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厚生労働科学研究費補助金

第3次対がん総合戦略研究事業

消化器がん個別化医療におけるファンクショナルゲノミクス、プロテオミクス、メタボロミクスの臨床応用と治療体制の確立

平成19年度　総括・分担研究報告書

主任研究者　門田　守人

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厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
総括研究報告書

消化器がん個別化医療におけるファンクショナルゲノミクス、プロテオミクス、メタボロミクスの臨床応用と治療体制の確立

主任研究者　門田守人　大阪大学　副学長

研究要旨：本研究は臨床試験と分子生物学的情報解析のスパイラル的発展を主軸とし、消化器がんの個別化医療を確立するために計画された。一定のプロトコールにより集積された大規模症例を対象に、がん組織の遺伝子・タンパク発現プロファイルによるがんの転移・再発の予測診断系とがん患者の末梢血からメタボローム解析によるバイオマーカーを探索した。

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関本貢嗣　大阪大学消化器外科 准教授
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松原謙一　株式会社 ＤＮＡチップ研究所 代表取締役
西村紀　大阪大学蛋白質研究所 特任教授
山崎泰代　Phenomenome Discoveries Inc. 日本総括ディレクター

A. 研究目的

消化器がんの大規模症例を対象に、がん組織の遺伝子・タンパク発現プロファイルによるがんの転移・再発の予測診断系と、がん患者の末梢血からメタボローム解析によるバイオマーカーを探索し、臨床応用化することを目的とする。

B. 研究方法

消化器がん大規模症例の集積コンソーシアム遺伝子発現はDNAチップ、タンパク発現は NBS(2-nitrobenzenesulfenyl)法を用いてそれぞれの発現プロファイルを取得し、がんの転移・再発に関わる予測診断系を構築した。またがん患者の末梢血からフーリエ変換質量分析計を用いたメタボロミクス技術を用いてメタボライトバイオマーカを探索した。

（倫理面への配慮）

本研究ではゲノムは扱わないが、３省合同の「ゲノムに関する指針」に準じた情報管理を行い、大阪大学の倫理規定に従って、患者の同意が得られたサンプルを使用した。

C. 研究結果

症例集積コンソーシアムにより約 4000 例の消化器がん組織と臨床情報が集積された。このうち大腸癌 170 例、肝臓癌 100 例、胃癌 170 例の遺伝子発現プロファイルより、それぞれ異時性の転移予測が 75%、80%、75％可能な遺伝子群を同定した。また大腸癌 12

例の NBS 解析により、128 種類の関連蛋白を同定した。さらに大腸癌 83 例の血清メタボローム分析より、特異度 97%、感度 74% 結果が得られた。

D. 考察

遺伝子発現は RT-PCR 法で、タンパク発現はウェスタンブロット法でそれぞれデータの信頼性を実証した。今後は遺伝子とタンパクの相互関係をネットワーク解析することでより正確な予測診断系が期待される。また血清レベルでもメタボライトマーカーによる早期結腸直腸癌リスクを評価する可能性が示され、OMICS 技術の臨床応用が期待される。

E. 結論

本研究では、がんの遺伝子・タンパクの両者から得られた基礎的研究の成果と、がん患者の末梢血のメタボローム解析によって得られた特異的メタボライト発現パターンの結果を、臨床研究デザインに合わせ prospective に解析することで、トランスレーショナルリサーチとして十分な evidence が得られることが期待される。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
特になし

2. 実用新案登録
特になし

3. その他
特になし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

消化器がん個別化医療におけるファンクショナルゲノミクス、プロテオミクス、メタボロミクスの臨床応用と治療体制の確立

分担研究者　松原謙一　株式会社ＤＮＡチップ研究所　代表取締役社長

研究要旨：消化器がん個別化医療におけるファンクショナルゲノミクスの確立

A.　研究目的

大腸がんを対象に用いてファクショナルゲノミクスを行う。

B.　研究方法

切除大腸がん試料からＲＮＡを抽出し発現プロファイルを解析する。

C.　研究結果

正常組織と対比することにより、がん特異的遺伝子発現プロファイルを入手した。

D.　考察

本解析を進めることにより、がん切除患者の予後予測が行えるようになると考える。

E.　結論

大腸がん切除試料のＲＮＡを解析しそのプロファイルの特徴を入手した。これをもとに将来予後予測判別に進むことができると考える。

F.　健康危険情報

なし

G.　研究発表

1.　論文発表

なし

2.　学会発表

なし

H.　知的財産権の出願・登録状況

1.　特許取得

なし

2.　実用新案登録

なし

3.　その他

なし

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

NBS法による大腸癌組織の蛋白質発現解析

分担研究者　西村 紀　大阪大学蛋白質研究所　特任教授

研究要旨： NBS(2-nitrobenzenesulfenyl)法による大腸癌組織(12症例)でのプロテオミクス解析を行い、大腸癌関連蛋白質を128種類同定した。同定した大腸癌関連蛋白質のうち、6種類(Zyxin(ZYX), RAS related nuclear protein(RAN), Reticulocalbin 1(RCN1), S-adenosylhomocystein dehydrogenase(AHCY), Galectin-1(LGALS1), Vimentin(VIM))について、ウェスタンブロッティング、および免疫組織化学染色を行い、これら6種類の蛋白質が癌関連蛋白質であることを実証した。

A. 研究目的

新規大腸癌関連蛋白質を同定し、バイオマーカー(診断、予後予測、治療ターゲットなど)への応用を目的とする。

B. 研究方法

NBS(2-nitrobenzenesulfenyl)法を用いて大腸癌組織(12症例)の蛋白質発現解析を行った。新規に同定された大腸癌関連蛋白質のうち、6種類の蛋白質(ZYX, RAN, RCN1, AHCY, LGALS1, VIM)についてウェスタンブロッティングおよび、免疫組織化学染色によるバリデーション解析を行った。

