of *KRAS* was found in 6 of the 44 cases (13.6%), but no *BRAF* V600E mutation was detected. These data suggest that the *BRAF* V600E mutation is not the target gene for abnormal MMR in carcinogenesis in patients with sporadic endometrial cancer, unlike in colon cancer. This is supported by the relatively few previous reports indicating a correlation between endometrial cancer and the *BRAF* V600E mutation. Identification of new candidates for the target gene for abnormal MMR in endometrial cancer requires further work.

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Key words: endometrial cancer, *BRAF*, *KRAS*, *hMLH1*, microsatellite instability

Introduction

A cancer may develop as a result of repeated mutation of genes involved in differentiation or proliferation. Such a multi-step mechanism of carcinogenesis with mutation of multiple cancer-related genes is often observed in patients with colon cancer. The correlation between colon cancer carcinogenesis and point mutation of *RAS/RAF* genes in the *MAP* kinase pathway suggests that these genes have an important role at an early stage of malignant alteration of colon cancer (1).

Endometrial cancer has many similarities with colon cancer and is detected at high rates as a double cancer of hereditary non-polyposis colon cancer (HNPCC). Germline mutation of *hMLH1*, a DNA mismatch repair (MMR) gene, occurs at high rates in HNPCC patients (2), and decreased expression of *hMLH1* due to aberrant hypermethylation has also been found in patients with sporadic colon cancer and endometrial cancer (3). Decreased expression of *hMLH1* due to epigenetic changes may facilitate gene replication errors and cause gene instability, which can be detected as microsatellite instability (MSI) (4). Microsatellite DNA is a region with short repeated sequences of 1-2 bases, and PCR-based detection of replication errors in this region has been used widely as a clinical test to examine gene instability. Such instability may cause mutation of cancer-related genes, and a correlation between MSI due to decreased *hMLH1* expression and point mutations of *KRAS* and *BRAF* genes has been proposed in patients with colon cancer (5,6).

Mutation of the *BRAF* gene has been found in many human cancers, including colon cancer, malignant melanomas, thyroid carcinoma and ovarian carcinoma (7-9). *BRAF* is one of the 3 subtypes of *RAF* family genes and encodes a tyrosine kinase involved in mitogenic signaling in the *RAS-RAF-MEK-ERK-MAP* kinase pathway. The function of *RAF* is regulated by *RAS*, and an activating point mutation of *BRAF* causes unregulated constitutive activation of the tyrosine kinase activity and facilitates cell proliferation via the *MAP* kinase pathway. The V600E mutation in exon 15 of *BRAF* is of particular interest, since tyrosine kinase activity 10-fold that of wild-type has been found in tumor tissue with this mutation (10). The V600E mutation is found in about 15% of patients with sporadic colon cancer and can be used for clinical diagnosis of non-inheritive sporadic colon cancer (10). Furthermore, since *BRAF* V600E is observed in 32% of cases of MSI-positive

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

Gene	PCR method	Sense	Antisense	Size (bp)	Annealing (°C)
hMLH1	Methylated	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	112	60
	Unmethylated	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124	60
KRAS	Codons 12, 13	GCCTGCTGAAAATGACTGAAT	TTATCTGTATCAAAGAATGGTC	180	64
BRAF	Codon 600	TCATAATGCTTGCTCTGATAGGA	GGCCAAAAATTTAATCAGTGGA	150	60

sporadic colon cancer and 75% of cases with sporadic colon cancer with aberrant hypermethylation of *hMLH1*, *BRAF* has been proposed as the target gene of abnormal MMR (11).

In contrast to colon cancer, only a few reports have shown mutation of *BRAF* in patients with endometrial cancer. Feng *et al* found *BRAF* mutations in 21% of patients with endometrial cancer and suggested that the mutation correlated with decreased *hMLH1* expression (12). However, Salvesen *et al* found a *BRAF* mutation in only 2% of patients with endometrial cancer (13). Therefore, it is unclear whether mutation of *BRAF* is important in carcinogenesis of endometrial cancer and whether the mutation may be linked to abnormal expression of the *hMLH1* gene. In this study, we analyzed aberrant hypermethylation of *hMLH1*, MSI, and mutations of *KRAS* and *BRAF* in patients with sporadic endometrial cancer to examine correlations among point mutations in *RAS/RAF* family genes, abnormal MMR caused by aberrant *hMLH1* hypermethylation, and carcinogenesis of sporadic endometrial cancer.

Materials and methods

Cell lines. Eight cell strains were used in the study: HEC108, Ishikawa (a human endometrial cancer-derived cultured cell line supplied by Dr Hiroyuki Kuramoto); HOOUA and HHUA (supplied by Dr Isamu Ishiwata); and SNG-II, SNG-M, HEC-1B 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., Tokyo, Japan). All other cells were cultured in 10% FBS-supplemented F12 medium (Sigma, St. Louis, MO, USA). The cells were incubated in a dish of 10 cm in diameter under 5% CO_2 at 37°C.

Clinical specimens. The subjects were 44 patients with endometrial cancer (G1, 20; G2, 11; G3, 13) who gave informed consent to collection of cancer specimens. Of these patients, 37 had endometrioid adenocarcinoma and 7 had adenosquamous carcinoma. The grade of histological 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.

DNA extraction and methylation-specific PCR (MSP) in the hMLH1 promoter region. DNA was extracted from the 44 endometrial cancer 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 and 5.5 μl of 3 N NaOH solution was added. After mixing, the solution was incubated at 37°C for 15 min, and then 520 μl of 3 M sodium bisulfite (Sigma) 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, 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. The column was rinsed with 2 ml of 80% isopropanol and then centrifuged at 15,000 rpm for 3 min to remove the isopropanol completely. Next, 50 μl of distilled water (70°C) was added directly to the column, which was then 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. After mixing, the solution was incubated at 37°C for 20 min, after which 66 μl of 5 N ammonium acetate solution and 243 μl of 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 DNA. Approximately 50 μl of the supernatant was left in the tube. 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 PCR analysis and DNA was analyzed using a GeneAmp PCR System 9700 (Applied Biosystems). The PCR primer sequences are shown in Table I. DNA extracted from the cultured cell lines was also used in MSP analysis of *hMLH1* (14).

Microsatellite instability analysis. Genomic DNA extracted from normal and tumor tissue samples from the 44 patients with endometrial cancer was PCR amplified at the microsatellite repeat loci D2S123, D5S346, D17S250, BAT26, BAT25, MSH3, MSH6, TGF-ßRII, BAX, MBD4A10 and MBD4A6, which include 3 dinucleotide (CA) and 8 mononucleotide repeats as microsatellite markers. PCR reactions were performed in a total volume of 25 μl containing 10X buffer, 0.125 mM deoxynucleoside triphosphate, 0.2 μM of each primer, and 0.25 units of TaqDNA polymerase. The PCR

conditions were as follows: 94°C for 10 min; 30 cycles at 94°C for 45 sec, 58°C for 45 sec, and 72°C for 40 sec; followed by a final extension step at 72°C for 10 min. After PCR, 1 μl of the product was mixed with 12 μl of loading buffer containing formamide and Rox size standards. This mixture was denatured at 95°C for 2 min and cooled on ice before loading onto an ABI PRISM 310 sequencer (Applied Biosystems). The results were analyzed using GeneScan software (Applied Biosystems). Tumors were classified as MSI-H when ≥30% of these markers showed MSI, in accordance with the recent recommendation of the National Cancer Institute Workshop. Low-frequency MSI (<30% of 11 markers) was included in the category of MSI-L and alteration of even one microsatellite region led to definition of the patient as MSI-positive (15).

