〔文　献〕
1)東口高志，他：臨床外科 60：565. 2)da Costa MA, et al：J Parenter Enteral Nutr 27：182, '03. 3)Exner R, et al：Ann Surg 237：110, '03. 4)Bansal V, et al：J Parenter Enteral Nutr 29：75. 5)Koller M, et al：Clin Nutr 22：59, '03. 6)Matheson PJ, et al：Surgery 132：673, '02. 7)Jho DH, et al：J Parenter Enteral Nutr 26：291, '02. 8)福島亮治，他：静脈経腸栄養 20：151. 9)Braga M, et al：Nutrition (in press).

## 八、腹腔鏡下手術

内視鏡外科手術は、導入から一〇年以上が経過し、広い範囲で臨床応用されている。ここ一年で報告された論文は、大腸癌に対する妥当性の評価、simulatorによる教育、肥満に対する手術の有用性の報告が多かった。内視鏡外科手術の悪性疾患への導入は大腸癌が現状で最も普及していることから長期成績の検討も進んでおり、他分野の悪性疾患への導入に大きな影響を持っている。この分野においては、二〇〇四年までに欧米で大規模なRCTの結果が出た。

二〇〇四年の米国Nelsonらの大腸癌切除における腹腔鏡下と開腹の多施設RCTの結果[1]では、四八施設八七二名の参加による解析で、大腸癌の再発率に両群で差がなかったことから、結腸癌のために腹腔鏡のアプローチが開腹術に代わりうることを示唆していると結論づけていた。スペインのLacyら[2]、香港Leungら[3]の報告でも、腹腔鏡下手術は開腹手術と遜色ない成績を示しており、また、Lacyら[2]のCOLOR groupは、再び多国籍多施設のRCTにて腹腔鏡下群での短期成績の有用性を報告しており[4]、今後より一層大腸癌に対する腹腔鏡下手術が普及していくことと思われる。

しかしコスト面では、腹腔鏡下手術において問題があることをJansonら[5]が指摘している。手術と術後一二週間目までの費用の比較で、特に手術費用や器械代などで腹腔鏡下手術が有意に高く、労業などに伴う社会的費用は差がなかったことから、腹腔鏡下手術を選択することは術後の疼痛の軽減や早い回復をその費用で買うかどうかの問題となるであろう。本邦においては、二〇〇四年から日本臨床腫瘍研究グループ(JCOG)にて多施設RCTの登録が開始されている[6]が、結果は二〇一〇年以降になる。胃癌においては単独施設での報告がほとんどで、多施設でのRCTや長期成績に関しての報告はまだない。

内視鏡外科手術はモニター画面上での二次元での手術であり、hands on handsのトレーニングの重要性が不可欠である。その点から、virtual reality(VR) simulatorによるトレーニング(VS)が注目されるようになり、論文報告も増加している。Saymourら[7]やGrantcharovら[8]は、いずれも外科修練者を対象に腹腔鏡下胆嚢摘出術におけるVSの有効性を報告している。また、Kordorfferら[9]は、腹腔鏡下での縫合においてVSの有効性を報告している。今後ますますVSの機器の進歩により有効な訓練がなされる可能性は高い。しかし従来のtraining BoxでのトレーニングとVRの間に差がなく[10]、training Boxが明らかに廉価である[11]ことも指摘されている。なお外科初心者においては、市販のテレビゲームの得意なものが腹腔鏡下手術手技の得意な傾向を示したとの論文[12]も報告されている。

肥満手術については、本邦において対象が未だ少ないので割愛する。

（斉田 芳久、炭山 嘉伸）

〔文　献〕
1)Clinical Outcomes of Surgical Therapy Study Group：N Engl J Med 350：2050, '04. 2)Lacy AM, et al：Lancet 359：2224, '02. 3)Leung KL, et al：Lancet 363：1187, '04. 4)Veldkamp R, et al：Lancet Oncol 6：477. 5)Janson M, et al：Br J Surg 91：409, '04. 6)Kitano S, et al：Jpn J Clin Oncol 35：475. 7)Saymour NE, et al：Ann Surg 236：458, '02. 8)Grantcharov TP, et al：Br J Surg 91：146, '04. 9)Kordorffer JR Jr, et al：Am Coll Surg 201：23. 10)Munz Y, et al：Surg Endosc 18：485, '04. 11)Fried GM, et al：Ann Surg 240：518, '04. 12)Rosenberg BH, et al：J Endourol 19：372.

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# Rapid and Simple Detection of Hot Spot Point Mutations of Epidermal Growth Factor Receptor, BRAF, and NRAS in Cancers Using the Loop-Hybrid Mobility Shift Assay

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# Rapid and Simple Detection of Hot Spot Point Mutations of Epidermal Growth Factor Receptor, BRAF, and NRAS in Cancers Using the Loop-Hybrid Mobility Shift Assay

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**A simple and rapid method to detect the epidermal growth factor receptor hot spot mutation L858R in lung adenocarcinoma was developed based on principles similar to the universal heteroduplex generator technology. A single-stranded oligonucleotide with an internal deletion was used to generate heteroduplexes (loop-hybrids) bearing a loop in the complementary strand derived from the polymerase chain reaction product of the normal or mutant allele. By placing deletion in the oligonucleotide adjacent to the mutational site, difference in electrophoretic mobility between loop-hybrids with normal and mutated DNA was distinguishable in a native polyacrylamide gel. The method was also modified to detect in-frame deletion mutations of epidermal growth factor receptor in lung adenocarcinomas. In addition, the method was adapted to detect hot spot mutations in the B-type Raf kinase (BRAF) at V600 and in a Ras-oncogene (NRAS) at Q61, the mutations commonly found in thyroid carcinomas. Our mutation detection system, designated the loop-hybrid mobility shift assay was sensitive enough to detect mutant DNA comprising 7.5% of the total DNA. As a simple and straightforward mutation detection technique, loop-hybrid mobility shift assay may be useful for the molecular diagnosis of certain types of clinical cancers. Other applications are also discussed.** ***(J Mol Diagn 2006, 8:504–512; DOI: 10.2353/jmoldx.2006.060030)***

Rapid and accurate detection of mutations in various cancer-related genes has become increasingly important to provide molecular diagnostic information about clinical cancers. For example, information of the mutated states of particular genes is crucial for successful chemotherapy with certain gene-targeting drugs. Namely, gleevec (imatinib) has been shown to be effective for gastrointestinal stromal tumor with specific mutations in KIT[1,2] and PDGFRA,[3] as well as for chronic myelogenous leukemia carrying the chimeric gene BCR/ABL1.[4] Recently, a subset of lung adenocarcinomas with specific mutations in epidermal growth factor receptor (EGFR) has been reported to respond remarkably well to Iressa (gefitinib).[5–9]

To detect mutations in these and other oncogenes, there are several long-standing methods available such as direct sequencing of polymerase chain reaction (PCR)-amplified DNA, single-strand conformation polymorphism (SSCP),[10] SSCP/duplex analyses,[11] mutant allele-specific amplification,[12] and denaturing high performance liquid chromatography.[13,14] These techniques have been successfully applied for the detection of mutational changes in various cancer genes. Each method has its own advantages as well as disadvantages or difficulties in practical situations. Direct sequencing of heterozygous point mutations and deletions may produce results requiring sophisticated data analysis for heterozygous mutations, especially in the presence of contaminating normal tissue DNA. SSCP, widely used for its simplicity, requires strict temperature control during a long electrophoretic time and radiolabeling in standard detection. SSCP/duplex and denaturing high performance liquid chromatography analyses necessitate sophisticated separation equipment such as capillary electrophoresis or high performance liquid chromatography with temperature control. Mutant allele-specific amplification requires several primers with mutational sites at the 3′ ends to discriminate the mutational base changes by the lack of polymerase extension beyond the mis-

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matched end, and occasional read-through can cause ambiguous results.

Heteroduplex analysis using universal heteroduplex generator (UHG) technology[15,16] is based on the retarded mobility in native polyacrylamide gel electrophoresis (PAGE) of a heteroduplex between the test PCR fragment and the PCR fragment termed the heteroduplex generator. Heteroduplex generators contain small deletions in the vicinities of mutational sites and generate four kinds of heteroduplexes with mutant and normal strands by hybridization, which are differentiated by the mobility changes. The method was applied to detect point mutations in sickle-cell diseases and phenylketonuria. Recently, UHG technology was adapted for detection of point mutations in NRAS at codons 12, 13, and 61.[17,18] In UHG technology, band patterns of four different retarded bands in PAGE are analyzed to determine mutational states. We simplified UHG technology by using single-stranded oligonucleotides with internal deletions as the generators of the loop-bearing heteroduplexes. This modification yields two bands for heterozygosity and one band for homozygosity, enabling more straightforward data analysis. Our method, designated the loop-hybrid mobility shift assay (LH-MSA), was developed to detect the point mutation L858R of EGFR exon 21 in lung adenocarcinoma that is associated with responsiveness to gene-targeted kinase inhibitors such as gefitinib. Adaptations of LH-MSA to detect in-frame deletions of EGFR exon 19 in lung adenocarcinoma and hot spot point mutations of BRAF and NRAS in thyroid carcinoma are also described.

## Materials and Methods

### DNA Preparations and Plasmid Clones

DNA from fresh tumor tissues (16 cases of lung adenocarcinoma, 25 cases of papillary thyroid carcinoma, and 19 cases of follicular thyroid carcinoma) was prepared according to standard protocols after obtaining informed consent. Formalin-fixed, paraffin-embedded (FFPE) tissue sections of lung adenocarcinoma and papillary thyroid carcinoma were also used for preparing DNA as follows. Thin-sectioned tissues (15 to 20 μm thick) were deparaffinized with xylene followed by ethanol series and air-dried. Tumor tissues, identified in the hematoxylin and eosin-stained serial sections (2 μm), were applied with a pinpoint solution (Pinpoint slide DNA isolation system; Zymo Research, Orange, CA), air-dried, and cut out together with the overlaid dried film of the pinpoint solution. The excised tissues (3-mm square) were digested in proteinase K buffer solution at 55°C for 4 hours, heat inactivated at 95°C for 15 minutes, and used as PCR template directly, or after purification with a spin column.

PCR-amplified DNA fragments were ligated to the TOPO-TA ligation vector pCR4TOPO (Invitrogen, Carlsbad, CA), electroporated into *Escherichia coli*, and cloned. Cloned bacterial cells were suspended in lysis solution (CloneChecker; Invitrogen), heat-lysed at 98°C for 30 seconds, and used as the cloned plasmid DNA solutions. DNA of the cloned plasmids was amplified with Phi29 polymerase (GE Health Care Bio-Science, Piscataway, NJ) and used for sequencing the inserts of tumor DNA fragments (CEQ8000 sequence analysis system; Beckman Coulter, Fullerton, CA). Direct sequencing of the PCR products from EGFR exon 21 was performed as described by Lynch and colleagues.[6]

### Loop-Hybrid Formation in the LH-MSA

The LH-MSA consisted of two parts: hybridization of PCR products to the loop-hybrid generator (LH-G) probes made from synthetic oligonucleotides (70 to 99 mers) to generate loop-hybrids (Figure 1A) and analysis of mobility shifts of the loop-hybrids after native PAGE as described below. Nucleotide sequences of PCR primers and LH-G probes used to detect point mutations (7R, 18R, 9F, and 10K) and deletions (19JWTF) are described in Table 1. Each LH-G probe was designed to overlap with one of the PCR primer pairs at the 5′ end, and a stretch of up to 18 nucleotides was deleted in the region adjacent to the mutational hot spot for detection of point mutations. To detect deletion mutations in EGFR exon 19, the LH-G probe (19JWTF) was an oligonucleotide with no internal deletion that extended 26 nucleotides beyond the region of deletion mutations so that a loop forms only when it hybridizes with PCR products containing a deletion in EGFR exon 19 (Figure 3A). LH-G probes were purified with high performance liquid chromatography. PCR was performed with Accuprime *Taq* polymerase containing primer-template hybridization-enhancing reagent (Invitrogen). Generation of loop-hybrids was conducted at the end of the PCR amplification cycles by adding a specific LH-G probe into the PCR reaction solution at a final concentration of 500 nmol/L, ie, in large excess to the initial concentration of primer pairs (200 nmol/L). The mixture was then subjected to the loop-hybrid formation (LH-F) steps, consisting of 1) denaturation at 94°C for 2 minutes, 2) hybridization of the LH-G probe to the complementary strand at 55°C for 15 seconds, and 3) extension of the 3′ end of the LH-G probe in loop-hybrids by *Taq* polymerase at 68°C for 4 minutes. After the LH-F steps, reaction products were analyzed by PAGE to detect migration shift of the loop-hybrid bands by the mutations.

