Carcinogenesis vol.26 no.5 pp.916–922, 2005
doi:10.1093/carcin/bgi044
Advance Access publication February 24, 2005

Evolution of intratumoral genetic heterogeneity during colorectal cancer progression

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Evolution of intratumoral genetic heterogeneity during colorectal tumor progression has not been investigated so far. Multiple sample areas in colorectal adenocarcinoma at early and advanced stages and in metastases were studied for the well-known genetic alterations: *K-ras* and *p53* point mutations and loss of heterozygosity (LOH) on chromosomes 5q and 18q. In primary colorectal cancers (CRCs), intratumoral genetic heterogeneity was more often observed in early than in advanced stages, at 90 and 67%, respectively. All but one of the advanced CRCs were composed of one predominant clone and other minor clones, whereas no predominant clone has been identified in half of the early cancers. At the early stage, the last events that were produced, the *p53* mutation and LOH of 18q, were also the most heterogeneous. At the advanced stage, the LOH of 5q and 18q were the most frequent heterogeneous events (67 and 58%, respectively). The intratumoral heterogeneity for mutations was significantly reduced, from the early to the advanced stages (from 60 to 20% for *K-ras* and from 70 to 20% for *p53*). On the other hand, a quasi absence of intratumoral genetic heterogeneity was observed for *K-ras* and *p53* in distant metastasis. In conclusion, colorectal adenocarcinomas are characterized by marked intratumoral genetic heterogeneity. A reduction of the intratumoral genetic heterogeneity for point mutations and a relative stability of the heterogeneity for allelic losses indicate that, during the progression of CRC, clonal selection and chromosome instability continue, while an increase cannot be proven.

Introduction

After lung and breast cancer, colorectal carcinoma (CRC) is the most common cause of mortality in Western countries. Colorectal carcinogenesis is intensively studied at the molecular genetic level. Consequently, CRC is one of the best-characterized models of multistep process, associated with the progressive acquisition of a variety of genetic changes in neoplastic cells. Among these genetic alterations, the most common genes involved are oncogenes, such as *K-ras* gene (1) and tumor suppressor genes, such as *APC*, *DCC* and *p53* (2–4). Alterations in *APC* and *K-ras* genes are considered to be early events, whereas loss of heterozygosity (LOH) on chromosome 18q (related to the *DCC* or *SMAD4* genes) and *p53* gene mutations are thought to be late events in colorectal carcinogenesis (5,6).

In the current model of stepwise progression of colorectal carcinogenesis (7), each new step is initiated by the acquisition of an additional alteration contributing to the progression of the lesion. This leads to the clonal expansion of the abnormal cell through the acquisition of a proliferative advantage. Consequently, this model implies a certain genetic heterogeneity between the individual cells in a tumor. As in different human cancer tissues (8–10), intratumoral genetic heterogeneity has been demonstrated in colorectal tumor tissue for *K-ras* and *p53* gene mutations (11). Recently, we have found a considerable intratumoral genetic heterogeneity in advanced CRC for the combination of *K-ras* and *p53* gene mutations, as well as for 5q and 18q losses (12).

In the past few years, the prognostic significance of allelic losses and gene mutations have been investigated in the different stages of CRC (13–16). Although some of these main genetic alterations occur frequently in CRC, their impact on prognosis remains contradictory (17–19). Only one sample/tumor was used for molecular genetic analysis. This could explain, considering the intratumoral genetic heterogeneity found in colorectal tumors (12), the lack of concordance described in the literature in these studies between prognostic significance and genetic alterations. By increasing the number of tumor samples, the intratumoral heterogeneity should be evaluated for *K-ras*, *p53*, *APC* and *DCC* genes for each stage of the colorectal carcinogenesis.

In this study, we examined the existence of intratumoral genetic heterogeneity for *p53* and *K-ras* point mutations, and for LOH on chromosomes 5q and 18q in sporadic CRC, at early and advanced stages and in metastases. An evolution pattern of intratumoral genetic heterogeneity through different stages of the tumor progression was established to better understand the genetics of the colorectal carcinogenesis.

