早期大腸癌 2005 Vol.9 No.2

II 大腸の新しい pit pattern 分類
——箱根合意に基づいた VI，VN 型 pit pattern

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はじめに

pit pattern 診断[1]が大腸に応用され，腫瘍，非腫瘍の鑑別，腺腫癌の鑑別，そしてm，$sm_{1a,b}$とsm_{1c}以深の massive 癌との鑑別が VI，VN の亜分類[2]によって成されるようになってきた．

しかし，pit pattern 診断[1]が各施設間で微妙に異なることはこれまでも指摘されていた．とくに VI 型と VN 型[2]の境界については施設による多少の違いは否めなかった．pit pattern 診断の普及のためには，診断基準の統一化が必要であることは明らかである．これまでも 2001 年の「早期大腸癌」の座談会[3]で V 型の用語の統一がなされ，各種研究会および 2002 年より開始された厚生労働省「大腸腫瘍性病変における腺口構造の診断学的意義の解明に関する研究」班（以下，工藤班）で討論が繰り返され各種研究会でも議論されてきた．さらに今回 2004 年 4 月の「箱根 pit pattern シンポジウム」にて，V 型の亜分類の概念の統一化がなされた（**図 1**）[4]．V 型の統一に向けて，① 簡便である，② 理解しやすい，③ 分類に意味がある，④ 長年の研究に基づいた知見を反映させる，などを基本理念として，初学者でも外国人でも理解できる国際性を重視し，箱根 pit pattern シンポジウムのコンセンサス（箱根合意）が得られた（**表 1**，図 1）．これにより VI 型と VN 型の境界が明瞭とな

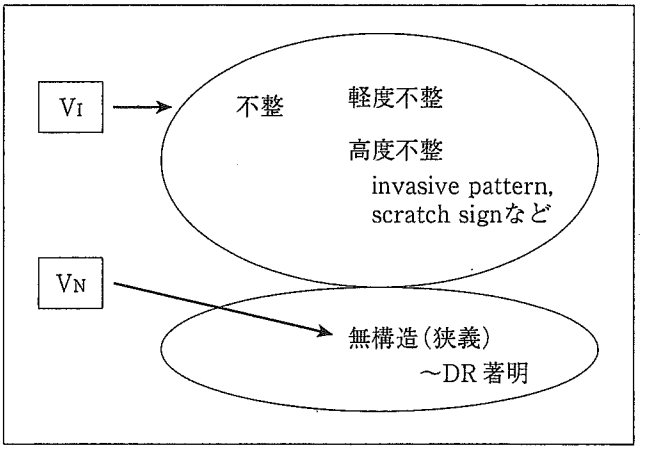

図 1 箱根合意に基づく V 型 pit pattern の亜分類
DR : desmoplastic reaction

表 1 箱根合意

1. 不整腺管構造を VI とする．
2. 明らかな無構造領域を有するものを VN とする．
3. sm 癌の指標としての invasive pattern・高度不整腺管群・scratch sign は付記してもよい．

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表2　従来のV型pit patternと病理組織診断

	adenoma		cancer			total
	mild-mod	severe	m	$sm_{1a,b}$	$sm_{1c\sim3}$	
V_I	29 (13.4%)	28 (13.0%)	112 (51.9%)	17 (7.9%)	30 (13.9%)	216
V_N	0	0	1 (1.6%)	7 (11.5%)	53 (86.9%)	61
total	29	28	113	24	83	277

(2001. 4～2004. 6)

り，理解しやすい分類になると考えられた．

本稿では従来のV型と箱根合意後のV型の比較，およびこれからのpit patternの問題点について考察する．

I. 従来の診断基準でのpit pattern診断の成績

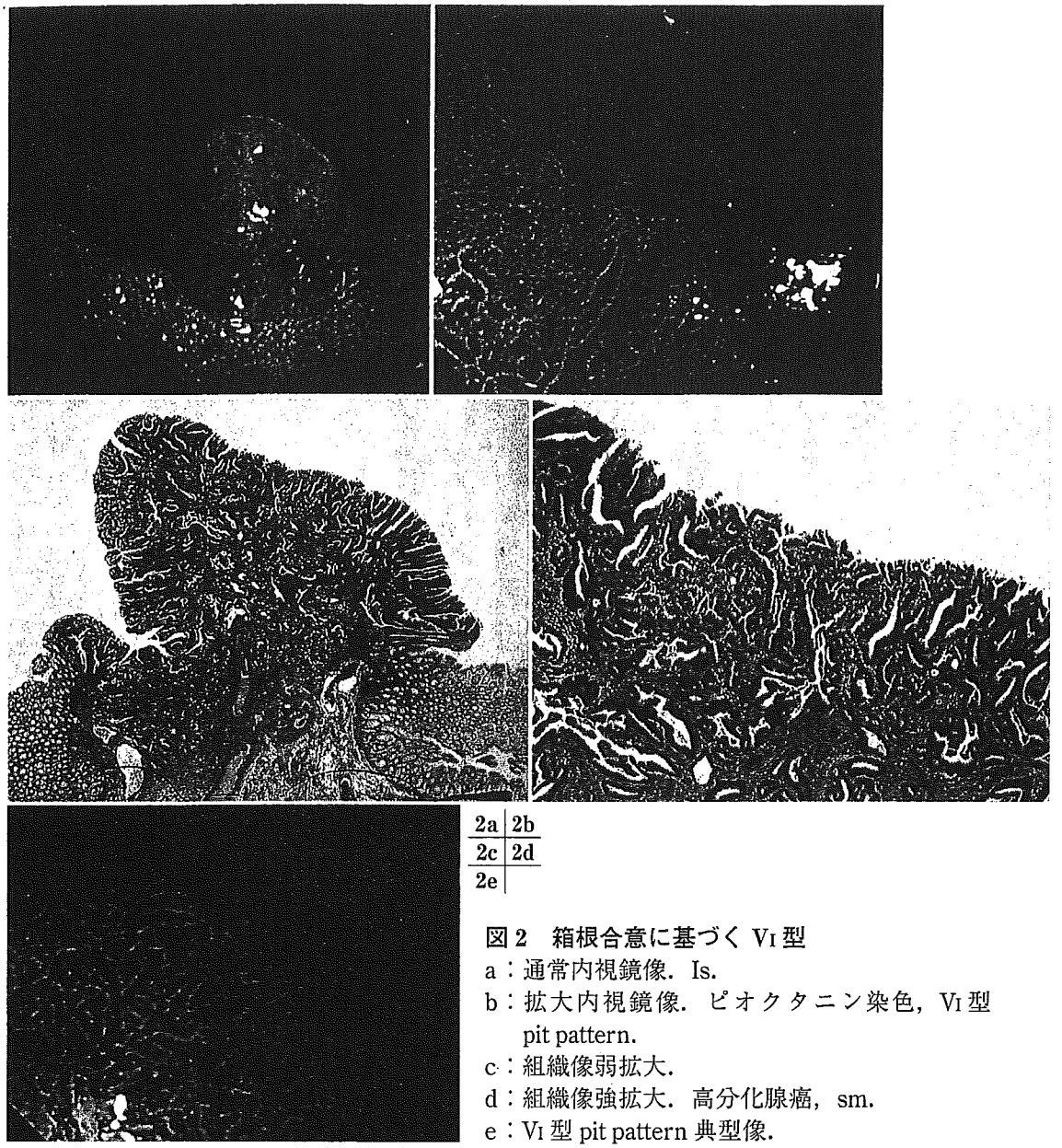
まず従来の診断基準でのV型pit patternの成績であるが，2001年4月から2004年6月までに昭和大学横浜市北部病院消化器センターで観察され，内視鏡的切除または外科手術された大腸腫瘍性病変のうち，V型pit patternを呈した277病変を対象に，内視鏡観察時のpit pattern診断と切除標本の病理組織診断の対比を行った(**表2**)．V_I型を呈した病変は216病変認め，高度異型腺腫が28病変(13.0%)，m癌が112病変(51.9%)，sm癌が47病変(21.8%)であり，深達度が$sm_{1a,b}$までの病変が186病変(86.1%)であった．V_N型を呈した病変は61病変認め，m癌が1病変(1.6%)，sm癌が60病変(98.4%)であり，深達度sm_{1c}以深のsm massive invasionの病変が53病変(86.9%)であった．これによりV_I型を呈するものは内視鏡治療の適応，V_N型を呈するものは手術を視野に入れて治療方針を決定するべきであるといえる．

II. 箱根合意に基づいたV_N型pit patternとsm massive癌

次に箱根合意に基づいて検討を行った．2001年4月から2004年6月までに昭和大学横浜市北部病院消化器センターでV_N型pit patternと診断された大腸腫瘍性病変のうち，クリスタルバイオレット染色にて詳細な検討が可能であった47病変を対象とし，箱根合意に基づいて再分類を行った(**表3**)．再分類によりV_I型に変更されたのは12病変(25.5%)であり，m癌が1病変，$sm_{1a,b}$が3病変，sm massive癌が8病変であった．箱根合意に基づいた分類でもV_N型と診断されたものは35病変(74.5%)であり，$sm_{1a,b}$が2病変，sm massiveが33病変であった．V_N型をsm massive癌の指標と考えた場合これまでの正診率が86.9%であるのに対して94.3%と上昇しており，箱根合意によりV_N型がsm massive癌を診断するうえでより有用な指標となったといえる．また同時にsm癌全体を考えると，箱根合意のV_Nは100%すべてsm癌ということになる．すなわち，V_N型を呈するものにはm癌がなくすべてsm癌であったことは深達度診断における拡大内視鏡pit pattern診断の有用性を示していると考えられる．ただ拡大内視鏡観察を始めて間もない初学者を含めた検討では，明らかな無構造領

