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GENETIC ANALYSIS OF THE PROCESS OF COLORECTAL CARCINOGENESIS

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要旨

Multi-stage carcinogenesis is an important concept in cancer biology. Each new stage is triggered by the acquisition of an additional genetic aberration, leading to clonal expansion of the cancer cell. The resulting tumor mass consists of cancer cells with all the acquired genetic aberrations, but may also include precursor cells at an earlier stage of carcinogenesis. In the present study I focused on a typical example of multi-stage tumorigenesis - the Vogelstein model on the chronology of genetic events in colorectal cancer. I aimed to analyze the process of carcinogenesis through a study of genetic aberrations and their heterogeneity, a common biological feature of human cancer.

Eighty-six bulk colorectal cancer tissue samples and thirteen metastases were examined for mutations in APC, CTNNB1, K-ras *and* TP53, *and for loss of heterozygosity in chromosome 18q. Primary cancer tissues with mutated* APC, K-ras *and* TP53 *genes were selected for the next experiments. To analyze intratumor heterogeneity of mutations in the three genes, 50 regions (100x100x40 μm) consisting of only tumor cells were microdissected from each primary tumor*

sample, and their mutational status was determined by SNaPshot assay, a primer extension assay. Available metastatic tissues with three mutated genes were also examined.

Simultaneous occurrence of mutations in APC, K-ras *and* TP53 *genes was found in 13 out of 86 cases (15.1%). In 10 out of these 13 tumor tissues, I further verified the existence of intratumor heterogeneity and identified cancer cell subpopulations corresponding to putative precursors: cells having mutations in one or two of the three genes. Intratumor heterogeneity in human cancer is probably due to such precursor cancer cells. Furthermore, samples were likely to be of monoclonal origin, and temporal sequences of mutations could be deduced from the mutation patterns of putative precursors. The order of mutation events was variable. The results with two tumor tissues that included not only carcinoma but also adenomatous part did not contradict the core of the Vogelstein model, i.e.,* APC *is the first gene to be mutated. The analysis also revealed considerable heterogeneity in allele ratios of one or two of the chromosomes. By comparing colorectal tumor tissue and its metastasis, I found that metastases were likely to develop from the major population of the primary site.*

To my knowledge, this is the first study of colorectal tumorigenesis, based on heterogeneity of three mutated genes, in individual cancer tissues. The approach used in this study should help to uncover the process of carcinogenesis in other cancers.

List of Abbreviations

APC	adenomatous polyposis coli
β-TrCP	β-transducin repeat containing protein
CaMP	cancer mutation prevalence
CGH	comparative genomic hybridization
CIN	chromosomal instability
CRC	colorectal cancer
Dvl	Dishevelled protein
GAP	GTPase activating protein
GEF	guanine-nucleotide exchange factor
GSK-3β	glycogen synthase kinase 3β
HE	hematoxylin-eosin staining
LRP5/6	LDL-receptor-related protein
LOH	loss of heterozygosity
MCR	mutation cluster region of *APC* gene
MSI	microsatellite instability
NES	nuclear export signal
NLS	nuclear localization signal
RTK	receptor tyrosine kinase
SNP	single-nucleotide polymorphism
Tcf/Lef1	T-cell specific transcription factor/lymphoid enhancer-binding factor

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INTRODUCTION

CARCINOGENESIS

How does cancer develop? This is one of the main questions in cancer biology. According to the widely accepted "clonal evolution" model of tumor progression, proposed by Nowell (1976), tumor initiation occurs by an induced change in a previously normal cell, which makes it neoplastic and provides it with a selective advantage over adjacent normal cells. Neoplastic proliferation then proceeds, during which more mutations are introduced as a result of genetic instability in the expanding tumor. Almost all variants are eliminated due to metabolic or other disadvantages - clonal selection occurs. Only a few cells carry enough selective advantages to enable them to go on dividing, and these cells become the dominant clone. Therefore, carcinogenesis is a multi-stage process in which an initial population of slightly abnormal cells, descendants of a single mutant ancestor, evolves through successive cycles of mutation and natural selection (Nowell, 1976; Alberts, 2002).

In Nowell's model, tumors are of monoclonal origin. However, more recent data are controversial. One study, in which the clonality of tumors was examined by analyzing the status of tumors in females who are mosaic for X-linked genes, found that tumors develop from one cell (Vogelstein et al., 1985). Monoclonal origin was also reported by Fearon et al.

(1987) and Kuwabara et al. (1998), who studied colorectal carcinoma. However, others who studied intestinal tumors claim polyclonal origin (Novelli et al., 1996; Halberg et al., 2007). Thus, still there is no clear answer to the question of cancer clonality.

The model of colorectal carcinogenesis proposed by Fearon and Vogelstein (1990) is a typical example of the Nowell's clonal evolution theory of tumorigenesis.

COLORECTAL CANCER

Colorectal cancer (CRC) is the fourth most common cancer in men and the third in women. New colorectal cancer cases for 2007 were estimated to be nearly 1.2 million; about 600,000 deaths worldwide were caused by this type of cancer in 2007 (ACS, http://www.cancer.org/). The highest incidence rates are found in North America, Japan, parts of Europe, New Zealand, and Australia. Rates are low in Africa and South-East Asia. Rates are substantially higher in men than in women.

Symptoms and factors related to CRC are summarized in Table 1.

Table 1. *Main characteristics of CRC*

Symptoms (Ballinger et al., 2007) * Blood in the stool * Change in bowel habits * Abdominal pain
Risk factors (ACS, http://www.cancer.org/) * Age * Genetic mutations (Hardy et al., 2000) * Chronic inflammatory bowel disease * Obesity * Smoking * Alcohol consumption * Diet high in red or processed meat
Preventive factors (ACS, http://www.cancer.org/) * Physical activity * Diet high in fruits and vegetables * NSAID - nonsteroidal anti-inflammatory drugs

Colorectal cancer classification

Colorectal cancer cases can be divided into three groups: hereditary, sporadic and familial (The Human Genome - http://genome.wellcome.ac.uk/). About 85% of colorectal tumors are sporadic (Soreide et al., 2006). Hereditary colon cancer accounts for about 5-10% of CRC cases. The commonest hereditary syndromes are familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC). Familial cases occur in people with a family history of the disease. The exact reason for increased risk of CRC in these families is unknown.

Genetic pathways in sporadic CRC

There are two groups of molecular alterations in sporadic CRC. In about 15% of cases, a defect in DNA mismatch repair leads to microsatellite instability (MSI), characterized by widespread deletions and insertions in microsatellite repeats (Soreide et al., 2006; Treanor et al., 2007). In the other 85%, chromosomal instability (CIN) is observed. CIN refers to an increased rate of loss or gain of a whole chromosome or parts of chromosomes during cell division as a result of aberrations in certain genes (Lengauer et al., 1998). The consequences of CIN are an imbalance in the number of chromosomes per cell (aneuploidy) and an enhanced rate of loss of heterozygosity (LOH).

Mechanism of sporadic colorectal carcinogenesis

In 1990, a stepwise model of colorectal carcinogenesis was developed (Fearon et al., 1990) – Figure 1. According to this model, colorectal tumorigenesis proceeds through a series of genetic alterations involving oncogenes (*K-ras*) and tumor suppressor genes (*APC*, *TP53* and *DCC/SMAD*). Mutations in the *APC* gene are proposed to occur early during the development of polyps; *K-ras* mutations arise during the adenomatous stage, followed by LOH on chromosome 18q (*DCC/SMAD*), and mutations in the *TP53* gene at a later stage (Fearon et al., 1990). However, this model is based on studies of patient populations and

lacks direct support from observations of individual tumor tissues. Moreover, many papers have shown that mutations in *TP53* or *K-ras* are found in CRC patients in the absence of *APC* mutation (Smith et al., 2002; Frattini et al., 2004; Samowitz et al., 2007). Samowitz et al. (2007) identified sequence variations in all three genes in 11.1% of examined specimen. Furthermore, Smith et al. (2002) found three genes mutated in only 6.6% of the analyzed cases, and suggested that multiple alternative genetic pathways to CRC exist. Besides, Frattini et al. (2004) reported two different pathways, depending on the tumor localization: *K-ras*-dependent (*APC*-*K-ras*-*TP53*-chr18q) and *K-ras*-independent (*APC*-*TP53*-chr18q). Decreased rates of K-ras mutations were also found in one type of CRC, nonpolypoid (flat) growth type, in which height of the lesion is less than width (Speake et al., 2007). Flat lesions are thought to develop through "de novo" pathway because no adenoma stage was identified in them (Soetikno et al., 2006). The mechanism of colorectal carcinogenesis needs further investigation.

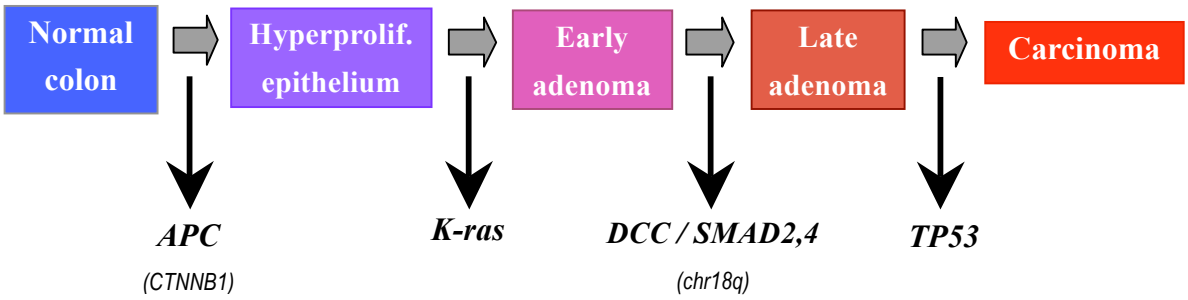


Figure 1. *Model of colorectal carcinogenesis, proposed by Fearon and Vogelstein (1990). The process of tumorigenesis includes numerous steps; each of them is triggered by an aberration in a particular gene.*

GENES INVOLVED IN SPORADIC CRC

According to The Wellcome Trust Sanger Institute's Catalogue of Somatic Mutations in Cancer - COSMIC (http://www.sanger.ac.uk/genetics/CGP/cosmic/), the top three mutated genes in colorectal adenocarcinoma are *TP53* (39%), *APC* (38%) and *K-ras* (35%). Furthermore, in a recent report about mutations in colon and breast cancer (Wood et al., 2007), genes with alterations were ordered according to their cancer mutation prevalence score (CaMP), which reflects the probability that a gene is mutated at a higher frequency than the passenger mutation rate. According to this report, the five commonest mutated genes in CRC are *APC*, *K-ras*, *TP53*, *PIK3CA* and *FBXW7*. The first three genes in both this report and COSMIC are components of the model of colorectal carcinogenesis proposed by Vogelstein and Fearon (1990). *APC* and *TP53* are also believed to be CIN genes, thus explaining the high frequency of CIN observed in CRC (Fukasawa et al., 1996; Fodde et al., 2001).

APC (adenomatous polyposis coli)

The *APC* gene is located on chromosome 5q21 and includes 16 exons. It encodes a 2843-amino acid protein (310 kDa) with multiple functional domains that mediate both

oligomerization and binding of many intracellular proteins: β-catenin, γ-catenin, GSK-3β (glycogen synthase kinase), axin, tubulin, EB1 (end binding protein), hDLG (human homologue of the Drosophila discs large tumor suppressor protein), etc. - Figure 2A. Specific localization of APC at the end of microtubules implies an important role in regulation of the cytoskeleton and cellular migration (Hanson et al., 2005). Interacting with many proteins, APC is also thought to play role in cell-cell adhesion, regulation of mitotic spindle assembly and chromosome segregation, cell cycle progression and cell death (Leslie et al., 2002; Hanson et al., 2005).

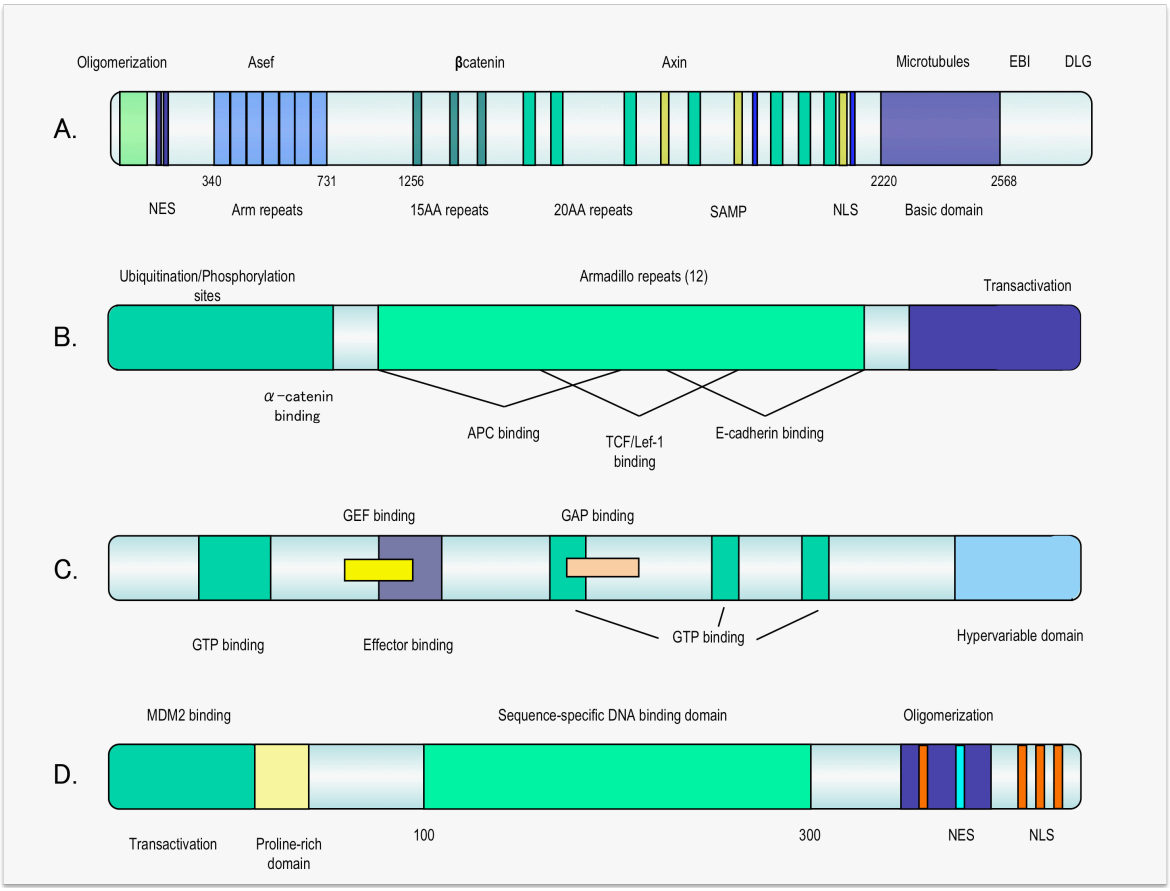


Figure 2. *Structure of the protein encoded by:* ***A.*** *APC gene;* ***B.*** *CTNNB1 gene;* ***C.*** *K-ras gene;* ***D.*** *TP53 gene. Functional domains of the proteins are shown.*

APC has two nuclear localization signals (NLSs) and several nuclear export signals (NESs) (Hanson et al., 2005). It was shown that APC binds nuclear β-catenin and stimulates its nuclear export and subsequent cytoplasmic degradation (Neufeld et al., 2000). Phosphorylation sites near one of the NLSs were shown to be critical for regulation of APC distribution inside the cell (Zhang et al., 2000).

Mutations in *APC* were reported in 40 to 95% of CRC cases (Chung, 2000; Smith et al., 2002; Michor et al., 2005; Samowitz et al., 2007). Most were found in the so-called mutation cluster region (MCR), spanning codons 1286 to 1585 (Miyaki et al, 1994; Smith et al., 2002; Thorstensen et al., 2005; Klaus et al., 2008). About 90% of the mutations in *APC* are nonsense mutations, C-to-T transitions, leading to a truncated protein (Chung, 2000; Segditsas et al., 2006).

APC is part of the Wnt signaling pathway. In about 50% of the colon tumors with intact *APC*, mutations in the *CTNNB1* gene – another protein from Wnt pathway, are found (Chung, 2000).

- ***CTNNB1 (β-catenin)***

Beta-catenin was first described in humans as an adherent junction protein which interacts with the cytoplasmic domain of E-cadherin and with α-catenin, anchoring the cadherin complex to the actin cytoskeleton (Kemler et al., 1989). Later, its role in Wnt

signaling was found (Gumbiner, 1995). *CTNNB1* encodes a 781-amino acid protein (85 kDa). The N terminus of β-catenin is important for regulation of its stability (it includes phosphorylation sites at Ser 33, Ser 37, Thr 41 and Ser 45), whereas the C terminus works as a transcriptional activator domain, binding Tcf/Lef1 and activating transcription of target genes - Figure 2B. Beta-catenin interacts with many proteins: β-TrCP, α-catenin, E-cadherin, axin, APC, Tcf/Lef1, etc. APC, Tcf and E-cadherin bind the middle part of β-catenin in a mutually exclusive manner (Clevers, 2006).

Activating mutations in *CTNNB1* occur in less than 5% of CRC (Segditsas et al., 2006), and in about 1% of sporadic CRC (Klaus et al., 2008). These mutations mainly affect the phosphorylation sites of β-catenin within exon 3, and thereby impede its degradation.

- ***Canonical (β-catenin-dependent) Wnt pathway***

Both APC and β-catenin are part of the Wnt signaling pathway. This pathway is essential in many biological processes in normal tissues (cell differentiation, signaling, proliferation, adhesion and stem cell self-renewal) (Klaus et al., 2008) but also in cancers. A schematic view of the Wnt pathway without/with Wnt ligand is presented in Figure 3A/3B, respectively. As well as APC and β-catenin, other proteins also take part in Wnt signaling (Clevers, 2006; Gordon et al., 2006). Although genetic variations in some of the genes

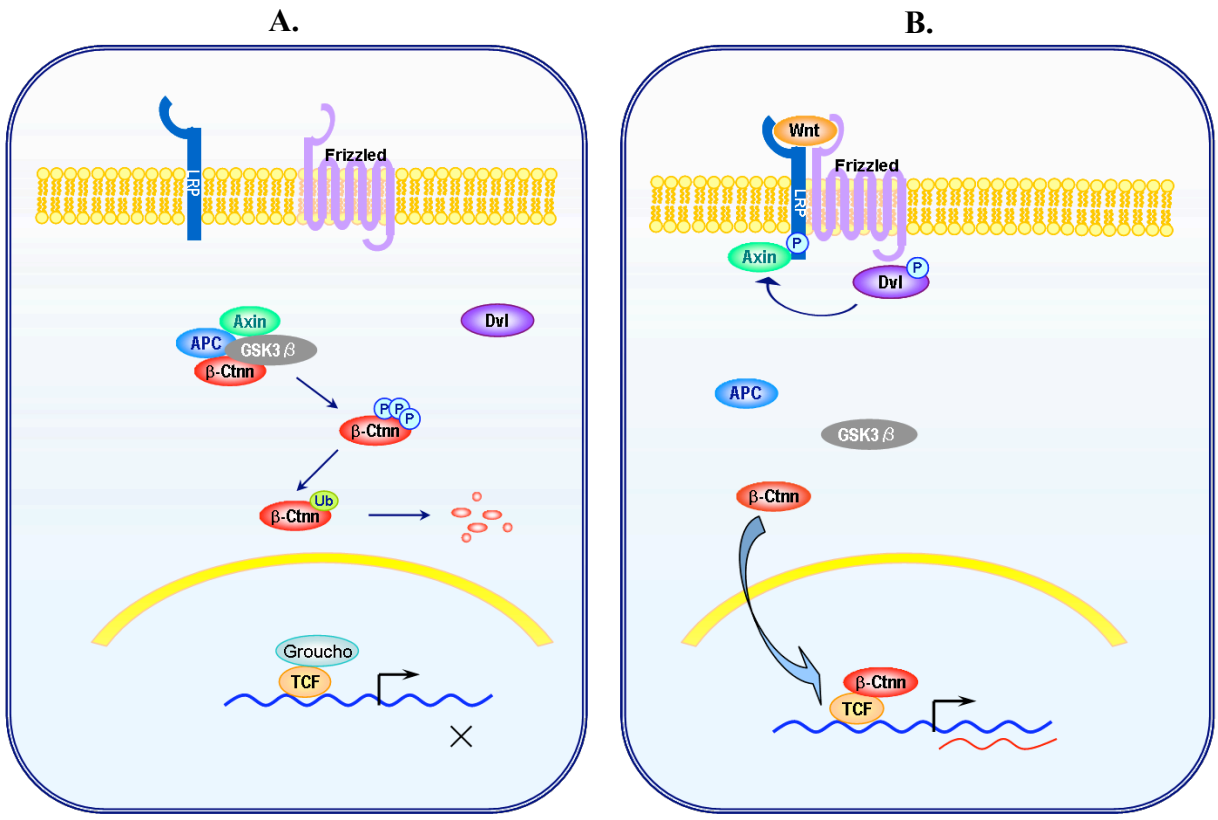
encoding these proteins are reported in CRC, it is not clear whether these changes are functional (Segditsas et al., 2006). These proteins include:

- Frizzled – transmembrane, serpentine receptor; contains extracellular cysteine-rich domain, binds Wnt;
- LRP5/6 (LDL-receptor-related protein) – single-pass transmembrane protein; acts as a co-receptor for Wnt;
- Dvl (Dishevelled) – cytoplasmic phosphoprotein; positive mediator of Wnt signaling;
- GSK3β (Glycogen synthase kinase) – member of the Ser/Thr family of protein kinases; phosphorylates β-catenin and LRP (works together with CK1 - casein kinase);
- Axin – scaffold protein; negative regulator of Wnt signaling;
- E3/β-TrCP (β-transducin repeat containing protein) – component of E3 ubiquitin ligase complex;
- Tcf/Lef1 (T-cell specific transcription factor/lymphoid enhancer-binding factor) – high-mobility group transcription factor; regulates transcription of Wnt target genes.

In the absence of Wnt ligand (Figure 3A), Dvl is inactive and cannot prevent formation of the "destruction complex" of axin, APC and GSK3β. Upon binding this complex, β-catenin is phosphorylated and then ubiquitylated by β-TrCP. Thus, β-catenin is targeted for degradation by the proteasome. Tcf/Lef1 partners with Groucho, which inhibits transcription of target genes (Gordon et al., 2006).

In the presence of Wnt (Figure 3B), it binds Frizzled and LRP. This binding activates Dvl; phosphorylation of Dvl is involved. LRP is also phosphorylated; it recruits axin and the whole "destruction complex" is inhibited. Free β-catenin accumulates in the cytoplasm and then translocates to the nucleus, where it displaces Groucho from Tcf/Lef1 and activates transcription of target genes (c-myc, cyclin D1, survivin, PPARδ, etc.).

If the Wnt cascade is mutationally activated in colon epithelium, cells maintain their progenitor status indefinitely. This also provides the opportunity for acquisition of further mutations (Reya et al., 2005).