Materials and methods

Patients and tissues

Forty-five patients, who were treated surgically for primary sporadic CRC at the Centre Hospitalier Universitaire Vaudois in Lausanne, were separated into three groups. Tumors were staged according to the criteria of UICC classification (20). The first group was constituted of 10 cases diagnosed as being early CRCs invading the submucosa or the muscularis propria without any regional lymph node metastasis (T1N0M0 or T2N0M0). The second group included 15 cases considered as advanced CRCs invading beyond the muscularis propria and all the patients presented regional lymph node metastasis (T3N1M0 or T4N1M0). The third group consisted of distant and/or peritoneal metastases from 20 patients; the metastases, multiples in 5 patients, were found at the time or after the surgery. The analyzed metastases were only from the liver or the

Abbreviations: CIN, chromosomal instability; CRC, colorectal carcinoma; LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism.

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peritoneum in 9 and 6 cases, respectively. In the other 5 cases, the analyzed metastases were from multiple localizations: peritoneal, liver, pelvis, ovary, ileal, stomach and/or vagina.

Tissue preparation

Tumors were fixed in formalin, sampled *in toto*, embedded in paraffin, and serially sectioned at 7 μm. The sections were stained with hematoxylin and eosin for histopathological diagnosis. Depending on the size of each tumor, 10–20 areas, including one from normal mucosa, were selected and care was taken to randomly sample throughout the tumor.

Microdissection and DNA extraction

Deparaffinized and rinsed sections were stained in 0.1% toluidine blue for 30 s, washed, air dried and stored until microdissection. The selected areas were microdissected and extracted as described previously (21). In brief, stromal tissue was scraped from the slide using a scalpel blade under microscopic control. After washing the sections with water, a pathologist (H.B.) confirmed the complete removal of normal tissue before collection of the tumor cells. This control guaranteed that the collected tissue contained at least 95% tumor cells. Extracted DNA was resuspended in 25 μl of 10 mM Tris–HCl, pH 8.0 and kept at −20°C until used.

Single-strand conformation polymorphism (SSCP) analyses of the K-ras and p53 genes

Exons 5 to 8 of the *p53* gene and exon 1 of the *K-ras* gene were separately amplified by PCR (12). Aliquots of 5 μl of PCR products were mixed with 5 μl of denaturing buffer (0.1 M NaOH and 2 mM EDTA) and heated at 50°C for 10 min. After addition of 1 μl of formamide dye, the samples were immediately loaded onto a 40% MDE gel (FMC BioProducts, Rockland, ME) and electrophoresed in 0.5 × TBE buffer at 20°C and 300 V (20 V/cm) for 5–7 h (*K-ras* exon 1 and *p53* exon 5B) or 220 V (14.5 V/cm) overnight (*p53* exon 5A, 6, 7 and 8). The gels were stained with a SYBR® Gold gel stain (Molecular Probes, Eugene, OR) diluted 1:10 000 in 1 × TBE buffer and visualized under UV light using a CCD camera. After SSCP analysis, the mutation-specific bands observed on the 40% MDE gel were cut out and reamplified using the same primers. The reamplified PCR products were purified using QIA-quick PCR purification Kit (Qiagen, Germany) and sequenced on an ABI PRISM 310 automatic sequencer.

Restriction fragment length polymorphism (RFLP) analysis of the APC gene

A polymorphic stretch in the 3′-UTR of the *APC* gene was chosen as the target for the detection of LOH of the *APC* gene. The 215 bp amplified fragments contained two SspI restriction sites, the first site based upon a polymorphism and the second site, constitutional, which served as an internal control for complete enzymatic digestion (12). Each PCR product was enzymatically hydrolyzed at 37°C for 3 h with SspI (Promega, Madison, MA). An aliquot (5 μl) of the resulting digested PCR product was loaded onto a 6% non-denaturing polyacrylamide gel and electrophoresed at 20°C and 500 V (33.5 V/cm) for 45 min. The gel staining and result analysis were performed the same way as in the SSCP analyses section. From the intragenic RFLP analysis of the completely digested PCR products, heterozygous cases displayed four fragments: 186, 135, 51 and 29 bp. Homozygous cases displayed either two (186 and 29 bp), or three fragments (135, 51 and 29 bp).

LOH analysis of chromosome 18q

Three microsatellite loci on chromosome 18q (D18S68, D18S69 and D18S58) were separately amplified by PCR (12). A 5-μl aliquot of each PCR product was mixed with 2 μl of formamide dye, denatured for 10 min at 90°C, immediately loaded onto a 6% denaturing polyacrylamide gel that contained 8 M urea and electrophoresed at 40°C and 500 V (33.5 V/cm) for 90 min. The gel staining and results analysis were performed as detailed in the SSCP section.