表3　従来のV_N型を箱根合意に基づいて再分類した結果

	m	$sm_{1a,b}$	$sm_{1c\sim3}$	計
V_I型	1	3	8	12(25.5%)
V_N型	0	2	33	35(74.5%)
計	1	5	41	47

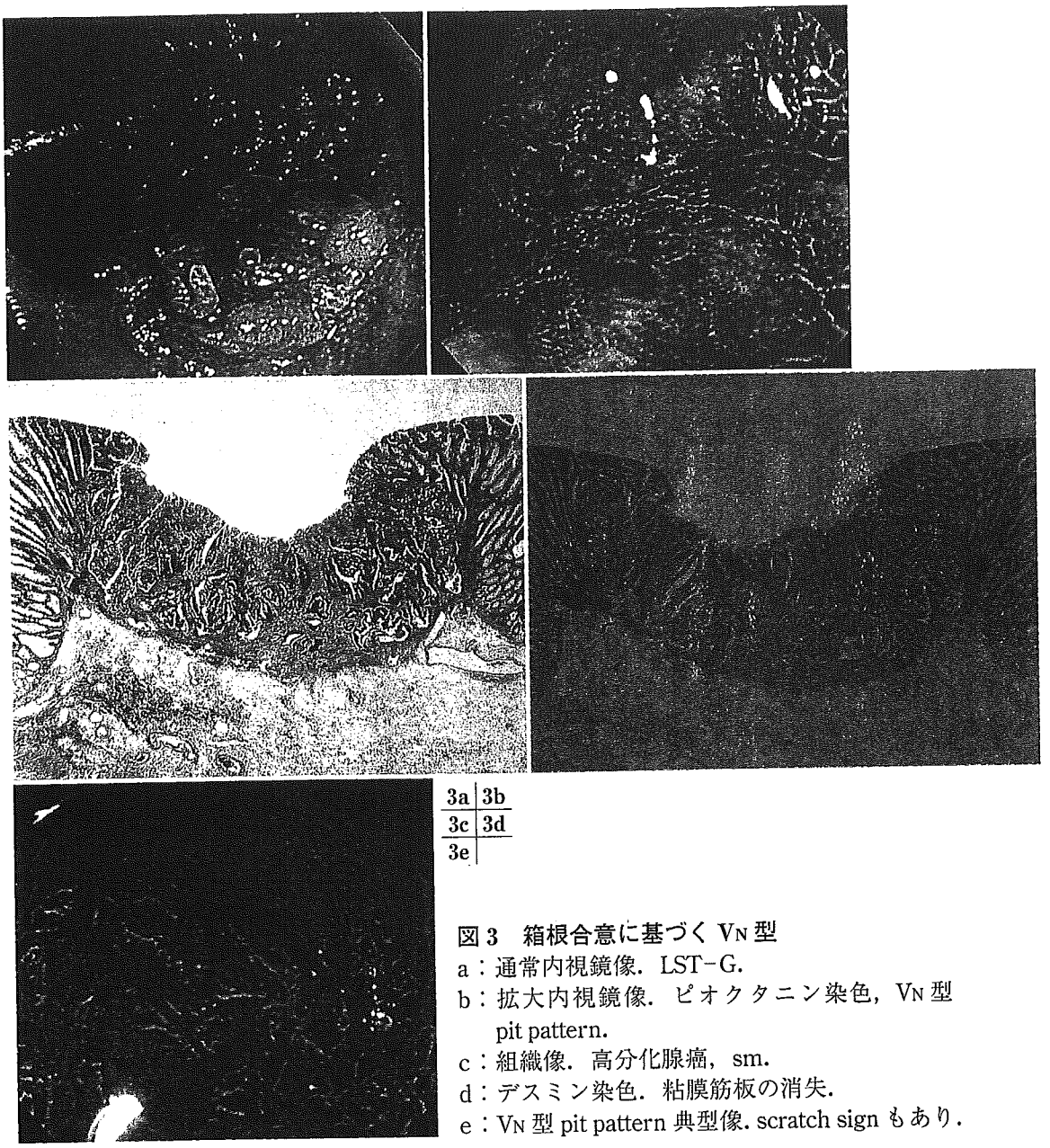


2a	2b
2c	2d
2e	

図2　箱根合意に基づくVI型
a：通常内視鏡像．Is.
b：拡大内視鏡像．ピオクタニン染色，VI型 pit pattern.
c：組織像弱拡大.
d：組織像強拡大．高分化腺癌，sm.
e：VI型 pit pattern 典型像.

域と判定できVN型と診断できるかどうかは人により多少のばらつきがあった．やはりある程度の経験をつまないことには明らかな無構造領域の判定にも微妙な違いが認められた．また，VI型 pit pattern を示す病変に sm 癌が増加することからscratch sign や pit の破壊，高度不整などの sm 癌の指標となる所見をさらに明確にすることが，今後の課題である．箱根合意に基づくVI型(**図2**)，VN型(**図3**)の典型例を呈示する．

前号(Vol.9 No.1)で記述したように[4]，箱根合意後に行われた2004年11月の工藤班班会議での討論では，施設間での診断基準の相違はあるものの，VI型を軽度不整と高度不整に亜分類したほうが，混乱が少ないのではないかとの意見が出た．しかしV型の亜分類であるVI型のなかでさらに亜分類を行うことは，また新たに軽度と高度の境界の問題が残り，分類がさらに複雑となり，簡便で理解しやすいという箱根合意の主旨に反すると考えられるので，VI，VNと大別し，VIについてはまずはあくまで所見の一つとしてsm癌あるいはsm massive癌の指標の検討を重ねることとした．今後各施設間でのV型の検討を行い，sm massive癌の指標となるような所見について統一できるなら，正式にVI型を高度不整と軽度不整



3a	3b
3c	3d
3e	

図3　箱根合意に基づくVN型
a：通常内視鏡像．LST-G.
b：拡大内視鏡像．ピオクタニン染色，VN型 pit pattern.
c：組織像．高分化腺癌，sm.
d：デスミン染色．粘膜筋板の消失.
e：VN型 pit pattern 典型像．scratch sign もあり．

として亜分類する可能性もある．

従来，内視鏡診断とはあくまでも病理の予測であり，治療方針決定のために，あるいは，診断と治療を兼ねて生検やポリペクトミー，EMRなどを行ってきた．生検はあくまでも腫瘍の検索の一部にすぎず，深達度診断には無力であり，sm massive 癌の明確な指標にはならなかった．その点で拡大内視鏡による pit pattern 診断は，深達度診断をよく反映しており，この新たな箱根合意に基づくVN型の診断により臨床の場で第一選択として内視鏡治療か手術的治療かを決定することがより容易になり，内視鏡診断が治療と直結した．このことは内視鏡診断の医療における位置付けの大きな進歩になったと考えられる．

そもそもV型は癌の指標として分類[1]されたものであり，腺口形態の不整，無構造から組織学的な不整さ，構造異型を反映したものである．

癌の診断基準は病理学者にとっても多少の違いがあり，とくに粘膜癌に関してはかなりの判定の違いを認める現状である．したがってV型を癌の指標としても無構造を呈さない不整の判断に関しては病理学的診断と明確に対応するか否かは問題が残る．しかし，pit pattern 診断で明らかな無構造領域を示す病変はきわめて高い頻度で sm 癌

図4

であることが示されたことは従来の病理診断により，ある面では利点もあることが示唆された．明らかな無構造領域は粘膜病変が脱落し粘膜下層に浸潤したdesmoplastic reactionを反映していると考えられる．VI型pit patternは明らかな無構造領域を認めない不整なpit pattern病変であるが，不整の程度は軽度から高度までさまざまである．従来のsm癌の指標とされていた爪で引っかいたようなscratch sign[5]や藤井らのいうinvasive pattern[6]，そして明らかなsm癌の浸潤腺管とみなされる大型の高度不整腺管などがVI型に含まれることにより，VI型にsm癌が多くなり，sm癌の可能性をより考慮する必要が生じてきた．それに対し，VN型は従来の基準より狭くなり，sm癌，とくにsm massive癌の指標としてより正確度が高まった．そもそもscratch signを呈するものは密在した癌腺管，間質の亀裂，巨大sm浸潤腺管などを示しておりsm_2以深の所見であるが，そのscratch signにはしばしば連続してVN無構造領域を認めることが多い(図3b，e)．VNの判定によりsm-massiveの診断精度が向上したことは手術の適応に直接的につながることになり，拡大内視鏡が腹腔鏡手術かEMR，EPMR，ESD(endoscopic submucosal dissection)などの内視鏡治療が可能かの治療方針の決定に有力な判断材料になることを示している．

おわりに

箱根合意によりV型の概念の統一化がはかられた．しかし，境界に関しては施設間の診断基準の微妙なばらつきは存在している．病理の問題がそうであるように，あくまでも人間の判断の経験と印象の違いは存在する．しかし症例を重ねるにつれて，経験差は縮まっていく．VIとVNの境界についても箱根合意に基づく基準が習慣化，一般化されれば，その違いは少なくなっていくと考えられる．その意味で今後も新しい症例の討議がさらに必要であると考えられる．また2004年12月には第1回拡大内視鏡研究会も新たに発足した．箱根合意に基づくVI，VNの概念は国内のみならず，今後拡大内視鏡のpit pattern診断が世界中に広まるとともに，国際的な概念になっていくことが予測される．

さくらの花満開(**図4**)の箱根シンポジウム合意がpit pattern診断のさらなる幕開けとなることを祈念し，さらなる検証が成されていくことを願う．

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Summary

New classification of pit patterns based on Hakone consensus

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Subdivisions of type V pit patterns were consolidated at the Hakone Pit Pattern Symposium, in April, 2004. It was determined that type VN pit patterns only showed the area of apparent nonstructure. Therefore, the classification of pit patterns became easy to understand by abecedarian and foreigners. 13.9 % of colorectal lesions which showed type VI pit patterns and 86.9 % of colorectal lesions which showed type VN pit patterns, which had invaded deeply into the submucosal layer (so-called sm massive cancers) by the former classification. With a classification based on the Hakone consensus, the lesions which showed new type VN pit patterns and were considered sm massive cancers. Because the number of sm massive cancers with VI pit patterns has increased, we must examine these indexes which represent sm massive cancers.

