A Novel Multiplexing, Polymerase Chain Reaction-Based Assay for the Analysis of Chromosome 18q Status in Colorectal Cancer

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Chromosome 18q allelic loss has been reported to have prognostic significance in stage II colorectal carcinoma. We have developed a fluorescent multiplex polymerase chain reaction assay to analyze five microsatellite markers (D18S55, D18S58, D18S61, D18S64, and D18S69) for allelic loss at the long arm of chromosome 18. Amplicon detection and evaluation was accomplished by capillary electrophoresis using an ABI 310 genetic analyzer. Robustness of the assay when performed on DNA extracted from formalin-fixed, paraffin-embedded tissue sections was confirmed by analyzing its repeatability and reproducibility. Allelic loss was assessed in 61 stage II colorectal tumors and was detected in 58% (31 of 53) of tumors not showing instability. As part of the study, results of 207 previous polymerase chain reaction/polyacrylamide-based assays were re-evaluated by two independent observers to determine the degree of concordance of visual evaluation. In the case of stage II colorectal tumors, when electropherogram results were compared with those obtained from visual evaluation of the same markers after polyacrylamide gel electrophoresis, discrepancies between observers were detected in 16.4% of determinations. In conclusion, we have developed a robust and reliable assay for multiplexed loss of heterozygosity determination that improves assessment of chromosome 18q allelic loss in colorectal tumors processed as routine formalin-fixed, paraffin-embedded specimens. (J Mol Diagn 2005, 7:478–485)

The prognosis of colorectal cancer is mainly based on tumor stage at time of diagnosis. However, other clinico-pathological features such as tumor location, perineural invasion, lymphatic vessel invasion and differentiation, as well as certain molecular alterations such as microsatel-lite instability, have also been reported to be predictors of tumor recurrence and patient outcome.[1,2] Survival for patients with tumors confined to the muscularis propria (stage I) or with extensive metastatic disease (stage IV) is predictable; however, tumor biological behavior after surgical treatment for stage II and III tumors is not well established. Approximately 30% of patients with stage II colon cancer will relapse and die of the disease.[3–6] More significantly, up to 65% of patients with stage III tumors will succumb to colon cancer. The use of adjuvant therapies in patients with stage II lesions is not exempt of controversy. These therapies have side-effects, and should be given to those patients who would benefit from them.[7] Prognostic markers that complement standard clinical and pathological staging further stratify stage II patients into high-risk and low-risk groups of relapse after surgery, and better guide adjuvant therapy. Biological factors that account for the different outcome among patients presenting with the same clinical stage are still poorly understood, but several studies have revealed the prognostic significance of 18q allelic loss in stage II colorectal carcinomas.[3,5,6,8–10] Loss of this region, from which several tumor suppressor genes have been cloned and characterized, such as *DCC*, *SMAD2*, and *SMAD4*, can be detected in ~60 to 70% of colorectal cancer cases.[8,11] Within the group of stage II tumors, 18q21-22 loss correlates significantly with appearance of recurrent disease and poor survival.[3,5,6,8–10] Thus, examination of 18q21-22 loss of heterozygosity (LOH) in primary stage II colorectal cancers can assist in identifying patients prone to recurrence and candidates for further treatment.

The aim of this study was to analyze chromosome 18q LOH by means of a new multiplex polymerase chain reaction (PCR) assay and GeneScan analysis after capillary electrophoresis developed in our laboratory. After the reproducibility of the multiplex assay using DNA obtained from paraffin-embedded tissues had been evaluated, the new multiplex assay was used to assess 18q LOH in 61 stage II colorectal carcinomas and to deter-

Table 1.   Primers and Fragment Length of PCR Products from Microsatellite Loci Analyzed