Results

Intratumoral genetic heterogeneity in early CRCs

Ten early CRCs (T1 or T2) were investigated. Depending on the size of the tumor, 9–14 areas were selected. In all the tumor areas sampled, the target genes were evaluated by molecular analyses. *K-ras* mutation was detected in 6 cases (60%) and all of them showed an intratumoral genetic heterogeneity (Tables I and II). A *p53* mutation was observed in 9 tumors (90%) and 7 of them showed an intratumoral genetic heterogeneity (Table III). Different *K-ras* and *p53* mutations were found within the same tumor, in 1 and 5 cases, respectively.

Table I. Frequency of genetic alteration[a] in colorectal cancer and colorectal metastasis

Genes	Early colorectal cancer	Advanced colorectal cancer[c]	Colorectal metastasis
K-ras	6/10 (60%)	8/15 (53%)	10/20 (50%)
p53	9/10 (90%)	5/15 (34%)	7/20 (35%)
5q locus[b]	7/10 (70%)	8/12 (67%)	NA
18q locus[b]	8/10 (80%)	7/12 (58%)	NA
At least one	10/10 (100%)	11/15 (73%)	14/20 (70%)

[a]Genetic alteration frequency is the number of cases presenting a given gene alteration within the number of cases analyzed.
[b]For 5q and 18q locus analysis, not all the cases were taken into account since some of them were not informative.
[c]According to our previously published data (12).

Table II. Frequency of intratumoral genetic heterogeneity[a] in primary colorectal cancer and colorectal metastasis

Genes	Early colorectal cancer	Advanced colorectal cancer[c]	Colorectal metastasis
K-ras	6/10 (60%)	3/15 (20%)	0/20 (0%)
p53	7/10 (70%)	3/15 (20%)	1/20 (5%)
5q locus[b]	5/10 (50%)	8/12 (67%)	NA
18q locus[b]	7/10 (70%)	7/12 (58%)	NA
Mutations	8/10 (80%)	5/15 (33%)	1/20 (5%)
Allelic losses	9/10 (90%)	9/12 (75%)	NA
At least one	9/10 (90%)	10/15 (67%)	1/20 (5%)

[a]Intratumoral genetic heterogeneity frequency is the number of cases exhibiting at least two different genotypes for a given gene alteration within the number of areas analyzed in the primary tumor.
[b]For 5q and 18q locus analysis, not all the cases were taken into account since some of them were not informative.
[c]According to our previously published data (12).

Allelic loss at the *APC* gene locus was detected in 7 cases (70%) and 5 of them were in heterogeneous tumors. Allelic loss at the *DCC* locus was detected in 8 cases (80%) and 7 of them were found in heterogeneous tumors (Tables I and III).

When considering all the genetic alterations, the intratumoral genetic heterogeneity was observed in 9 cases (90%) (Table II). Only one case exhibited the same genotype in all the areas examined (Table III, case 5). The heterogeneous tumors presented three to eight different genotypes, which we consider as subclones of variable size (Table III). A dominant clone, which was defined as the group of samples exhibiting the most frequently occurring genotype within a tumor, was observed in 5 cases (1, 3, 4, 5 and 8). The intratumoral genetic heterogeneity was observed at a similar frequency for allelic losses (50 and 70% for 5q and 18q, respectively) and for point mutations (60 and 70% for *K-ras* and *p53*, respectively) (Table II).

Detailed analysis is shown for the tumor 7 (Figures 1 and 2). The totality of the CRC was fixed and paraffin-embedded in one block. Ten areas were selected on the stained section (Figure 1B). After microdissection and molecular analysis, allelic loss at 5q was observed for all the samples. In contrast, mutations for *K-ras* and *p53* genes, as well as allelic loss at 18q, were heterogeneous (Figure 2). The samples were regrouped according to their respective genotypes, which enabled the visualization of four putative genotypic subclones (Figure 1C).

