Differences in 20q13.2 Copy Number between Colorectal Cancers with and without Liver Metastasis

Shigekazu Hidaka, Toru Yasutake, Hiroaki Takeshita, Masamichi Kondo, Takashi Tsuji, Atsushi Nanashima, Terumitu Sawai, Hiroyuki Yamaguchi, Tohru Nakagoe, Hiroyoshi Ayabe, and Yutaka Tagawa

The First Department of Surgery, Nagasaki University School of Medicine, Nagasaki 852-8501 [S. H., T. Y., H. T., M. K., T. T., A. N., T. S., H. Y., T. N., H. A.], and School of Allied Medical Sciences, Nagasaki University, Nagasaki 852-8520 [Y. T.], Japan

ABSTRACT

Frequent gains of 20q have been identified recently in many neoplasias, including breast, bladder, ovarian, pancreatic, and colon cancers. A high level of 20q13.2 amplification is associated with poor prognosis in breast cancer. We investigated the copy number of the 20q13.2 region including the ZNF217 oncogene in 17 nonmetastatic colorectal cancers (CRCs), 18 primary CRC tumors with liver metastasis, and 18 metastatic lesions by two-color fluorescence in situ hybridization to evaluate the significance of an increased copy number of 20q13.2 in CRC, especially in those cases with liver metastasis. The frequency of increased relative copy number of the 20q13.2 region was higher in primary and liver metastatic lesions of CRC than in CRC lesions without liver metastasis. In particular, a high-level increase (>3.0-fold) in the relative copy number of 20q13.2 was observed in 2 of 18 (11%) primary CRC lesions with liver metastasis, 7 of 18 (39%) liver metastatic lesions, and in none of the cases of primary CRC without liver metastasis. The absolute and relative copy number of chromosome 20q13.2 was higher in CRCs with metastasis than in CRCs without metastasis. The percentage of cells with high-level 20q13.2 amplification was also higher in both lesions with metastasis per specimen than without metastasis. Our results suggest that the level of 20q13.2 amplification correlates with the metastatic potential and tumor progression of CRC. The results also suggest that 20q13.2 amplification with ZNF217 is associated with increased metastatic potential.

INTRODUCTION

Advanced CRC is associated with poor prognosis, and surgical resection is the best available treatment at present. However, recurrence and metastasis are the major causes of mortality in patients treated by surgical resection. This behavior of CRC is believed to be dependent on multiple genetic aberrations. A molecular genetic model of preferential sequence has been proposed (1). However, even with the present knowledge of the cellular and molecular mechanisms of CRC, no biological parameter can predict the behavior of cancers.

Recently, gain of chromosome 20q sequences has been reported in many types of malignant tumors, including breast (2, 3), bladder (4), ovarian (5), and pancreatic cancer (6). Karyotypic/cytogenetic data regarding CRC have been accumulated over the last 10 years (7–9), and a gain of the long arm of chromosome 20 has been described as a common genetic aberration (10–12).

We recently reported the numerical chromosome aberration for CRC (13). In that study, we demonstrated that gain of chromosome 20 is a frequent aberration in primary and metastatic lesions in patients with CRC associated with liver metastasis (13). Recently, gain of 20q was also identified in 85% of metastatic lesions of the liver using CGH (14). These findings suggest that aberration of chromosome 20q is associated with metastasis in CRC.

In human breast carcinoma, DNA amplification at chromosomal region 20q13, which was identified by CGH, was detected in 12–18% of lymph node-negative primary breast cancers and 40% of cell lines (3). Furthermore, a high level of 20q13.2 amplification was found in 6.8% of primary breast cancers (15). Amplification of the 20q13.2 region was associated with poor prognosis in lymph node-negative breast cancers (16–18). These data suggest that the chromosomal segment 20q13 harbors one or more novel oncogenes. To our knowledge, the relationship between aberration of this region and the behavior of CRC has not been investigated previously. The aim of the present study was to investigate the significance of an increased copy number of 20q13.2 in CRCs, especially in those CRCs with liver metastasis.