（倫理面への配慮）

大阪大学の倫理規定に従って、患者の同意が得られたサンプルを使用した。

C. 研究結果

12症例の大腸癌組織の蛋白質発現解析により大腸癌関連蛋白質を128種類同定した。同定された大腸癌関連蛋白質のうち、絞り込んだ6種類の蛋白質について、ウェスタンブロットによる解析でプロテオミクスデータとの相関が取れ、更には、免疫組織化学染色により癌組織における間質細胞および癌細胞での高発現が確認された。

D. 考察

ウェスタンブロット解析により、プロテオミクスデータの信頼性を実証できた。本解析では、特に細胞外や細胞膜に発現する蛋白質が多数同定されたことから、本研究で同定された蛋白質にはバイオマーカーに応用可能な分子が多数含まれていると考えられる。更には、今回選んだ6種類の蛋白質については、大腸癌と蛋白質の発現相関を新規に実証することができた。

E. 結論

NBS 法によるプロテオミクス解析により新規マーカー候補になりうる癌関連蛋白質を同定できることを実証できた。更には、6 種類の蛋白質の発現が大腸癌と正の相関があることが示され、今後のバイオマーカーへの応用が期待される。

F. 健康危険情報

特になし

G. 研究発表

1. 論文発表

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H. 知的財産権の出願・登録状況

(予定を含む。)

1. 特許取得

特になし

2. 実用新案登録

特になし

3. その他

特になし

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

結腸直腸癌と関連する各種バイオマーカーの評価・探索

分担研究者　山崎泰代　Phenomenome Discoveries Inc.（PDI）ディレクター

研究要旨：臨床血清試料を用いて、結腸直腸癌と関連する各種バイオマーカーの評価と探索を行う。

A.　研究目的

結腸直腸癌患者の予後に、肝転移、肺転移が与える影響は大きい。転移が予測できるバイオマーカーが発見できれば、臨床的意義は非常に大きい。

B.　研究方法

肝転移、肺転移する結腸直腸癌患者の血清試料を用いて、PDI 独自のフーリエ変換質量分析計を用いたメタボロミクス技術を用いてデータ解析を行い、転移に関与している代謝物を探索する。

またすでに、PDI にて発見･開発された結腸直腸癌に特異な新規バイオマーカー（新規ビタミン E 誘導体）の三連四重極質量分析計を用いたスクリーニング法を用いて、結腸直腸癌患者、手術前の 46 例、手術後の 37 例と正常対象群 35 例の計 118 例の血清試料の分析を行い、その評価を行った。

（倫理面への配慮）

試料回収は大阪大学医学部にて倫理指針に沿って行われている。

C.　研究結果

肝転移患者、手術前・後、各 30 例、肺転移患者、手術前・後、各 20 例、転移を起こさない結腸直腸癌患者、手術前･後、各 50 例、正常対象群、50 例の、計 250 例の血清試料の回収が 1 年間で完了した。

PDI の新規バイオマーカーを用いた評価では、特異度 97%、手術前試料の感度 74%、手術後試料の感度 68%という結果が得られた。

D.　考察

PDI の新規バイオマーカーは、腫瘍除去によっても、代謝システムの欠乏が元に戻らないことを示している。これは、臨床的に検出される腫瘍の発生以前に、このシステムの欠乏が先行していることを示し、さらに、早期結腸直腸癌リスクを評価する新規の方法である可能性がある。

E.　結論

転移予測のバイオマーカー探索については、今後、サンプル解析が行われる。メタボロミクスという生体内低分子化合物の研究を網羅的に行うことにより、バイオマー

カーとしての利用のみならず、生化学的に病気のシステムを理解することまで可能になると考えられる。

F. 健康危険情報

特になし

G. 研究発表

1. 論文発表

特になし

2. 学会発表

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H. 知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得

特になし

2. 実用新案登録

特になし

3. その他

特になし

別紙4

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

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14) 九山浩樹,他	総説: 安定同位体技術 プロテオーム解析のための安定同位体標識試薬	RADIOISOTOPES	56(6)	303-314	2007

Gene expression of colorectal cancer: Preoperative genetic diagnosis using endoscopic biopsies

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Abstract. In colorectal cancer, to predict the response to chemo- and/or radio-therapy or the existence of lymph node metastasis preoperatively, a more competent diagnostic system is required, in addition to conventional diagnosis based on morphology and pathology. The application of gene expression profiling to preoperative cancer diagnosis using endoscopic biopsies could enable the selection of a more appropriate therapy for patients. In this study, we evaluated the feasibility of gene expression profiling using preoperative biopsies of colorectal tumors in a clinical setting, by investigating the influence of intra-tumor heterogeneity on the profiles and testing the prediction ability of tumor malignancy. Under endoscopic examination, two biopsies were sampled from each of 10 colorectal cancers and 10 adenomas, and their gene expression data were obtained using cDNA microarrays. The intra- and inter-tumor heterogeneities of the profiles were compared with unsupervised clustering analysis. Molecular prediction of tumor malignancy using biopsies was performed with the supervised classification algorithm. In clustering analysis, almost all paired biopsies from the same tumors joined each other. Pearson's correlation coefficients of the profiles between biopsies from the same tumors (mean, 0.83) were significantly greater than those of the profiles between biopsies from other cancers (mean, 0.58) ($p<0.0001$). In the supervised classification method, malignancy was correctly predicted in 39 out of 40 biopsies with 8-71 informative genes. Gene expression profiling using endoscopic biopsies of colorectal tumors revealed that the intra-tumor heterogeneity was smaller than the inter-tumor heterogeneity and tumor malignancy was correctly predicted. Our findings suggest that the technique of gene expression profiling accurately represents the biological properties of colorectal cancer and could help the preoperative diagnosis of this disease.

