Analysis of Colorectal Cancer by Comparative Genomic Hybridization: Evidence for Induction of the Metastatic Phenotype by Loss of Tumor Suppressor Genes

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ABSTRACT
Current models suggest that colon cancer initiation and progression are secondary to both the activation of oncogenes and the deletion of tumor suppressor genes. The role of each, however, is still poorly understood, particularly with regard to the induction of metastasis. We hypothesized that genetic differences exist between tumors that metastasize distantly and those that do not, and that oncogenes and tumor suppressor genes participate equally in this process. To address this hypothesis, human tumor specimens from localized [tumor-node-metastasis (TNM) stage I-III] and primary colon cancers (n = 10) were directly compared with metastatic (TNM stage IV) lesions (n = 10) using comparative genomic hybridization analysis. Although several alterations were shared equally between primary tumors and metastases (+7q, +19q, and +20q), two patterns of distinguishing alterations were observed: (a) alterations that were more extensive in liver metastases than in primary tumors (+8q, +13q, −4p, −8p, −15q, −17p, −18q, −21q, and −22q); and (b) alterations that were unique to metastatic lesions (−9q, −11q, and −17q). Overall, genetic losses were more common than gains, and, more importantly, the number of losses/tumor was significantly higher for metastases than for primary tumors (9.3 ± 1.3 versus 4.1 ± 0.7; P = 0.00062, Wilcoxon’s rank-sum test). The distinct predominance of genetic losses in the metastatic lesions when compared with the primary localized tumors provides evidence that the metastatic phenotype is induced by the deletion of tumor suppressor genes and permits the construction of physical maps targeting regions where novel tumor suppressor genes are likely to exist.

INTRODUCTION
Carcinoma of the large bowel is a common disease that is frequently lethal because of metastatic spread to the liver (1). For many years, it has been suggested that colon cancer tumorigenesis involves multiple steps (2, 3). More recently, Fearon and Vogelstein (4) suggested that these steps involve the mutation or gain of tumor-promoting oncogenes and the mutation or loss of tumor suppressor genes. Although no particular order of genetic events was considered necessary, early genetic alterations were thought to include mutations in the APC and RAS genes, and later alterations were thought to include mutations and deletions of the p53 and DCC genes (5, 6).

The majority of genetic studies involving colon cancer have focused on the early changes (7–9) responsible for tumor initiation. Many studies have addressed the differences between normal mucosa and primary cancers, but little is known regarding the genetic steps (10) that promote the later stages of tumor progression and the development of the metastatic phenotype. Current models (11) for the induction of metastasis suggest that multiple molecules must be differentially expressed to produce a cancer cell that is capable of producing its own blood supply, invading blood and lymphatic vessels, adhering to end-organ endothelial cells, and growing in distant organ sites. The process likely requires multiple genetic alterations and seems to be quite complex, and its regulation is poorly understood (12).

We hypothesized that alterations in oncogenes and tumor suppressor genes equally account for the genetic differences postulated to exist between primary loco-regional (stage I-III) tumors and tumors that have spread to distant organ sites (stage IV) such as the liver. This hypothesis was based on the clinical observation that nearly 70% of patients with loco-regional disease are curable, whereas few patients survive once distant spread has occurred (13). To test this hypothesis, we used CGH, a relatively new molecular cytogenetic technology designed to assay the entire genome for chromosomal gains and losses (14–16). We attempted to specifically address the hypothesis by directly comparing primary loco-regional cancers with liver-metastatic cancers. By focusing on the interface between cancers that grow locally but do not metastasize to distant organ sites versus those that have already spread to the liver, we have been able to identify and map specific chromosomal loci that are more likely to be altered in the process of conversion to the metastatic phenotype. Both losses that are shared with primary tumors and losses that are unique to metastatic tumors were identified. Our data provide compelling evidence that...
conversion to the metastatic phenotype may be largely based on the predominance of specific genetic losses, a finding that suggests a primary role for tumor suppressor genes in this complex process.

MATERIALS AND METHODS

Human Specimens. The material consisted of 20 fresh specimens (at least 2 mm³/specimen), 10 colorectal adenocarcinomas (tumor specimens 1–10) and 10 liver metastases (tumor specimens A–I), that were surgically removed from 18 patients at the Moffitt Cancer Center and Research Institute at the University of South Florida. No patients received preoperative chemotherapy. All specimens were carefully trimmed of all normal adjacent tissues. A frozen section of the specimens was done to confirm the histological diagnosis and the absence of normal adjacent contaminating tissue. The specimens were snap-frozen in liquid nitrogen immediately after resection and stored at −80°C in the Tissue Procurement Laboratory until analysis.