### Detection of Loop-Hybrid DNA with PAGE

PCR products subjected to LH-F steps in the presence of LH-G probes were separated by electrophoresis in a native 10% polyacrylamide gel (6 cm × 6 cm preformed compact gels; ATTO Inc., Tokyo, Japan) in Tris-glycine buffer (37.5 mmol/L Tris, 288 mmol/L glycine) at 20 mA for 30 minutes at room temperature. Using a native 8% polyacrylamide gel in TBE buffer (89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L ethylenediaminetetraacetic acid), electrophoresis of the loop-hybrid DNA performed at 10 mA for 1 hour at room temperature yielded equivalent results. After electrophoresis, gels were stained for 10 minutes with SYBER Green I (Cambrex Bio Science, Rockland, ME) diluted to 1/10,000 in distilled water and

**Table 1.** PCR Primers and LH-G Probes Used for Detection of Mutations in EGFR, BRAF, and NRAS

| Gene | Mutation | Amplicon | PCR primer† | LH-G probe‡ (probe name) |
|---|---|---|---|---|
| EGFR | In-frame deletion in exon 19 | 212bp | (F) GGACTCTGGATCCCAGAAGGTG | GGACTCTGGA TCCCAGAAGG TGAGAAAGTT |
| | | | | AAAATTCCCG TCGCTATCAA GGAATTAAGA |
| | | | (R) CATTTAGGATGTGGAGATGAGC | GAAGCAACAT CTCCGAAAGC CAACAAGGAA |
| | | | | ATCCTCGAT (19JWTF) |
| EGFR | Point mutation in exon 21 at L858 | 161bp | (F) GGCATGAACTACTTGGAGGAC | CTTACTTTGC CTCCTTCTGC ATGGTATTCT |
| | | | | TTCTCTTCCG CACCCAGCAG *******AGC |
| | | | (R) CTTACTTTGCCTCCTTCTGCATG | CCAAAATCTG TGATCTTGAC ATGCTGCG |
| | | | | (7R) |
| | | | | CTTACTTTGC CTCCTTCTGC ATGGTATTCT |
| | | | | TTCTCTTCC* ********** *******AGC |
| | | | | CCAAAATCTG TGATCTTGAC ATGCTGCG |
| | | | | (18R) |
| BRAF | Point mutation in exon 15 at V600 | 157bp | (F) ATTTCTTCATGAAGACCTCACAG | ATTTCTTCAT GAAGACCTCA CAGTAAAAAT |
| | | | | AGGTGATTTT GGTCTAGCTA CAGT****** |
| | | | (R) GGCCAAAAATTTAATCAGTGGA | ***ATGGAGT GGGTCCCATC AGTTTG |
| | | | | (9F) |
| NRAS | Point mutation at 061 | 149bp | (F) GTGAAACCTGTTTGTTGGAC | GTGAAACCTG TTTGTTGGAC ATACTGGATA |
| | | | | CAGCTGGACA ********** AGTGCCATGA |
| | | | (R) CCTGTAGAGGTTAATATCCG | GAGACCAATA CATGAGGACA GG (10K) |

†(F), forward primers; (R), reverse primers.
‡*, deleted nucleotides; underlined nucleotides indicate the hot spots of mutations to be detected.

placed in deionized water to remove excess dye. After the staining, DNA was detected with a laser-scanning imager (STORM860; GE Health Care Bio-Science) using 450-nm excitation and a 520-nm long path filter. A 100-bp ladder (Promega, Madison, WI) was used to distinguish the bands of loop-hybrid DNA showing retarded migration from the homoduplex bands showing size-dependent migration.

### *Estimation of Mutant Copy Numbers with LH-MSA*

Plasmid clones of PCR products from the L858R mutant and normal alleles of EGFR exon 21 were mixed together at mutant to normal ratios ranging from 1 to 0.05. After PCR of these mixed samples with Accuprime *Taq* High Fidelity (Invitrogen), an aliquot was examined directly with LH-MSA using the 18R LH-G probe to determine the sensitivity of mutation detection and the remaining portion was used for TOPO-TA ligation and cloning as described above. Forty-eight to ninety-six bacterial clones were genotyped with LH-MSA using the 18R LH-G probe to estimate copy numbers of the mutant allele in PCR products from the mixed samples. No mutant was found in the 48 clones of the normal control. Dideoxy sequencing of the mixed plasmid DNA was performed as described above.

## *Results*

### *Detection of the EGFR Hot Spot Mutation L858R in Lung Adenocarcinoma Using LH-MSA*

For simple and easy detection of the point mutation at L858 in EGFR exon 21, a method similar to the universal heteroduplex generator technology[15–18] was developed. As illustrated in Figure 1A, the heteroduplex with a loop (hereafter referred to as loop-hybrid) can be generated by hybridization of the PCR product from EGFR exon 21 with a synthetic oligonucleotide (LH-G probe) having a stretch of nucleotides deleted adjacent to the mutation hotspot at L858. A series of LH-G probes expected to generate loops of various nucleotide lengths (Figure 1B) was examined. Migration of the loop-hybrids in PAGE was markedly retarded compared to the size-dependent migration of the homoduplex, and the degree of retarded mobility depended on the size of loop in the loop-hybrid (Figure 1, B and C). The loop-hybrid band of the mutant allele was markedly shifted from that of the normal allele when the same LH-G probe was used (Figure 1B). The shift was particularly pronounced for the LH-G probes 4R, 7R, 14R, and 18R. Faint secondary bands were visible beside the major LH band when the LH-G probes 3R, 6R, and 8R were used. DNA from the retarded bands excised from the gel was amplified with PCR and cloned into plasmids. When these clones were sequenced, both the original PCR fragment and the derivative of the LH-G probe used to generate the loop-hybrid were identified (data not shown), confirming our model (Figure 1A). When the faint secondary band produced by LH-G probe 8R was similarly analyzed, a mutation (one base deletion five bases upstream of the internally deleted site) was detected in the sequence corresponding to the 8R LH-G probe, whereas the expected sequence of 8R was found in the main loop-hybrid band. Therefore, the faint secondary band seemed to be generated by a contaminating mutant form of the 8R LH-G probe. Purification of LH-G probes with PAGE may be necessary to reduce such inadvertent contaminants. The LH-G probes 7R and 18R showed single, discrete loop-hybrid bands, well distinguishing the mutant from the normal allele and distinctly separated from the homoduplex band. Therefore, these LH-G probes were adopted in the following LH-MSA analysis to detect the L858R mutation in EGFR exon 21. Heterozygous mutations are detected as the double bands of the normal and the shifted mutant loop-hybrid bands.

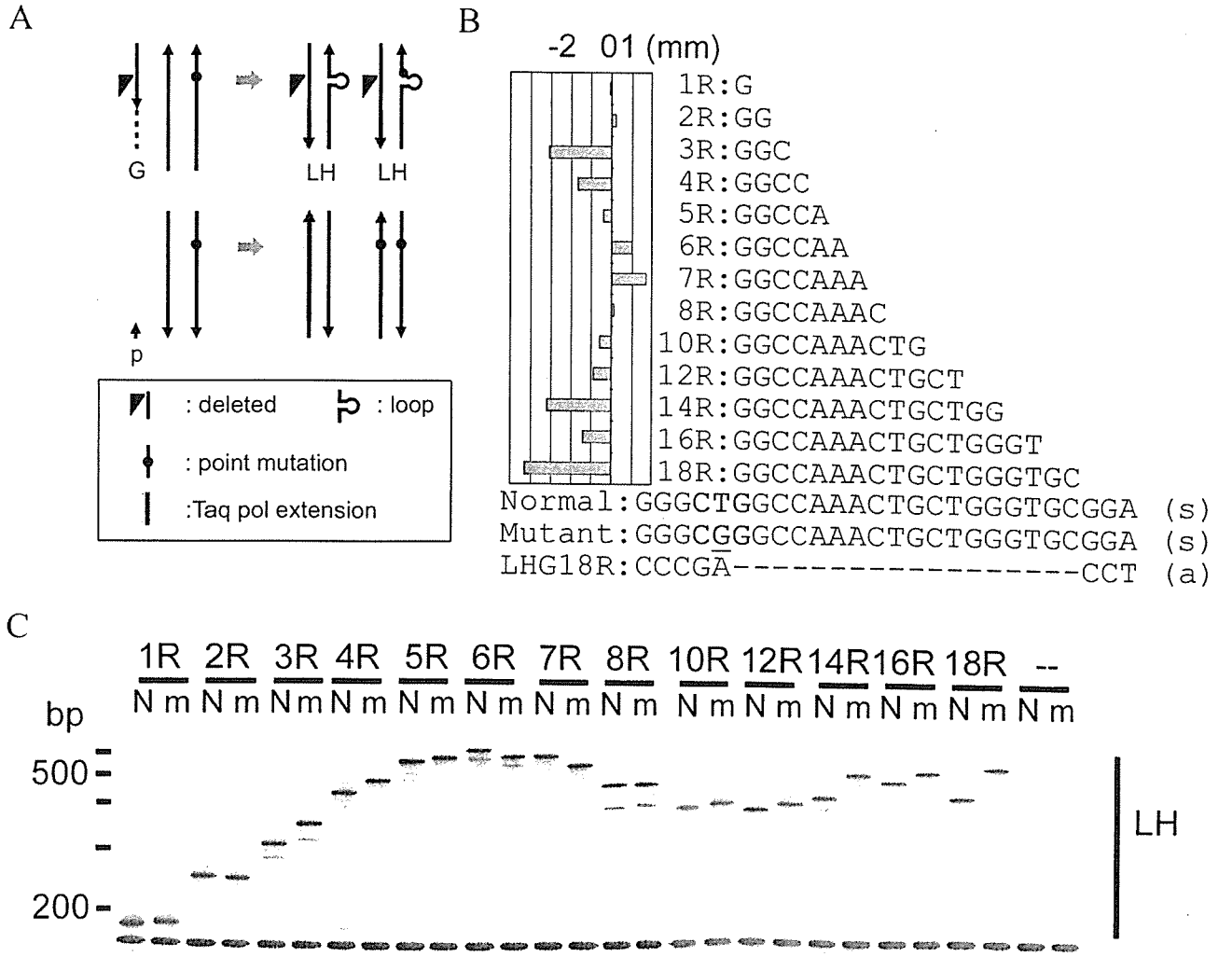


**Figure 1.** Detection of the hot spot point mutation L858R (CTG>CGG) of EGFR exon 21 by LH-MSA using various LH-G probes. **A:** Schematic representation of loop-hybrids (LH) generated by an LH-G probe (G) in the PCR product heterozygous for a point mutation. Homoduplexes of the normal and mutant alleles are produced by polymerase extension of the hybridized PCR primer (p). The broken line indicates the 3' region of the UH-G probe to be extended by *Taq* polymerase after hybridization. **Gray arrows** indicate the hybridizing reaction. **B:** Mobility shifts of the loop-hybrid bands caused by the mutant allele are shown as the difference (mm) in mobility in the gel between the loop-hybrid band of the mutant allele and that of the normal allele for each LH-G probe, together with the sequence of the loop generated by the LH-G in the sense strand of the normal allele. Partial sequences of the normal and mutant alleles in the sense (s) strand are shown, with the codon L858 in bold. Also shown is the partial reverse anti-sense sequence (a) of the LH-G probe 18R with deleted nucleotides (−). **C:** Paired PCR products from the template DNA of the plasmid clones of the normal (N) and the mutated (m) alleles were examined with a series of LH-G probes as denoted for each lane and analyzed with PAGE. Deletions in these LH-G probes generated the loops of 1 to 18 nucleotide lengths in the loop-hybrids. Control PCR products (−) hybridized with no LH-G probe are also shown.