MATERIALS AND METHODS

Patients and Tumor Samples. A total of 53 samples were obtained from 35 patients (24 males and 11 females) who underwent surgery between 1989 and 1997 at the First Department of Surgery, Nagasaki University School of Medicine (Nagasaki, Japan). None of the cases received adjuvant therapy before surgery. Seventeen samples of Dukes’ stage B or Dukes’
stage C primary CRC without liver metastasis or recurrence for 5 years after surgical resection of the primary tumor were selected. Thirty-six samples were paired samples from 18 patients (18 samples were obtained from primary tumors with liver metastasis, and the other 18 samples were obtained from metastatic lesions of the same patients). Five synchronous cases and 13 metachronous cases were included in these samples. Synchronous cases were diagnosed with metastasis within 12 months of the diagnosis of the primary tumor. Metachronous cases were diagnosed with metastasis more than 12 months after diagnosis of the primary tumor. These cases were designated as primary CRC without liver metastasis, primary CRC with liver metastasis, and metastatic CRC. The study protocol was approved by the Human Ethics Review Committee of our hospital and the Nagasaki University School of Medicine.

**Cell Preparation.** All fresh tissue samples were stored at –80°C. Tissue samples were examined histologically. Specimens were stamped on a glass slide. We ascertained that malignant cells formed more than 60% of the cells in these samples. The slides were fixed with a 3:1 methanol:acetic acid solution at –20°C until use.

**FISH.** Two-color FISH was performed using a 20q13.2 locus-specific probe and a chromosome 20-specific α-satellite DNA biotin-labeled reference probe (D20Z1; Oncor, Gaithersburg, MD). The 20q13.2 locus-specific probe including the novel zinc finger gene ZNF217 was labeled with Spectrum Orange by the vendor (Vysis, Downers Grove, IL). Tumor samples were denatured with 70% formamide and 2× SSC at 73°C for 2.5 min and treated with 1.5 μg/ml proteinase K at 37°C for 7.5 min, followed by dehydration using serial concentrations of 70%, 85%, and 100% ethanol solutions. The probe mixtures were denatured at 70°C for 5 min and incubated on glass slides at 37°C for hybridization. After hybridization for 16 h, the samples were washed three times with 50% formamide at 45°C. The reference probe was stained immunohistochemically with fluorescein-avidin DCS (Vector Laboratories, Burlingame, CA). The slides were counterstained with 0.2 μM 4′,6-diamidino-2-phenylindole in an antifade solution. At least 50 nonoverlapping interphase nuclei were examined under a fluorescence microscope with a dual bandpass filter and a ×400 lens. Each signal was counted with a single bandpass filter. Control hybridization to normal peripheral blood lymphocytes and normal epithelial cells was performed to confirm that the hybridization efficiency of the test and reference probes was similar.

**Data Analysis and Statistical Methods.** Data were expressed as the mean ± SE. The relative copy number was expressed as the mean level of amplification (the ratio of the number of signals from the 20q13.2 probe to the number of signals from the reference probe per individual cell). Low-level amplification was defined as more than 1.5-fold of the relative copy number, whereas high-level amplification was defined as more than 3.0-fold of the relative copy number. The ratio of the number of cells with high-level 20q13.2 amplification (>3.0-fold of the relative copy number) to the total number of cells counted was the percentage of high-level amplification. Differences in the frequency of amplification among the three groups were tested for statistical significance using ANOVA and multiple comparison tests (Scheffe method). P < 0.05 denoted the presence of a statistically significant difference.

**RESULTS**

Fig. 1 shows representative signals of the chromosome 20q13.2 locus-specific probe and the chromosome 20 centromere reference probe in one case of CRC with liver metastasis by using two-color FISH. The mean level of increased 20q13.2 copy number per case was determined. An increased copy number of the 20q13.2 region was seen in a total of 7 of 17 (41%) primary CRC lesions without liver metastasis, 16 of 18 (89%) primary CRC lesions with liver metastasis, and 17 of 18 (94%) metastatic CRC lesions (Table 1). High-level increased copy number of 20q13.2 was observed in 2 of 18 (11%) primary CRC lesions with liver metastasis and 7 of 18 (39%) metastatic CRC lesions. There was no high-level increased copy number of the 20q13.2 region in cases of primary CRC without liver metastasis. The frequency of increased copy number of the 20q13.2 region was significantly higher in primary CRC lesions with liver metastasis than in primary CRCs without liver metastasis (CRCs without liver metastasis versus primary CRCs with liver metastasis, P < 0.01; CRCs without liver metastasis versus metastatic CRCs, P < 0.001; Table 1).