Determination of KRAS and BRAF mutations. DNA was extracted from the 8 endometrial cancer-derived cell lines and 44 endometrial cancer specimens using liquid-based cytology with a Get Pure DNA Kit (Dojindo Molecular Technologies). Individual point mutations of the *KRAS* and *BRAF* genes were documented using two gene-specific oligonucleotide primer pairs designed for PCR amplification of the region of the *KRAS* gene harboring codons 12 and 13 and the region of exon 15 of the *BRAF* gene encompassing codon 600, respectively. The oligonucleotide primers for sequencing of *KRAS* and *BRAF* are shown in Table I. Each exon was amplified by PCR using 0.5 Ag of template DNA, sense and antisense primers, and an AmpliTaq Gold PCR kit (Applied Biosystems). A total of 50 μl of reaction mixture was prepared according to the manufacturer's instructions and PCR was commenced at 94°C for 3 min; followed by 35 cycles of 94°C for 30 sec, 64°C or 60°C for 30 sec, and 72°C for 1 min; with a final extension step for 5 min. The PCR products were purified using an UltraClean PCR Clean-up kit (Mobio Laboratories, Solana Beach, CA) and subjected to direct sequencing using purified products and the same sets of primers in a capillary automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). Sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) software located at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov) (12).

Statistical analysis. Correlation of *KRAS* mutations 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 *KRAS* mutations with patient age was also examined, after establishing that age had a normal distribution in the groups of patients with and without *KRAS* mutations. Mann-Whitney test was used to examine whether the population medians of the two independent groups differed significantly. Correlation of aberrant DNA hypermethylation of *hMLH1* with MSI was analyzed by the χ^2 test.

Results

MSP analysis of samples of endometrial cancer showed aberrant *hMLH1* hypermethylation in 17 of the 44 cases (38.6%) (Fig. 1, Table II). In MSI analysis, 31.8% (14 samples), 6.8% (3 samples), and 61.4% (27 samples) of the cases were categorized as MSI-H, MSI-L and MSS (microsatellite stability), respectively; that is, 38.6% were judged to be MSI-positive. Aberrant hypermethylation of the *hMLH1* gene was found in a higher number of MSI-positive cases, with a statistically significant positive correlation (p=0.02) between abnormal *hMLH1* methylation and MSI (Table III).

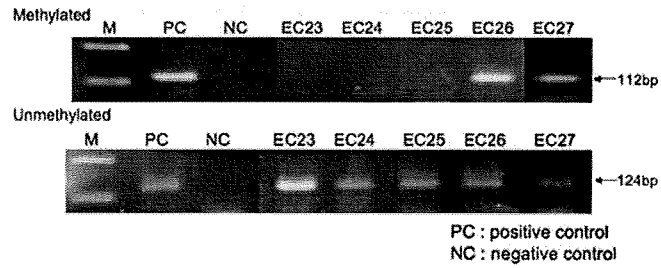

Figure 1. Detection of abnormal hypermethylation of the *hMLH1* gene in endometrial cancer using MSP analysis. The 112 bp band indicating abnormal hypermethylation was found in EC26 and EC27.

A point mutation at codon 12 of *KRAS* was found in 3 (HEC-1B, HHUA and SNG-M) of the 8 endometrial cancer-derived cell lines that were examined. These changes resulted in a G-D mutation in one cell line and G-V mutations in the other 2 cell lines. None of the cell lines had a point mutation at codon 13 of *KRAS* or at codon 600 of *BRAF* (Table IV). A point mutation at codon 12 of *KRAS* was observed in 6 of the 44 samples of endometrial cancer (13.6%) (Fig. 2, Table II), with a similar mutation to those in the cultured cell lines (G-D or V) in 5 of the 6 cases (83.3%). The point mutation at codon 12 of *KRAS* showed no correlation with clinicopathological characteristics of endometrial cancer or with age upon development of cancer, but tended to occur more frequently in well-differentiated adenocarcinoma (p=0.1, Table V). There were no correlations among aberrant *hMLH1* hypermethylation, MSI, and point mutation at codon 12 of *KRAS*. No point mutation at codon 13 of *KRAS* (Table II) or at codon 600 of *BRAF* (Table VI) was found in the 44 clinical samples of endometrial cancer.

Discussion

Carcinogenesis of colon cancer has been correlated with point mutation of the *RAS/RAF* family of genes in the MAP kinase pathway, suggesting the importance of mutation of these genes in an early stage of malignant change in colon cancer (1). Since mutations of *KRAS* and *BRAF* are observed in many MSI-positive cases of sporadic colon cancer with aberrant hypermethylation of the *hMLH1* gene, a correlation with MSI caused by decreased expression of hypermethylated *hMLH1* has been suggested (5). Similar decreased expression of *hMLH1* due to aberrant hypermethylation has been reported in endometrial cancer (14), but the correlation with point mutations of *KRAS* and *BRAF* remains unclear.

In the present study, aberrant hypermethylation of *hMLH1* was found in 38.7% of cases of sporadic endometrial cancer. Expression of *hMLH1* is significantly reduced by aberrant hypermethylation (14) and this may induce gene instability that can be detected as microsatellite instability (MSI). Previous studies have shown that about 13% of cases of sporadic colon cancer are MSI-positive (16) and that 84% of cases of MSI-

Table II. Results of MSI analysis, MSP analysis, and analysis of *BRAF* and *KRAS* gene mutations in cases of endometrial cancer.