Primer	Sequence	5′- Modification for ABI analysis	Molar concentrations	Size of fragment (bp)
D18S55 A	5′-GGGAAGTCAAATGCAAAATC-3′	Tail	0.2 μmol/L	134–152
D18S55 M	5′AGCTTCTGAGTAATCTTATGCTGTG-3′	6-FAM	0.2 μmol/L	
D18S58 A	5′-GCTCCCGGCTGGTTTT-3′	Tail	0.08 μmol/L	144–160
D18S58 M	5′-GCAGGAAATCGCAGGAACTT-3′	TET	0.08 μmol/L	
D18S61 A	5′-ATTTCTAAGAGGACTCCCAAACT-3′	HEX	0.28 μmol/L	157–183
D18S61 M	5′-ATATTTTGAAACTCAGGAGCAT-3′	Tail	0.28 μmol/L	
D18S64 A	5′-ATACTGGTGGTGGTTATACAACAT-3′	6-FAM	0.16 μmol/L	188–208
D18S64 M	5′-AAATCAGGAAATCGGCA-3′	Tail	0.16 μmol/L	
D18S69 A	5′-CATTAGCAGTCTGGAAATCCTC-3′	TET	0.2 μmol/L	194–210
D18S69 M	5′-CGCTATTGTACTGAAAACCTGA-3′	Tail	0.2 μmol/L	

mine the contribution of each marker to LOH detection. Finally, also as a part of our experimental design, the degree of discordance between different observers when analyzing gel electrophoresis results was determined by re-evaluating retrospective LOH results from a cohort of 207 colorectal carcinomas.

Materials and Methods

Patient Characteristics and Tissues

Between February 1996 and November 2002, a total of 207 colorectal adenocarcinomas were collected after surgical excision from patients in the area of Barcelona, Spain. Demographic data on this group of patients may be summarized as follows: 119 patients were males and 88 females; mean age was 67 ± 12 years (range, 36 to 98 years). Staging according to the TNM (International Union against Cancer) system[12] revealed 5 stage 0, 29 stage I, 72 stage II (65 IIA and 7 IIB), and 101 stage III to IV tumors. Regarding histological grade,[13] 81 tumors were classified as low-grade lesions (grade 1), 80 as intermediate grade (grade 2), and 45 as high-grade tumors (grade 3). One tumor was not graded due to previous treatment with radiotherapy. Specimens were processed for routine histopathology by formalin fixation (10%) and paraffin embedding. Representative hematoxylin and eosin-stained sections of each case were examined microscopically by a pathologist to confirm the presence of tumor and associated normal mucosa.

Tissue Macrodissection, DNA Extraction, and Control Amplification

Ten 5-μm-thick unstained sections were used for each paired case (normal and tumor samples) to perform tissue scrapping. DNA was extracted from the macrodissected tissue using a proteinase K-phenol/chloroform protocol.[14] A 268-bp fragment of the human β-globin gene was also amplified and detected in 2% agarose gels to test the quality of the genomic DNA extracted.[15]

Design of the Multiplex PCR Assay

Amplification of microsatellite markers of the chromosome 18q region involving the DCC gene was performed in a single multiplexed PCR assay using well-characterized primers (D18S55, D18S58, D18S61, D18S64, and D18S69).[8] The PCR reaction was set up with 200 ng of genomic DNA as template and 1.05 U of the Expand high-fidelity PCR system (Boehringer Mannheim Corp., Indianapolis, IN). Primer molar concentrations were optimized to obtain similar yields of marker amplification. The fluorescent labels were chosen such that the potential amplicons were sufficiently separated in size to prevent overlapping between PCR products. Table 1 summarizes primer sequences, fluorescence dyes, molar concentration, and product sizes obtained. To avoid polymerase stuttering, only one primer of each pair was labeled, and a 5′ tail was added at the nonlabeled primer (PE Applied Biosystems, Foster City, CA). PCR was performed in a GeneAmp PCR System 9600 thermal cycler (PE Biosystems) under the following cycling conditions: denaturation at 94°C for 5 minutes followed by 30 cycles consisting of 94°C denaturation for 30 seconds, 55°C annealing for 30 seconds, and 72°C extension for 30 seconds. A final 30-minute extension was performed to avoid incomplete 3′ adenine nucleotide addition (+A peaks).

