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Chromosomal Imbalances in Barrett's Adenocarcinoma and the Metaplasia-Dysplasia-Carcinoma Sequence

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To characterize cytogenetic alterations found in Barrett's adenocarcinoma (BA) and, more importantly, its premalignant stages, we studied chromosomal imbalances in various lesions in the histologically proposed metaplasia-dysplasia-carcinoma sequence using comparative genomic hybridization (CGH). Using 30 esophageal adenocarcinoma resection specimens, we were able to study 30 areas of Barrett's adenocarcinoma and 8 lymph node metastases (LN). In addition, we investigated 25 premalignant lesions adjacent to BA derived from a subset of 14 resection specimens including 11 areas of high grade dysplasia (HGD), 8 areas of low grade dysplasia (LGD), and 6 areas of intestinal metaplasia (IM), which were laser-microdissected and studied with CGH. To validate the CGH findings, fluorescence *in situ* hybridization analysis on 13 BA with probes specific for HER-2/*neu* and 20q13.2 were performed. The chromosomal alterations most often identified in BA were: gains on 8q (80%), 20q (60%), 2p, 7p and 10q (47% each), 6p (37%), 15q (33%) and 17q (30%). Losses were observed predominantly on the Y-chromosome (76%), 4q (50%), 5q and 9p (43% each), 18q (40%), 7q (33%) and 14q (30%). High-level amplifications were observed on 8q23-qter, 8p12-pter, 7p11-p14, 7q21–31, 17q11-q23. Recurrent chromosomal changes were also identified in metaplastic (gains on 8q, 6p, 10q, losses on 13q, Y, 9p) and dysplastic epithelium (gains on 8q, 20q, 2p, 10q, 15q, losses on Y, 5q, 9p, 13q, 18q). Novel amplified chromosomal regions on chromosomes 2p and 10q were detected in both Barrett's adenocarcinoma and premalignant lesions. An increase of the average number of detected chromosomal imbalances from IM (7.0 ± 1.7), to LGD (10.8 ± 2.2), HGD (13.4 ± 1.1), BA (13.3 ± 1.4), and LN (22 ± 1.2) was seen. Although the detection of common chromosomal alterations in premalignant lesions and adjacent carcinomas suggest a process of clonal expansion, the occurrence of several chromosomal changes in an apparently random order relative to one another is striking evidence that clonal evolution is more complex than would be predicted by linear models. This is probably a reflection of the existence of many divergent neoplastic subpopulations and highlights one of the main problems associated with surveillance of Barrett's patients, namely sampling error. *(Am J Pathol 2000, 156:555–566)*

As a result of chronic duodeno-gastro-esophageal reflux, the normal squamous epithelium of the distal esophagus is often replaced by a columnar or intestinalized epithelium with goblet cells.[1,2] This metaplastic (Barrett's) epithelium is a predisposing condition for the development of adenocarcinoma, through a well defined series of steps from intestinal metaplasia (IM) to low grade dysplasia (LGD) to high grade dysplasia (HGD) and then to carcinoma (BA). The risk for developing adenocarcinoma is estimated to be 30- to 125-fold greater in patients with IM than in patients without IM. In the Western world, BA has the most rapidly increasing incidence of all malignancies.[3,4] So far, the best predictor of BA in Barrett's esophagus is the histopathological detection of HGD in Barrett's metaplasia, because it has been shown that invasive cancer is frequently coincident or develops within a short time in patients in whom HGD has been identified.[5] However, the histopathological grading of dysplasia in endoscopic biopsies from Barrett's esophagus is moderately subjective, resulting in relatively high interobserver disagreement.[6] For this reason, it is recommended that the diagnosis of HGD, which is, in most institutions, regarded as an indication for prophylactic esophagectomy, be agreed on by two experienced pathologists.[7,8] Thus, because the identification of HGD in

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Table 1. Summary of Clinical and Histopathological Characteristics of the 30 Barrett's-Associated Esophageal Adenocarcinomas

Case	Age/sex*	pTNM†	G	R	Status	Survival‡
1	62 /F	T1N1Mx	4	0	Dead	31
2	75/M	T2N1M1a	3	0	Alive	11
3	62/M	T1N0Mx	3	0	N/A	N/A
4	61/M	T1N0Mx	3	0	Alive	36
5	60/M	T1N0Mx	2	0	N/A	N/A
6	72/M	T1N0Mx	2	0	Alive	41
7	72/M	T1N0Mx	3	0	Alive	21
8	73/M	T2N1Mx	3	0	Alive	6
9	70/M	T1N0Mx	2	0	Alive	29
10	72/M	T1N0Mx	4	0	Dead	0
11	34/M	T1N0Mx	2	0	Alive	15
12	53/M	T3N1Mx	2	1	Dead	0
13	70/M	T1N0Mx	3	0	Dead	4
14	68/M	T2N1Mx	3	0	Dead	14
15	58/M	T1N0M1a	2	0	Dead	1
16	76/M	T1N0Mx	2	0	Alive	85
17	50/M	T1N0Mx	3	0	Alive	19
18	62/M	T1N0Mx	2	0	Alive	7
19	68/M	T1N0Mx	2	0	Alive	4
20	59/M	T1N0Mx	2	0	Alive	71
21	75/M	T1N0Mx	3	0	Alive	40
22	75/M	T3N1Mx	3	0	Alive	8
23	64/M	T1N0Mx	2	0	Alive	80
24	58/M	T3N0Mx	1	0	Alive	23
25	65/M	T3N1Mx	3	0	Dead	43
26	55/M	T3N1Mx	3	x	Dead	27
27	59/M	T3N1Mx	3	0	Dead	16
28	79/M	T1N0Mx	3	0	Dead	1
29	55/M	T1N0Mx	2	0	Alive	1
30	61/M	T3N1Mx	3	1	Dead	0

*Age at diagnosis in years.
†UICC classification.
‡Survival in number of months from diagnosis.
M, male; F, female; N/A, data not available; G, histological tumor differentiation grade; R, residual tumor; X, unknown.

endoscopic biopsies is problematic and the consequences of failing to identify a BA are great, independent biomarkers for the prediction of subsequent carcinoma development would be very helpful as an adjunct to dysplasia identification for the surveillance of Barrett's esophagus.

