A Simple Method for the Routine Detection of Somatic Quantitative Genetic Alterations in Colorectal Cancer

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Background & Aims: Several quantitative genetic alterations have been suggested to have in colorectal cancer (CRC) either a prognostic or a therapeutic predictive value. Routine detection of these alterations is limited by the absence of simple methods. **Methods:** The somatic quantitative multiplex polymerase chain reaction of short fluorescent fragments (QMPSF) is based on the simultaneous amplification under quantitative conditions of several dye-labeled targets both from tumor and nonmalignant tissues. For each patient, the resulting QMPSF fluorescent profiles are superimposed, and quantitative changes are simply detected by an increase or decrease of the corresponding fluorescent peaks. Two assays were developed and applied to 57 CRC: a "bar code" exploring several loci with known prognostic value and a "kinogram" studying the copy number change of kinase genes, against which inhibitors have been developed. **Results:** The bar code revealed that the most frequent alterations were the gain of *AURKA*/20q13 (53%) and *MYC*/8q24 (39%) and heterozygous deletion of *DCC*/18q21.3 (39%) and *TP53*/17p13 (23%). The kinogram detected a gene copy number increase for *AURKA*, *PTK2*, *MET*, and *EGFR* in 53%, 37%, 33%, and 28% of the tumors, respectively. QMPSF results were validated by comparative genomic hybridization and multiplex real-time polymerase chain reaction on genomic DNA. **Conclusions:** The somatic QMPSF is a simple method able to detect simultaneously on a routine basis several quantitative changes in tumors. Its flexibility will allow the integration of clinically relevant genes. This high throughput method should be a valuable complementary tool of fluorescent in situ hybridization and comparative genomic hybridization.

The optimization of clinical management of cancer patients requires to integrate progressively in pathology or molecular genetics laboratories the systematic screening of tumors for somatic molecular alterations with demonstrated prognostic or predictive value. In colorectal cancers (CRC), somatic genetic alterations whose detection has been recently suggested to be clinically relevant include (1) mutations of the *TP53* tumor suppressor gene, which have been recently associated with a shorter survival in stage III CRC patients[1,2]; (2) microsatellite instability (MSI), which is associated with a favorable clinical outcome[2–4]; and (3) quantitative alterations including loss or gain of genetic material. Loss of heterozygosity (LOH) at 18q and 17p detected in 49%–70% and 51%–66% of CRC, respectively, is a negative prognostic factor in stages II and III CRC.[5–12] In particular, the prognostic value of LOH in the vicinity of or at the *DCC* locus on chromosome 18q has been suggested in several studies.[13,14] Gains in the 20q13 region, reported in 53%–75% of CRC, have been associated with faster progression and worse survival.[8,15] Moreover, gene dosage alterations might also have a predictive value for targeted chemotherapy based on kinase inhibitors. This has been suggested by recent reports showing that, in CRC, increase in *EGFR* gene copy number is associated with a clinical response to anti-EGFR agents.[16,17] Considering the number of antikinase agents that are investigated in clinical trials, and the urgent need to identify reliable predictive molecular markers, the systematic evaluation of gene dosage alterations of targeted kinases might also be of interest. However, it still remains unclear which marker or combination of markers will be clinically relevant to predict outcome or treatment sensibility.

One of the main pitfalls limiting, at the present time, the screening of tumors for quantitative alterations with potential or predictive value is the absence of simple methods, which can be used on a routine basis. In the future, high-resolution comparative genomic hybridization (CGH) array will probably represent the most attractive tool able to

provide a molecular portrait of the tumor. Nevertheless, the cost of the equipment and analysis of each sample hampers its use in routine laboratories. Furthermore, confirmation of CGH results in larger series of patients, and fine delineation of the boundaries of recurrent quantitative alterations require having simple methods able to analyze specifically target regions.