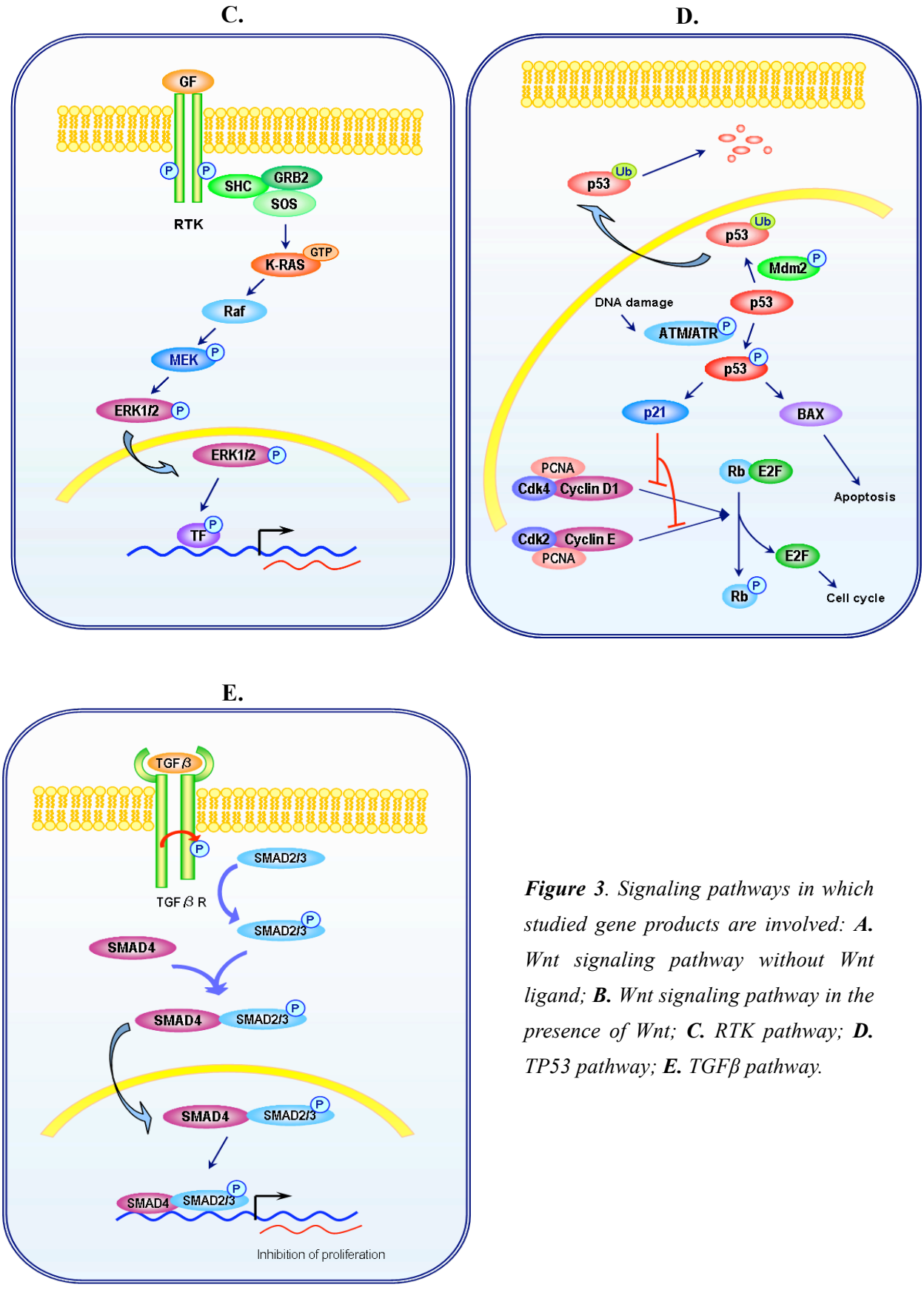


Figure 3. *Signaling pathways in which studied gene products are involved:* ***A.*** *Wnt signaling pathway without Wnt ligand;* ***B.*** *Wnt signaling pathway in the presence of Wnt;* ***C.*** *RTK pathway;* ***D.*** *TP53 pathway;* ***E.*** *TGFβ pathway.*

K-ras

K-ras is a member of the Ras family of ~21-kDa monomeric, membrane-localized GTPases (Friday et al., 2005). *Ras* genes have the ability to transform cells and *K-ras* is analogous to the oncogene of the Kirsten sarcoma virus. K-ras exists in two alternatively spliced forms, 4A and 4B (188 and 189 amino acids, respectively). K-ras acts as a molecular switch, transmitting signals from the extracellular to the intracellular environment. K-ras has a highly conserved N terminus and a variable C terminus. Several regions are involved in GTP-binding – Figure 2C. There are also motifs binding the effector, GEF (guanine-nucleotide exchange factor) and GAP (GTPase activating protein).

K-ras activation is triggered by upstream receptors; the best-described pathway is the one involving receptor tyrosine kinases (RTK) - Figure 3C. Briefly, binding of the ligand induces oligomerization of RTK and transphosphorylation. This results in adaptor protein recruitment of GEF to the cell membrane. K-ras is activated and transmits the signal to specific effectors (BRAF, PIK3-K, etc.). Several processes are thereby regulated, including cell growth, survival, differentiation and angiogenesis (Friday et al., 2005).

K-ras genetic alterations are reported in 30 to 50% of colorectal tumors (Chung, 2000; Smith et al., 2002). Mutations in *K-ras* appear to be more frequent in rectal than in colon cancers (Smith et al., 2002). *K-ras* point mutations most commonly occur in codons 12, 13

and 61 (Karnoub et al., 2008) and render the Ras protein insensitive to GAP-induced hydrolysis of GTP to GDP, which results in constitutive activation of K-ras (Leslie et al., 2002; Friday et al., 2005).

TP53

TP53 is one of the most commonly mutated genes in cancer; sequence variations in it are detected in approximately 50% of all tumors (Soussi, 2007). It encodes a 394-amino acid nuclear protein, which plays an essential role in cell cycle regulation, specifically in the transition from G0 to G1. P53 is a DNA-binding protein containing three domains: an N-terminal transcription activation domain, a core region that binds DNA in a sequence-specific manner, and a C-terminal oligomerization domain (Vousden et al., 2007) – Figure 2D. P53 is found at very low levels in normal cells. A ubiquitin ligase, Mdm2, which is the product of a p53-inducible gene, interacts with p53, targeting it for degradation in the proteasome. However, in a variety of transformed cell lines, *TP53* is expressed in high amounts (Kufe et al., 2003). When activated (for example, as a result of DNA damage), p53 binds as a tetramer to p53-binding sites and activates (*p21, BAX*, etc.) or suppresses (*Nanog, Survivin*, etc.) the expression of downstream genes, thus regulating the cell cycle, cell survival and apoptosis, differentiation and senescence (Xu, 2008) – Figure 3D. P53 is

also involved in the Wnt pathway, where it inhibits accumulation of β-catenin (Sadot et al., 2001).

TP53 genetic alterations occur in about 50% of colorectal tumors (Chung, 2000). Almost 80% of mutations in *TP53* are missense mutations leading to the synthesis of a stable protein (Soussi, 2007). *TP53* mutations are predominantly found in the core region of the protein (exons 5 to 8). Two classes have been distinguished: class I mutations (DNA contact-defective mutants; for example, mutations at codon 248 or 273) affect amino acids involved in sequence-specific DNA binding, and class II mutations (structure-defective mutants, exemplified by mutations at codons 175 and 179) alter conformation and thus indirectly affect binding to DNA (Soussi, 2007; Strano et al., 2007).

Allelic loss on chromosome 18q

Previous studies have demonstrated LOH on chromosome 18q in 58-70% of colorectal cancers (Thiagalingam et al., 1996; Chung, 2000; Losi et al., 2005). Originally, the gene on 18q involved in carcinogenesis was thought to be *DCC* (deleted in colorectal cancer; Leslie et al., 2002). *DCC* encodes a cell surface receptor for the ligand netrin and is believed to participate in regulation of apoptosis (Chung, 2000). Later, two other genes in the same region were identified: *SMAD2* and *SMAD4*. SMAD2 and SMAD4 are part of the TGFβ

pathway - Figure 3E. Binding of TGFβ ligand to the TGFβR heterodimer stimulates phosphorylation of TGFβR, which in turn phosphorylates SMAD2/3. SMAD2/3 then forms a heterodimer with SMAD4 and is translocated to the nucleus, where it regulates transcription of genes involved in cell proliferation (Chung, 2000).

Analysis of genetic changes in colon cancer

Most previous studies performed mutation screening on bulk cancer tissues (Fearon et al., 1990; Sjoblom et al., 2006; Wood et al., 2007). However, human tissues are composed of different cell types and analysis of bulk tissues gives average information on genetic events in it. Cancer tissues include not only tumor cells, but also normal cells and stroma. Thus, collection of precise information about genetic changes in tumor cells is obscured when bulk tissue is used. Recently, improved methods have been developed which enable investigators to examine the mutation status of populations of cells inside the tissue (Wild et al., 2000). Therefore, more accurate information about the features of tumor tissues can be acquired. One such biological feature is their internal heterogeneity.

GENETIC HETEROGENEITY

Intratumor heterogeneity

Solid tumors are constrained by limited space and resources. Thus, any cell clone that acquires a somatic mutation which brings it a growth or survival advantage will tend to spread in the tumor at the expense of other clones and normal cells. Eventually, this leads to clonal expansion of the cell and overgrowth over the other cells. The clonal evolution model would imply a relative homogeneity inside the tumor tissue. On the other hand, this process may yield a tumor mass that includes cancer cells at an earlier stage of carcinogenesis as a minor population, leading to intratumor heterogeneity in mutation patterns. There have been a considerable number of studies demonstrating intratumor genetic heterogeneity in human colorectal (Baisse et al., 2001; Barnetson et al., 2000; Giaretti et al., 2000; Gonzalez-Garcia et al., 2002; Lips et al., 2008; Losi et al., 2005; Samowitz et al., 1999; Tsao et al., 2000), prostate (Konishi et al., 1995), esophageal (Maley et al., 2006), breast (Wild et al., 2000), ovarian (Takeshima et al., 2001), and cervical (Lyng et al., 2004) cancers. In these studies, sections were excised from different areas of the tumor mass and mutation or chromosomal aberration patterns were compared. Lips et al. (2008) suggested that it is essential to analyze several tumor fractions per patient for an accurate assessment of genetic changes. One of the studies correlated the degree of

heterogeneity with the evolutional process of cancer (Losi et al., 2005).

In sporadic CRC, contradictory results are reported. While most reports find intratumor genetic heterogeneity (Baisse et al., 2001; Losi et al., 2005), a few indicate that heterogeneity exists in early stages of tumor progression – adenomas, but not in advanced stages, adenocarcinomas (Shibata et al., 1993).

A recent review by Adams et al. (2008) discusses intratumor heterogeneity in the light of current models for tumor propagation. The authors suggest that if tumor growth is sustained by rare cancer stem cells, then heterogeneity within the tumor should be a result of aberrant differentiation from the cancer stem cell. On the other hand, if the dominant clone model is true, differences in phenotype within a tumor may reflect subclones at different stages of neoplastic transformation, each having a growth advantage over the normal cells, but to varying extents. Although several studies have addressed intratumor heterogeneity, its origin is not yet clear.

Heterogeneity between primary tumor and its paired metastasis

It is widely accepted that metastasis is the next step in tumor progression after the formation of a carcinoma (Alberts, 2002). According to the clonal selection hypothesis, cell populations with all of the prerequisites for metastatic capacity are the ones that metastasize

(Talmadge, 2007). However, other models of the metastatic process exist (Scheel et al., 2007; Talmadge, 2007). One of them, the "parallel evolution" model, suggests that metastasis occurs early in tumor progression followed by parallel evolution of primary and metastatic tumors (Gray et al., 2003; Schmidt-Kittler et al., 2003). This model is based on a study of genetic heterogeneity between disseminated breast cancer cells within the bone marrow and the primary breast tumor. Such heterogeneity is reported in a number of articles (Zhang, 1997; Al-Mulla et al., 1998; Baisse et al., 2001; Albanese et al., 2004). According to Albanese et al. (2004) there is a higher probability of finding the same mutation both in a primary tumor and its liver metastasis for *TP53* than for *K-ras,* suggesting that mutations in *K-ras* do not preferentially mark the neoplastic cells which are the origin of the liver metastasis. This assumption raises doubts about *K-ras* aberration as an early event in colorectal carcinogenesis.

However, when considered within the life history of a tumor, the parallel evolution model and most of the other hypotheses seem to be consistent with the clonal selection model (Talmadge, 2007). More studies are needed to clarify the issue of heterogeneity between primary tumor and its metastasis.

A model of colorectal carcinogenesis, based on Nowell's hypothesis of multistage carcinogenesis, was proposed by Vogelstein and Fearon in 1990.

However, a number of articles demonstrated the existence of various pathways to colorectal tumorigenesis. Moreover, this model is based on mutation frequencies in tumors of different histological stages and cannot explain the heterogeneity observed in cancer tissues and between the primary site and metastases. Characterizing carcinogenesis only by the presence/absence of a mutation at a bulk-tissue level can obscure the genetic diversity that drives both tumor progression and therapeutic resistance. These facts point to new questions concerning tumorigenesis mechanisms. Therefore, I aimed to study the carcinogenesis process in individual sporadic CRC tissues through analysis of genetic heterogeneity. To find out more about the process of tumor progression, I also compared data from bulk tissue analysis with those from analysis of intratumor and primary tumor-to-metastasis heterogeneity.

MATERIALS AND METHODS

SAMPLES

Tissue samples from eighty-six colorectal carcinomas, plus twelve liver and one lymph node metastasis, were obtained from the Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases. Clinical characteristics of the patients are shown in Table 2. All samples were divided into two parts, one to be used for mutation screening and the other for study of heterogeneity. DNA was extracted from bulk tumor tissues by QIAamp DNA Micro kit (Qiagen, Germany). Samples with DNA concentration of 20 ng/μl were prepared. The study was approved by the ethical committee of Osaka Medical Center for Cancer and Cardiovascular Diseases.

Table 2. *Clinical parameters of the patients.*

	Number of patients, n=86 (%)
Age (median)	40-85 years (64.2)
≤60	30 (35%)
>60	56 (65%)
Gender	
Female	38 (44%)
Male	48 (56%)
Location	
Proximal	15 (17%)
Distal	18 (21%)
Rectal	47 (55%)
Stage	
I	8 (9%)
II	30 (35%)
III	24 (28%)
IV	24 (28%)

MUTATION SCREENING

Mutation screening was performed by High-Resolution Melting on a LightScanner (Idaho Technology Inc., USA), followed by direct sequencing. High-Resolution Melting includes 3 steps: 1) PCR in the presence of a fluorescent dye, LCGreen Plus; 2) melting of the PCR product; and 3) analysis of the data. The main feature of the method is that samples with sequence variation are detected by changes in the shape of their melting curves compared to a reference sample, or wild type. Detection is made possible by the addition of LCGreen Plus to the PCR mixture. This dye binds dsDNAs in a saturating manner; thus when DNA melts, the number of dye molecules bound to DNA is reduced and the fluorescence decreases. High-Resolution Melting has a sensitivity of 97.8% and a specificity of 99.7% for 50-1000-bp products (Reed et al., 2004).

The coding regions of *APC*, *K-ras*, *TP53* and exon 3 of *CTNNB1* gene were amplified in 10-μl reactions on a GeneAmp PCR System 9700 (Applied Biosystems, USA). The PCR mixture included 1xPCR Buffer (Applied Biosystems), 200 μM each dNTP, 2 mM $MgCl_2$, 1x LCGreen Plus dye (Idaho Technology Inc.), oligonucleotide primers (0.3 μM each; designed with LightScanner Primer Design Software; Table 3), and 1 U AmpliTaqGold polymerase (Applied Biosystems). Amplicons with CG content of 60% or more required addition of 0.1% BSA (TaKaRa, Japan) to the mixture. Cycling conditions were as follows: denaturing at 95°C for 5 min; 45 cycles of denaturing at 95°C for 30 sec, annealing at

60-66°C for 30 sec and synthesis at 72°C for 30 sec; and final synthesis at 72°C for 5 min. After cycling, denaturation and reannealing were performed – at 95°C for 30 sec and 25°C for 30 sec, respectively.

Table 3. *Sequences of the primers for High-Resolution Melting.*

Gene/exon	Bp	%GC	Forward Primer	Reverse Primer	T_{an}
APC					
Exon 2	245	36	TAAAACAAGCAGCCACTAAAT	AAACTACAATTAAAAGTCACAGTCT	60
Exon 3	217	29	GAAATACAGAATCATGTCTTGAAGT	CTACACACCTAAAGATGACAATTTG	60
Exon 4	292	38	AGACTGCTTAAAGCAATTGTTGTAT	GAGTACAAGGCAATGTTTACTA	64
Exon 5	200	31	TCTGCAGTCTTTATTAGCATTGTT	AGTTTCAAATAAGTTGTACTGCCA	60
Exon 6	234	34	GCTTTTTTGCTTTTACTGATTAACG	TTTATTCCTAATAGCTCTTCGCTG	62
Exon 7	200	32	CTTTTAAGTGGTAGCCATAGTATGA	TACCCACAAACAAGAAAGGCA	60
Exon 8	207	36	GCATGTACTGATGTTAACTCCA	AGAACCATCTTGCTTCATACTTT	64
Exon 9	207	39	TGGAGTACCTTAACATGATGTTATC	TTAGTGACCAGGGTTTGTAATCTT	62
Exon 10	509	46	TTGGCTTTTGGATATTAAAGTCGTA	ACATGCACTACGATGTACAC	62
Exon 11	177	34	ACAAAGCATTATGGTTTATGTTGAT	CCACCAGTAATTGTCTATGTCA	60
Exon 12	227	37	TGATTGTCTTTTTCCTCTTGCC	AAATTAACTCATACCTGAGCTATCT	62
Exon 13	197	38	TAAAGCTTGGCTTCAAGTTGTC	GTGAGACCCTGCCTCAAA	62
Exon 14	286	31	AGATTTCTATTCTTACTGCTAGCAT	ATCTCATGGCTAAAAGAAGGC	64
Exon 15	377	39	CCAACTCTAATTAGATGACCCA	GGTCTTTTTGAGAGTATGAATTCTG	64
Exon 16_1	391	39	TACTGCATACACATTGTGACCTTA	GCATCTAATTCTGCTTCTAGGG	64
Exon 16_2	405	40	AGCTTGCCATCTCTTCATGTTA	GGTGGAGATCTGCAAACC	64
Exon 16_3	358	40	TCCAGGAACTTCTTCAAAGCG	TTCAGAATAGGATTCAATCGAGGG	64
Exon 16_4	466	38	CAGTAGTAGTGATGGTTATGGTAA	ATTAATTCCATGATTAGAACCCAC	64
Exon 16_5	361	36	CCAATGGTTCAGAAACAAATCG	CTCTTGGCATTAGATGAAGGTG	64
Exon 16_6	380	42	CTTCAAGCAGTGAGAATACG	CTGCAGTCTGCTGGATTT	62
Exon 16_7	409	47	GTGTCACAGCACCCTAGA	TTTCAGCAGTAGGTGCTTTATT	64
Exon 16_8	434	39	AAACCAAGCGAGAAGTACC	TGCTTTACGTGATGACTTTGT	64
Exon 16_9	351	44	AAGAATGTATTATTTCTGCCATGC	CTGTAGGAATGGTATCTCGTT	62
Exon 16_10	409	39	GGCACAGTCAGGTGAATTT	CTGTTTCTTTGAATCTTTGTTGTC	64
Exon 16_11	366	35	ACTGAATATAGGACACGTGTAAG	TTGGTTGGAGGTTAGTTCTG	64
Exon 16_12	337	42	GGCTAAAGTTACCAGCCAC	TTGAGGTTTACTTGGTTCTCCC	64
Exon 16_13	407	41	CCTATCAAAGAGACTGAGCCC	GCAGCAGCAGCTTGATGTAAA	66

Exon 16_14	382	38	GAAAGCTATTCAGGAAGGTGC	CCTGCCTCGAGAGATTG	64
Exon 16_15	334	49	GCCCCTTCAAGCAAACAT	TCGGCCAGGAGACTGTAT	60
Exon 16_16	407	40	TTCAAGACCTGCCCAGC	TGATGAAAGTTGACTGGCG	64
Exon 16_17	374	45	CTGATAGATCAGAAAGACCTGTA	TTTGCTGTGCTCACGTT	62
Exon 16_18	352	39	CCTTCTAGACTTCCAATCAATAGG	TAGAAACAGCAGGTGCCA	62
Exon 16_19	410	42	TACAAATGGTGCTGAATCAAAGAC	TGTGTTTGCTTGAGCTGCTA	64
Exon 16_20	402	40	GAAAGTTCTATAGTGGAACGTACC	ACCCTCTAACAAGAATCAAACC	64
CTNNB1					
Exon 3	363	45	AGTAACATTTCCAATCTACTAATGC	CTGACTTTCAGTAAGGCAATG	63-64
K-ras					
Exon 2	225	36	ATTAACCTTATGTGTGACATGTTCT	GAATGGTCCTGCACCAGTA	64
Exon 3	256	39	CACTGTAATAATCCAGACTGTGTT	ACTCCTTAATGTCAGCTTATTATA	62
Exon 4	257	34	ATTTGTGTTACTAATGACTGTGCTA	CAGTTATGATTTTGCAGAAAACAGA	64
Exon 5	221	34	TTACTCTTACCAATGCAACAGACT	AGACATCTGCTTTCTGCCA	64
Exon 6	284	29	ATGAAGCCATCGTATATTCACA	GTCTGCATGGAGCAGGA	64
TP53					
Exon 2	174	56	AGGGTTGGAAGTGTCTCAT	CCTGCCCTTCCAATGGAT	60
Exon 3+4	491	60	CATGGGACTGACTTTCTGC	GCCAGGCATTGAAGTCTC	60
Exon 5	248	60	TGACTTTCAACTCTGTCTCCTT	CTGTCGTCTCTCCAGCC	64
Exon 6	177	53	AGGCCTCTGATTCCTCAC	CCTCCTCCCAGAGACCC	64
Exon 7	202	57	TCATCTTGGGCCTGTGTTATC	GGTCAGAGGCAAGCAGAG	64
Exon 8	212	56	TTACTGCCTCTTGCTTCTCTTT	CTTGGTCTCCTCCACCG	62
Exon 9	127	49	TCACCTTTCCTTGCCTCTTT	CTTGATAAGAGGTCCCAAGACT	62
Exon 10	302	63	GCATGTTGCTTTTGTACCGTC	CATGAAGGCAGGATGAGAATGG	62
Exon 11	228	51	TGATGTCATCTCTCCTCCCTG	TGACGCACACCTATTGCAAG	64

Samples with aberrant melting curves were analyzed by direct sequencing with a BigDye Terminator Cycle Sequencing Kit (version 3.1, Applied Biosystems) on an ABI PRISM 3730 (Applied Biosystems) according to the supplier's standard protocol. Results were analyzed by AutoAssembler software (Applied Biosystems).