No.	Age	Type	Stage	Grade	MSI	*hMLH1*	*BRAF* mutation Codon 600 GTG(V)	*KRAS* mutation Codon 12 GGT(G)	Codon 13 GGC(G)
EC1	52	EM	Ib	G3	MSI-H	M	GTG	GGT	GGC
EC2	51	EM	IIIc	G1	MSI-H	U	GTG	GGT	GGC
EC3	54	AS	IIIc	G3	MSI-H	M	GTG	GGT	GGC
EC4	53	EM	Ib	G3	MSI-H	U	GTG	GGT	GGC
EC5	69	EM	IIIc	G2	MSI-H	M	GTG	GGT	GGC
EC6	55	EM	IIIc	G2	MSI-H	M	GTG	GGT	GGC
EC7	54	EM	Ia	G1	MSI-H	U	GTG	GGT	GGC
EC8	63	EM	Ia	G1	MSI-H	M	GTG	GGT	GGC
EC9	58	EM	Ib	G2	MSI-H	M	GTG	GGT	GGC
EC10	50	EM	IIIa	G3	MSI-H	U	GTG	GGT	GGC
EC11	61	EM	Ib	G1	MSI-H	M	GTG	GGT	GGC
EC12	55	AS	IVb	G2	MSI-H	U	GTG	GGT	GGC
EC13	78	EM	Ib	G3	MSI-H	U	GTG	GGT	GGC
EC14	65	EM	Ib	G2	MSI-H	M	GTG	GGT	GGC
EC15	61	EM	IIb	G1	MSI-L	U	GTG	GGT	GGC
EC16	57	EM	Ib	G3	MSI-L	U	GTG	GGT	GGC
EC17	41	EM	Ib	G1	MSI-L	M	GTG	GGT	GGC
EC18	50	EM	Ia	G1	MSS	U	GTG	GGT	GGC
EC19	61	EM	Ib	G1	MSS	M	GTG	GAT(D)	GGC
EC20	70	EM	IIIc	G2	MSS	U	GTG	GGT	GGC
EC21	62	AS	IIIa	G2	MSS	U	GTG	GCT(A)	GGC
EC22	40	EM	IIa	G1	MSS	U	GTG	GGT	GGC
EC23	59	EM	IIa	G3	MSS	U	GTG	GGT	GGC
EC24	80	EM	IIIc	G3	MSS	U	GTG	GGT	GGC
EC25	54	AS	Ib	G1	MSS	U	GTG	GGT	GGC
EC26	42	EM	IIb	G1	MSS	M	GTG	GGT	GGC
EC27	71	EM	IIIc	G3	MSS	M	GTG	GGT	GGC
EC28	60	EM	Ib	G1	MSS	U	GTG	GGT	GGC
EC29	57	EM	IIIa	G2	MSS	U	GTG	GGT	GGC
EC30	71	EM	IIa	G1	MSS	U	GTG	GTT(V)	GGC
EC31	37	EM	IIa	G2	MSS	M	GTG	GGT	GGC
EC32	47	EM	IIIb	G1	MSS	M	GTG	GAT(D)	GGC
EC33	67	EM	Ic	G2	MSS	U	GTG	GGT	GGC
EC34	53	EM	Ia	G1	MSS	M	GTG	GGT	GGC
EC35	62	AS	Ib	G1	MSS	M	GTG	GGT	GGC
EC36	56	EM	IIIc	G3	MSS	U	GTG	GGT	GGC
EC37	71	EM	Ib	G2	MSS	U	GTG	GAT(D)	GGC
EC38	53	EM	Ib	G3	MSS	U	GTG	GGT	GGC
EC39	42	AS	IIIc	G3	MSS	U	GTG	GGT	GGC
EC40	55	EM	Ic	G3	MSS	U	GTG	GGT	GGC
EC41	34	AS	IIIc	G1	MSS	U	GTG	GTT(V)	GGC
EC42	61	EM	Ic	G1	MSS	U	GTG	GGT	GGC
EC43	61	EM	Ic	G1	MSS	U	GTG	GGT	GGC
EC44	59	EM	Ib	G1	MSS	U	GTG	GGT	GGC

Table III. Correlation between MSI and abnormal hypermethylation of the *hMLH1* gene in cases of endometrial cancer.

	hMLH1		
	M	U	
MSI	10	7	
MSS	7	20	p=0.02

MSI, microsatellite instability; MSS, microsatellite stability; M, methylated; U, unmethylated.

Table IV. *KRAS* and *BRAF* gene mutations in human endometrial cancer-derived cell lines.

	KRAS		*BRAF*
	Codon 12 GCT(G)	Codon 13 GGC(G)	Codon 600 GTG(V)
Cell lines			
Hec108	GGT	GGC	GTG
SNG-II	GGT	GGC	GTG
Ishikawa	GGT	GGC	GTG
Hec-1B	GAT(D)	GGC	GTG
HHUA	GTT(V)	GGC	GTG
SNG-M	GTT(V)	GGC	GTG
HOOUA	GGT	GGC	GTG
KLE	GGT	GGC	GTG

Table V. Correlation of *KRAS* gene mutations with clinicopathological factors in cases of endometrial cancer.

	Grade		Stage		Age
	G1, 2	G3	I, II	III, IV	(average)
KRAS codon 12					
Mut	6	0	3	3	57.7
Wt	25	13	26	12	57
% Mut	19.4	0	10.3	20	
	p=0.1		p=0.32		p=0.88

Statistical analysis was performed with the χ^2 test and Mann-Whitney test. Mut, mutation; Wt, wild-type.

Table VI. Correlation of abnormal *BRAF* V600E genes with abnormal MMR and mutated *KRAS* genes.

	MSI		*hMLH1*		*KRAS* codon 12	
	Positive	Negative	M	U	Mut	Wt
BRAF						
Mut	0	0	0	0	0	0
Wt	17	27	17	27	6	38

MSI, microsatellite instability; M, methylated; U, unmethylated; Mut, mutation; Wt, wild-type.

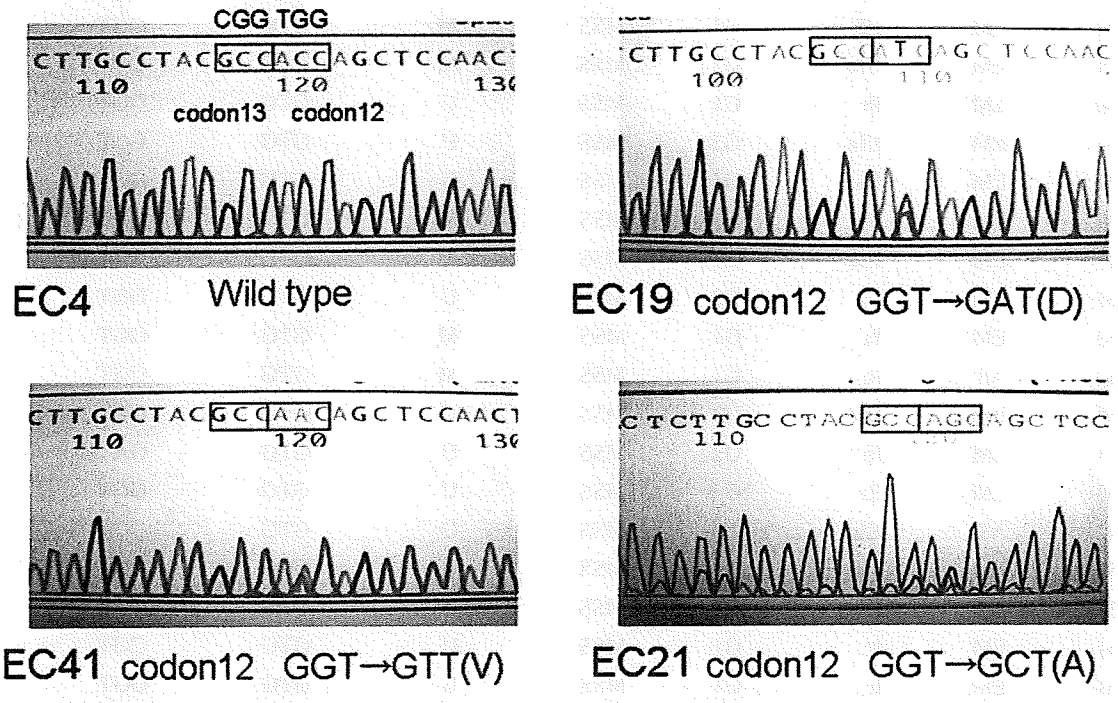

Figure 2. Analysis of point mutations of the *KRAS* gene in clinical samples of endometrial cancer. Three types of *KRAS* point mutation were detected at codon 12. No point mutation was observed at codon 13.

positive colon cancer have aberrant *hMLH1* hypermethylation (17). In our analysis, MSI-positive cases accounted for 38.6% of all cases of sporadic endometrial cancer. Mutch *et al* reported an incidence of MSI-positive cancer of 29% (18), with MSI occurring at higher rates in endometrial cancer than in colon cancer, suggesting that gene instability caused by an abnormal MMR gene is important in carcinogenesis of endometrial cancer. Our analysis showed aberrant *hMLH1* hypermethylation in 58.8% (10/17) of MSI-positive cases, with a significant positive correlation between aberrant *hMLH1* hypermethylation and MSI-positive cases of sporadic endometrial cancer (p=0.02). Based on this, we suggest that aberrant *hMLH1* hypermethylation causes MSI in endometrial cancer, as also seen in colon cancer.