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Abbreviations: CRC, colorectal cancer; HCA, hierarchical clustering analysis; SNR, signal-to-noise ratio; IHC, immunohistochemical staining; pAb, polyclonal antibody; mAb, monoclonal antibody; FNAB, fine needle aspiration biopsy

Key words: colorectal cancer, gene expression profile, microarray, biopsy, tumor heterogeneity

Introduction

The incidence of colorectal cancer (CRC) is increasing and it is one of the leading causes of cancer death in Japan (1). Conventional diagnosis based on morphology and pathology, such as Dukes' classification and the tumor-node-metastasis (TNM) staging system, has played an important role in the clinical decision-making and evaluation of prognosis for CRC (2-4). However, it is difficult to differentiate the response to chemo- and/or radio-therapy or the existence of lymph node metastasis preoperatively by conventional diagnosis. To predict such individual heterogeneous cancers' characteristics preoperatively, a more competent diagnostic system is required.

Comprehensive gene expression assay using microarray technology has provided insights into cancer pathogenesis and is expected to help to fulfil the clinical demands for individualized medicine (5). The discovery of a set of new molecular markers, which can classify cancers according to their properties using surgical specimens, has been reported in various cancers (6). In CRC, this technology has been used to elucidate the mechanisms involved in carcinogenesis (7-16) as well as to predict various clinicopathological aspects, such as recurrence after surgery (17-22). However, in clinical practice, this fruitful molecular prediction using surgical specimens will be limited to the selection of postoperative medicine such as adjuvant therapy and follow-up schedules. On the other hand, analysis using preoperative endoscopic biopsies, instead of

Table I. Patients and tumor characteristics.

Case	Age (years)	Sex	Tumor location	Tumor size (cm)	Tumor type	TNM staging		
						T	N	Stage
Ca1	67	M	D	2.0	Well differentiated adenocarcinoma	T1	N0	I
Ca2	55	F	R	1.5	Well differentiated adenocarcinoma	T1	N0	I
Ca3	69	F	R	5.6	Well differentiated adenocarcinoma	T2	N0	I
Ca4	59	F	R	3.5	Moderately differentiated adenocarcinoma	T2	N0	I
Ca5	66	M	R	5.5	Moderately differentiated adenocarcinoma	T2	N0	I
Ca6	48	M	S	2.8	Moderately differentiated adenocarcinoma	T2	N0	I
Ca7	73	F	S	5.7	Well differentiated adenocarcinoma	T3	N0	II
Ca8	55	M	R	3.8	Moderately differentiated adenocarcinoma	T3	N0	II
Ca9	67	F	R	3.4	Moderately differentiated adenocarcinoma	T3	N0	II
Ca10	60	F	R	3.0	Well differentiated adenocarcinoma	T2	N1	III
Ad1	60	M	S	4.0	Tubulovillous adenoma			
Ad2	74	M	S	2.5	Tubular adenoma			
Ad3	77	M	A	2.5	Tubulovillous adenoma			
Ad4	68	M	T	1.1	Tubular adenoma			
Ad5	62	F	A	1.0	Tubulovillous adenoma			
Ad6	60	M	D	2.0	Tubulovillous adenoma			
Ad7	72	M	R	0.8	Tubulovillous adenoma			
Ad8	61	M	R	0.8	Tubular adenoma			
Ad9	64	F	S	1.2	Tubular adenoma			
Ad10	78	F	S	1.8	Tubular adenoma			

M, male; F, female; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum.

surgically resected samples, would widen the utility of microarray technology.

In rectal cancer, preoperative rather than postoperative chemo- and/or radio-therapy reduces local recurrence after surgery (23). Using preoperative biopsies under colonoscopic examination, prediction of the response to preoperative chemo- and/or radio-therapy would be useful for the selection of patients who would most benefit from preoperative therapies aimed at a better prognosis or improved chances of sphincter preservation (24,25). The prediction of lymph node metastasis could contribute to the avoidance of unnecessary surgery for early invasive CRC. This is clinically important since lymph node metastasis is found in only approximately 10% of early invasive CRCs (26); the remaining 90% without metastasis are more suited to undergo local excision such as colonoscopic resection or transanal endoscopic microsurgery.

To determine the clinical importance of diagnosis based on gene expression profiles using preoperative endoscopic biopsies in CRC, we investigated the data quality from low volume samples and the influence of intra-tumor heterogeneity on the profiles. Wide differences in profiles within a tumor would hinder the adoption of this technique. There are no reports that compared intra- and inter-tumor heterogeneity in expression profiles in colorectal tumors. In the present study, we sampled two biopsy specimens from each of 10 cancers and 10 adenomas obtained under colonoscopic examinations, and determined their gene expression profiles using cDNA microarrays. By comparing their profiles, we assessed the intra-tumor heterogeneity in colorectal tumors. Moreover, as a first step for clinical applicability, by testing the molecular prediction ability of tumor malignancy, we investigated whether this technique, employing preoperative endoscopic biopsies, allows characterization of the biological properties of colorectal cancer.

Materials and methods

Patients and sample collection. From each of the 10 cancers and 10 adenomas, two biopsy specimens were obtained under colonoscopic examination. The clinicopathological data of patients and their tumors are summarized in Table I. Six bulky samples of the same cancers and 8 normal colorectal epithelium tissues were obtained from surgically resected specimens. None of the adenomas contained a cancerous component. Samples were stored in RNAlater (Qiagen, Valencia, CA) at -20°C after sampling until RNA extraction. This study was approved by the Institutional Review Board of Osaka University and Minoh City Hospital, and informed consent was obtained from all patients.

RNA extraction. Total RNA was extracted from biopsy samples using an RNeasy kit (Qiagen). The average volume of extracted total RNA from one biopsy was 21.1 μg in cancers and 22.6 μg in adenomas. From bulky samples, total RNA was extracted

using Trizol reagent (Invitrogen, Carlsbad, CA) and cleaned using the RNeasy kit. The quality of extracted total RNA was checked by 0.8% agarose-gel electrophoresis and a LabChip kit (Agilent Technologies, Palo Alto, CA), and the quantity was determined with a spectrophotometer. The total RNA solution was stored at -80°C until use.