A comparison of the mean level of increased copy number showed that the mean ratio of 20q13.2 locus copy number to chromosome 20 centromere copy number was 1.48 ± 0.20, 2.11 ± 0.54, and 2.66 ± 0.65 in primary CRCs without liver metastasis, primary CRCs with liver metastasis, and CRC metastatic lesions, respectively (Fig. 2). Furthermore, the mean ratio...
of the absolute 20q13.2 locus copy number was 4.28 ± 2.00, 5.88 ± 2.20, and 6.48 ± 2.30 in primary CRCs without liver metastasis, primary CRCs with liver metastasis, and CRC metastatic lesions, respectively (Fig. 2). These mean absolute and relative copy numbers of 20q13.2 were significantly higher in primary lesions of CRC with liver metastasis and CRC metastatic lesions than in primary CRC lesions without liver metastasis [CRCs without liver metastasis versus primary CRCs with liver metastasis, \( P < 0.01 \) (relative copy number) and \( P < 0.05 \) (absolute copy number); CRCs without liver metastasis versus CRC metastatic lesions, \( P < 0.001 \) (relative copy number) and \( P < 0.01 \) (absolute copy number)]. The mean relative copy numbers of the 20q13.2 region were also higher in metastatic CRC lesions than in primary CRC lesions with liver metastasis (\( P < 0.01 \); Fig. 2). The mean absolute copy number of 20q13.2 was higher in primary CRC lesions with liver metastasis and CRC metastatic lesions than in primary CRC lesions without liver metastasis (Fig. 2). These mean absolute and relative copy numbers of 20q13.2 were significantly higher in primary lesions of CRC with liver metastasis and CRC metastatic lesions than in primary CRC lesions without liver metastasis [CRCs without liver metastasis versus primary CRCs with liver metastasis, \( P < 0.01 \) (relative copy number) and \( P < 0.05 \) (absolute copy number); CRCs without liver metastasis versus CRC metastatic lesions, \( P < 0.001 \) (relative copy number) and \( P < 0.01 \) (absolute copy number)]. These mean absolute and relative copy numbers of 20q13.2 were significantly higher in primary lesions of CRC with liver metastasis and CRC metastatic lesions than in primary CRC lesions without liver metastasis [CRCs without liver metastasis versus primary CRCs with liver metastasis, \( P < 0.01 \) (relative copy number) and \( P < 0.05 \) (absolute copy number); CRCs without liver metastasis versus CRC metastatic lesions, \( P < 0.001 \) (relative copy number) and \( P < 0.01 \) (absolute copy number)]. The mean relative copy numbers of the 20q13.2 region were also higher in metastatic CRC lesions than in primary CRC lesions with liver metastasis (\( P < 0.01 \); Fig. 2). The relative copy number of the 20q13.2 region in all cases of primary CRC without liver metastasis was less than 2.0-fold. In contrast, >2.0-fold of the relative copy number of 20q13.2 was seen in 11 of 18 (61%) primary CRC lesions with liver metastasis and 16 of 18 (89%) metastatic CRC lesions. The frequency of cases showing >2.0-fold of the relative copy number was also higher in metastatic CRC lesions than in primary CRC lesions with liver metastasis. However, the frequency did not differ significantly between primary CRC lesions with liver metastasis and metastatic CRC lesions. The mean copy number was higher in the metastatic lesions in the liver than in the primary tumors in all but two cases (data not shown).