Point mutations of the *KRAS* gene at codons 12 have been reported to occur in 0-46% of endometrial cancers and the most frequent codon 12 *KRAS* mutations are transitions from G to D, to V (19). Point mutations of the *KRAS* gene at codons 12 and 13 have been reported in 5.9% and 2.9% of patients with endometrial cancer, respectively, and the mutation showed a positive correlation with age upon development (20). Mutch *et al* found point mutations at codons 12, 13, and 61 of *KRAS* in 19.9%, 3.4% and 0.7% of cases of endometrial cancer, respectively, with a correlation with age upon development and a high rate of mutation in MSI-positive cases (18). In our analysis, point mutation at codon 12 was confirmed in 14% of cases, but none were observed at codon 13 and *KRAS* mutation showed no correlation with age. The incidence of well-differentiated adenocarcinoma tended to be high among cases with a mutation of *KRAS*, but the relationship was not significant, and there was no tendency for a higher rate of mutation of *KRAS* in MSI-positive cases. Point mutation of *KRAS* has been found in 51% of cases with colon cancer, and the rate in endometrial cancer is much lower (1). Mutation of *KRAS* may have some correlation with carcinogenesis in patients who develop sporadic endometrial cancer at an old age, but the current and previous results suggest that this mutation is not important for carcinogenesis in other cases of sporadic endometrial cancer.

Feng *et al* found mutation of the *BRAF* gene in 21% of cases of endometrial cancer, and proposed a correlation with decreased expression of the MMR gene (12). In contrast, Salvesen *et al* found the activating *BRAF* V600E mutation in only 2% of cases of endometrial cancer, and a consensus has not been obtained regarding the correlation between carcinogenesis of endometrial cancer and *BRAF* mutation (13). In our analysis, no *BRAF* V600E mutation was observed in cases of sporadic endometrial cancer. Collectively, these data suggested that the *BRAF* V600E mutation occurs at an extremely low rate in endometrial cancer, and thus may not be important for carcinogenesis of sporadic endometrial cancer. In contrast, the *BRAF* V600E mutation occurs at a high rate in sporadic colon cancer, and may be useful diagnostically to rule out the possibility of a hereditary tumor. However, this mutation is not useful in diagnosis of sporadic endometrial cancer.

Since we did not find a *BRAF* V600E mutation in our analysis, there was clearly no correlation between the *BRAF* V600E mutation and aberrant hypermethylation of *hMLH1* or MSI. Decreased expression of *hMLH1* due to aberrant hypermethylation could cause gene instability, with a high rate of mutation of a target gene such as *BRAF*. However, our results suggest that *BRAF* is not the target of abnormal MMR in sporadic endometrial cancer. On the other hand, since aberrant hypermethylation of *hMLH1* and MSI were detected at high rates in sporadic endometrial cancer patients, an abnormal MMR system is clearly associated with the mechanism of carcinogenesis in endometrial cancer. Identification of the new target gene for abnormal MMR will be extremely important for clarification of this mechanism.

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Analysis of candidate target genes for mononucleotide repeat mutation in microsatellite instability-high (MSI-H) endometrial cancer

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Abstract. Microsatellite instability (MSI) is an indicator of DNA instability and is caused by abnormalities in DNA mismatch repair (MMR) genes such as *hMLH1*, *hMSH2* and *hMSH6*. MSI occurs frequently in endometrial cancer (in approximately 30% of cases), and accumulation of gene mutations due to MSI may therefore have a major role in the mechanism of malignant transformation. However, a responsible target gene has not been identified in endometrial cancer. In this study, we analyzed mutations in 11 cancer-related genes with mononucleotide repeats susceptible to MSI in a coding region [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6 in exon 7), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] in 22 patients with MSI-H sporadic endometrial cancer. Mutations in *hMSH6* (C8) and *TGF-βRII* (A10) were found most frequently, at rates of 36.3% (8/22) each. Mutations of *BAX* (G8) and *TCF-4* (A9), which are common in MSI-positive colorectal cancer, occurred at rates of 22.7 and 0%, respectively, which suggests that the MSI target gene may differ between endometrial and colorectal cancers. Mutations in *hMSH6* (C8) were correlated with reduced protein expression (p=0.042) and patients with these mutations had significantly more mutations in mononucleotide repeats in other cancer-related genes compared to patients without *hMSH6* (C8) mutations (p=0.042). This suggests the possibility of a novel cascade in carcinogenesis of endometrial cancer in which MSI mutates *hMSH6* (C8), increases gene instability, and leads to accumulation of mutations in other cancer-related genes. To our knowledge, this is the first report to show that *hMSH6* (C8) has an important role as an MSI target gene in sporadic endometrial cancer.

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Key words: endometrial cancer, microsatellite instability, *hMSH6*, mutation, mononucleotide repeat

Introduction

Microsatellite instability (MSI) is an indicator of genetic instability at the DNA level (1,2). MSI can be evaluated by PCR-based detection of errors in replication of DNA sequences called microsatellites, which consist of repeating units of 1-2 base pairs. MSI has been found in many carcinomas and is particularly common in patients with hereditary non-polyposis colorectal cancer (HNPCC), a familial colon and endometrial cancer that is frequently MSI-positive (3). The mutated genes associated with HNPCC, *hMLH1* (4,5), *hMSH2* (6,7), *hMSH3* (8), *hMSH6* (9,10), *hPMS1* and *hPMS2* (11), are mismatch repair (MMR) genes that repair errors during DNA replication. In HNPCC patients, germline mutations in these genes cause abnormalities in the MMR system, which results in frequent errors in target genes. In addition, approximately 15% of patients with non-hereditary sporadic colon cancer are MSI-positive (3). This may be due to inactivation of the *hMLH1* gene promoter by aberrant hypermethylation, which causes abnormalities in the MMR system similar to that in HNPCC and results in unstable MSI-positive genes (12,13). About 30% of patients with sporadic endometrial cancer are also MSI-positive (14,15) and this may also be due to inactivation of hypermethylated *hMLH1* (16).

In somatic cells, replication errors are likely to occur in DNA regions including repeat sequences. MSI-based mutations accumulate in target genes with repeat sequences, resulting in malignant transformation of cells. In particular, mutation of tumor suppressor genes with a mononucleotide or dinucleotide repeats (repeating unit of one or two base pairs, respectively) may be strongly associated with malignant transformation of cells. Cancer-related genes including mononucleotide repeats (i.e., candidate MSI-target genes) include *TGF-βR II* (17) and *PTEN* (18), which are related to cell growth inhibition; apoptosis-related *BAX* (19) and *Caspase-5* (20); *TCF-4* (21), *EPHB2* (22) and *AXIN2* (23), which are components of the Wnt-signaling pathway; and *HDAC2* (24), which codes for a histone deacetylase. *hMSH3* (25) and *hMSH6* (26), which are MMR genes, and *MBD4* (27), which codes for the methyl-CpG binding protein, also have a mononucleotide repeat sequence and are also candidate MSI-target genes. In MSI-positive sporadic colorectal cancer, mutations of *TGF-βRII* (A10) and *BAX* (G8) have been found in 90% (28) and 45%

Table I. Primer sequences used in gene mutation analysis.

