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Unique genetic profile of sporadic colorectal cancer liver metastasis *versus* primary tumors as defined by high-density single-nucleotide polymorphism arrays

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Most genetic studies in colorectal carcinomas have focused on those abnormalities that are acquired by primary tumors, particularly in the transition from adenoma to carcinoma, whereas few studies have compared the genetic abnormalities of primary *versus* paired metastatic samples. In this study, we used high-density 500K single-nucleotide polymorphism arrays to map the overall genetic changes present in liver metastases ($n = 20$) from untreated colorectal carcinoma patients studied at diagnosis *versus* their paired primary tumors ($n = 20$). *MLH1, MSH2* and *MSH6* gene expression was measured in parallel by immunohistochemistry. Overall, metastatic tumors systematically contained those genetic abnormalities observed in the primary tumor sample from the same subject. However, liver metastases from many cases (up to 8 out of 20) showed acquisition of genetic aberrations that were not found in their paired primary tumors. These new metastatic aberrations mainly consisted of (1) an increased frequency of genetic lesions of chromosomes that have been associated with metastatic colorectal carcinoma (1p, 7p, 8q, 13q, 17p, 18q, 20q) and, more interestingly, (2) acquisition of new chromosomal abnormalities (eg, losses of chromosomes 4 and 10q and gains of chromosomes 5p and 6p). These genetic changes acquired by metastatic tumors may be associated with either the metastatic process and/or adaption of metastatic cells to the liver microenvironment. Further studies in larger series of patients are necessary to dissect the specific role of each of the altered genes and chromosomal regions in the metastatic spread of colorectal tumors.
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Occurrence of distant metastasis in sporadic colorectal cancer (eg, liver metastasis) confers a poor prognosis. In fact, metastatic disease is the main cause of death in colorectal carcinoma patients, and the liver is the most common site for metastatic spread of colorectal carcinoma.[1,2] Current knowledge about the genetic pathways of clonal evolution

in colorectal carcinoma suggest that development of colorectal cancer could be triggered by the clonal expansion of cells that carry mutations, which most frequently involve the *APC, RAS, TP53* and/or *DCC* genes, and lead to a growth and/or survival advantage of tumor cells.[3] As metastatic cells derive from primary tumor cells, specific genomic alterations driving these ultimate steps of the metastatic cascade are expected to be acquired over the genomic profile of neoplastic cells from the primary tumor.[4] The genomic abnormalities, which are potentially characteristic of such advanced stages of the disease, are complex and so far, poorly described and partially understood. This relates to the fact that most genetic studies in colorectal cancer have focused on those abnormalities that are acquired in primary tumors, particularly, in the transition from adenoma to carcinoma, and few studies have compared these abnormalities with those observed in paired metastatic samples.[5–7] Despite this, multiple recurrent chromosomal abnormalities, which are found in primary tumors have been associated with metastatic colorectal carcinoma. Among others, these mainly include numerical changes such as gains of chromosomes 8q, 13q and 20q, and losses of the 1p, 8p, 17p and 18q chromosomal regions.[8–10] However, the molecular mechanisms underlying the association of such genetic profiles with metastatic colorectal carcinoma remain largely unknown.

Previous studies using conventional karyotyping,[5] comparative genomic hybridization (CGH),[5,7,11] fluorescence *in situ* hybridization (FISH)[9,11] or microsatellite markers to detect regions of loss of heterozygosity (LOH),[12] have largely failed in identifying recurrent chromosomal abnormalities acquired in metastatic *versus* primary colorectal tumors. This could be explained, at least in part, because of the relatively limited resolution of these techniques. More recently, the availability of high-density single-nucleotide polymorphism (SNP) arrays has facilitated the identification of small regions of chromosomal gains and losses because of its higher resolution (down to 2.5 kb),[13] and provides new opportunities in the identification of novel cancer genes involved in the metastatic process of colorectal cancer. However, previous reports in which high-density SNP arrays have been used to investigate the genetic profiles of colorectal carcinoma have specifically focused on primary tumor samples,[14] and to the best of our knowledge, no study has been reported so far in which high-density SNP arrays are employed to investigate the potential genetic differences between paired primary and metastatic tumors from colorectal carcinoma patients.

In the present study, we applied high-density (500 K) SNP mapping arrays—mean distance between the interrogated SNPs of 5.8 kb (median intermarker distance of 2.5 kb)—to map the overall genetic changes present in liver metastases from 20 untreated colorectal carcinoma patients studied at diagnosis *versus* their paired primary tumors ($n = 40$ samples). Our goal was to search for recurrent genetic differences between paired primary *versus* metastatic tumor samples that might contain candidate genes highly characteristic of metastatic liver disease.

Patients and methods

Patients and Samples

Tissue specimens from 20 sporadic colorectal adenocarcinomas and 20 paired liver metastases ($n = 40$ samples) were obtained from 20 patients (13 males and 7 females; median age of 70 years, ranging from 49 to 80 years) after informed consent had been given by each subject. It should be noted that only patients with metastatic lesions able to be resected were included in this cohort, which, therefore, is not representative of the whole colorectal cancer patient population. All patients underwent surgical resection of both tumor tissues at the Department of Surgery of the University Hospital of Salamanca (Salamanca, Spain). All tumors were diagnosed and classified according to the WHO criteria,[15] and they were all studied before any treatment was given. According to the tumor grade, 11 cases were classified as well-differentiated tumors, 8 as moderately- and one as poorly differentiated carcinomas. In all cases, histopathological grade was confirmed in a second independent evaluation by an experienced pathologist. Median follow-up at the moment of closing the study was of 37 months (range: 36–96 months). The study was approved by the local ethics committee of the University Hospital of Salamanca (Salamanca, Spain).

Seven primary tumors were localized in the rectum, and the other 13 were localized either in the right (cecum, ascending or transverse) or the left (descending and sigmoid) colon. The mean size of the primary tumors was of 5.3 ± 1.9 cm with the following distribution according to their TNM stage at diagnosis:[16] T3N0M0, two cases; T3N1M1, four cases; T3N1M0, four cases; T3N2M1, four cases; T4N0M1, one case; T4N0M0, three cases; T4N1M1, one case and; T4N2M1, one case. Liver metastases were identified either at the time of colorectal surgery ($n = 11$) or during the first year after initial diagnosis ($n = 9$); to date, patients have not shown any other metastasis. The mean size of the liver metastases was of 4.3 ± 2.2 cm.