Although numerous cytogenetic and molecular genetic studies have been performed on esophageal adenocarcinomas, fundamental data, especially pertaining to precursor lesions, which could substantially clarify our understanding of the tumorigenesis of BA are not available. Cytogenetic studies using G-banding, interphase fluorescence *in situ* hybridization (FISH), and comparative genomic hybridization (CGH) have revealed a complex pattern of structural and numerical chromosomal aberrations in BA of the distal esophagus and gastric cardia.[9–13] On the molecular genetic level, microsatellite analyses in previous studies have revealed frequent loss of heterozygosity (LOH) as well as allelic imbalances on chromosomes 5q, 17p, and 18q[14] and on chromosomes 3q, 4q, 5q, 6q, 9p, 9q, 12p, 12q, 17p, and 18q.[15] The latter studies have provided support for the proposed metaplasia-dysplasia-carcinoma sequence, in that they demonstrate a sequential accumulation of alterations and microsatellite changes in metaplasia and dysplasia. Other molecular studies of esophageal carcinoma have focused on alterations in specific candidate genes such as the fragile histidine triad (*FHIT*) gene on chromosome 3p14.2[16,17] or the *DPC4* gene on chromosome 18q21.1.[18] Despite the identification of molecular alterations in these chromosomal regions, *FHIT* and *DPC4* do not seem to be important for the development of these carcinomas, and it appears that the genes which play key roles in carcinoma development in Barrett's epithelium have not yet been identified.

To provide fundamental cytogenetic data in a large number of cases, we investigated 30 Barrett's-associated adenocarcinomas, 25 premalignant lesions (6 IM, 8 LGD, 11 HGD), and 8 regional lymph node metastases by CGH. Using an approach that combined laser-assisted microdissection and CGH, we were able to identify recurrent chromosomal changes in the histologically proposed metaplasia-dysplasia-carcinoma sequence.

Materials and Methods

Patient Samples

Thirty patients (1 female, 29 males) with BA of the distal esophagus diagnosed between 1990 and 1998 were studied. Follow-up data were available for 28 cases (mean follow-up, 23 months; range, 0–85 months). All patients underwent an esophagectomy without preoperative radiotherapy or chemotherapy. Clinical and histopathological data for the study group are summarized in Table 1. The analyses were performed on archival

Table 2. DNA Copy Number Changes in Intestinal Metaplasia (IM), Low-Grade Dysplasia (LGD), High-Grade Dysplasia (HGD), Adenocarcinoma (BA), and Regional Lymph Node Metastasis (LN)