Gene	Repeat	Sense	Antisense
hMSH3	A8	AGATGTGAATCCCCTAATCAAGC	ACTCCCACAATGCCAATAAAAAT
hMSH6	C8	GGGTGATGGTCCTATGTGTC	CGTAATGCAAGGATGGCGT
TGF-βRII	A10	CTTTATTCTGGAAGATGCTGC	GAAGAAAGTCTCACCAGG
MBD4	A10	TGACCAGTGAAGAAAACAGCC	GTTTATGATGCCAGAAGTTTTTTG
BAX	G8	ATCCAGGATCGAGCAGGGCG	ACTCGCTCAGCTTCTTGGTG
PTEN	A6	CCTGTGAAATAATACTGGTATG	CTCCCAATGAAAGTAAAGTACA
HDAC2	A9	ACCTCCGATTCCGAGCTTT	CCGCTCACCGTCGTAGTAGT
EPHB2	A9	CACGAGACGTCACCAAGAAA	CGCAAGAACAGTCATTGCTTT
Caspase-5	A10	CAGAGTTATGTCTTAGGTGAAGG	ACCATGAAGAACATCTTTGCCCAG
TCF-4	A9	GCCTCTATTCACAGATAACTC	GTTCACCTTGTATGTAGCGAA
Axin2	G7	CCTACCCCTTGGAGTCTGC	CAGGGTCCTGGGTGAACA

(29) of cases, respectively, which suggests that MSI plays an important role in malignant transformation in this cancer. However, the mutation frequency of target genes varies between carcinoma types and a responsible MSI-target gene has not been identified in endometrial cancer. Mutation of the tumor suppressor gene *PTEN* has been found in MSI-positive endometrial cancer (18). However, genes including mononucleotide repeats have not been investigated in endometrial cancer.

In this study, we analyzed mutations of 11 cancer-related genes with mononucleotide repeat sequences [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6 in exon 7), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] in MSI-positive sporadic endometrial cancer, in order to identify MSI-target genes that contribute to the pathogenic mechanism of endometrial cancer.

Materials and methods

Clinical specimens. The subjects were 69 patients with endometrial cancer (G1, 32; G2, 17 and G3, 20) who gave informed consent to collection of tissue specimens. Of these patients, 59 had endometrioid adenocarcinoma and 10 had adenosquamous carcinoma. The grade of histological 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.

Microsatellite instability (MSI) analysis. Genomic DNA was extracted from normal and tumor tissue samples collected from the 69 patients with endometrial cancer using a Get Pure DNA kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The genomic DNA was PCR amplified at the microsatellite repeat loci D2S123, D5S346, D17S250, BAT26 and BAT25. PCR reactions were performed in a total volume of 25 μl containing 10X buffer, 0.125 mM deoxynucleoside triphosphate, 0.2 μM of each primer and 0.25 Units of TaqDNA polymerase. The PCR conditions were as follows: 94°C for 10 min; 30 cycles at 94°C for 45 sec, 58°C for 45 sec, and 72°C for 40 sec; followed by a final extension step at 72°C for 10 min. After PCR, 1 μl of the product was mixed with 12 μl of loading buffer containing formamide and Rox size standards. This mixture was denatured at 95°C for 2 min and cooled on ice before loading onto an ABI 310 Prism sequencer (Applied Biosystems, Foster City, CA). The results were analyzed using Genescan software (Applied Biosystems). Tumors were classified as MSI-H when ≥30% of the markers showed MSI in accordance with the recent recommendation of the National Cancer Institute Workshop. Tumors in which <30% of the markers showed MSI were included in the MSI-L category. Alteration of even one microsatellite region led to definition of the patient as MSI-positive.

Determination of frameshift mutations of mononucleotide repeats in 11 cancer-related genes. DNA was extracted from tumor tissue from patients with MSI-H endometrial cancer using a Get Pure DNA kit (Dojindo Molecular Technologies). Somatic frameshift mutations in 11 cancer-related genes [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] were determined using two gene-specific oligonucleotide primer pairs designed for PCR amplification of mononucleotide repeat regions. The oligonucleotide primers for sequencing of the 11 genes are shown in Table I. Each mononucleotide region was amplified by PCR using 0.5 μg of template DNA, sense and antisense primers, and an AmpliTaq Gold PCR kit (Applied Biosystems). A 50-μl reaction mixture was prepared according to the manufacturer's instructions and PCR was started at 94°C for 3 min; followed by 35 cycles of 94°C for 30 sec, 64°C or 60°C for 30 sec, and 72°C for 1 min; with a final extension step for 5 min. The PCR products were purified using an UltraClean PCR Clean-up kit (Mobio Laboratories, Solana Beach, CA) and subjected to direct sequencing using purified products and the same sets of primers in a capillary automatic sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). Sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) software located at the National Center for Biotechnology Information web site (http:/ www.ncbi.nlm.nih.gov).

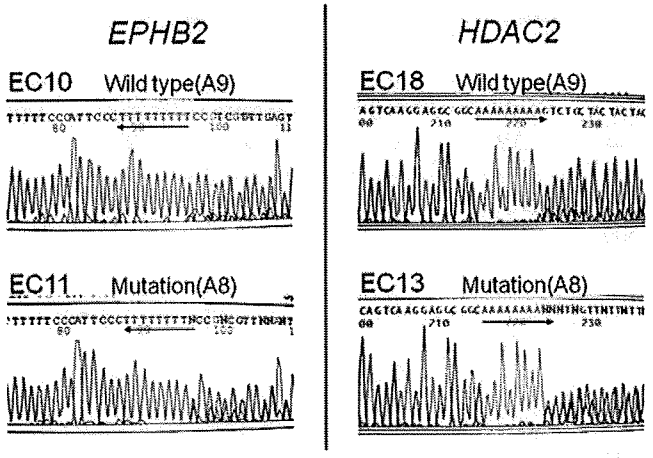

Figure 1. Analysis of mutations in MSI-H endometrial cancer. Frameshift mutations were observed in *EPHB2* (A9) in case EC11 and *HDAC2* (A9) in EC13.

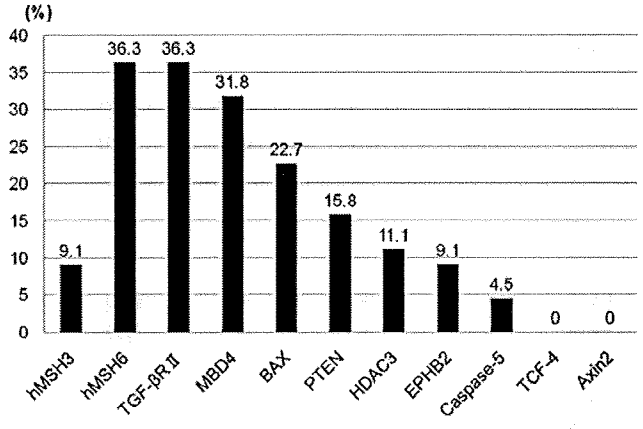

Figure 2. Frequency of mutations in mononucleotide repeats in cancer-related genes in tissue samples from patients with MSI-H endometrial cancer. Mutations in *hMSH6* and *TGF-βRII* were found most frequently (36.3%).