Intratumor heterogeneity was seen frequently in individual tumor cells. We examined the percentage of cells with a high-level increase in relative copy number (>3.0-fold) in each group. The average percentage of cells with a high-level increase in relative copy number was 6.09 ± 4.20%, 21.15 ± 16.36%, and 38.60 ± 19.45% in primary CRCs without liver metastasis, primary CRCs with liver metastasis, and CRC metastatic lesions, respectively (Fig. 3). The average percentage of cells with a high-level increase in relative copy number was significantly higher in metastatic lesions and in primary tumors with metastasis than in primary tumors without metastasis (CRCs without liver metastasis versus primary CRCs with liver metastasis, \( P < 0.01 \); CRCs without liver metastasis versus CRC metastatic lesions, \( P < 0.001 \); primary CRCs with liver metastasis versus CRC metastatic lesions, \( P < 0.01 \)).
cases, although the difference was not statistically significant. The percentage of 20q13.2 amplification in synchronous metastatic and metachronous metastases was higher than that in metachronous primary CRCs with liver metastasis and in CRC primary lesions without metastasis. In the metastatic lesion, the results were similar to those obtained by CGH. The present study showed that not only numerical aberration of chromosome 20 but also a change in copy number of the chromosome 20q13.2 region is associated with liver metastasis in CRC.

The major finding of the present study was the significantly high mean relative copy number of chromosome 20q13.2 in CRCs with metastasis as compared with that in CRCs without metastasis. We also showed that the mean relative copy number was higher in metastatic lesions than in primary lesions. These results suggest that the mean level of the relative copy number of the 20q13.2 locus correlates with tumor progression of CRC. Only high-level gains of the 20q13.2 region may play an important role in the metastatic potential of CRC.

The percentage of cells showing 20q13.2 amplification per specimen was higher in metastatic lesions and primary tumors with metastasis than in CRCs without liver metastasis. Thus, cancer cells with >2.0-fold 20q13.2 amplification as well as a >20% high-level amplification may have a higher metastatic potential. It seems that evaluation of the copy number of the chromosome 20q13.2 region by FISH may be useful for identification of CRCs with a high metastatic potential.

Comparison of the mean amplification level and percentage of amplification of the chromosome 20q13.2 region in primary and metastatic lesions in cases with liver metastasis showed that the mean level of the relative copy number was higher in almost all cases in metastatic lesions than in primary lesions. These results indicate that the number of tumor cells with the same clonality and 20q13.2 amplification was higher in metastatic lesions than in primary lesions. Interestingly, no amplification case of primary CRC with liver metastasis showed a high percentage of amplification in the metastatic lesion. These results suggested that a subpopulation with 20q13.2 amplification was selected more in the metastatic lesion under intratumor heterogeneity. Therefore, we postulate that cancer cells with a high level of relative copy number of chromosome 20q13.2 have a high metastatic potential and that metastatic lesions of the liver consisted of a clonal cell population with high-level amplification of this region. However, in a few cases, the level of 20q13.2 amplification in primary lesions was almost similar or lower than that in metastatic lesions. These results suggest that, in addition to 20q13.2, other unknown factors are involved in liver metastasis of CRC.

Several known genes, including the Src oncogene, CAS (cellular apoptosis susceptibility; Ref. 22), AIB1 (amplified in breast cancer; Ref. 23), and BTAK (breast tumor amplified kinase; Ref. 24), are located on chromosome 20q. Recently, novel zinc finger gene ZNF217, which is amplified in breast cancer, was found on 20q13.2 (25). According to the recent analysis of FISH mapping of 20q in CRC metastases, the most frequent region with high-level gains was located on 20q13.1–

**Fig. 3** Comparison of the percentage of cells with a high-level increase in the copy number of the 20q13.2 region per total cells among the three groups. **Bars**, average values. The percentage of amplification was significantly higher in primary CRCs with liver metastasis and in CRC metastases than in CRCs without liver metastasis (CRCs without liver metastasis versus primary CRCs with liver metastasis, \( P < 0.01 \); CRCs without liver metastasis versus metastasis, \( P < 0.01 \)).

Discussions

A gain of the long arm of chromosome 20 has been described in many human tumors. It has been reported that low-level 20q gain is associated with immortalization, whereas high-level 20q13.2 amplification is associated with chromosomal instability (19), and that gene amplification and overexpression of STK15/BTAK mapped on 20q13 induce centrosome amplification, chromosomal instability, and transformation (20). These findings suggest that 20q13.2 amplification may lead to genome instability in other human epithelial cell types and may contribute to tumor progression and, ultimately, to metastasis.