Table III. Genetic alterations in different areas of early colorectal cancer

Case	Total areas per tumor	No. of areas with the same subclone	*K-ras* mutation	*p53* mutation	LOH pattern[a]
1	**15**	**12**	**wt**	**141 TAC**	**18q**
		1	wt	141 TAC	—
		2	12 AGT	141 TAC	—
2	9	4	wt	141 TAC	5q, 18q
		4	wt	141 TAC	5q
		1	12 GCT	141 TAC+ 289 CAC	5q, 18q
3	**14**	**10**	**wt**	**wt**	**5q**
		1	wt	303 ACC	5q
		1	wt	301 GCA	5q
		1	12 GTT/GAT	wt	5q
		1	12 AGT	303 ACC	5q
4	**14**	**8**	**12 GTT**	**wt**	**18q**
		4	12 GTT	wt	5q, 18q
		1	12 GTT	141 TAC	5q, 18q
		1	wt	wt	5q, 18q
5	**14**	**14**	**wt**	**289 GTC**	—
6	14	5	wt	wt	5q
		2	wt	wt	18q
		2	wt	243 ACG	5q
		2	wt	217 ATG	5q, 18q
		1	wt	148 GAC + 243 ACG	5q
		1	13 GAC	wt	18q
		1	13 GAC	248 CGT	5q, 18q
7	10	4	13 GAC	wt	18q
		3	13 GAC	wt	—
		2	wt	wt	18q
		1	13 GAC	153 CGC + 222 CTG	18q
8	**14**	**8**	**wt**	**wt**	**5q**
		4	wt	245 AGC	5q, 18q
		2	wt	245 AGC	5q
9	14	4	wt	wt	18q
		3	wt	wt	5q, 18q
		2	wt	wt	5q
		1	wt	244 GAC	—
		1	wt	156 CTC	5q
		1	wt	185 AGA	5q
		1	wt	162 ATG	5q, 18q
		1	wt	176 TGT	5q, 18q
10	14	5	wt	wt	5q
		4	wt	wt	5q, 18q
		3	wt	wt	—
		2	wt	wt	18q

The tumors that contained a dominant clone, as well as this dominant clone, are indicated in bold.
[a]Only chromosomal loci with LOH are listed.

Intratumoral genetic heterogeneity in advanced CRCs

Fifteen advanced CRCs (T3N1M0 or T4N1M0) were investigated (12). Depending on the size of the tumor, 12–17 areas were selected. *K-ras* mutation was detected in 8 tumors (53%) and 3 of these tumors were found to be heterogeneous (Tables I and II). A *p53* mutation was observed in 5 (34%) cases and 3 of them were found in heterogeneous tumors. Different mutations within the same tumor were observed for *K-ras* (Table IV, case 2) but not for *p53*. Allelic losses at the *APC* and *DCC* loci were detected in 8 (67%) and 7 cases (58%), respectively, among the 12 informative cases, and all of them were in heterogeneous tumors (Tables I and II).

Considering all the four genetic alterations investigated, intratumoral genetic heterogeneity was observed in 10 cases (67%) (Tables II and IV). In 5 tumors, only one genotype was present in all of the analyzed areas, whereas two to five different genotypes were identified in the heterogeneous tumors (Table IV). One dominant clone was observed in all but one of the heterogeneous tumors. The intratumoral genetic heterogeneity was more frequently observed for allelic losses (67 and 58% for 5q and 18q, respectively) than for point mutations (20% for *K-ras* and *p53*) (Table II). Detailed analysis is shown for the tumor 4 (Figure 3).

Intratumoral genetic heterogeneity in colorectal metastases

Distant and/or peritoneal colorectal metastases from 20 patients were investigated for *K-ras* and *p53* mutations. Depending on the size and the number of localizations of the metastases, 6–20 areas were selected from each case. Owing to the low proportion and dispersion of tumor cells in the metastases, a microdissection of these tissues was unfeasible. Thus, the allelic loss analysis was not performed on metastases. *K-ras* and *p53* mutations were detected in 10 (50%) and 7 cases (35%), respectively (Table I). Genetic heterogeneity in all the metastases available per patient was observed in only one case. For this unique heterogeneous case, the peritoneal metastasis detected concomitantly with the primary tumor was wild-type *p53*, whereas the peritoneal metastasis detected 16 months later harbored a *p53* mutation. Nevertheless, in each individual