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Key words : Consensus of Hakone Pit Pattern Symposium, type V pit pattern, magnifying colonoscopy

展望

大腸腫瘍に対するポリペクトミーの歴史と未来

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はじめに

大腸ポリペクトミーが開発され，ポリープを見つけて診断と治療が同時に行われるようになり，多くのポリペクトミーがなされてきた。しかし，診断と治療が同時に行われるのはメリットもあるが，ポリペクトミーが可能かどうかのみの診断学に終始し，本来の腫瘍の診断学が発展することがなかったとも言え，診断学が軽んじられる時代があった。その後，陥凹型早期癌やLSTなどの内視鏡診断学が重視されるようになり，1990年頃より，大腸Ⅱcを中心とする表面型早期癌の診断が積極的になされるようになった。同時に拡大pit patternによる精密診断が導入されるようになり，腫瘍・非腫瘍の鑑別，良性・悪性の鑑別がより正確になされるようになった。そして，EMRも導入され，overポリペクトミーの風潮が少しずつ是正されるようになってきた。

最近はESDも大腸においても一部で行われるようになり，深達度診断や範囲診断がより重視されるようになった。すなわち，正確な診断に基づいて本来の内視鏡治療の適応病変のみを切除するようになってきた。

本稿では，ポリペクトミーに始まる内視鏡治療の歴史と未来について概説する。

ポリペクトミー

高周波電流が生体内で用いられ始めたのは1920年代のことである。内視鏡的にスネアを用いてポリープを切除するようになったのは1950年代といわれており，最初は硬性の直腸鏡下に施行されていた。1968年常岡[1]は，初めてflexible fiber scope下に胃のポリープを切除したが，この際は高周波電流を使用していなかった。同じころ，丹羽[2]が高周波電流を用いた胃のポリープ切除を試みている。ポリープの発生頻度は圧倒的に大腸において高いため，ポリペクトミーも大腸病変に応用されていった。

高周波電流を用いたポリペクトミーを世界で最初に行ったのは1969年のDr. Shinyaといわれている。1970年代に入ってからポリペクトミーの報告が相次ぎ，技術的にも確立されていった。

ホット・バイオプシー

大腸のポリープは多発していることがあり，そのすべてをポリペクトミーするのは困難な場合がある。5 mm以下の微小なポリープに対する簡便な治療法としてホット・バイオプシーがWilliamsにより開発された[3]。しかし，5 mm以下の腺腫を治療することの必要性に関しては議論のあるところである。

ピースミール・ポリペクトミー

1回のポリペクトミーでは切除しきれないような大きなvillous tumorなどに対し，分割して切除するピースミール・ポリペクトミーを施行することがある。

The future of polypectomy based on its history of development
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内視鏡的粘膜切除術(endoscopic mucosal resection: EMR)

スネアによる切除は有茎性や亜有茎性のポリープには大変有用だが，表面型の病変には施行困難である。そのため，人工的に病変をいったん持ち上げてからスネアで切除する手技が開発された。2チャンネルスコープを用いる方法もあったが，大腸病変に対しては，生理食塩水などを局注してからスネアで切除するのが一般的である。EMRは，1973年ドイツのDeyhleらにより初めて報告され[4]，1984年多田ら[5~7]によりストリップ・バイオプシーとして主に胃病変を対象に本格的に臨床応用され，現在，消化管全般にわたり広範に活用されている内視鏡治療手技である。

内視鏡的分割粘膜切除術(endoscopic piecemeal mucosal resection: EPMR)

20 mmを超えるようなlaterally spreading tumor (LST)など，大型の表面型腫瘍に対しては，一括切除が困難であることが多い。術前診断が腺腫あるいは粘膜内癌であればLSTに対してEMRを繰り返し行い，分割して病変の完全摘除を行う方法がEPMRである。遺残・再発が10%程度あるとされているが，再発形式は腺腫，あるいは粘膜内癌であるため，追加内視鏡治療で対処可能である[8,9]。また，切除断端のpit patternを観察し，腫瘍残存がないことを確認することにより，さらに再発が抑えられることが認められている。

内視鏡的粘膜下層剝離術(endoscopic submucosal dissection: ESD)

ESDとは病変の周囲を粘膜切開し，さらに粘膜下層を直接剝離していくことにより切除していく方法である。この方法により腫瘍径20 mm以上の病変でも一括切除が可能となった。胃では，多くの施設でESDが行われるようになったのは周知のとおりであるが，大腸では腸管壁が薄いため，穿孔のリスクが高いこと，管腔が狭く屈曲があるため難易度が高く，大腸では限られた施設で行われているのが現状である[10~14]。

現時点での適応は，LST-NG(pseudo-depressed type)とされている。ヒアルロン酸を局注剤として使用することが必須であることが提唱され，さらにグリセオール®で希釈して使用することにより，硬度が増すことが追加されている。根治性がきわめて高いこと，正確な病理組織診断が可能であることから，今後は技術に基づいた安全性が確保されれば，局注剤，処置具の改良などをもって，適応拡大の可能性も含めて発展していくことが期待される。