LASER MICRODISSECTION AND DNA EXTRACTION

Sections (40 μm thick) from frozen cancer tissues were prepared on a Leica CM1900 cryostat (Leica Microsystems, Germany). After mounting on a film-covered glass slide, the sections were stained with Mayer's hematoxylin (Wako, Japan). Microdissection was performed using Leica AS LMD system (Leica Microsystems). Genomic DNA was extracted by prepGEM kit (ZyGEM, New Zealand) according to the supplier's protocol; 20 μl DNA mixture was prepared from each sample.

PCR AMPLIFICATION AND SNAPSHOT ASSAY

DNA fragments containing mutations were amplified by multiplex PCR on a GeneAmp PCR System 9700 (Applied Biosystems). The PCR mixture included 5 μl DNA (about 250 pg), 1x PCR buffer (Applied Biosystems), 2 mM $MgCl_2$, 200 μM each dNTP, Primer mix (0.2 μM each primer, Table 4) and 1 U AmpliTaqGold polymerase (Applied Biosystems) in a 10-μl reaction. Cycling conditions were as follows: denaturing at 94°C for 5 min; 40 cycles of denaturing at 94°C for 30 sec, annealing at 54–56°C for 30 sec and synthesis at 72°C for 40 sec; and final synthesis at 72°C for 5 min.

The SNaPshot assay is a primer extension assay: each primer is designed to bind to a complementary template immediately adjacent to the mutation site. The reaction is carried

out in the presence of fluorescently labeled ddNTPs, and DNA polymerase extends the primer by one nucleotide, adding a single ddNTP to its 3′ end. Fluorescent dyes used for dideoxynucleotides are as follows: A, dR6G; C, dTAMRA; G, dR110; and T, dROX.

PCR fragments for primer extension were prepared by incubating 7.5 µl PCR product with 0.5 U shrimp alkaline phosphatase (TaKaRa) and 1 U exonuclease I (TaKaRa) for 40 min at 37°C in a final volume of 10 µl, followed by inactivation of the enzymes for 20 min at 80°C. Primer extension was carried out in a 5-µl reaction containing 2 µl treated PCR product, 2.5 µl ABI Prism SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems) and 0.5 µl extension primers mix (0.2 µM each primer). Primer sequences are shown in Table 4. Cycling conditions were according to the manufacturer's protocol: 25 cycles of 10 sec at 96°C denaturation, 5 sec at 50°C annealing, and 30 sec at 60°C extension. To remove unincorporated ddNTPs 5 µl SNaPshot products were incubated for 40 min at 37°C with 0.5 U shrimp alkaline phosphatase (TaKaRa) in a final volume of 6 µl, and the enzyme was inactivated as described above. A 1-µl aliquot of the treated SNaPshot reaction was denatured in 9 µl distilled water in the presence of GeneScan 120 LIZ Size Standard (Applied Biosystems) for 5 min at 95°C, and was analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Fragment analysis was performed with Peak Scanner Software v1.0 (Applied Biosystems).

Table 4. *Sequences of the primers for multiplex PCR and SNaPshot assay.*

Gene	Exon	Locus	Forward PCR primer	Reverse PCR primer	Bp	SNaPshot Primer	Length
APC	**14**	c.1690 C>T R564Stop	AGATTTCTATTCTTACTGCTAGCAT	ATCTCATGGCTAAAAGAAGGC	286	CTTGTACTAATCTTGCTTTCACACTTCCAACTTCTC	36
	16	c.2626 C>T R876Stop	AGCTTGCCATCTCTTCATGTTA	GGTGGAGATCTGCAAACC	405	TCTAGTATCTAATCTAATCCAGGAACTTCTTCAAAG	36
	16	c.2755 A>T R919Stop	TCCAGGAACTTCTTCAAAGCG	TTCAGAATAGGATTCAATCGAGGG	358	TTGTACTAATCTGCATTACATTGTGTGACAGATGAG	36
	16	c.2932 C>T Q978Stop	TCCAGGAACTTCTTCAAAGCG	TTCAGAATAGGATTCAATCGAGGG	358	CTATGTACTAATCTTGATGGTTATGGTAAAAGAGGT	36
	16	c.3927 C>A S1276Stop	CTTCAAGCAGTGAGAATACG	CTGCAGTCTGCTGGATTT	380	TGTACTGATCTCCAATATGTTTTTCAAGATGTAGTT	36
	16	c.3956_6 delC	CTTCAAGCAGTGAGAATACG	CTGCAGTCTGCTGGATTT	380	TTGTACTAATCGATTGGAACTAGGTCAGCTGAAGATC	37
	16	c.4043_4 insA	GTGTCACAGCACCCTAGA	TTTCAGCAGTAGGTGCTTTATT	409	TTGACTACTAATCTGTTTATCTTCAGAATCAGCCAG	36
	16	c.4124_37 del13bp/insTA	GTGTCACAGCACCCTAGA	TTTCAGCAGTAGGTGCTTTATT	409	TAGATCCTACTATCTAATCAGACACCCAAAAGTCCACCTGAACA	44
	16	c.4146_7 insA	GTGTCACAGCACCCTAGA	TTTCAGCAGTAGGTGCTTTATT	409	CTATACTATCTCACTATGTTCAGGAGACCCCACTCA	36
	16	c.4285 C>T Q1429Stop	GTGTCACAGCACCCTAGA	TTTCAGCAGTAGGTGCTTTATT	409	CTTATTACTAATCTTGATCTTCCAGATAGCCCTGGA	36
	16	c.4348 C>T R1450Stop	GTGTCACAGCACCCTAGA	TTTCAGCAGTAGGTGCTTTATT	409	TTACTAAATATTCATTCCTCAAACAGCTCAAACCAAG	37
	16	c.4359_9 delT	GTGTCACAGCACCCTAGA	TTTCAGCAGTAGGTGCTTTATT	409	TAGATCCTACTATCTAATCAGCTCAAACCAAGCGAGAAGTACC	42
	16	c.4393_4 delAG	AAACCAAGCGAGAAGTACC	TGCTTTACGTGATGACTTTGT	434	TTGCTACTAATCTAACCTACTGCTGAAAAGAGAGAG	36
K-ras	**2**	c.34 G>A/T G12S	ATTAACCTTATGTGTGACATGTTCT	GAATGGTCCTGCACCAGTA	225	TTACTAAGTAACTTGTGGTAGTTGGAGCT	29
	2	c.35 G>A/T G12D	ATTAACCTTATGTGTGACATGTTCT	GAATGGTCCTGCACCAGTA	225	TACTAATAACTTGTGGTAGTTGGAGCTG	28
	2	c.37 G>T G13C	ATTAACCTTATGTGTGACATGTTCT	GAATGGTCCTGCACCAGTA	225	TCACTAATTTGTGGTAGTTGGAGCTGGT	28
	2	c.38 G>A G13D	ATTAACCTTATGTGTGACATGTTCT	GAATGGTCCTGCACCAGTA	225	TACTAATTTGTGGTAGTTGGAGCTGGTG	28
	3	c.173 C>T T58I	CACTGTAATAATCCAGACTGTGTT	ACTCCTTAATGTCAGCTTATTATA	256	TACTAATGTCTCTTGGATATTCTCGACA	28
	3	c.183 A>T Q61H	CACTGTAATAATCCAGACTGTGTT	ACTCCTTAATGTCAGCTTATTATA	256	CTCTTAGATATTCTCGACACAGCAGGTCA	29
	3	c.204 G>C R68S	CACTGTAATAATCCAGACTGTGTT	ACTCCTTAATGTCAGCTTATTATA	256	TACTAATAGAGGAGTACAGTGCAATGAG	28
TP53	**4**	c.374 C>T T125M	ACTGCTCTTTTCACCCATC	GCCAGGCATTGAAGTCTC	350	AGCCAAGTCTGTGACTTGCA	20
	5	c.524 G>A R175H	TGACTTTCAACTCTGTCTCCTT	CTGTCGTCTCTCCAGCC	248	ACATGACGGAGGTTGTGAGGC	21
	5	c.526 T>A C176S	TGACTTTCAACTCTGTCTCCTT	CTGTCGTCTCTCCAGCC	248	TGACGGAGGTTGTGAGGCGC	20
	6	c.659 A>G Y220C	AGGCCTCTGATTCCTCAC	CCTCCTCCCAGAGACCC	177	ACATAGTGTGGTGGTGCCCT	20
	7	c.681_8 ins 8bp	TCATCTTGGGCCTGTGTTATC	GGTCAGAGGCAAGCAGAG	202	GTAGTGGATGGTGGTACAGT	20
	7	c.733 G>A G245S	TCATCTTGGGCCTGTGTTATC	GGTCAGAGGCAAGCAGAG	202	TGTAACAGTTCCTGCATGGGC	21
	7	c.755_63 del 9bp	TCATCTTGGGCCTGTGTTATC	GGTCAGAGGCAAGCAGAG	202	GCATGAACCGGAGGCCCATC	20
	7	c.767 C>A T256K	TCATCTTGGGCCTGTGTTATC	GGTCAGAGGCAAGCAGAG	202	GCCCATCCTCACCATCATCA	20
	7	c.776_7 AC>TT D259V	TCATCTTGGGCCTGTGTTATC	GGTCAGAGGCAAGCAGAG	202	TCACCATCATCACACTGGAAG	21
	8	c.818 G>A R273H	TTACTGCCTCTTGCTTCTCTTT	CTTGGTCTCCTCCACCG	212	ACGGAACAGCTTTGAGGTGC	20
	8	c.824 G>T C275F	TTACTGCCTCTTGCTTCTCTTT	CTTGGTCTCCTCCACCG	212	ACAGCTTTGAGGTGCGTGTTT	21
	8	c.916 C>T R306Stop	TTACTGCCTCTTGCTTCTCTTT	CTTGGTCTCCTCCACCG	212	TGCCCCCAGGGAGCACTAAG	20
TGFR2	**5**	rs2228048 C>T	CAATACTGGCTGATCACCG	GGCCTGGCTCAAGGTAAA	342	CTCCAATATCCTCGTGAAGAA	21

The mutated allele ratio, - M/(M+N), was calculated, where M is mutant allele peak height and N is normal peak height. Reproducibility of the amplification and the SNaPshot assay was checked in two experiments. The results were plotted on 3D graphs using Graphis software (ver.2.9.22, Kylebank Software Ltd., UK).

LOH ANALYSIS

Chromosome 18q allelic loss in bulk tissue was assessed by multiplex PCR-based assay (Erill et al., 2005) on five microsatellite markers – D18S478, D18S64, D18S68, D18S55 and D18S61. The PCR mixture included 20 ng DNA, 1x PCR buffer (Applied Biosystems), 2 mM $MgCl_2$, 200 µM each dNTP, Primer mix (Table 5) and 1 U AmpliTaqGold polymerase (Applied Biosystems) in a 10-µl reaction. Cycling conditions were as follows: denaturing at 94°C for 5 min; 30 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and synthesis at 72°C for 30 sec; and final synthesis at 72°C for 30 min.

Amplification of microsatellite markers on chromosome 5q (D5S107, D5S82 and D5S346) and on chromosome 17p (D17S796 and D17S786) from microdissected areas was performed in two separate multiplex PCR reactions. The PCR mixture included 5 µl DNA, 1x PCR buffer (Applied Biosystems), 2 mM $MgCl_2$, 200 µM each dNTP, Primer mix (Table 5) and 1 U AmpliTaqGold polymerase (Applied Biosystems) in a 10-µl reaction. Cycling

conditions were as follows: denaturing at 94°C for 5 min; 40 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec and synthesis at 72°C for 30 sec; and final synthesis at 72°C for 30 min. The fluorescent products were analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Fragment analysis was performed with Peak Scanner Software v1.0 (Applied Biosystems).

Table 5. *Sequences of the primers for LOH analysis.*

Gene	Locus	Forward PCR primer	Reverse PCR primer	Bp
Chr.5	D5S107	GATCCACTTTAACCCAAATAC*	GGCATCAACTTGAACAGCAT	133-149
	D5S82	ATCAGAGTATCAGAATTTCT*	CCCAATTGTATAGATTTAGAAGTC	169
	D5S346	ACTCACTCTAGTGATAAATCGGG*	AGCAGATAAGACAGTATTACTAGT	96
Chr.17	D17S786	TACAGGGATAGGTAGCCGAG*	GGATTTGGGCTCTTTTGTAA	135-157
	D17S796	CAGTCAATGGAACCAAATGTGGTC*	GCTCAGTAGTCCGATAATGCCAGGATG	154-184
Chr.18	D18S478	ACATCATCAAGTAGATTTCCATT*	GACCAACTCAAGTGTTCCAC	240-252
	D18S64	ATACTGGTGGTGGTTATACAACAT*	CAAATCAGGAAATCGGCA	189-209
	D18S68	ATGGGAGACGTAATACACCC*	AATGCTGCTGGTCTGAGGT	271-291
	D18S55	AGCTTCTGAGTAATCTTATGCT*	GGGAAGTCAAATGCAAAATC	134-152
	D18S61	ATTTCTAAGAGGACTCCCAAACT*	ATATTTTGAAACTCAGGAGCAT	157-183

* 5'FAM

RESULTS

In the present study, I focused on genetic analysis of CRC samples at a bulk-tissue level and a cell-population level. By examining genetic aberrations in the tumor tissue, I aimed to acquire information about the carcinogenic process. To accomplish that, first mutation screening of bulk tissue using High-Resolution Melting and Direct Sequencing, and LOH analysis of chromosome 18q were performed. Then, samples with simultaneously mutated *APC*, *K-ras* and *TP53* genes were selected and examined for intratumor heterogeneity by multipoint microsampling. Available metastases were also screened in a search for hallmarks of this step in carcinogenesis. The workflow is summarized in Figure 4.

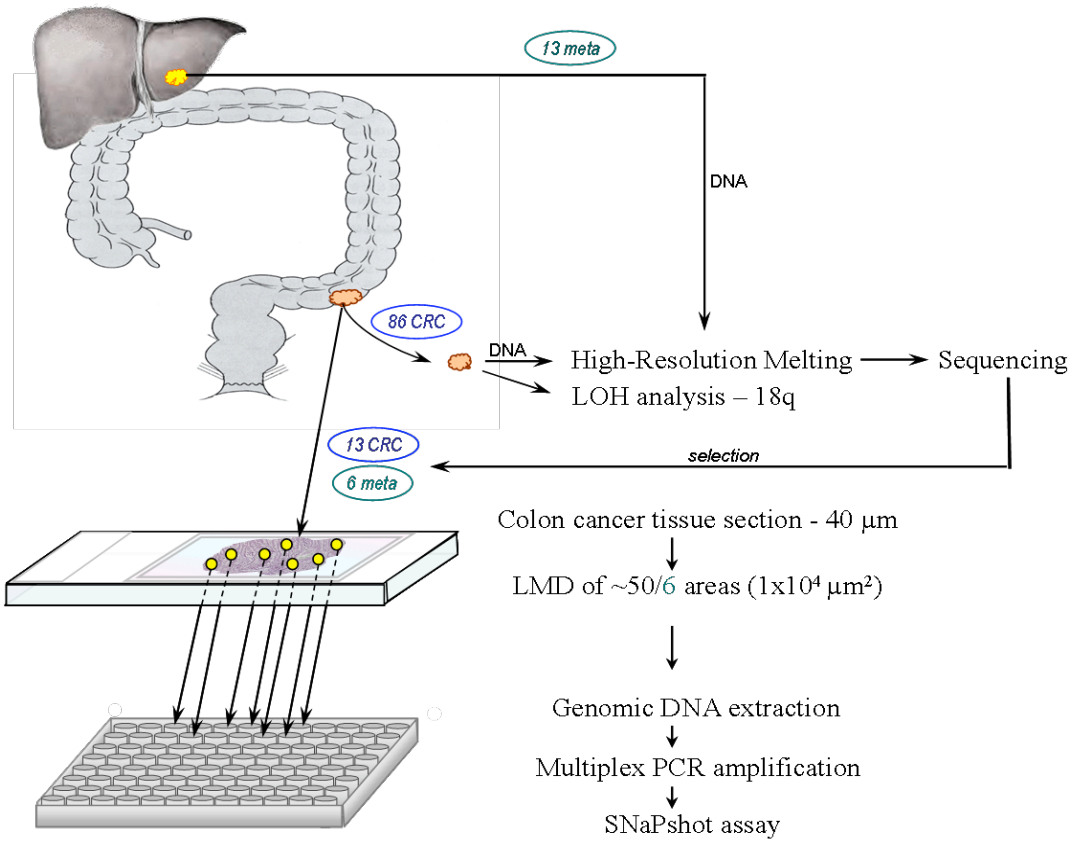


Figure 4. *Graphic representation of the workflow. Ninety-nine DNA samples extracted from bulk tissues of 86 patients with CRC and 13 metastatic tissues were screened for mutations in APC, CTNNB1, K-ras and TP53 genes by High-Resolution Melting and Sequencing. LOH status of 18q was also determined. Samples with simultaneously mutated APC, K-ras and TP53 genes were selected for the next experiments. Tissue sections were cut and about 50 (6 for metastases) areas ($1\times10^4\mu m^2$) were microdissected from each sample. After multiplex PCR amplification, mutation status was examined by SNaPshot assay.*

GENETIC ANALYSIS OF BULK TUMOR TISSUES

Ninety-nine samples were screened for mutations in the coding regions of the *APC*, *K-ras* and *TP53* genes and in exon 3 of the *CTNNB1* gene. First, the presence of sequence variants was assessed by High-Resolution Melting on a LightScanner. Next, Sanger

sequencing was used to determine the exact genetic aberration. Primary tumor samples were also examined for allelic loss on chromosome 18q.

Most of the samples (n=86) were primary cancer tissues. However, both primary site and metastatic tissues were available from 13 patients. To distinguish the two types of samples, I used the case number (for example, sample 1) for primary CRC samples and the case number plus M (for example, sample 1M) for paired metastatic samples.

LightScanner – optimization and screening

In the present study, the recently introduced and highly sensitive High-Resolution Melting method for mutation scanning was applied (Graham et al., 2005; Vandersteen et al., 2007). The most critical step in High-Resolution Melting is obtaining a robust single PCR product in the presence of a fluorescent dye, LCGreen Plus. Because LCGreen Plus binds all double stranded DNA present in a reaction – the specific amplification product, undesired products, and dimer products - the concentrations of individual reaction components and thermal cycling parameters were optimized. PCR optimization was done according to the recommendations from Idaho Technology.

Scanning for mutations using the LightScanner requires the addition of a fluorescent dye, which elevates the annealing temperature for PCR. Not all available polymerases can work in this condition; hot-start procedures are recommended. PCR optimization was done with KOD-plus (Toyobo, Japan) and two hot-start polymerases, AmpliTaq Gold (Applied Biosystems, USA) and Titanium Taq polymerase (TaKaRa, Japan). AmpliTaq Gold showed the best results and was chosen for subsequent PCR reactions – Figure 5.

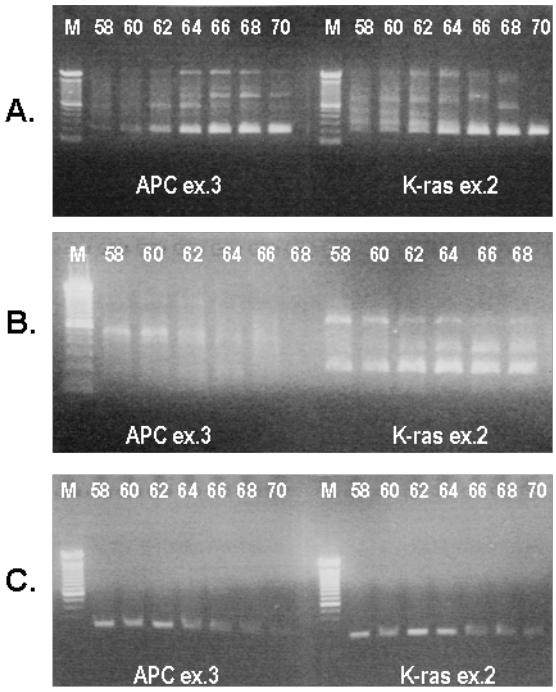


***Figure 5**. Optimization of PCR amplification for High-Resolution Melting. PCR reaction was performed with three different polymerases – KODplus (**A**), Tithanium Taq (**B**) and AmlpiTaq Gold (**C**). The results of amplification of APC exon 3 and K-ras exon 2 are shown.*

Primers were designed using LightScanner Primer Design Software and purchased from Hokkaido System Science. Optimal amplification was achieved with 0.3 mM primers and 1.5-2 mM $MgSO_4$.

High-Resolution Melting compares melting curves from independent PCR reactions; therefore, it is important to minimize reaction-to-reaction variability by using the same

amount of template in each reaction. Twenty ng DNA template were applied in a 10-µl reaction volume. Optimized conditions for PCR reactions are summarized in the Materials and Methods section and Table 3.

Ninety-nine samples were examined for genetic variation in 49 amplicons, so that 4,851 melting reactions (1,527,273 bp) were performed. Figure 6 shows melting profiles of some of the examined genetic regions. No aberrant melting curve was detected in exon 3 of the *APC* gene (Figure 6A), and only wild type curves were found also in *APC* exons 2, 5, and 8, and the 3' terminus of exon 16. No alterations were detected in *K-ras* exons 4 and 5 or in *TP53* exon 11. According to the data of The Wellcome Trust Sanger Institute, COSMIC (http://www.sanger.ac.uk/genetics/CGP/cosmic/), no pathological mutations have been detected in *APC* exons 2, and 3, or the end of coding sequence in exon 16; in *K-ras* exon 5; and in *TP53* exon 11. These data confirm the correctness of present results.

4,851 reactions were performed on the LightScanner and 749 aberrant melting curves were found. However, High-Resolution Melting does not give information about the type of genetic aberration; DNA sequencing is required. Results from High-Resolution Melting and sequence analysis are presented in the next section.