High molecular weight tumor DNA was isolated from 200 mg of frozen tumor sections using Trizol reagent (Life Technologies, Inc., Grand Island, NY). Normal DNA was isolated from the peripheral blood lymphocytes of genetically normal males, using the Puregene extraction system (Gentra, Inc., Triangle Park, NC). The samples were checked for purity by nondenaturing agarose gel electrophoresis.

Nine of the primary tumors were tumor-node-metastasis (TNM) stage I–III. One stage IV tumor (tumor 4) was also included for purposes of comparison with its synchronous liver metastasis (tumor H). Seven primary tumor specimens were derived from the rectosigmoid region, and three specimens were derived from the right colon. Tumor diameters ranged from 1–7 cm. Eight primary tumors were well-differentiated to moderately differentiated, and two were mucinous (tumors 4 and 9). One of the nine primary tumors (tumor 10) represented a local recurrence that was resected for cure. The liver metastasis specimens included seven lobectomies (four of the right lobes and three of the left liver lobes, respectively), one left lateral segmentectomy, and two right wedge resections. Table 1 shows a summary of the clinical and pathological data. All liver lesions were resected for cure, but only 30% were alive without disease at 2 years median follow-up.

CGH. CGH was performed using directly labeled fluorescent dUTP in extracted DNA according to specific protocols (Vysis, Inc., Downers Grove, IL). DNA samples from tumors were labeled with Spectrum Red dUTP (Vysis, Inc.), and reference DNA was labeled with Spectrum Green dUTP (Vysis, Inc.) with the nick translation technique. The nick translation reaction was monitored to obtain a probe size of 300–3000 bp. The probe size was checked through nondenaturing agarose gel electrophoresis.

In Situ Hybridization. Spectrum Red-labeled tumor DNA and Spectrum Green-labeled reference DNA (200–400 ng) and 10 μg of human Cot-1 DNA were combined and precipitated in the presence of 3 M sodium acetate and 2.5 volumes of 100% ethanol. The hybridization mix was then centrifuged, and the supernatant was removed, resuspended in CGH buffer, allowed to preanneal at 37°C for 30 min, and denatured at 73°C for 5 min. The hybridization probe was immediately applied to previously denatured normal chromosome metaphase slides (Vysis, Inc.), coverslipped, sealed with rubber cement, and placed in a sealed moist hybridization chamber in a 37°C incubator for 72 h. After hybridization, the slides were washed two times in 0.4× SSC and 0.3% NP40 wash.
solution at 74°C ± 1°C for 2 min and once in 2× SSC and 0.1% NP40 at ambient temperature for 5 s to 1 min. After air drying, the slides were counterstained with 4',6-diamidino-2-phenylinodole (Vysis, Inc.) in antifade solution.

**Digital Image Analysis.** Three successive images/metaphase were captured with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) mounted on a Leitz Orthoplan 2 epifluorescence microscope interfaced to a Power Macintosh 8100/100. Ten to 15 three-colored digital images of metaphase chromosomes were collected per hybridized tumor. Of these, the best 8–10 smooth high-quality images were selected for further analysis. Nearly 10,000 individual chromosomes were analyzed. Relative DNA copy number changes were found by analyzing the fluorescent ratio of tumor and reference DNA along the axis of each chromosome, as described previously (14, 17). After background correction and normalization of the red: green ratios to 1.0, red:green fluorescence intensity ratio profiles were calculated along the length of each chromosome using IPLabs and QUIPS software (Vysis, Inc.). Hybridizations of Spectrum Red-labeled and Spectrum Green-labeled normal male DNAs disclosed a mean ratio and corresponding SD between 0.9 and 1.1. Positive control experiments using the MPE-600 breast cancer cell line were performed, and the results were directly compared with described chromosomal alterations (18). Two negative control CGH experiments in which aliquots of normal DNA were labeled differentially with green and red fluorochromes, respectively, were performed to identify chromosomal regions with artifactually abnormal fluorescent profiles. Regions immediately adjacent to and including heterochromatic segments were excluded from analysis, because they typically showed very low red and green fluorescence intensities, due to partial hybridization suppression with Cot-1 DNA. A threshold of 1.2 and 0.8 for the red:green ratio values was set for gains and losses of test DNA material.

**Statistical Analysis.** The statistical significance between the number of gains and losses of primary and metastatic tumors and the frequency of alterations in selected chromosome arms was calculated using Wilcoxon’s rank-sum test and the two-tailed Fisher’s exact test, respectively.