Immunohistochemistry. Immunohistochemical staining was performed on 2-μm sections of formalin-fixed, paraffin-embedded tissues using standard procedures. Slides were cleaned in xylene and dehydrated in graded alcohols. Antigen retrieval was performed with 10-min microwave treatment in 10 mM citrate buffer (pH 7.0). Endogenous peroxidase was blocked by dipping sections in 0.3% H_2O_2 in methanol for 10 min. Slides were incubated with mouse monoclonal antibody to hMSH6 (clone44; BD Transduction Laboratories, San Jose, CA) (1:500) for 90 min at room temperature. Immunostaining was performed by the avidin-biotin-peroxidase complex technique with an Elite ABC kit (Vector Laboratories, Burlingame, CA), using 3,3-diaminobenzidine as a chromogen and H_2O_2. Slides were counterstained with hematoxylin, dehydrated in graded alcohol, dried and cover-slipped. The normal staining pattern for hMSH6 is nuclear, and nuclei in stromal cells were used as internal positive controls. For the purpose of the study, staining of tumor nuclei for hMSH6 was evaluated as positive (+) or negative (-).

Statistical analysis. The association of frameshift mutations in the mononucleotide repeat region of *hMSH6* (C8) in MSI-H endometrial cancer specimens with mutations in the other 10 genes was analyzed using a Mann-Whitney test. The statistical association between mutations in *hMSH6* (C8) and hMSH6 protein expression was analyzed using a Fisher's exact test.

Results

MSI was determined by PCR in 69 patients with endometrial cancer and 22 cases (31.8%) were diagnosed as MSI-H. Mutations in mononucleotide repeats in 11 cancer-related genes [*hMSH3* (A8), *hMSH6* (C8), *TGF-βRII* (A10), *MBD4* (A10), *BAX* (G8), *PTEN* (A6), *HDAC2* (A9), *EPHB2* (A9), *Caspase-5* (A10), *TCF-4* (A9) and *Axin2* (G7)] were examined in the 22 cases of MSI-H endometrial cancer. Mutations in *hMSH6* (C8) and *TGF-βRII* (A10) were found most frequently, each in 36.3% (8/22) of the cases. For the other genes, the percentages of cases with mutations were 9.1% (2/22) for

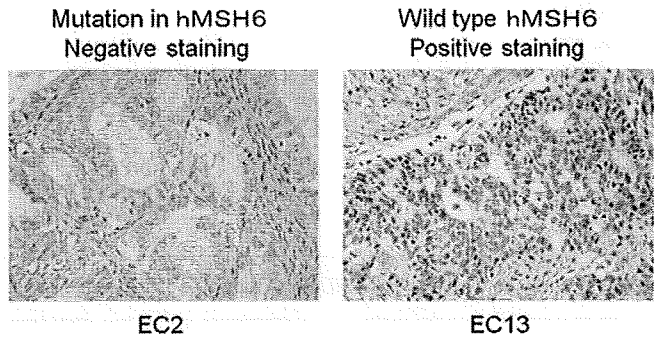

Figure 3. Immunohistochemical analysis of hMSH6 protein in endometrial cancer. Reduced expression of hMSH6 in tumor regions was found in case EC2, which had mutations in *hMSH6*. In contrast, hMSH6 showed clear staining in tumor cell nuclei in EC13, in which there were no mutations in *hMSH6*. In both specimens, normal nuclei surrounding the tumor are normally stained.

hMSH3 (A8), 31.8% (6/18) for *MBD4* (A10), 22.7% (5/22) for *BAX* (G8), 15.8% (3/19) for *PTEN* (A6), 11.1% (2/18) for *HDAC2* (A9), 9.1% (2/22) for *EPHB2* (A9), and 4.5% (1/22) for *Caspase-5* (A10). No mutation was found in the mononucleotide repeat regions of *TCF-4* (A9) or *Axin2* (G7) (Figs. 1 and 2, Table II).

Mutations were found most frequently in mononucleotide repeats in *hMSH6* (C8) among the 11 genes that were analyzed. Further analysis in patients with mutations in *hMSH6* (C8) showed a statistically significant tendency for accumulation of mutations in mononucleotide repeats in one or more genes other than *hMSH6* (p=0.012, Tables II and III). Furthermore, tumors with mutations in *hMSH6* showed significant negative immunostaining for hMSH6 protein (p=0.042, Fig. 3, Table IV), indicating that mutations in hMSH6 correlated with reduced *hMSH6* protein expression.

Discussion

Microsatellite instability (MSI) is an indicator of genetic instability at the DNA level. MSI can be evaluated by detecting errors in replication of DNA regions referred to as

Table II. Analysis of mutations in mononucleotide repeats in 11 cancer-related genes in tissue samples from patients with MSI-H endometrial cancer.

Case	hMSH3 A8	hMSH6 C8	TGF-ßRII A10	MBD4 A10	BAX G8	PTEN A6	HDAC2 A9	EPHB2 A9	Caspase-5 A10	TCF-4 A9	Axin2 G7
EC1	-	+	-	+	-	ND	ND	-	-	-	-
EC2	-	+	+	+	-	ND	-	-	-	-	-
EC3	-	+	-	-	-	+	-	-	-	-	-
EC4	-	+	+	ND	-	-	-	+	-	-	-
EC5	-	+	+	ND	+	-	-	-	-	-	-
EC6	-	+	+	+	-	+	-	-	-	-	-
EC7	+	+	+	-	-	+	-	+	-	-	-
EC8	-	+	+	-	+	-	-	-	-	-	-
EC9	-	-	-	-	+	ND	+	-	-	-	-
EC10	-	-	+	-	-	-	-	-	-	-	-
EC11	-	-	+	-	-	-	ND	-	-	-	-
EC12	-	-	-	+		-	ND	-	-	-	-
EC13	-	-	-	-	-	-	+	-	-	-	-
EC14	-	-	-	-	-	-	-	-	-	-	-
EC15	-	-	-	-	-	-	-	-	+	-	-
EC16	-	-	-	-	-	-	-	-	-	-	-
EC17	+	-	-	-	-	-	-	-	-	-	-
EC18	-	-	-	ND	-	-	-	-	-	-	-
EC19	-	-	-	-	-	-	-	-	-	-	-
EC20	-	-	-	ND		-	ND	-	-	-	-
EC21	-	-	-	+	+	-	-	-	-	-	-
EC22	-	-	-	+	+	-	-	-	-	-	-

+, mutated; -, wild-type and ND, not done.

Table III. Association of mutations in *hMSH6* in MSI-H endometrial cancer with mutations in 10 other cancer-related genes (p=0.012, Mann-Whitney test).

	No. of mutations in 10 genes (other than hMSH6)				
	0	1	2	3	4
Mutation in hMSH6	0	2	4	0	2
No mutation in hMSH6	4	7	2	1	0

Statistical analysis was performed by Mann-Whitney test (p=0.012).

microsatellites, which consist of a sequence of repeating units of 1 or 2 base pairs. HNPCC is a familial tumor that is very frequently MSI positive and is probably caused by germline mutations in DNA mismatch repair (MMR) genes that cause abnormalities in the MMR system. This results in frequent replication errors of various target genes followed by malignant transformation. In MSI-positive colorectal cancer, mutations of *TGF-ßRII* and *BAX* tumor suppressor genes are frequently found and these genes are considered to be MSI target genes. *TGF-ßRII* and *BAX* include mononucleotide repeats susceptible to MSI and are likely to be mutated in MSI-positive tumors; therefore, these mutations are suspected to be involved in malignant transformation of cells.