After PCR amplification, the fluorescent products were directly diluted in formamide, mixed to 0.5 μl of GS350 TAMRA size standard, and then separated by capillary electrophoresis using an ABI Prism 310 automated sequencer (PE Applied Biosystems). Analysis was performed using the GeneScan 3.1.2 software (PE Applied Biosystems). To calculate the LOH, peak heights were measured in relative fluorescent units. For each informative marker (showing two alleles in normal tissue), the height of the major peak of each allele was considered, being consistent with the highest one. A ratio between normal and tumor tissue peaks was calculated as follows:

$$LOH = \left(\frac{\text{(peak height of normal allele 2)/}}{\text{(peak height of normal allele 1)}} \middle/ \frac{\text{(peak height of tumor allele 2)/}}{\text{(peak height of tumor allele 1)}} \right)$$

A LOH event was considered when the ratio was less than 0.5 or higher than 2.0.[16] Ratios outside this range would correspond to a frequency of at least 50% of tumor cells displaying allelic imbalance in a pure 100% tumor sample.

Analysis of the Repeatability and Reproducibility of the Multiplexed LOH Assays

Several experiments were performed to assess intra- and interassay variability regarding LOH determination by multiplexed PCR. The variability of differences among distinct replicates of the same sample analyzed in the same experiment (intra-assay precision) is in this study referred to as "repeatability." The variability of differences between the samples analyzed in independent experiments (interassay variability) is in this study referred to as "reproducibility." Three paired normal-tumor samples were randomly selected for assay reproducibility experiments, and were analyzed in triplicate in three independent assays (*n* = 27). Injection of samples in the automated sequencer was done in random order to minimize putative effects associated to run conditions. For each pair of samples, five independent variables (microsatellite markers) were examined, on which LOH ratio was calculated as described above.

Assay for DNA Concentration

To determine whether DNA concentration was critical for the assay, two additional concentrations (100 ng and 50 ng) were tested for each pair of samples used in reproducibility assays in an independent experiment.

LOH Determination in Stage II Colorectal Carcinomas

LOH at the chromosome 18q region involving the DCC gene was analyzed by multiplex PCR in a subset of 61 stage II A (pT3N0M0) tumors. LOH was evaluated for each marker, and chromosome 18q allelic loss was considered when LOH was detected in at least one marker. Unstable and noninformative results for each marker were also recorded.

Gel Electrophoresis LOH Determination and Visual Evaluation of Band Intensities

In our laboratory, LOH at chromosome 18q region had been prospectively analyzed using independent PCR assays followed by gel electrophoresis of the same five markers described above.[8] Results were available for 207 routine determinations. PCR products had been run in GeneGel Excel 12.5/24 polyacrylamide nondenaturing precast gels (Amersham Pharmacia Biotech, Uppsala, Sweden), and finally developed by silver staining. Results were obtained by visual assessment of band intensities. Two independent observers (N.E. and A.C.) examined *de novo* the acrylamide gels and blindly assigned results for each marker. The criterion for the presence of chromosome 18 LOH was at least one marker showing LOH (50% reduction of the signal or more). Tumors expressing LOH at least in one marker were considered LOH-positive. In the present study, LOH results of stage II colorec-
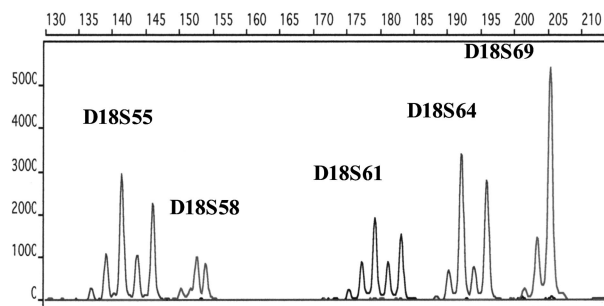

Figure 1. Representative electropherograms for the five multiplexed chromosome 18q markers. The fluorescent labels were chosen such that the amplification products were sufficiently separated in size to prevent overlapping, and primer molar concentrations were also adjusted to achieve similar peak amplitudes for all markers.

tal tumors were compared with those obtained by capillary electrophoresis.

Statistical Analyses

The repeatability and reproducibility of the multiplexed LOH assays were studied modeling the global data set with a two random factor linear model for each analyzed variable. To normalize the original five variables, the LOH ratio measures were transformed by using the decimal logarithm. In our model, the first factor is the assay date (or experiment number), and the second factor is the subject (samples). Therefore, the first factor is associated to the reproducibility of the assays, whereas the repeatability is associated to the residual variance component of the model. The statistical power for the assay factor analysis was also computed.