Case	Dx	DNA losses	DNA gains and high-level amplifications
1	BA	2p23-pter, 2q21-23, 4, 5q14-21, 6q21-qter, 8p21-pter, 9p13-pter, 13q12-33, 14q12, 18q21-qter, 21q11-21	3p14-pter, 3q21-qter, 7p 7q22, 9q33-qter, 15q24-qter, 16q 17q, 20, 22q12-qter
1	LN	2p24-25, 4 5q12-23, 6q22-23, 8p12-pter, 9p21-pter, 11p12-15, 11q14-24, 13q12-31, 18q12-qter	2p14-22, 3p14-pter, 3q21-qter, 7p14-pter, 8q24, 10p14-pter, 15q21-qter, 16q, 17q, 20
2	BA	1q25-31, 3p23-pter, 4, 5p11-14, 5q11-23, 6q11-23, 7q21-31, 8p12-22, 9p11-23, 10q21-24, 12q21-23, 14q13-22, 15q11-22, Y	2p, 2q11-22, 3q23, 3q26-qter, 5q31-q534, 6p, 7p, 8q/**8q23-qter**, 9q22-qter, 10p, 11p, 11q23-qter, 13q, 16p, 16q 17/**17q11-23**, 18, 20, 22q12-qter
2	LN1	1q31-qter, 2q21-34, 4q, 5q11-23, 6q16-23, 7q22-31, 9p11-22, 12q15-22, 14q13-21, 15q15-21, Y	2p23-pter, 5q31-qter, 6p, 7p, 8q, 9q32-qter, 10p13-pter, 10q23-qter, 11p13-pter, 11q23-qter, 13q, 16p, 17p, 17q 18p, 20, X
2	LN2	4q21-qter, 5p13-14, 5q12-23, 7q21-32, 8p, 9p11-21, 15q, 16q13-qter, 18q14-qter, Y	2p23-pter, 3p14-21, 6p, **7p**, 8q, 9q32-qter, 10p, 10q23-qter, 11p, 11q23-qter, 13q, 16p, 17q, 20, 21q21-qter
3	BA•	2q22-31, 4q11-31, 5q11-23, 9p, 12q14-15, 13q14-31, 15q21-22, 18q12-22,	2q32-qter, 6p12-22, 7p, 8p, 8q23-qter, 10q22-qter, 20q
3	HGD•	2q22-24, 3q26-28, 11q23-24, 12q14-24, 13q14-qter, 14q24-qter, 15q23-qter, 17q22-24, 18q	18p, X
3	LGD•*	7q21-31, 12q11-23, 13q13-22, 14q13, 18q12-22, Y	3p21-pter, 2p24-pter, 8p22-pter, 8q23-qter, 10p14-pter, 10q24-qter, 15q23-qter, 17q, 20q
3	IM•*	12q12-22, 13q14-32, 14q13-22, 18q12-22, Y	3p21-pter, 15q23-qter
4	BA	4q, 5q11-23, 6q15-23, 7q21, 9p, 13q21-31	1q31-qter, 2p16-pter, 2q34-qter, 6p, 8p, 8q23-qter, 9q, 10q22-qter, 11, 15q21-qter, 16, 17, 20, X
4	LGD	9p11-21, 17p, 18p, Y	2p22-pter, 6p12-21, 6q21-25, 11q23-qter, 12p, 13q, 14q23-qter, 15q22-qter, 17q22-23, 18q,
5	BA	4q11-27, 5q11-21, 7q21-22, 9p11-23, 14q11-23, 15q12-21, 16p11-12, 18q12-qter, Y	2p23-pter, 7p, 8q23-qter, 10p14-pter, 10q22-qter, 20, Xq21-qter
5	HGD	5p14-pter, 13q21-qter, 15q22-qter, 18q, Y	18p, 21, Xq21-qter
6	BA	4q, 5q11-23, 9p11-22, 12q14-21, Y	2p23-pter, 6p1-23, 7p, 7q31-qter, 8q23-qter, 9q31-qter, 10q23-qter, 13q13-14, 14q23-qter, 15q23-qter, 17q, 20
6	HGD	2q22-23, 4q, 5q13-22, 6q13-24, 9p11-22, Y	2p24-pter, 3p24-pter, 3q23-qter, 6p, 7p, 7q33-qter, 8q23-qter, 10q25-qter, 13q21-32, 15q24-qter, 17q, 18p, 20p, 20q
6	LGD	Y	8q23-qter
7	BA	4q22-28, 5q12-23, 9q, 16q, 17q, 18q, Y	6p, 6q16-24, **7p11-14**, **7q11-31**, 8q, 10q22-23, 11p, 12p, 14q22-qter, 17p, 18p, 20, Xq
7	HGD•	9p11-22, 14q21-22, 15q11-15, 17q21-24, 18q12-22, 21q, 22q, Y	1q22-qter, 6q13-21, 7p11-21, 7q21-22, 8q, 11p, 20p, 20q,
7	LGD•	7q11-22, 9p11-23, 17q21-24, 22q, Y	1q25-32, 2p23-pter, 3p24-pter, 6q14-15, 7p14-15, 8q, 10q24-qter
8	BA	Y	8p21-pter, 8q13-qter, 12p11-12, 13q12-21
9	BA	5q12-21, 8p21-pter, 18q12-22, Y	7p, 7q11-22, 8q22-qter, 20, X
10	BA	8p, 16, 17p, Y	3q21-qter, 5p, 6, 7p12-21, 8q, 11p, 13q,
10	LGD	15q11-24, 17p11-12, Y	2p24-pter, 8p22-pter, 8q23-qter,
10	IM	9p11-13, 13q14-31, 17q11-22, Y	1q31-qter, 2p24-pter, 6p21-pter, 8p22-pter, 8q23-qter, 10q24-qter
11	BA	13q, Y	1q23-qter, 6p, 8p12-pter, 8q21-qter, 20
11	HGD	13q12-22, 14q11-21, 15q11-21, 16p11-12, 17p, 22, Y	1q, 2p23-pter, 6p21-pter, 7p11-15, **8p12-pter**, **8q22-qter**, 20p, 20q,
12	BA	4q, 5q11-23, 7q31, 9p11-21, 12q15-21, 18	2p23-pter, 3q22-qter, 7p, 7q22, 8q23-qter, 16p12-pter, 17q, 20q
12	LN	2q22-32, 4, 5q12-23, 6q11-23, 9p11-23, 13q14-31, Y	2p24-pter, 3p14-pter, 6p23-pter, 8p, 8q23-qter, 9q32-qter, 10q25-qter, 11p14-pter, 15q23-qter, 16p12-pter, 16q22-qter, 17, 20q, 22q,
13	BA	4p14-15, 4q25-27, 7q11-22, 10p13-14, 13q14-31, 15q11-21, 21q11-22	8q23-qter, 9q22-qter, 10q24-qter, Xp
14	BA	2q21-31, 4, 5p11-14, 5q11-22, 6q11-15, 13q14-32, 14q22-23, 15q14-15, 18q, Y	2p23-pter, 3p21-pter, 6p, 7q34-qter, 8p, 8q23-qter, 10q25-qter, 11q, 12q22-qter, 15q23-qter, 16p23-pter, 16q22-qter, 17, 20, 22q
14	LN	2q33-31, 4q, 5q14-23, 12q14-21, 13q14-31, 15q21-23, Y	3p24-pter, 6p, 7p12-14, 7q31-qter, 8p, 8q22-qter, 10p, 10q24-qter, 12q22-qter, 16, 20q
15	BA	5q14-22, 14q11-21, 17p, 21q11-21, 22q, Y	8q24, 13q, 17q11-23, 18p, 20q
15	HGD	5q13-22, 7q21-22, 9p, Y	2p24-pter, 8q23-qter, 10q25-qter, 16q, 17q, 20p, 20q,
15	LN	2q21-32, 7q22-31, 11p12-14, 11q21-23, 13q21-31, 14q11-21, 15q11-21, Y	1q31-qter, 3p, 7p13-15, 8q22-qter, 9q33-qter, 10q25-qter, 12p, 16p, 17q11-q23, 20
16	BA	9p11-21, 14q11-22, Y	1q, 2+, 3p24-pter, 3q, 5+, 7p15, 8q23-qter, 10q24-qter, 12q11-qter, 13q, 18q
16	IM	1q32-qter, 2q13-23, 8p21-pter, 13q21-qter, 18q12-22	8q13-21, 17p 18p, X
17	BA	—	7p13, 8q23-qter, 10q25-qter, 13q21-22
17	HGD	4q, 5q11-23, 13q14-31, Y	2p23-pter, 7p12-14, 8q23-qter, 17p, 20
17	LGD•	7q21-22, 17q22-24, Y	—
17	IM•	13q14-22, Y	6p23-pter, 7p12-13, 8q23-qter
18	BA	2q11-31, 4q,7q22-35, 9p, 9q11-31, 18q, Y	7q11-21, 10q21-qter, 11q13, 13q11-21, 15q22-qter, 16q, 18p, 20q
18	HGD	2q11-24, 4q, 5q13-23, 7q31-32, 9p, 15q11-15, 18q, 22q, Y	2p23-pter, 7q11-22, 8q23-qter, 10q21-qter, 11p, 13q11-14, 15q21-qter, 18p, 20p,