a|b

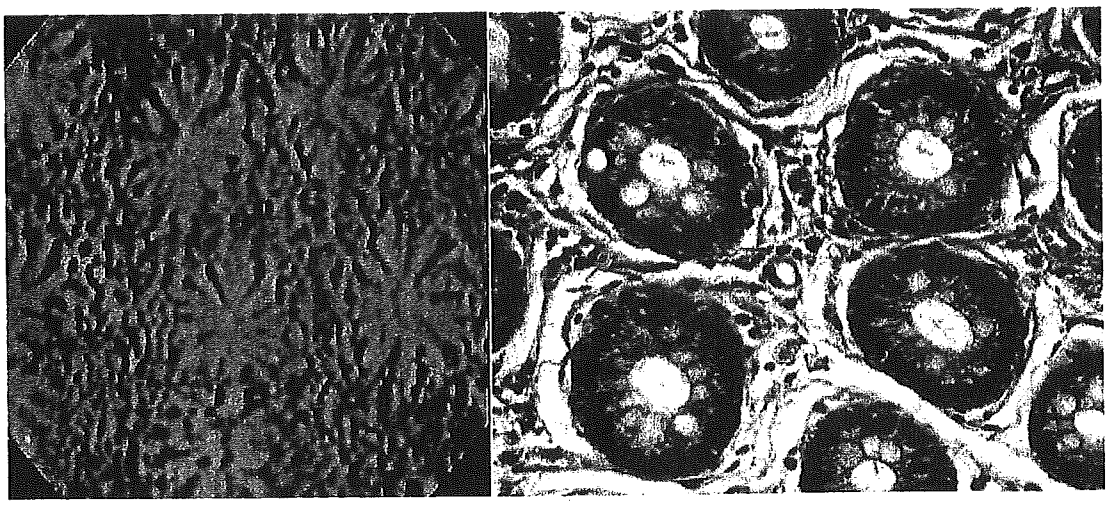

図1 通常粘膜

a. endo-cytoscope像　　b. HE像

a|b

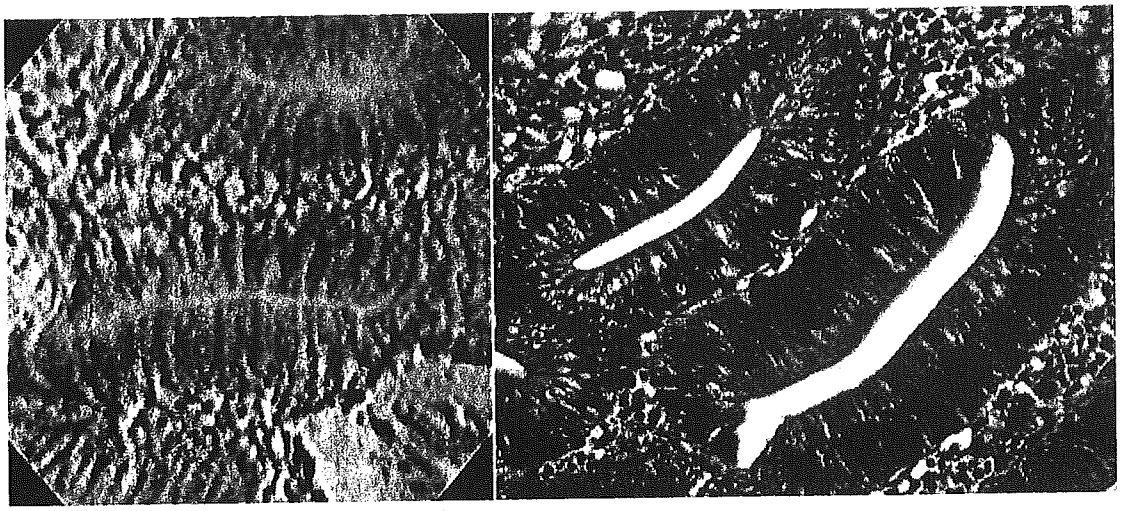

図 2　腺腫

a. endo-cytoscope像　　b. HE像

a|b

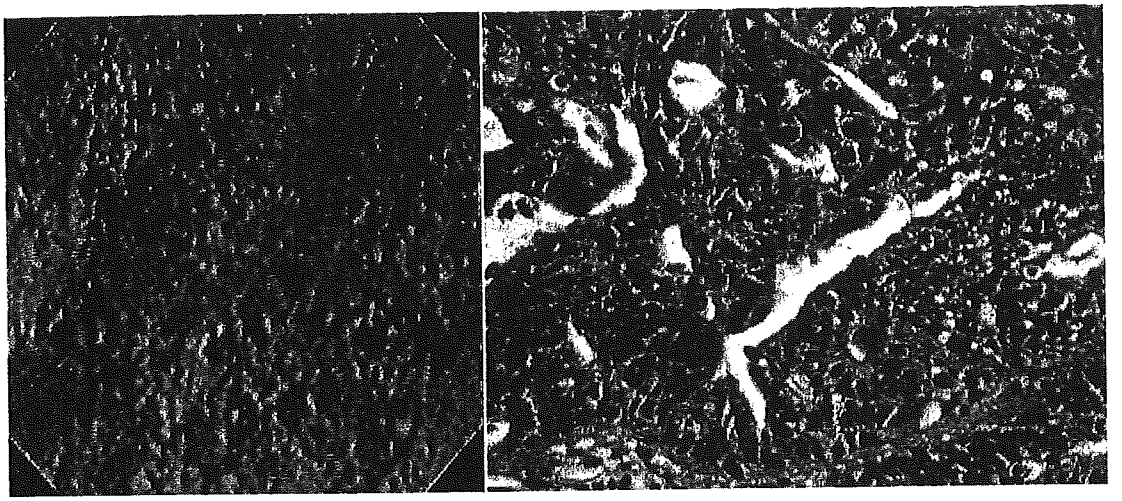

図 3　腺癌

a. endo-cytoscope像　　b. HE像

ポリペクトミーの未来

内視鏡治療の歴史は単なる手技の進歩ではなく，診断学の進歩に伴い必然的に生まれてきたものである。かつては手術でover treatmentされていたような早期癌が内視鏡治療できるようになってきた。しかし，そのためには腫瘍病変に対する診断学が重要となる。そして今後もますます重視されていくであろう。色素内視鏡，NBI，拡大pit pattern診断に加え，endo-cytoscopeによる500～1,000倍超拡大観察(**図1，2，3**)による細胞および核の診断も，*in vivo*で発達していくであろう[15]。

実際に，生体内で細胞・核のレベルの診断学を行うことにより，限りなく顕微鏡によるHE診断に近い診断が可能となりつつある。生体内でそこまでの診断学が必要かどうかは今後の問題ではあるが，症例を重ねて生きた状態での動きのなかでの腫瘍の診断学は，確実に治療に直結するものとなるだろう。

一部の施設でのESDは，腫瘍径が大きくても一括切除ができるきわめて根治性の高い手技であるが，大腸においてはリスクが高く，手技的に難易度が高いことから，広く普及するかどうか，大幅な器具の

開発が望まれ，もう少し様子をみていく必要がある。

内視鏡的全層切除(EFTRD)も適切な器具の出現により近い将来現実化されるであろう。そのためには腹腔鏡とのコンビネーションも必要となるだろう。そして，いずれは現在の内視鏡とは全く異なったタイプの治療ロボットのような機械が開発され，ポリペクトミーや内視鏡治療が行われる時代が来るだろう。

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A New Method for Isolating Colonocytes From Naturally Evacuated Feces and Its Clinical Application to Colorectal Cancer Diagnosis

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Background & Aims: **The early detection of colorectal cancer is desired because this cancer can be cured surgically if diagnosed early. The purpose of the present study was to determine the feasibility of a new methodology for isolating colonocytes from naturally evacuated feces, followed by cytology or molecular biology of the colonocytes to detect colorectal cancer originating from any part of the colorectum.** *Methods:* **Several simulation studies were conducted to establish the optimal methods for retrieving colonocytes from any portion of feces. Colonocytes exfoliated into feces, which had been retrieved from 116 patients with colorectal cancer and 83 healthy volunteers, were analyzed. Part of the exfoliated colonocytes was examined cytologically, whereas the remainder was subjected to DNA analysis. The extracted DNA was examined for mutations of the APC, K-*ras*, and p53 genes using direct sequence analysis and was also subjected to microsatellite instability (MSI) analysis.** *Results:* **In the DNA analysis, the overall sensitivity and specificity were 71% (82 of 116) of patients with colorectal cancer and 88% (73 of 83) of healthy volunteers. The sensitivity for Dukes A and B was 72% (44 of 61). Furthermore, the sensitivity for cancers on the right side of the colon was 57% (20 of 35). The detection rate for genetic alterations using our methodology was 86% (80 of 93) when the analysis was limited to cases in which genetic alterations were present in the cancer tissue.** *Conclusions:* **We have developed a new methodology for isolating colonocytes from feces. The present study describes a promising procedure for future clinical evaluations and the early detection of colorectal cancers, including right-side colon cancer.**

Colorectal cancer is one of the most common malignancies worldwide. In Japan, colorectal cancer is the third and second leading cause of death from cancer in men and women, respectively.[1] However, colorectal cancer is curable by surgical resection if diagnosed at a sufficiently early stage. This incentive has prompted investigators to develop new methods enabling the early diagnosis of colorectal cancer and has led to the introduction of cancer screening programs in many countries. For mass cancer screenings, a simple, economic, and noninvasive method of cancer detection is desired. The Hemoccult test is currently used in many countries for this purpose.[2–6] However, this test is nonspecific and is not sufficiently sensitive to detect early stage colorectal cancer, although a higher sensitivity has been reported for advanced-stage colorectal cancer.[7] Radioimmunoassays using tumor markers, such as carcinoembryonic antigen, also are not suitable for the detection of early cancer, although such tests can be used to monitor patients for an increasing tumor burden or tumor recurrence. Diagnosis by barium enema study and fiberoptic colonoscopy is accurate but time-consuming, expensive, and invasive. Therefore, an urgent need exists to establish a sensitive, reliable, and noninvasive method for the detection of colorectal cancer at an early stage.

To date, several screening methods for colorectal cancer based on the detection of mutated DNA in feces have been reported.[8–20] These methods, however, are time-consuming and are not sufficiently sensitive. The major reason for this inaccuracy is the fact that

Abbreviations used in this paper: APC, adenomatous polyposis coli; MSI, microsatellite instability; OMIM, Online Mendelian Inheritance in Man.

0016-5085/05/$30.00
doi:10.1053/j.gastro.2005.10.007

nucleic acids in feces are derived from an enormous number and variety of bacteria and normal cells. Accordingly, the proportion of genes derived from cancer cells in feces is as low as 1%, at most.[9] This makes the application of gene-detecting methods difficult in clinical practice.

We previously reported that the expression of CD44 variants in exfoliated colonocytes isolated from feces according to the Percoll centrifugation method could serve as a noninvasive diagnostic marker for early colorectal cancer.[21] However, the repetition of the Percoll centrifugation method was found to distort the morphology of the exfoliated colonocytes. Accordingly, the sensitivity of this method also appeared to be unsatisfactory because of the low retrieval rate of the exfoliated colonocytes. Another study described a processing method that involved scraping or washing the stool's surface with a buffer to collect exfoliated colonocytes.[22] In the ascending colon, however, the feces remains unformed. Therefore, most cancer cells exfoliated from the walls of the ascending colon would be incorporated into the inner core of the feces during the course of its formation. Thus, recovering cancer cells that originated from the ascending colon might be difficult using methods that involve scraping or washing solid feces.

Under these circumstances, we succeeded in developing a new, very effective methodology that allows the simple isolation of exfoliated colonocytes from not only the surface but also the central portion of feces while maintaining the colonocytes' initial morphology. Currently, we are attempting to apply a molecular biologic tool to purified colonocytes exfoliated into feces to detect cells from early colorectal cancers, including right-side colon cancer.

Materials and Methods

Study Design

This was a prospective study conducted between December 2002 and August 2004. The study protocol was reviewed and approved by the Institutional Review Board of the National Cancer Center, Japan. Written informed consent was obtained from all patients and healthy volunteers. No modifications to the protocol procedures were made during the course of the study.

Study Population

A total of 116 patients with histologically confirmed colorectal cancer and 83 healthy volunteers were enrolled. The healthy volunteers consisted of 37 men and 46 women with no apparent abnormalities, such as adenoma or carcinoma (including hyperplastic polyps), found during a total colonoscopy performed at the National Cancer Center Research Center for Cancer Prevention and Screening. The median age of these volunteers was 58.4 years (range, 40–70 years). The characteristics of the patients and healthy volunteers are summarized in Table 1. All the patients with colorectal cancer had undergone surgical resection of their primary tumor at the National Cancer Center Hospital, Tsukiji, or at Hospital East, Kashiwa, Japan. The median age of the patients was 62.0 years (range, 32–82 years). There were 69 men and 47 women patients. The primary tumors were located in the following sites: rectum in 53 patients, sigmoid colon in 21 patients, descending colon in 7 patients, transverse colon in 6 patients, ascending colon in 23 patients, and cecum in 6 patients. The clinical stage of the patients according to Dukes' classification was as follows: Dukes' stage A in 30 patients, stage B in 31 patients, stage C in 53 patients, and stage D in 2 patients.