**RESULTS**

**Controls.** To assess the sensitivity and validity of the CGH methodology, two normal negative control male DNAs (Vysis, Inc.) and a positive breast cancer DNA specimen (MP-600; Vysis, Inc.) were studied (data not shown). The CGH analysis of the normal DNA specimens (negative controls) identified no genetic alterations using the parameters outlined in “Materials and Methods,” but analysis of the positive control found all of the previously reported (18) alterations (+1q, −9p, +11q, −11pter, +13pter, −16q, +17q, +X, and −Y).

**Overview of Genetic Changes.** Figs. 1 and 2 summarize the chromosomal abnormalities (gains and losses, respectively) found in the primary and metastatic colorectal carcinomas. All evaluated specimens showed DNA copy number changes by CGH. The tumors harbored at least 3 chromosomal alterations, with a range of 3–16 and 7–20 chromosomal alterations in the primary and metastatic tumors, respectively. Four of 10 primary tumors (40%) showed more relative DNA sequence gains than losses, 5 of 10 tumors (50%) had more losses than gains, and 1 of 10 tumors (10%) had equal numbers of gains and losses. On average, there were 9.6 alterations/primary tumor: 5.3 gains (range, 2–12 gains) and 4.3 losses (range, 1–8 losses; Fig. 3). Metastatic tumors harbored more chromosomal abnormalities than primary tumors, and all had more relative DNA sequence losses than gains, with an average of 12.6 alterations/tumor (9.3 losses (range, 5–17 losses) and 3.3 gains (range, 0–6 gains)). A representative three-color CGH image demonstrating visually detectable gains and losses in a liver metastasis is shown in Fig. 4. The mean number of losses/tumor was greater in the metastatic neoplasms than in the primary neoplasms (Fig. 3), with a highly significant P of 0.00062 (Wilcoxon’s rank-sum test). The total number of gains and losses was also greater in the metastatic neoplasms, but the difference was not statistically significant. The values for chromosomes X and Y were consistent with the gender of the patients.

**Primary Tumors.** All patients bearing primary localized tumors that were resected for cure were alive without evidence of disease at follow-up (median follow-up, >2 years). All of the tumors showed gains (Fig. 1) and losses (Fig. 2) collectively affecting all of the chromosomes with the exception of chromosome 2. Four tumors showed sequence copy number gains, and seven tumors showed gains at one or more chromosomal region. Frequent gains involved 20q (four tumors), 13q (four tumors), and 19 (three tumors). The minimal overlapping regions of gain were 9q21.3, 20q11.23–q13.1, 13q14–q21, and 7cen–q11.2. Frequently lost chromosomal regions were 18q, 17p, 18q, 8p, and 5q. The minimal common regions of loss were 18q21–qter, 17pter–p12, 18pter–p11.3, 8pter–p23, and 5q21.2–q21.3. There was no relationship between the tumor size and the number of chromosomal alterations.

**Metastatic Tumors.** Consistent with national statistics for hepatic resection of colorectal liver metastases, 30% of all patients who underwent resection of liver metastases were alive at the time of follow-up (median follow-up, >2 years). Eight of the 10 metastatic tumors showed both regional gains (Fig. 1) and losses (Fig. 2), and two tumors had only losses. Losses were more common than gains, and the total number of losses/tumor for metastatic lesions was significantly greater than the total number of losses/tumor for primary lesions. Two tumors (tumors F and G) showed copy number losses of chromosome 18. Common regional losses included regions involving 18q (90%), 17p (90%), 8p (80%), and 22q (70%), and 40% of the tumors had a regional loss at 4p, 9q, 15q, and 17q. The minimal overlapping regions of loss for chromosome 18 were 18q22–qter in 100%, 18q21–qter in 80%, and 18q12–qter in 70% of the tumors. Chromosome 17 had overlapping losses involving 17p23–cen (four tumors), 17pter–p12 (four tumors), and 17pter–p13 in six tumors. The remaining chromosomes had the following common overlapping regions of loss: (a) 8pter–p22; (b) 22q11.2–q12; (c) 22q12.3–q13.1; (d) 22q13.3; (e) 4p16; (f) 9q21; (g) 9q34.3; and (h) 15q26.

Two distinct patterns of common chromosomal losses were observed: (a) losses in metastases that involved the same chromosome arms as the primary tumors but were more frequent or extensive (4p, 8p, 15q, 17p, and 18q); and (b) regional losses unique to the metastatic tumors (9q, 11q, and 17q). Regional gains per tumor most commonly affected 13q (50%), 8q (40%),
7q, and 20q (30%). High-level amplifications (red:green ratios > 1.5) of the 13q whole arm were observed in three cases, and high-level amplifications of a smaller chromosomal region were observed in two cases.