Approximately 30% of MSI-positive endometrial cancer is defined as MSI-H, but a responsible MSI-target gene has not been identified in endometrial cancer. In this study, we analyzed MSI in 69 patients with endometrial cancer and 22 (31.8%) were diagnosed as MSI-H. This result is similar to those in previous studies. Mutations in mononucleotide repeats in 11 cancer-related genes were analyzed in the 22 cases of MSI-H endometrial cancer. Mutations in *hMSH6* (C8) and *TGF-ßRII* (A10) were found most frequently (36.3%), whereas no mutation was found in *TCF-4* (A9) or *Axin2* (G7), which are components of the Wnt-signaling pathway. Mutations in *PTEN* (A6), which has a high frequency of mutations in MSI-positive endometrial cancer, were found in 15.8% of the 22 cases.

TGF-ß inhibits growth of epithelial cells and TGF-ßRII transmits growth inhibitory signals; therefore, a loss of the function of these proteins may lead to malignant transformation of cells. In a previous study, mutations of *TGF-ßRII* (A10) were found in 90% of MSI-positive colorectal cancer, whereas no mutation was found in MSI-negative colorectal cancer, which suggests that *TGF-ßRII* plays an important role in malignant transformation as an MSI-target gene (28). Similarly, mutations of *BAX* (G8), which is involved in apoptosis, have been found in 45% of cases of MSI-positive colorectal cancer and *BAX* is thought to be related to malignant

Table IV. Association of mutations in *hMSH6* in MSI-H endometrial cancer with reduced hMSH6 protein expression.

	Positive	Negative	Total
Mutation in hMSH6	2	6	8
Wild-type in hMSH6	11	1	12
Total	13	7	20

Statistical analysis was performed by Fisher's exact test (p=0.042).

transformation in this cancer as an MSI target gene (29). The mutation rates of *TGF-βRII* (A10) and *BAX* (G8) in MSI-H endometrial cancer have been shown to be 12 and 33%, respectively (29), whereas we found rates of 36.3 and 22.7%, respectively, with these rates being the highest and third highest among the 11 genes analyzed. However, both rates are much lower than those found in MSI-positive colorectal cancer. Mutations in *TCF-4* (A9), a component of the Wnt-signaling pathway, were not found in our specimens, but occur at a frequency of 39% in MSI-positive colorectal cancer (29). This suggests that the frequency of mutations in mononucleotide repeats differs substantially between colorectal and endometrial cancers, and that MSI target genes and the mechanism of malignant transformation may also differ between these cancers.

Mutations in *PTEN* are found in about 60% of cases of MSI-positive endometrial cancer and about 30% of cases of MSI-negative endometrial cancer (30,31). The significantly higher rate in MSI-positive endometrial cancer suggests an association with MSI. Mutations in mononucleotide A repeats in exons 7 and 8 of *PTEN* are found in 27% of cases of MSI-positive endometrial cancer, which suggests that *PTEN* is an MSI-target gene (31), but PTEN mutation patterns vary and another study found mutations in the mononucleotide A repeats in only 3% of cases of endometrial cancers with microsatellite instability (32). The results of our study showed a 15.8% mutation rate for *PTEN* (A6), which was lower than those for *hMSH6* and *TGF-βRII*.

The function of hMSH6 is to detect deletion or insertion of a base pair in a mononucleotide repeat sequence and to initiate repair by forming a complex with hMSH2, hPMS2 and hMLH1. Reduced expression of hMSH6 due to mutation of *hMSH6* damages MMR function and induces MSI, which may result in malignant transformation of cells. Hendriks *et al* investigated families with germline mutations in *hMSH6* and showed that carriers of these mutations had a significantly higher risk of endometrial cancer than carriers of an *hMSH1* or *hMSH2* mutation (33). Furthermore, 69% of cases of endometrial cancer among *hMSH6* mutation carriers were MSI-H and immunohistochemistry showed that 97.5% were negative for hMSH6, indicating reduced expression of *hMSH6* (33). These results suggest that reduced expression of *hMSH6* caused by germline mutation induces MSI and is associated with development of hereditary endometrial cancer. Somatic mutations in *hMSH6* in MSI-positive sporadic endometrial cancer patients have been shown in several studies, but it is unclear if these mutations have an important role (34,35). Goodfellow *et al* found somatic mutations in *hMSH6* in 16 of 60 patients (26.6%) with MSI-H endometrial cancer and frameshift mutations in C8 in 12 of 16 patients with a somatic mutation, but no somatic mutation in *hMSH6* in MSI-negative patients. In the current study, frameshift mutations in *hMSH6* (C8) were found in 36.3% of patients, higher than the rate in Goodfellow *et al*, and immunohistochemical analysis showed that mutation of *hMSH6* correlated with reduced protein expression. These results suggest that mutation in *hMSH6* plays an important role in development of MSI-H sporadic endometrial cancer, similarly to hereditary endometrial cancer.

The current results also showed that patients with mutations in *hMSH6* (C8) had a tendency for accumulation of mutations in mononucleotide repeats in other genes. This tendency was found only in patients with mutations in *hMSH6* (C8). In MSI-positive colorectal cancer, Ikeda *et al* found that mutations in *E2F4* (CAG13), which codes for a transcriptional activator, were often associated with mutations in *hMSH3* (A8), an MMR gene that repairs dinucleotide and trinucleotide repeats, and proposed an interesting hypothesis in which mutations in trinucleotide repeats in *E2F4* are induced by mutations in *hMSH3*, with a subsequent reduction in expression (36). A cascade of malignant transformation with a similar mechanism to this hypothesis may also occur in MSI-H endometrial cancer, with mutations in *hMSH6*, a repair gene for mononucleotide repeats, inducing mutations in tumor suppressor genes that include mononucleotide repeats, such as *TGF-βRII* (A10), *BAX* (G8) and *β* (A6).

Collectively, the results of this study suggest the possibility of a novel cascade in endometrial cancer, in which MSI caused by reduced expression of *hMLH1* due to aberrant hypermethylation (epigenetic change) leads to mutation of *hMSH6* (C8), an MSI target gene, and reduced expression of hMSH6 subsequently increases gene instability and leads to accumulation of mutations in other cancer-related genes (genetic change), resulting in malignant transformation. This is the first study to show that *hMSH6* (C8) has an important role in the mechanism of malignant transformation in MSI-H sporadic endometrial cancer as a target gene, and further studies on the proposed cascade may provide new drugs and preventive approaches for endometrial cancer.

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Cyr61, a member of ccn (connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed) family, predicts survival of patients with endometrial cancer of endometrioid subtype☆

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Abstract

Objectives. It has been reported that expression of Cyr61 decreased in endometrial cancers and cancer cell lines compared with normal endometrium, and forced expression of Cyr61 could suppress the growth of human endometrial cancer cells. However, in another report, Cyr61 was immunostained in most of endometrial cancer tissues analyzed. Thus, the aim of this study was to examine the expression of Cyr61 in endometrial cancer and to correlate Cyr61 expression with cinicopathologic factors in a larger cohort.