After histopathological diagnosis was established, part of the primary tumor and its paired liver metastasis (both corresponding to a macroscopically tumoral region) were used to prepare single-cell suspensions. Once prepared, single-cell suspensions were resuspended in methanol/acetic acid (3/1; vol/vol) and stored at $-20\,^{\circ}C$ for further interphase FISH analyses, as recently described.[17] The remaining tissue was either fixed in formalin

and embedded in paraffin, or frozen in liquid nitrogen and stored at room temperature or at −80 °C, respectively. All tissues were evaluated after hematoxylin–eosin staining to confirm the presence of tumor cells and to evaluate their quantity in each individual sample. For SNP array studies, tumor DNA was extracted from representative areas of freshly frozen tumor tissues (primary tumors and liver metastases), which contained ≥65% epithelial tumor cells, localized mirror-cut to those used for iFISH analyses. In turn, normal DNA was extracted from peripheral blood leukocytes from the same patient. For the three types of samples (primary tumors, paired liver metastases and peripheral blood leukocytes), DNA was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer.

SNP Array Studies

Each DNA sample derived from primary tumors and liver metastases and normal peripheral blood leukocytes was hybridized to two different 250 K Affymetrix SNP Mapping arrays (*Nsp*I and *Sty*I SNP arrays, Affymetrix, Santa Clara, CA, USA); for this purpose, 250 ng of DNA per array was used, according to the instructions of the manufacturer. Fluorescence signals were detected using the Affymetrix GeneChip Scanner 3000 (Affymetrix), and average genotyping call rates of 94.4, 91.5 and 97.3% were obtained for primary tumors, liver metastases and normal peripheral blood DNA samples, respectively.

To identify copy number changes throughout the whole tumor genome, the aroma.affymetrix algorithm was used, following the CRMA v2 method described elsewhere[18] (R-software package, http://www.aroma-project.org) and the following sequential steps: (i) calibration for crosstalks between pairs of allele probes; (ii) normalization for probe nucleotide-sequence effects; and (iii) normalization for PCR fragment-length and probe localization effects. Then, data from the 250K *Sty*I and 250K *Nsp*I arrays was integrated into a single database, and raw copy number values were calculated as transformed $\log_2$ values of the primary tumor/normal peripheral blood, liver metastasis/normal peripheral blood, liver metastases/primary tumor ratios calculated for each individual patient.

To identify DNA regions with similar copy number values, we used Circular Binary Segmentation as implemented in the DNAcopy Bioconductor package[19] with the default parameters; a *P*-value ≤0.01 for ≥5 markers per DNA segment was used to define points with changes. We used the smoothed value by assigning the median segment value to each probe. For the identification of altered (gained or lost) DNA regions, a threshold was established on the basis of the changes observed in the fluorescence intensity of sequential DNA segments for primary tumor *versus* peripheral blood, liver metastasis *versus* peripheral blood, and for liver metastases *versus* primary tumor samples, for each of the 20 patients studied. $\log_2$ ratio values >0.09 and <−0.09 were used as cut-off thresholds to define the presence of increased and decreased copy number values, respectively. High-level gains (DNA amplification) were defined as regions with a mean $\log_2$ copy number ratio ≥0.25. The specific frequencies of both copy number gains and losses per SNP were established and plotted along individual chromosomes for each tumor sample analyzed, for all individual cases studied. On the basis of the empirical frequency distribution of gains and losses among the 20 primary and the 20 metastatic tumor samples, respectively, we took the common altered regions grouping the contiguous SNPs with adjusted *P*-values <0.01 (false discovery rate correction, based on the Benjamini and Hochberg procedure).[20] Minimal common regions were defined as the smallest subset of SNPs in the altered regions with the highest frequency of gains and losses. At least five contiguous SNPs were required to define a region. Genes in these regions were identified using Ensembl release 53 (http://www.ensembl.org). The pattern of copy number changes of the primary tumors analyzed here has been previously reported in detail in a recent study.[21]

Interphase FISH Studies

To evaluate the reproducibility of the SNP array results and to assess background noise impact of this technique, interphase FISH analyses of the same tumor samples was performed in parallel, using 24 probes directed against an identical number of regions from 20 different human chromosomes, which are frequently altered in sporadic colorectal carcinomas. Overall, our results showed a high degree of correlation between both methods; this also holds true when such analysis was restricted to the most frequently altered regions, as previously described.[21]

Immunohistochemistry

One block of formalin-fixed paraffin wax-embedded adenocarcinoma tissue was selected in each case. In all cases, this block comprised an area of normal colonic mucosa adjacent to the tumor. Sections (4 μm) were affixed to Superfrost-plus slides (CML, Nemours, France) and dried overnight at 37 °C. Paraffin was removed and the tissue rehydrated using xylene and ethanol. Slides were subjected to microwave antigen retrieval in 10 mM citrate buffer (pH 6) at 85 °C for 35 min and cooled in phosphate-buffered saline, pH 7.4 (Sigma). Endogenous peroxidase activity was blocked with 2% hydrogen peroxide in methanol, and slides were washed with phosphate-buffered saline before overnight incubation with the appropriate antibody at a dilution of

1:100. Commercially available monoclonal antibodies against the nuclear proteins MLH1 (Clone G168-15; BD Biosciences, San Jose, CA, USA), MSH2 (Clone FE11; Biocare Medical, CA, USA) and MSH6 (Clone BC/44; Biocare Medical) were applied, followed by staining with Strept ABC complex/HRP Duet kit (DAKO, Copenhagen, Denmark) in conjunction with diamino benzedene 180 mg in 300 ml phosphate-buffered saline with 300 ml hydrogen peroxide. Sections were washed under running tap water and then lightly counterstained in Mayer's hematoxylin. Loss of expression was recorded when nuclear staining was absent from all malignant cells, but preserved in normal epithelial and stroma cells. Two observers assessed all cases independently.