The differences between observers and the differences between GeneScan and each observer were studied for the global 18q status and for every individual marker by computing the asymptotic normal confidence interval (at 95% confidence level) of the frequency discordance. C18q status was also cross-tabulated with different combinations of the markers in several tables. Five of these tables cross simply the individual markers with C18q. For the rest of the tables, including therefore several markers simultaneously as a factor, the combined allelic loss is defined as the loss of any of the individual markers.

Results

The designed multiplexing of the five microsatellite markers at chromosome 18q, using DNA obtained from paraffin-embedded tissues, was successfully achieved in all paired normal tumor samples analyzed (Figure 1). A problem inherent to microsatellite-based assays is the presence of stutter products generated by slipped strand mispairing during the PCR process. In general, loci with shorter repeat units have a higher incidence of stutter. Markers used in this study are dinucleotide repeats that, when amplified using a 5′ tail in the nonlabeled primer, display a maximum of two additional stutter bands clearly distinguishable in height of the real allele peak. Moreover,

Table 2.   Analyses of Variance Table for the Five Markers (Log_{10} of the LOH Ratio Values)

Source*	D18S55		D18S58		D18S61		D18S64		D18S69	
	MS	P value	MS	P value	MS	P value	MS	P value	MS	P value
Date	0.016	0.121	0.012	0.439	0.009	0.067	0.031	0.144	0.049	0.030
Subject	5.569	<0.001	0.683	<0.001	0.980	<0.001	3.753	<0.001	0.945	<0.001
Error	0.007		0.014		0.003		0.013		0.010	

*Date refers to the assays and subject to the microsatellite marker analyzed.

the +A peaks, one nucleotide longer than the allele peak that frequently appears as PCR artifacts, have been eliminated by performing a final longer extension. Thus, electropherograms obtained with the selected panel of markers are simple and easy for LOH analysis.

Reliability on LOH determination was evaluated in experiments performed with three paired normal tumor samples selected at random. LOH of the five microsatellite markers was determined in one pair of samples, and in four markers in two cases on which one marker was noninformative. Four of the analyzed markers (Table 2) did not show statistical significance for the first factor in the statistical analysis, that of the date in which the assay was run. This indicates that for these markers the assay is reproducible. Data from D18S69 microsatellite analysis did show small differences within experiments, being the least reproducible marker. Notice the small residual variability of all variables. The experimental design let us detect standard deviations of experiment factor 1.26 times the interassay SD, with statistical power $\beta = 0.8$. The error measure of the technique is summarized in Table 3. The five variables showed similar results, with errors in log scale ranging between 0.08 (D18S61) and 0.14 (D18S69). Thus, for a given marker, any log_{10} ratio measure can be considered as the observed value $\pm$ its error measure with 95% confidence level. Ratios obtained with all markers when performing the experiment using lower DNA concentrations (50 and 100 ng) were consistent with those achieved using 200 ng of DNA, being the final LOH status determination not influenced by the DNA concentrations tested (Figure 2).

Multiplexed LOH Analysis in Stage II Colorectal Carcinomas

LOH at chromosome 18q21 region was determined in 61 stage II A (pT3N0M0) tumors by multiplex amplification and capillary electrophoresis. Table 4 summarizes the relative frequencies obtained crossing all possible results of the five microsatellite markers. No constitutional homozygosity was detected, and tumors with a minimum of two informative markers were evaluated for chromosome 18q status. In our series, 8 of the 61 tumors (13%) showed additional bands in three or more microsatellite markers, and were classified as unstable. Besides unsta-

Table 3.   Individual Errors of the Log_{10} LOH Ratio Measures

	D18S55	D18S58	D18S61	D18S64	D18S69
Accuracy	0.082	0.131	0.075	0.098	0.148

ble tumors, allelic loss was detected in 31 of the 53 (58.5%) colorectal stage II A tumors. Four tumors displayed LOH in only one marker, whereas 27 lesions showed loss in two or more markers. Allelic loss of D18S55 and D18S69 markers was never detected independently of loss of other markers. The combined information of both D18S58 and D18S64 analysis was sufficient to predict chromosome 18q status in 95% of cases. The addition of D18S61 raised the prediction to 100% of cases. Mean informativeness was of 51 tumors per marker. When individual markers were analyzed, D18S61 and D18S64 turned out to be the most and the least informative markers, respectively (Table 4). Regarding the distribution of instability results, all markers showed a frequency between 11% and 13% (Table 4). The presence of individual instability of one marker was identified in five additional cases, two without chromosome 18 allelic loss and three with LOH.