DNA prepared from fresh lung adenocarcinoma tumor tissues was screened for the L858R mutation using LH-MSA (Figure 2A). The expected double bands for the putatively heterozygous mutation were clearly shown for 3 of 16 examined cases. The L858R mutation in these three cases was confirmed by direct sequencing. For comparison DNA from FFPE tissues of 50 cases in pathological archives of operated lung adenocarcinoma were analyzed by LH-MSA and by direct sequencing. PCR products from those cases in which mutations were detected by LH-MSA were cloned in plasmids and screened with LH-MSA, and mutant clones were sequenced. As summarized in Table 2, 26% of the cases (13 of 50) exhibited the L858R mutation. Nine of these were consistent with the direct sequencing results, but the remaining four were not confirmed because of insufficient quality of the direct sequence data. One mutation (2%) other than L858R was detected by LH-MSA and determined to be A859T by sequence analysis of the mutant clone and by the direct sequencing. When FFPE tissue DNA from an additional 68 lung adenocarcinoma cases was examined by LH-MSA, the mutation L858R was again observed at a high frequency (27.9%, Table 2). Mutations other than L858R (L861R) were detected at a low frequency (2.9%, 2 of 68) by LH-MSA and sequence analysis of the mutant clones. One of the rare mutations (L861R) produced a mutant loop-hybrid band that shifted differently from that of L858R (Figure 2B). These rare mutations were close in proximity to L858 (Figure 2C). In the present analysis, the mutational state of EGFR exon 21 at L858 was diagnosed by LH-MSA with a high accuracy of 97.5%, taking into account mutations other than L858R (2.5%). Mutations other than

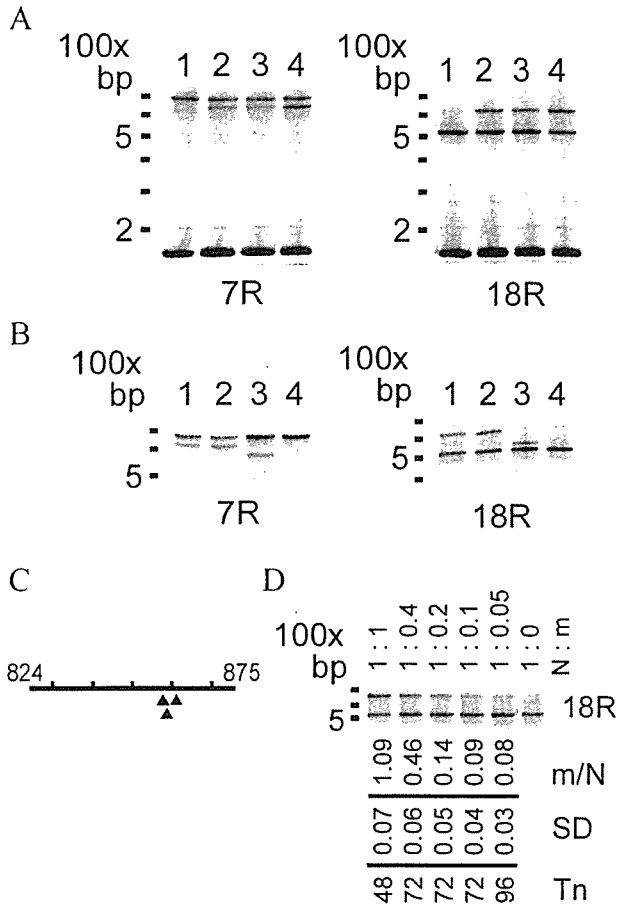


**Figure 2.** Detection of point mutations at or near the hot spot L858 of EGFR exon 21. **A:** The electropherogram after LH-MSA using LH-G probes 7R and 18R to detect the L858R mutation (CTG>CGG) of EGFR exon 21 in DNA from fresh lung adenocarcinoma tissues. **Lane 1**, the normal allele; **lanes 2** to **4**, putative heterozygous mutations. The homoduplex bands were 161 bp in length. **B:** LH-MSA with probes 7R and 18R to detect the following heterozygous mutations in EGFR exon 21 of L858R (CTG>CGG, **lane 1**), A859T (GCC>ACC, **lane 2**), and L861R (CTG>CGG, **lane 3**). **Lane 4** is the normal allele. **C:** Positions of point mutations in exon 21 detected by LH-MSA (L858R, A859T, L861R; closed triangles). **D:** Detection of the mutated allele (m) of EGFR L858R present at decreasing ratios to the normal allele (N) with LH-MSA using the LH-G probe 18R. The template DNA was constituted by mixing the diluted DNA of cloned plasmids of the mutated and normal alleles. Loop-hybrid bands are shown, together with the observed mutant to normal ratios (m/N) in the PCR products, the standard deviations (SDs), and the total numbers of clones examined (Tn).

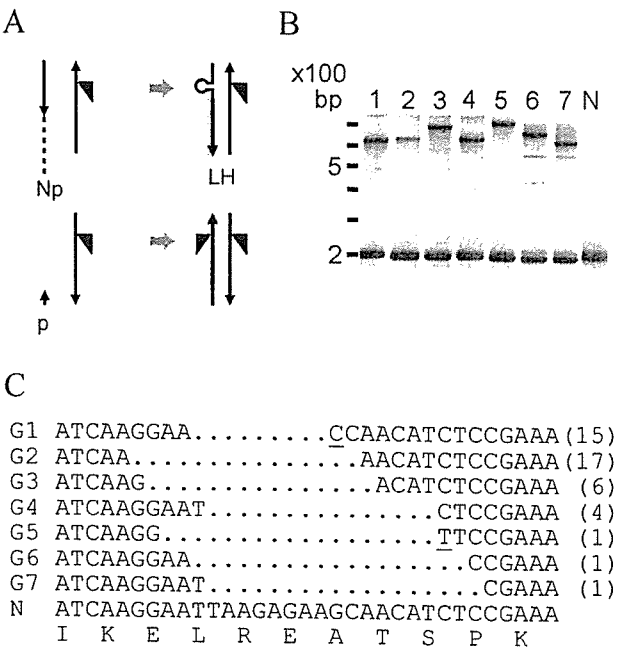


**Figure 3.** Detection of deletion mutations in EGFR exon 19 with LH-MSA. **A:** Schematic representation of loop-hybrids (LH) generated by the normal LH-G probe (Np) hybridized to the anti-sense strands of the PCR products of deleted mutant alleles. Homoduplexes of the deleted mutants are reproduced in LH-MSA by extension of the PCR primer (p) hybridized to the sense strands of the PCR products. The symbols are the same as in Figure 1A. **B:** LH-MSA of PCR products from the plasmid clones of deletion mutant alleles G1 to G7 (**lanes 1** to **7**) and of the normal allele (N) treated with the normal LH-G probe 19JWTF. **C:** Partial nucleotide sequences of the deletion mutants (G1 to G7) and the normal allele (N) of EGFR exon 19, as well as the corresponding normal amino acid sequence. Deleted nucleotides are indicated by dots, and nucleotide displacements are underlined. Incidences of the single deleted mutations in 118 cases of lung adenocarcinoma are shown in parentheses.

L858R that were undetected by LH-MSA could occur, but their presence was not verified in this study.

LH-MSA may be used semiquantitatively, as shown in Figure 2D. The mutant allele in the mixed sample at the mutant to normal ratio 0.05 (0.08 by the observed ratio) was detected with LH-MSA but not detected by dideoxy sequencing (data not shown). Our results show that LH-MSA was able to detect a mutant allele comprising 7.5% of the total DNA, suggesting that tumor cells with a heterozygous mutation that comprise 15% of the total cell mixture might be detected by LH-MSA.

## *Detection of In-Frame Deletion Mutations in Lung Adenocarcinoma Using LH-MSA*

In-frame deletion mutations in EGFR exon 19 involving 9 to 18 bases (including the overlapping region L747 to E749; Figure 3C) were detected frequently in lung adenocarcinoma.[5–9] For heterozygous deletion mutations, heteroduplexes between the normal and the deleted mutant alleles of the PCR product were detected in PAGE as

**Table 2.** Point Mutations in EGFR Exon 21 Detected by LH-MSA in FFPE Tissue DNA from Lung Adenocarcinomas

| Genotype of EGFR exon 21 | Experiment 1 | | Experiment 2 | Total |
|---|---|---|---|---|
| | LH-MSA (%) | Direct sequence (%) | LH-MSA (%) | LH-MSA (%) |
| Normal | 36 (72) | 35 (70) | 47 (69.1) | 83 (70.3) |
| L858R | 13 (26) | 9 (18) | 19 (27.9) | 32 (27.1) |
| A859T | 1 (2) | 1 (2) | | 1 (0.8) |
| L861R | | | 2 (2.9) | 2 (1.7) |
| Undetermined | | 5 (10) | | |
| Total | 50 | 50 | 68 | 118 |

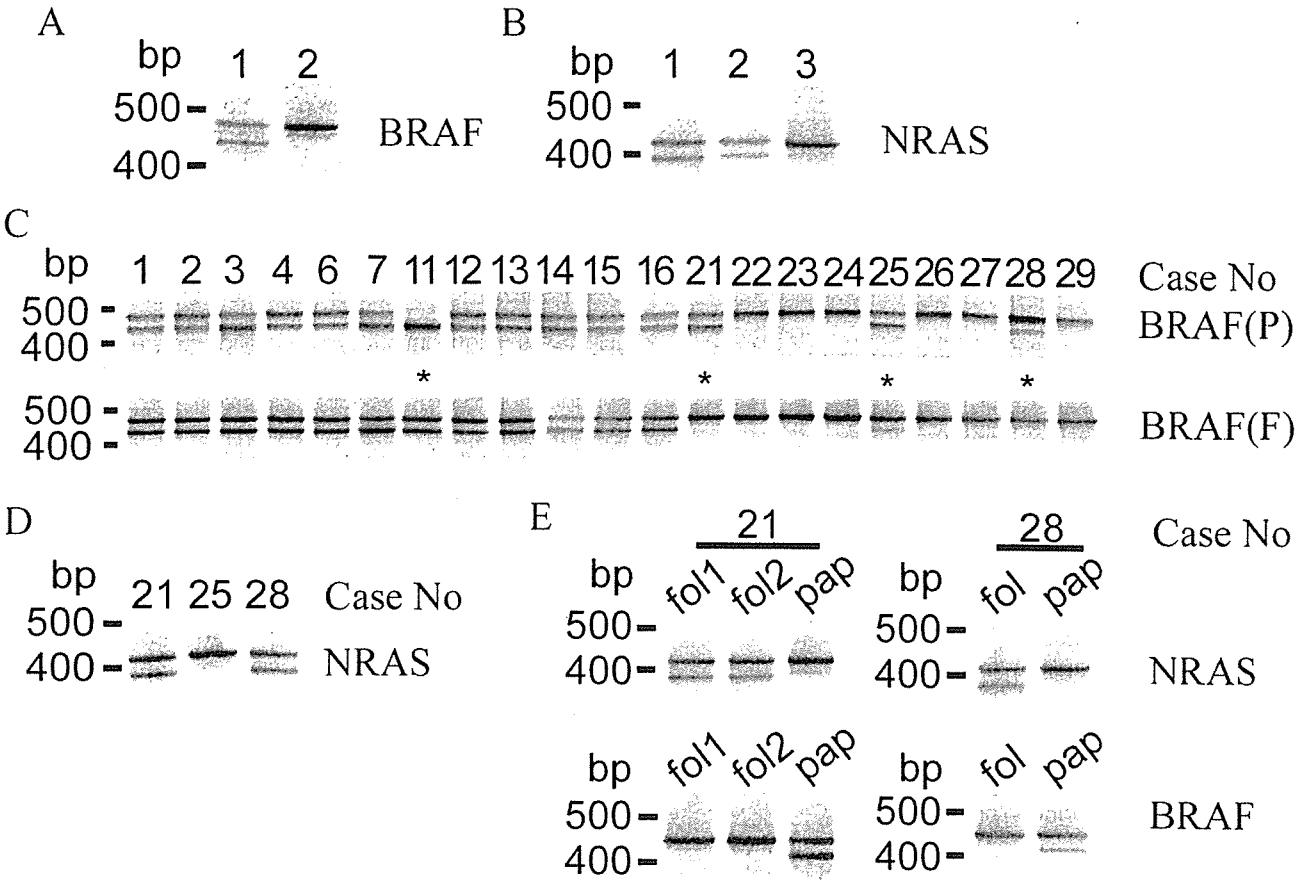


**Figure 4.** Comparison of the mutations in BRAF at V600 and NRAS at Q61 detected by LH-MSA in DNA derived from bulk fresh tumor tissues versus FFPE tumor tissues of thyroid carcinoma. **A:** Loop-hybrid bands after LH-MSA with the LH-G probe 9F for detection of the mutation V600E (GTG>GAG) in BRAF exon 15, using the DNA from fresh tumor papillary thyroid carcinoma tissue. **Lane 1**, the putative heterozygous BRAF mutation; **lane 2**, the normal allele. **B:** Loop-hybrid bands after LH-MSA with the LH-G probe 10K for detection of mutations in NRAS at Q61, using DNA from fresh tumor follicular thyroid carcinoma tissue. **Lanes 1** and **2**, the putative heterozygous NRAS mutations Q61R (CAA>CGA) and Q61K (CAA>AAA), respectively; and **lane 3**, the normal allele. **C:** Loop-hybrid bands for detection of the BRAF mutation V600E, using DNA from FFPE tumor tissues (P) and from bulk fresh tumor tissues (F) of 21 thyroid papillary carcinoma cases. **Asterisks** indicate discrepant results. **D:** Loop-hybrid bands after LH-MSA for detection of the NRAS mutation Q61R in bulk fresh tumor DNA of the discrepant cases with respect to the BRAF mutation. The heterozygous NRAS mutation was evident in cases 21 and 28. **E:** Loop-hybrid bands indicating the heterozygous NRAS mutation Q61R (top) and the heterozygous BRAF mutation V600E (bottom) in DNA derived from follicular-type (fol1, fol2, and fol) and papillary-type (pap) tumors of FFPE tissue sections in the discrepant cases 21 and 28.