High level amplification is presented in boldface; shared chromosomal imbalance within distinct histopathological lesion from one patient are underlined. Samples in close proximity <0.5 cm) are marked by a •.

*In case 3 IM was in proximity to LGD and HGD was in proximity to BA, whereas IM/LGD and HD/BA derive from separate tissue blocks.

Table 2. Continued

Case	Dx	DNA losses	DNA gains and high-level amplifications
18	LGD	4p11-14, 4q11-31, 5q13-23, 7q21-22, 9p11-23, 12q14-15, 13q14-22, 14q11-22, Y	2p23-pter, 3p14-pter, 6p 8p, 8q23-qter, 9q22-qter, 10q22-qter, 15q22-qter, 20q
18	IM	—	—
19	BA	3p, 4, 9p, 18q, Y	2p14-pter, 7, 8q13-qter, 9q, 11q23-qter, **17q11-q24**
20	BA	2q23-33, 4q, 9p11-22, Y	6p, 8p22-pter, 8q23-qter, 9q22-qter, 10q25-qter,13q11-21, 15q23-qter, 16p, 20q
20	HGD•	2q22-31, 4q11-31, 13q21-31, Y	2p24-pter, 3p23-pter, 3q21-25, 6p23-pter, 7p14-15, 8q22-qter, 10p22-pter, 10q22-qter, 15q23-qter, 20p, 20q
20	LGD•	9p11-pter, 13q14-31, Y	2, 3p, 6p, 6q, 11, 17, 18, 20
21	BA	7q11-31, 18q12-22,	8p22-pter, 8q23-qter, 18p, 20q
22	BA	13q14-22, 16p11-12, Y	8q23-qter, 11q24-qter,
22	LN	2q31-32, 4q27-31, 13q14-23, Xq	1q22-qter, 2p23-pter, 2q11-21, 5p, 6p, 7q32-qter, 8p21-pter, 8q22-qter, 9q22-qter, 11q, 16, 17, 20, 22
23	BA	2q21-31, 4q, 5q11-23, 6p, 7q31, 12q11-21, 14q11-22, Y	2p24-pter, 8q22-qter, 10q22-qter, 15q22-qter, 16p, 17q, 20q
23	HGD	4q11-31, 5q11-23, 9p, 12p13-21, 14q11-21, Y	2p23-pter, 6p22-pter, 7p, 8, 9q, 10q25-qter, 15q21-qter, 20p, 20q
23	IM	9p11-21, 12q15-21, 13q21-33, 18q11-22,	6p, 7p12-15, 8p21-pter, 8q22-qter, 9q, 10q25-qter, 17p, 20q
24	BA	7q31-34, 9p, 10p11-14, 12p11-12, 14q11-22, 15q11-14, 18q, Y	1q31-qter, 2p24-pter, 6p, 7p12-15, 8q22-qter, 11p, 13q, 16q, 20q,
25	BA	Y	2p24-pter
25	LN	2q23-32, 3q24-26, 4q, 5q14-22, 9q22-32, 13q13-31, 14q12-22, 18q	1q31-qter, 2p22-pter, 3p, 6p, 7p, 8q22-qter, 10p, 15q22-qter, 16q, 17, 18p, 20, 22
26	BA	3p11-13, 4−, 7q11-33, 9p, 13q14-31, Y	1q23-qter, 2p, 3q21-qter, 5q13-23, 6p, 7p13, 8q22-qter, 10q25-qter, 14q23-qter, 15q23-qter, 20p, 20q
27	BA•	18q, Y	8q/**8q21-qter**, 10p, 13q22-qter
27	HGD•	4p11-15, 5q12-23, 7p31, Y	1q31-qter, 3p21-pter, 8p22-pter, 8q21-qter, 10p13-pter, 10q22-qter, 20q
28	BA	14q11-23, 17p, Y	2, 4p, 4q24-qter, 7q21-32, 10p, 15q21-qter, 18q.
29	BA	17p, Y	2,4
30	BA	17p	3p, 4p, 5p, 10q25-qter, 13q14-31, 15q21-qter

material from formalin-fixed tissues embedded in paraffin. Hematoxylin and eosin-stained slides from the resection specimens were re-evaluated independently by two pathologists for the identification of intestinal metaplasia (IM), columnar epithelial dysplasia (low-grade dysplasia - LGD or high-grade dysplasia - HGD), tumor differentiation grade (well, moderate, poor), depth of tumor invasion, and regional lymph node metastasis (LN). The adenocarcinomas (BA) were staged according to the Union Internationale Contre le Cancer TNM system.[19] The most BA in our series were classified as pT1 and pT2, reflecting common surgical strategies which exclude advanced cases from esophagectomy. Paraffin blocks of BA (n = 30) were obtained from 30 resection specimen including LN (n = 8). In addition, from a subset of 14 resection specimen 25 premalignant lesions consisting of HGD (n = 11), LGD (n = 8), and nondysplastic intestinal metaplasia IM (n = 6) were selected for laser microdissection and subsequent DNA extraction. Several samples were in close proximity to each other (<0.5 cm) and are marked by a • in Table 2.