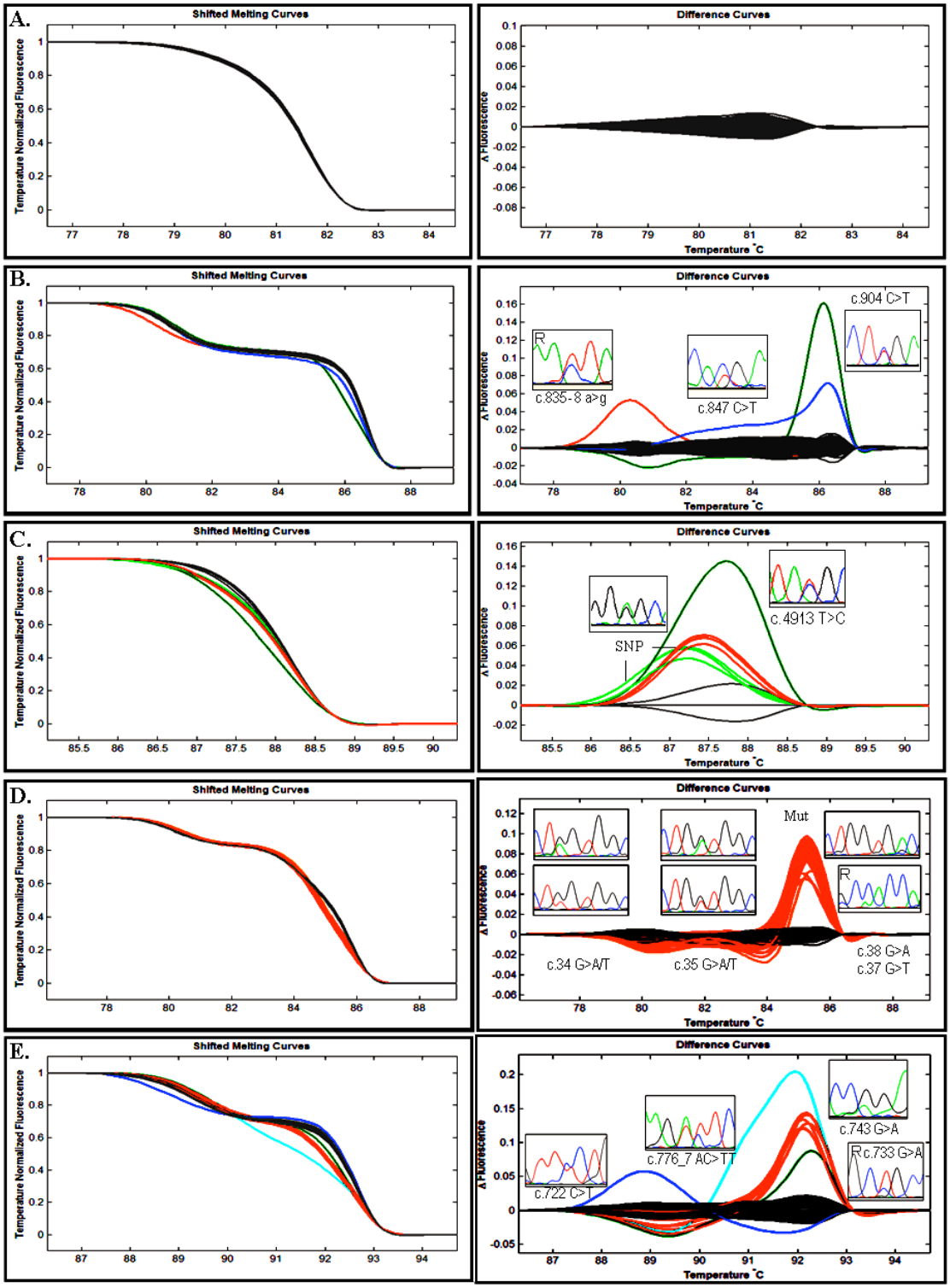


Figure 6. *Melting profiles and sequencing results. Grey curves represent wild type* ***A.*** *APC ex.3 – all samples were wild type;* ***B.*** *APC ex.9 – three mutations were detected - c.835-8 a>g (red), c.904 C>T R302Stop (green), c.847 C>T R283Stop (blue);* ***C.*** *APC ex.16_9: SNP - c.5034 G>A G1678G heterozygous (light green), homozygous (red); Mutation - c.4913 T>C M1638T;* ***D.*** *K-ras ex.2 mutations: c.34 G>A G12S, c.34 G>T G12C, c.35 G>A G12D, c.35 G>T G12V, c.37 G>T G13C, c.38 G>A G13D;* ***E.*** *TP53 ex.7 c.722 C>T S241F (dark blue), c.776_7 AC>TT D259V (light blue), c.742 C>T R248W and c.743 G>A R248Q (red), c.733 G>A G245S (green).* R *– reverse sequence.*

Sequencing confirmation

Samples that showed abnormal melting curves were analyzed by DNA sequencing of the PCR product scanned by High-Resolution Melting. Mutations in *APC*, *CTNNB1*, *K-ras* and *TP53* were found in 57 (66.3%), 2 (2.3%), 30 (34.9%) and 46 (53.5%) patients, respectively.

Figure 6B shows one example of mutations found in exon 9 of the *APC* gene. Three samples had deviant melting profiles. DNA sequencing identified three different mutations: two nonsense mutations (c.904 C>T R302Stop in sample 43 and c.847 C>T R283Stop in sample 48), and one mutation in intron 8 (c.835-8 a>g in sample 37) which created a new splicing site. C-to-T transitions are the most common mutations in the *APC* gene. This kind of nonsense mutation is found mostly in exon 16 of the *APC* gene, in MCR (Chung, 2000; Smith et al., 2002; Michor et al., 2005; Thorstensen et al., 2005).

High-Resolution Melting does not give information about the type of genetic aberration. Thus, in some exons not only mutations but also single-nucleotide polymorphisms (SNPs) were detected – Figure 6C. Samples with homozygous SNPs (c.5034 G>A G1678G; red curves) had melting curves with peaks shifted slightly to the right of the heterozygous curves (light green curves). In sample 54 a homozygous SNP together with a mutation (c.4913 T>C M1638T) was found; its melting curve differed

significantly from the curves of other samples.

Figure 6D and 6E show results from screening of *K-ras* exon 2 and *TP53* exon 7, respectively. All aberrant melting profiles were proven to be results of mutations. Genetic variations in exon 2 of *K-ras* were as follows: c.34 G>T G12C, c.34 G>A G12S, c.35 G>T G12V, c.35 G>A G12D, c.37 G>T G13C and c.38 G>A G13D. Although there were six different mutations in *K-ras* exon 2, they all showed similar melting profiles (Figure 6D). This fact could be explained by the close proximity of the alterations in the genetic sequence. The same was observed in the case of *TP53* exon 7: mutation c.742 C>T R248W (found in samples 53, 67 and 76) and that of the next nucleotide (c.743 G>A R248Q in samples 26, 36, 45 and 66) could not be identified by their melting curves (Figure 6E).

However, not all samples with shifted melting curves showed genetic variations. One example is shown in Figure 7A. When exon 10 of the *APC* gene was sequenced, no mutation or SNP was found in it.

It should also be noted that the wild type did not always appear as wild type – Figure 7B. The software defines the most abundant group as wild type. In exon 14 of *APC* there was SNP in most of the samples, so that the real wild type appeared as an aberrant curve (blue curves on Figure 7B). This result suggests that some of the samples appearing as wild type on the LightScanner should be sequenced along with those with aberrant melting

curves in order not to miss a mutation. In sequencing wild type samples for confirmation, I found three mutations in exon 16 of *APC* that were not observed by High-Resolution Melting (mutations in samples 4, 6 and 24).

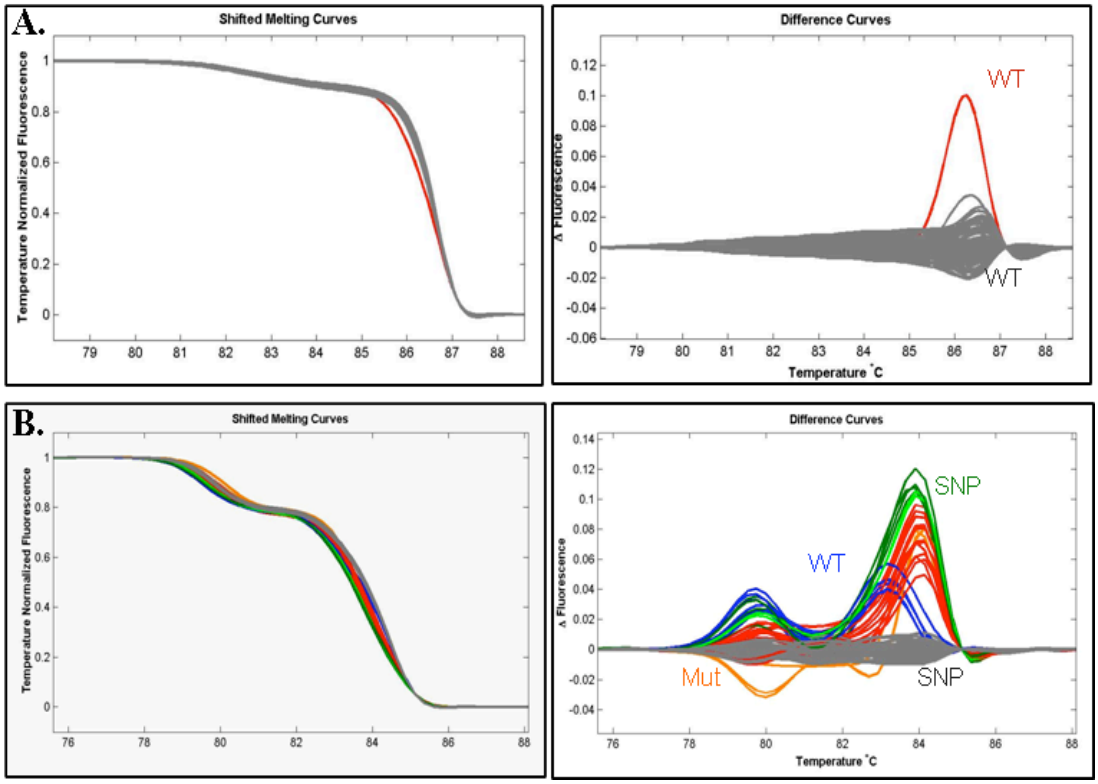


Figure 7. *Examples of discrepancy between High-Resolution Melting and Sequencing results.* ***A.*** *APC gene ex.10 – no variation was found in the sample with deviant melting profile;* ***B.*** *APC gene ex.14. WT – blue curves; SNP - c.1635 G>A A545A (heterozygous – red and green curves; homozygous – grey curves); Mutation (c.1660 C>T R554Stop) – orange curves, was found together with heterozygous SNP.*

The details of the mutations that were detected in 99 bulk tissue samples are listed in Table 6.

Table 6. *List of genetic aberrations and LOH status*

Sample	APC									CTNNB1	K-ras		TP53			LOH
1	c.4348 C>T R1450Stop	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	3'UTR 112207808 C>A			c.34 G>T G12C		c.818 G>A R273H	c.215 C>G P72R		-
2	c.2413 C>T R805Stop	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A							c.993+19 t>a		-
3	c.3956_6 delC	c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P			c.34 G>A G12S		c.818 G>A R273H	c.215 C>G P72R		+
4	c.4592_3 insA	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	3'UTR 112207808 C>A			c.35 G>T G12V			c.215 C>G P72R		+
5		c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.1958+8 t>c						c.215 C>G P72R		-
6	c.4616_7 insA	c.2720 G>A G907E	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A			c.35 G>A G12D			c.215 C>G P72R		+
7		c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.1958+8 t>c						c.215 C>G P72R		-
8	c.3925 G>T E1309Stop	c.3139 G>T E1047Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S							c.569_71 delCCT	c.215 C>G P72R		-
9	c.4222 G>T E1408Stop												c.817 C>T R273C	c.215 C>G P72R		-
10		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.35 G>A G12D	c.519 T>C D173D	c.817 C>T R273C	c.215 C>G P72R		-
11	c.2932 C>T Q978Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.35 G>A G12D		c.767 C>A T256K			-
12	c.2829 C>G S943Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.35 G>A G12D			c.215 C>G P72R		-
13	c.2767 A>T R923Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.35 G>A G12D			c.215 C>G P72R		-
14		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S							c.519 T>C D173D		c.215 C>G P72R		-
15		c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A							c.215 C>G P72R		-
16	c.3862G>C G1288Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S									c.215 C>G P72R		-
17	c.3991 A>T R1331Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S								c.811 G>T E271Stop	c.215 C>G P72R		-
18	c.1213 C>T R405Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.35 G>T G12V	c.519 T>C D173D		c.993+19 t>a		+
19	c.4285 C>T Q1429Stop	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.1958+8 t>c			c.173 C>T T58I		c.776_7 AC>TT D259V	c.215 C>G P72R		+
20	c.3944 C>A S1315Stop	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A						c.524 G>A R175H			-

21	c.2626 C>T R876Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.38 G>A G13D			c.215 C>G P72R		-
22		c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P				c.519 T>C D173D		c.215 C>G P72R		-
23		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S								c.31 G>C E11Q			-
24	c.4393_4 delAG	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.38 G>A G13D		c.916 C>T R306Stop	c.215 C>G P72R		+
25		c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P			c.35 G>T G12V		c.524 G>A R175H	c.215 C>G P72R		+
26		c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A				c.35 G>T G12V		c.743 G>A R248Q	c.215 C>G P72R		-
27	c.4219_27 ins8bp	c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S							c.215 C>G P72R		-
28	c.4192_3 delAG	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S							c.519 T>C D173D	c.775_5 delG	c.215 C>G P72R		ND
29	c.3991 A>T R1331Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S									c.215 C>G P72R		-
30		c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S					c.34 G>A G12S			c.215 C>G P72R		-
31		c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S							c.215 C>G P72R		+
32		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S									c.215 C>G P72R		-
33	c.2755 A>T R919Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.38 G>A G13D		c.824 G>T C275F	c.215 C>G P72R		+
34		c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A						c.722 C>T S241F	c.215 C>G P72R		+
35		c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A							c.108 G>A P36P	c.215 C>G P72R	-
36	c.4062_3 delTT	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S								c.743 G>A R248Q	c.215 C>G P72R		-
37	c.835-8 a>g	c.5465 T>A V1822D	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	3'UTR 112207808 C>A						c.215 C>G P72R		-
38		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5465 T>A V1822D	c.5625 C>T S1875S				c.35 G>A G12D			c.215 C>G P72R		-
39	c.4729_9 delA	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A					c.519 T>C D173D	c.637 C>T R213Stop	c.215 C>G P72R		-
40	c.2981_2 delTA	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P					c.519 T>C D173D	c.586 C>T R196Stop	c.215 C>G P72R		-
41	c.4043_4 insA	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A				c.204 G>C R68S		c.659 A>G Y220C	c.215 C>G P72R		-

42		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S									c.215 C>G P72R		ND
43	c.904 C>T R302Stop	c.3922 A>T K1308Stop	c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P				c.733 G>A G245S	c.215 C>G P72R		+
44	c.4132 C>T Q1378Stop													c.215 C>G P72R		+
45	c.4285 C>T Q1429Stop	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.1958+8 t>c					c.743 G>A R248Q	c.215 C>G P72R		+
46	c.4479_9 delG	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.1958+8 t>c					c.31 G>C E11Q	c.434T>C L145P	c.215 C>G P72R	-
47		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.38 G>A G13D		c.524 G>A R175H	c.215 C>G P72R		-
48	c.847 C>T R283Stop	c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P					c.844 C>T R282W	c.215 C>G P72R		-
49	c.3827 C>A S1276Stop	c.4124_37 del13bp	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A			c.37 G>T G13C		c.526 T>A C176S	c.215 C>G P72R		-
50		c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P						c.215 C>G P72R		-
51	c.2626 C>T R876Stop	c.4359_9 delT	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P			c.35 G>T G12V	c.519 T>C D173D	c.818 G>A R273H	c.215 C>G P72R		+
52	c.1660 C>T R554Stop	c.3925 G>T E1309Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S								c.215 C>G P72R		-
53	c.4099 C>T Q1367Stop	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A						c.742 C>T R248W	c.215 C>G P72R		+
54	c.4913 T>C M1638T	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034G>A G1678G	c.5268 T>G S1756S								c.215 C>G P72R		-
55	c.3115 G>T G1039Stop	c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P			c.38 G>A G13D			c.215 C>G P72R		+
56		c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A							c.215 C>G P72R		-
57	c.2937_8 GA>TT K980Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.34 G>T G12C			c.215 C>G P72R		+
58		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S									c.215 C>G P72R		+
59	c.1549-8 a>g	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S									c.215 C>G P72R		+
60		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S								c.524 G>A R175H	c.215 C>G P72R		+
61		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S								c.764_6 delATC	c.215 C>G P72R		-
62		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	3'UTR 112207808 C>A	c.1958+8 t>c				c.519 T>C D173D		c.215 C>G P72R		-
63	c.403_3 delG	c.4128_34 del7bp/insGCA	c.5465 T>A V1822D	3'UTR 112207808 C>A	c.1958+8 t>c								c.1045 G>T E349Stop	c.215 C>G P72R		+

64											c.35 G>A G12D		c.31 G>C E11Q			+
65	c.1690 C>T R564Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.35 G>A G12D		c.659 A>G Y220C	c.215 C>G P72R		+
66	c.4099 C>T Q1367Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S								c.743 G>A R248Q	c.215 C>G P72R		-
67	c.4135 G>T E1379Stop	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.519 T>C D173D	c.742 C>T R248W	c.215 C>G P72R		-
68	c.4099 C>T Q1367Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S									c.215 C>G P72R		+
69	c.3935_5 delG	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S									c.215 C>G P72R		-
70	c.4200_16 del17bp	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S						c.519 T>C D173D	c.844 C>T R282W	c.215 C>G P72R		+
71	c.3412 C>T Q1338Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P							c.637 C>T R213Stop	c.981 T>A Y327Stop	c.993+19 t>a	-
72		c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A			c.133 T>C S45P			c.472 C>T R158C	c.215 C>G P72R		-
73		c.1635 G>A A545A	c.5034 G>A G1678G	c.5034 G>A G1678G	c.5268 T>G S1756S					c.75_113 del38bp/74_5 insG/82 A>C	c.35 G>A G12D			c.215 C>G P72R		-
74	c.2626 C>T R876Stop	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	3'UTR 112207808 C>A				c.35 G>T G12V		c.374 C>T T125M	c.215 C>G P72R		-
75	c.646 C>T R216Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S								c.733 G>A G245S	c.215 C>G P72R		+
76	c.1660 C>T R554Stop	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S							c.519 T>C D173D	c.742 C>T R248W			+
77		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S											+
78	c.3625 G>T E1209Stop	c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P					c.469 G>T V157F	c.215 C>G P72R		-
79		c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.7065 A>G T2355T	3'UTR 112207808 C>A	c.1958+8 t>c			c.519 T>C D173D		c.215 C>G P72R		-
80	c.2626 C>T R876Stop	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P							c.215 C>G P72R		-
81	c.1690C>T R564Stop	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.1958+8 t>c			c.183 A>T Q61H		c.524 G>A R175H	c.215 C>G P72R		-
82	c.2626 C>T R876Stop	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.1958+8 t>c				c.519 T>C D173D	c.755_63 del9bp			+
83	c.4146_7 insA	c.5465 T>A V1822D	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P			c.38 G>A G13D		c.681_8 ins8bp	c.215 C>G P72R	c.993+19 t>a	-
84	c.884G>C S295T	c.1369_70 insT	c.3907 C>T Q1303Stop	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P		c.182 A>T Q61L					-
85	c.2626 C>T R876Stop	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P						c.733 G>A G245S			-
86		c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P									+

Meta																	
1M	c.4348 C>T R1450Stop	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	3'UTR 112207808 C>A			c.34 G>T G12C		c.818 G>A R273H	c.215 C>G P72R		
5M		c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.1958+8 t>c						c.215 C>G P72R		
40M	c.2981_2 delTA	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P					c.519 T>C D173D	c.586 C>T R196Stop	c.215 C>G P72R		
44M	c.4132 C>T Q1378Stop													c.215 C>G P72R		
51M	c.2626 C>T R876Stop	c.4359_9 delT	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P			c.35 G>T G12V	c.519 T>C D173D	c.818 G>A R273H	c.215 C>G P72R		
71M	c.3412 C>T Q1338Stop	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P						c.637 C>T R213Stop	c.981 T>A Y327Stop	c.993+19 t>a	
80M		c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P							c.215 C>G P72R		
81M	c.1690C>T R564Stop	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.1958+8 t>c			c.183 A>T Q61H		c.524 G>A R175H	c.215 C>G P72R		
82M		c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P	c.1958+8 t>c			c.34 G>A G12S	c.519 T>C D173D	c.755_63 del9bp			
83M	c.4146_7 insA	c.5465 T>A V1822D	c.1458 T>C Y486Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P			c.38 G>A G13D		c.681_8 ins8bp	c.215 C>G P72R	c.993+19 t>a	
84M	c.884G>C S295T	c.1369_70 insT	c.3907 C>T Q1303Stop	c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P		c.182 A>T Q61L					
85M		c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P				c.35 G>T G12V		c.733 G>A G245S			
86M		c.1458 T>C Y489Y	c.1635 G>A A545A	c.4479 G>A T1493T	c.5034 G>A G1678G	c.5268 T>G S1756S	c.5880 G>A P1960P									

Identified genetic changes were classified in 4 groups after search in human mutation databases – reported functional mutations (red), non-reported mutations (yellow), reported non-functional alterations (blue) and SNP (green).

Identified genetic aberrations were classified into four groups (functional mutations, non-reported mutations, non-functional missense mutations and SNP) after searches of mutation databases – The Human Gene Mutation Database, HGMD (http://www.hgmd.cf.ac.uk/ac/index.php), The Sanger Institute, COSMIC (http://www.sanger.ac.uk/genetics/CGP/cosmic/) and the IARC TP53 Mutation Database (http://www-p53.iarc.fr/). Incidence of different types of genetic aberrations is displayed in Table 7. Missense mutations were the most common genetic changes observed (excluding SNPs) in the primary tumor site.

Table 7. *Summary of the found genetic alterations.*

Found genetic variations:	
SNP	520
Mutations:	149
- *missense*	74
- *nonsense*	50
- *deletions*	18
- *insertions*	7

Next, the mutation status of metastases and their paired primary tumors were compared. The details of the mutations found in the primary sites and in the paired metastatic tissues are listed in Table 8. In most cases both tissues carried the same mutations, implying that cancer cells with all the mutations in the primary site gave rise to the metastatic lesion. However, in three patients there were differences in the mutation status of the primary site and metastasis (patients 80, 82 and 85 in Table 8).

Table 8. *Sequence alterations identified in primary tumor tissues (T) and paired metastases (M).*

Patient		APC mut			K-ras mut	TP53 mut	
1	T	c.4348 C>T R1450Stop			c.34 G>T G12C	c.818 G>A R273H	
	M	c.4348 C>T R1450Stop			c.34 G>T G12C	c.818 G>A R273H	
5	T						
	M						
40	T	c.2981_2 delTA				c.586 C>T R196Stop	
	M	c.2981_2 delTA				c.586 C>T R196Stop	
44	T	c.4132 C>T Q1378Stop					
	M	c.4132 C>T Q1378Stop					
51	T	c.2626 C>T R876Stop	c.4359_9 del T		c.35 G>T G12V	c.818 G>A R273H	
	M	c.2626 C>T R876Stop	c.4359_9 del T		c.35 G>T G12V	c.818 G>A R273H	
71	T	c.3412 C>T Q1338Stop				c.637 C>T R213Stop	c.981 T>A Y327Stop
	M	c.3412 C>T Q1338Stop				c.637 C>T R213Stop	c.981 T>A Y327Stop
80	T	c.2626 C>T R876Stop					
	M	-					
81	T	c.1690C>T R564Stop			c.183 A>T Q61H	c.524 G>A R175H	
	M	c.1690C>T R564Stop			c.183 A>T Q61H	c.524 G>A R175H	
82	T	c.2626 C>T R876Stop			-	c.755_63 del 9bp	
	M	-			c.34 G>A G12S	c.755_63 del 9bp	
83	T	c.4146_7 insA			c.38 G>A G13D	c.681_8 ins 8bp	
	M	c.4146_7 insA			c.38 G>A G13D	c.681_8 ins 8bp	
84	T	c.884G>C S295T	c.1369_70 insT	c.3907 C>T Q1303Stop	c.182 A>T Q61L		
	M	c.884G>C S295T	c.1369_70 insT	c.3907 C>T Q1303Stop	c.182 A>T Q61L		
85	T	c.2626 C>T R876Stop			-	c.733 G>A G245S	
	M	-			c.35 G>T G12V	c.733 G>A G245S	
86	T						
	M						

Allelic loss on chromosome 18q

Eighty-six primary cancer tissues were screened for allelic loss on chromosome 18q using a panel of five microsatellite markers. LOH was found in 30 cases (39%) – Table 6. The results from two tissues are shown in Figure 8.