Hybridization to cDNA microarray. We used cDNA microarrays containing 4608 clones which were derived from 30,000 clones expressed in CRC tissues (10,16,22). The Pearson's correlation coefficient of the data from replicated samples using our microarrays was 0.95. Gene expression data were obtained using a previously described method (10), with some improvements. As a standard normal control reference, a mixture of total RNA extracted from 40 normal colorectal epithelia was used. Labeled cDNA targets for hybridization were synthesized by reverse transcription from standard- and sample-total RNA respectively, with the indirect labeling method.

For each reverse transcription, 12 μg of total RNA was mixed with 1 μg of oligo-dT primer (Invitrogen) in a total volume of 15.5 μl, heated to 70°C for 10 min and cooled for 5 min. The mixture consisted of 6 μl of 5X first strand buffer, 3 μl of 0.1 M DTT, 3 μl of nucleotide cocktail (5 mM each dATP, dCTP, and dGTP, 3 mM dTTP and 2 mM aminoallyl-dUTP) and 0.5 μl of 40 U/μl RNase inhibitor was added. After incubation for 2 min at 42°C, 2 μl of 200 U/μl Superscript II reverse transcriptase (Invitrogen) was added. After incubation for 1 h at 42°C, 3.3 μl of 0.5 M EDTA was added and the RNA strand was degraded with 3.3 μl of 2 N NaOH and incubation at 70°C for 20 min. The mixture was neutralized with 3.3 μl of 2 N HCL and 60 μl of distilled water. This cDNA was purified using a QIAquick kit (Qiagen) and dried cDNA was dissolved in 9 μl of 0.2 M $NaHCO_3$-Na_2CO_3 (pH 9.0). Cy-dye solution (1 μl) (Amersham, Piscataway, NJ), Cy3 for the standard target, and Cy5 for the tested target, were added and incubated under shade at room temperature for 1 h, respectively. After purification using Micro Bio-Spin Columns P-30 Tris (Bio-Rad, Hercules, CA), the separately synthesized Cy3- and Cy5-labeled targets were combined and purified using Microcon YM-30 (Millipore, Billerica, MA), concentrated to a volume of 16.5 μl. To the concentrated target, 3.5 μl of human COT-1 DNA (Invitrogen), 7 μl of 20X SSC, 7 μl of 20X Denhart's solution, and 1 μl of 10% SDS were added. The 35 μl of target mixture was denatured by heating for 2 min at 95°C and cooled on ice. After the incubation at 50°C, the target was placed on the array. The array was incubated at 50°C for 14 h in a humid chamber. After hybridization, the slides were washed in 2X SSC with 0.1% SDS for 10 min, 0.1X SSC with 0.1% SDS for 10 min, and 0.1X SSC for 5 min at 30°C.

Scan and data processing. The array was scanned with ScanArray Lite (Perkin-Elmer, Wellesley, MA). The images were analyzed with QuantArray software (GSI Lumonics, Billerica, MA), converting signal intensities of each spot into numerical data. Data was processed after background subtraction, as described previously (10,16,22). Cy5/Cy3 ratios were log-transformed, and the global normalization was performed. Genes with >15% missing values in each group of cancers and adenomas were excluded from further analysis.

Statistical analysis. First, gene expression profiles of 20 cancer biopsies and 8 normal epithelia were compared. Hierarchical clustering analysis (HCA) was performed with the software GeneMaths version 2.0 (Applied Maths, Inc., Austin, TX) using all 1966 genes after data processing. Pearson's correlation was used as the similarity coefficient and the unweighted pair group method using arithmetic average as the clustering algorithm. In HCA, similarity in the expression pattern of paired biopsies from the same cancer was compared with those from other cancers, to define the difference between intra- and inter-tumor heterogeneity of profiles in cancers. Statistical significance of the difference was determined using t-test. To verify the validity of the list of differentially expressed genes involved in colorectal carcinogenesis using biopsy samples, examined genes in cancer biopsies and normal epithelia were ranked according to the signal-to-noise ratio (SNR) (5) and they were compared with previous reports using tissue samples. The p-value of SNR was calculated by performing the random permutation test 1,000 times (5).

Next, using 20 cancer biopsies and 20 adenoma biopsies, the prediction of malignancy in colorectal tumors by their gene expression profiles was tested. Genes differentially expressed in cancer and adenoma biopsies were ranked according to SNR, and differential diagnosis was performed using the weighted-votes method (5). The prediction accuracy was determined with the leave-one-out cross validation (5). Positive prediction strength was judged to be a cancer and negative to be an adenoma. Using the gene set with the highest accuracy, HCA was performed to identify the difference between the intra- and the inter-tumor heterogeneity of the profiles in cancers and adenomas.

Immunohistochemical staining. Immunohistochemical staining (IHC) was performed to investigate the translation of mRNA of differentially expressed genes to each coding protein and to examine the intra-tumor heterogeneity of expression patterns in cancers at the protein level. Among differentially expressed genes between cancer biopsies and normal epithelia in our transcriptional analyses, 7 genes whose antibodies were commercially available were selected. Buffered formalin-fixed (10%), paraffin-embedded sections were prepared from 10 surgically resected cancers, whose biopsy specimens were included in the microarray analyses. The streptavidin-biotin immunoperoxidase complex method (27) was used for IHC. Primary antibodies used were as follows; polyclonal antibody (pAb) to human peroxiredoxin 1 (PRDX1, Alexis Biochemicals, Lausen, Switzerland), pAb to high-mobility group box 1 (HMGB1, Santa Cruz Biotechnology, Santa Cruz, CA), pAb to DEK oncogene (DEK, Santa Cruz Biotechnology), pAb to poly(A) binding protein, cytoplasmic 1 (PABPC1, Santa Cruz Biotechnology), monoclonal antibody (mAb) to heat shock 60-kDa protein 1 (HSPD1, Sigma Aldrich, St. Louis, MO), mAb to nucleolin (NCL, Santa Cruz Biotechnology) and mAb to carbonic anhydrase II (CA2, Rockland, Gilbertsville, PA). Sections for negative control were tested by using normal mouse serum instead of primary