Statistical Methods

For all continuous variables, mean values and their s.d. and range were calculated using the SPSS software package (SPSS 12.0. Chicago, IL USA); for dichotomic variables, frequencies were reported. To evaluate the statistical significance of differences observed between groups, the Mann–Whitney *U*-test and the χ^2-test were used for continuous and categorical variables, respectively (SPSS).

Results

Frequency and Chromosomal Localization of Copy Number Changes in Liver Metastasis from Colorectal Carcinoma

Overall, liver metastases from the 20 colorectal cancer patients analyzed systematically contained those chromosomal abnormalities that were identified in their paired primary colorectal carcinomas; (please note that the later have been previously described in detail for a larger series of patients).[21] Despite this, some aberrations were either newly acquired or more frequently found in liver metastases than in their paired primary tumors, which could reflect an increased genetic instability of neoplastic cells from metastatic *versus* primary tumor samples (Figure 1).

In detail, all liver metastases showed copy number changes in at least one chromosomal region. The highest frequency of copy number losses detected corresponded to chromosomes 1p ($n=16$; 80%), 17p ($n=18$; 90%) and 18q ($n=19$; 95%); in turn, copy number gains more frequently involved chromosomes 7p ($n=18$; 90%), 8q ($n=15$; 75%), 13q ($n=14$; 70%) and 20q ($n=15$; 75%) (see Supplementary Table S1). Interestingly, each of these regions has been previously found to contain

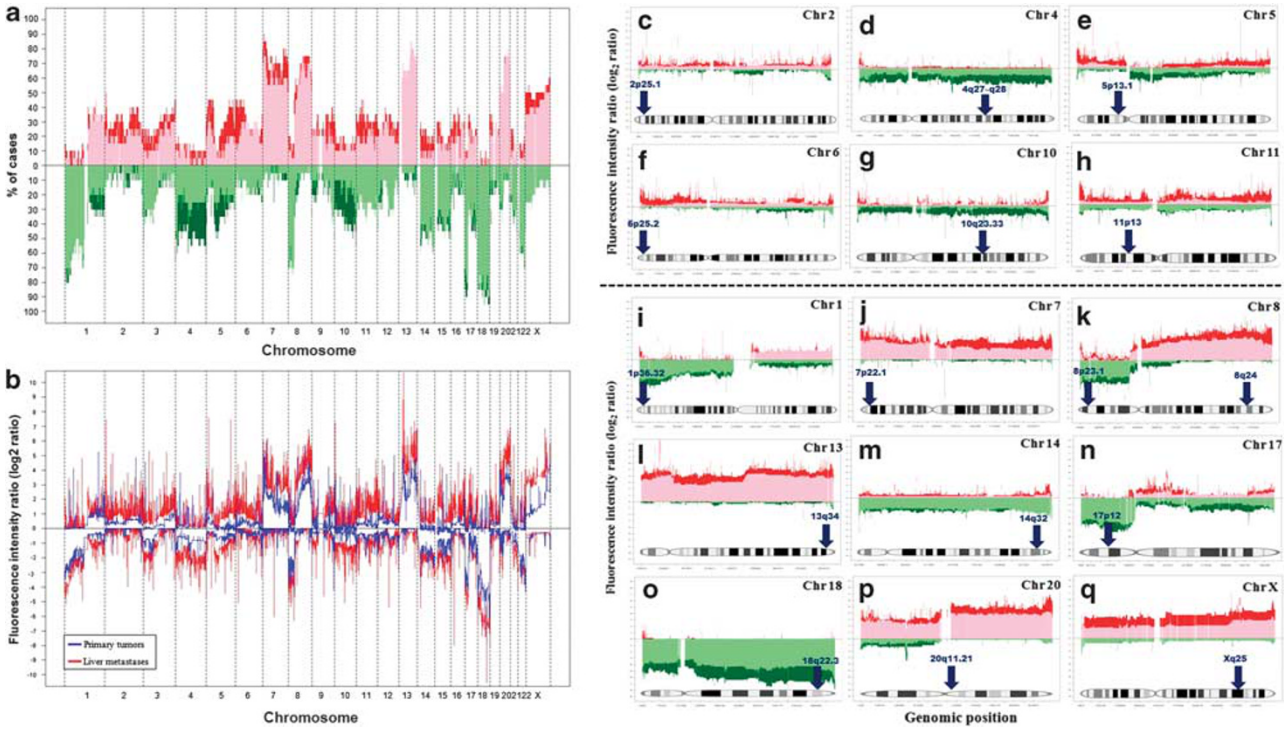

Figure 1 Metastatic colorectal cancer genome for the 20 colorectal carcinoma patients genotyped on the Affymetrix 500 K SNP array platform. A summary plot showing the frequency (**a** (in the left)) and fluorescence intensity $\log_2$ ratios (**b** (in the left)) of those copy number gains (plotted in red above zero values in the *x* axis) and losses (plotted in green below zero values in the *x* axis) identified in primary sporadic colorectal tumors (light colors), and their paired liver metastases (dark color) are displayed for the whole genome. The panels in the right show abnormalities identified in primary sporadic colorectal tumors (light colors) and their paired liver metastases (dark color) for individual chromosomes, which showed new abnormalities in metastatic *versus* primary tumor samples (**c–h**), or displayed an increased frequency of abnormalities in metastatic samples, which were already detected in primary tumors (**i–q**). Arrows point to regions of interest.

genes, which are altered/involved in colorectal cancer (eg, *ANGPT2, UBR5, KLF10, EIF3H, NOV, DCT, ABCC4, SLC15A1, EFNB2, IRS2, ING1, MAP2K4* (mitogen-activated protein kinase kinase-4)*, ID1, BCL2L1, MYLK2, CBFA2T2* and *E2F1*) and/or genes that are relevant to the metastatic process (eg, *ANGPT2, RRM2B, KLF10, RAD21, NOV, POU4F1, SPRY2, DCT, CLDN10, EFNB2, IRS2, COL4A2, ING1, MYH8, MAP2K4, ID1, BCL2L1, TPX2, MYLK2* and *E2F1*), in addition to genes associated with other malignancies (ie, *TRPS1, BTF3L1, DNAJC3, STK24, TM9SF2, LIG4, ARHGEF7, SCO1, MYOCD, GALR1, HCK* and *SMC1B*; Table 1). From them, the *ANGPT2, MAP2K4, E2F1, ID1* and *BCL2L1* genes have been reported to be involved in mechanisms that lead to increased cell proliferation and angiogenesis, and they have been found to be altered in both colorectal cancer and the metastatic events.