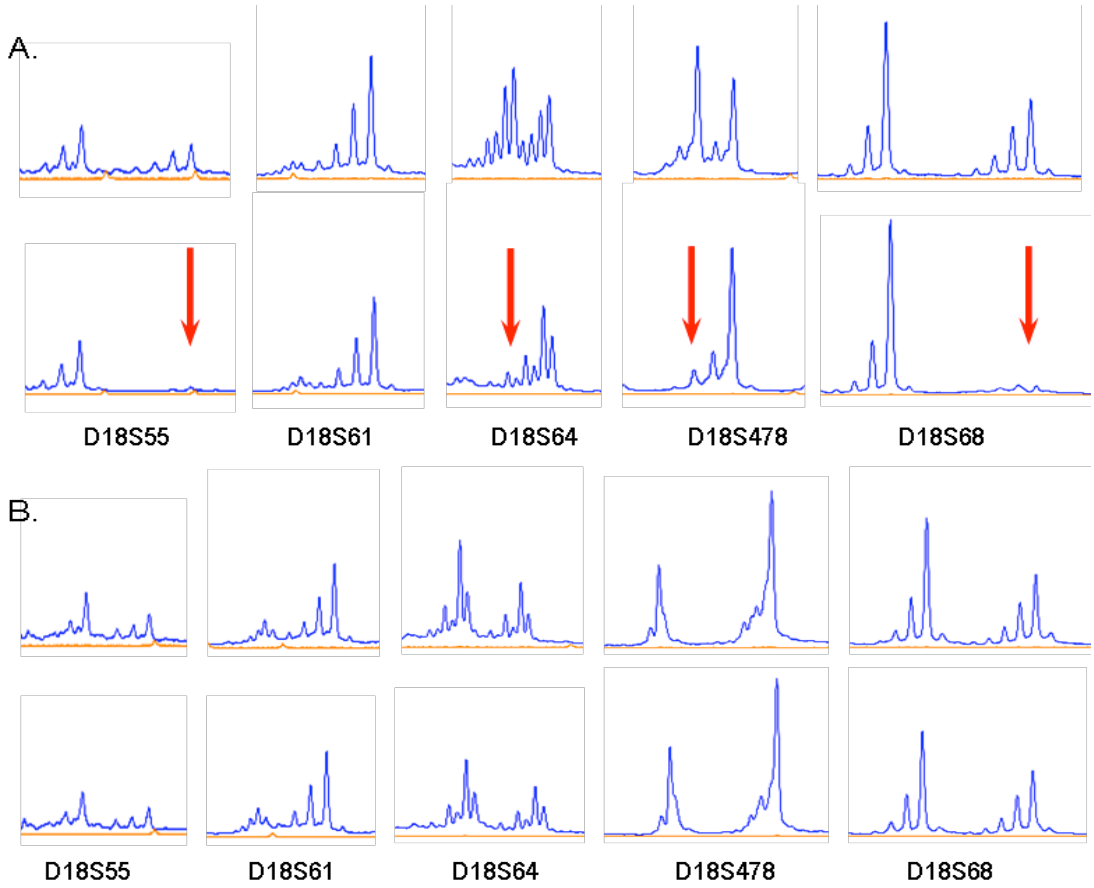


Figure 8. *Microsatellite analysis of chromosome 18q. Normal tissue and cancer tissue profiles are shown.* ***A.*** *LOH in sample 6 (red arrows);* ***B.*** *no LOH was detected in sample 23.*

GENETIC HETEROGENEITY ANALYSIS

Sample selection

As I intended to test the model of carcinogenesis proposed by Vogelstein and Fearon, I screened samples for sequence aberrations in genes that are involved in the model. Next aim was to assess this model at a cell-population level. To do so, samples in which *APC*, *K-ras* and *TP53* were all mutated were selected for the next experiments. Samples were not

chosen by their LOH status, as allelic loss could be a result of more than one event. Thirteen out of 86 samples (15.1%) were selected for study of genetic heterogeneity. Mutations found in these patients are summarized in Table 9. Although there may be additional mutations not detected by the bulk tissue analysis, I focused on these mutations for detailed analysis.

Table 9. *Sequence aberrations identified in CRC tissues of 13 patients.*

Sample No.	APC exon	Genetic variation	K-ras exon	Genetic variation	TP53 exon	Genetic variation
1	16	c.4348 C>T R1450Stop	2	c.34 G>T G12C	8	c.818 G>A R273H
3	16	c.3956_6delC	2	c.34 G>A G12S	8	c.818 G>A R273H
11	16	c.2932 C>T Q978Stop	2	c.35 G>A G12D	7	c.767 C>A T256K
19	16	c.4285 C>T Q1429Stop	3	c.173 C>T T58I	7	c.776_7 AC>TT D259V
24	16	c.4393_4delAG	2	c.38 G>A G13D	8	c.916 C>T R306Stop
33	16	c.2755 A>T R919Stop	2	c.38 G>A G13D	8	c.824 G>T C275F
41	16	c.4043_4insA	3	c.204 G>C R68S	6	c.659 A>G Y220C
49	16	c.3827 C>A S1276Stop c.4124_37del13bp	2	c.37 G>T G13C	5	c.526 T>A C176S
51	16	c.2626 C>T R876Stop c.4359_9delT	2	c.35 G>T G12V	8	c.818 G>A R273H
65	14	c.1690 C>T R564Stop	2	c.35 G>A G12D	6	c.659 A>G Y220C
74	16	c.2626 C>T R876Stop	2	c.35 G>T G12V	4	c.374 C>T T125M
81	14	c.1690 C>T R564Stop	3	c.183 A>T Q61H	5	c.524 G>A R175H
83	16	c.4146_7 insA	2	c.38 G>A G13D	7	c.681_8 ins 8bp

Liver metastatic tissues were available from four of the selected cases (patients 1, 51, 81 and 83) and they were also analyzed.

Outline of multipoint microsampling

For the analysis of genetic heterogeneity, 40–50 small areas containing only cancer cells were excised from the frozen primary tumor tissue sections; areas including only

normal cells from the same sections were microdissected for comparison, and genomic DNA was extracted. The sampling was random, but repeated sampling from the same cryptic region was avoided. To compare tumor cells from the colorectal neoplasm with those from metastasis, I microdissected six areas from metastatic tissues where these were available. As reference material, two areas were cut from normal liver tissue/lymph node stroma regions in the vicinity of the metastasis. After simultaneous amplification of three genes by multiplex PCR, mutation status was quantitatively determined with the SNaPshot assay, a primer extension assay.

In a previous study on lung cancer (Taniguchi et al., 2008), the smallest size of tissue sample that enabled stable and unbiased PCR amplification was determined. I set the size of my sections as 100×100×40 μm, a slight increase in thickness from the 35 μm used in that study. This volume was less than 1/100 of those used in previous studies (for example, 5×5×5 mm^3 in Lyng et al., 2004). Twenty-five percents of the genomic DNA extracted from each excised section were used for a single multiplex PCR reaction.

- ***Method validation***

To confirm the quantitative recovery of PCR products in the protocol, the following experiment was performed. Genomic DNA was extracted from colorectal tumor areas (0.01 mm^2 each) from two other patients, one with homozygous T for SNP in *TGFR2*

(rs2228048) and one with homozygous C. Samples with various ratios of the two genomic DNAs (1:0, 3:1, 1:1, 1:3, 0:1) were prepared, maintaining the total amount of DNA as that used for the single multiplex PCR reaction. To simulate the multiplex PCR reaction, I simultaneously amplified *TGFR2*, *K-ras* and *APC*, and determined allele ratios of amplified *TGFR2* gene by the SNaPshot assay.

The protocol for the SNaPshot assay previously applied to lung cancer samples (Taniguchi et al., 2008) was optimized by reducing the dilution of the PCR product. When this optimized protocol was applied, a good correlation between the ratios in the templates and in the amplified products was found, assuring unbiased amplification - Figure 9A.

Although I set the size of the excised section as small as possible, it nevertheless consisted of 180–200 cells. One might argue that contamination with normal cells could lead to erroneous conclusions. To address this concern, the following experiment was performed: from a CRC tissue, I excised twenty areas (0.01 mm^2 each), which included various numbers of normal cells. This CRC tissue had mutations in all three genes. After isolation of genomic DNAs, mutated exons of *APC*, *K-ras* and *TP53* were amplified and allele ratios were quantitated with the SNaPshot assay. The relative allele ratios of *APC* and *K-ras* were plotted (Figure 9B). In the case of normal cell contamination, the ratio of the normal allele should be proportional to the fraction of normal cells included in the

microdissected region. The ratios of normal/mutant alleles of the two genes are indeed proportional, as shown in Figure 9B. The same correlation was observed with *APC–TP53* and *K-ras–TP53* (data not shown). In general, a data point from a mixture of mature cancer cells and normal cells would lie on or near to the line connecting data points derived from each cell type. I can thus exclude normal cell contamination by this plotting. As shown below, no areas had this characteristic.

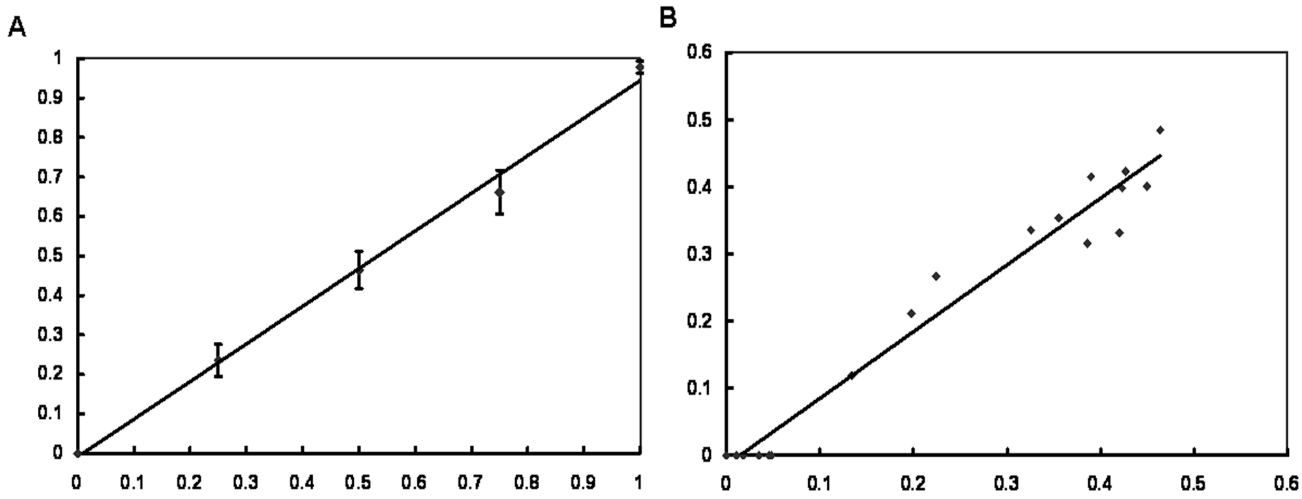


Figure 9. *Method validation.* ***A.*** *Quantitative accuracy of the SNaPshot assay of amplified products. Horizontal axis indicates the ratio of the mutant allele (T at rs2228048) of TGFR2 in the template; vertical axis indicates the ratio of the mutant allele of TGFR2 in the amplified product, i.e., M/(M+N), where M is mutant peak height, and N is normal peak height. The error bars correspond to the standard deviations of ten experiments.* ***B.*** *Contamination of normal cells in the excised section areas. Data obtained from areas including various fractions of normal cells among cancer cells were plotted. Horizontal axis indicates mutant allele ratios (M/(M+N)) of APC; vertical axis indicates that of K-ras.*

Genetic heterogeneity in primary tumors

Thirteen CRC tissues were examined and it was found that 11 contained cancer cells with mutation types different from that of the major population. Figure 10 shows an

example (sample 11). Six areas are presented. Electropherograms of three areas (A, D, F) revealed mutations in three genes, i.e., *APC*, *TP53* and *K-ras*. One area (B) had mutations in *APC* and *K-ras*, whereas the other two areas (C, E) had mutations only in *APC*. No mutation was found in normal cells microdissected from the same tissue. All other tissues, except for sample 51, also showed no germline mutation. Therefore, all these genetic events occurred in the colon.

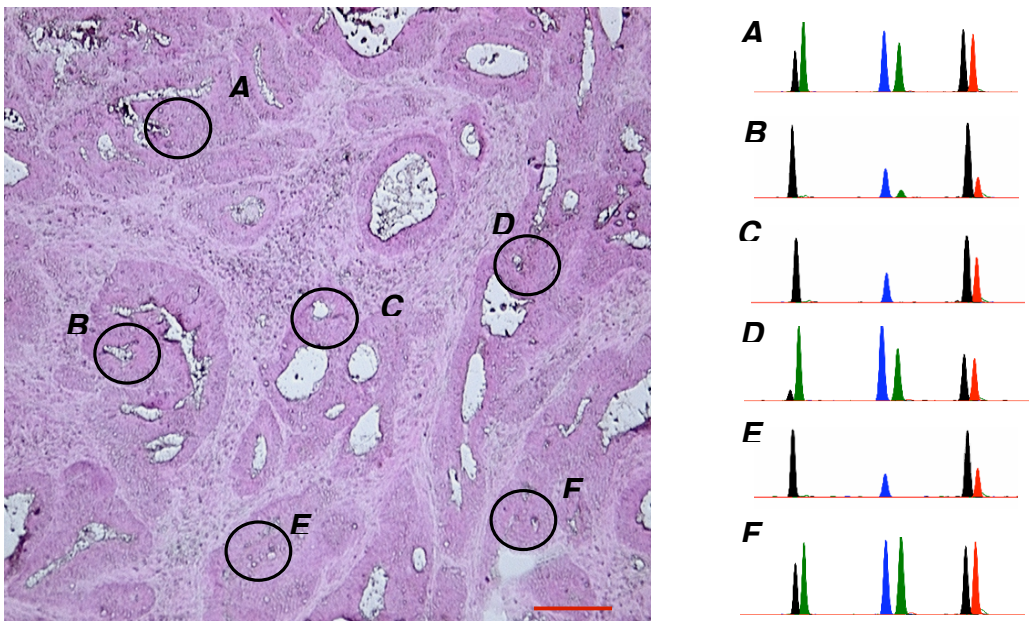


Figure 10. *An example of intratumor genetic heterogeneity (Sample 11). A microscopic view of a colorectal cancer tissue section (left), black circles indicate microdissected areas; (right), electropherograms of the SNaPshot assay. The first two peaks represent the mutation status of TP53 (C>A); third and fourth – that of K-ras (G>A); and the last two peaks – that of APC (C>T). Black peak, fragment amplified with ddC; green peak, fragment amplified with ddA; blue peak, fragment amplified with ddG; red peak, fragment amplified with ddT. Red bar, 100μm.*

Table 10. *Mutant allele ratios in the selected 13 CRC tissues.*

	Sample 1				Sample 3				Sample 11				Sample 19		
Area	**TP53**	**K-ras**	**APC**		**TP53**	**K-ras**	**APC**		**TP53**	**K-ras**	**APC**		**TP53**	**K-ras**	**APC**
1	1.00	0.62	0.81		0.78	0.62	0.93		***0.00***	***0.00***	***0.33***		0.95	0.50	0.96
2	1.00	0.71	0.65		0.88	0.48	1.00		0.97	0.44	0.55		0.98	0.51	0.92
3	1.00	0.52	0.85		0.94	0.62	1.00		0.88	0.39	0.47		0.99	0.55	1.00
4	1.00	0.62	1.00		0.97	0.69	0.96		0.97	0.50	0.52		0.96	0.48	0.97
5	1.00	0.36	1.00		0.59	0.81	1.00		0.97	0.45	0.50		0.98	0.54	0.98
6	1.00	0.55	0.81		0.64	0.62	0.97		0.11	0.40	0.32		0.97	0.53	0.96
7	1.00	0.58	0.76		0.93	0.63	0.98		0.94	0.42	0.49		0.95	0.51	0.96
8	1.00	0.61	0.74		0.68	0.58	0.97		0.76	0.47	0.56		0.98	0.54	0.98
9	1.00	0.79	0.80		0.94	0.70	0.96		0.98	0.45	0.55		0.48	0.35	0.49
10	1.00	0.66	0.96		0.77	0.52	1.00		***0.02***	***0.00***	***0.22***		0.98	0.53	0.99
11	1.00	0.69	0.82		0.59	0.74	1.00		***0.02***	***0.16***	***0.33***		1.00	0.50	1.00
12	1.00	0.62	0.79		0.90	0.65	1.00		0.92	0.50	0.51		***0.98***	***0.53***	***0.00***
13	1.00	0.73	1.00		0.67	0.61	0.96		***0.02***	***0.00***	***0.29***		0.98	0.53	0.98
14	1.00	0.66	0.93		0.72	0.69	1.00		0.90	0.50	0.52		0.95	0.49	0.96
15	1.00	0.62	0.95		0.63	0.66	0.97		***0.02***	***0.00***	***0.18***		0.97	0.49	0.99
16	1.00	0.65	0.97		0.57	0.73	1.00		0.93	0.54	0.47		0.70	0.43	0.76
17	1.00	0.64	0.68		0.65	0.63	0.95		0.87	0.42	0.50		0.98	0.47	0.97
18	1.00	0.73	0.77		0.52	0.51	0.83		0.92	0.52	0.57		0.97	0.54	0.98
19	1.00	0.11	0.54		0.64	0.39	0.91		0.99	0.46	0.53		0.99	0.53	0.98
20	1.00	0.70	0.83		0.68	0.58	0.96		0.94	0.47	0.51		***0.97***	***0.43***	***0.00***
21	1.00	0.75	0.66		0.91	0.60	1.00		0.16	0.64	0.31		0.98	0.51	1.00
22	1.00	0.54	0.73		0.68	0.71	0.96		0.75	0.45	0.48		0.97	0.54	0.98
23	1.00	0.79	0.67		0.65	0.64	0.96		***0.02***	***0.00***	***0.30***		0.99	0.46	0.98
24	1.00	0.71	0.86		0.66	0.63	0.94		***0.03***	***0.20***	***0.39***		0.74	0.50	0.95
25	1.00	0.56	0.70		0.90	0.56	0.96		0.46	0.49	0.55		0.92	0.51	0.97
26	1.00	0.59	0.69		0.74	0.57	0.96		0.65	0.45	0.48		0.94	0.49	1.00
27	1.00	0.11	1.00		***0.30***	***0.28***	***0.01***		***0.02***	***0.21***	***0.16***		0.00	1.00	0.00
28	1.00	0.54	0.78		0.98	0.62	1.00		0.42	0.53	0.49		0.98	0.55	0.99
29	1.00	0.55	0.91		0.60	0.45	0.90		0.67	0.46	0.51		0.95	0.53	0.96
30	1.00	0.59	0.80		***0.47***	***0.21***	***0.02***		0.57	0.50	0.57		0.99	0.51	0.99
31	1.00	0.68	0.89		0.86	0.59	1.00		***0.03***	***0.00***	***0.28***		0.94	0.53	0.98
32	***1.00***	***0.00***	***1.00***		0.91	0.62	1.00		0.13	0.40	0.41		0.99	0.54	1.00
33	***1.00***	***0.00***	***0.00***		0.69	0.59	0.95		0.63	0.43	0.55		0.89	0.44	0.93

34	1.00	0.49	0.87		0.64	0.55	1.00		***0.04***	***0.00***	***0.17***		0.99	0.49	0.99
35	1.00	0.61	1.00		0.56	0.40	0.93		0.84	0.49	0.54		0.95	0.53	0.98
36	1.00	0.60	1.00		0.92	0.63	0.98		0.58	0.52	0.52		0.81	0.45	0.87
37	1.00	0.54	1.00		0.86	0.63	1.00		0.56	0.57	0.44		0.99	0.54	1.00
38	1.00	0.77	1.00		0.71	0.59	0.96		0.38	0.39	0.53		0.97	0.53	0.99
39	1.00	0.57	0.94		0.69	0.60	1.00		0.12	0.61	0.41		0.86	0.29	0.47
40	***0.76***	***0.07***	***0.88***		0.80	0.62	0.97		0.32	0.39	0.57		0.87	0.53	0.94
41	***0.25***	***0.00***	***0.00***		0.59	0.43	0.92		***0.03***	***0.18***	***0.32***		0.90	0.53	0.87
42	***0.43***	***0.00***	***0.03***		0.76	0.64	1.00						0.94	0.51	0.97
43					0.91	0.67	1.00						0.97	0.55	0.97
44					0.59	0.33	0.99						0.96	0.51	0.94
45					0.88	0.57	1.00						0.94	0.49	0.96
46					0.83	0.55	1.00								
47					0.84	0.73	1.00								
48					0.74	0.53	0.92								
49															
50															