a pair of retarded mobility bands easily distinguishable from the homoduplex band showing size-dependent migration (data not shown). LH-MSA was adapted to detect not only heterozygous but also mono-allelic deletion mutations (Figure 3A). Namely, a normal oligonucleotide, 19JWTF, was added as the LH-G probe to the PCR products from plasmid clones of several deleted mutant alleles and hybridized using LH-F steps. Analysis of these reaction products in PAGE revealed various retarded mobility bands of loop-hybrids at specific positions for each of the deleted mutant alleles (Figure 3B). No retarded mobility band was observed for the PCR product of the normal allele treated with this LH-G probe. Using this LH-MSA adapted for the detection of deletion mutations in EGFR exon 19, 49 deletion mutations (41.5%) were detected in DNA samples from FFPE tumor tissues of 118 lung adenocarcinoma cases and these deletions were confirmed by sequencing the cloned mutants. Although most of the cases (45 of 49, 92%) were uniquely associated with one of the deletion mutations shown in Figure 3C, composite mutations of two different in-frame deletions were also found after sequencing mutant clones. They were composed of G1 and G2 (two cases), G1 and G3 (one case), and G1 and G4 (one case) (Figure 3C). These observations implicated multiple mutations in these tumor cases.

### *Detection of BRAF and NRAS Hot Spot Mutations in Thyroid Carcinoma Using LH-MSA*

The kinase-activating mutation V600E in BRAF was shown to occur at high frequencies in papillary thyroid carcinoma,[19–21] whereas the activating mutation at Q61 in NRAS is prevalent in follicular thyroid carcinoma.[22,23] LH-MSA was adapted for detection of these mutations. DNA samples prepared from fresh tumor tissues of papillary thyroid tumors were examined by LH-MSA for the V600E BRAF mutation. Distinct double bands (Figure 4A), presumably indicating the heterozygous point mutation, were observed in 64% (16 of 25) of cases, consistent with the previous direct sequencing results. Similarly, DNA samples prepared from fresh tumor tissues of follicular thyroid carcinoma were screened for the NRAS

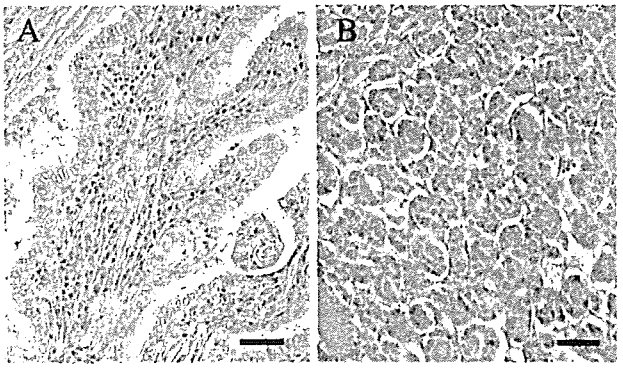


**Figure 5.** Different histopathological types of the thyroid tumor in case 28 in tissue sections stained with H&E. **A:** Tumor tissue of the papillary type. **B:** Tumor tissue of the follicular type. Scale bars = 50 μm.

Q61 mutations using LH-MSA. As shown in Figure 4B, distinct double bands were observed for the putative heterozygous mutations of Q61R and Q61K (26 and 5% of 19 cases, respectively), consistent with predetermined direct sequencing results.

DNA from fresh papillary thyroid carcinoma tumor tissues and from FFPE tumor tissues from the same cases was compared for consistency of the mutations detected by LH-MSA. Of 21 cases compared, 17 yielded the same results using both DNA samples, namely, the BRAF heterozygous V600E mutation in 11 cases and no such mutation in six cases. Discrepant results were observed in four cases (Figure 4C). In one case (no. 11), a single mutant loop-hybrid band was predominant in the FFPE tissue DNA, but heterozygous double bands were observed at equal densities in the fresh tumor DNA. Such differences may be attributed to the loss of heterozygosity in some portions of tumor tissues, including the sampled area of the paraffin section within the tumor heterozygous for the BRAF mutation. The faint shifted loop-hybrid band of the mutant allele in case 25 may suggest that the fresh tumor DNA was contaminated by DNA from normal cells, because equally dense double bands indicating the heterozygous BRAF mutation were detected in the FFPE tumor tissue DNA. In the other two cases (nos. 21 and 28), the normal BRAF in the bulk fresh tumor tissue DNA were discrepant with the apparently heterozygous BRAF mutation in the FFPE tissue DNA. Interestingly, NRAS mutation Q61R was detected in the bulk fresh tumor DNA in these two cases (Figure 4D) but not in the other 19 cases. Histopathological examinations of the tumors documented the presence of both of papillary and follicular tissue types as separate components in the same tumors in these two particular cases, but not in the others (Figure 5, A and B). Tumor DNA from these papillary and follicular tissue types were separately sampled from the FFPE tissue sections and examined for the mutations in BRAF and NRAS using LH-MSA. As shown in Figure 4E, double bands indicating the heterozygous BRAF mutation were detected in the papillary-type but not in the follicular-type tumor tissues. On the other hand, the double bands indicating the heterozygous NRAS mutation were detected in the follicular-type but not in the papillary-type tumor tissues. Because NRAS mutations tended to be associated with follicular thyroid carcinoma,[22,23] DNA prepared from fresh bulk tumors before histological diagnosis in the above two thyroid tumor cases seemed to be derived from the follicular-type tumors carrying the mutation in NRAS but not from the papillary-type tumors carrying the mutation in BRAF.

## *Discussion*

LH-MSA is unique in that synthetic oligomers are used for the generation of heteroduplexes bearing small loops by hybridization to PCR products. Differential mobility of the loop-hybrids in native PAGE that reflects a slight change in the nucleotide adjacent to or within the loop was exploited to detect mutational changes present in the DNA. LH-MSA, along with the original UHG technology,[15–18] may provide a simple and useful system for detection of mutations in various cancers with fixed mutational hot spots. However, detection of widespread loss-of-function mutations in TP53, BRCA1, MLH1, and other cancer-related genes may elude straightforward application of LH-MSA in the present simple format. As shown in this study, LH-MSA is particularly useful for the molecular diagnosis of lung adenocarcinoma because it can easily detect in-frame deletion mutations in exon 19 and the hot spot point mutation L858R in exon 21 of EGFR that is associated with therapeutic responses to EGFR kinase inhibitors such as gefitinib.[5–9] The V600E point mutation in BRAF could be diagnostically important for thyroid carcinoma[19–21] and melanoma[24–26] if specific kinase inhibitor drugs for mutated BRAF become commonly available for therapeutic use.[27] LH-MSA was able to detect mutant alleles present at low proportions in the sampled DNA. This might be a valuable feature of LH-MSA for cases in which tumor cells are intermingled with varying amounts of normal cells in available diagnostic samples. Similar sensitivity in the detection of NRAS mutations at low proportions was also achieved using the standard UHG technology.[17,18]

Point mutations detectable with LH-MSA may not be limited to the genes presently described. Adaptations of LH-MSA to detect various point mutations of the Ras-oncogene KRAS at codons G12 to G13 in various cancers may be pursued. Genotyping of single nucleotide polymorphisms (SNPs) can also theoretically be achieved by LH-MSA at any loci, and adaptation of LH-MSA for various intragenic SNP loci is under investigation. LH-MSA could be incorporated in SNP analyses of risks such as hypersensitivity to certain therapeutic drugs[28,29] and susceptibility to cancers.[30]

LH-MSA is applicable to any unique genomic sequences amplified with PCR. LH-G probes to detect point mutations or to genotype SNPs can be designed using a simple hypothesis. Namely, a mismatched base at the site adjacent to the loop in the loop-hybrid will induce a shift in its mobility. Nucleotide lengths of the loop and the positions of the loop relative to the mutational site can be varied to attain optimal shifts of the loop-hybrid band in the presence of the mutation. A faint secondary band that

might confuse interpretation of the results may be virtually eliminated using PAGE-purified LH-G probes. Mutational changes occurring within the loops were detected by LH-MSA as shown for the mutations in EGFR, A859T, and L861R. These instances indicated that there might be some sensitive positions in the loop sequences such that a single nucleotide change therein may affect loop conformation and lead to a mobility shift of the loop-hybrid. In the present study, PAGE was executed at room temperature, and the influence of ambient temperature fluctuation on the retarded mobility of loop-hybrid DNA during electrophoresis appeared to be low. However, detection of mutations within the loops by LH-MSA seemed to be improved by lowering the temperature during electrophoresis (our preliminary observation).

To assess the reliability of LH-MSA for detecting mutations in the DNA of fixed tumor tissues, DNA prepared from fresh tumor tissues and from FFPE tissue sections was compared. Identical results were obtained for the deletion mutations in EGFR exon 19 of lung adenocarcinoma (data not shown). However, a few discrepant results were observed for the point mutation of BRAF in papillary thyroid carcinoma. In two cases, discrepancy was attributed to the presence of two distinct lines of differentiation in thyroid tumor development. The different histopathological types of tumors and their association with distinct mutations in the above two cases may indicate the existence of tumors that were initiated by a common cause but subsequently followed separate courses of tumor development by the mutations of different genes in RAS-RAF signaling pathway. Our detailed analysis not only resolved the discrepancies but also affirmed the tissue-type-specific associations of the BRAF mutation to papillary[19–21] and the NRAS mutation to follicular[22,23] thyroid carcinoma. The results for the unusual cases shown above clearly indicated that the DNA derived from FFPE tissues, although low in quality and quantity, could provide precise and important molecular data associated with histopathological diagnosis.

Single LH-bands for each of the mutant and normal alleles were sharp and well separated, and therefore seemed amenable to semiquantitative approaches such as copy number estimation. Simplicity of LH-MSA may allow instantaneous detection of mutations in comparison to other mutation detection systems such as SSCP and UHG, in which four bands derived from normal and mutant strands are usually exhibited. Compared with denaturing high performance liquid chromatography, LH-MSA showed a fourfold higher sensitivity in detection of the L858R mutation in EGFR when serially mixed samples were applied at a low concentration (our preliminary results). The simple LH-MSA detection system may enable rapid and low-cost screening of hot spot mutations in certain types of cancers and genotyping of SNPs in clinical risk assessment. LH-MSA and silver staining of the gel after PAGE may facilitate genetic examinations in minimal laboratory settings. Use of LH-MSA for genotyping analysis may not be limited to humans but might be extended to any organism. Current genome-wide searches for SNP loci associated with specific phenotypes and for mutations responsible for hereditary diseases in humans and domestic animals may be greatly facilitated by simple genotyping tools such as LH-MSA.