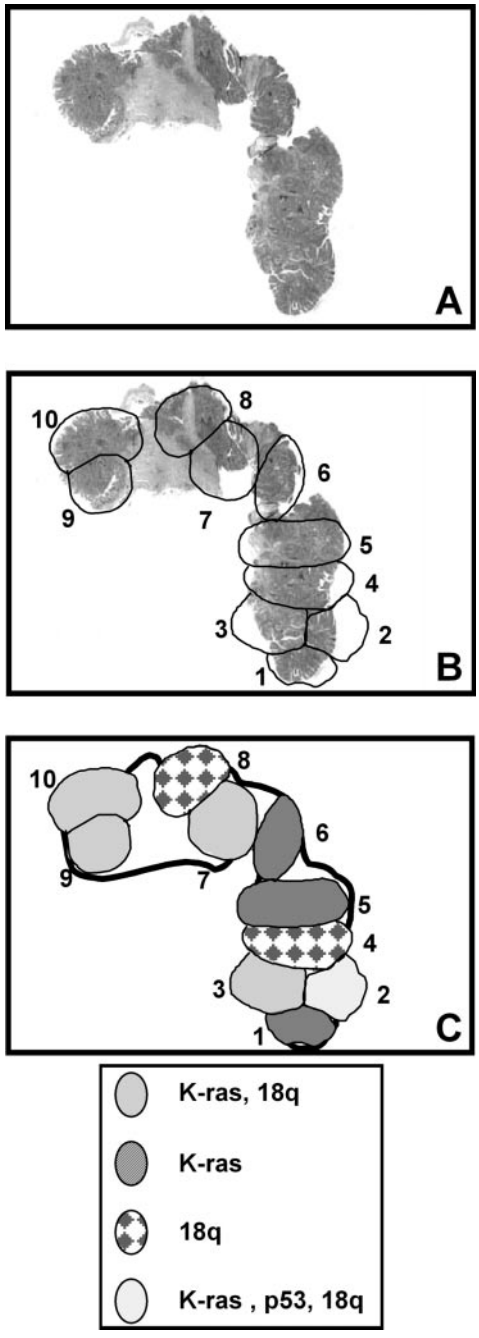

Fig. 1. Intratumoral genetic heterogeneity in an early primary colorectal cancer. The tumor 7, described in Table III, was embedded in one paraffin block. (**A**) First section stained with hematoxylin and eosin and used for histological control. (**B**) Same section with the 10 selected areas intended to be microdissected and analyzed for *K-ras* and *p53* mutations and allelic losses of 5q and 18q. (**C**) Schematic representation of the different genotypes observed for this tumor. The four different genotypes observed for this tumor are shown in the right of the figure.

metastasis, all the analyzed areas harbored the same genetic alterations.

Genetic alterations in the different stages of CRCs

The number of tumors with a *K-ras* mutation, at least in a subclone, is almost constant during the tumor progression from early stage to metastasis (Table I). In contrast, the occurrence of *p53* mutation decreased significantly ($P < 0.007$, Fisher test) from the early to the advanced stages, then remained stable during the metastatic process. During tumor progression, from the early to the advanced stages, the occurrence of LOH at 5q and 18q loci did not change significantly.

Intratumoral genetic heterogeneity in the different stages of CRCs

In the early stage of CRC, the intratumoral heterogeneity was observed at a similar level for all the genetic alterations investigated (Table II). In contrast, in advanced stages, the occurrence of intratumoral heterogeneity was significantly lower for the mutations than for allelic losses (cf. Figures 1 and 3). During tumor progression, from early stages to metastases, the intratumoral heterogeneity for *K-ras* and/or *p53* mutations was significantly reduced ($P < 0.0001$, χ^2-test) (Figure 4). In contrast, the occurrence of intratumoral heterogeneity for losses at the 18q and 5q loci was found to be relatively stable during tumor progression.

Discussion

The data presented in this study confirm the frequent occurrence of intratumoral genetic heterogeneity for point mutations and allelic losses in CRCs. This result is in accordance with several reports published to date (11,12,22,23). However, the evolution of intratumoral genetic heterogeneity during tumor progression has not yet been investigated. Among the four genetic alterations (*K-ras* and *p53* mutations, and allelic losses at the *APC* and *DCC* loci) we investigated, 19/25 (76%) of the analyzed primary colorectal tumors were heterogeneous for at least one of these biomarkers. This heterogeneity was more pronounced in the early than in the advanced stages (90 and 67%, respectively). Furthermore, all but one of the advanced CRCs were composed of one predominant clone and other minor clones, whereas no predominant clone has been identified in half of the early cancers. Separate analysis of the two different types of genetic alterations showed that the occurrence of intratumoral genetic heterogeneity was relatively stable during the tumor progression for allelic loss, whereas a significant reduction was observed for point mutations. In metastases, intratumoral heterogeneity was uncommon for both *K-ras* and *p53*. In summary, we observed a strong and linear reduction in the intratumoral heterogeneity for point mutations during tumor progression through the early stage, late stage and metastasis (80, 33 and 5%, respectively).