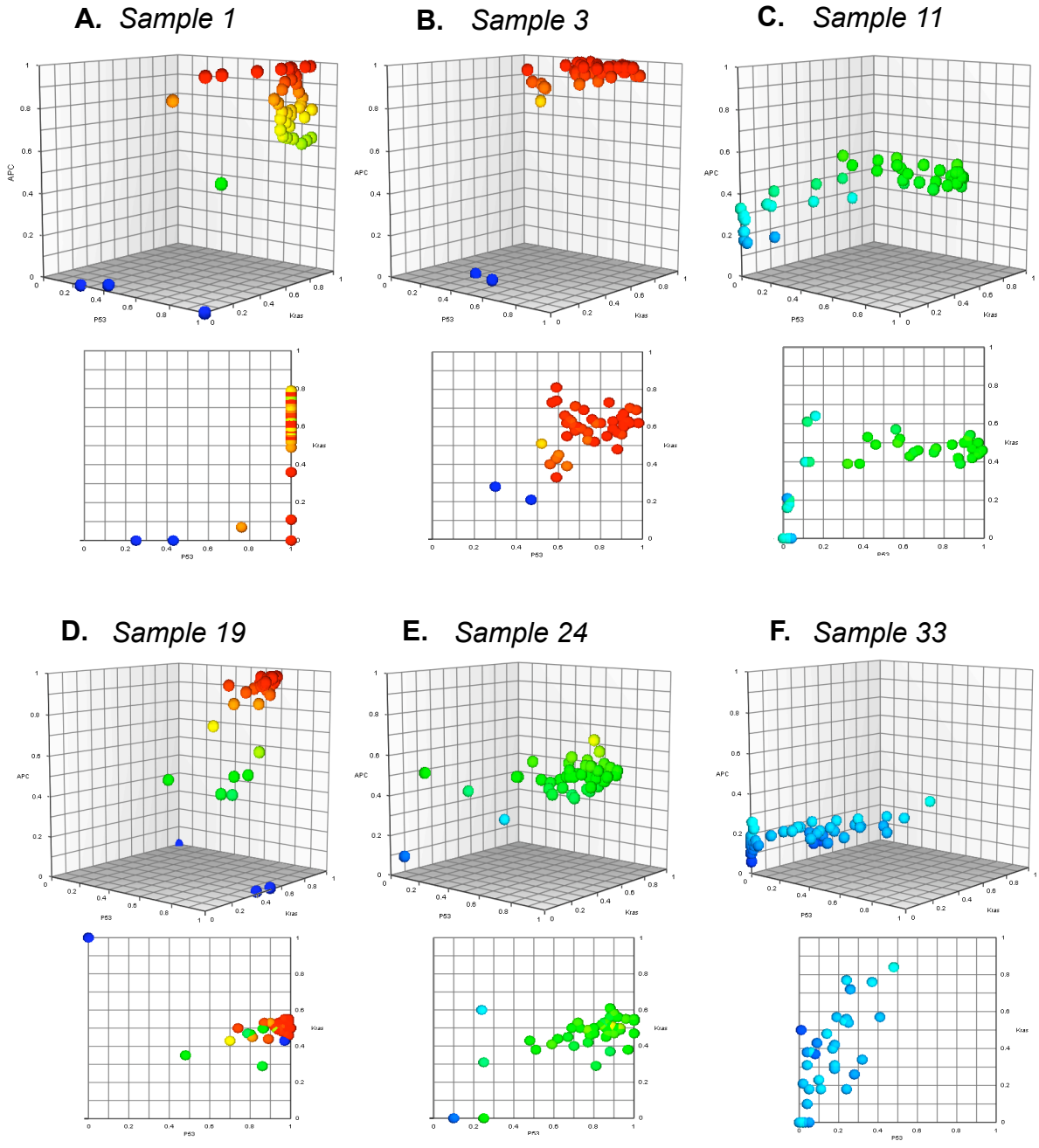
	Sample 24				**Sample 33**				**Sample 41**				**Sample 49**			
Area	**TP53**	**K-ras**	**APC**		**TP53**	**K-ras**	**APC**		**TP53**	**K-ras**	**APC**		**TP53**	**K-ras**	**APC1**	**APC2**
1	0.25	0.31	0.41		***0.01***	***0.00***	***0.12***		0.96	0.21	0.88		0.97	0.26	0.58	0.58
2	***0.10***	***0.00***	***0.11***		***0.01***	***0.00***	***0.26***		0.92	0.19	0.96		0.96	0.30	0.49	0.52
3	1.00	0.55	0.56		***0.10***	***0.23***	***0.21***		0.77	0.18	0.81		1.00	***0.00***	0.51	0.52
4	0.78	0.46	0.52		0.23	0.55	0.22		0.85	0.21	0.37		1.00	0.24	0.58	0.49
5	0.48	0.43	0.49		***0.03***	***0.00***	***0.16***		0.99	0.18	0.92		0.81	0.16	0.25	0.48
6	0.51	0.38	0.50		***0.00***	***0.00***	***0.19***		0.73	0.18	0.79		0.97	0.34	0.39	0.58
7	0.59	0.41	0.58		0.18	0.29	0.18		0.92	0.20	0.66		0.68	0.16	0.09	0.81
8	0.62	0.44	0.49		0.19	0.57	0.18		0.90	0.21	0.90		0.85	0.16	0.51	0.48
9	0.77	0.42	0.47		0.14	0.48	0.21		0.91	0.22	0.91		0.99	0.24	0.15	0.90
10	0.84	0.52	0.55		***0.01***	***0.00***	***0.16***		0.87	0.18	1.00		0.99	0.20	0.56	0.61
11	0.70	0.40	0.46		***0.04***	***0.10***	***0.18***		0.89	0.17	0.90		1.00	0.12	0.35	0.74
12	0.78	0.46	0.55		0.24	0.18	0.18		***0.18***	***1.00***	***0.00***		0.96	0.29	0.45	0.63
13	***0.25***	***0.00***	***0.54***		***0.00***	***0.00***	***0.11***		0.97	0.18	0.79		0.98	0.27	0.54	0.52
14	1.00	0.47	0.54		0.11	0.18	0.20		0.71	0.20	0.58		0.99	0.21	0.54	0.55
15	1.00	0.54	0.54		0.41	0.57	0.17		0.76	0.16	0.80		0.97	0.20	0.54	0.53

16	0.77	0.49	0.61		***0.02***	***0.21***	***0.18***		0.66	0.24	0.86		0.97	0.19	0.53	0.55
17	0.81	0.29	0.46		0.18	0.42	0.17		0.82	0.25	0.95		0.98	0.26	0.57	0.52
18	0.67	0.45	0.48		0.25	0.54	0.18		***1.00***	***0.00***	***0.00***		0.96	0.24	0.51	0.44
19	0.91	0.56	0.50		***0.05***	***0.18***	***0.19***		0.83	0.17	0.83		0.96	0.26	0.54	0.54
20	0.72	0.53	0.57		***0.03***	***0.00***	***0.17***		0.87	0.18	0.88		0.92	0.23	0.41	0.62
21	0.80	0.50	0.53		0.32	0.34	0.17		0.90	0.22	0.88		0.93	0.24	0.23	0.83
22	0.86	0.45	0.54		***0.00***	***0.00***	***0.11***		***1.00***	***0.00***	***0.00***		0.93	0.25	0.51	0.48
23	0.85	0.51	0.60		***0.02***	***0.00***	***0.14***		1.00	0.14	0.86		0.96	0.25	0.57	0.44
24	0.85	0.53	0.46		***0.06***	***0.38***	***0.21***		0.99	0.21	0.96		1.00	0.19	0.44	0.58
25	0.91	0.50	0.52		0.28	0.26	0.15		0.91	0.18	0.64		0.96	0.21	0.65	0.48
26	0.86	0.50	0.53		0.24	0.56	0.18		***1.00***	***0.00***	***0.00***		0.98	0.12	0.44	0.75
27	0.89	0.51	0.70		***0.02***	***0.00***	***0.23***		0.92	0.19	0.93		0.95	0.22	0.74	0.42
28	0.90	0.58	0.58		***0.00***	***0.00***	***0.16***		***1.00***	***0.00***	***0.00***		0.96	0.17	0.37	0.74
29	0.85	0.51	0.46		***0.00***	***0.00***	***0.14***		0.80	0.26	0.94		0.87	0.23	0.42	0.73
30	0.73	0.50	0.51		***0.04***	***0.38***	***0.15***		0.93	0.21	0.89		1.00	0.20	0.53	0.54
31	0.88	0.61	0.55		***0.05***	***0.00***	***0.15***		***1.00***	***0.00***	***0.02***		0.99	0.20	0.55	0.62
32	0.88	0.37	0.44		***0.00***	***0.00***	***0.14***		0.82	0.18	0.90		0.98	0.19	0.69	0.52
33	0.24	0.60	0.22		0.24	0.77	0.20		0.98	0.18	0.85		0.95	0.24	0.54	0.64
34	0.93	0.50	0.65		0.48	0.84	0.30		1.00	0.20	0.72		0.98	0.19	0.57	0.60
35	0.87	0.52	0.51		0.37	0.76	0.21		0.89	0.26	0.95		0.95	0.19	0.70	0.49
36	0.90	0.47	0.58		***0.00***	***0.00***	***0.15***		0.66	0.21	0.89		0.96	0.19	0.61	0.59
37	0.93	0.49	0.56		0.26	0.72	0.16		0.86	0.23	0.89		0.98	0.22	0.67	0.50
38	0.86	0.53	0.57		***0.00***	***0.00***	***0.06***		0.79	0.20	0.74		0.96	0.21	0.57	0.46
39	0.86	0.52	0.45		***0.09***	***0.43***	***0.13***		***1.00***	***0.00***	***0.00***		0.97	0.17	0.47	0.72
40	0.69	0.50	0.49		***0.00***	***0.00***	***0.15***		***1.00***	***0.00***	***0.00***		1.00	0.27	0.48	0.49
41	0.79	0.47	0.52		***0.00***	***0.00***	***0.10***									
42	0.79	0.47	0.43		***0.02***	***0.00***	***0.23***									
43	0.97	0.38	0.50		0.17	0.40	0.19									
44	0.88	0.56	0.47		***0.04***	***0.31***	***0.19***									
45	0.96	0.55	0.57		***0.00***	***0.00***	***0.17***									
46	0.92	0.56	0.50		***0.08***	***0.37***	***0.10***									
47	0.87	0.50	0.52		0.18	0.31	0.19									
48	0.87	0.47	0.58		***0.01***	***0.50***	***0.08***									
49	0.80	0.47	0.52													
50																

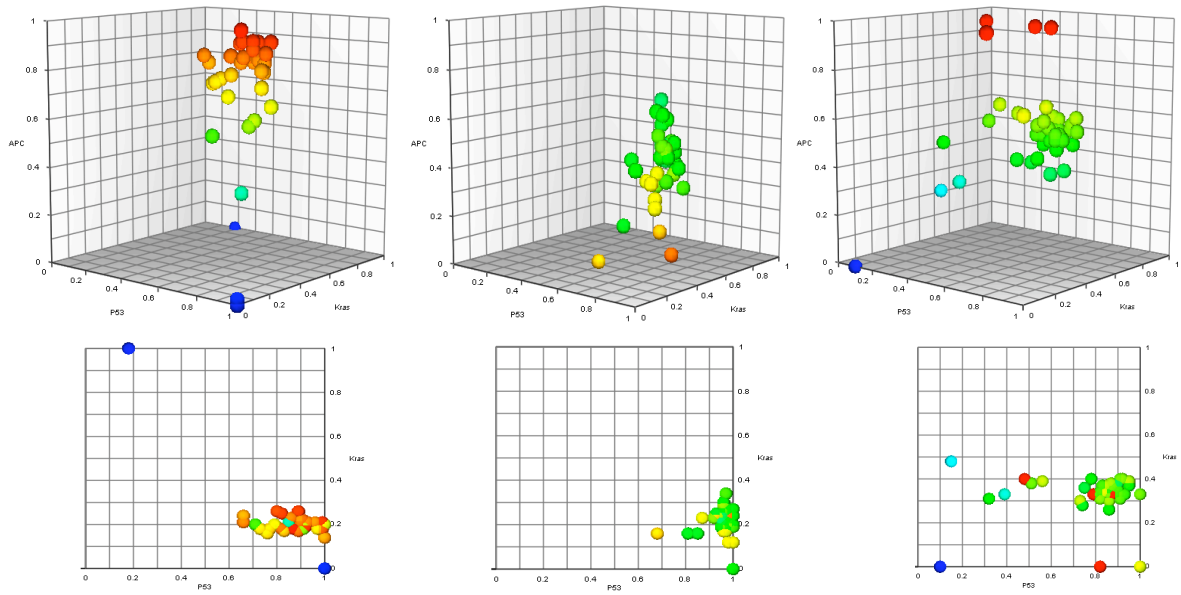
	Sample 51					Sample 65				Sample 74				Sample 81				Sample 83		
Area	TP53	K-ras	APC1	APC2		TP53	K-ras	APC		TP53	K-ras	APC		TP53	K-ras	APC		p53	Kras	APC
1	0.90	0.38	0.44	0.94		0.79	0.47	0.92		1.00	0.27	0.55		0.60	0.84	0.92		***0.04***	***0.60***	***1.00***
2	0.87	0.33	1.00	1.00		0.81	0.40	1.00		0.96	0.29	0.62		0.12	0.31	0.64		***0.00***	***0.48***	***1.00***
3	0.87	0.36	0.60	0.77		0.98	0.42	0.98		0.56	0.12	0.45		***0.03***	***0.13***	***0.11***		0.90	0.55	1.00
4	0.95	0.37	0.55	0.81		0.99	0.39	0.91		0.97	0.30	0.59		0.73	0.91	0.97		***0.06***	***0.47***	***1.00***
5	0.78	0.40	0.53	1.00		0.93	0.45	0.96		1.00	0.28	0.57		0.08	0.00	0.00		0.97	0.72	1.00
6	0.93	0.33	0.57	0.86		0.89	0.43	0.90		0.94	0.25	0.51		0.68	0.90	0.98		0.94	0.55	1.00
7	0.90	0.35	0.58	0.82		0.81	0.33	1.00		1.00	0.26	0.53		0.74	0.91	0.95		0.28	0.34	1.00
8	0.90	0.33	0.53	0.83		1.00	0.44	0.89		1.00	0.27	0.50		0.68	0.92	0.96		0.92	0.55	0.94
9	0.92	0.40	0.62	0.77		0.91	0.36	0.94		1.00	0.28	0.69		0.72	1.00	0.96		0.97	0.66	0.96
10	1.00	0.33	0.61	0.86		0.89	0.42	0.96		0.96	0.29	0.70		0.72	0.89	0.96		0.70	0.65	1.00
11	0.48	0.40	1.00	1.00		0.71	0.33	0.68		0.86	0.16	0.41		***0.00***	***0.20***	***0.33***		0.97	0.68	1.00
12	0.84	0.34	0.69	0.83		0.82	0.38	0.74		0.82	0.15	0.46		***0.00***	***0.50***	***0.96***		0.94	0.64	0.94
13	0.87	0.38	0.64	0.80		0.95	0.40	0.96		0.94	0.26	0.53		0.66	0.86	0.92		0.76	0.58	1.00
14	0.87	0.33	0.43	0.87		0.75	0.47	0.88		0.95	0.29	0.54		0.71	0.38	0.98		0.96	0.68	1.00
15	0.91	0.31	0.59	0.85		0.95	0.40	0.96		0.94	0.26	0.58		0.63	0.96	0.91		0.94	0.72	1.00
16	***0.82***	***0.00***	***1.00***	1.00		1.00	0.48	0.99		1.00	0.29	0.53		0.50	0.80	0.89		0.98	0.59	1.00
17	0.86	0.33	0.61	0.86		0.99	0.42	0.96		0.97	0.23	0.48		0.66	0.70	0.85		0.96	0.56	1.00
18	0.82	0.31	0.62	1.00		0.98	0.40	0.85		0.97	0.29	0.56		0.72	0.89	0.92		0.94	0.65	0.96
19	0.39	0.33	0.34	0.91		0.91	0.41	0.90		1.00	0.28	0.33		0.74	0.91	0.91		0.98	0.68	1.00
20	0.91	0.40	0.60	0.79		0.95	0.42	0.91		1.00	0.27	0.55		0.73	1.00	1.00		0.97	0.66	1.00
21	0.75	0.36	0.46	0.88		0.90	0.37	0.88		0.98	0.29	0.57		0.66	0.93	0.90		***0.09***	***0.36***	***1.00***
22	0.83	0.37	0.58	0.82		***0.01***	***0.00***	***1.00***		0.90	0.27	0.47		0.67	0.94	0.85		0.94	0.63	1.00
23	0.15	0.48	0.25	0.57		1.00	0.41	1.00		0.94	0.22	0.39		0.04	0.00	0.03		***0.03***	***0.29***	***1.00***
24	0.80	0.33	0.58	1.00		0.93	0.44	0.98		0.98	0.26	0.56		0.75	0.36	0.96		0.93	0.65	0.97
25	0.51	0.38	0.60	1.00		0.99	0.39	0.97		0.93	0.25	0.36		0.65	0.85	0.92		0.95	0.67	1.00
26	***0.10***	***0.00***	***0.00***	0.49		0.94	0.31	0.91		0.98	0.29	0.66		***0.03***	***0.37***	***1.00***		0.93	0.64	1.00
27	0.79	0.33	1.00	0.67		0.88	0.48	0.98		0.96	0.24	0.41		0.77	0.33	0.94		0.96	0.68	1.00
28	0.74	0.28	0.48	0.88		***0.52***	***0.00***	***1.00***		1.00	0.23	0.72		0.58	0.33	0.92		0.90	0.64	1.00
29	0.88	0.37	0.60	0.80		0.93	0.37	0.96		0.93	0.26	0.52		0.81	0.34	0.94		0.97	0.70	1.00
30	0.91	0.33	0.59	0.79		0.88	0.37	0.95		0.94	0.24	0.62		0.70	0.28	0.85		***0.04***	***0.54***	***1.00***
31	0.85	0.37	0.65	0.88		0.92	0.34	0.98		0.95	0.30	0.45		0.68	0.36	0.95		***0.06***	***0.49***	***1.00***
32	0.91	0.31	0.57	0.85		0.94	0.40	0.93		0.96	0.27	0.44		0.59	0.40	0.90		0.95	0.74	1.00
33	0.95	0.39	0.65	0.75		0.93	0.40	0.93		0.97	0.26	0.41		0.69	0.29	0.90		0.98	0.56	1.00
34	0.86	0.26	0.50	0.85		0.95	0.42	0.92		1.00	0.29	0.44		0.60	0.35	0.98		***0.06***	***0.34***	***1.00***
35	0.32	0.31	0.50	0.85		***0.02***	***0.00***	***1.00***		0.96	0.26	0.42		0.57	0.31	0.99		0.08	0.05	0.10

36	0.86	0.34	0.56	0.81		0.86	0.36	0.97		0.92	0.25	0.56		0.50	0.23	0.67		0.98	0.71	1.00
37	0.73	0.30	0.66	0.82		0.96	0.41	0.99		0.96	0.30	0.52		0.35	0.23	0.61		0.98	0.67	1.00
38	0.56	0.39	0.67	1.00		0.99	0.35	1.00		0.95	0.26	0.58		0.57	0.23	0.73		0.97	0.66	1.00
39	0.86	0.30	0.64	0.76		0.97	0.40	0.98		0.93	0.28	0.56		0.61	0.31	0.95		0.98	0.68	1.00
40	***1.00***	***0.00***	***0.71***	0.69		0.94	0.42	0.97		0.86	0.32	0.44		0.64	0.33	0.96		0.95	0.54	1.00
41	0.82	0.34	0.58	0.85		0.89	0.43	0.85						0.65	0.33	0.92		0.97	0.56	1.00
42	0.90	0.33	0.57	0.69		1.00	0.43	0.98						0.60	0.32	1.00		0.98	0.55	1.00
43						0.95	0.39	0.95						0.75	0.96	0.87		0.97	0.51	1.00
44						0.72	0.45	0.90						0.86	0.97	0.95		0.97	0.70	1.00
45						0.69	0.39	0.95										0.93	0.64	1.00
46						0.78	0.44	0.93												
47						0.70	0.38	0.96												
48						0.79	0.45	0.95												
49						***0.52***	***0.00***	***1.00***												
50																				

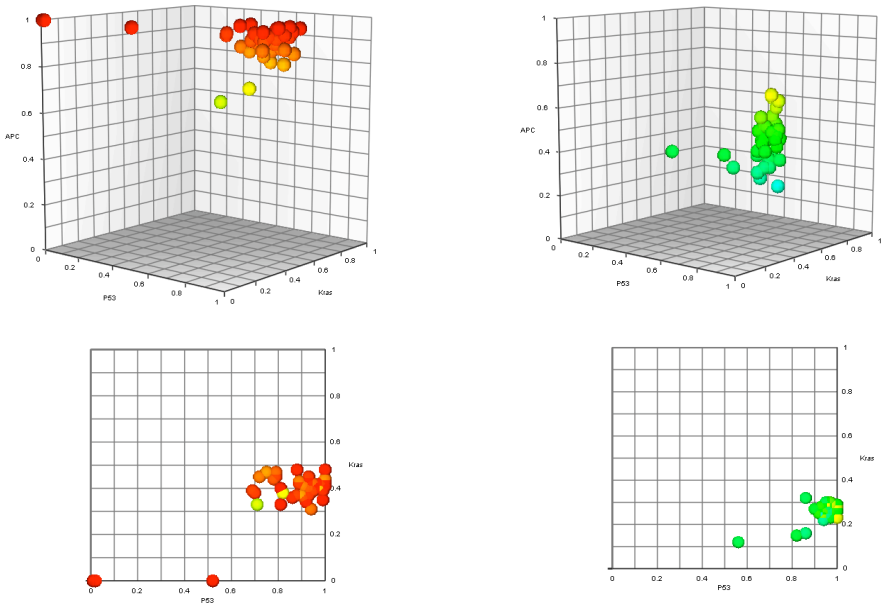
In present experimental system, relative ratios of mutated and normal alleles were measured (Table 10). Areas were plotted in three-dimensional space created by mutated allele ratios of *APC*, *K-ras* and *TP53* - Figure 11, top graphs. Two-dimensional plots with color gradation for the *APC* allele ratio were also presented - Figure 11, bottom graphs.



G. *Sample 41* **H.** *Sample 49*[*] **I.** *Sample 51*[†]



J. *Sample 65* **K.** *Sample 74*



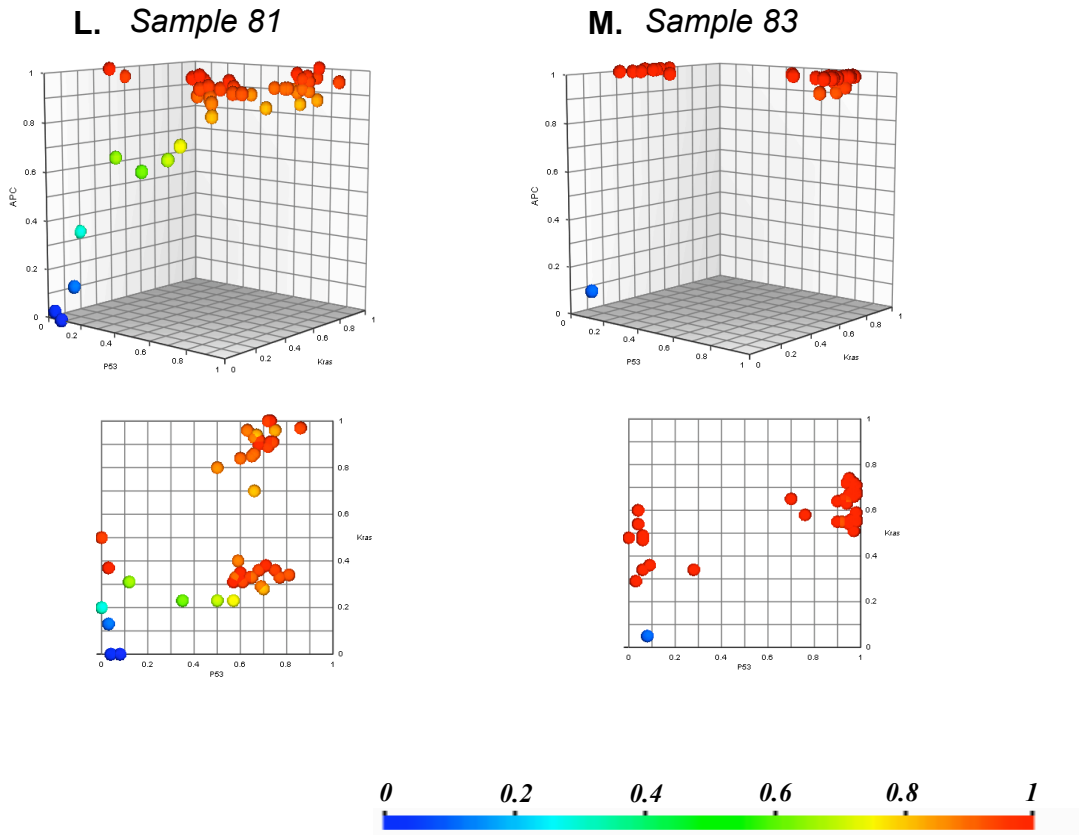


Figure 11. *Genetic heterogeneity inside the colorectal cancer tissues. The mutant allele ratios (M/(M+N)) of TP53, K-ras and APC genes are plotted in the x, y and z axis, respectively. Each sphere represents a single area; the color indicates the mutant allele ratio of APC gene. The top graphs are 3D graphs; the bottom graphs show TP53 and K-ras only (the color is the same as in the 3D graph).* ***A.*** *Sample 1;* ***B.*** *Sample 3;* ***C.*** *Sample 11;* ***D.*** *Sample 19;* ***E.*** *Sample 24;* ***F.*** *Sample 33;* ***G.*** *Sample 41;* ***H.*** *Sample 49, * the color indicates the allele ratio of the second APC mutation (c.4124_37 del13bp);* ***I.*** *Sample 51, † germline APC mutation (c.4359_9 delT) is not shown;* ***J.*** *Sample 65;* ***K.*** *Sample 74;* ***L.*** *Sample 81;* ***M.*** *Sample 83.*

The major population of sample 1 (37/42 areas) is characterized by mutations in all three genes (Figure 11A). I also found one subpopulation with both *TP53* and *APC* mutated (2/42), and another with a single *TP53* mutation (3/42). Mutant allele ratio of *TP53* in most of the areas was 1.0; loss of the normal allele is the most probable reason for it.