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## *References*

1. Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad Tunio G, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y: Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science 1998, 279:577–580
2. Joensuu H, Roberts P, Sarlomo-Rikala M, Andersson L, Tervahartiala P, Tuveson D, Silberman S, Capdeville R, Dimitrijevic S, Druker B, Demetri G: Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. N Engl J Med 2001, 344:1052–1056
3. Heinrich M, Corless C, Duensing A, McGreevey L, Chen C, Joseph N, Singer S, Griffith D, Haley A, Town A, Demetri G, Fletcher C, Fletcher J: PDGFRA activating mutations in gastrointestinal stromal tumors. Science 2003, 299:708–710
4. Savage D, Antman K: Imatinib mesylate—a new oral targeted therapy. N Engl J Med 2002, 346:683–693
5. Arao T, Fukumoto H, Takeda M, Tamura T, Saijo N, Nishio K: Small in-frame deletion in the epidermal growth factor receptor as a target for ZD6474. Cancer Res 2004, 64:9101–9104
6. Lynch T, Bell D, Sordella R, Gurubhagavatula S, Okimoto R, Brannigan B, Harris P, Haserlat S, Supko J, Haluska F, Louis D, Christiani D, Settleman J, Haber D: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004, 350:2129–2139
7. Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Camplese P, Iarussi T, Mucilli F, Mezzetti A, Cuccurullo F, Sacco R, Buttitta F: EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. J Clin Oncol 2005, 23:857–865
8. Paez J, Janne P, Lee J, Tracy S, Greulich H, Gabriel S, Herman P, Kaye F, Lindeman N, Boggon T, Naoki K, Sasaki H, Fujii Y, Eck M, Sellers W, Johnson B, Meyerson M: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004, 304:1497–1500
9. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M, Varmus H: EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA 2004, 101:13306–13311
10. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T: Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci USA 1989, 86:2766–2770
11. Kozlowski P, Krzyzosiak W: Combined SSCP/duplex analysis by capillary electrophoresis for more efficient mutation detection. Nucleic Acids Res 2001, 29:E71
12. Takeda S, Ichii S, Nakamura Y: Detection of K-ras mutation in sputum by mutant-allele-specific amplification (MASA). Hum Mutat 1993, 2:112–117
13. Underhill P, Jin L, Lin A, Mehdi S, Jenkins T, Vollrath D, Davis R, Cavalli-Sforza L, Oefner P: Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. Genome Res 1997, 7:996–1005
14. Liu W, Smith D, Rechtzigel K, Thibodeau S, James C: Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. Nucleic Acids Res 1998, 26:1396–1400
15. Wood N, Standen G, Hows J, Bradley B, Bidwell J: Diagnosis of

sickle-cell disease with a universal heteroduplex generator. Lancet 1993, 342:1519–1520
16. Wood N, Tyfield L, Bidwell J: Rapid classification of phenylketonuria genotypes by analysis of heteroduplexes generated by PCR-amplifiable synthetic DNA. Hum Mutat 1993, 2:131–137
17. Belli C, De Brasi C, Larripa I: Rapid detection of exon 1 NRAS gene mutations using universal heteroduplex generator technology. Hum Mutat 2003, 21:132–137
18. Belli C, Bowen D, De Brasi C, Larripa I: A single, multiplex analysis for all relevant activating NRAS gene mutations using heteroduplex generators. Br J Haematol 2004, 126:602–605
19. Cohen Y, Xing M, Mambo E, Guo Z, Wu G, Trink B, Beller U, Westra W, Ladenson P, Sidransky D: BRAF mutation in papillary thyroid carcinoma. J Natl Cancer Inst 2003, 95:625–627
20. Kimura E, Nikiforova M, Zhu Z, Knauf J, Nikiforov Y, Fagin J: High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 2003, 63:1454–1457
21. Xu X, Quiros R, Gattuso P, Ain K, Prinz R: High prevalence of BRAF gene mutation in papillary thyroid carcinomas and thyroid tumor cell lines. Cancer Res 2003, 63:4561–4567
22. Vasko V, Ferrand M, Di Cristofaro J, Carayon P, Henry J, de Micco C: Specific pattern of RAS oncogene mutations in follicular thyroid tumors. J Clin Endocrinol Metab 2003, 88:2745–2752
23. Nikiforova M, Lynch R, Biddinger P, Alexander E, Dorn IIG, Tallini G, Kroll T, Nikiforov Y: RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. J Clin Endocrinol Metab 2003, 88:2318–2327
24. Davies H, Bignell G, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett M, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson B, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins G, Bigner D, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho J, Leung S, Yuen S, Weber B, Seigler H, Darrow T, Paterson H, Marais R, Marshall C, Wooster R, Stratton M, Futreal P: Mutations of the BRAF gene in human cancer. Nature 2002, 417:949–954
25. Brose M, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, Einhorn E, Herlyn M, Minna J, Nicholson A, Roth J, Albelda S, Davies H, Cox C, Brignell G, Stephens P, Futreal P, Wooster R, Stratton M, Weber B: BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res 2002, 62:6997–7000
26. Curtin J, Fridlyand J, Kageshita T, Patel H, Busam K, Kutzner H, Cho K, Aiba S, Brocker E, LeBoit P, Pinkel D, Bastian B: Distinct sets of genetic alterations in melanoma. N Engl J Med 2005, 353:2135–2147
27. Strumberg D, Seeber S: Raf kinase inhibitors in oncology. Onkologie 2005, 28:101–107
28. Lee W, Lockhart A, Kim R, Rothenberg M: Cancer pharmacogenomics: powerful tools in cancer chemotherapy and drug development. Oncologist 2005, 10:104–111
29. Michael M, Doherty M: Tumoral drug metabolism: overview and its implications for cancer therapy. J Clin Oncol 2005, 23:205–229
30. Bond G, Hu W, Bond E, Robins H, Lutzker S, Arva N, Bargonetti J, Bartel F, Taubert H, Wuerl P, Onel K, Yip L, Hwang S, Strong L, Lozano G, Levine A: A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell 2004, 119:591–602

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REVIEW ARTICLE

Hiroshi Kashida · Shin-ei Kudo

# Early colorectal cancer: concept, diagnosis, and management

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**Abstract** In colorectal cancers, although flat and depressed-type lesions are found by regular endoscopic view, magnification and pit-pattern observation are vital parts of the precise diagnosis of the lesion. The depressed-type lesions appear to have a prominent tendency to show malignant characteristics, and the recognition and timely treatment of such lesions is important for improving the morbidity and mortality of colorectal cancer. Chromoscopy is mandatory for an accurate diagnosis of these lesions. The pit-pattern classification correlates well with actual histological findings and can provide important additional information prior to endoscopic treatment of the lesion.

**Key words** Depressed colorectal cancer · Chromoscopy · Magnifying colonoscopy · Pit pattern · Endoscopic mucosal resection

## Introduction

Colorectal cancer is increasing and is expected to become the first cause of death from diseases of the digestive system in Japan within 10 years. Therefore it is important to prevent, to detect, and to treat colorectal cancer early enough. In this article, the present concept, diagnosis, and management of early colorectal cancer are reviewed.

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## Concept of early colorectal cancer

In carcinogenesis, a single cell transforms into a cancer, proliferates through frequent mitoses, and gradually forms a mass. It takes a certain time for a cancer to be diagnosed as such after its birth. Early colorectal cancer is defined by the Japanese rule[1] as being limited to the mucosa or invading only to the submucosa, regardless of the presence or absence of lymph node metastases. The term "early" does not always imply that the lesion is detected right after its birth, but only means that it is early enough for the tumor to be completely cured. Thus, the term does not reflect the time course of the tumor, nor does it fully represent its biological nature.

According to the classification published by The World Health Organization (WHO),[2] only epithelial tumors that have penetrated through the muscularis mucosae into the submucosa are considered malignant in the colon or rectum. Japanese pathologists do not agree with this definition.[3] Western and Japanese pathologists have discussed the matter and have published a new classification in the hope that it would be accepted universally.[4]

The Japanese rule[5] divides the gross appearance of early colorectal cancers into: type I, protruded (Ip, Isp, Is); type II, superficial (IIa, IIb, IIc); and type III, excavated. The Paris endoscopic classification of superficial gastrointestinal neoplasms[6] is largely based on the Japanese classification. Colorectal adenomas and early carcinomas can be grossly divided into three groups; protruded or polypoid, flat-elevated, and depressed.[7] The depressed-type colorectal cancers can be absolutely depressed or can be accompanied by a slightly elevated margin. Some flat-elevated adenomas spread extensively and circumferentially along the colonic wall although they are very short in height. These large flat-elevated adenomas are sometimes malignant, but not so advanced in spite of their large diameter. We[8] advocated a category "laterally spreading tumor (LST)", which is defined as lesions larger than 10 mm in diameter but extending circumferentially rather than vertically. This category is further divided into a granular type, which is composed of fine

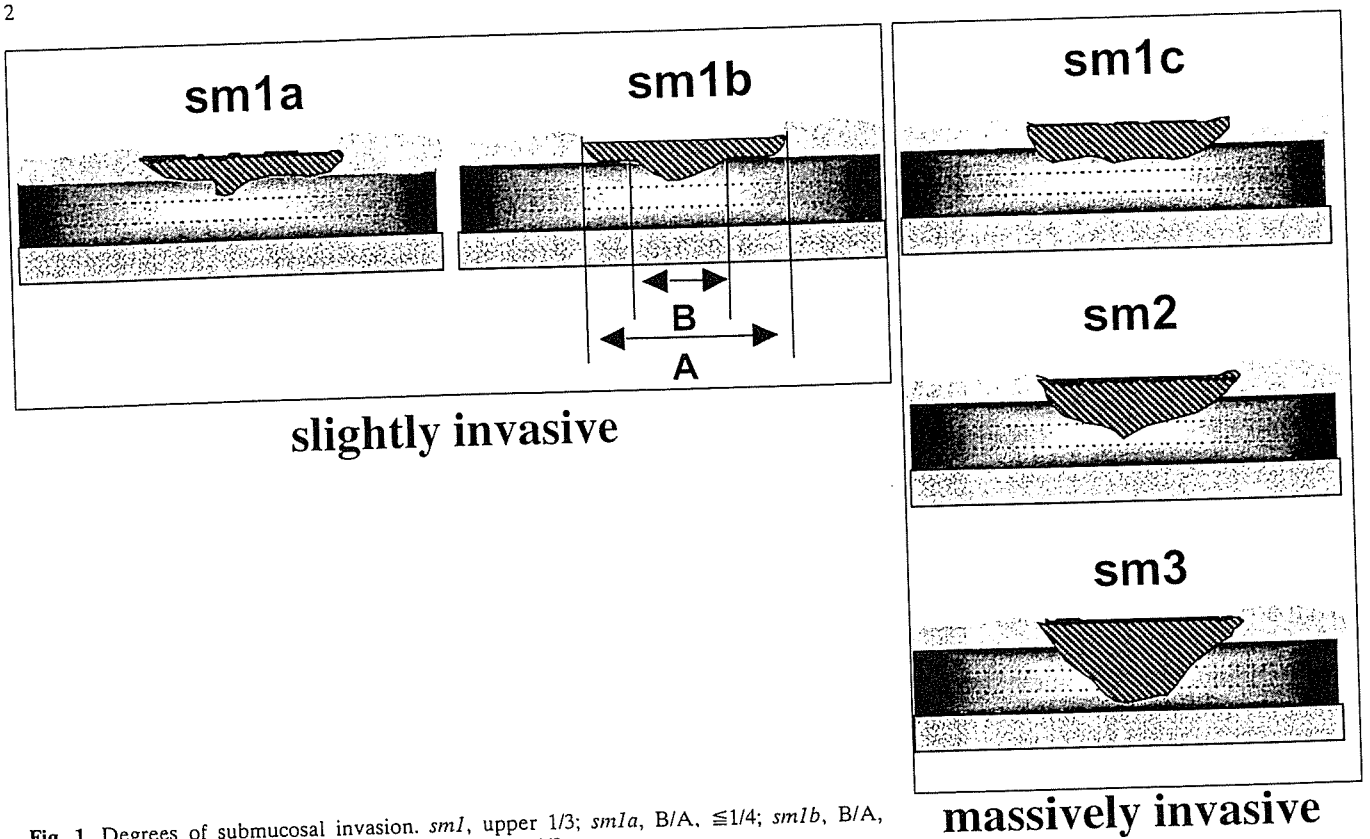


**Fig. 1.** Degrees of submucosal invasion. *sm1*, upper 1/3; *sm1a*, B/A, ≦1/4; *sm1b*, B/A, 1/4–1/2; *sm1c*, B/A, ≧1/2; *sm2*, middle 1/3; *sm3*, lower 1/3

**Table 1.** Rates of submucosal cancer in colorectal neoplasms, excluding advanced cancers (April 1985–June 2005)

| Gross appearance | Size (mm) | | | | | Total |
|---|---|---|---|---|---|---|
| | ≦5 | 6–10 | 11–15 | 16–20 | ≧21 | |
| Depressed | 20/253 | 70/156 | 51/73 | 21/24 | 15/17 | 177/523 |
| | 7.9% | 44.9% | 69.9% | 87.5% | 88.2% | 33.8% |
| Flat-elevated | 2/6848 | 3/1197 | 14/550 | 22/201 | 65/307 | 106/9103 |
| | 0.03% | 0.25% | 2.5% | 10.9% | 21.2% | 1.2% |
| Protruded | 0/6234 | 62/4732 | 89/1163 | 68/414 | 68/233 | 287/12776 |
| | 0% | 1.3% | 7.7% | 16.4% | 29.2% | 2.2% |
| Total | 22/13335 | 135/6085 | 154/1786 | 111/639 | 148/557 | 570/22402 |
| | 0.16% | 2.2% | 8.6% | 17.4% | 26.6% | 2.5% |

granules, and a nongranular type, which is devoid of apparent nodules or granules.