In the model of stepwise progression of colorectal carcinogenesis, each new step is initiated by the acquisition of an additional genetic abnormality, conferring a growth advantage to the targeted cell (5,7). This leads to a rapid clonal expansion and finally an overgrowth of the tumor by this new dominant cell clone. This model would imply a relative homogeneity in CRC for the main known genetic alterations (*APC*, *K-ras* and *p53* point mutations and allelic losses at 5q and 18q). Our results, as well as those published earlier, indicate that several subclones can be present in the same lesion and thus, clonal expansion is probably less dominant as predicted. Nevertheless, we observed that the intratumoral genetic heterogeneity, at least for the point mutations, decreases in parallel to the tumoral progression.

The genetic alterations, *p53* point mutations and LOH on chromosome 18q, occur late during the colorectal carcinogenesis (5,7). For these alterations, it is expected that the intratumoral genetic heterogeneity must be significant in the early

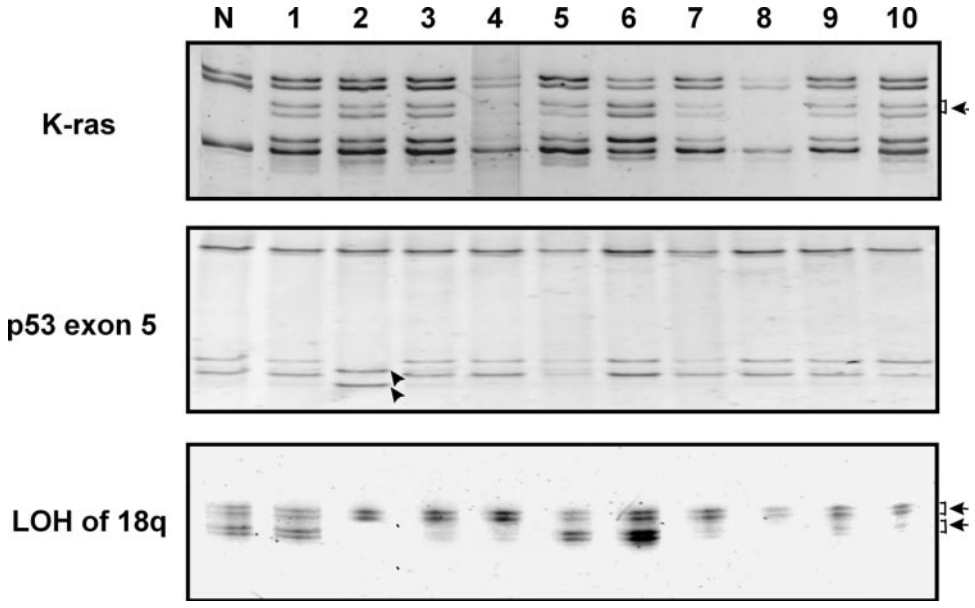

Fig. 2. Genetic analysis corresponding to the early primary colorectal cancer shown in Figure 1. DNA sample was obtained from the normal mucosa area (lane N) and 10 different colorectal microdissected tumor areas (lanes 1–10). *K-ras* point mutations analysis by PCR–SSCP: the mobility of tumor samples was compared with that of the normal sample (N). Arrows showed mobility shifts, for all but one of the samples, corresponding to a codon 12 GGT → GAT transition. *P53* point mutations were detected by PCR–SSCP, arrows showed mobility shifts, which occurred for the second tumor sample. LOH of 18q (D18S69 marker), arrows showed normal mobility of both the alleles. Allelic loss within a sample was defined as the absence or significant reduction of one allele.