Most of the excised areas from sample 3 carry mutations in all three genes – 46 out of

48 areas (Figure 11B). However, two areas with mutations in both *K-ras* and *TP53* but not in the *APC* gene were found.

The major population of sample 11 (30/41 areas) has the genetic variations in all three genes (Figure 11C). Chromosomal status is stable for *APC* and *K-ras*, but there is a distinct heterogeneity for *TP53*. Besides this major population, there are two subpopulations with either *APC* and *K-ras* mutations (4/41) or with the *APC* mutation alone (7/41).

Most areas microdissected from sample 19 (43/45) carry the three mutated genes (Figure 11D). However, I found two areas with both *TP53* and *K-ras* mutated, but not *APC*. There was also one area which carried a homozygous mutation of *K-ras*; here loss of the normal allele is suspected.

Sample 24 also contained a major population (47/49 areas) with all three genes mutated and a subpopulation with two mutated genes (*APC* and *TP53*, 2 areas) – Figure 11E. Heterogeneity in the *TP53* mutation was observed.

Sample 33 included three cell populations, one with all three genes mutated (18/48), a subpopulation with *APC* and *K-ras* mutations (9/48) and another with the *APC* mutation alone (21/48). Interestingly, the population with all genes mutated was not the biggest one. Furthermore, *TP53* and *K-ras* alleles have marked heterogeneity (Figure 11F).

In sample 41, there are two minor populations, one with the *TP53* mutation alone and one with *K-ras* and *TP53* mutations - Figure 11G. The former lost the normal *TP53* allele, and the latter lost the normal *K-ras* allele, whereas the major population always maintained the normal *K-ras* allele, and often also the normal *TP53* allele. Subsequent loss of normal alleles after divergence of the major population is suspected in this case.

Sample 51 is characterized by two mutations in the *APC* gene. When the mutation status of normal colon tissue was examined, one of these mutations (c.4359_9 delT) was found to be present not only in tumor cells, but also in normal tissues (Figure 11I). I continued the analysis of this sample for the other *APC* mutation and found a minor subpopulation carrying both *APC* and *TP53* mutations (2/42 areas). The only somatic mutation detected in one tumor area was found in the *TP53* gene.

There are three subpopulations of cancer cells in sample 65 (Figure 11J). *APC* alleles are highly homogeneous –the *APC* mutation was detected in all 49 areas. A subpopulation with only the *APC* mutation (2/49) and another carrying both *APC* and *TP53* aberrations (2/49) were identified.

In sample 81, only one minor subpopulation (4/44 areas) with both *APC* and *K-ras* mutations was detected – Figure 11L. Interestingly, two groups could be differentiated according to *K-ras* mutation status, one with a *K-ras* mutant allele ratio of 0.3-0.4 and the

other with a ratio of 0.8-1.0. The latter one may be the result of LOH on chromosome 12p.

All microdissected areas from sample 83 contained mutated *APC* and *K-ras* genes – Figure 11M. However, eight areas had only these two genetic aberrations, but not the one in *TP53* that was observed in the other 37 areas. As the *APC* mutant allele ratio in all areas was close to 1.0 (homozygous mutation), a germline mutation was suspected, but the analysis of normal colon tissue revealed that the *APC* gene was wild type. Subsequent loss of the normal allele in tumor cells is the most probable explanation of these data.

Although minor subpopulations were identified in the above cases, samples 49 and 74 showed no heterogeneity in the mutation pattern (Figure 11H and 11K, respectively).

- ***Order of genetic events***

In the case of the *APC*, *K-ras* and *TP53* genes, mutations in various loci of the gene evoke similar biological effects in the cells that harbor them. Because the likelihood that a second mutation is introduced into the same locus is very low, these mutations will have been introduced by single events. Therefore, subpopulations within each sample should have been derived from a single ancestor cell, and the order of mutation events can be deduced from the mutation patterns of subpopulations. If mutations are present throughout a tumor, they are believed to have occurred early in carcinogenesis (Barnetson et al., 2000), while mutations that are present in a heterogeneous pattern may have arisen later in tumor

progression. For example, sample 11 had a subpopulation with *APC* and *K-ras* mutations, and another with *APC* mutations alone. The order of mutation events in this sample is therefore *APC*→*K-ras*→*TP53*. The deduced orders of mutation events are shown in Table 11.

Table 11. *Summary of the order of genetic events in the samples.*

Sample	Precursor genotype *N,normal; M,mutation*	Order of genetic events	Adenoma histology
1	$APC^{N}Kras^{N}TP53^{M}$ (3/42) $APC^{M}Kras^{N}TP53^{M}$ (2/42)	*TP53*→*APC*→*Kras*	No
3	$APC^{N}Kras^{M}TP53^{M}$ (2/48)	*TP53/Kras*→*APC*	No
11	$APC^{M}Kras^{N}TP53^{N}$ (7/41) $APC^{M}Kras^{M}TP53^{N}$ (4/41)	*APC*→*Kras*→*TP53*	Yes
19	$APC^{N}Kras^{M}TP53^{M}$ (2/45)	*TP53/Kras*→*APC*	No
24	$APC^{M}Kras^{N}TP53^{M}$ (2/49)	*APC/TP53*→*Kras*	No
33	$APC^{M}Kras^{N}TP53^{N}$ (21/48) $APC^{M}Kras^{M}TP53^{N}$ (9/48)	*APC*→*Kras*→*TP53*	No
41	$APC^{N}Kras^{N}TP53^{M}$ (7/40) $APC^{N}Kras^{M}TP53^{M}$ (1/40)	*TP53*→*Kras*→*APC*	No
51	$APC^{N}Kras^{N}TP53^{M}$ (1/42) $APC^{M}Kras^{N}TP53^{M}$ (2/42)	*APC*→*TP53*→*APC*→*Kras*	No
65	$APC^{M}Kras^{N}TP53^{N}$ (2/49) $APC^{M}Kras^{N}TP53^{M}$ (2/49)	*APC*→*TP53*→*Kras*	Yes
81	$APC^{M}Kras^{M}TP53^{N}$ (4/44)	*APC/Kras* → *TP53*	-
83	$APC^{M}Kras^{M}TP53^{N}$ (8/45)	*APC/Kras* → *TP53*	-

A major concern is the possibility that the absence of mutation is due to loss of the mutated allele. Loss of a mutant allele was reported in the mismatch repair region (Sanchez De Abajo et al., 2006), although it is unknown for *APC* and *TP53*. I performed LOH analysis of 5q and 17p with relevant areas using microsatellite markers, and detected both alleles in all cases, thus eliminating the possible loss of the mutated allele (Figure 12).

During a review of archival HE sections, I found that in two samples adenocarcinoma was accompanied by adenoma regions (Table 11).

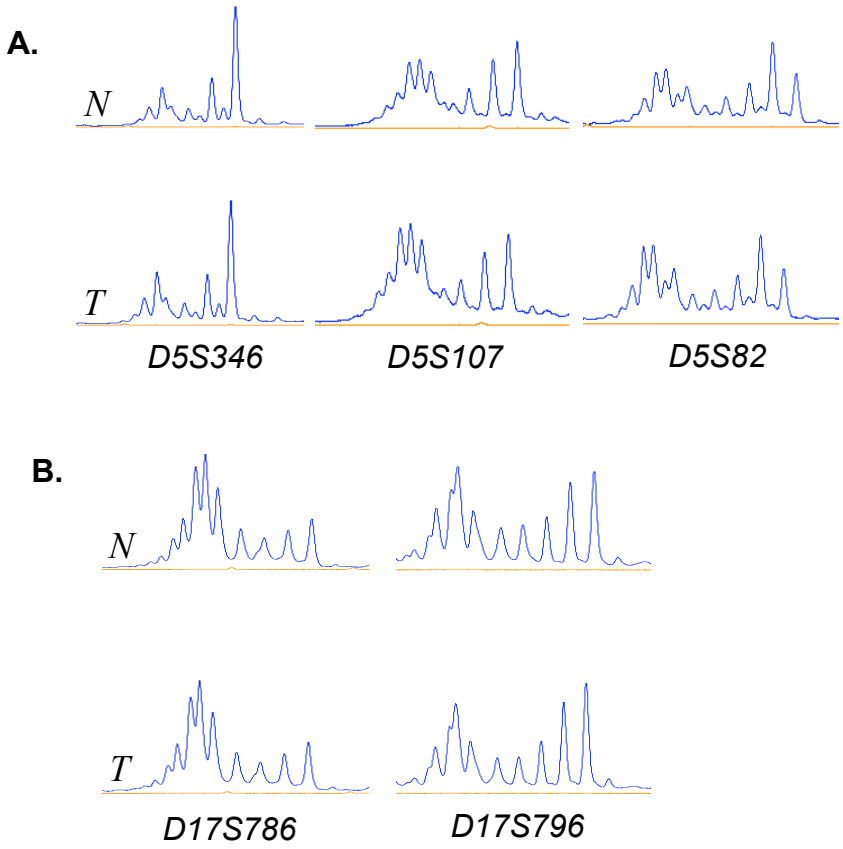


Figure 12. *Microsatellite analysis of chromosome 5q and 17p.* ***A.*** *5q LOH analysis of sample 85;* ***B.*** *17p LOH analysis of sample 51. Areas with mutated only APC or TP53 gene were examined for allelic loss on chromosome 17p or 5q, respectively.*

Genetic heterogeneity analysis of liver metastases

The previous section shows the results of intratumor genetic heterogeneity analysis. Liver metastases samples from 13 patients were also obtained. Bulk tissue analysis showed

that four patients (Nos. 1, 51, 81 and 83) carry genetic aberrations in the studied three genes in both tissues; their genetic heterogeneity was analyzed. When DNA from excised metastatic areas of samples 1M, 51M, 81M and 83M was examined, I found mutation patterns that were highly similar to those of samples 1, 51, 81 and 83, respectively - Figure 13. Mutated allele ratios of specimens taken from the liver metastasis overlapped with those of the major population from the primary site, which supports the clonal selection hypothesis (Talmadge, 2007).

Two areas microdissected from sample 51M were found to have only wild type *K-ras*, and another two had only the mutant allele of *K-ras*; loss of an allele of the *K-ras* gene is suspected.

Screening bulk tissue samples for sequence variations, I found that mutation patterns of three metastatic samples (80M, 82M and 85M) did not match those of the primary tumors (80, 82 and 85, respectively). There was only one mutation, in the *APC* gene, identified in sample 80, while samples 82 and 85 showed mutations in both *APC* and *TP53* – Table 6, Figure 14. On the other hand, samples 82M and 85M had mutations in *K-ras* and *TP53* – Figure 14. These two samples and their paired primary site samples were analyzed in detail by multipoint microsampling. The results are presented in Figure 15.

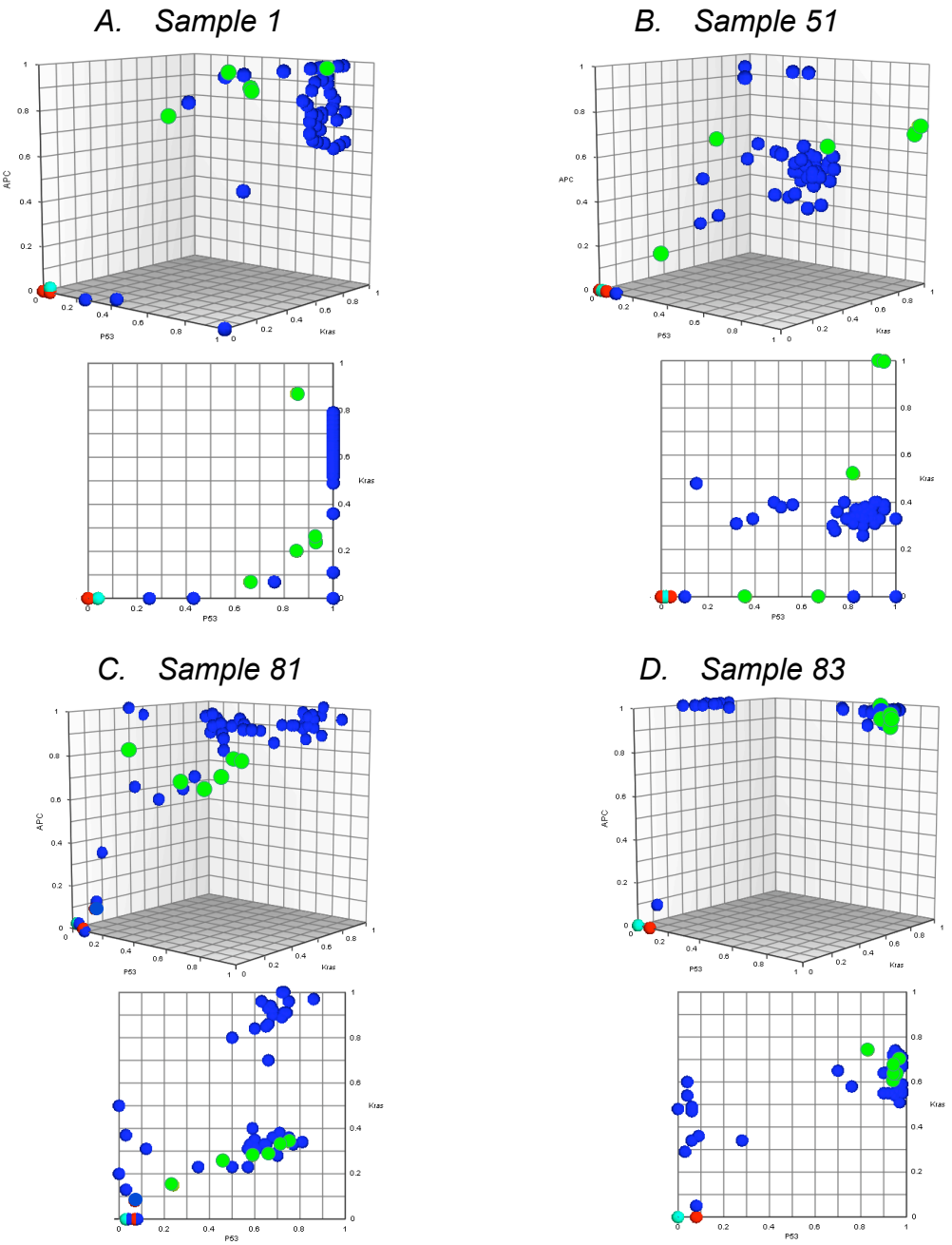


Figure 13. *Comparison of genetic heterogeneity in primary site and paired metastases.* ***A.*** *Patient 1;* ***B.*** *Patient 51;* ***C.*** *Patient 81;* ***D.*** *Patient 83. Each sphere represents a single area; the color indicates as follows: tumor tissue – dark blue; liver metastasis – light green; normal colon tissue – red; normal liver tissue – light blue.*

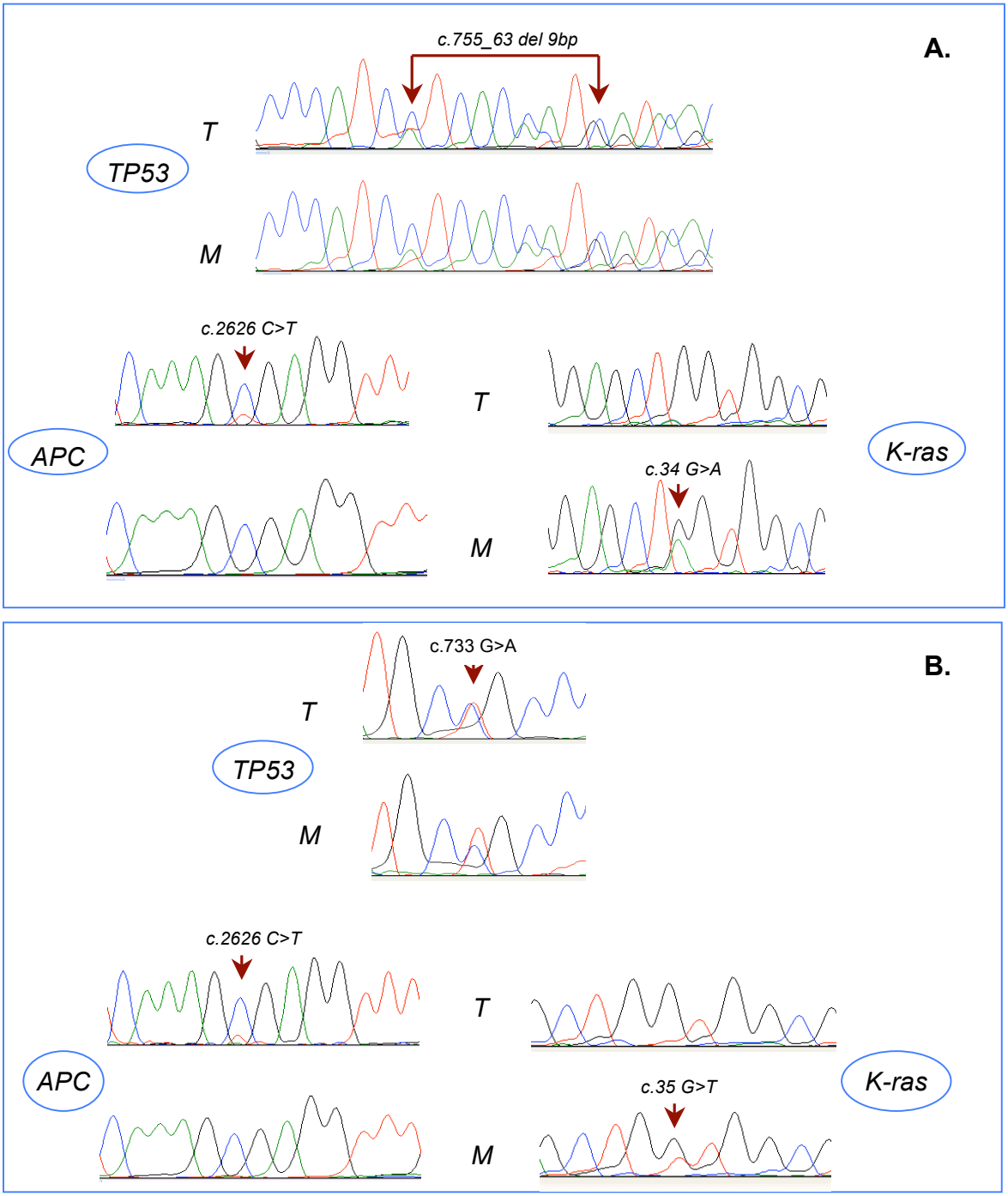


Figure 14. *Sequencing of bulk cancer tissues from tumor (T) and liver metastasis (M) of patient 82 (**A**) and 85 (**B**).*

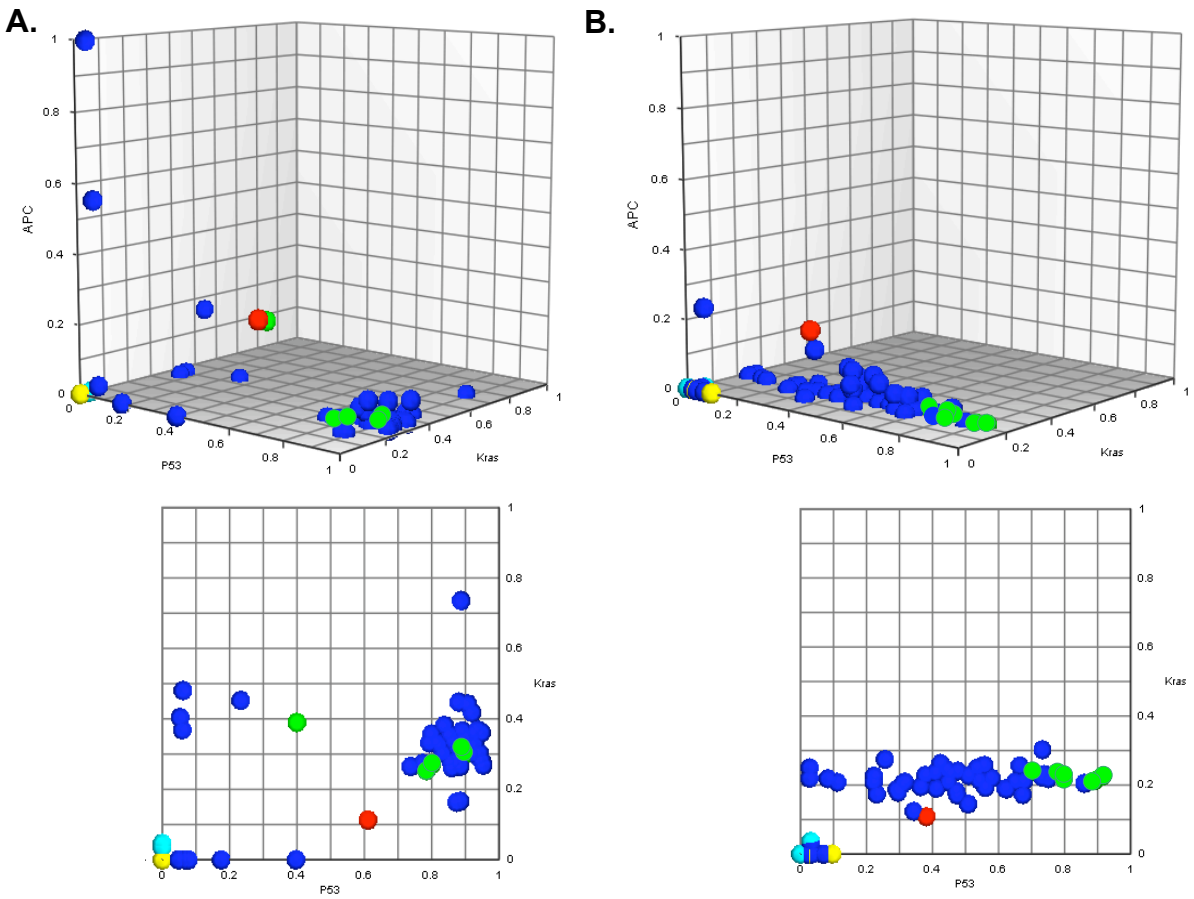


Figure 15. *Genetic heterogeneity in:* ***A.*** *Sample 82 and 82M;* ***B.*** *Sample 85 and 85M. Each sphere represents a single area; the color indicates as follows: tumor tissue – dark blue; liver metastasis – light green; normal colon tissue – yellow; normal liver tissue – light blue; bulk tumor tissue - red.*

Although sequencing data of bulk tissue showed that sample 82 carried mutations only in the *APC* and *TP53* genes, mutation in *K-ras* was also detected when microdissected samples were used (Figure 15A). Moreover, more than three subpopulations were found inside the cancer tissue. One is characterized by mutation only in *TP53* (3/49 areas), another carries the *APC* mutation alone (2/49 areas) and the third one has only *K-ras* mutated (3/49 areas). Besides, the major population carries two mutations – in *K-ras* and *TP53*, but not in

APC. These results imply that the mutation in the *APC* gene occurred in different cells from those having mutations in *K-ras* and *TP53*. When I compared samples 82 and 82M, I found that the mutation profile of the areas that were microdissected from liver metastasis overlapped with that of the major population of sample 82 (Figure 15A).

