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Advances in Brief

Consistent Genetic Alterations in Xenografts of Proximal Stomach and Gastro-Esophageal Junction Adenocarcinomas[1]

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Abstract

The genetic alterations underlying the development of gastric and gastro-esophageal carcinoma remain largely undefined. DNA copy number changes were determined by comparative genomic hybridization in eight xenografts of proximal gastric and gastro-esophageal junction adenocarcinomas of the intestinal type. All tumors exhibited DNA copy number changes, with a total of 139 changes detected (range, 11–24 per tumor; mean = 17), indicating numerous and widespread alterations within these cancers. Gains (65%) in DNA copy number were more frequent than losses (35%).

Our most striking finding was gain (all eight cases) or high-level amplification (four cases) in 20q, with a minimal common overlapping region at 20q13. Other frequent gains were observed at 6p, 7q, and 17q (six cases each) and at 1q, 2q, and 8q (five cases each). Frequent losses were observed at 4q and 5q (six cases each) and at 9p (five cases). No differences in DNA copy number changes were seen in tumors arising from the gastro-esophageal junction compared to those of the proximal stomach. The presence of common and consistent DNA copy number changes in these tumors implicate a number of chromosomal regions that may harbor important genes that are involved in tumorigenesis of the proximal stomach and gastro-esophageal junction.

Introduction

GC[3] is one of the most common malignancies worldwide. Moreover, the incidence of gastro-esophageal junction adenocarcinomas appears to be on the rise for reasons that are unclear (1). These junctional adenocarcinomas are generally recognized to arise from Barrett's esophagus. The molecular alterations that underly GC or gastro-esophageal cancer development are largely unknown. Karyotype analysis of GC has shown several chromosomal aberrations, but no consistent specific changes have been reported thus far.

CGH is a powerful tool for molecular cytogenetic analysis of neoplasms. It enables the screening of entire tumor genomes for gains and losses of DNA copy number and consequent mapping of aberrations to chromosomal subregions (2). Thus far, only one CGH report on primary carcinomas of the stomach has been noted, with DNA copy number changes in the range of 4–50% of cases (3). This is the first report of molecular cytogenetic changes in gastro-esophageal junctional adenocarcinoma.

For CGH analysis, xenografts are ideal material. Xenografting of human tumors has been used to produce optimal samples that are enriched for neoplastic cells for subsequent molecular analyses. Studies of xenografted tumors generated from both human colon and pancreatic adenocarcinomas have led to the discovery of important genetic alterations underlying these malignancies (4, 5). To identify important genomic areas possibly involved in the oncogenesis of cancers of the upper gastrointestinal tract, we used CGH to identify significant DNA copy number changes in xenografts grown from resected human adenocarcinomas.

Materials and Methods

Xenografts. Fresh tissue from surgically resected adenocarcinomas of the stomach or gastro-esophageal junction were processed to generate xenografts according to Insitutional Research Board approved protocols at two academic institutions, the Indiana University Medical Center and the University of Virginia Health Sciences Center. Specimens were collected in RPMI medium and stored temporarily on ice until further processing for xenografts. An adjacent portion of tissue from the resected primary tumor specimen was embedded and used to confirm the histopathology of the xenografted samples.

Xenografting was performed as described previously (6). Briefly, small pieces (1–2 mm) of the fresh tissue in RPMI medium were subsequently soaked in Matrigel (Collaborative Biomedical Research) for 30 min and used for implantation. Immunodeficient mice were anesthetized for s.c. implantation of small pieces of Matrigel-soaked tissue in the subscapular and flank regions, and incisions were closed. Mice were subsequently examined for tumor growth, and neoplasms were harvested and frozen when they reached approximately 1 cm in diameter. High molecular weight genomic DNA was prepared from these frozen xenografted tumors by standard organic extraction methods. Histological confirmation of the xenografted tumors was performed on cryostat-sectioned slides stained with H&E.