To facilitate the routine detection of multiple gene dosage alterations in cancer, we have developed the somatic quantitative multiplex polymerase chain reaction (PCR) of short fluorescent fragments (QMPSF). QMPSF is a simple method relying on the simultaneous amplification of short genomic sequences under quantitative conditions, using dye-labeled primers, and on the comparison of profiles generated from tested and control DNA. We had initially developed QMPSF to optimize the molecular diagnosis of Mendelian diseases and, in particular, of hereditary nonpolyposis colorectal cancer (HNPCC)[18,19]; systematic screening of 332 French HNPCC families by QMPSF allowed us to show that germ-line MSH2 and MLH1 quantitative alterations are present in 20% and 7%, respectively, of the HNPCC families without any detectable MSH2 or MLH1 point mutations.[20] QMPSF was then applied to numerous monogenic or chromosomal disorders. All these applications have demonstrated that QMPSF is a highly sensitive method for the detection of both deletion and gain of genetic material[21–23] and that this method indeed optimizes the molecular diagnosis of inherited diseases. In the somatic version of QMPSF, several short fragments corresponding to target genes are PCR-amplified, both from patient tumors and normal tissues, and the QMPSF profiles are then compared.

We present in this study 2 applications of somatic QMPSF specifically designed for CRC: a "bar code" exploring several loci, the loss or gain of which has been shown to have a potential prognostic value, and a "kinogram" comparing, between tumor and nonmalignant tissue, the copy number of kinase genes.

Materials and Methods

Samples

Colorectal tissues from 57 patients (39 males, 18 females) who underwent surgery were collected immediately after surgical excision and stored at −80°C until use. The distribution of the tumors was as follows: 2 (3.5%) stage I (T1N0M0 + T2N0M0), 33 (57.9%) stage II (T3N0M0 + T4N0M0), 17 (29.8%) stage III (all T, N1 to N3, M0), and 5 (8.8%) stage IV (metastatic disease). Their MSI status was as follows: MSI+ (6 patients, 10.5%), MSI− (51 patients, 89.5%). For each patient, both tumor and nonmalignant tissues distant from the tumor were sampled; control H&E-stained section facing the tumor sample showed at least 50% malignant cells. DNA was extracted by standard methods, ie, proteinase K digestion and phenol-chloroform extraction.

Table 1. Percentage of Quantitative Gene Alterations Detected Using the QMPSF-Based Bar Code in 57 Colorectal Cancers

Gene	Location	Percentage of tumors with a gene copy number increase[a]	Percentage of tumors with a gene copy number decrease[b]
APC	5q21-q22	5	10.5
EGFR	7p12	28	0
BLK	8p23-p22	5	23
MYC	8q24.12-q24.13	39	0
TP53	17p13.1[c]	2	23
ERBB2	17q11.2-q12	9	3.5
DCC	18q21.3[c]	0	39
AURKA	20q13.2-q13.3[c]	53	0

[a]As detected by a peak ratio <1.2 (see Materials and Methods section).
[b]As detected by a peak ratio >0.8 (see Materials and Methods section).
[c]The prognostic value of quantitative alteration in this region has been previously demonstrated (see opening text).

Somatic QMPSF

Short genomic sequences (<260 base pairs [bp]) corresponding either to 8 target genes/regions with potential prognostic value in CRC (Table 1, *bar code*) or to 16 targeted kinase genes (Table 2, *kinogram*) were simultaneously amplified in a single tube, using dye-labeled primers (primer sequences are given in Tables 3 and 4). Both QMPSF also included 2 control amplicons corresponding to PCBD2 and HMBS, respectively, located on chromosomes 5q31 and 11q23. Genomic DNA (100 ng) was amplified by PCR in a final volume of 25 μL after an initial step of denaturation at 94°C for 3 minutes using the following conditions: for the bar code, 26 PCR cycles consisting of denaturation at 94°C for 10 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 20 seconds, followed by a final extension step at 72°C for 5 minutes; for the kinogram, 25 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 30 seconds, followed by a final extension step at 72°C for 5 minutes. DNA amplicons generated by QMPSF were separated on an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA), and the resulting fluorescent profiles were analyzed using the GeneScan 3.7 software (Applied Biosystems). For each patient, the QMPSF pattern generated from tumor DNA was superimposed to that generated from nonmalignant tissue DNA, after adjustment of control amplicons (PCBD2 and HMBS) to the same heights. For each targeted genomic region, the copy number ratio was determined by dividing the normalized peak height obtained from tumor tissue by that observed on nonmalignant tissue. All QMPSF analyses were performed at least twice. Based on an extensive number of