Table 1. Characteristics of Patients and Healthy Volunteers

Characteristic	Patient (N = 116)	Healthy volunteer (N = 83)
Age, *y*		
Mean	62.0	58.4
Range	32–82	40–70
Sex, no (%)		
Male	69 (59.5)	37 (44.6)
Female	47 (40.5)	46 (55.4)
DNA, ng/gram of stool		
Mean	570.8	175.3
Range	2.0–7462.8	0.2–1907.5
Tumor location, no (%)		
Cecum	6 (5.2)	
Ascending colon	23 (19.8)	
Transverse colon	6 (5.2)	
Descending colon	7 (6.0)	
Sigmoid colon	21 (18.1)	
Rectum	53 (45.7)	
Size, mm		
Mean	40.0	
Range	4.0–120.0	
Histology, no (%)		
W/D	55 (47.4)	
M/D	56 (48.3)	
P/D	2 (1.7)	
Mucinous carcinoma	2 (1.7)	
Carcinoid tumor	1 (0.9)	
Depth, no (%)		
T1	10 (8.6)	
T2	32 (27.6)	
T3	71 (61.2)	
T4	3 (2.6)	
Dukes' stage, no (%)		
A	30 (25.9)	
B	31 (26.7)	
C	53 (45.7)	
D	2 (1.7)	

W/D, Well-differentiated adenocarcinoma; M/D, moderately differentiated adenocarcinoma; P/D, poorly differentiated adenocarcinoma.

Stool Samples

Before surgical resection, stool samples were obtained from 116 patients with colorectal cancer. Stool sam-

ples were also obtained from 83 healthy volunteers a few weeks after they had undergone a total colonoscopy. Naturally evacuated feces from subjects who had not taken laxatives were used as stool samples. Each patient was instructed to evacuate into a polystyrene disposable tray (AS one, Osaka, Japan) measuring 5 × 10 cm in size at home and bring the sample to the reception counter at the outpatient clinic or the Cancer Prevention and Screening Center of the National Cancer Center. The samples were collected and transferred to a laboratory at which they were allowed to stand at room temperature. Preparation of the stool samples for examination was conducted within 1–6 hours after the evacuation.

Magnetic Beads

Dynabeads Epithelial Enrich are uniform, superparamagnetic, polystyrene beads (4.5-μm diameter) coated with a mouse IgG1 monoclonal antibody (mAb Ber-EP4) specific for the glycopolypeptide membrane antigen Ep-CAM, which is expressed on most normal and neoplastic human epithelial tissues (Dynal, Oslo, Norway). Ep-CAM is widely expressed in the highly proliferative cells of the intestinal epithelium, from the basal cells to cells throughout the crypts at the basolateral membranes, and only the apical membrane facing the lumen is negative. The development of adenomas has been reported to be associated with increased Ep-CAM expression, and Ep-CAM over expression (mAb GA733) has frequently been demonstrated in colorectal carcinomas.[23–25]

Simulation Studies

A series of simulation studies were conducted to establish the optimal conditions for retrieving HT-29 colorectal cancer cells from feces. Feces from healthy volunteers were divided into several portions, each of which was seeded with 100 μL HT-29 cells (1×10^6/approximately 5 g feces). The cells were retrieved under several different conditions as follows: use of a Hank's solution and 25 mmol/L Hepes buffer (pH 7.35); processed feces of 5, 10, or 30 g volume; filter with a pore size of 48, 96, 512, or 1000 μm; incubation of homogenized solution with magnetic beads at 4°C or room temperature; application of 20, 40, 80, 200, or 400 μL magnetic beads; incubation of homogenized solution with magnetic beads under gentle rolling at 15 rounds/minute in a mixer for 10, 20, 30, or 40 minutes; and the reaction time between the cell-magnetic bead complexes and a magnet on a shaking platform for 0, 2, 10, 20, 30, 40, 50, or 60 minutes. Finally, the cell retrieval rate calculated for the magnetic beads method under the conditions determined to be the most suitable for this simulation study was compared with that calculated for the Percoll centrifugation method. The retrieval rate was calculated by dividing the number of cells that bound to the retrieved beads by the number of cells initially added to the feces. The cells were counted using a NucleoCounter (ChemoMetec A/S, Allerød, Denmark).

Isolation of Exfoliated Cells From Feces

The procedure was conducted using the most suitable and optimal conditions determined by the simulation study (Figure 1). Approximately 5–10 g of naturally evacuated feces were used to isolate exfoliated cells. Feces were collected into Stomacher Lab Blender bags (Seward, Thetford, United Kingdom). The stool samples were homogenized with a buffer (200 mL) consisting of Hank's solution, 10% fetal bovine serum (FBS), and 25 mmol/L Hepes buffer (pH 7.35) at 200 rpm for 1 minute using a Stomacher (Seward). The homogenates were then filtered through a nylon filter (pore size, 512 μm), followed by division into 5 portions (40 mL each). Subsequently, 40 μL of magnetic beads were added to each homogenized solution portion, and the mixtures were incubated for 30 minutes under gentle rolling in a mixer at room temperature. The samples on the magnet were then incubated on a shaking platform for 15 minutes at room temperature. Colonocytes isolated from 5 tubes were smeared onto slides and then stained using the Papanicolaou method. The remainder of the samples was centrifuged, and the sediments were stored at −80°C until DNA extraction.

Extraction of DNA

Fresh tissue samples were obtained from the surgically resected specimens of 116 patients with colorectal cancer. The samples were snap frozen in liquid nitrogen within 20 minutes of their arrival at the pathologic specimen reception area and were stored in liquid nitrogen until analysis.

Genomic DNA was extracted from each tumor tissue specimen using a DNeasy kit (QIAGEN, Valencia, CA). Genomic DNA was also extracted from colonocytes isolated from feces using the SepaGene kit (Sanko-Junyaku, Tokyo, Japan).

Direct Sequence Analysis

Direct sequencing was conducted to identify mutations in the APC codon 1270–1594, in codons 12 and 13 of the K-*ras* gene, and in exons 5, 6, 7, and 8 of the p53 gene.

The PCR primers used in this study were as follows: APC (5′-AAACACCTCAAGTTCCAACCAC-3′, 5′-GGTAATTTTGAAGCAGTCTGGGC-3′); K-*ras* (5′-CTGGTGGAGTATTTGATAGTG-3′, 5′-CCCAAGGAAAGTAAAGTTC-3′); p53 exon 5 (5′-GCCGTCTTCCAGTTGCTTTAT-3′, 5′-CCAAATACTCCACACGCAAAT-3′); p53 exon 6 (5′-CATGAGCGCTGCTCAGATAG-3′, 5′-TGCACATCTCATGGGGTTATAG-3′); p53 exon 7 (5′-CTTGGGCCTGTGTTATCTCCTA-3′, 5′-AAGAAAACTGAGTGGGAGCAGT-3′); and p53 exon 8 (5′-ACCTCTTAACCTGTGGCTTC-3′, 5′-TACAACCAGGAGCCATTGTC-3′).

The sequence primers used in this study were as follows: APC (5′-CAAAAGGCTGCCACTTGCAAAG-3′, 5′-AAAATAAAGCACCTACTGCTG-3′, 5′-GAATCAGCCAGGCACAAAGC-3′); K-*ras* (5′-CTGGTGGAGTATTTGATAGTG-3′); p53 exon 5 (5′-CCAAATACTCCACACGCAAAT-3′); p53 exon 6 (5′-CATGAGCGCTGCTCAGATAG-3′); p53 exon 7 (5′-AAGAAAACTGAGTGGGAGCAGT-3′); and p53 exon 8 (5′-