Laser-Assisted Microdissection and Cell Pretreatment

An UV laser microbeam (P.A.L.M, Wolfratshausen, Germany) was used to excise precisely defined tissue areas on unmounted H&E-stained 5-μm serial sections. At least 1 to 5 $\times$ 10^3 microdissected cells from 3 to 6 serial sections were sampled from normal squamous epithelium (control), IM, LGD, and HGD. From BA, 10^5 to 10^6 microdissected cells were sampled, representing the complete BA area of 1 to 3 serial sections. The cells were lysed in 50 μl of 100 mmol/L Tris-HCl (pH 7.5), 1 mg/ml proteinase K for 24 hours at 55°C.

Amplification and Labeling of Test DNA

Degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) was performed on DNA extracts from microdissected tissue according to a previously published method.[20–23] DOP-PCR-amplified DNA as well as non-amplified DNA from tumor and control samples were labeled with biotin-16-dUTP (Boehringer Mannheim, Mannhein, Germany) using standard nick translation.

CGH and Image Analysis

CGH was performed on test DNA amplified by DOP-PCR according to published procedures.[21–23] For all CGH preparations 300 ng of labeled test DNA and Spectrum-Red direct-labeled normal female or male total human genomic DNA (Vysis, Inc., Downers Grove, IL), plus 25 μg *Cot*IDNA were cohybridized to denatured metaphases for 72 hours at 37°C. After hybridization, biotin-labeled test DNA was detected with Cy2-conjugated streptavidin (Dianova). For CGH analysis, at least ten metaphases were imaged and karyotyped after visualization with a Zeiss Axioplan 2 fluorescence microscope equipped with filter sets (single-band excitation filters) for

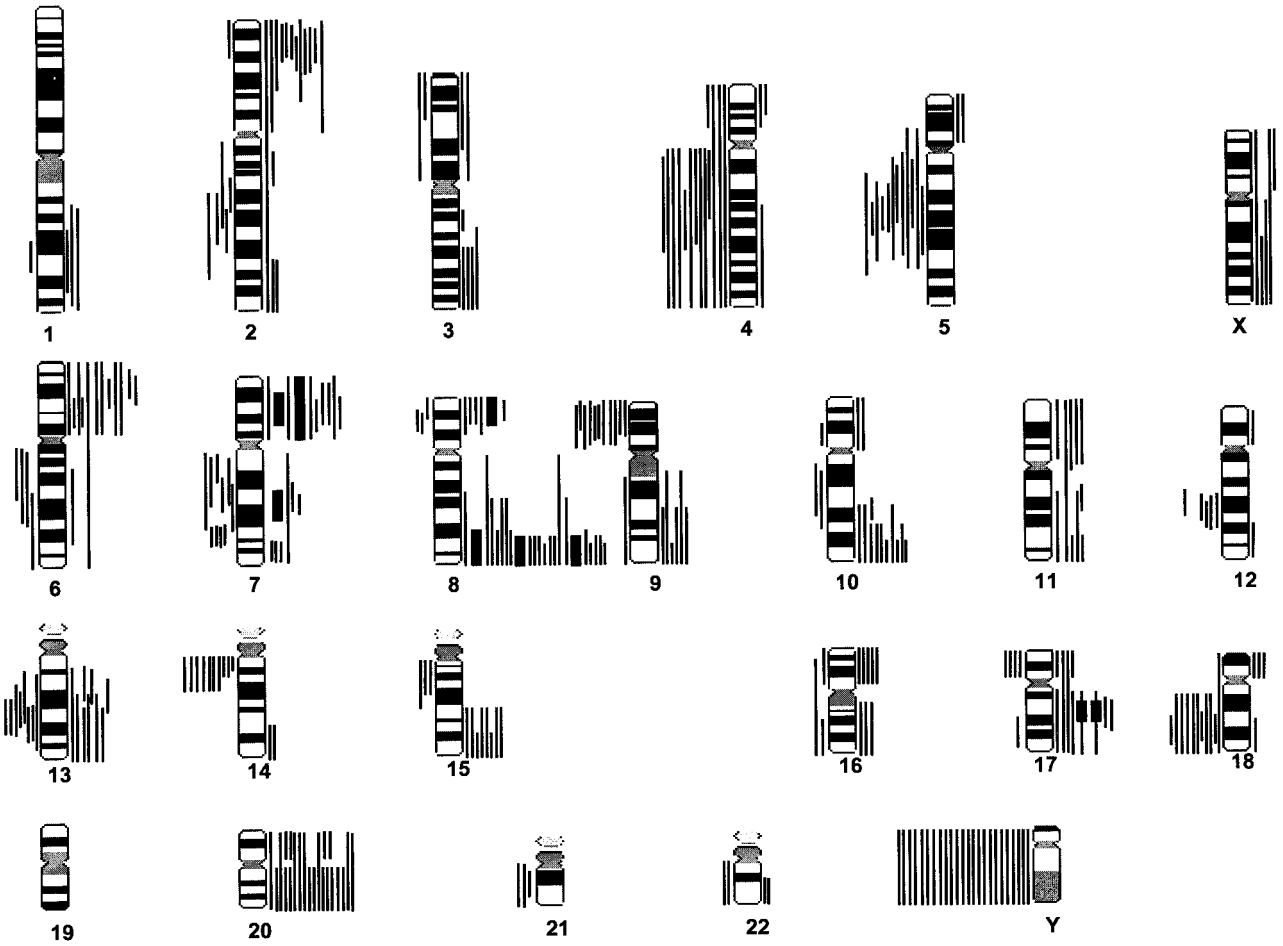

Figure 1. Summary of DNA copy number changes detected by CGH in 30 BA. Lines to the left and the right of the chromosomes indicate the regions lost and gained, respectively. Thick bars represent regions of high-level gain. Imbalances found on chromosome 1p and chromosome 19 were not scored, as detailed in Material and Methods.