CGH. CGH was performed according to previously reported procedures (7), with a modification using fluorochromes conjugated to a mixture of dCTP and dUTP for standard nick translation (8). Hybridizations, washings, and ISIS digital image analysis (Metasystems GmbH, Altlussheim, Germany) were performed as described elsewhere (9). Three-color images (red for reference DNA, green for tumor DNA, and blue for counterstaining) were acquired from 8–10 metaphases per sample. Only metaphases of good quality with strong uniform hybridization were included in the analysis. Chromosomes that were not suitable for CGH analysis (*i.e.*, chromosomes were heavily bent, overlapped, or had overlying artifacts) were excluded. On the basis of our earlier reports, we used 1.17 and 0.85 as cutoff levels for gains and losses, respectively. Thus, a signal greater than 1.17 was considered a gain and a signal less than 0.85 was considered a loss. A threshold value of 1.5 or greater was used for high-level amplification, corresponding to greater than five extra copies. All of the results were confirmed using a 99% confidence interval. In each CGH experiment, a negative control (peripheral blood DNA from a healthy donor) and a positive control were included. The positive control was a tumor with known changes in DNA copy numbers.

Results

Eight cases of xenografted tissue from freshly resected adenocarcinomas of the stomach and gastro-esophageal junction grew tumors

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[3] The abbreviations used are: GC, gastric carcinoma; CGH, comparative genomic hybridization.

Table 1 *Clinical and histopathological data of xenograft cases studied*

Case no.	Age (yr)	Sex	Race[a]	Primary site[b]	Histopathology[c]	Grade[d]	TNM stage	Barrett's mucosa[e]
1	41	F	C	GEJ	Intestinal	Well differentiated	IIIb/$T_3N_2M_0$	Yes
2	66	M	C	GEJ	Intestinal	Moderately differentiated	Ib/$T_1N_1M_0$	Yes
3	69	F	C	GEJ	Intestinal	Poorly differentiated	IIIb/$T_3N_2M_0$	Yes
4	67	M	C	GEJ	Intestinal	Moderately differentiated	IIIa/$T_3N_1M_0$	Yes
5	50	M	C	GEJ	Intestinal	Poorly differentiated	IIIa/$T_3N_1M_0$	No
6	70	M	C	Proximal	Intestinal	Poorly differentiated	Ib/$T_2N_0M_0$	No
7	57	M	C	Proximal	Intestinal	Moderately differentiated	Ia/$T_1N_0M_0$	No
8	67	F	C	Proximal	Intestinal	Moderately differentiated	Ib/$T_2N_0M_0$	No

[a] C, Caucasian.
[b] GEJ, primary tumor arose at gastro-esophageal junction; proximal, primary tumor arose in proximal stomach, fundus portion.
[c] Histopathology of the primary tumor according to the Lauren classification.
[d] Primary cancer's differentiation grade as judged by a pathologist (O. W. C. or H. F. F.).
[e] Refers to the presence (yes) or absence (no) of specialized intestinal epithelium detected in esophageal portions of resected specimens.

that were further processed for our molecular studies. The primary resected cancers and xenografted tumors were all classified histopathologically as intestinal-type adenocarcinomas with one well-differentiated, four moderately differentiated, and three poorly differentiated cases (Table 1). Five cases had resections of carcinomas arising from the gastro-esophageal junction, and four of these specimens exhibited Barrett's specialized epithelium within the esophagus portion. The other three cases had tumors arising from the proximal portion of the stomach, with the malignant growth margin noted 1–3 cm below the gastro-esophageal junction. All cases were Caucasian, with five males and three females. The ages of these patients at the time of resection ranged from 41 to 67 years. There were four early-stage cancers (stages Ia and Ib) and four more advanced carcinomas (stages IIIa and IIIb).

Complex changes of DNA copy numbers were detected in all cases. DNA copy number changes were found in all chromosomes (Y chromosome was excluded from the analysis). A total of 139 DNA copy number changes was detected (range, 11–24 per case; mean, 17). Gains (65%) were more frequent than losses (35%). Twenty high-level amplifications in different chromosomal areas were seen. Changes were not uniquely associated with tumor location (*i.e.*, gastro-esophageal junction *versus* proximal stomach), pathological stage, sex, or histopathological subtype.

Frequent and consistent chromosomal changes occurred with the following minimal common overlapping regions. Gains were detected in 20q (all cases); 6p11–p21.3, 7q21.2–q22.1, and 17q (six cases each, 75%); and in 1q, 2q11-q21.2, and 8q22.2-qter (five cases each, 63%). Gains with a frequency of 50% (four cases) were found in 3q21–qter and 12q13–q14. Moreover, high-level amplification was detected in 20q13.1–qter (four cases, 50%). Losses were shown in 4q23–q26 and 5q14–q22 (six cases each, 75%) and 9p21–pter (five cases, 63%). In addition, several other less frequent changes were found in all of the chromosomes, as shown in Table 2 and Fig. 1. All negative controls were normal at cutoff values of 0.85 for losses and 1.17 for gains. The positive controls showed the same aberrations that had been detected originally.