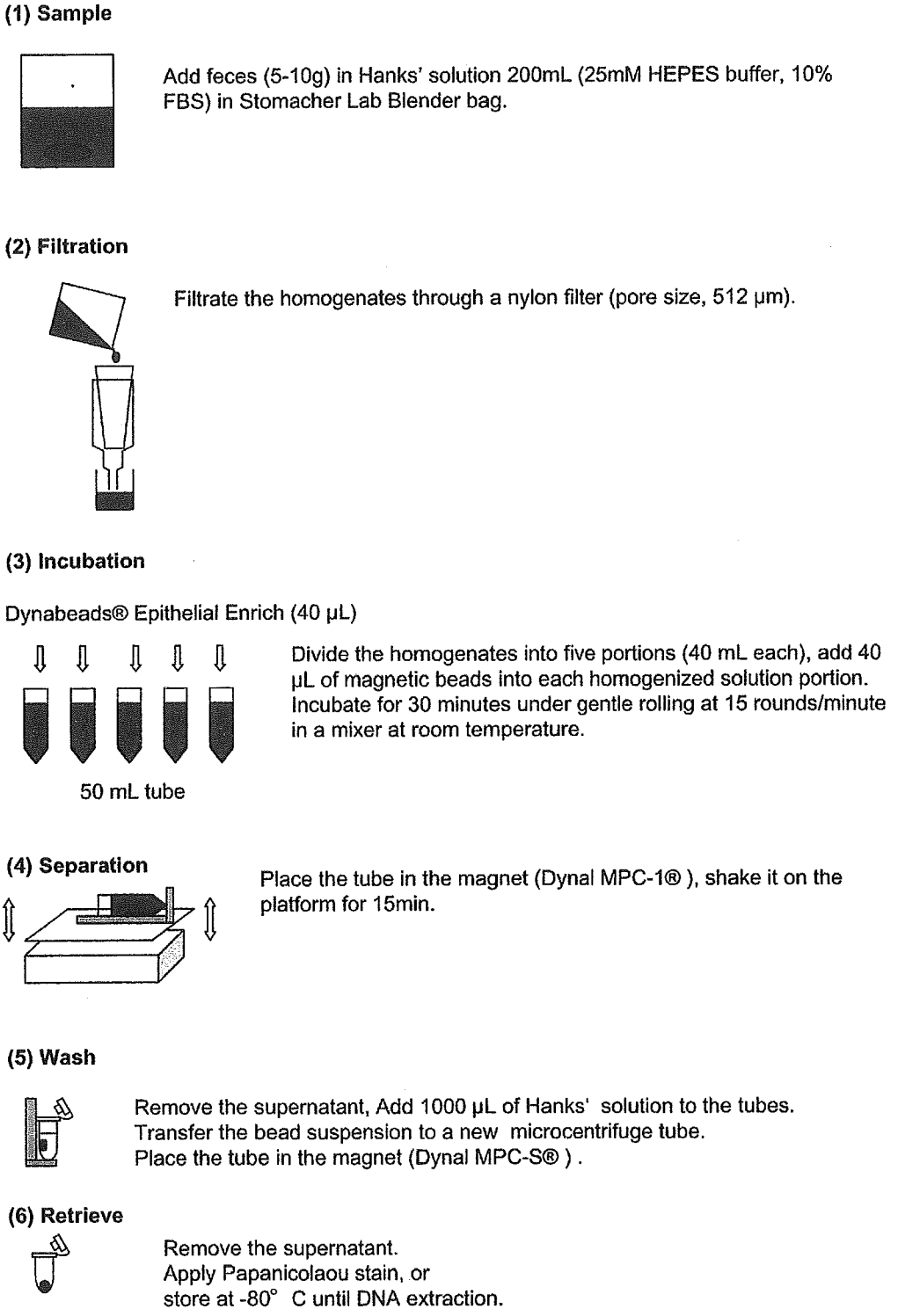

Figure 1. Schematic of procedure for isolating colonocytes from feces.

ACCTCTTAACCTGTGGCTTC-3′). Each fragment was sequenced by direct sequencing using the Big Dye Terminator v 3.1/1.1 cycle kit (Applied Biosystems, Forester City, CA).

All obtained sequences were aligned with previously published sequences (National Center for Biotechnology Information [NCBI] Genbank accession No. M74088 [APC], M54968 [K-*ras*], and X54156 [p53]) for each of the target genes and were analyzed using Phred/Phrp/DNASIS pro (Hitachi Software Engineering, Tokyo, Japan). The presence and nature of each mutation were confirmed by repeated PCR and sequencing.

BAT26

The BAT26 gene, an indicator of microsatellite instability (MSI), was amplified by PCR. Each fragment was elec-

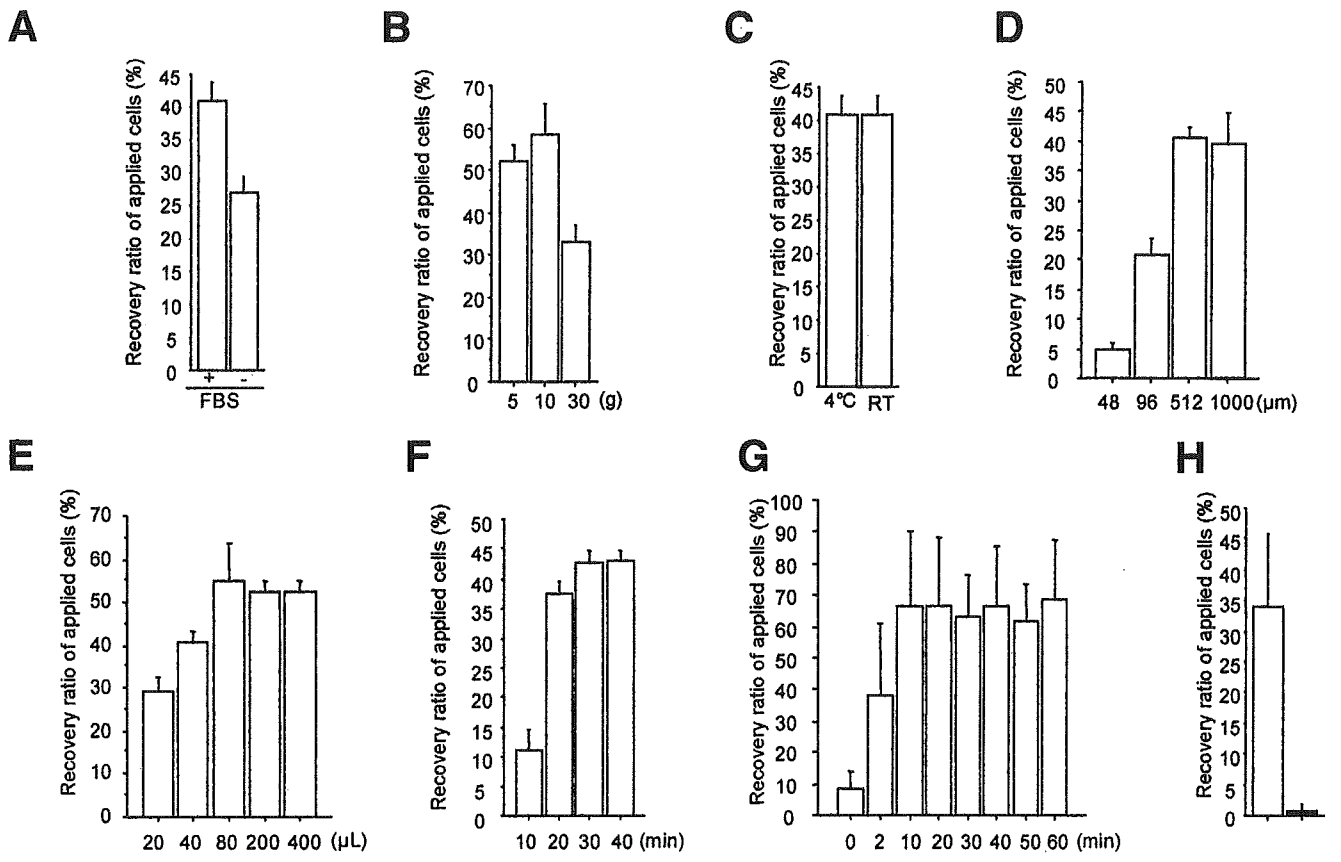

Figure 2. Simulation study to establish the optimal conditions for retrieving HT-29 colorectal cancer cells from feces and to compare the cell retrieval rates for the magnetic beads methods and the Percoll centrifugation method. Feces from healthy volunteers were divided into several portions, each of which was seeded with 100 μL HT-29 colorectal cancer cells (1×10^6/approximately 5 grams of feces). The procedure for retrieving the HT-29 cells was conducted under various conditions as follows: (*A*) homogenizing buffer with or without FBS; (*B*) stool weight (5, 10, or 30 g); (*C*) temperature during the cell-yielding procedure (4°C or room temperature); (*D*) filter pore size (48, 96, 512, or 1000 μm); (*E*) volume of applied magnetic beads (20, 40, 80, 200, or 400 μL); (*F*) incubation time of the homogenized solution with the magnetic beads under gentle rolling in a mixer (10, 20, 30, or 40 minutes); and (*G*) reaction time for the cells-magnetic bead complexes and the magnet on the shaking platform (0, 2, 10, 20, 30, 40, 50, or 60 minutes). The cell retrieval ratio (%) was calculated using the following formula: 100 × number of HT-29 cells retrieved/number of applied HT-29 cells. (*H*) Comparison of cell retrieval rates for the magnetic beads methods (*open column*) and the Percoll centrifugation method (*solid column*).

trophoresed using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) and then analyzed by GeneScan v 3.7 (Applied Biosystems). The PCR primers used in this study were 5′-TGACTACTTTTGACTTCAGCC-3′ and 5′-AACCATTCAACATTTTTAACCC-3′.

Cytology

Colonocytes isolated from feces were examined by 2 experienced cytotechnologists after Papanicolaou staining.

Study Blinding

We followed the guidelines of our medical institution for preparing blinded samples. Technicians processed the stool samples and prepared the slides for cytology and the cell pellets for DNA extraction. The samples were blinded to prevent the identification of individuals and the samples' origins. Two cytologists assessed the blinded samples, and the Life Science Group of Hitachi, Ltd, analyzed the DNA sequences.

Statistical Analysis

A Fisher exact test was used to compare all proportions. All reported P values are 2-sided. A value of $P < .05$ was considered statistically significant.

Results

Simulation Studies

The cell retrieval rate was found to decrease when Hank's solution without FBS was used, thus indicating the effectiveness of adding serum to the homogenizing buffer (Figure 2*A*). The cell retrieval rate was found to decrease when more than 30 g of feces were processed (Figure 2*B*). The cell retrieval rates were similar when incubation was conducted at room temperature and at 4°C (Figure 2*C*). Filtering of the stool suspension with the 48- or 96-μm filter resulted in significant clogging and thus hampered cell retrieval. However, a lot of fecal

residue remained after filtering with the 1000-μm filter, hindering the handling of the stool suspension thereafter. We therefore decided to use the 512-μm filter (Figure 2*D*). The dose of the magnetic beads applied was also examined. The cell retrieval rate increased in a dose-dependent manner up to 80 μL. In reality, a sufficient amount of genomic DNA derived from exfoliated colonocytes was obtained, even when 40 μL of magnetic beads were used (Figure 2*E*). Regarding the optimal incubation time of the magnetic beads for the complete binding of HT-29 cells to the beads, 30 minutes of incubation was found to be sufficient for the satisfactory binding of HT-29 cells to the beads (Figure 2*F*). For the retrieval of the cell-magnetic bead complexes on the magnet, a 10-minute reaction period was sufficient (Figure 2*G*).

