

Tissue sampling for mutation analysis in colorectal cancer: K-ras is homogeneously distributed throughout the tumor tissue

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Abstract. The prognostic value of gene abnormalities in colorectal cancer is unclear, varies between investigators, and may be unreliable due to tumor heterogeneity. We analyzed tumors completely divided into biopsies to characterize the overall distribution of LOH at 5q, 17p, 18q, K-ras mutations, and methylation of the p16 and MGMT genes. Seventeen tumors (stage II-III) were completely divided into biopsy cubes from which DNA was sequentially analyzed. The results from fragment analysis for LOH at 5q, 17p, 18q, TGGE of the K-ras, and MSP for p16 and MGMT genes were used to characterize the occurrence of these aberrations. LOH at 18q, 17p, and LOH 5q were present in 17/17, 15/17, and 15/16 tumors, respectively. p16 and MGMT were methylated in 16/17 and 14/17, respectively. The frequency of these aberrations varied largely between tumors. K-ras-mutations, present in 8/17 tumors, were much more consistently distributed in mutated tumor. Methylation of p16 and MGMT and LOH at 18q, 17p, and 5q are present within the tumor mass in a large majority of CRC tumors and are of no or little prognostic value. K-ras mutations appear more homogeneously distributed. This has clinical relevance for biopsing to predict anti-EGFR response.

Introduction

Since the Fearon/Vogelstein model of multistep carcinogenesis was first presented in 1990 (1), new discoveries have continuously increased our insights into the development of colorectal carcinoma (CRC). An increasing number of both genetic and epigenetic changes have been described. The

original theory has somewhat been remodeled (2) as further knowledge on mutations of specific genes, epigenetic modulation of tumor suppressors and oncogenes, and allelic imbalance has been explored. Currently, the development of non-inherited CRC is broken down into three distinct tracts of genomic instability governing tumor development. These pathways, consisting of chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) probably overlap to a large extent (2). As of yet, none of these discoveries specifically has had any major implementation on clinical work.

Curative treatment of CRC is based on careful surgical removal of all tumor-bearing tissue. In addition, today it is generally accepted that adjuvant chemotherapy and (for rectal carcinomas) preoperative radiotherapy improves survival in advanced tumor stages (3,4). The basis for the utilization of adjuvant therapies in addition to surgery, and the intensity of follow-up programs, still depend on histology and the Dukes and TNM classifications. This undoubtedly leads to some degree of over-treatment of patients in advanced stages (5,6) and, reciprocally, omission of a number of patients in earlier stages that may benefit from adjuvant chemotherapy (7).

Accordingly, many attempts have been made to subclassify CRC further, and a range of intratumoral genetic and chromosomal abnormalities have been evaluated with this purpose (2,8-13). In CRC, the presence or not of loss of heterozygosity (LOH) at different chromosomes (14-19) as well as mutations in, for example, the p53 and K-ras genes (20-24) as well as methylational status (25) have been investigated in relation to prognosis. However, despite a large number of studies of putative genetic markers of relevance for the outcome after surgery, conflicting results are at hand. For instance, differential conclusions of the prognostic significance of K-ras mutations and of LOH at chromosome 18q have been presented (11,14-16,26,27) despite similar methods and study material. In addition, and explaining different conclusions, the frequency of a given aberration varies relatively widely between investigators.

We, and others, previously described that the presence or not of LOH 18q and 17p in different invasive areas of the same colorectal tumors varies in a random fashion (28-30). Thus, single biopsies, which are almost exclusively used in this type of studies, do not guarantee the true status of genetic aberrations in a given tumor, even if the specimen

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Table I. LOH at 5q, 17p, and 18q in the 17 colorectal tumors (number of abnormal biopsies/total number of analyzed biopsies).