Frequency and Chromosomal Localization of High-Level Copy Number Gains in Liver Metastases from Colorectal Carcinomas

Chromosome 7 showed 17 regions, which displayed high level genetic amplification (Table 2) with mean $\log_2$ ratio fluorescence intensities of 0.28 (range: 0.25–0.36). These 17 regions were distributed along the whole chromosome 7 at the 7p22, 7p21, 7p15, 7p12, 7q22 and 7q36 chromosome bands, and they were all found to be altered (amplified) in $\geq$14/20 cases studied (70%; see Supplementary Table S2). These regions contain multiple genes, which have been recurrently associated with the pathogenesis of colorectal cancer and the metastatic process: *FSCN1, TWIST1, ITGB8, DFNA5, HOXA7, GRB10, EGFR, AZGP1, MCM7, EPHB4* and *MUC3A* (Table 2). In turn, for chromosome 8, only two regions of high-level genetic amplification (mean fluorescence intensities of 0.42 and 0.37, respectively) were detected; both regions were localized at the 8q24 chromosome band, and they involved the *MTSS1* and *ASAP1* genes (Table 2). Additionally, chromosomes 13 and 20 also displayed three regions (two in chromosome 13 and one in chromosome 20) with high level genetic amplification (mean fluorescence intensities of 0.45 and 0.43, respectively), containing genes potentially involved in the pathogenesis of colorectal cancer and the metastatic process, the *KLF5* and *IRS2* and the *MMP9* genes coded in the 13q22.1, 13q34 and 20q13.12 chromosomal regions, respectively (Table 2).

Acquired Chromosomal Abnormalities in Liver Metastases

In individual patients, primary tumors and their paired liver metastases frequently revealed the same chromosomal changes at both sites (Figure 1). However, liver metastases from 8 out of 20 cases showed acquisition of new genetic abnormalities that were not found in their paired primary tumors.

Table 1 Chromosomal regions, which most frequently displayed copy number alterations by SNP arrays, in colorectal liver metastases and that contain genes commonly associated with colorectal cancer and the metastatic process ($n = 20$)

Altered chromosomal regions (bp)	*Region length (bp)*	*Number of SNPs*	*Chromosome band*	*Event*	*Altered cases (%)*	*Cancer-associated genes*
Chr8: 6 319 564–6 393 980	74 416	41	8p23.1	Deletion	70	***ANGPT2***[a]
Chr8: 102 281 574–104 598 943	2 317 369	449	8q22.3	Gain	75	***RRM2B***, *UBR5*[a], ***KLF10***,
Chr8: 116 722 193–118 020 419	1 298 226	222	8q23.3	Gain	75	*TRPS1*, *EIF3H*[a], ***RAD21***
Chr8: 120 491 103–120 508 049	16 946	8	8q24.12	Gain	75	***NOV***[a]
Chr13: 75 649 333–83 553 225	7 903 892	1469	13q22.3	Gain	70	*BTF3L1, POU4F1, SPRY2*
Chr13: 93 770 996–94 062 605	291 609	72	13q31.3	Gain	70	***DCT***[a]
Chr13: 94 410 370–94 792 167	381 797	118	13q31.3	Gain	70	*ABCC4*[a]
Chr13: 94 803 129–95 581 085	777 956	80	13q31.3	Gain	70	***CLDN10,*** *DNAJC3*
Chr13: 96 635 106–99 063 992	2 428 886	530	13q32.1	Gain	70	*STK24*, *SLC15A1*[a], *TM9SF2*
Chr13: 105 576 623–106 900 640	1 324 017	327	13q33.2	Gain	70	***EFNB2***[a]
Chr13: 107 631 285–107 678 245	46 960	15	13q33.3	Gain	70	LIG4
Chr13: 109 092 251–110 808 910	1 716 659	406	13q34	Gain	70	***IRS2, COL4A2, ING1***[a], *ARHGEF7*
Chr17: 10 134 845–11 066 755	931 910	178	17p13.1	Deletion	90	***MYH8***, *SCO1*
Chr17: 11 124 244–12 787 020	1 662 776	312	17p12	Deletion	90	***MAP2K4***[a], *MYOCD*
Chr18: 71 151 155–73 487 286	2 336 131	645	18q22.3	Deletion	95	*GALR1*
Chr20: 29 314 247–30 386 296	1 072 049	103	20q11.21	Gain	75	***ID1***, ***BCL2L1***[a], ***TPX2***, *HCK, MYLK2*[a]
Chr20: 31 377 143–32 096 987	719 844	81	20q11.21	Gain	75	*CBFA2T2*[a], ***E2F1***[a]
Chr22: 44 114 817–44 172 947	58 130	16	22q13.2	Deletion	70	*SMC1B*

[a]Genes that have been described to be involved/altered in colorectal cancer, and genes that have been commonly associated with the metastatic process are shown in bold italics.

Table 2 Chromosomal regions, which most frequently displayed high-level genetic amplification by SNP arrays, and which contained genes commonly involved/altered in colorectal cancer and/or associated with the metastatic process ($n=20$)