## Staging of early colorectal cancer

The degree of submucosal invasion (Fig. 1)[8] is classified into three stages, based on the depth of invasion; sm1, sm2, and sm3. Stage sm1 is subclassified into 1a, 1b, and 1c, according to the horizontal extension of the invaded area. Sm 1a or 1b cancers (slightly or minimally invasive cancers) without vessel permeation never metastasize to the lymph nodes, liver, or other organs. In contrast, sm1c and sm2 or sm3 lesions (massively invasive submucosal cancers) show a substantial proportion (around 10%) of nodal metastasis.

## Biological behavior of colorectal neoplasia

The invasive rates (Table 1) in depressed types of tumors were 7.9% in lesions not exceeding 5 mm, 44.9% in those of 6–10 mm, and 69.9% in those of 11–15 mm. Depressed lesions are invasive even when they are very small, strongly suggesting that they grow rather rapidly at an early stage,[9–14] to become advanced cancer. In contrast, invasive rates in flat-elevated adenomas were 0.03% for those less

than 5 mm, 0.25% for those of 6–10 mm, and 2.5% for those of 11–15 mm, with these rates being even lower than those for protruded polyps. Therefore, flat lesions are usually benign or only focally malignant and grow very slowly, not becoming invasive until they are rather large. Some studies[15] have suggested that, on following, many small adenomas either do not grow, or sometimes diminish in size.

## Development of colorectal cancer

Concerning the development of colorectal carcinoma, two main doctrines have been advocated; one, called the "adenoma-carcinoma sequence" theory,[16] maintains that a cancer develops from normal mucosa through a stage of adenoma, while the other, called the "de-novo" theory,[17] claims that a carcinoma emerges directly from normal epithelium without going through a stage of adenoma.

The majority of advanced colon cancers are depressed rather than protruded. Few researchers have witnessed an intermediate-stage colon cancer between a benign polyp and an advanced depressed carcinoma, while a significant number of small colonic cancers without any adenomatous remnant have been reported,[18–20] and benign adenomatous tissue is seen in only about 20% of advanced colorectal carcinomas.[21,22] The periphery is usually covered with the normal mucosa and is elevated because of compression by the carcinoma or because of the submucosal proliferation of the tumor cells. The transition from the carcinoma to the adjacent normal colonic mucosa is abrupt and there is no evidence of "residual" adenoma. Occasionally, there can be a few glands which show adenoma-like changes, but these charges could be reactive to the tumor and not related to carcinogenesis.

The supporters of the adenoma-carcinoma theory assert that the adenomatous polyp has been ulcerated or sloughed away during the development. But this may not be the case, because most small early cancers without an adenomatous remnant are not ulcerated. There is speculation that a carcinoma may overgrow a polyp, destroying the evidence of a precursor adenomatous polyp. Shimoda et al.[21] described that, in early colorectal cancers which showed polypoid growth, about 90% contained an adenomatous element; while in those which showed nonpolypoid growth, none contained any adenomatous remnants, although they were significantly smaller (average diameter, 8.7 mm) than those with polypoid growth (average diameter, 16.8 mm). If an adenomatous precursor is to be gradually replaced by a carcinoma, a higher incidence of adenomatous remnants should be recognized in smaller lesions.

Meanwhile, there is no evidence, either, that colon cancers without an adenomatous component really arise de novo from an absolutely normal mucosa. An alternative explanation may be that these lesions arise from very small microadenomas, which are rapidly replaced by the expanding carcinoma. It is difficult to prove which is true – whether a cancer arises from a microadenoma that is replaced rapidly by carcinomatous tissue, or whether it arises strictly de novo from an isolated dysplastic cell that degenerates readily without going through an adenomatous step. In any case, such a rapid development of cancer looks quite different from the conventional polyp-cancer concept. It would be natural to believe that there is a de-novo pathway also in colorectal cancer development. De-novo carcinogenesis from flat mucosa, without going through an adenomatous stage, is not rare in the gastrointestinal tract, other than the colorectum. Studies of chemically induced colonic carcinomas in experimental animals[23,24] have shown evidence in support of de-novo carcinogenesis. It is only in the human colorectum that the adenoma-carcinoma sequence is regarded as the main pathway of carcinogenesis.

## Genetic alterations in colorectal neoplasia

Genetic studies also seem to support the idea that there are at least two ways of colonic cancer development. The adenoma-carcinoma sequence theory in the colorectum was supported by Vogelstein et al.[25] Multistep carcinogenesis had been assumed in other organs as well, but the study by Vogelstein's group was epoch-making, in that they connected the genetic alterations to morphological changes in colorectal tumors. The first genetic alteration to be recognized in colorectal tumorigenesis is that of the *APC* (adenomatous polyposis coli) gene. Mutational activation of the K-*ras* oncogene is considered to play a role in the progression of the size and grade of atypia in the adenoma-carcinoma sequence. In studies comparing the K-*ras* mutation and the configuration of colorectal tumors, K-*ras* mutations were detected in as many as 70% of protruded adenomas, whereas they were rare in superficial adenomas.[26] Superficial colorectal tumors are regarded as candidates for de-novo carcinogenesis and seem to be associated with specific genetic alterations different from those in the adenoma-carcinoma sequence. It is speculated that, once a lesion becomes overt carcinoma, the consecutive changes are similar regardless of the lesion's initial form or whichever pathway it has followed. P53 alteration is not only frequently witnessed in the development of carcinoma through the adenoma-carcinoma sequence but has also been shown to be a frequent event in de-novo carcinogenesis. It would be important to determine the genetic profiles of early colorectal cancers and precancerous lesions classified by their gross configuration.

## Detection of colorectal adenomas and early cancers

The immunological fecal occult blood test (FOB) is used in Japan as a method of screening for colorectal cancers. In actuality, however, subjects positive for FOB turn out to have polyps more often than cancers. It is not rare that a flat adenoma or a depressed lesion is found in FOB-negative subjects. These lesions are also notoriously difficult to detect with a barium enema study. Therefore, total colono-

scopy is important for the diagnosis of early colorectal cancers.

To detect a protruded polypoid lesion is not so difficult. In order to detect a depressed or a flat-elevated lesion, finding a small area with slight color change is important.[27] Some lesions look faintly red; others are slightly pale. A minimal deformation of the bowel wall, interruption of the mucosal capillary network pattern, or spontaneous bleeding suggests the existence of a depressed lesion. It is also important to observe the suspected lesion with air inflation and deflation. In benign lesions, both the lesion and the surrounding normal mucosa are elevated after the deflation of air, and so no significant shape change occurs. In intramucosal or minimally invasive cancers, the surrounding mucosa becomes elevated when the air is deflated, emphasizing the depression. The sign is not observable in more invasive lesions, as they are hard, and fixed to the colonic wall.

Chromoendoscopy is very useful for clarifying the depression within a lesion. But you should bear in mind that lesions should be detected with ordinary views and the dye be sprayed only after the detection of a lesion. Kariya et al.[28] reported the first case of depressed early colon carcinoma in the Japanese language in 1977. Kudo and Muto[29] reported their own cases, in Japanese, in 1986, and Kudo made a report in English in 1993.[30] Depressed-type early colorectal cancers, as well as flat adenomas, were first thought to be unique to a localized area in Japan or the East, but, recently, an increasing number of similar lesions has also been witnessed in Western countries.[31–34] Though depressed early

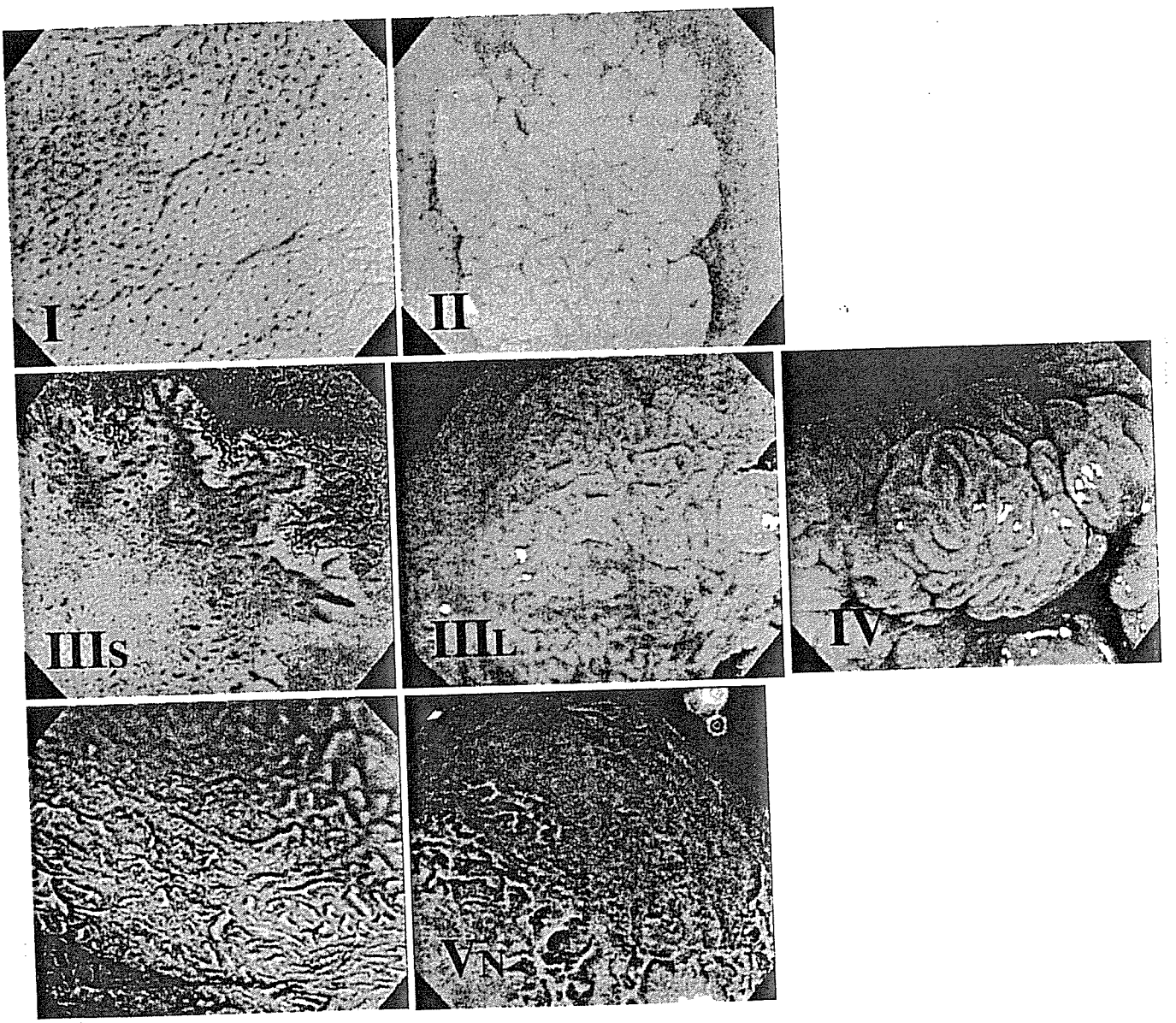


**Fig. 2.** Classification of pit patterns. *I*, *II*, *IIIs*, small; *IIIL*, large; *IV*, *VI*, irregular; *VN*, nonstructural

colonic cancers are still found only rarely, taking into account the difficulty of endoscopic detection of such tumors and their rapid growth, there could be still far more that are being overlooked than detected.