Patient no.	LOH 18q		LOH 17p		LOH 5q	
	D18S58	D18S67	D17S796	D17S1832	D5S299	D5S495
1	1/36	25/36	19/37	33/37	2/40	2/40
2	H	12/25	1/40	H	6/10	ND
3	2/24	8/26	H	H	14/28	12/20
4	0/23	10/23	6/10	10/18	0/40	2/40
5	17/40	6/39	H	H	12/40	21/40
6	8/20	14/20	14/20	H	H	H
7	1/42	4/42	H	8/19	6/20	12/20
8	6/18	H	4/18	12/18	19/38	H
9	2/34	8/34	H	2/48	8/35	6/35
10	H	12/20	11/20	6/20	H	14/20
11	16/19	7/17	2/38	3/38	15/19	H
12	15/18	8/18	14/18	H	11/17	12/18
13	5/18	0/18	2/38	H	2/18	MI
14	H	8/19	6/20	H	1/20	MI
15	20/20	19/19	H	19/19	H	17/19
16	10/18	14/18	17/20	19/20	H	0/19
17	15/20	20/20	16/20	H	16/20	20/20
Total	13/14	15/16	12/12	9/9	12/13	12/13
Both microsatellite	17/17		15/15		15/16	

We used automated fragment analysis (Gene Scan[®]) with two different microsatellite markers for each chromosome. As seen, 18q and 17p were LOH positive somewhere in 100% of the eligible tumors; 5q was positive in 15 of 16 tumors (94%). H, homozygotes for the chosen microsatellites; MI, microsatellite instability; ND, not done.

is microdissected, due to intratumoral heterogeneity (31-33). A recent finding exemplifying the importance of this is the utilization of K-ras analysis to predict the responsiveness to anti-epidermal growth factor antibody therapy in patients with metastatic CRC (34).

To evaluate the natural rates of occurrence, and to establish a more correct figure of the frequencies of allelic loss, methylation of genes and point mutations in CRC, 17 colorectal tumors were completely divided into biopsies, which were analyzed consecutively. We chose to analyze LOH 5q, 17p, 18q, methylation of the p16 and MGMT genes and mutations in K-ras, as these aberrations have been widely studied in CRC.

Materials and methods

Patients, specimens, and DNA extraction. Seventeen patients (9 female, 8 male) with CRC, 37-88 years of age (median 76) were studied. Informed consent was obtained from each patient prior to surgery. The study was approved by the Ethics committee at Karolinska University Hospital, Stockholm. There were 10 patients with stage II and 7 with stage III disease. The material consisted of 4 rectal, 5 left-sided and 8 right-sided colonic tumors.

Staging was carried out according to the UICC TNM classification on the basis of histological and clinical findings.

The specimens were thoroughly examined macroscopically and only a limited part of the tumor mass were excised to maintain adequate microscopical examination, usually 0.5-1.5 g. Stage I tumors were excluded from the study. With the specimens on ice, the whole tumors were divided into ~3-mm cubes which were snap-frozen separately in liquid nitrogen, and stored in -70°C until analysis. DNA was extracted from the biopsies, and from normal mucosa at least 10 cm away from the tumor, with Qiagen tissue kit (Qiagen, Hilden, Germany). All samples were analyzed in sets of 6-40 until one or more aberrations were detected; 2 µl, with the concentration of 25 ng/µl, was used for the polymerase chain reaction (PCR).

Microsatellite markers and amplification. Oligonucleotide primers for microsatellite markers from the short arm of chromosome 17 and the long arm of chromosome 5 and 18 were designed on the basis of published sequences (35). The loci tested were D17S1832 (3 cM telomeric to the p53 gene), D17S796 (1 cM telomeric to the p53 gene), D18S58 (7 cM telomeric to the DCC gene), D18S67 (1 cM centromeric to the DCC gene), D5S299 (7 cM telomeric to the APC gene) and D5S495 (6 cM centromeric to the APC gene). Each of the PCR reaction cycles consisted of denaturation for 30 sec at 94°C and annealing for 30 sec at 52°C for most of the primers except D18S67, 5q299 and 5q495 (60°C) and a final extension at 72°C for 30 sec (25 cycles).

Table II. Analyses of K-ras and methylation status of the p16 and MGMT genes throughout the tumors.