Prospective Visual Re-Evaluation—Differences between Observers and Comparison of Gel and Capillary Electrophoresis Results

Results of 207 routine chromosome 18q determinations by acrylamide electrophoresis were re-evaluated. A discrepant result was considered when estimation of LOH from the two observers was different (loss versus no loss). Interobserver discordances for LOH assessment of individual markers ranged from 8.7 to 14.5%, and marker D18S58 accounted for the highest frequency of discrepancies. Results for all markers are presented in Table 5. When the global result of chromosome 18q status was considered, interobserver discordance was 9.2% (CI, 5.3 to 13.1).

Results obtained from electropherograms were compared with those obtained after visual re-evaluation of the stage II subset of cases (Figure 3). Because there were interobserver differences, comparison with the GeneScan results was performed versus both of them. When considering individual markers, mean differences between the two techniques were 19.9% (CI, 5.85 to 35.40) and 18.6% (CI, 2.36 to 37.27) for the two observers. For both observers, the most misleading markers were D18S58 and D18S61. When global chromosome 18q status was considered, discrepancies between the two assays were detected in 16.39% (CI, 7.10 to 25.68) and 8.2% (CI, 1.31 to 15.08) of determinations, for the two observers.
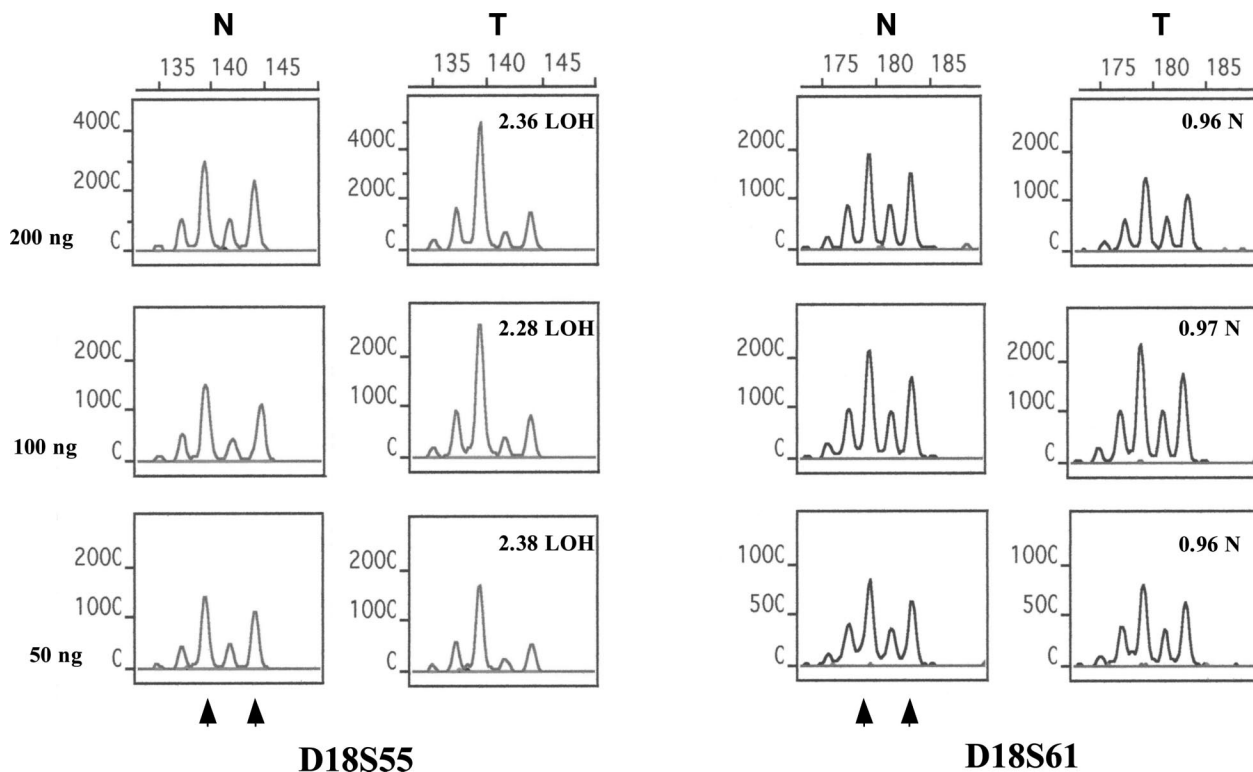
Figure 2. GeneScan electropherograms of two different microsatellites using three decreasing DNA concentrations. For each informative marker, the major peak of each allele selected for LOH analysis, consistent with highest one, is pointed by an **arrow**. The relative ratios between normal and tumor allele for each experiment are exposed.