So-called flat adenomas are not perfectly flat, but are usually slightly elevated. Their color is often faintly reddish, which helps to detect them. Chromoendoscopy is useful for clarifying the lesion, but sometimes, with this modality, flat lesions resemble depressed lesions, because they appear to have a depression. The depression in a depressed lesion is rather extensive and clearly demarcated. In contrast, the "depression" in flat adenomas is actually an ill-defined pseudodepression with only a thorny or groove-like appearance. Adenomas with a pseudodepression should be differentiated from truly depressed lesions, because the former are almost invariably benign.[35,36] Some depressed cancers are somewhat elevated as a whole, though they definitely contain a discrete depression.[37] In such cases, the lesions look simply polypoid at first sight, but, after the spraying of dye, the depression emerges. These polyp-mimicking

**Table 2.** Pit patterns and histology of lesions (April 2001–June 2005)

| Pit pattern | Adenoma (dysplasia) | | Submucosal cancer | Total |
|---|---|---|---|---|
| | Low-grade | High-grade | | |
| IIIL | 2714 (83.3%) | 546 (16.7%) | 0 | 3260 |
| IV | 400 (51.1%) | 364 (46.5%) | 19 | 783 |
| IIIs | 29 (55.8%) | 22 (42.3%) | 1 | 52 |
| V | | | | |
| I | 35 | 165 (59.1%) | 79 (28.3%) | 279 |
| N | 0 | 8 (9.9%) | 73 (90.1%) | 81 |
| Total | 3178 | 1105 | 172 | 4455 |

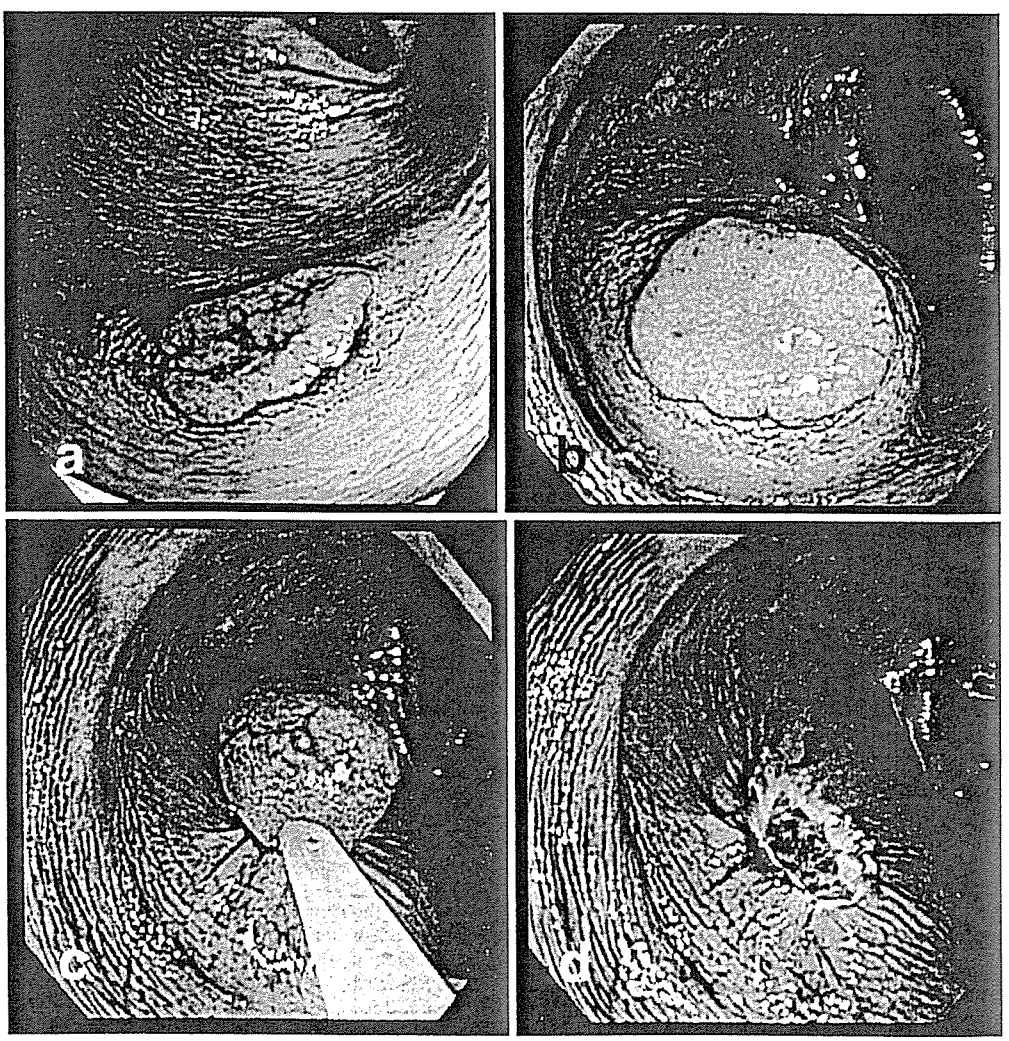


**Fig. 3a–d.** Endoscopic mucosal resection technique. **a** A laterally spreading tumor. **b** After the injection of normal saline. **c** The lesion is captured with a snare and is cut off. **d** After the resection

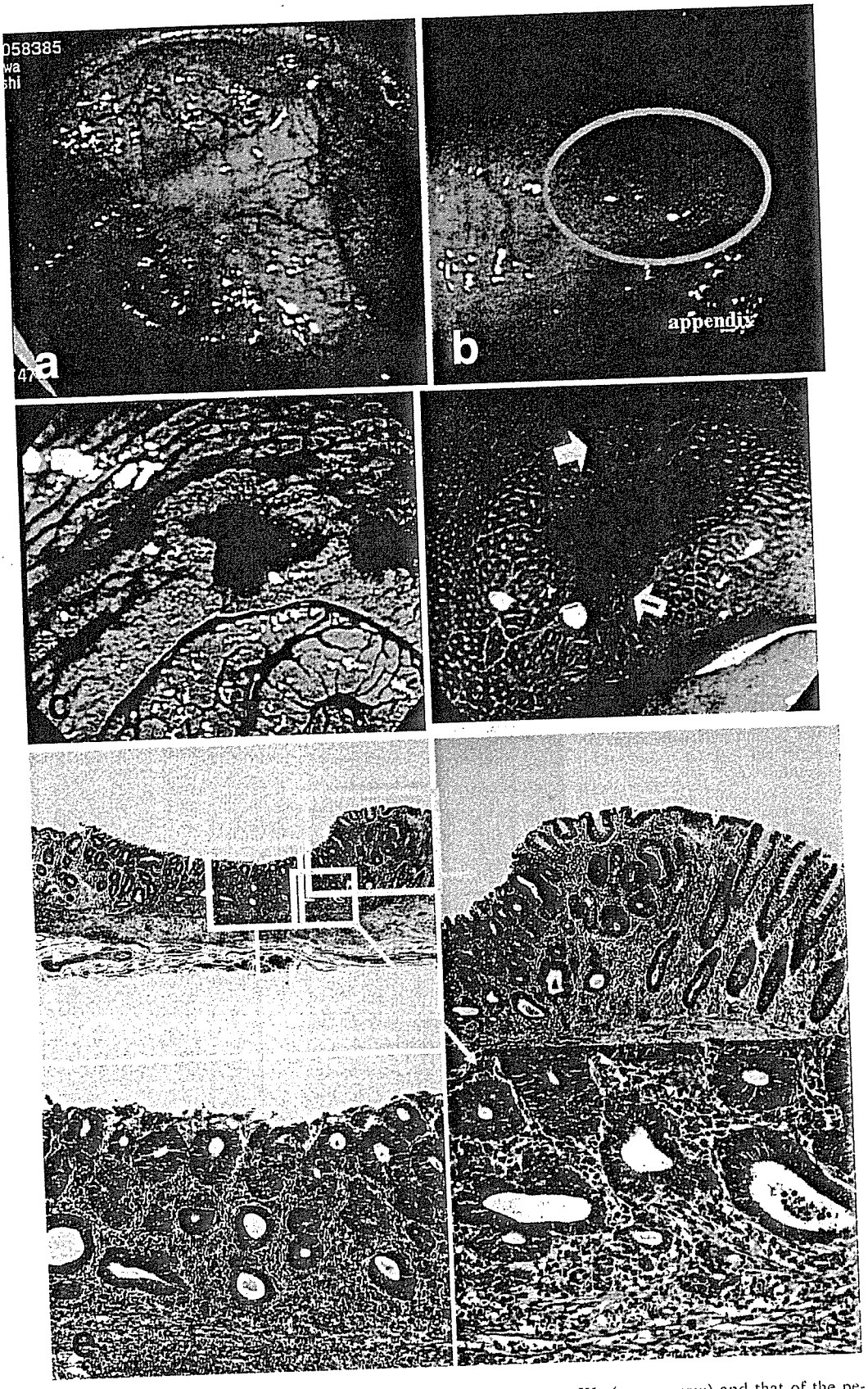


**Fig. 4a–e.** Depressed lesion. **a** Ordinary view of the cecum and the appendiceal orifice. **b** At low magnification, there was a tiny reddish area (*circle*), 5 mm in diameter, near the appendiceal orifice. **c** Indigocarmine dye spraying clarified a depression within the lesion. **d** In a high-magnification view after crystal violet staining, the pit pattern within the depression was type IIIs (*open arrow*) and that of the periphery was type I (*closed arrow*). **e** The lesion was endoscopically treated and was diagnosed pathologically as an intramucosal carcinoma, Vienna category 5.1

cancers with a depression may represent a transient and intermediate stage of the "depressed lesion-advanced carcinoma" sequence.

Despite their large diameters, LSTs are often overlooked because they are extremely short and almost normal in color. The keys to detect them are minimal color change and slight irregularity of the colonic wall. Dye-spraying helps in clarifying the boundary and the extent of the tumor.

In summary, chromoendoscopy is useful for confirming small colorectal lesions, for determining their lateral extent, and for clarifying their gross configuration; this is especially useful in determining the presence or absence of depression within the lesions.

## Endoscopic prediction of histology

The openings of colonic crypts are referred to as "pits", and the specific arrangement of the openings of the glands in various kinds of lesions (which can be observed with a magnifying endoscope) is called the "pit-pattern".[38,39] We have documented all resected lesions, with pit-pattern findings, diameter, and final pathological findings.[40,41] The pit patterns (Fig. 2) were classified as I, II, IIIs (small), IIIL (large), IV, VI (irregular), and VN (nonstructural). On magnifying colonoscopy, most (95.7% and 95.7%) of the protruded and flat neoplasms showed a type IIIL or IV pit pattern. Eighty-six percent of the depressed lesions were characterized by type IIIs, VI, or VN pit patterns. The pit patterns correlated well with the final histological diagnosis (Table 2). Type IIIs, IIIL, and IV pit patterns were typical of adenomas, and were only rarely seen in invasive cancers (1.9%, 0%, and 2.4%, respectively). On the other hand, 90.1% of type VN lesions were invasive.