Patient no.	K-ras-mutated/ total	p16 methylated/ total	MGMT methylated/ total
1	0/25	11/25	25/25
2	23/23	25/25	0/25
3	0/27	15/19	18/20
4	0/20	16/20	16/20
5	1/20	9/20	5/20
6	0/33	12/19	1/19
7	0/22	21/21	3/21
8	15/17	18/19	13/18
9	0/23	19/19	17/19
10	17/17	19/19	5/19
11	0/27	4/20	19/20
12	17/18	17/17	12/13
13	26/26	1/19	0/19
14	0/20	19/20	18/20
15	0/30	0/20	19/20
16	19/19	8/20	0/20
17	20/20	9/20	17/20
Total ^a	8/17	16/17	14/17
Mean% ± SD ^b	40.4±12.0	65.4±8.5	57.0±10.1

The number of mutated K-ras/methylated samples per total number of analyzes of each tumor is indicated. The mutated or wild-type form of K-ras was homogeneously present in 14/17 cases. In patients 5, 8, and 12 the results of the K-ras analysis were homogeneous to 95, 88, and 94% respectively. ^aNo. of tumors with at least one positive biopsy. ^bMean percent positive biopsies/tumor (mean ± SEM).

Calculation of LOH. LOH was estimated as described by Cawkwell *et al* (36) with minor modifications as described (28). After electrophoresis of the PCR products, the fluorescence was detected in a laser scanning region and stored using Gene Scan Collection Software (Applied Biosystems, Foster City, CA). Normal tissue displayed allele ratios between 0.7 and 1.3 for 17p and 18q, while 5q rated between 0.8 and 1.4. Values outside these ranges indicated LOH (28).

Temperature gradient gel electrophoresis (TGGE). The amplification of the K-ras gene was performed between codons 9 through 30, thus enabling detection of mutations within codons 12 and 13. TGGE of the purified amplification products was performed in a TGGE system (37) patented by Qiagen and produced by Biometra (Göttingen, Germany). We used horizontal 8% gels with 4.2 g urea, 2.0 ml acryl amide (stock solution 37.5:1, 40%), 0.1 ml TBE (10X), 0.5 ml glycerol (50%), 22.5 µl TEMED and 42 µl APS (4%) in distilled water, filled up to 10 ml. An initial perpendicular TGGE made it possible to identify the different alleles by their individual melting behavior yielding the temperature at which the different DNA strands would separate. The

screening of the multiple samples was done on plates with pre-fixed slots for 8 or 12 samples by using parallel TGGE.

Methylation-specific PCR (MSP). DNA methylation patterns in CpG islands of the promotor region of the p16-gene were determined by sodiumbisulfite modification of the unmethylated, but not the methylated, cytosine to uracil, and subsequent PCR using primers specific for either the methylated or the modified unmethylated DNA as described by Herman *et al* (38). Primer sequences of p16 were for the unmethylated reaction 5'-TTA TTA GAG GGT GGG GTG GAT TGT-3' (sense) and 5'-CAA CCC CAA ACC ACA ACC ATA A-3' (antisense) and for the methylated modified reaction 5'-TTA TTA GAG GGT GGG GCG GAT CGC-3' (sense) and 5'-GAC CCC GAA CCG CGA CCG TAA-3' (antisense). The annealing temperature was 65°C for the methylated and 60°C for the unmethylated reaction; 35 PCR cycles were carried out. Methylated and unmethylated DNA sequences were detected on 2.5% agarose gels. Samples giving signals approximately equivalent to the positive methylated control were designated as methylated. Samples giving no signals with positive methylated control, but demonstrating presence of unmethylated DNA, similar to the negative control, were designated as unmethylated.

Statistics. The significance of differences in LOH frequency between K-ras mutated and wild-type tumors were tested using Mann-Whitney U test.

Results

Loss of heterozygosity and microsatellite instability (MI). LOH at 18q was present in all 17 tumors as detected by at least one of the two primers employed. LOH 17p was undetectable due to homozygosity with both primers in two patients (3 and 5), but positive in all the 15 remaining tumors. LOH at 5q was homozygous in one patient (6), and in another patient no loss was seen (16). In all the other 15 patients LOH 5q was present. Clear-cut signs of MSI, revealing multiple fragments on electrophoresis, were seen with one of the primers (5q) in two patients. Furthermore, in three patients signs of allelic gain (18q) were detected (data not shown).