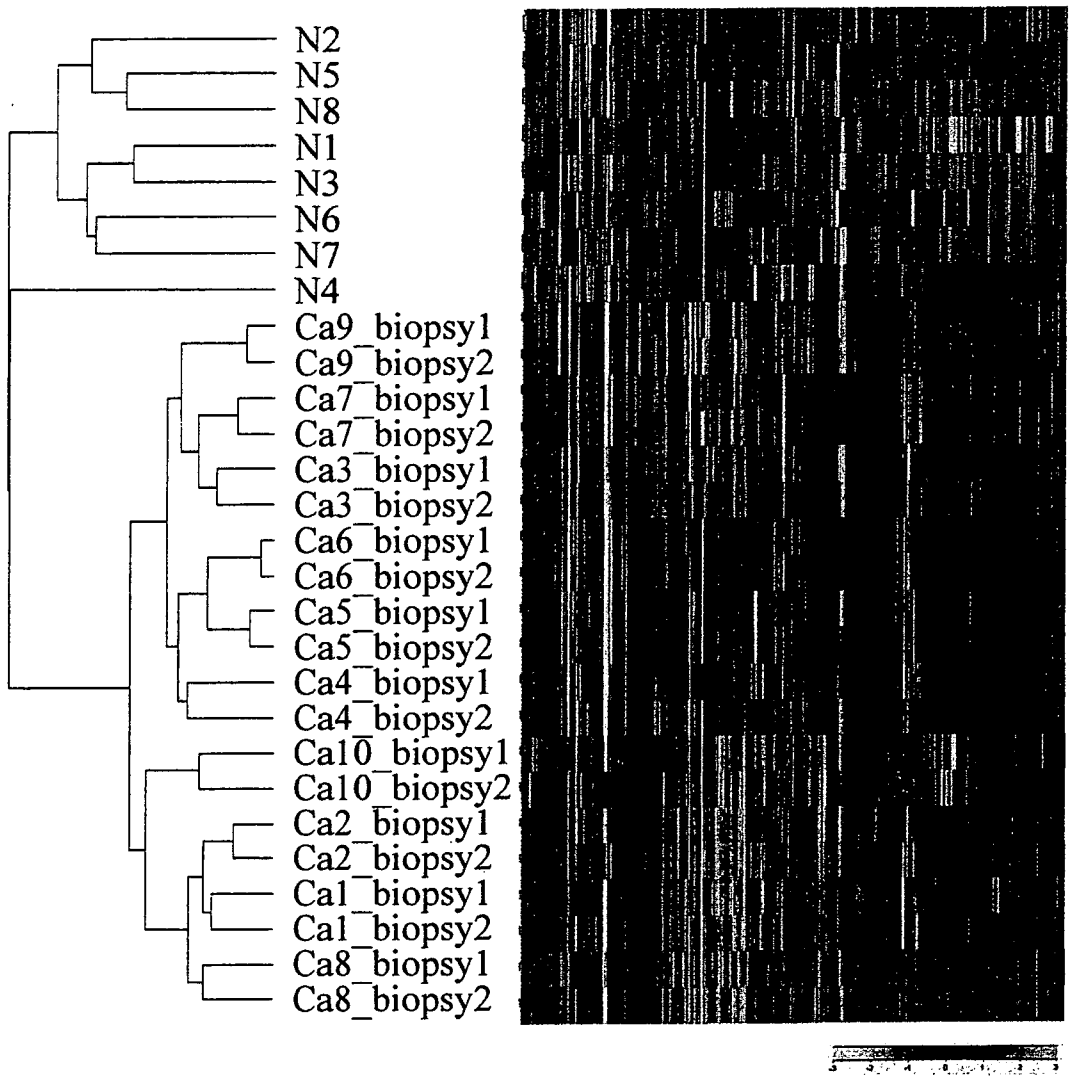

Figure 1. Hierarchical clustering analysis using full genes. Samples consisted of cancer biopsies (n=20; 10 pairs) and normal epithelia (n=8). Pearson's correlation was used as the similarity coefficient and the unweighted pair group method using the arithmetic average as the clustering algorithm. Red indicates overexpression, and green indicates underexpression. The respective paired biopsies from different areas of the same tumors are joined together.

antibody. Tissue sections of thyroid adenoma (for PRDX1), poorly differentiated gastric adenocarcinoma (for HMG1 and DEK), uterine cervical carcinoma (for HSPD1 and NCL), normal testis (for PABPC1) and normal colon (for CA2) were prepared as positive controls according to the recommendations of the manufacturer and previous publications on IHC. All slides were evaluated by a pathologist who was blinded to the microarray data. For each immunohistochemical analysis, the mean intensity in epithelial or tumor cells was evaluated in comparison with the positive controls as follows: weak, 1^+; moderate, 2^+; and strong, 3^+.

Results

Intra-tumor heterogeneity in colorectal cancers. Comparison of gene expression profiles between cancers and normal epithelia by HCA showed clear separation of the 20 cancer biopsies from the 8 normal tissues using all genes (Fig. 1). Moreover, the respective paired biopsies from different areas of the same cancers were joined together. Pearson's correlation coefficients of paired biopsies from the same cancers (mean, 0.83; SD, 0.08) were significantly greater than those of unpaired biopsies from other cancers (mean, 0.58; SD, 0.12; $p<0.0001$). Pearson's correlation coefficients between biopsies and their parent surgical bulky samples (mean, 0.60; SD, 0.15) were significantly greater than those between biopsies and surgical bulky samples from other cancers (mean, 0.37; SD, 0.16; $p<0.0001$).