**DISCUSSION**

Colon cancer that has not metastasized distantly (stages I-III) is often frequently cured by surgical resection; however, when metastasis to the liver and other organs has occurred (stage IV), the chances for cure are dramatically reduced (1). It is well known that there are subgroups of patients whose tumors grow locally without ever spreading distantly; however, some patients present with synchronous, small primary tumors and incurable, distant metastatic disease. Whereas our current understanding of the genetic mechanisms underlying the process of familial (19-21) and sporadic colon cancer initiation is now quite lucid (10), the mechanisms responsible for progression to the metastatic phenotype are still poorly understood.

Based on current models of colon cancer tumorigenesis (12, 22), we hypothesized that genetic alterations exist that may account for differences in biological behavior between tumors that metastasize distantly and those that do not, and that both oncogenes and tumor suppressor genes would play an equal role (23, 24) in the process of metastasis. Our data, however, provide evidence that the primary difference between tumors that metastasize and those that grow locally without distant spread is the genetic loss of chromosomal loci. These observations suggest that tumor suppressor genes, rather than oncogenes, play a predominant role in the development of the metastatic phenotype. The recent report associating PTEN (25), a novel tumor suppressor gene located at 10q23, with aggressive growth and metastasis of brain, breast, and prostate cancer and reports of other similar genes located at 18q (26), 8p22 (16), and 11p11.2 (27, 28) support this hypothesis. Interestingly, the existence and location of the 10q23 gene was accurately predicted by CGH (29) and allelic mapping (30) with polymorphic markers. This locus, although not frequently altered, is among several that were found in our metastatic tumors. Because a number of the mapped regions of loss associated with our metastatic tumors are not currently associated with specific tumor suppressor genes, novel genes linked to the metastatic process are postulated to exist.

This is the first study comparing the genetic alterations of primary (loco-regional) tumors with those of metastatic colorectal adenocarcinomas using CGH. With the exception of one tumor, all of the primary tumors tested were localized at the time of resection and had not yet metastasized distantly within more than 2 years of median follow-up. We purposely chose to study nonsynchronous cases of colon cancer primary tumors and liver metastases, because we wanted to maximize the potential for...
Fig. 2  Summary of DNA losses in primary loco-regional colorectal adenocarcinomas (left) and in liver metastases (right).

Fig. 3  Comparison of genetic alterations between primary and metastatic colon cancers. Data are represented as the mean number of genetic changes ± SD/tumor. For a comparison of losses between primary and metastatic lesions, \( P = 0.00069 \) (Wilcoxon’s rank-sum test).

identifying significant differences that might exist between tumors with and without metastatic potential. The inherent risk in this experimental design is that genetic changes in metastases may not accurately reflect the primary events associated with the process of metastasis but rather the microenvironmental effects of tumor growth in the liver. Previous studies have examined Astler Collier Stage D primary cancers (primary cancers that have metastasized distantly) in relation to earlier stage lesions, but significant differences were not reported (31, 32), perhaps because of tumor heterogeneity that could have resulted in sampling errors. Moreover, despite the use of microdissection techniques, previous reports identifying no alterations in some locally advanced colon cancers suggest the potential for sampling errors (32). By selecting liver metastases, we ensured that our sampling procedure was not influenced by heterogeneity known to exist in the primary tumor simply because these tumors were presumed to be clonally derived from one or more cells with metastatic potential. To test this principle, a primary tumor (tumor 4) and its respective liver metastasis (tumor H) that occurred synchronously in the same patient were included in this analysis for the purpose of determining whether significant differences could be detected between two tumors that were genetically related but growing in different organ sites. Interestingly, we found some, but not all, of the alterations identified in the primary tumor in its respective liver metastasis (losses on 10q and 18q), and in addition, we found new alterations in the metastasis (losses on 8p and 17p and gains of 8q and 12p). In addition, a primary tumor (tumor 3) and its local recurrence (tumor 10) were obtained from the same patient and found to share losses on 8p and 18q and gains on 13q. Alterations present
only in the recurrent neoplasm were losses on 1p, 4q, and 17q and gains in 6q, 9q, and 20q. These cases demonstrate the principle that secondary metastatic tumors derived from primary tumors, although sharing a number of common alterations, also harbor new alterations as part of the process of tumor progression.