The cell retrieval rates were 0.8% and 33.5% using the Percoll centrifugation method and the magnetic beads method, respectively, thus underscoring the advantage of the magnetic beads method (Figure 2*H*).

Cytology

Atypical cells were observed in colonocytes isolated from the feces of 32 of 116 patients with colorectal cancer, with a sensitivity rate of 28% (95% CI: 20–37; Table 2, Figure 3*A* and 3*B*). No atypical cells were observed in any of the 83 healthy volunteers, with a specificity rate of 100% (95% CI: 96–100). A significant difference ($P < .0001$) was found in the positivity rate between the patient group and the healthy volunteer group. The sensitivity rates for Dukes' A, B, and C or D colorectal cancers were 23% (7 of 30; 95% CI: 10–42), 32% (10 of 31; 95% CI: 17–51), and 27% (15 of 55; 95% CI: 16–41), respectively. No significant differences in the positivity rates were found among any of the stages. Furthermore, the sensitivity rates for cancers on the right side of the colon, including the cecum, ascending colon, and transverse colon, and for those on the left side of the colon, including the descending colon, sigmoid colon, and rectum, were 9% (3 of 35; 95% CI: 2–23) and 36% (29 of 81; 95% CI: 25–47), respectively. Therefore, the positivity rate was significantly higher for cancers on the left side of the colon ($P < .01$).

DNA Analysis

Overall analysis of stool samples. Sequence analysis showed distinct mutations in each of the analyzed genes in the tumor tissue and colonocytes isolated from feces (Figure 3*C–F*). Genetic alterations were observed in the colonocytes isolated from the feces of 82 of the 116 patients with colorectal cancer, yielding a sensitivity rate of 71% (95% CI: 62–79; Table 2). However, 10 of the 83 healthy volunteers were also positive for genetic alterations, producing a specificity value of 88% (95% CI: 79–94). A significant difference ($P < .0001$) was noted in the positivity rates of the patient group and the healthy volunteer group.

Genetic alterations were observed in 18 of the 30 patients with Dukes' A colorectal cancer, yielding a sensitivity rate of 60% (95% CI: 41–77). Furthermore, genetic alterations were observed among 26 of the 31 patients with Dukes' B colorectal cancer (84%; 95% CI: 66–95) and 38 of the 55 patients with Dukes' C or D colorectal cancer (69%; 95% CI: 55–81). No significant difference in sensitivity was found among any of the stages.

Genetic alterations were observed in colonocytes isolated from feces in 20 out of 35 patients with cancers originating on the right side of the colon (57%; 95% CI: 39–74) and in 62 out of 81 patients with cancers originating on the left side of the colon (77%; 95% CI: 66–85). No significant differences in the sensitivity rates were observed, although the sensitivity rate tended to be higher for cancers on the left side of the colon.

DNA analysis limited to colonocytes isolated from the feces of patients with colorectal cancer tissue involving genetic alterations. We assessed the performance of the present methodology for isolating cancer cells by examining the positivity rate of genetic alterations in colonocytes isolated from the feces of patients who showed alterations in their cancer tissues (Table 3). Among the 116 patients, a total of 93 (80%; 95% CI: 72–87) exhibited genetic alterations in the APC, K-*ras*, or p53 genes or BAT26 positivity in their cancer tissue: 51 patients exhibited APC mutations (44%, 95% CI: 35–53), 33 patients exhibited K-*ras* mutations (28%; 95% CI: 20–38), 62 patients exhibited p53 mutations (53%; 95% CI: 44–63), and 6 patients exhibited BAT26 positivity (5%; 95% CI: 2–11). Among the 93 patients with genetic alterations in their cancer tissues, the alterations were also successfully detected in colonocytes isolated from the feces of 80 patients (86%; 95% CI: 77–92). Among the 39 patients with Dukes' C or D advanced cancer who exhibited a genetic alteration in their cancer tissues, 36 patients exhibited genetic alterations in colonocytes isolated from their feces (92%; 95% CI: 79–98). Furthermore, genetic alterations were detected in colonocytes isolated from the feces of 18 of 24 patients with Dukes' A cancer (75%; 95% CI: 53–90) and 26 of 30 patients with Dukes' B cancer (87%; 95% CI: 69–96). No statistically significant difference was found among these 3 groups. In addition, genetic alterations could be detected in colonocytes isolated from the feces of 20 of 27 patients with cancers originating on the

Table 2. Incidences of Genetic Alterations of the APC, K-*ras*, p53, and MSI (BAT26) Genes as Well as Results From Cytology in all Patients and Healthy Volunteers

		Patient				Healthy volunteer	
		Tumor tissue		Isolated cell		Isolated cell	
	Marker	No.	Positivity (%) (95% CI)	No.	Sensitivity (%) (95% CI)	No.	Specificity (%) (95% CI)
Overall Patients (n = 116), healthy volunteers (n = 83)	Combined marker	93	80 (72–87)	82	71 (62–79)	10	88 (79–94)
	APC	51	44 (35–53)	47	41 (32–50)	1	99 (93–100)
	K-*ras*	33	28 (20–38)	33	28 (20–38)	1	99 (93–100)
	p53	62	53 (44–63)	45	39 (30–48)	6	93 (85–97)
	BAT26	6	5 (2–11)	4	3 (1–9)	3	96 (90–99)
	Cytology			32	28 (20–37)	0	100 (96–100)
Dukes' stage A (n = 30)	Combined marker	24	80 (61–92)	18	60 (41–77)		
	APC	14	47 (28–66)	11	37 (20–56)		
	K-*ras*	6	20 (77–39)	5	17 (6–35)		
	p53	6	20 (77–39)	9	30 (15–49)		
	BAT26	1	3 (1–17)	1	3 (1–17)		
	Cytology			7	23 (10–42)		
Dukes' stage B (n = 31)	Combined marker	30	97 (83–100)	26	84 (66–95)		
	APC	17	55 (36–73)	17	55 (36–73)		
	K-*ras*	10	32 (17–51)	9	29 (14–48)		
	p53	18	58 (39–75)	13	42 (25–61)		
	BAT26	2	6 (1–21)	1	3 (1–17)		
	Cytology			10	32 (17–51)		
Dukes' stages C and D (n = 55)	Combined marker	39	71 (57–82)	38	69 (55–81)		
	APC	20	36 (24–50)	19	35 (22–49)		
	K-*ras*	17	31 (19–45)	19	35 (22–49)		
	p53	27	49 (35–63)	23	42 (29–56)		
	BAT26	3	5 (1–15)	2	4 (0–13)		
	Cytology			15	27 (16–41)		
Right-sided colon cancer (n = 35)	Combined marker	27	77 (60–90)	20	57 (39–74)		
	APC	11	31 (17–49)	8	23 (10–40)		
	K-*ras*	16	46 (29–63)	12	34 (19–52)		
	p53	17	49 (31–66)	11	31 (17–49)		
	BAT26	2	6 (1–19)	1	3 (1–15)		
	Cytology			3	9 (2–23)		
Left-sided colon cancer (n = 81)	Combined marker	66	81 (71–89)	62	77 (66–85)		
	APC	40	49 (38–61)	39	48 (37–60)		
	K-*ras*	17	21 (13–31)	21	26 (17–37)		
	p53	45	56 (44–67)	34	42 (31–53)		
	BAT26	4	5 (1–12)	3	4 (1–10)		
	Cytology			29	36 (25–47)		

right side of their colon (74%; 95% CI: 54–89) and 60 of 66 patients with cancers originating on the left side of their colon (91%; 95% CI: 81–97). A statistically significant difference was found between the right- and left-side colon cancer patient groups ($P = .03$).