Differentially expressed genes in cancer biopsies. Differentially expressed genes in cancer biopsies and normal epithelia were ranked according to SNR. Among them, 692 up-regulated and 219 down-regulated genes in cancer biopsies had SNR ($p<0.001$). The top 20 up-regulated and 20 down-regulated genes, excluding 4 down-regulated genes with no definition, are listed in Table II. PABPC1 and HSPD1 in the up-regulated genes and CA2, carboxylesterase 2 (CES2) and one EST in the down-regulated genes in cancer biopsies were also included in differentially expressed genes involved in colorectal carcinogenesis in our previous reports (10,16). Nine of the 40 differentially expressed genes were reported previously with respect to colorectal carcinogenesis with DNA microarray

Table II. Differentially expressed genes in colorectal cancer biopsies and normal colorectal epithelia.

Accession no.	Symbol	Gene definition	Previous report[a]	SNR
Up-regulated genes in cancer biopsies				
NM_181697	**PRDX1**	**peroxiredoxin 1, transcript variant 3**		**2.645**
NM_002128	**HMGB1**	**high-mobility group box 1**	**8,11,15**	**2.364**
NM_003472	**DEK**	**DEK oncogene (DNA binding)**	**11**	**2.341**
NM_001009	RPS5	ribosomal protein S5		2.052
NM_002568	**PABPC1**	**poly(A) binding protein, cytoplasmic 1**	**11,16**	**1.994**
NM_199440	**HSPD1**	**heat shock 60-kDa protein 1 (chaperonin), nuclear gene encoding mitochondrial protein, transcript variant 2**	**10,11,16,19**	**1.985**
NM_021130	**PPIA**	**peptidylprolyl isomerase A (cyclophilin A), transcript variant 1**		**1.957**
BE564899		EST		1.930
NM_001011	RPS7	ribosomal protein S7		1.894
NM_000978	RPL23	ribosomal protein L23		1.878
NM_002954	RPS27A	ribosomal protein S27a		1.860
AK090536		EST		1.826
NM_198829	**RAC1**	**ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1), transcript variant Rac1c**		**1.814**
NM_000971	RPL7	ribosomal protein L7		1.795
NM_003908	EIF2S2	eukaryotic translation initiation factor 2, subunit 2 ß, 38 kDa		1.772
NM_003756	**EIF3S3**	**eukaryotic translation initiation factor 3, subunit 3 γ, 40 kDa**		**1.763**
NM_000998	RPL37A	ribosomal protein L37a		1.732
NM_021034	**IFITM3**	**interferon induced transmembrane protein 3 (1-8U)**		**1.730**
NM_005381	**NCL**	**nucleolin**	**9**	**1.727**
NM_002106	H2AFZ	H2A histone family, member Z		1.721
Down-regulated genes in cancer biopsies				
NM_174977	SEC14L4	SEC14-like 4 (S. cerevisiae)		-2.518
NM_000067	**CA2**	**carbonic anhydrase II**	**8-11,15,16**	**-2.280**
NM_017958	PLEKHB2	pleckstrin homology domain containing, family B (evectins) member 2		-2.272
NM_005578	**LPP**	**LIM domain containing preferred translocation partner in lipoma**	**18**	**-2.236**
BF826364		EST		-1.926
BC051280		EST		-1.921
NM_003869	**CES2**	**carboxylesterase 2 (intestine, liver), transcript variant 1**	**10,14,16**	**-1.897**
BX647478		EST		-1.834
XM_375330		EST		-1.811
NM_002518	NPAS2	neuronal PAS domain protein 2		-1.789
CD652660		EST		-1.766
NM_020746	KIAA127	KIAA1271 protein		-1.742
NM_000355	TCN2	transcobalamin II; macrocytic anemia		-1.731
NM_005242	F2RL1	coagulation factor II (thrombin) receptor-like 1		-1.728
BM987276		EST		-1.698
XM_370781		**EST**	**16**	**-1.684**
AK129631		FLJ26120		-1.657
NM_002096	GTF2F1	general transcription factor IIF, polypeptide 1, 74 kDa		-1.623
NM_000014	A2M	α-2-macroglobulin		-1.617
NM_020675	Spc25	kinetochore protein Spc25		-1.606

Top 20 up-regulated and 20 down-regulated genes excluding 4 down-regulated genes with no definition, among 692 up-regulated and 219 down-regulated genes in cancer biopsies with SNR ($p<0.001$). Bold: genes reportedly involved in colorectal carcinogenesis based on microarray analysis or genes reportedly involved in carcinogenesis of other cancer types. [a]Previous report on colorectal carcinogenesis using microarray analysis (ref. no.). SNR, signal-to-noise ratio.

Table III. Immunohistochemical staining.

Gene name	Symbol		Expression grade[a]				Staining pattern[b]
			-	1^+	2^+	3^+	
Peroxiredoxin 1	PRDX1	Normal	10				
		Cancer		6	4		Homogeneous
High-mobility group box 1	HMGB1	Normal		10			
		Cancer		1	7	2	Homogeneous
DEK oncogene	DEK	Normal			7	3	
		Cancer		4	6		Heterogeneous
Poly(A) binding protein, cytoplasmic 1	PABPC1	Normal		10			
		Cancer		3	7		Heterogeneous
Heat shock 60-kDa protein 1	HSPD1	Normal	10				
		Cancer			2	8	Homogeneous
Nucleolin	NCL	Normal	4	6			
		Cancer			5	5	Homogeneous
Carbonic anhydrase II	CA2	Normal			4	6	
		Cancer		8	2		Homogeneous

Microarray analyses revealed up-regulation of PRDX1, HMGB1, DEK, PABPC1, HSPD1 and NCL and down-regulation of CA2 in cancers. [a]The mean intensity of immunohistochemical staining in the epithelial or tumor cells evaluated relative to the positive controls as follows: weak, 1^+; moderate, 2^+; strong, 3^+. [b]Intra-tumoral heterogeneity in immunohistochemical staining pattern.

assay. Among the 20 up-regulated genes, 6 genes of ribosomal proteins were included; this finding was in agreement with previous observations (7,9,11,18). In addition, relationships with carcinogenesis of other cancers have been reported regarding PRDX1 (28), peptidylprolyl isomerase A (PPIA) (29), ras-related C3 botulinum toxin substrate 1 (RAC1) (30), eukaryotic translation initiation factor 3, subunit 3 γ (EIF3S3) (31) and interferon induced transmembrane protein 3 (IFITM3) (32).