Discussion

The prognostic value of chromosome 18q LOH in colon cancer was first described by Jen and colleagues,[8] using a panel of 10 microsatellite markers that spanned the entire long arm of the chromosome. The utility of chromosome 18q allelic loss as a prognostic indicator was soon confirmed in larger series of colorectal carcinomas[3,5,6,9,10] by PCR-based microsatellite analysis of different markers of the chromosome 18q21-22 region.

We have developed and validated a multiplex-fluorescent PCR assay for the detection of allelic loss using five microsatellite markers spanning the chromosome 18q21-22 area that had been shown to be closely associated with the presence of metastatic disease.[8] Our approach is a simple, rapid, and accurate method of LOH assessment. PCR multiplexing allows a faster analysis of different microsatellite markers in a single assay.[17,18] Because reliability of LOH-multiplexed assays has been controversial when using DNA obtained from paraffin-embedded tissues,[19] we performed reproducibility experiments to determine the robustness of this technique, demonstrating its high reliability for LOH anal-

Table 4.   Description of ABI Results

	D18S55	D18S58	D18S61	D18S64	D18S69	Overall cr.18q status
Allelic loss (%)	23.0	32.8	26.2	31.2	24.6	50.8*
No loss (%)	45.9	32.8	50.8	27.9	39.3	34.4
Instability (%)	11.5	13.1	11.5	13.1	13.1	13.1
Noninformative (%)	14.8	21.3	11.5	27.9	21.3	1.6
Not valuable (%)	4.9	0	0	0	1.6	0
Total (*n*)	61	61	61	61	61	61

*This is the overall frequency. If unstable tumors are not considered, the frequency of allelic loss rises to 58.5%.

Table 5.   Discrepancies between Two Observers: Frequencies for Each Marker and Confidence Limits (*n* = 207)

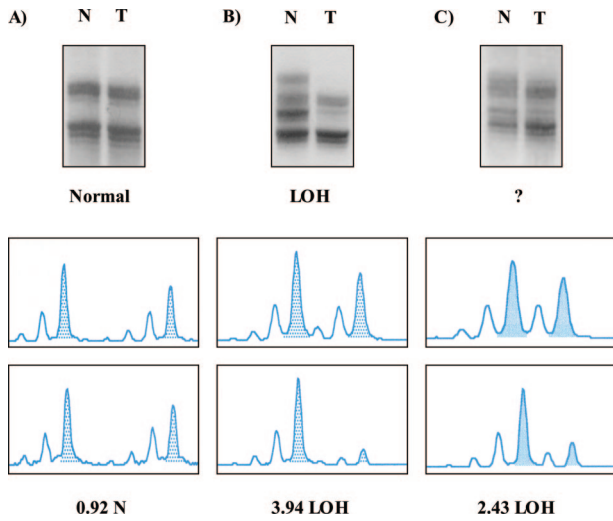| % Discrepancies	D18S55	D18S58	D18S61	D18S64	D18S69	Cr.18q status
LOH versus no LOH	9.2	14.5	8.7	9.7	10.1	9.2
% Inferior limit	5.3	9.7	4.9	5.6	6.0	5.3
% Superior limit	13.1	19.3	12.5	13.7	14.3	13.1

Figure 3. Polyacrylamide gel images and their corresponding electropherograms of marker D18S55. Representative cases for three different results are shown. The results given by the two methods in examples **A** and **B** are coincident, whereas the ambiguous result of acrylamide gel shown in example **C** is resolved by GeneScan analysis.

evidenced by results obtained comparing Gene Scan data and previous visual estimation results, fluorescent technology achieves superior resolution and avoids subjectivity, thus becoming a more accurate methodology for LOH determination.