In patients with only a minor number of samples positive, analyzes were re-run, verifying the results (Table I). As seen, the frequency of biopsies with allelic loss varied widely throughout the individual tumors, ranging between 0 and 100%. Overall, the mean percentage of biopsy samples among the tumors displaying allelic loss ranged from 37.5±7.8% (D5S299) to 54.5±1.2% (D17S1832). Altogether, counting only tumors possible to analyze with at least one of the primers, 47 of 48 (98%) screenings for 18q, 17p, and 5q were found to harbor LOH in this material.

Methylation of p16 and MGMT. In one of the tumors none of the biopsies were found to contain methylation of p16 (Table II). The MGMT gene was unmethylated throughout the tumor in three patients. All the other tumors, 16/17 (94.1%) for p16 and 14/17 (82.4%) for the MGMT gene contained a variable proportion of positive MSP assays. Of the total number of biopsies analyzed, 65.4% (±) were

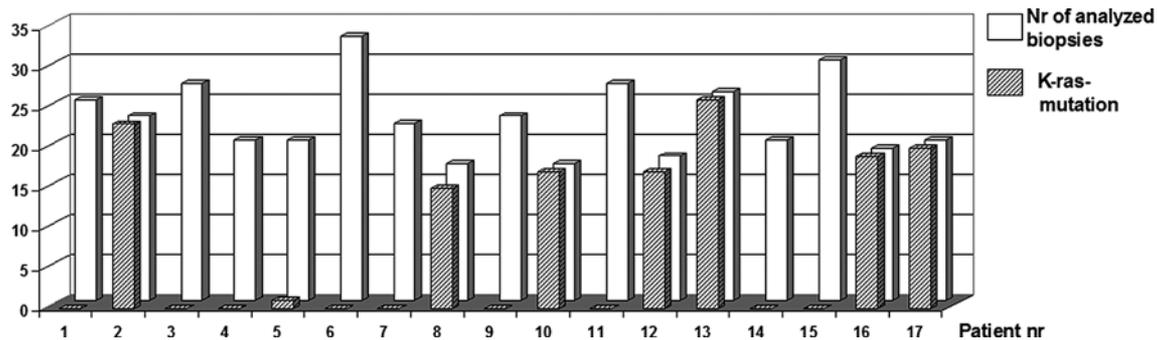


Figure 1. K-ras mutations in the biopsies of the 17 tumors. A clear-cut pattern of distribution is seen: in 10 of the tumors virtually all biopsies, taken throughout the whole tumor mass, contained only wild-type K-ras whereas in 7 tumors practically all biopsies harbored mutations, indicating that K-ras mutation is a very early and thereafter highly conserved aberration in CRC development.

positive for p16 and 57.0% for MGMT. There was no correlation between the relative numbers of biopsies with a methylation and the K-ras status or the degree of allelic loss among the tumors; $p=0.07$ (data not shown). In the two patients displaying MSI (on 5q) in our set of assays (Table I), one had a high degree of methylation and the other tumor did not.

K-ras mutations. K-ras-mutations were found to be distributed in a different pattern (Table II). While the occurrence of LOH and methylation positive biopsies seemed randomly distributed throughout the tumors, K-ras in general were homogeneously mutated or not. The mean numbers of analyzed biopsies were 21.6 per tumor, tested in duplicate. In 9/17 tumors only the wild-type K-ras gene was detected. In one tumor, one single biopsy out of 20 harbored a mutation. Of the remaining 7 tumors, 5 were found to homogeneously contain only mutated K-ras; one were mutated in 15/17 biopsies, and one in 17/18. There were no differences between the tumors with/without mutations in correlation to size, histology, localization, or clinical behavior (data not shown). In this limited cohort of patients, a trend towards a higher rate of LOH in the tumors displaying K-ras mutations was seen; however, this was not significant. In the group of tumors with mutated K-ras, the frequency of biopsies positive for LOH 18q was $55.0 \pm 9.1\%$ (mean \pm SEM) compared to $33.0 \pm 8.8\%$ among the wild-type K-ras tumors ($p=0.24$). The results for 17p and 5q were 44.4 ± 13.6 vs. $40.1 \pm 11.8\%$ ($p=0.5$) and $55.6 \pm 12.3\%$ vs. $29.8 \pm 10.8\%$ ($p=0.43$), respectively.