Discussion

We have devised a simple, highly reliable methodology for isolating colorectal cancer cells from nonlaxative-induced, naturally evacuated feces from most patients with colorectal cancer. To date, several methods of isolating colorectal cancer cells from feces have been reported.[21,22,26,27]

Our new funnel-shaped filter system extensively improved the filtration efficiency of the stool suspension by enlarging the filtration area and selecting the optimal pore size; the system was capable of filtrating the entire stool suspension without filter clogging. These properties permit the omission of centrifugation and simplify the overall process because all steps can be performed at room temperature. Furthermore, the use of serum successfully increased the cell retrieval rate. We presume that this increase may be attributed to the suppression of protease activity or the inhibition of nonspecific reactions of the antibodies on the bead surface. Consequently, our new methodology also allows the extraction of high-quality DNA or RNA from exfoliated colonocytes. Very recently, Imperiale et al compared a panel of fecal DNA markers and Hemoccult II as screening tests for colorectal cancer. It is worth noting that, in their study, colonoscopy as a reference standard was used

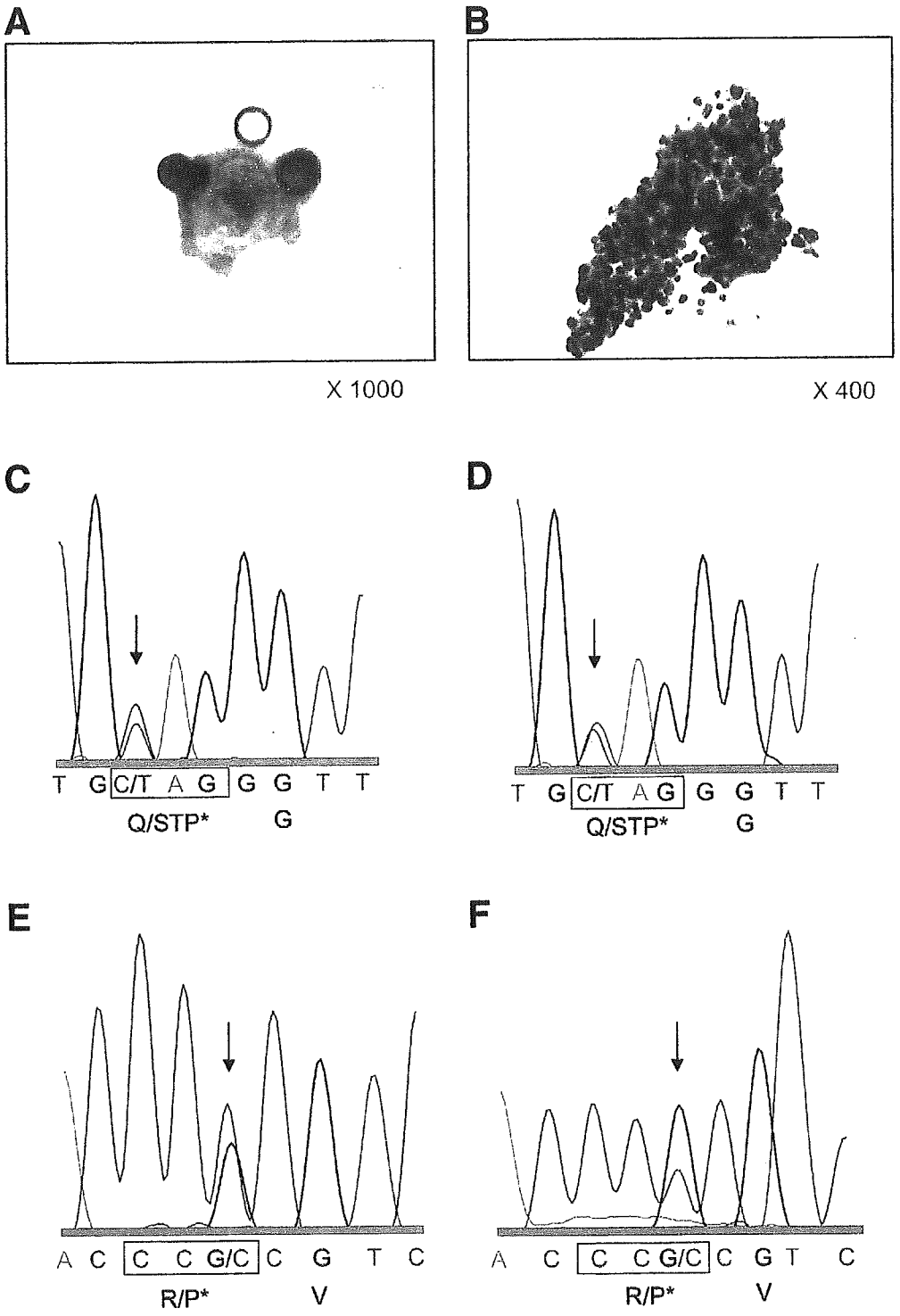

Figure 3. Cytology and DNA sequencing. Papanicolaou staining of colonocytes isolated from the feces of patients with colorectal cancer. (*A*) A patient with ascending colon cancer, Dukes' stage A. (*B*) A patient with rectal cancer, Dukes' stage C. Detection of mutations in tumor tissues and colonocytes isolated from the feces of patients with colorectal cancer. (*C*) A point mutation of the APC gene in a tumor tissue specimen obtained from a patient with rectal cancer, Dukes' stage B. (*D*) An identical mutation was detected in colonocytes isolated from the feces of the patient. (*E*) A point mutation of the p53 gene in a tumor tissue specimen obtained from a patient with ascending colon cancer, Dukes' stage A. (*F*) An identical mutation was detected in colonocytes isolated from the feces of the patient. *Wild/mutant.

in all subjects. They conducted those tests in a blinded fashion and showed that sensitivity of DNA analysis was 4-fold higher than that of Hemoccult test.[28] We believe that this report may prompt a study of fecal DNA test for colorectal cancer screening.

The idea to isolate cancer cells from feces originally derived from a study that described the abnormal expression of the CD44 gene in many tumors, including colon cancer and bladder cancer.[21,29,30] In the course of a series of studies, we predicted that normal mucous cells would die and be exfoliated during turnover and that the cancer cells would likely survive for a long time in the feces.

Although cytology is highly specific compared with direct sequence analysis, its sensitivity, especially for cancers on the right side of the colon is relatively low. From a technical aspect, our cytology method does not allow the

Table 3. Incidences of Genetic Alterations in Colonocytes Isolated From the Feces of Patients With Colorectal Cancer Tissue Involving Genetic Alterations of the APC, K-*ras*, p53, or MSI (BAT26) Gene

	Combined marker		APC		K-ras		p53		BAT 26	
	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)	No.	% (95% CI)
Overall	80/93	86% (77–92)	46/51	90% (79–97)	29/33	88% (72–97)	42/62	68% (55–79)	4/6	67% (22–96)
Dukes' stage A	18/24	75% (53–90)	11/14	79% (49–95)	5/6	83% (36–100)	5/6	83% (36–100)	1/1	100% (3–100)
Dukes' stage B	26/30	87% (69–96)	16/17	94% (71–100)	9/10	90% (56–100)	12/18	67% (41–87)	1/2	50% (1–99)
Dukes' stages C and D	36/39	92% (79–98)	19/20	95% (75–100)	15/17	88% (64–99)	21/27	78% (58–91)	2/3	67% (9–99)
Right-sided	20/27	74% (54–89)	8/11	73% (39–94)	12/16	75% (48–93)	11/17	65% (38–86)	1/2	50% (1–99)
Left-sided	60/66	91% (81–97)	38/40	95% (83–99)	17/17	00% (81–100)	31/45	69% (53–82)	3/4	75% (19–99)

NOTE. Number of positive cases in tumor tissue and colonocytes isolated from feces/number of positive cases in tumor tissue, with 95% confidence interval.

observation of cells unless there are 5×10^4 cells per slide. Technical improvements might increase the benefits of feces cytology. However, we believe that cytology is not suitable as a method for identifying cancer because of its low sensitivity, at least at present. From a practical point of view, we have conducted a study to determine the effect of the time and temperature after evacuation on the recovery rates of fecal colonocytes, and we have found that we can obtain almost the same number of colonocytes from stool materials 3 days after evacuation in comparison with 6 hours after evacuation if fecal material is kept at 4°C (data not shown). This observation may be important for the potential clinical application of this method.

Direct sequence analysis of colonocytes isolated from the feces of 83 healthy volunteers revealed mutations in 8 subjects (9%; 95% CI: 4–18), the breakdown of which was as follows: 1 APC1 mutation, 1 K-*ras* mutation, and 6 p53 mutations. Points of mutations identified of the p53, APC, and K-*ras* genes observed in the 83 healthy volunteers in this study were identical to that reported previously in tumors. These mutations of p53, APC, and K-*ras* in tumors are recorded in the database of OMIM. PCR errors were unlikely because multiple PCR reactions and sequence reactions were separately conducted. However, genetic alterations in precancerous lesions may have been present, although endoscopy findings macroscopically verified the absence of adenoma and carcinoma. The individuals in whom the present methodology revealed genetic alterations should be monitored to assess whether these findings were false-positive results or a predictor of tumorigenesis.

Oncogenes in feces are presumably derived from cancer cells exfoliated from the cancer tissue, and genetic alterations would not be detected in colonocytes isolated from feces if the original cancer tissue did not contain genetic alterations. In fact, among the 93 patients who exhibited genetic alterations in their cancer tissues, alterations were detected in colonocytes from the stools of 80 patients, producing a true sensitivity rate of 86% (80 of 93), although the present overall sensitivity was 71%. Furthermore, our methodology allows the isolation and retrieval of colorectal cancer cells from both early stage cancer and right-side colon cancer. Because the methodology allows processing at room temperature, we are currently constructing an automated, mechanized processing system on a commercial basis. A problem of our test was its relatively low specificity for a screening test as described previously. We consider that mutations observed in the healthy subjects might be attributable to the fact that they belonged to a high-risk group for colorectal cancer because these 83 volunteers were selected from among colonoscopy examinees recruited by the newly established National Cancer Center Research Center for Cancer Prevention and Screening, and the detection rate of cancers appeared to be considerably higher in the all examinees at the center than in the general population in Japan (unpublished observation). Therefore, we speculate that precancerous lesions with mutations of the genes tested might have been present in the colorectal epithelium of some of these healthy volunteers. We think that a prospective randomized study would be needed to determine the actual specificity of our method in a real screening population and to verify its clinical usefulness.

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Received May 24, 2005. Accepted August 31, 2005.

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Supported in part by a grant for research on advanced medical technology from the Ministry of Health, Welfare, and Labor and a research and development project of the Industrial Science and Technology Program supported by the New Energy and Industrial Technology Development Organization (NEDO) of Japan.