Amplified chromosomal regions (bp)[a]	*Chromosomal band*	*Mean log_2 ratio*	*Maximum log_2 ratio*	*Altered cases (%)*	*Cancer-associated genes*
Chr7: 2 888 108–2 965 622	7p22.2	0.26	1.40	80	*CARD11*
Chr7: 4 624 574–5 634 592	7p22.1	0.26	1.13	85	***FSCN1***[b]
Chr7: 19 099 597–19 164 657	7p21.1	0.29	1.33	80	***TWIST1***[b]
Chr7: 20 315 252–20 348 199	7p15.3	0.26	0.93	80	***ITGB8***[b]
Chr7: 24 288 850–24 294 445	7p15.3	0.28	1.68	80	*NPY*[b]
Chr7: 24 761 544–24 764 289	7p15.3	0.27	1.45	80	***DFNA5***[b]
Chr7: 27 110 282–27 278 326	7p15.2	0.36	1.67	70	*HOXA5, HOXA7*[b], *HOXA9, HOXA11, HOXA13*
Chr7: 28 169 667–28 211 202	7p15.2	0.28	1.19	70	*JAZF1*
Chr7: 50 163 751–50 752 627	7p12.2	0.27	1.93	75	*IKZF1, DDC*
Chr7: 50 797 965–50 839 403	7p12.2	0.32	1.41	75	*SLC4A2, FASTK*
Chr7: 50 797 965–50 839 403	7p12.2	0.33	1.42	75	***GRB10***[b]
Chr7: 54 954 150–56 213 585	7p11.2	0.27	1.10	75	***EGFR***[b], *PSPH*
Chr7: 99 301 754–101 811 250	7q22.1	0.25	0.70	70	***AZGP1***[b], ***MCM7***[b], ***CUX1***, ***EPHB4***[b], ***MUC3A***[b], *MUC12*[b]
Chr7: 105 711 183–105 715 751	7q22.2	0.25	0.88	70	*PBEF1*
Chr7: 154 677 722–155 005 086	7q36.3	0.27	0.91	70	EN2
Chr7: 156 357 544–156 630 253	7q36.3	0.28	0.63	70	*MNX1, UBE3C*
Chr7: 156 893 472–158 147 850	7q36.3	0.29	1.34	70	*PTPRN2*
Chr8: 125 800 442–125 834 484	8q24.13	0.42	1.19	85	MTSS1
Chr8: 131 064 043–131 191 826	8q24.21	0.37	0.98	70	***ASAP1***[b]
Chr13: 72 497 695–72 659 497	13q22.1	0.41	0.73	70	***KLF5***[b]
Chr13: 79 810 102–79 825 947	13q31.1	0.46	1.39	70	SPRY2
Chr13: 90 792 026–90 811 945	13q31.3	0.47	1.50	70	*MIRHG1*
Chr13: 98 007 816–98 035 844	13q32.2	0.43	1.49	70	*STK24*
Chr13: 109 205 907–109 255 030	13q34	0.44	1.20	70	***IRS2***[b]
Chr13: 109 743 976–109 764 350	13q34	0.47	1.47	70	*COL4A1, COL4A2*
Chr13: 110 549 062–110 578 598	13q34	0.45	1.79	70	*ARHGEF7*
Chr20: 41 247 578–41 278 159	20q12	0.41	1.10	70	*PTPRT*
Chr20: 44 007 866–44 178 129	20q13.12	0.41	0.88	70	***MMP9***[b]
Chr20: 59 241 454–59 268 793	20q13.33	0.47	1.8	70	*CDH4*[b]

[a]Only those regions, which were recurrently amplified in at least 14 out of 20 cases analyzed (>70%) are listed.
[b]Genes that have been described to be involved/altered colorectal cancer, and genes that have been commonly associated with metastatic processes are shown in bold italics.
High-level genetic amplification was defined *versus* those with an average log_2 copy number ratio ≥ 0.25.

These new metastatic aberrations included copy number gains at chromosomes 2p, 5p, 6p, 7q and 11p, together with copy number losses of chromosomes 4, 5q and 10q (Table 3). The specific abnormalities, which were recurrently detected in 8 out of 20 colorectal carcinomas metastasis for those chromosomal regions that showed a normal diploid profile in their corresponding (paired) primary tumors, are shown in Figure 2. As illustrated, these metastatic abnormalities involved chromosomal regions which harbor i) tumor suppressor genes that have a key role in the metastatic process (eg, the *ANXA5*, *CCNA2*, *IL2* and *IL21* genes at chromosome 4q27; the *PLK4, IL15, GAB1, HHIP* and *SMAD1* genes coded at the 4q28.1 chromosome regions and the *PTEN* gene coded at the 10q23.33 chromosomal region) and; (ii) oncogenes (eg, the *PTGER4* and *PRKAA1* genes coded at chromosome 5p13.1, and both the *RIPK1* and *NQO2* genes coded at chromosome 6p25.2); copy number gains of the former two oncogenes have been associated with advanced colorectal carcinoma. Many other genetic aberrations were present in liver metastases from colorectal carcinoma analyzed, but at lower frequencies (Figure 1).

Correlation Between the Chromosomal Changes Detected by Interphase FISH and SNP Array Studies

Overall, the chromosomal abnormalities identified by interphase FISH in liver metastases showed profiles similar to those found by SNP array studies, also when such analysis was restricted to the most frequently altered regions. Thus, gains/amplification at 7q were detected in 60% of the cases by interphase FISH *versus* 70% by SNP array studies ($r^2=0.67$; $P<0.001$); similarly, gains/amplification of chromosomes 8q (found in 70% of cases by interphase FISH *vs* 75% by SNP array studies;

Table 3 Metastatic colorectal cancer genome for the 20 colorectal cancer patients genotyped on the Affymetrix 500 K SNP array platform: chromosomal abnormalities identified exclusively in liver metastases (and not in their paired primary tumors), which involved chromosomal regions that contain genes commonly associated with cancer and/or the metastatic process