4′−6-diamidino-2-phenylindole, Cy2 and Texas Red. Averaged profiles were generated by CGH analysis software (ISIS 3, V2.84; MetaSystems, Altlussheim, Germany) from at least 10 to 15 homologous chromosomes and interpreted according to published criteria[21,24] using statistical confidence limits based on *t*-statistics. An overexpressed area was classified as a high-level amplification when the CGH ratio exceeded a value of 1.5, or when the Cy2 fluorescence showed a strong, distinct signal by visual inspection and the corresponding ratio profile was diagnostic of overrepresentation.[25] Regions of high level amplification are shown as thick bars in Figures 1 and 2.

Control Experiments

DOP-PCR-amplified DNA obtained from morphologically normal-appearing esophageal squamous epithelium was hybridized with non-amplified male or female reference DNA (SpectrumRed) to metaphase preparations. In these experiments no chromosomal changes were detected except for chromosome region 1p34–36 and chromosome 19. These regions are known to show artifactual results by CGH.[22,26,27] Therefore, chromosomes 1p and 19 were excluded from further analysis. In addition, three cases of BA were comprehensively analyzed using both DOP-PCR amplified and nonamplified DNA, with the same chromosomal changes being detected by both methods. CGH results were further validated by comparison with FISH analysis.

FISH Analysis

FISH analysis with specific probes was essential to complement the CGH analysis both as validation of the CGH findings and as a control of the degree of intratumor heterogeneity. Thirteen cases with known DNA copy number changes on chromosomes 17q and 20q from CGH experiments were selected to confirm these changes in the BA samples (Table 5). Serial 5-μm sections of the tissue blocks were used for FISH analysis which investigated areas corresponding to those examined by CGH. For FISH analysis a PathVysion HER-2 DNA probe kit (Vysis, Inc.) was used according to the manufacturer's recommendations. The kit consists of directly labeled, fluorescent DNA probes specific for the HER-2/*neu* gene locus (17q11.2-q12) and a DNA probe specific for the α satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1). DNA probes for