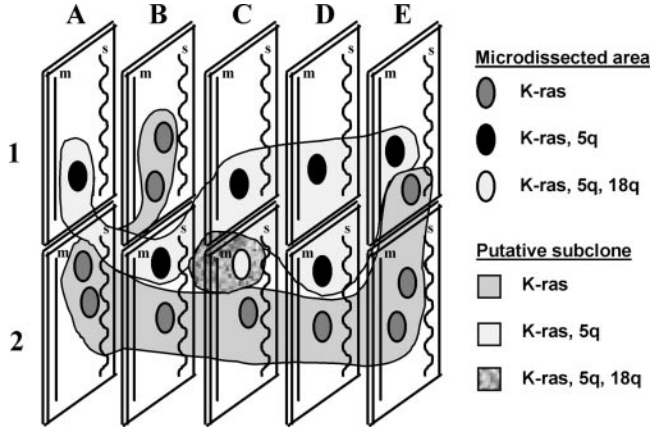

Fig. 3. Intratumoral genetic heterogeneity in an advanced primary CRC. Three-dimensional reconstruction of a tumor (Table IV, case 4) that was divided into two parts (1 and 2) and then serially sectioned into five slices (**A**–**E**) of ~4.5 mm thickness. The samples selected in the superficial and in the profound parts of the tumor are shown, in this reconstruction, near the mucosa (m) and the serosa (s), respectively. Each oval represents a microdissected sample site analyzed for *K-ras* and *p53* mutations and allelic losses of 5q and 18q. The genetic alterations observed in these microdissected samples are shown in the right of the figure. A representation of the three putative genotypic subclones observed is designed.

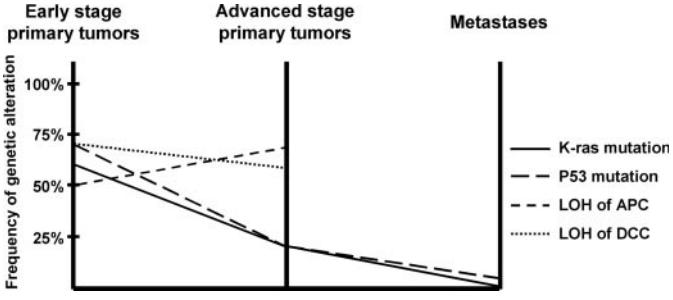

Fig. 4. Evolution of intratumoral genetic heterogeneity during CRC progression.

stage of CRC. On the other hand, the level of intratumoral heterogeneity for *K-ras* mutations and LOH of 5q, events that occur early during the colorectal carcinogenesis, have to be much lower. In this study, intratumoral heterogeneity was found to be particularly pronounced in the early stage of primary tumors and, surprisingly, observed at a high frequency for each of the four genetic alterations investigated in this study. To be in line with the previous proposal, the level of intratumoral heterogeneity for *K-ras* and 5q loss has to be much lower than that we observed for the *p53* and 18q loss. A lower number of alterations/cell should be expected in adenomas than in tumors. Furthermore, certain alterations in adenomas should be expected to affect only a subpopulation of the cells before clonal expansion. These small lesions resulting from isolated mutations will progress to invasive cancer only when allowed to evolve. Thus, premalignant lesions should demonstrate more genetic heterogeneity than the subsequent invasive cancer. The result could be a mosaic of different genotypes within the evolving premalignant lesion. In this condition, the level of intratumoral genetic heterogeneity has to remain relatively high in the early stage of primary tumors, even for the genetic alterations that occur early during carcinogenesis. This is precisely what we observed for the *K-ras* and *APC* genetic alterations.

In our series of CRC, the incidence of point mutations and allelic loss at 18q locus decreased from the early to the advanced stage. This phenomenon was particularly marked for the p53 mutation. These data are in contradiction with those reported in the literature (24,25) and could eventually suggest that normal genotypes reappear during tumor progression. Since different areas were analyzed within a tumor in this study, small subclones could be detected, particularly in early CRC. Furthermore, the proliferative capacity or the occurrence

Table IV. Genetic alterations in different areas of advanced colorectal cancer[a]