In addition, gain of 20q including this region is likely to play an important role in many other tumors. According to several CGH analyses in colorectal tumors, gains of chromosome 20q were observed more often in carcinomas than in adenomas (10, 11). Frequent gains of chromosome 20q were identified in both primary and metastatic lesions of CRC with metastasis (21). Furthermore, it seems that aberration of chromosome 20 occurs relatively early and contributes to tumorigenesis in CRC. However, the relationship between clinicopathological features and aberration of chromosome 20q remains unknown. Our analysis of numerical chromosome aberration in CRC showed that gain of chromosome 20 is a frequent aberration in primary and metastatic lesions in patients with liver metastasis (13). The total number of cases with gain of the chromosome 20q13.2 region detected by two-color FISH was markedly higher in both primary lesions of CRC with liver metastasis and CRC metastatic lesions than in CRC primary lesions without metastasis. In the metastatic lesion, the results were similar to those obtained by CGH. The present study showed that not only numerical aberration of chromosome 20 but also a change in copy number of the chromosome 20q13.2 region is associated with liver metastasis in CRC.
20q13.2 (14). However, the candidate genes that are clearly associated with the metastatic potential of CRC in this region (20q13.2) remain unknown. Our results suggest that the increased copy number of the ZNF217 gene may be associated with liver metastasis in CRC.

In the present study, the level of 20q13.2 in CRC was not very high (1.3–3.8-fold) compared with the levels reported previously in breast cancer and CRC (14, 18). We also found the increased copy number of chromosome 20 α-satellite DNA reference probe as described previously (13). These findings may be due to differences in the reference probe selected for analysis. The increased relative copy number at 20q13.2 in CRC may reflect the coamplification of a different region in the same chromosome arm or part of large 20q aberrations. The FISH probe used in our study includes a more than 300-kb sequence in 20q13.2 region according to information provided by the vendor; thus, it may also cover other genes, in addition to ZNF217. Therefore, our results may not be specific for the ZNF217 oncogene. Further analysis of FISH mapping using a larger number of CRCs would narrow down the critical region on 20q for localization of a gene or genes associated with liver metastasis in CRC.

The relative copy number in cases of synchronous metastasis was higher, albeit insignificantly, than that in metachronous cases. Liver metastasis occurred within 1 year in almost all metachronous cases. These findings suggest that occult micro-metastasis undetected before surgery may occur in metachronous cases.

In conclusion, we demonstrated that the mean level of the relative copy number of 20q13.2 with the ZNF217 oncogene and the frequency of amplification of this region were higher in both primary and metastatic colorectal tumors with liver metastasis than in CRCs without liver metastasis. Our results suggest that the increased level of copy number of chromosome 20q13.2 is associated with metastasis in CRC. Further functional analysis of candidate genes on this region could reveal the mechanism of metastasis in CRC.

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REFERENCES

Brinkley, B. R., and Sen, S. Tumour amplified kinase STK15/BTAK 
induces centrosome amplification, aneuploidy and transformation. Nat. 

G. D. Comparative genomic hybridization analysis of primary colorectal 
carcinomas and their synchronous metastases. Genes Chromosomes 

The human CAS (cellular apoptosis susceptibility) gene mapping on 
chromosome 20q13 is amplified in BT474 breast cancer cells and part 
of aberrant chromosomes in breast and colon cancer cell lines. Genome 

M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., 
and Meltzer, P. S. AIB1, a steroid receptor coactivator amplified in 
breast and ovarian cancer. Science (Washington DC), 277: 965–968, 
1997.

kinase encoding gene BTAK on chromosome 20q13 is amplified and 
overexpressed in human breast cancer cell lines. Oncogene, 14: 2195– 
2200, 1997.

25. Collins, C., Rommens, J. M., Kowbel, D., Godfrey, T., Tanner, M., 
Hwang, S. I., Polikoff, D., Nonet, G., Cochran, J., Myambo, K., Jay, 
K. E., Froula, J., Cloutier, T., Kuo, W. L., Yaswen, P., Dairkee, S., 
Giovanola, J., Hutchinson, G. B., Isola, J., Kallioniemi, O. P., Palazzolo, 
M., Martin, C., Ericsson, C., Pinkel, D., and Gray, J. W. Positional 
cloning of ZNF217 and NABC1: genes amplified at 20q13.2 and over-
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