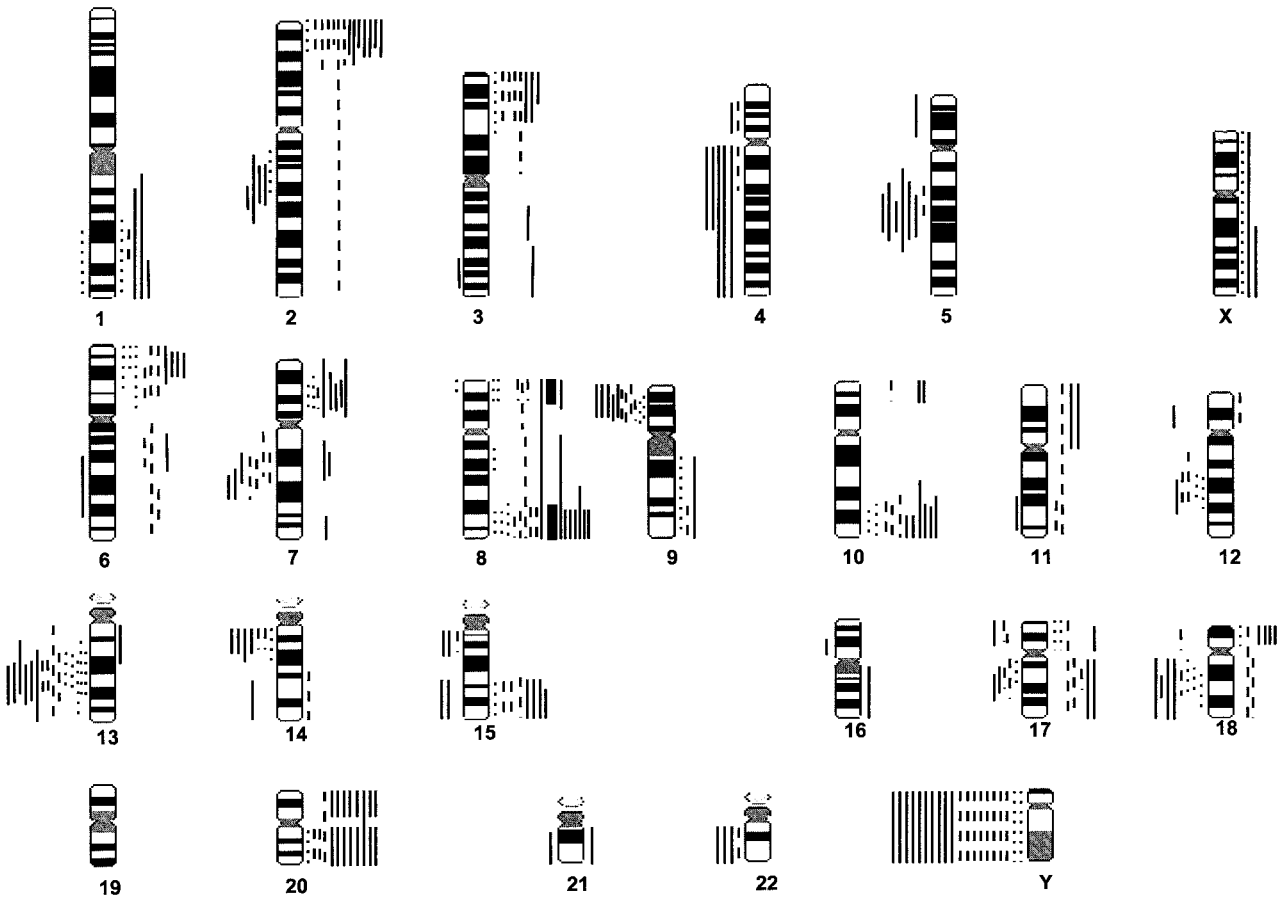

Figure 2. Summary of DNA copy number changes detected by CGH in six metaplastic (·····), eight low grade dysplastic (– – – – –), and 11 high grade dysplastic (———) areas. Lines to the left and the right of the chromosomes indicate the regions lost and gained, respectively. Thick bars represent regions of high-level gain. Imbalances found on chromosome 1p and chromosome 19 were not scored, as detailed in Material and Methods.

the subchromosomal region 20q13.2 (Spectrum Orange-labeled, Vysis, Stuttgart, Germany) and for the centromeric region of chromosome 20 (D20Z1, biotin-labeled, Oncor Appligene, Heidelberg Germany) were used as described elsewhere.[28] Signals from 100 to 150 tumor cell nuclei were counted using confocal laser scanning microscopy (Zeiss LSM 410). According to published criteria,[29] gene amplification was detected if the ratio of locus-specific signals to centromeric signals per cell was at least 3 in >10% of tumor cells or tight clusters of >10 signals in multiple cells. Further controls consisted of adjacent, phenotypically normal squamous epithelium and lymphocytes.

Statistical Analysis

For comparison of average aberration frequencies in each entity, the SEM was calculated. A pairwise comparison analysis was applied to compare the occurrence of chromosomal imbalances in different stages of the metaplasia-dysplasia-carcinoma sequence. For this purpose, 14 cases were selected with available data for at least one precursor stage (IM, LGD, or HGD) and the corresponding carcinoma. The data were compared pairwise in Table 4, indicating concordant results for each chromosome aberration. Concordance was classified into two groups: 1–1 (aberration present in both lesions) and 0–0 (aberration absent from both lesions). This comparison resulted in four pairs each for IM *versus* LGD and IM *versus* HGD, six pairs each for IM *versus* BA and LGD *versus* BA, eight pairs for LGD *versus* BA, and eleven pairs HGD *versus* BA.

Results

The clinical and histopathological data for the individual cases are presented in Table 1. A statistical correlation between cytogenetic and clinicopathological data could not be observed using Fisher's exact test ($P > 0.05$ for gender, survival, and TNM stage). The detailed DNA gains and DNA losses for each of the 63 samples investigated are presented in Table 2. The chromosomal imbalances which were found are presented in Figure 1 for the adenocarcinomas and in Figure 2 for the areas of IM, LGD, and HGD. A comparison of the results from FISH and CGH analysis is presented in Table 5.

BA and LN

An average of 13.3 ± 1.4 chromosomal imbalances per case were detected in the 30 BAs. The chromosomal

Table 3. Frequent Chromosomal Imbalances from CGH Analysis of IM, LGD, HGD, BA, and LN Areas

Chromosomal changes	IM	LGD	HGD	BA	LN
amp8q [8q23-24]	4/6	5/8	9/11	24/30 (80%)	8/8
amp20q	1/6	3/8	8/11	18/30 (60%)	8/8
amp2p [2p23-24]	1/6	6/8	7/11	14/30 (47%)	6/8
amp7q	0/6	0/8	3/11	14/30 (47%)	2/8
amp10q [10q25-26]	2/6	3/8	5/11	14/30 (47%)	5/8
amp6p	3/6	3/8	4/11	11/30 (37%)	6/8
amp15q [15q24-26]	1/6	3/8	4/11	10/30 (33%)	3/8
amp17q [17q11-22]	0/6	3/8	2/11	9/30 (30%)	7/8
del#Y	3/6	8/8	10/11	23/30 (76%)	5/8
del 4q	0/6	1/8	5/11	15/30 (50%)	7/8
del5q [5q13-23]	0/6	1/8	6/11	13/30 (43%)	6/8
del9p [9p13-pter]	2/6	4/8	5/11	13/30 (43%)	4/8
del18q [18q12-22]	3/6	1/8	4/11	12/30 (40%)	3/8
del7q	0/6	4/8	2/11	10/30 (33%)	3/8
del14q [14q11-22]	1/6	2/8	4/11	9/30 (30%)	3/8

The aberrations listed were most often found in BA areas. Minimal common regions are given in brackets.

alterations most often identified were gains on 8q (80%), 20q (60%), 2p, 7p and 10q (47% each), 6p (37%), 15q (33%), and 17q (30%). Losses were observed predominantly on the Y-chromosome (76%), 4q (50%), 5q and 9p (43% each), 18q (40%), 7q (33%), and 14q (30%) (Figure 1). High-level amplifications were observed on 8q23-qter, 8p12-pter, 7p11-p14, 7q21–31, and 17q11-q23. In the eight LN, an average of 22.0 ± 1.2 chromosomal imbalances per case were detected, involving namely gains on 8q and 20q (8/8), 7p and 17q (7/8 each), 2p, 3p, 6p, and 16p (6/8 each), and 9q, 10p, 10q, and 16q (5/8 each) and losses on 4q (7/8), 2q, 5q, and 13q (6/8 each), Y chromosome (4/8), 9p and 15q (4/8 each), and 18q, 14q, and 6q (3/8 each).