Case	Total areas per tumor	No. of areas with the same subclone	*K-ras* mutation	*p53* mutation	LOH pattern[b]
1	**13**	**8**	**12 GTT**	**wt**	**NI**
		3	12 GTT	202 AGT	NI
		2	wt	wt	NI
2	**13**	**6**	**13 GAC**	**wt**	**5q**
		3	13 GAC	wt	5q, 18q
		2	13 GAC	wt	—
		1	13 GAC	wt	18q
		1	12 TGT	wt	5q, 18q
3	**13**	**11**	**wt**	**wt**	**—**
		2	wt	wt	18q
4	**17**	**10**	**12 GTT**	**wt**	**—**
		6	12 GTT	wt	5q
		1	12 GTT	wt	5q, 18q
5	**17**	**17**	**12 GAT**	**wt**	**—**
6	**17**	**13**	**12 TGT**	**248 CAG**	**18q**
		3	12 TGT	248 CAG	5q, 18q
		1	12 TGT	248 CAG	—
7	**17**	**17**	**wt**	**wt**	**NI**
8	**17**	**8**	**12 GTT**	**245 AGC**	**18q**
		4	12 GTT	245 AGC	—
		4	12 GTT	245 AGC	5q
		1	12 GTT	245 AGC	5q, 18q
9	**17**	**17**	**wt**	**wt**	**NI**
10	**17**	**9**	**wt**	**199 GAA**	**—**
		4	wt	199 GAA	18q
		2	wt	199 GAA	5q
		1	wt	199 GAA	5q, 18q
		1	wt	wt	—
11	**13**	**7**	**wt**	**wt**	**5q**
		6	wt	wt	—
12	12	4	wt	175 CAC	—
		3	wt	wt	—
		2	wt	175 CAC	5q
		2	12 GTT	175 CAC	5q
		1	12 GTT	175 CAC	—
13	**12**	**9**	**13 GAC**	**wt**	**18q**
		1	13 GAC	wt	5q
		1	13 GAC	wt	18q
		1	13 GAC	wt	5q, 18q
14	**12**	**12**	**wt**	**wt**	—
15	**13**	**13**	**wt**	**wt**	—

The tumors that contained a dominant clone, as well as this dominant clone, are indicated in bold.
[a]According to our previously published data (12).
[b]Only chromosomal loci with LOH are listed.
NI, non informative.

of a dominant clone (50% of the early tumors had no dominant clone) will depend on the number and the type of its genetic alterations. In the present study, only four genetic markers were investigated, whereas many other alterations have been described in CRC. All these findings show that there is obviously no reappearance of a normal genotype in advanced CRC. In fact, there was no evident selection between the different subclones in early CRC and thus, many subclones with numerous genetic alterations could coexist in the same tumor. In conclusion, most of the subclones, independent of their genetic alterations, will disappear during the clonal selection that occurs during tumor progression.

The high level of the intratumoral heterogeneity for the allelic losses in advanced CRC could be explained by the increase of chromosomal instability (CIN) that occurs during tumor progression. CIN refers to the increased rate of lost or gained whole or large parts of chromosomes during cell division. Short telomeres of tumor cell chromosomes, loss of normal centrosome regulation and function, partial inactivation of DNA repair pathways and demethylation of chromosomal segments could explain this chromosomal instability (26). More than 80% of CRC and most other solid tumors have CIN (27,28). During colorectal carcinogenesis, CIN could play a major role in the allelic losses of a number of genes of interest (29). According to our data, the clonal selection that occurs during the tumoral progression would not be sufficient to compensate the high level of CIN in CRC.

The occurrence of intratumoral genetic heterogeneity could explain the controversial data concerning the prognostic significance of potential biomarkers in CRC. In fact, the analysis of only one small area from a tumor could not be representative of the whole tumor and could, in part, explain the disagreement in the literature concerning the utility of these biomarkers (18,19,30). Thus, it seems reasonable to conclude from the present study that each genetic alteration intended to be used for prognosis in patients with CRC should be evaluated carefully for intratumoral genetic heterogeneity.

In conclusion, our study confirms the existence of intratumoral genetic heterogeneity during the progression of CRC. In primary CRCs, intratumoral genetic heterogeneity was more often observed in the early than in the advanced stages. Indeed, due to the clonal selection, intratumoral genetic heterogeneity, at least for the point mutations, decreases significantly during tumor progression. On the other hand, the relative stability of the LOH intratumoral heterogeneity along the tumor progression is probably a consequence of the high level of CIN without a sufficient compensation by the clonal selection.

Acknowledgements

We thank Gabrielle Gallagher for the critical reading of the manuscript. This work was supported by grants from the Swiss National Fund (31-51025.97) and the Progetti di Ricerca Avanzata (Italy).

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Received September 23, 2004; revised February 2, 2005; accepted February 6, 2005