Discussion

We analyzed eight cases of xenografted human intestinal-type adenocarcinomas from the proximal stomach and gastro-esophageal junction for DNA copy number changes by CGH. A number of alterations were observed, some of which were consistently apparent in these cases. Xenografted tumors provide optimal samples for comparative analyses because they are enriched for neoplastic cells and consist almost entirely of neoplasic cells, with only minimal contaminating mouse stromal tissue present, as confirmed histologically. Previous studies have demonstrated that genetic changes found in these xenografted tumors are stable and correlate well with those from the corresponding primary tumors (6). Additional genetic alterations which might occur during propagation of these human tissues in immunodeficient mice appear to occur only rarely.

Consistent changes identified in these samples include: gains or high-level amplifications in 20q (eight cases, 100%); 17q, 6p, and 7q (six cases each, 75%); and 8q (five cases, 63%); and losses in 4q and 5q (six cases each, 75%) and 9p (five cases, 63%). These findings agree with those of a previous CGH study on primary GCs (3) that showed 17q and 20q gains in intestinal-type cases but at much lower frequencies (36% and 50%, respectively). Our results clearly highlight these chromosomal regions, as well as others that are commonly altered in a defined subgroup of carcinomas, specifically those of intestinal-type arising from the gastro-esophageal junction or proximal stomach.

DNA amplification at 20q was demonstrated in all eight cases. A minimal region of consistent amplification occurred at 20q13.1–qter. Gains and high-level amplifications in 20q have been reported in breast, colon, and ovarian adenocarcinomas (10–12), and such changes have been noted to correlate with poor prognosis in breast

Table 2 *DNA copy number changes in xenografts from eight proximal gastric and gastro-esophageal junctional cancers*

Case no.	DNA copy number changes	
	Losses	Gains and high-level amplifications[a]
1	2q14.2–qter, 4pter–q28, 5q, 12, 14q12–qter, 17p12–pter	1q, 3q21–qter, 5p, 6p11–p21, 7pter–q22, 8q21.2–qter, 10q24–qter, 17q, 18, 20q (**20q13.1–qter**), 21
2	4q, 8pter–q21.3, 9p21–pter, 12q21–q23, X	8q22–qter, 9q, 10q21–q23, 12p, 17q22–qter, 20
3	3p21.3–pter, 4q, 5q15–q22, 8p12–pter, 9, 10p, 11q14–qter, 13q22–q32, 14q23–qter, 18q, 21, X	2q (**2q32–q34**), 3q, 6p, **7q11–q31**, 10q, 12, 13q13–q21, 17, 18p, 20
4	1p21–p31, 4q11–q26, 5q, 7q31–qter, 10, 18q	1q, 2pter–q14.3, 3p22–pter, 3q21–qter (**3q24–q26.3**), 4p, 5p, 6q22–qter, 7q21.2–q22.1, **8p** (**8p21–pter**), **8q21.1–qter**, 11q11–q21 (**11q13**), 12pter–q15, **12q23–qter**, 14q13–qter, 15q22–qter, 17q, **20q**, 22
5	3p11–p21, 4pter–q31.1, 5q12–q14, 9, 13, 17p, 18pter–q21	3p21–pter, 7p14–q31 (**7q21–q31**), 8, **14q11–q24.1**, 17q, 20, 22
6	4q23–qter, 5q14–q23, 8p21–pter, 9pter–q22, 13q21–qter	1q, 2pter–q14.3, Xq, 6p, 7p, 10p, 11q11–q23.2 (**11q14–q23.2**), 12q13, 16p, 17 (17q), 19, 29 (**20q**), 21q22, Xq
7	5q14–q23, 6q11–q21, 12p	1q, 2, 3q, 5p, 6p, 7p (**7p14–pter**), 7q21–q22, **8q**, 10p, 11p13–pter, **13**, 16, **20**, X
8	5q13.2–q31, 9p21–pter, 10, Xpter–q25	1q, 2p12–q21, 6p, 7p14–qter, 9q, 12q13–q21, 15q23–qter, 17q, 19, 20

[a] High-level amplifications are presented in boldface.