Altered chromosomal regions (bp)	*Chromosome band*	*Event*	*Number of altered cases*[a]	*Mean log_2 ratio in liver metastases*	*Cancer-associated genes*
Chr2: 11 301 969–11 420 624	2p25.1	Gain	7	1.13	—
Chr4: 11 217 752–12 280 681	4p15.33	Deletion	7	−23.76	—
Chr4: 12 402 361–14 483 843	4p15.33	Deletion	7	−53.43	—-
Chr4: 14 537 247–16 743 438	4p15.33	Deletion	7	−48.17	***BST1***[b], ***FGFBP1***[b], ***PROM1***[b]
Chr4: 95 174 065–97 996 020	4q22.3	Deletion	7	−58.56	***BMPR1B***[b]
Chr4: 98 021 823–101 566 164	4q22.3	Deletion	7	−55.19	***EIF4E***[b], *ADH5*, ***MTTP***[b]
Chr4: 122 461 059–126 659 227	4q27	Deletion	8	−61.43	***ANXA5***[b], ***CCNA2***[b], ***IL2***[b], ***IL21***[b], *NUDT6*[b], *FAT4*[b]
Chr4: 126 668 154–147 205 681	4q28.1	Deletion	8	−324.84	***PLK4***[b], ***SLC7A11***, *NARG1*, *SETD7*, ***IL15***[b], *INPP4B*, ***GAB1***[b], ***SMARCA5***, ***HHIP***[b], ***SMAD1***[b]
Chr4: 147 218 521–148 654 896	4q31.22	Deletion	7	−26.13	*EDNRA, POU4F2*, *LSM6*
Chr4: 148 658 165–150 007 154	4q31.23	Deletion	7	−24.35	***EDNRA***, *ARHGAP10*, ***NR3C2***[b]
Chr4: 151 081 476–169 411 644	4q31.3	Deletion	7	−317.79	*LRBA*, *MAB21L2*[b], ***FBXW7***[b], ***SFRP2***[b], ***ANXA10***[b], ***LRAT***, ***PDGFC***[b], *PPID*[b], ***CPE***[b]
Chr4: 171 433 995–173 576 567	4q33	Deletion	7	−37.39	-
Chr4: 175 432 179–176 373 292	4q34.1	Deletion	8	−17.93	*HPGD*[b]
Chr4: 176 393 349–181 031 240	4q34.2	Deletion	7	−93.92	***VEGFC***[b]
Chr4: 181 036 139–183 912 221	4q34.3	Deletion	8	−81.56	-
Chr4: 183 917 599–185 620 740	4q35.1	Deletion	7	−28.21	***IRF2***[b], ***DCTD***[b], ***ING2***[b]
Chr4: 188 066 517–189 969 188	4q35.2	Deletion	8	−31.94	*ZFP42*
Chr5: 31 602 359–32 770 165	5p13.1	Gain	7	17.03	*NPR3*, ***PDZD2***
Chr5: 40 710 736–40 901 620	5p13.1	Gain	8	1.55	***PTGER4***[b], ***PRKAA1***[b]
Chr5: 42 953 413–43 243 995	5p12	Gain	7	3.87	—
Chr5: 43 501 986–43 901 209	5p12	Gain	7	2.68	—
Chr5: 58 935 951–59 201 448	5q12.1	Deletion	6	−6.29	—
Chr5: 141 299 822–141 552 149	5q31.3	Gain	7	2.14	*RNF14*[b]
Chr6: 1 310 265–1 608 630	6p25.3	Gain	7	7.89	—
Chr6: 2 713 210–3 252 280	6p25.2	Gain	8	9.74	***RIPK1***[b], ***NQO2***[b], *SERPINB1*, *SERPINB6*[b], *SERPINB9*
Chr6: 3 391 923–3 411 021	6p25.2	Gain	8	0.98	—
Chr6: 3 663 243–3 673 023	6p25.2	Gain	8	0.90	—
Chr6: 4 026 261–4 342 058	6p25.2	Gain	7	5.45	—
Chr6: 4 840 846–5 220 281	6p25.1	Gain	7	7.36	—
Chr6: 5 493 557–5 966 877	6p25.1	Gain	7	7.00	—
Chr6: 6 476 360–6 654 484	6p25.1	Gain	7	4.22	—
Chr6: 7 647 658–7 683 958	6p24.3	Gain	8	1.18	***BMP6***[b]
Chr6: 10 495 977–10 532 295	6p24.3	Gain	7	0.96	***TFAP2A***[b]
Chr6: 13 593 062–13 758 950	6p23	Gain	7	2.08	—
Chr6: 13 857 081–14 361 384	6p23	Gain	7	6.40	—
Chr6: 15 143 396–15 884 421	6p23	Gain	7	8.47	***CD83***[b]
Chr6: 16 085 720–16 448 755	6p22.3	Gain	7	4.71	—
Chr6: 17 210 737–18 442 309	6p22.3	Gain	7	12.44	*NRN1*, ***DEK***, *BPHL*, ***RIPK1***[b]
Chr7: 158 430 322–158 640 662	7q36.3	Gain	7	3.79	—
Chr10: 71 077 186–71 425 507	10q13.2	Deletion	6	−10.26	—
Chr10: 83 990 316–84 564 355	10q23.1	Deletion	6	−9.53	—
Chr10: 89 574 656–89 676 489	10q23.2	Deletion	7	−1.89	***PTEN***[b]
Chr10: 97 903 413–100 323 090	10q23.33	Deletion	6	−21.38	*BLNK*, *DNTT*, ***FRAT1***[b], ***LOXL4***[b], ***PGAM1***[b], ***SFRP5***[b]
Chr11: 32 104 370–34 401 072	11p13	Gain	6	17.60	***LMO2***, *HIPK3*, ***WT1***[b]
Chr11: 43 653 493–45 414 563	11p11.2	Gain	6	19.81	***CD82***[b], ***EXT2***[b], *ALKBH3*

[a]Number of cases with chromosomal abnormalities identified exclusively in liver metastases and not in their paired primary tumors.
[b]Genes that have been described to be altered/involved in colorectal cancer, and genes that have been commonly associated with the metastatic process are shown in bold italics.

$r^2 = 0.79$; $P < 0.001$), 13q (80 *vs* 70%; $r^2 = 0.78$; $P < 0.001$) and 20q (80 *vs* 75%; $r^2 = 0.80$; $P < 0.001$), as well as deletions of chromosomes 8p (65 *vs* 70%; $r^2 = 0.81$; $P < 0.001$), 17p (75 *vs* 90%; $r^2 = 0.64$; $p = 0.02$) and 18q (75 *vs* 95%; $r^2 = 0.63$; $p = 0.03$) were detected at similar frequencies with both methods.

Microsatellite Status

All primary tumors examined ($n = 20$) showed a normal expression of the MLH1, MSH2 and MSH6 mismatch repair proteins in the nucleus and adjacent non-neoplastic tissue elements.