To confirm that there is a mutation in the *K-ras* gene in the DNA extracted from the bulk cancer tissue (even though it was not detected by direct sequencing), I performed a SNaPshot assay. The DNA was first diluted to the concentration of a microdissection sample, and the SNaPshot assay was performed according to the protocol used for mictodissected specimens. I found the mutation in *K-ras*, but its ratio was 0.11 (11%). Sequencing sensitivity is reported to be about 10% (Loeb et al., 2008), which explains why the mutation was missed. The genetic aberration in *APC* was found in only three microdissected areas and was not present in the major population of the tumor tissue; however, its ratio in the bulk tissue was 0.28 or 28%, which is high enough to be detected by sequencing.

Similar results were observed with samples 85 and 85M. The major population of sample 85 carries mutations in *K-ras* and *TP53* (Figure 15B). Besides this major population, there are two subpopulations, one with the *K-ras* mutation alone (3/49), and the other with the *APC* mutation alone (1/49). Chromosomal status is stable for *K-ras*, but I detected

distinct heterogeneity with *TP53* (Figure 15B). Sample 85M has the same mutations as the major population of sample 85. Analysis of bulk tumor tissue confirmed the presence of the *K-ras* mutation in 11% and that of the *APC* genetic alteration in 20% of the cells.

DISCUSSION

MUTATION SCREENING

Tumorigenesis is a result of alterations in oncogenes and tumor-suppressor genes. In the present study, by applying High-Resolution Melting and sequencing, mutations in *APC*, *CTNNB1*, *K-ras* and *TP53* were found in 57 (66.3%), 2 (2.3%), 30 (34.9%) and 46 (53.5%) CRC patients, respectively. These results support previously published data - Table 12. Moreover, mutations in the *CTNNB1* gene were found in two patients whose *APC* genes were wild type (Table 6). According to previous reports, *CTNNB1* gene mutations occur in 50% of CRC patients lacking *APC* mutations (Chung, 2000; Leslie et al., 2002).

Table 12. *Mutations found in colorectal cancer patients in previous reports and in the present study.*

	Mutations in genes:			
	APC	*K-ras*	*TP53*	*CTNNB1*
Sanger Institute (COSMIC)	38%	35%	39%	6%
Chung, 2000	>70%	50%	≥50%	4-15%
Smith et al., 2002	60%	30%	61.3%	-
Luchtenborg et al., 2005	37%	36%	-	1%
Thorstensen et al., 2005	72%	-	54%	-
Michor et al.,2005	95%	70%	95%	-
Segdistas et al., 2006	-	30-40%	-	<5%
Samowitz et al., 2007	59.1%	-	-	-
Present study	**66.3%**	**34.9%**	**53.5%**	**2.3%**

C-to-T transitions of the *APC* gene were detected in 25/42 (59.5%) CRC patients with nonsense mutations in *APC*, which is also in accordance with previously reported 55% (Samowitz et al., 2007). Most of them (16/42 or 38.1%) were found in one particular codon (CGA, coding for arginine). High frequency of C-to-T transition within arginine codon has been reported not only for *APC*, but also for *TP53* (Greenblatt et al., 1994); I found C>T mutation in 4/7 (57.1%) CRC cases with nonsense mutation in *TP53*. This transition is thought to be the result of methylation of cytosine by endogenous methyltransferases with subsequent deamination to thymine (Samowitz et al., 2007).

Data about the position of the most common mutations in *APC* gene are controversial (Samowitz et al, 2007). According to Miyaki et al. (1994), 77 to 94% of the mutations in *APC* are located in the MCR, which overlaps with domains involved in β-catenin and axin binding (Figure 2A). I found 67 mutations in the *APC* gene; 35 of them (52.2%) were in the MCR – Figure 16A, while 32 (47.8%) were outside. The prevalence of mutations in the MCR is clear, but searching for genetic alterations only in the MCR is not sufficient for assessing the *APC* mutation status of CRC patients.

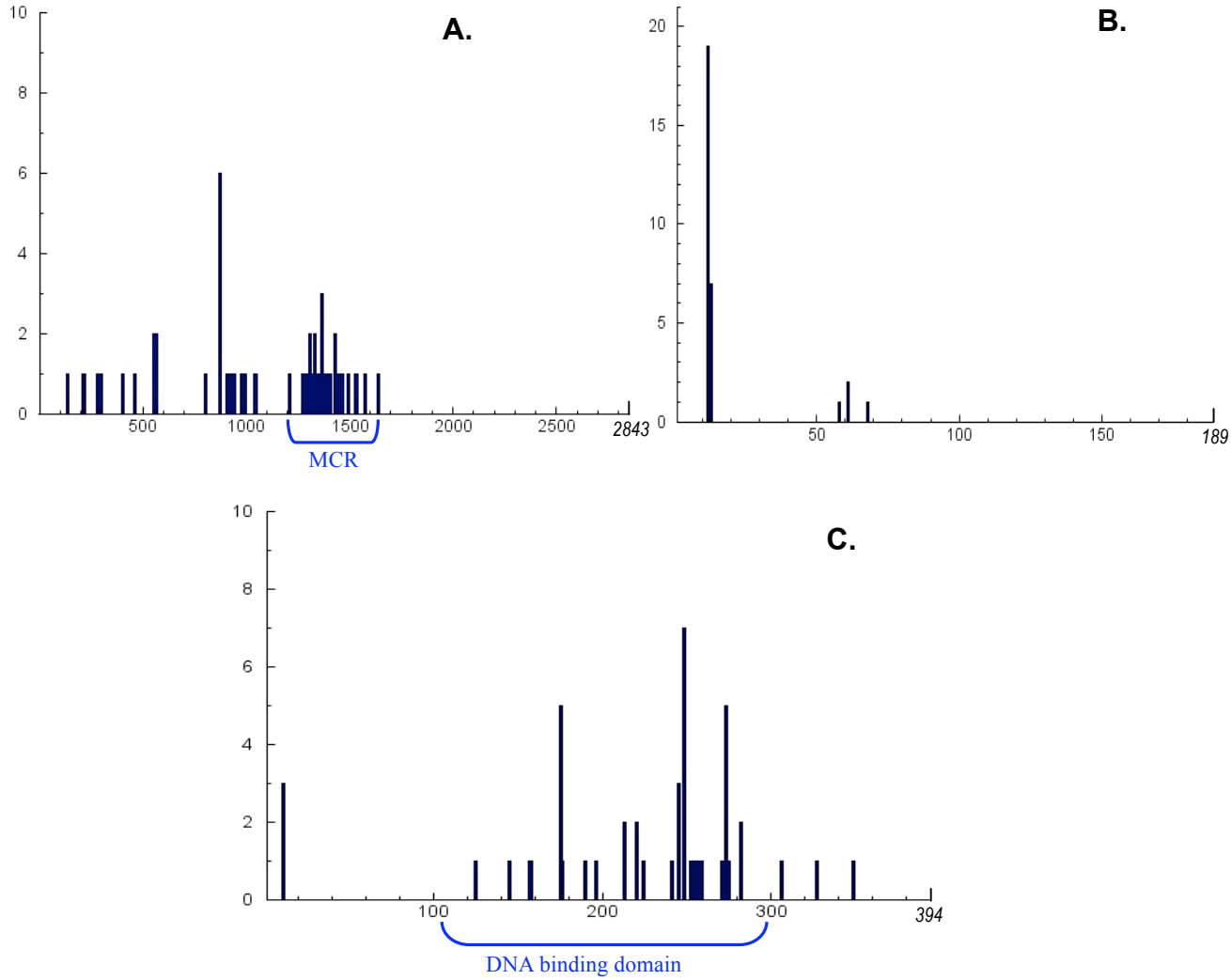


Figure 16. *Distribution of mutations along the length of the gene:* ***A.*** *APC;* ***B.*** *K-ras;* ***C.*** *TP53. Horizontal axis indicates the codon number; vertical axis indicates the number of patients with the same mutation.*

Mutations in the *K-ras* gene are reported predominantly in codons 12, 13 and 61 (Friday et al., 2005). In the present study *K-ras* aberrations in these codons were detected in 28 patients (93.3% of all mutations in the *K-ras* gene) – Figure 16B.

Most of the mutations found in the *TP53* gene were in the core domain (42 out of 46; 91.3%) – Figure 16C. According to Xu et al. (2008), mutations at four hot-spot Arg residues

175, 248, 249 and 273 account for over 25% of all missense mutations identified in human cancers. I detected genetic aberrations at these four codons in 17 patients, or 37% of all *TP53* mutations. Missense mutations, which are unlikely to occur in most other tumor suppressor genes, were found in 78.3% of cases with *TP53* alterations – very close to the previously reported frequency of 80% (Soussi, 2007).

Chromosome 18q LOH analysis, which was performed on bulk tissue samples, identified allelic loss in 30% of the cases (Table 6). This proportion is lower than the 58-70% range in published reports (Chung, 2000; Erill et al., 2005; Losi et al., 2005). One factor that may explain this discrepancy is that it is difficult to unequivocally identify LOH in DNA extracted from bulk cancer tissue because there is a variable degree of normal cell contamination in each tumor specimen.

The model of colorectal carcinogenesis proposed by Vogelstein and coworkers suggests that often in the carcinoma of large intestine all three genes (*APC*, *K-ras* and *TP53)* are mutated. In the present study, simultaneous occurrence of mutations in *APC*, *K-ras* and *TP53* genes in the same sample was found in 13 out of 86 (15.1%) tumor tissue samples, comparable to previous findings of 11.1% (Samowitz et al., 2007) and 6.6% (Smith et al., 2002). Present results are in agreement with previous articles indicating that accumulation of multiple mutations in these three genes does not apply to all sporadic CRC patients.

In 37 patients (43%), mutations in two genes were detected; the most common combination was *APC* and *TP53,* found in 23 cases (26.7%), very similar to the value of 27.1% reported by Smith et al. (2002). Twenty patients (23.3%) had only one gene mutated: *APC* alone carried genetic aberration in 12 cases (14%), *K-ras* in three (3.5%) and *TP53* in five cases (5.8%). Moreover, in 16 patients (18.6%) no mutation in any of the examined genes was detected. One possible explanation is that microsatellite instability is the cause of these carcinoma cases (Soreide et al., 2006).

GENETIC HETEROGENEITY

The study of human carcinogenesis has been a difficult task due to the unavailability of tumor samples of different stages, especially early stages, from the same patient. Thus, most models have been based on indirect evidence obtained from studies with patient populations (Fearon et al., 1990; Diep et al., 2006). In the present study, I demonstrated that primary CRC tissues are highly heterogeneous and contain cells with a subset of the mutations found in the major population, which are likely to be precursors of the major population. They were named precursor cancer cells. In addition, from their mutation patterns, the order of genetic events was deduced (Table 11). The presence of precursor cancer cells is likely to be a common feature of CRC, because 11 out of 13 cases (84.6%)

had such minor populations. This proportion of samples showing heterogeneity is higher than published estimates (67%, according to Baisse et al., 2001), perhaps because of the smaller size of tissue samples used in the present experiments.

Intratumor genetic heterogeneity previously reported in colorectal carcinoma (Giaretti et al., 2000; Baisse et al., 2001; Losi et al., 2005) is probably due to these precursor cancer cells. In colorectal adenoma, genetic heterogeneity of carcinogenic mutations is well established in the context of tumor evolution (Shibata et al., 1993; Shih et al., 2001). The current findings imply that the heterogeneity found in early adenoma still remains in the later carcinoma stage, although the proportion of heterogeneous clones is reduced. My results also agree with the data presented by Losi et al. (2005), namely that intratumor genetic heterogeneity is more frequent in early than in advanced stages. It should be noted, however, that mutation patterns are not necessarily consistent in adjacent adenoma and carcinoma regions: *K-ras* mutation status in the adenoma region and in the adjacent carcinoma was reported to be different in 24% of the cases examined (Zauber et al., 1999). Lips et al. (2008) also found more aberrations in adenoma than in carcinoma counterparts of early rectal cancer. This suggests that even adjacent carcinoma and adenoma can arise from different ancestors and that their comparison may not provide precise information about the process of carcinogenesis. Moreover, contrary to the classical adenoma-carcinoma sequence, adenomas were found to be nearly as old as carcinomas

(Tsao et al., 2000). Taking into account “de novo” carcinogenesis (Shimoda et al., 1989), it appears that collecting information directly from carcinoma tissues is the best way to study the process of tumor formation and progression.

Numerous studies indicate that tumors consist of cancer cell subpopulations of different genotypes (Baisse et al., 2001; Barnetson et al., 2000; Giaretti et al., 2000; Gonzalez-Garcia et al., 2002; Konishi et al., 1995; Losi et al., 2005; Lyng et al., 2004; Samowitz et al., 1999; Takeshima et al., 2001). All of these reports depended on co-amplification of mutated and normal alleles by PCR, but none of them presented any evidence that amplified products maintained stable ratios of alleles in genomic DNA. Confirmation of unbiased amplification is especially important, because most of the studies did not use frozen tissues but formalin-fixed, paraffin-embedded sections (Baisse et al., 2001; Barnetson et al., 2000; Gonzalez-Garcia et al., 2002; Losi et al., 2005; Takeshima et al., 2001). Formaldehyde facilitates a number of physicochemical changes affecting DNA, including breaks, base substitutions, base losses and formation of unstable forms of DNA (Srinivasan et al., 2002). These changes may greatly reduce the amount of unaltered DNA available for PCR templates, and introduce artifacts during amplification (Bereczki et al., 2007). Thus, such experiments may lead to erroneous results in LOH analysis, and false negatives in mutation detection. It is not easy to evaluate the validity of a study, but one marked difference between the present study and the previous ones is that reproducibility of

the amplification was confirmed in one experiment (Figure 9A).

Because PCR from a small amount of DNA may lead to biased amplification, the whole experiment was carefully designed. In a previous study from the same laboratory (Taniguchi, 2008), the minimum size of a tissue specimen enabling unbiased quantitative amplification was determined. In the present study, quantitative recovery of amplified products under the chosen conditions was demonstrated (Figure 9A). This excludes the possibility that the observed loss of mutant/normal alleles is due to stochasticity in the PCR reactions. In addition, the chance of erroneous PCR identification of allelic loss due to the stochasticity was previously calculated: it was 3.5×10^{-5} and 6.04×10^{-7} in two different loci (Taniguchi, 2008). Moreover, except for one sequence variation pattern of sample 41 and another of sample 51 (Figure 11, Table 11), all mutation patterns of precursors were identified in more than two areas, demonstrating reproducibility of the patterns. By avoiding repeated sampling from the same crypt, which is believed to be a clonal population (Humphries et al., 2008), I was able to detect various subpopulations inside the tumor tissue. Also, the small size of the excised areas enabled me to identify different genotypes among cancer cell subpopulations.

Although there are a number of studies on intratumor heterogeneity in CRC, they examine only *K-ras* and *TP53* mutations (Baisse et al., 2001; Giaretti et al., 2000; Losi et al., 2005). Two of them also include LOH analysis of chromosomes 5q and 18q (Baisse et

al., 2001; Losi et al., 2005). The present study is the first to evaluate heterogeneity of genetic aberrations in *APC*, *K-ras* and *TP53* genes. Considering the mutation status of the three genes simultaneously enabled me to highlight the presence of multiple subpopulations in a single tumor tissue (Figure 11).

Two samples (49 and 74) were homogeneous: only one type of cell was found in each of them (Figure 11H and 11K, respectively). According to the widely accepted theory, cancer tissue includes a dominant clone, which overgrows the other cells (Alberts, 2002). Although advanced cancer growth could be an explanation, it is not clear whether there was any other type of cell in either of these samples. There may have been a heterogeneous pattern in another part of the section or the tissue.

One interesting finding in the present study is heterogeneity in allelic imbalance. It is important to note that the heterogeneity was usually restricted to particular chromosomes. In samples 11 and 85, heterogeneity was found only with *TP53* (Figure 11C and Figure 15B, respectively). In this context, the results of Comparative Genomic Hybridization (CGH) or array-CGH, which are methods for detection of copy number changes in DNA, should be interpreted with caution. CGH cannot discriminate cases with and without heterogeneity, presenting an averaged view for chromosome aberration with heterogeneity.

There appears to be no rule in the deduced order of mutation events. It should be noted

that “de novo” colorectal carcinoma is more frequent in Japanese than in other racial populations (Soetikno et al., 2006). Investigators believe that this neoplasm develops through a pathway different from the polypoid one (Soetikno et al., 2006; Speake et al., 2007; Uronis et al., 2007); this could explain current findings (Table 11). There were two cases (samples 11 and 65) in which carcinoma was accompanied by adenoma regions, and the *APC* mutation was the first event in these cases. Because the original Vogelstein model is for cancers developed from adenoma (Fearon et al., 1990), my results do not contradict the previous studies based on patient populations. Moreover, the authors note that the order of these genetic changes is not invariant and the accumulation rather than the order is most important (Fearon et al., 1990).

This approach for examining the temporal sequence of genetic events may be applied to other cancers, if precursor cancer cells exist. It should be noted that many genetic aberrations such as LOH occur frequently. Heterogeneity of LOH is reported to be more frequent than that of point mutations (Baisse et al., 2001; Losi et al., 2005). However, it is important to determine whether the aberration is from a single event or more than one event. Mutations in *APC* and *TP53* do not need such attention, because the chance of having mutations at the same base in both alleles is very low. For chromosomal aberrations, the use of multiple markers would probably provide more precise information.

Most models of carcinogenesis assume that tumors are monoclonal in origin. This

conclusion is based largely on studies using X chromosome-linked markers in females (Vogelstein et al., 1985). However, another study demonstrated relatively large sizes of X-inactivation patches in normal tissues, confounding the interpretation of early studies (Novelli et al., 2003). Intratumor heterogeneity could either be due to a polyclonal origin of the tumor cells or reflect their chromosomal instability (Lyng et al., 2004). However, a polyclonal origin is not a likely cause of the genetic heterogeneity observed in most of the samples here, because at least one mutation was common for all the areas of a tumor. The present results strongly suggest a monoclonal origin: the tumor tissues contained cancer cells with several different genetic patterns, but with one or two common mutations, which are thought to accumulate along a single lineage (Tsao et al., 2000). However, there is still possibility that the number of excised areas was not enough to detect other minor clones in the tumor tissue.

The current findings suggest that precursor cancer cells exist in the tumor mass surgically dissected, and molecular analysis can be performed by purifying and then culturing them. One intriguing question is whether they are the same as cells that differentiated into cancer cells with the three mutations in the past. As they still exist as a minor population, their growth rate is smaller than the major population, suggesting no additional mutation for growth advantage. The smaller number of replication cycles indicates fewer new mutations. Thus, I suspect that their biological properties would not

change. Another question is whether the precursor cancer cells are malignant or benign. Most adenoma cells accompany *APC* mutations, and precursor cancer cells with *APC* mutations might be reminiscent of adenoma cells.

Detailed comparison of mutations in the primary tumor and its metastasis in published articles reveals that a considerable number of cases have different mutation patterns (Albanese et al., 2004; Zhang et al., 1997). Unmatched mutation patterns indicate that metastatic lesions would be derived from a minor population in the primary tumor, suggesting the possible involvement of precursor cancer cells. However, by analyzing bulk tissues, I found that the mutation pattern of the primary tumor is sustained in its metastasis in 10/13 (76.9%) cases (Table 8). Analysis of heterogeneity in metastatic tissues of patients with three genes mutated confirmed this finding (Figure 13). Patients 82 and 85 showed tissue variations – *APC* mutation found in the primary tumor was not identified in the metastatic tissue and *K-ras* mutation in metastasis was not detected in the primary site (Figure 14). However, when data from microdissected areas were compared, the mutation status of samples 82M and 85M overlapped with that of samples 82 (Figure 15A) and 85 (Figure 15B), respectively, which supports the clonal selection theory of metastasis formation (Talmadge, 2007). The findings of the present study might explain unmatched mutation patterns detected in previous studies. As those studies analyzed DNA, extracted from bulk tumor tissue, they were not able to identify minor alleles.

I believe that finding putative precursor cancer cells is important for understanding carcinogenesis as well as for future drug discovery. If they have distinct molecular characteristics, it would be beneficial to develop anti-cancer drugs targeting them. Moreover, the present approach for analysis of genetic heterogeneity can be applied to the study of other types of carcinogenesis as well as to the verification of the recently proposed explanation for metastases by fusion of tumor cells with bone marrow-derived cells (Pawelek et al., 2008) or to cancer stem cell study.

Summary:

* Simultaneous occurrence of mutations in *APC, K-ras* and *TP53* genes is a rare event – 15.1% of cases.
* Precursor cancer cells exist in human colorectal cancer tissues. Intratumor heterogeneity of somatic mutations is likely to be the result of precursors.
* Chronology of genetic events can be deduced from precursor subpopulations. There is no common order of mutation occurrence in colorectal carcinogenesis.
* Tumor tissues show heterogeneity not only of somatic mutations, but also in allelic imbalance.
* Most colorectal adenocarcinomas with *APC*, *K-ras* and *TP53* genes mutated seem to be of monoclonal origin.
* There is a high similarity of mutation patterns in the major population of primary tumor and its paired metastasis.
* The proposed approach is applicable to colorectal carcinogenesis, but it is also likely to work with other cancer types.

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Teodora Goranova, Masayuki Ohue, and Kikuya Kato ***"Mutation Detection in Cancer Samples by High-Resolution Melting"***

2. *AACR Annual Meeting 2008*
Teodora Goranova, Masayuki Ohue, and Kikuya Kato ***"Order of genetic events during colorectal carcinogenesis revealed by analysis of cell subpopulations (Precursor cancer cells)"***