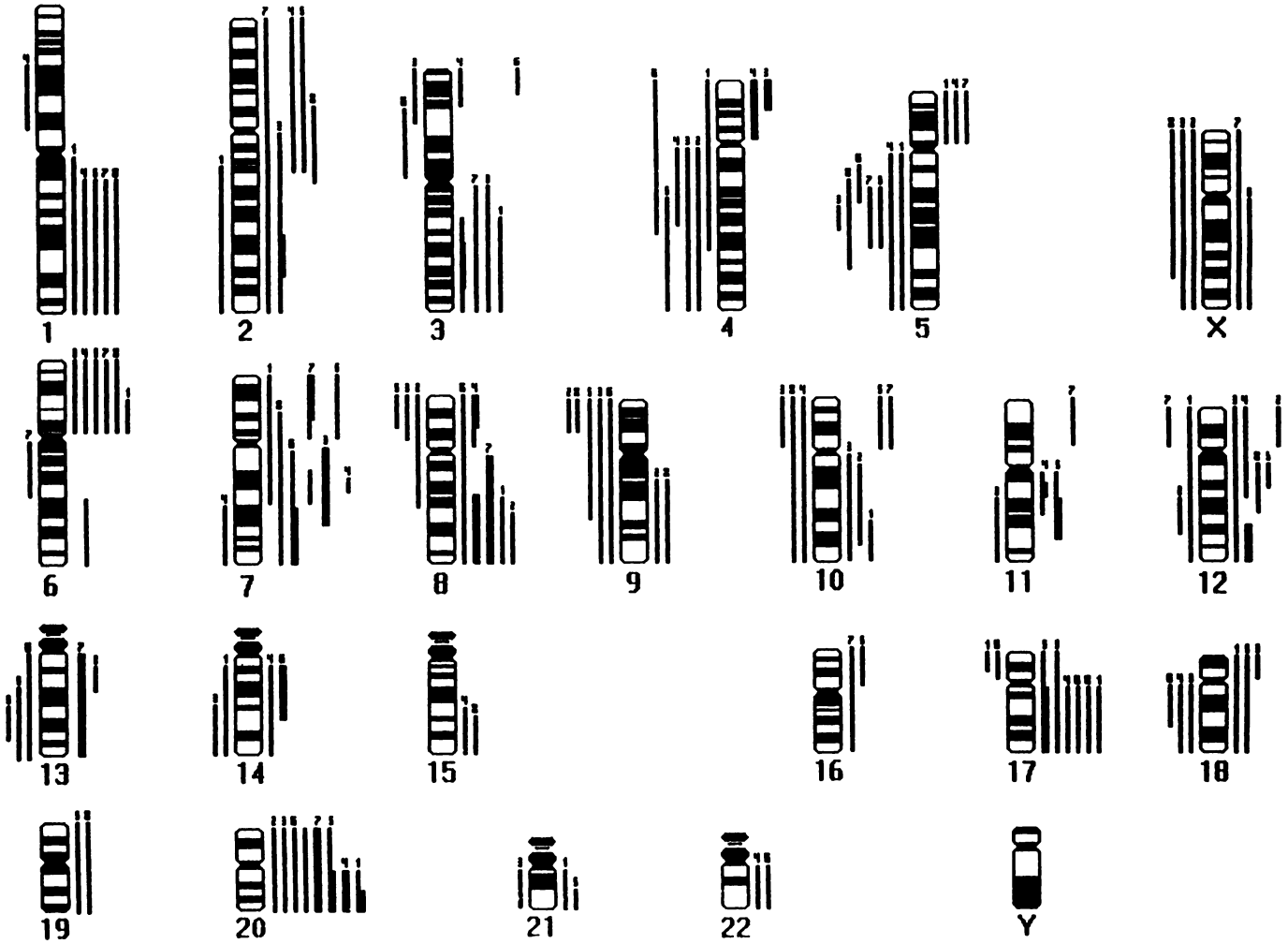

Fig. 1. Summary of DNA copy number gains and losses detected by CGH in xenografts of proximal stomach and gastro-esophageal intestinal-type carcinomas. *Bars*, individual tumor samples. *Right*, gains; *left*, losses. *Thick bars*, high-level amplifications.