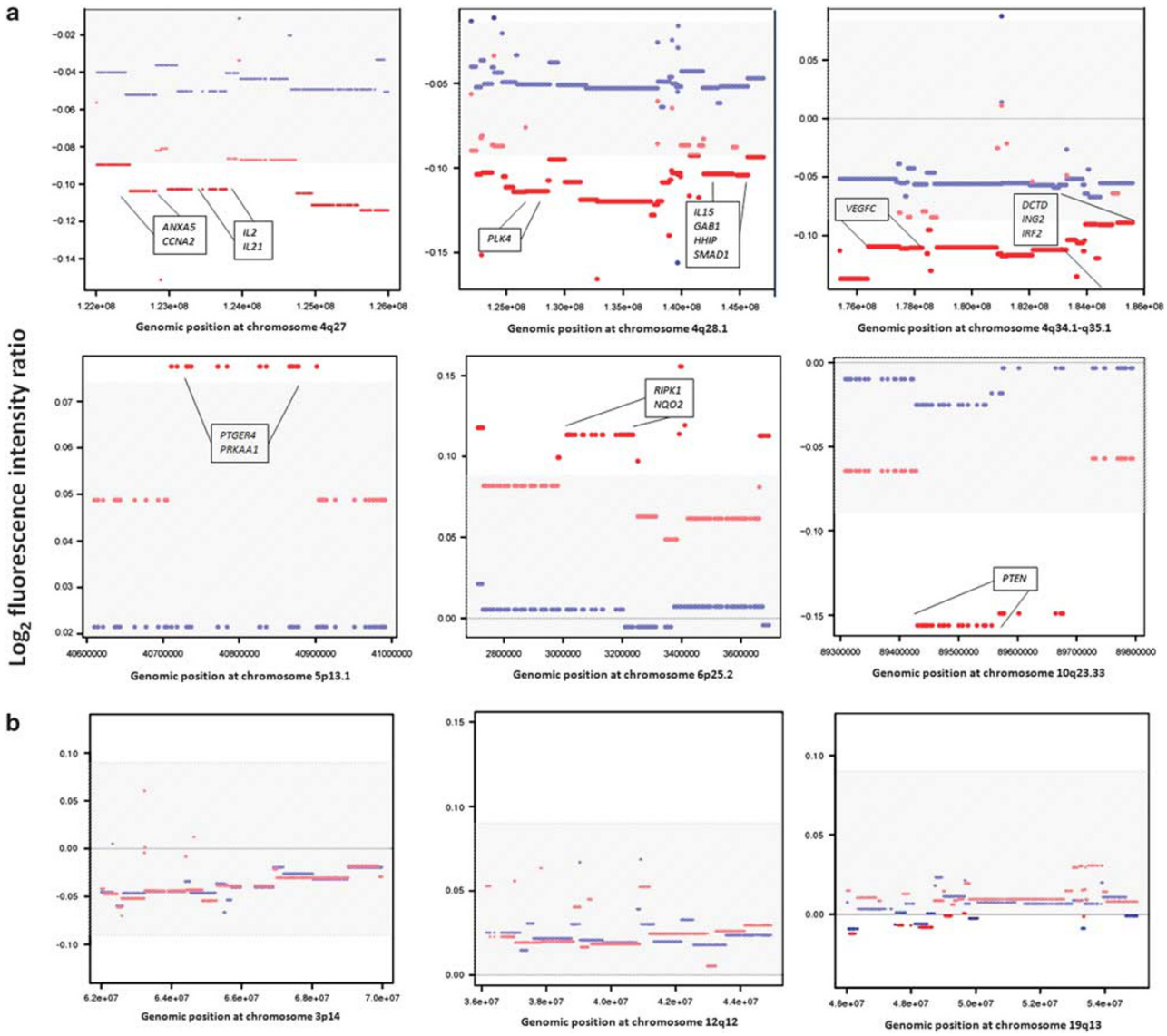

Figure 2 Metastatic colorectal cancer genome for the 20 colorectal carcinoma patients genotyped on the Affymetrix 500 K SNP array platform; copy number changes detected in liver metastases ($n = 8/20$ cases; red color) *versus* their paired primary tumors (blue color) for the 4q27, 4q28.1, 4q34.1, 5p13.1, 6p25.2 and 10q23.33 chromosomal regions (**a**). Genes contained in the newly altered chromosomal regions are listed in italics capital letters. Log_2 ratios >0.09 and <-0.09 (shown as colored background) were used as cut-off thresholds to define the presence of increased and decreased copy number values, respectively. All copy number changes detected between liver metastases *versus* their paired primary tumors showed statistically significant differences ($P<0.001$). As an example, the 3p14, 12q12 and 19q13 chromosomal regions, which did not show any differences between paired primary and metastatic lesions, are also shown (**b**).

Discussion

This study focused on the genetic characterization of liver metastases that occur in the context of primary colorectal carcinoma. To the best of our knowledge, this is the first study that compares the genetic abnormalities found in liver metastases *versus* paired primary colorectal tumors, in which high-resolution 500 K SNP arrays have been systematically used. Overall, primary tumors and their paired metastases from individual patients frequently revealed many common chromosomal changes at both sites; these findings support the existence of a close genetic relationship between primary colorectal tumors and their paired liver metastases, as previously suggested.[17] Genetic changes observed in common in both groups of samples included gains of chromosomes 7, 8q, 13q and 20 and losses of the 1p, 4, 8p, 17p, 18 and 22q chromosomes with normal expression of the MLH1, MSH2 and MSH6 mismatch repair proteins. In line with other studies, all our metastatic tumors showed a higher frequency of these chromosomal abnormalities than primary colorectal tumors,[7,22] and some of these abnormalities, together with deletions on chromosome 15q, have been associated with disease progression.[23] Previous studies in which the genetic abnormalities of colorectal carcinoma have been

investigated by conventional cytogenetics,[24] FISH,[17] CGH,[25] array CGH[26] and low-resolution 50 K SNP arrays[23] have also found that most of these genetic abnormalities are recurrently identified in primary tumors from metastatic colorectal carcinoma. On the basis of the high frequency of these chromosomal abnormalities in both primary and metastatic samples, it could be hypothesized that they reflect a metastatic genetic profile of colorectal carcinoma that could be of great clinical utility for the identification of colorectal carcinoma patients at higher risk of developing liver metastases, already at diagnosis.