Immunohistochemical staining. The expression status of encoding proteins from 7 genes in IHC is summarized in Table III. The up- and down-regulation of PRDX1, HMGB1, PABPC1, HSPD1, NCL, and CA2 at the protein level was not in conflict with observations at the transcription level (Table III, Fig. 2). PRDX1, HMGB1, HSPD1, NCL and CA2 showed a homogeneous staining pattern in cancer tissues regardless of the region in the tumors, while the others showed a heterogeneous pattern.

Molecular prediction of tumor malignancy. In the differential diagnosis between 20 cancer biopsies and 20 adenoma biopsies by their gene expression profiles using the supervised classification method, the highest prediction accuracy was 97.5% when 8-71 genes were used (Fig. 3). Comparison between intra- and inter-tumor heterogeneity of the profiles using HCA showed that respective paired biopsies from the same tumors tended to join each other. When the selected 71-gene set with the highest accuracy was used, cancer biopsies and adenoma biopsies were clearly separated, and 18 of 20 paired biopsies were clustered side by side though a small number of genes was used (Fig. 4). Among the 71-gene set, COL1A1, COL1A2, and EIF2S2 were also reported in other studies as useful discriminating genes between cancers and adenomas (11).

Discussion

In the present study, we applied comprehensive expression analysis and found that intra-tumor heterogeneity of the gene expression profiles was smaller than inter-tumor heterogeneity, using preoperative endoscopic biopsies of colorectal tumors. We also showed that tumor malignancy could be accurately diagnosed with the profile in a single biopsy. Such accuracy is a promising first step in the clinical application of this technique for various settings such as prediction of the response to preoperative chemo- and/or radio-therapy or the existence of lymph node metastasis. Our findings suggest that this technique can be potentially used to define accurately the biological properties of colorectal tumors.

In CRC, changes in gene expression profiles that occurred during chemotherapy were detected using rectal cancer biopsies (33). The possible prediction of response to preoperative chemo- and/or radio-therapy for rectal cancers by using gene expression profiling of a single biopsy has also been reported (24,25). However, in the application of diagnosis in the clinical field based on gene expression profiling using preoperative endoscopic biopsies, it is not favorable that the profiles of biopsies from a tumor are widely different from each other. In this regard, our results added support to those

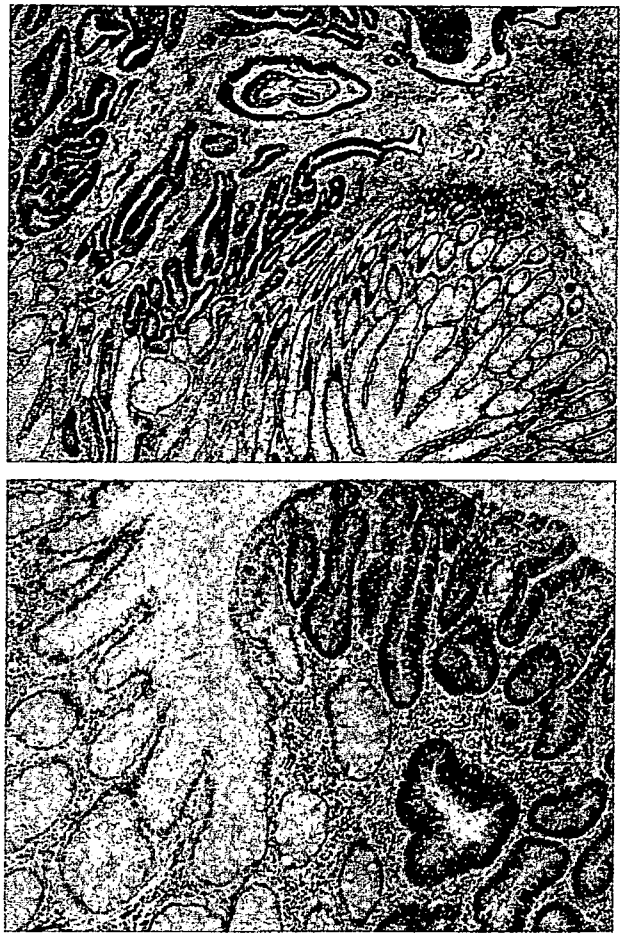

Figure 2. Immunohistochemical staining of HSPD1 (A) and NCL (B) in colorectal cancer tissues. (A) HSPD1 was expressed strongly in cancer cells while no HSPD1 expression was observed in adjacent normal epithelial cells (x4). (B) NCL was expressed strongly in cancer cells and weakly in normal epithelial cells in the lower portion of the colonic gland (x10).

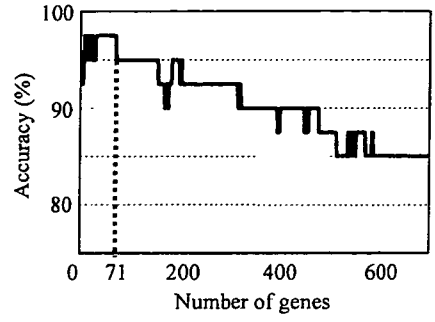

Figure 3. The accuracy curve of the differential diagnosis of 20 cancer biopsies and 20 adenoma biopsies using the supervised classification algorithm. Genes differentially expressed in cancer and adenoma biopsies were ranked according to the signal-to-noise ratio, and the accuracy was calculated with the weighted-votes method and the leave-one-out cross validation. The highest prediction accuracy was 97.5% when 8-71 genes were used.