cancer. This chromosomal region is likely to contain one or more genes that are overexpressed in several types of carcinoma. In breast cancer, the amplified 20q region is known to harbor specific amplified genes (*AIB1*, *AIB3*, and *AIB4*). Recently, *AIB1* was found to be a steroid receptor coactivator amplified in breast and ovarian cancer (13). The *PTP1B/PTPN1* gene, located at 20q12, is a nonreceptor tyrosine phosphatase that is involved in growth regulation and has been reported to be overexpressed in 72% of breast carcinomas (14). At 20q13, which is the minimal common overlapping region for high-level amplification in our cases, the *MYBL2* gene, which encodes a transcription factor, plays an important role in cell cycle progression. Furthermore, the human cellular apoptosis susceptibility gene (*CAS*) has been mapped to this same region.

Gains at 17q were found in three-fourths of our cases. 17q contains known genes that are potentially involved in tumorigenesis, such as *GAS*, which stimulates the growth of gastric and colon carcinoma cells *in vitro*. The *ERBB2* gene has been demonstrated to be amplified and overexpressed in a proportion of GCs (15). Kokkola *et al.* (3) reported 17q gains in 36% of intestinal-type GCs and further noted this change was conspicuously uncommon in diffuse-type GCs. Our results provide additional evidence that 17q gain is a consistent finding in intestinal-type cancers of the upper gastrointestinal tract, implying that an important gene that may be involved in tumorigenesis resides at this location.

Gains at 6p11–p21.3 and 7q21.2–q22.1 occurred in six (75%) of our cases. Amplifications at 6p21 have been described in ovarian carcinomas (16) and breast cancers (12). The proto-oncogene *PIMI*, which resides at 6p21, is a potential target of alteration in these cancers. Amplifications of the *MET* oncogene at 7q31 has been reported in GC (17); however, gains at 7q31 were only seen in three of our samples. We were able to demonstrate frequent gains at 7q21.2–q22.1 (six cases, 75%), the region where the multidrug resistance gene (*PGY1*) is located.

Loss of 4q23–q26 and 5q14–q22 occurred in three-fourths of our cases. Losses at 4q have been reported in mesotheliomas and have been related to tumor progression in gliomas (18, 19). Significant loss at 5q has been observed by loss of heterozygosity in GCs (20) and in leukemias, in which it is associated with a poor prognosis.

Gains and high-level amplifications at 1q, 3q21–qter, 8q22–qter, and 12q13–q14 and losses in 9p21–pter occurred in the majority of our samples. At 9p21–pter, two tumor suppressor genes are known, *CDKN2A* (p16) and *CDKN2B* (p15), and DNA copy number losses at these sites have been observed in mesotheliomas (19) and pancreatic carcinomas (21). The *p16* gene has been demonstrated to be altered in the majority of pancreatic cancers (22).

Gains and high-level amplifications at 1q have been found in sarcomas as well as in other malignancies (19, 23, 24). Several potentially important genes have been mapped to 1q, including *SKI*, which is related to metastasis in colon cancer (25), and the *CACY* gene, which has been associated with metastatic behavior of melano-

mas (26). Gains and high-level amplifications in 3q have been detected by CGH in several neoplasms and have been found to indicate tumor progression in cervical carcinoma and ovarian tumors (27, 28). The DNA copy gains and high-level amplifications seen at 8q occurred in the region of 8q24, which contains the proto-oncogene *CMYC*, previously shown to be amplified in numerous cancer types. At 12q, a complex amplicon was previously detected by CGH and loss of heterozygosity studies in sarcomas (29).

Specific CGH alterations were not observed to be uniquely associated with tumor location, pathological stage, histopathological grade, or patient demographics, such as sex or age. Because the number of cases studied was small, additional analyses examining more cases of adenocarcinomas, including those of various histopathological types and locations, will be required to conclusively determine whether or not DNA copy differences exist in various subgroups of GCs.

In summary, multiple DNA copy number imbalances at many locations in the genome were observed in proximal GCs of the intestinal type, suggesting its genetic complexity. Common gains and losses exhibited in several chromosomes (*i.e.*, 20q in all cases; 17q, 7q, 6p, 4q, and 5q in three-fourths of cases) suggest that these changes are critical in the development of these cancers. A consensus region of gain including significant high level amplification was noted at 20q13. Further molecular studies of the chromosomal sites implicated by these studies will likely identify genes that are important in proximal gastric tumorigenesis.

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