Genetic alterations were identified in all of the examined tumors, although previous studies using routine cytogenetic methodologies have identified normal karyotypes in up to 34% of the examined tumors (33–35). This increase in sensitivity is likely based on the technical advantages of CGH over routine cytogenetics, although occasionally, CGH fails to identify alterations in advanced tumors (32). Cytogenetic analysis is limited by the number of karyotypes that can be analyzed, with each karyotype representing the analysis of only a single cell. CGH, on the other hand, can provide a comprehensive analysis of a large portion of the tumor that includes billions of tumor cells. In addition, our study involved analyses of large numbers of metaphase spreads/tumor, and all experiments were performed with sizable tumor samples (because tumors were obtained from fresh frozen specimens rather than paraffin-fixed tissue sections). We believe that these procedures may reduce sampling errors that lead to false negative results.

Our analysis of primary tumors showed multiple chromosomal abnormalities collectively involving all of the chromosomes with the exception of chromosome 2. Whereas some of the chromosomal abnormalities were observed in the terminal regions of chromosomes such as 1p which may represent CGH artifact, we believe this not to be the case, because 1p has previously been shown to be commonly affected in colon cancer using cytogenetic studies (31), and our normal DNA controls did not demonstrate these changes. The most commonly gained chromosomal regions in the primary tumors involved 20q (50%), 9q (50%), 13q (40%), 8q (30%), and 19q (30%). These regions harbor genes that include c-SRC (20q), RB-1 (13q), and MYC (8q). Five of the primary tumors showed gains on 9q with a minimal overlapping region at 9q21.3, which has not yet been reported in colon cancer. This band was not amplified in any of the metastatic tumors but was lost in two. These findings are in agreement with previous cytogenetic colon cancer studies that have shown frequent gains of chromosome 20 and 13q (34). In a study by Bardi et al. (35), structural rearrangements of chromosomes 13 and 20 were seen in 3 of 49 colon cancer karyotypes, but with a loss of 20p material and isomerism of 13q. These results also confirm those of a recent CGH study in colon cancer demonstrating a similar pattern of gain involving 20q

Fig. 4 Three-color image of a representative liver metastasis illustrating high-level amplification of 13q, gain of 20q, and loss of 18q. These alterations involved the entire chromosome arm, making them identifiable by visual inspection. Histograms (right) display the mean red:green ratios (red lines) + SD (blue lines) along the entire length of the identified chromosome. Alterations were scored as gains when the mean red:green ratios exceeded 1.2 and losses when red:green ratios dropped below 0.8. Three gray transverse lines mark red:green ratios of 1.2 (top line), 1.0 (middle line), and 0.8 (bottom line).
(75% of the cases) and 13q (69% of the cases; Ref. 32). Schlegel et al. (36) similarly reported gains of chromosomes 20 and 13 in 16 and 27% of the microsatellite instability-negative colon tumors, respectively.

Although the primary and metastatic lesions shared a number of chromosomal alterations equally (+7q, +19q, and +20q), the metastatic tumors harbored more total alterations than the primary tumors, and these alterations were predominantly genetic losses ($P = 0.00062$). Some of these losses were shared with the primary tumors but were more extensive in the metastases (−4p, −8p, −15q, −17p, −18q, −21q, and −22q), and some were unique to the metastases (−9q, −11q, and −17q) and perhaps to the process of metastasis. We cannot, however, rule out the possibility that these unique differences were related, in part, to the growth of tumor in the liver microenvironment. These regions include specific gene loci reported to be commonly altered in advanced colorectal cancer as well as loci that have no mapped identity or function. For example, p53 is located in 17p13, and DCC is located in 18q21.3. Our data suggest that these genes would be deleted in 60 and 70% of metastases, respectively versus deletion in 30% and 50% of primary tumors, respectively. The metastatic tumors, however, showed a more extensive region of loss and more losses per tumor than the primary tumors for these regions. We believe this observation to be important, because it provides evidence that novel tumor suppressor genes exist that have not yet been identified (24). Vogelstein et al. (37) reported the statistically significant correlation of deaths from colorectal cancer metastasis with the loss of 17p and 18q. In our study, the number of losses involving 18q and 17p was statistically significant in liver metastatic lesions when compared to that in the colorectal primary neoplasms. Although the proportional regression hazard analysis of the number of losses per tumor and survival was not significant (probably because of the small sample size), six of the seven patients whose metastatic tumors harbored the loss in the DCC region have died. The association is softer for p53, because only three of six patients with regional loss of 17p13 have died. Our results verified a number of the findings that we identified (24). Vogelstein et al. (37) reported a statistically significant correlation of deaths from colorectal cancer metastasis with the loss of 17p and 18q. In our study, the number of losses involving 18q and 17p was statistically significant in liver metastatic lesions when compared to that in the colorectal primary neoplasms.

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