Interestingly, high-level genetic amplification was found at specific regions of chromosomes 7, 8, 13 and 20; overall, 43 genes commonly involved/altered in colorectal cancer and/or associated with the metastatic process are coded in these regions. Of note, 17 of these 43 genes have been associated with progression of hepatocellular carcinomas.[27–29] Altogether, these findings could suggest that these genetic abnormalities that are acquired by metastatic colorectal carcinoma cells in the liver could be associated with homing and/or adaptation to the liver microenvironment. Among these genes, overexpression of *TWIST1* has been demonstrated to induce angiogenesis; at the same time, it has been associated with both the development of metastasis in hepatocellular carcinomas[30] and an unfavorable outcome in colorectal carcinoma patients;[31] in turn, increased expression of *IRS2*—commonly found in human hepatocellular carcinoma specimens and hepatoma cell lines—[32] has been associated with colon tumorigenesis, in which it contributes to tumor progression and an aggressive clinical behavior.[33] In line with this hypothesis, preliminary studies on genomic differences detected in primary colorectal carcinomas *versus* paired brain metastases have described a genetic profile consisting of gains of 8q, 12p, 12q, 20p, and loss of 5q in brain metastasis,[34] which is clearly different from that observed in our liver metastases. The different genetic signature associated with liver *versus* brain metastases could mirror the unique adaptation process of metastatic tumor cells to each specific microenvironment. Despite these findings, those three chromosomal regions, which showed the highest levels of amplification, were 13q31.3, 13q34 and 20q13.33, where four known cancer genes (*MIRHG1, COL4A1, COL4A2* and *CDH4*) are coded. To the best of our knowledge, no specific association between amplification of these genes and colorectal carcinoma has been reported so far; conversely, deregulation of these genes has been associated with neuroblastoma,[35] esophageal squamous cell carcinoma[36] and glioblastoma multiforme.[37]

In the present study, we also show the existence of recurrent genetic changes between paired primary and metastatic colorectal tumor cells. Such changes mainly consist of (1) an increased frequency of genetic lesions of chromosomes that have been associated with metastatic colorectal carcinoma (1p, 7p, 8q, 13q, 17p, 18q, 20q) and, more interestingly, (2) acquisition of new chromosomal abnormalities (eg, losses of chromosomes 4 and 10q and gains of chromosomes 5p and 6p). Interestingly, the former abnormalities involved chromosomal regions that encode for up to 11 genes, which have been previously found to be involved in the metastatic process of colorectal carcinoma. As an example, the *ANGPT2* gene (localized in chromosome 8 at 8p23.1) is known to be involved in angiogenic processes and has been previously associated with an invasive/malignant potential;[38] in turn, the *E2F1* gene (20q11.21) has been shown to have a crucial role in the control of cell cycle through down-regulation of tumor suppressor proteins.[39] Similarly, the *ID1* and *BCL2L1* genes (both coded in the same chromosomal region at 20q11.21) are also known to have a role in cell growth, senescence and differentiation, and the carcinogenesis of human colorectal carcinoma,[40] whereas overexpression of the Id-1 protein has been associated with tumor progression in colorectal carcinoma.[41] In turn, Paredes *et al*[11] have recently described that losses of chromosome 17p in metastatic colorectal cancer samples cover larger regions than in primary tumors, suggesting that additional unknown suppressor genes could be present at 17p, in the newly deleted sequences. In line with these findings, we have recurrently identified loss of the *MAP2K4* gene at 17p12 in the great majority of the metastatic samples analyzed. The *MAP2K4* gene is a member of the stress-activated protein kinase signaling cascade involved in the regulation of multiple cellular processes, which among other associations, has been recently suggested to have a functional role as a metastasis-suppressor gene in several malignant tumors, for example, human prostatic cancer,[42] ovarian cancer,[43] as well as breast and pancreatic tumors.[44] Similarly, a potential role for 18q LOH in the development of colorectal cancer with associated liver metastases has been suggested,[10] as well as its potential independent prognostic value,[45] which may depend on the microsatellite instability status.[46] In this regard, chromosomal instability has been associated in colorectal cancer with a worse prognosis, and different groups of tumors have been defined on the basis of the chromosomal instability status.[47] Herein, we identified loss of the 18q22–q23 chromosomal region in the great majority (95%) of the metastatic samples analyzed; interestingly, no clear association could be found between 18q LOH and the microsatellite instability status, because of normal expression of the *MLH1, MSH2* and *MSH6* mismatch repair genes, and potentially, also the relatively limited number of cases studied. Similarly, the sample size and the presence of multiple structural and/or numerical chromosome changes in all liver metastases analyzed precludes the study of chromosomal instability subtypes.

Many patients also showed acquisition of new genetic aberrations, which were not detected in their paired primary tumors. These included gains of chromosomes 2, 5p, 6p, 7q and 11p, and losses of chromosomes 4 and 10q. These results suggest that these chromosomal regions may also have a relevant role in the metastatic process as supported by the fact that some of them—for example, del(4p15.33), del(4q22.3), del(4q27), del(4q28.1), del(4q31), del(4q35.1) and del(10q23)—are known to contain multiple tumor suppressor genes (eg, *PLK4* at 4q28.1, *SFRP2* at 4q31.3, *IRF2* at 4q35.1 and *PTEN* at 10q23.2)[48–51] and genes that are involved in the metastatic process.[52–54] In line with these findings, previous studies in which primary colorectal carcinomas were compared with liver metastases also reported a greater frequency of chromosome 4 losses in late *versus* early stages of the disease.[9,55] However, due to the limited sensitivity of the SNP array technique for the detection of small clones that could already be present in primary tumors, further studies in which such abnormalities are investigated at the single-cell level are required to confirm our findings.

In summary, here we show the existence of relevant genetic differences between paired primary and metastatic colorectal tumors, which mainly consist of (1) an increased frequency of genetic lesions of chromosomes that have been associated with metastatic colorectal cancer (1p, 7p, 8q, 13q, 17p, 18q, 20q) and, more interestingly, (2) acquisition of new chromosomal abnormalities (eg, losses of chromosomes 4 and 10q and gains of chromosomes 5p and 6p). These genetic changes acquired by metastatic tumors may be associated with either the metastatic process and/or adaption of metastatic cells to the liver microenvironment. Further studies in larger series of patients, in which cases with non-resecable liver metastasis are also analyzed, are necessary to dissect the specific role of each of the altered genes and chromosomal regions in the metastatic spread of colorectal tumors. Additional gene expression profile studies are required to validate the proteins associated with copy number alterations in the metastasis *versus* the primary tumor.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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