Methods. We used immunohistochemistry and RT-PCR to examine the expression of Cyr61 in 92 endometrial carcinomas of endometrioid subtype. We correlated the expression of Cyr61 with various clinicopathologic factors in patients with endometrioid adenocarcinoma. Survival analyses were performed by the Kaplan Meier curves and the log-rank test. Independent prognostic factors were determined by multivariate Cox regression analysis.

Results. Cyr61 expression was high in 21 of 92 cases of endometrioid adenocarcinoma (22.8%). High expression of Cyr61 was related to poor survival of patients with endometrioid adenocarcinoma. Multivariate analysis including Cyr61 expression revealed that Cyr61 expression and positive lymph node metastasis (LNM) were independent prognostic factors for survival. Survival of patients with endometrioid adenocarcinoma could be stratified into three groups by combination of Cyr61 expression and positive LNM with an estimated 5-year survival rate of 96.5% for no LNM irrespective of Cyr61 expression (group A), 85.7% for positive LNM with low/moderate expression of Cyr61 (group B), and 0% for positive LNM with high expression of Cyr61 (group C)($p=0.18$ for group A vs group B, $p=0.008$ for group B vs group C, and $p<0.0001$ for group A vs group C).

Conclusions. Cyr61 is highly expressed in some endometrial cancer of endometrioid subtype. Cyr61 expression and positive LNM were independent prognostic factors for patients with endometrioid adenocarcinoma. Cyr61 might be a new molecular marker to predict the prognosis of patients with endometrioid adenocarcinoma.

Keywords: Cyr61; Endometrial carcinoma; Immunohistochemistry; Survival; Prognostic factor

Introduction

Cyr61 (cysteine-rich 61/ccn1) belongs to the ccn (connective tissue growth factor/cysteine-rich 61/neuroblastoma overexpressed) family, which includes six members. The other five known members are connective tissue growth factor (CTGF/ccn2), nephroblastoma overexpressed (NOV/ccn3), Wnt-induced secreted protein-1, 2 and 3 (WISP-1/ccn4, WISP-2/ccn5, WISP-3/ccn6). The term "ccn family" was introduced by Bork in 1993, as Cyr61, CTGF and NOV were the three prototype members of this family [1]. Encoded by a growth factor-inducible immediate early gene, Cyr61 is a 40 kDa protein which is extremely cysteine-rich. This heparin-binding protein shares a 40 to 50% amino-acid homology with the other ccn family members. An important structural feature of ccn proteins is that they contain four conserved modules which exhibit similarities to the insulin-like growth factor-binding

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proteins (IGFBPs) , the von Willebrand factor type C (VWC), the thrombospondin type 1 (TSP1) and the carboxyl termini of several extra-cellular mosaic proteins (CT). However ccn5 is an exception, as it lacks the CT module.

The ccn proteins are involved in a variety of biological processes such as cell adhesion, proliferation, differentiation and migration; angiogenesis, chondrogenesis, wound-healing and tumorigenesis [2–4]. With respect to tumorigenesis, Cyr61 overexpression is associated with progression and formation of larger tumors in breast cancer [5,6]. Cyr61 can also stimulate the growth of gastric adenocarcinoma [7]. In non-small-cell lung cancer, prostate and papillary thyroid carcinoma, Cyr61 is found to be down-regulated [8–11]. These findings indicate that ccn proteins have variable biological functions which are dependent on the cellular contexts.

Difference of Cyr61 expression between endometrial cancer tissues and normal endometrium remains to be determined. Chien and colleagues reported that expression of Cyr61 decreased in endometrial cancers and cancer cell lines compared with normal endometrium, and forced expression of Cyr61 could suppress the growth of human endometrial cancer cells [12]. On the contrary, MacLaughlan et al. recently reported that Cyr61 was detected by immunohistochemistry and Western blot analysis in most of endometrial cancer tissues examined [13]. Thus, we used immunohistochemistry to examine the expression of Cyr61 in a larger cohort of endometrial carcinomas and to correlate Cyr61 expression with clinicopathological factors to explore the clinical significance of Cyr61 expression in endometrioid adenocarcinoma in this study.

Table 1
Cyr61 expression and clinicopathological factors of 92 endometrioid adenocarcinoma

Factor	Cyr61 expression		*p*-value
	Low/moderate	High	
Age			0.87
−49	19	6	
50-	52	15	
FIGO stage (1988)			0.87
I/II	46	14	
III/IV	25	7	
Architectural grade			0.38
1/2	60	16	
3	11	5	
Nuclear grade			0.16
1	25	11	
2/ 3	46	10	
Myometrial invasion			0.87
<=1/2	42	12	
>1/2	29	9	
Cervical invasion			0.58
negative	58	16	
positive	13	5	
Lymph-vascular space invasion			0.67
−/+	54	15	
++/+++	17	6	
Ovarian metastasis			0.88
Negative	65	19	
Positive	6	2	
Lymphnode metastasis			0.48
Negative	62	17	
Positive	9	4	

Materials and methods

Patients

A total of 92 endometrioid adenocarcinomas were obtained from archives of paraffin embedded tissues between January, 1994 and May, 2004 at the Department of Gynecology of Hokkaido University, Sapporo, Japan. We focused on endometrioid adenocarcinomas alone in this study because serous adenocarcinomas have different genetic abnormalities and biological properties from endometrioid adenocarcinomas. All subjects underwent modified radical hysterectomy, bilateral salpingo-oophorectomy and systematic retroperitoneal lymphadenectomy which consisted of complete dissection of pelvic and para-aortic lymph nodes from the femoral ring to the level of the renal vein. All lymphatic tissues that surrounded the arteries and veins were completely removed. Hematoxylin-Eosin (HE) sections were reviewed to confirm the pathological diagnosis of endometrioid carcinoma. The age of subjects ranged from 23 to 74 years with an average of 54.6 years. The following histopathologic prognostic factors were correlated with staining intensity of Cyr61 expression by immunohistochemistry : FIGO (1988) stage, architectural grade (AG), nuclear grade (NG), lymphvascular space invasion (LVSI), depth of myometrial invasion, cervical invasion (CI), ovarian metastasis, lymph node metastasis (LNM). All risk factors were determined as previously described [14]. Clinicopathological characteristics of endometrial cancer patients were shown in Table 1.

Immunohistochemistry

A standard streptavidin-biotin-peroxidase method was used for the immunohistochemistry (IHC) study. Briefly, 4 μm thick sections from each sample were deparaffinized in xylene and rehydrated through a sequence of alcohol. For antigen retrieval, the sections were boiled in 10 mM sodium citrate buffer (pH 6) for 6 min using autoclave. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 10 min. After phosphate buffered saline (PBS) wash for 5 min, the sections were treated with 10% normal goat serum at room temperature for 30 min to block the nonspecific binding. The sections were then incubated overnight in moist chamber at 4°C with a Cyr61 primary antibody, which was kindly provided by Dr Brahim Chaqour (State University of New York, Down state medical center, Brooklyn, New York, USA), at 1:100 working dilution. After washing away excess antibody with PBS (3 × 10 mins), the sections were incubated with biotinylated goat against anti-rabbit immunoglobulin for 30 min at 37°C. Following PBS washes(3 × 10 mins), the sections were then incubated with streptavidin-peroxidase conjugate for 30 min at room temperature and washed again with PBS (3 × 10 mins). 3′-3′-diaminobenzidine was used as a chromogen substrate. All sections were then washed in running tap water and