## Management of colorectal neoplasia

It is unnecessary to remove non-neoplastic lesions. Pit-pattern analysis is useful to differentiate between neoplastic and non-neoplastic lesions, because, if the pit pattern is type I or type II, the lesion is almost definitely non-neoplastic. In such a case you need neither take a biopsy nor wait for the pathological report before deciding whether or not to remove it. Lesions with type IIIs, IIIL, or IV pit patterns can be treated endoscopically because they are probably adenomatous but not invasive. Those with a type-VN pit pattern should be treated surgically because they are definitely invasive and may have nodal metastases. Lesions with a type-VI pit pattern encompass a variety of lesions, from benign adenoma to invasive carcinoma. Therefore, lesions with a type-VI pit pattern are first treated endoscopically, and additional surgical colectomy and lymph node dissection is considered after the histological analysis of the excised specimen; non-invasive or minimally invasive (sm1a or 1b) lesions without vessel infiltration can be merely observed. The Japanese Society for Cancer of the Colon and Rectum has produced a guideline for the treatment of colorectal carcinoma[42] and recommends additional surgery for submucosal cancers with a depth of invasion of more than 1000 μm. However, measuring the depth of invasion is neither always easy nor accurate, and you need to verify whether the guideline is really appropriate.

Endoscopic techniques for treating colorectal neoplasms include hot biopsy, polypectomy, and endoscopic mucosal resection (EMR)[30] (Fig. 3). Depressed and flat-elevated lesions which are suspected of showing cancer should be treated with an EMR technique which allows a sufficient safety margin and accurate pathological evaluation of the resected specimen (Fig. 4). Laterally spreading tumors (LSTs) are good indications for an EMR technique, but they are sometimes too large to be removed en bloc. Endoscopic piecemeal resection (EPMR) is performed for such large lesions. A newly developed technique which utilizes special devices and allows a large LST to be removed in one piece is called endoscopic submucosal dissection (ESD).[43] It is beneficial for the pathological confirmation of the cut margin, and for lower recurrence rates, but is time- and cost-consuming and is associated with a rather high rate of perforation. Therefore, it is not yet widely practiced for the treatment of colorectal lesions.

## Conclusion

Flat-elevated and depressed lesions, especially the latter, are important in the colorectum. Chromoscopy is mandatory for an accurate diagnosis of these lesions. Pit-pattern analysis helps to predict the histology of the lesions, and is therefore useful in determining treatment selection.

## References


1. Japanese Society for Cancer of the Colon and Rectum (1997) Japanese classification of colorectal carcinoma. Kanehara, Tokyo, p 20
2. Hamilton SR, Vogelstein B, Kudo S, et al (2000) Tumors of the colon and rectum. In: Hamilton SR, Aaltonen LF (eds) Pathology and genetics of tumors of the digestive system. IARC, Lyon, pp 103–143
3. Schlemper RJ, Itabashi M, Kato Y, et al (1998) Differences in the diagnostic criteria used by Japanese and Western pathologists to diagnose colorectal carcinoma. Cancer 82:60–69
4. Schlemper RJ, Riddell RH, Kato Y, et al (2000) The Vienna classification of gastrointestinal epithelial neoplasia. Gut 47:251–255
5. Japanese Society for Cancer of the Colon and Rectum (1997) Japanese classification of colorectal carcinoma. Kanehara, Tokyo, p 6
6. Lambert R, Lightdale CJ (2003) The Paris endoscopic classification of superficial neoplastic lesions: esophagus, stomach, and colon. Gastrointest Endosc 58:s5
7. Kashida H, Kudo S (2003) Magnifying colonoscopy, early colorectal cancer, and flat adenomas. In: Waye JD, Rex DX, Williams CB (eds) Colonoscopy: principles and practice. Blackwell, Malden, pp 478–486
8. Kudo S (1996) Early colorectal cancer: detection of depressed-types of colorectal carcinoma, Igaku-shoin, Tokyo, pp 34–36; 88–90

9. Hirata I, Tanaka M, Sugimoto K, et al (1991) Clinicopathological study on flat and depressed minute colorectal carcinomas. Dig Endosc 3:526–535
10. Iishi H, Tatsuta M, Tsutsui S, et al (1992) Early depressed adenocarcinomas of the large intestine. Cancer 69:2406–2410
11. Vas W, Somers S, Stevenson G (1982) Rapid growth of carcinoma of the colon. Gastrointest Endosc 28:19–21
12. Jelinek GP, Nava HR, Nime F (1983) Small primary de-novo adenocarcinoma of the colon with mesenteric lymphatic metastasis. J Surg Oncol 23:185–188
13. Herrera L, Hanna S, Castillo N, et al (1991) Primary de novo adenocarcinoma of the colon measuring 8mm in diameter with lymph node metastases: report of a case. Dis Colon Rectum 34:275–279
14. Minamoto T, Mai M, Ogino T, et al (1993) Early invasive colorectal carcinomas metastatic to the lymph node with attention to their nonpolypoid development. Am J Gastroenterol 88:1035–1039
15. Hoff G, Foerster A, Vatn MH, et al (1986) Epidemiology of polyps of the rectum and colon; recovery and evaluation of unresected polyps 2 years after detection. Scand J Gastroenterol 21:853–862
16. Morson BC (1974) The polyp cancer sequence in the large bowel. Proc R Soc Med 67:451–457
17. Helwig EB (1947) The evolution of adenomas of the large intestine and their relation to carcinoma. Surg Gynecol Obstet 84:36–49
18. Spjut HJ, Frankel NB, Appel MF, et al (1979) The small carcinoma of the large bowel. Am J Surg Pathol 3:39–46
19. Crawford BE, Stromeyer FW (1983) Small nonpolypoid carcinomas of the large intestine. Cancer 51:1760–1763
20. Kuramoto S, Oohara T (1988) Minute cancers arising de-novo in the human large intestine. Cancer 61:829–834
21. Shimoda T, Ikegami M, Fujisaki J, et al (1989) Early colorectal carcinoma with special reference to its development de-novo. Cancer 64:1138–1146
22. Hermanek P, Crall FP (1986) Early (microinvasive) colorectal carcinoma. Int J Colorect Dis 1:79–84
23. Maskens AP, Dujardin-Loits RM (1981) Experimental adenomas and carcinomas of the large intestine behave as distinct entities: most carcinomas arise de-novo in flat mucosa. Cancer 47:81–87
24. Chang WW (1984) Histogenesis of colon cancer in experimental animals. Scand J Gastroenterol 104:27–43
25. Vogelstein B, Fearon ER, Hamilton SR, et al (1988) Genetic alterations during colorectal tumor development. N Engl J Med 319:525–532
26. Fujimori T, Satonaka K, Yamamura-Idei Y, et al (1994) Non-involvement of ras mutations on flat colorectal adenomas and carcinomas. Int J Cancer 57:51–55
27. Kudo S, Kashida H, Nakajima T, et al (1997) Endoscopic diagnosis and treatment of early colorectal cancer. World J Surg 21:694–701
28. Kariya J, Mizuno K, Mayama M (1977) A case of early colonic cancer type IIc associated with familial polyposis coli (in Japanese with English abstract). Stomach and Intestine (I to Cho) 12:1359–1364
29. Kudo S, Muto T (1986) Superficial depressed type (IIc) of colorectal carcinoma (in Japanese with English abstract) Gastroenterol Endosc 28:2811–2813
30. Kudo S (1993) Endoscopic mucosal resection of flat and depressed types of early colorectal cancer. Endoscopy 25:455–461
31. Stolte M, Bethke B (1995) Colorectal mini-de-novo carcinoma: a reality in Germany too. Endoscopy 27:286–290
32. Fujii T, Rembacken BJ, Dixon MF, et al (1998) Flat adenomas in the United Kingdom: are treatable cancers being missed? Endoscopy 30:437–443
33. Hart AR, Kudo S, Mackay EH, et al (1998) Flat adenomas exist in asymptomatic people: important implications for colorectal cancer screening programs. Gut 43:229–231
34. Saitoh Y, Waxman I, West AB, et al (2001) Prevalence and distinctive biologic features of flat colorectal adenoma in a North American population. Gastroenterogy 120:1657–1665
35. Kudo S, Kashida H, Tamura S, et al (1997) The problem of "flat" colonic adenoma. Gastrointest Endosc Clin N Am 7:87–98
36. Sakashita M, Aoyama N, Maekawa S, et al (2000) Flat-elevated and depressed, subtypes of flat early colorectal cancers, should be distinguished by their pathological features. Int J Colorectal Dis 15:275–281
37. Kudo S, Kashida H, Tamura T, et al (1999) Colonoscopic diagnosis and management of non-polypoid ECC (early colorectal cancer). World J Surg 23:694–701
38. Kudo S, Hirota S, Nakajima T, et al (1994) Colorectal tumours and pit pattern. J Clin Pathol 47:880–885
39. Kudo S, Rubio CA, Teixeira CR, et al (2001) Pit pattern in colorectal neoplasia: endoscopic magnifying view. Endoscopy 33:367–373
40. Huang Q, Fukami N, Kashida H, et al (2004) Interobserver and intra-observer consistency in the endoscopic assessment of colonic pit patterns. Gastrointest Endosc 60:520–526
41. Kudo S, Kashida H (2005) Flat and depressed lesions of the colorectum. Clin Gastroenterol Hepatol 3:S33–36
42. Japanese Society for Cancer of the Colon and Rectum (2005) The guideline for the treatment of colorectal carcinoma (in Japanese). Kanehara, Tokyo, p 15
43. Yahagi N, Fujishiro M, Imagawa A, et al (2004) Endoscopic submucosal dissection for the reliable en bloc resection of colorectal mucosal tumors. Dig Endosc 16:s89–92

# Real-time in vivo virtual histology of colorectal lesions when using the endocytoscopy system

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**Background:** The histological findings of GI lesions are based on light-microscopic examination of H&E-stained thin-slice specimens. Recently, a concept of optical biopsy has been advocated. A study of the observation of colorectal lesions using endocytoscopy to obtain real-time histological images in vivo during endoscopy was performed.

**Design:** Prospective study.

**Aim:** To evaluate the usefulness of optical biopsy of colorectal lesions with the endocytoscopy (E-C) system.

**Patients:** The subjects were 113 consecutive patients who underwent a complete colonic examination, from April 2003 to March 2004, performed by a single colonoscopist.

**Setting:** Digestive Disease Center of Showa University Northern Yokohama Hospital.

**Results:** With E-C, it was possible to observe lesions at the cellular level and evaluate cellular atypia in addition to structural atypia in vivo. The correlation was statistically significant between the endocytoscopic diagnosis and the histological diagnosis.

**Limitations:** The endocytoscope had to be touched to the target colonic glands.

**Conclusions:** It was possible to distinguish neoplastic from non-neoplastic lesions, and also possible to distinguish invasive cancer from adenoma. 'Ultra-high' magnifying endoscopy, the E-C system, provides real-time histological images in vivo, which correspond well with those of H&E-stained microscopic images. (Gastrointest Endosc 2006;63:1010-7.)

With recent advances in endoscopic instruments and techniques, various new methods have been developed in addition to conventional endoscopy. Chromoendoscopy and magnifying endoscopy are now regarded as essential for diagnosing early colorectal lesions.[1] Our group has reported on pit-pattern diagnosis with magnifying endoscopy, describing the correlation between the pit-pattern findings and histologic diagnoses.[2,3] The pit-pattern analysis makes it possible to predict a histologic diagnosis of early colorectal lesions before resection.

There have been reports on laser-scanning confocal microscopy (LCM) imaging of untreated specimens from the GI tract, including the esophagus, the stomach, and the colon.[4,5] Sakashita et al[6] reported on virtual histology of colorectal lesions when using LCM and showed that LCM images corresponded well with H&E-stained microscopic images. Observation of the rectal mucosa in vivo was also performed by using a probe-type LCM endomicroscope, but the images obtained were not as clear as those provided by the in vitro LCM system.

The theory of contact endoscopy was applied to the endocytoscopy (E-C) system. Contact endoscopy was first described by Hamou[7,8] as microhysteroscopy to examine the surface of the genital tract at high magnification. Since 1979, the application of contact endoscopy has been extended to larynx, nose, and other sites. For the GI tract, Tada et al[9] first reported on ultrahigh magnifying endoscopy for colorectal lesions. Ooue,[10] Kumagai et al,[11,12] and Inoue et al,[13,14] independently reported on the experience of using prototype contact endoscopy for the digestive tract. The E-C system is a novel ultrahigh magnifying endoscopy, which enables microscopic observation at the cellular level and can be applied clinically.


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