Discussion

The results of the present study indicate an important difference in the rates and characteristics of occurrence of point mutations of the K-ras gene in comparison to both allelic imbalances at chromosome 5, 17, and 18 and methylation of the p16 and MGMT genes. Allelic loss and methylation were demonstrated to occur regularly and seemingly mandatory in advanced CRC tumors, while K-ras mutations in approximately half of the tumors occurred in the mutated form and were conserved as the wild-type gene in nine of the seventeen studied cases.

As depicted in Table I, the intratumoral frequency of allelic loss differed widely between the tumors in the present study. In the large number of studies on this issue, a definite variability of reported occurrence rates of LOH at different chromosomes, and their relative importance for the outcome of CRC, is at hand. The standard methodology involves analysis of very limited areas of the colorectal tumors, using one or a few biopsies (9,11,13-22,24,26,39-42). However, these tumors generally display a morphological mosaic-like pattern, representing different intratumoral subclones of tumor development, ultimately affecting the results of studies of tumor markers as point mutations (43,44), allelic imbalance (21,45), as well as aneuploidy (11,46,47) and methylation status (48). As a rule, histological invasiveness directly affects the results in terms of 'molecular morbidity' (29-31). Since the material investigated is generally extracted from very limited areas of CRC tumors, a considerable variation in the representativeness of tumor biopsies might be expected, even with the use of microdissection. Thus, biopsying for DNA aberrations that are heterogeneously distributed throughout tumors could result in uncertain and even clinically irrelevant data, and might also contribute to the variability in prognostic importance of certain markers found in different studies.

The results also suggest an important difference in the structural occurrence of a specific point mutation, K-ras, and the occurrence of allelic imbalance and methylational status in CRC. While LOH, although present in varying frequencies, seems to be mandatory throughout clinically advanced tumors, reflecting the loss of control mechanisms on a chromosomal level, K-ras mutations are not a prerequisite for the full development to advanced CRC. K-ras mutations, when present, are introduced early in the adenoma-carcinoma sequence (1), with a proportion of K-ras mutations in studies of colonic adenomas not differing very much from that of carcinomas (21,24,39,49,50). From our results, with only 3/17 tumors not containing homogeneously mutated or wild-type K-ras (Table II) it may be concluded that this early event is strikingly preserved throughout clonal expansion and tumor progression in CRC.

There were no significant correlation between the occurrence of K-ras mutations and the number of LOH positive biopsies among the tumors in this study, although

a trend towards this was seen. The mean follow-up time is only 21 (11-43) months and the number of patients (n=17) is too small to draw any conclusions on the prognostic effect of K-ras mutations (alone or in addition to allelic losses). In contrast to the case for LOH, however, the present study and earlier results from our group (31) theoretically supports studies of the impact of K-ras status for the outcome of CRC, as mutation in a single biopsy was far more representative for the whole tumor than allelic loss or the methylational status. In the majority of outcome reports, K-ras mutations have had a negative impact on survival and recurrence rates (20,21,46). In addition, our data support the clinical utilization of K-ras analysis from single, or a limited number of biopsies, for individual treatment guidance in clinical practice, and is already in use in decision making for anti-EGF therapy.

In summary, based on analysis of completely divided CRC tumors, our results suggest that chromosomal aberrations and deletions, here represented as LOH 5q, 17p, and 18q, and also methylation of MGMT and p16, are unevenly distributed throughout CRC's but practically always present in tumor tissue, or at least abundant in a much higher proportion than generally described. However, K-ras mutations seem to be imprinted early and then preserved throughout the development of sub-clones within the tumor. These findings are of general importance as such for the understanding of clonal expansion within CRC tumors, but also provide a demonstration of the potential pitfalls of genetic determination for clinical utilization. The study clearly supports the use of a single biopsy for the analysis of K-ras as a relevant clinical tool, but also implies careful characterization of other neoplastic genetic events in relation to colorectal 'tumor architecture' and tumor development before they can reach clinical relevance.

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