Allelic loss was detected in 58.5% of colorectal stage II A tumors that did not show microsatellite instability. This frequency is slightly higher than those described in the majority of similar studies,[3,5,6,8–10,20] although higher frequencies have recently been described[21] (Figure 4). The combination of analyzed markers, the technical approach used, and the criteria for microsatellite evaluation may account for these differences. In our study, the combined information of both D18S58 and D18S64 analysis was sufficient to predict chromosome 18q status in 95% of cases. The addition of D18S61 raised the prediction to 100% of cases. In a similar manner, Jen and colleagues[8] suggested that markers D18S58 and D18S61 were sufficient to determine the status of the chromosome in 80% of samples, and additional markers were only required when no heterozygosity or instability was present. Even though not always, these markers have been included in the majority of chromosome 18q prognostic studies (Figure 4). As it emerges, many markers can be used for LOH assessment of this area. Nevertheless it would be convenient to define a reference panel of markers for chromosome 18q LOH determination, similarly to that assent for microsatellite instability determination,[22] for which

ysis in paraffin-embedded tissues. Based on these experiments, D18S55, D18S58, D18S61, and D18S64 are reproducible markers for LOH evaluation, whereas D18S69 marker displays statistically significant differences on measures between experiments. Finally, as

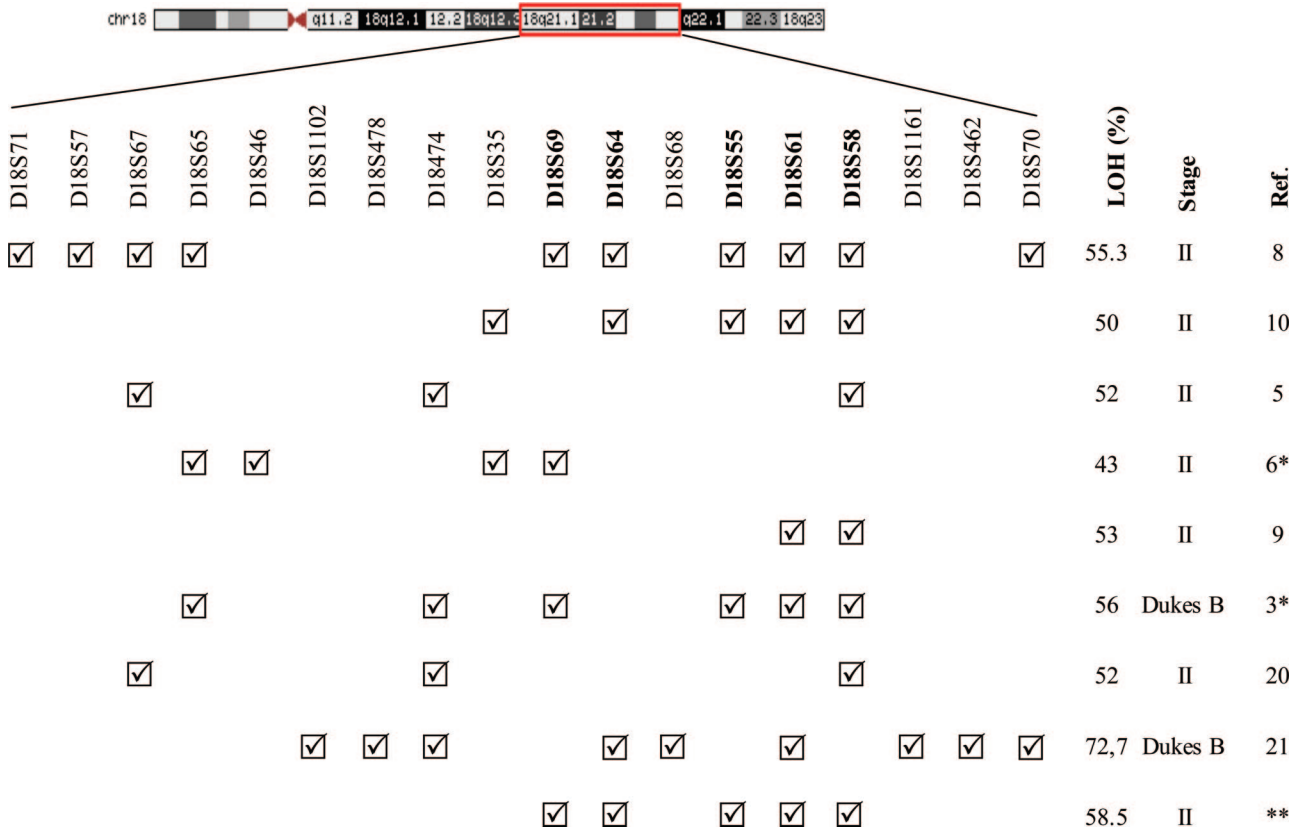| D18S71	D18S57	D18S67	D18S65	D18S46	D18S1102	D18S478	D18474	D18S35	D18S69	D18S64	D18S68	D18S55	D18S61	D18S58	D18S1161	D18S462	D18S70	LOH (%)	Stage	Ref.
☑	☑	☑	☑						☑	☑		☑	☑	☑			☑	55.3	II	8
								☑		☑		☑	☑	☑				50	II	10
		☑						☑						☑				52	II	5
			☑	☑					☑	☑								43	II	6*
													☑	☑				53	II	9
		☑				☑				☑		☑	☑	☑				56	Dukes B	3*
		☑				☑								☑				52	II	20
				☑	☑	☑			☑	☑		☑			☑	☑	☑	72,7	Dukes B	21
									☑	☑		☑	☑	☑				58.5	II	**