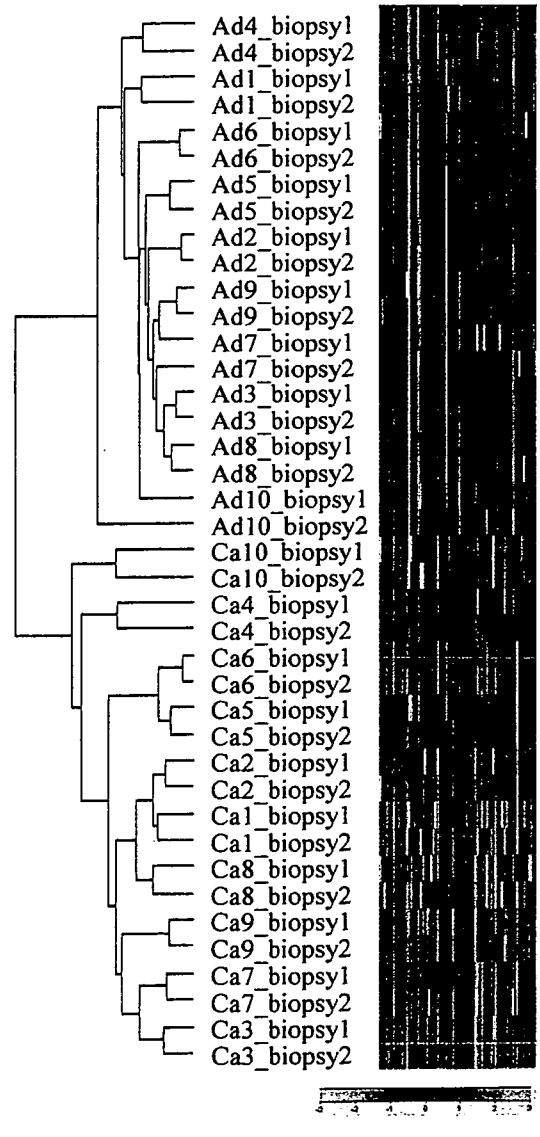

Figure 4. Hierarchical clustering analysis using 71 differentially expressed genes between cancer and adenoma biopsies. Samples consisted of cancer biopsies (n=20; 10 pairs) and adenoma biopsies (n=20; 10 pairs). Pearson's correlation was used as the similarity coefficient and the unweighted pair group method using arithmetic average as the clustering algorithm. Red indicates overexpression, and green indicates underexpression. Cancer biopsies and adenoma biopsies were clearly separated, and 18 of 20 paired biopsies were joined together.

of previous investigators (24,25), thus further promoting the technique.

Through using small-volume samples of a single biopsy of colorectal tumor, a gene expression profile was successfully obtained and a differentially expressed gene set related to colorectal carcinogenesis was detectable, as when using whole tissue samples. The feasibility of microarray-based study using fine-needle aspiration biopsy (FNAB) samples in some human tumors and endoscopically obtained tissues from precancerous lesions such as Barrett's esophagus was also reported (34,35). These results suggested that low-volume tissue samples such as endoscopic biopsy might give an accurate picture of gene expression in the whole tumor.

The histopathological features are not always homogeneous within a solid tumor. In CRC, the surface and invasive front of the tumor are sometimes histopathologically different. The influence of such morphological intra-tumor heterogeneity on gene expression profiles is not clear, although heterogeneity was detected in individual genes (36). Heterogeneity based on the superficial area of the tumor would have an unfavorable impact on molecular diagnosis when biopsy samples are used. In our study, the gene expression profiles of paired biopsies from the same tumors were identical in almost all tumors. At the protein level, IHC also showed a homogeneous staining

pattern in cancer cells at the superficial position in 5 out of 7 genes. The heterogeneity in some varied genes at the protein level did not affect the intra-tumor homogeneity of the profiles at the transcriptional level. This may be because the use of tens to thousands of genes in profiling compensated for the difference due to heterogeneity in some varied genes or because the data at the transcriptional level do not always parallel those at the protein level (37).

Concerning the intra-tumor heterogeneity of the profiles in other cancers, differences in gene expression between cancer cells in the periphery and those in the center of breast cancers were detected using microdissected samples (38). However, only in a few candidate genes was the difference in the expression levels more than two-fold. In breast cancer, the profile of FNAB was reported to resemble closely that of the corresponding source tumor (39). In a comparison between FNABs and core biopsies, the difference in profiles within the same tumor was not greater than that between other tumors (40). Also in soft tissue sarcomas, the intra-tumor heterogeneity of the profiles was smaller than the inter-tumor heterogeneity (41).

It is not clear how many biopsies are sufficient to represent the biological properties of a tumor with gene expression profiles. The first part of our study indicated that the profile of one biopsy could distinguish cancers from adenomas, beyond the intra-tumor heterogeneity. However, our next pursuit is the differential diagnosis of more delicate and important differences, such as the potential of metastasis and sensitivity to chemo- or radio-therapy. Any unfavorable variation based on the sampling skill and the experiment may be a drawback on the actual clinical application of this method. Multiple biopsies from a tumor may be required to balance the differences within a tumor. The number of biopsies for microarray analysis was studied using biopsies from rectal epithelia and two biopsies per person were recommended, based on the equality of the expression data within a person (42), though similar analyses using biopsies from colorectal tumors have not reported.

Sampling techniques are important. Every biopsy was sampled by a specialist in the colonoscopic unit and very reliable sampling was carried out in this study. Inaccurate sampling would give confusing results showing a wide gap of profiles even between biopsies from one tumor. We took care to avoid contamination of the normal epithelium or adenomatous component on the periphery of the cancer tissue and confirmed the histology with pathological diagnosis of simultaneously obtained biopsies and surgical samples.

Our results suggest that gene expression profiling using endoscopic biopsies can accurately describe the biological properties of colorectal cancer. Further studies of gene expression profiling using preoperative endoscopic biopsies may allow the development of new diagnostic systems for the selection of neoadjuvant therapy or the method most appropriate for tumor resection. Ultimately, it may lead to individualized therapy for colorectal cancer.

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