Figure 4. Microsatellite markers used for LOH determination at the long arm of chromosome 18 in stage II colorectal tumors. Revision of relevant results previously described. The frequencies of allelic loss detected with each panel of markers, altogether with the reference of the study are indicated. *An additional intragenic microsatellite located within the DCC (18qDCC-TA) is evaluated. **Inferior lane corresponds to the present study.

analogous multiplexed assays, including the most sensitive and specific consensus markers have been developed.[23,24] In this context, additional characteristics other than marker location, such as the repeat type, the frequency of heterozygosity, and the complexity of the peaks displayed, must be considered when deciding the markers to be included in a given assay.[24] In LOH analysis, the interval of the error measure should also be considered when electing markers. It is also important to settle the guidelines for defining the evaluation criteria for chromosome 18q determination in colorectal cancer. Several ratios have been used for LOH determination. In this study we have assigned LOH based on a ratio that allows detecting allelic imbalance when present at least in 50% of tumor cells in a pure tumor sample. This is a quite stringent ratio, being counterbalanced in our study by tissue scrapping of rich tumor areas and by considering LOH of the whole area when only one marker is lost. These parameters, together with the variability of the measures must be taken into account to perform an accurate LOH analysis. Thus, unifying the procedure for LOH determination is still under consideration.

Regarding instability results, in our series 13% of tumors were classified as chromosome 18q unstable. Because LOH cannot be determined in the presence of instability, unstable tumors are generally excluded from prognostic studies evaluating LOH. Interestingly, the inclusion of the microsatellite-unstable tumors in the group of tumors without 18q LOH has demonstrated a significant prognostic relevance in the largest patient series investigated so far for chromosome 18q status.[25] Moreover, a large number of studies have demonstrated that microsatellite instability phenotype is a predictive marker of good prognosis and differential response to adjuvant chemotherapy.[25] More recently, microsatellite instability status, as determined by immunohistochemistry, has been demonstrated as an independent predictive factor of good prognosis in T3N0M0 colon cancer.[26] In this context, microsatellite analysis of chromosome 18q may segregate patients into subsets of good and bad prognosis by detecting both LOH and microsatellite instability, in concert with other microsatellite instability analysis/criteria.

In conclusion, data from this study validate the fluorescent multiplex assay as an easy and reliable approach for chromosome 18q LOH determination. Evaluating previous published results, and despite methodological differences, the frequency of chromosome 18q allelic loss in stage II colorectal carcinoma seems to be around 50 to 60% of tumors, a frequency that should be taken into account when considering its prognostic value and putative impact in the choice of treatment. The establishment of consensus criteria for chromosome 18q status evaluation may contribute to a more accurate clinical management of stage II colorectal cancer patients.

Acknowledgments

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