HGD, LGD, and IM

In the 11 samples taken from HGD areas, an average of 13.4 ± 1.1 chromosomal imbalances per case were detected, with the following recurrent alterations: gains on 8q (9/11), 20p and 20q (8/11), 2p (7/11), 7p (6/11), 10q (5/11), 15q (4/11), and 6p(4/11), and losses on the Y chromosome (10/11), 5q (6/11), 9p, 13q (5/11 each), 2q, 4q, 14q, and 18q (4/11 each), and 8p (3/11). In the 8 LGD areas, an average of 10.8 ± 2.2 chromosomal imbalances per case was detected, with gains on 2p (6/8), 8q (5/8), 3p (4/8), and 15q and 20q (3/8 each) and losses on the Y chromosome (8/8), 7q and 9q (4/8 each), and 13q (3/8). Among the 6 samples taken from IM areas without dysplasia, there was an average of 7.0 ± 1.7 chromosomal imbalances per case, with gains on 8q (4/6), 6p (3/6), and 8p and 10q (2/6), and losses on chromosome 13q (5/6), Y chromosome (3/6), and 9p and 12q (2/6 each) being present.

Comparison of Chromosomal Changes between IM, LGD, HGD, BA, and LN Areas

The average number of chromosomal imbalances increased steadily from IM (7.0 ± 1.7) to LGD (10.8 ± 2.2) to HGD (13.4 ± 1.1) and BA (13.3 ± 1.4), and again to LN areas (22 ± 1.2). The largest differences in copy number changes were seen i) between BA and LN areas, in particular the frequency of DNA gain on 20q and 17q and DNA loss on 4q, in LN (Table 3); ii) between LGD and HGD areas, especially with DNA losses being more frequent in HGD, but without a particular chromosomal change which distinguished between them; and iii) between IM and LGD areas, with losses on 4q and 5q appearing first in LGD and remaining in all subsequent stages. In contrast, the average number and frequency of chromosome copy number changes were very similar in BA and HGD. In addition, a pairwise comparison analysis of 14 cases with at least one precursor stage and the corresponding carcinoma was performed to identify concordances for the occurrence of chromosomal aberrations between IM *versus* LGD, IM *versus* HGD, IM *versus* BA, LGD *versus* HGD, LGD *versus* BA, and HGD *versus* BA. This analysis of a subset of 14 cases revealed a stepwise occurrence of chromosomal imbalances in each stage of the metaplasia-dysplasia-carcinoma sequence (Table 4).

FISH Analysis

A total of 17 FISH experiments on 13 BA were performed. A comparison of the results from FISH and CGH analyses is presented in Table 5. Each of the tumors investigated showed striking heterogeneity, with several areas displaying different levels of Her-2/*neu* or 20q13.2 signals. Even in cases with only lower levels of amplification (eg, cases 6 and 14 for Her-2/*neu*) there were small subpopulations (<5%) with tight clusters of signals.

Discussion

This study is the first comprehensive analysis of DNA gains and losses associated with the metaplasia-dysplasia-carcinoma sequence in Barrett's esophagus. Using 30 esophageal adenocarcinoma resection specimens we were able to identify and study 30 BA and 25 premalig-

Table 4. Pairwise Comparison Analysis of 14 Carcinomas and Their Corresponding Precursor Lesions

Chromosomal aberrations	IM *vs.* LGD $n = 4$ pairs		IM *vs.* HGD $n = 4$ pairs		IM *vs.* BA $n = 6$ pairs		LGD *vs.* HGD $n = 6$ pairs		LGD *vs.* BA $n = 8$ pairs		HGD *vs.* BA $n = 11$ pairs	
	0–0	1–1	0–0	1–1	0–0	1–1	0–0	1–1	0–0	1–1	0–0	1–1
Amp 8q	0	1	1	2	1	4	0	3	0	4	0	8
Amp 20q	2	0	2	1	3	1	0	1	2	3	0	6
Amp 2p	1	1	1	0	3	0	0	2	1	1	2	2
Amp 7q	4	0	3	0	5	0	3	0	5	0	8	3
Amp 10q	1	0	2	1	0	1	2	1	1	2	1	3
Amp 6p	2	0	2	1	2	1	4	1	2	2	4	3
Amp 15q	2	1	1	0	3	0	2	1	3	2	6	4
Amp 17q	3	0	4	0	5	0	3	0	4	0	8	2
Del #Y	0	3	0	1	0	1	0	5	0	4	1	9
Del 4q	3	0	1	0	1	0	1	1	2	1	3	4
Del 5q	3	0	1	0	3	0	3	1	3	0	2	3
Del 9p	2	0	2	1	0	0	2	2	2	3	3	2
Del 18q	3	1	1	1	2	1	3	1	5	1	6	4
Del 7q	1	0	3	0	4	0	2	1	3	1	7	1
Del 14q	2	1	2	1	3	0	3	1	6	0	5	1

Classification of concordance: 0-0, aberration is absent from both stages; 1-1, aberration is present in both stages.

nant lesions adjacent to BA (11 HGD, 8 LGD, 6 IM) and 8 LN areas by combining laser assisted microdissection with CGH analysis. As a result, recurrent chromosomal regions involved in the neoplastic progression of Barrett's esophagus in discrete areas were able to be detected.

Tissue microdissection is particularly important for BA and its precursor lesions since they usually contain a significant component of non-neoplastic cells. As an alternate microdissection technique to previous CGH studies of BA,[11–13] we isolated histopathologically defined areas using laser assisted microdissection on each specimen. Using this technique, the chromosomal regions most frequently showing gains in the BA were 8q, 20q, 2p, 7p, 10q, 6p, 15q, and 17q. Losses were frequently observed on the Y chromosome, 4q, 5q, 9p, 18q, 7q, and 14q. These results are in agreement with previous CGH and LOH studies.[11–15] Furthermore, we were able to identify recurrent DNA gains on two chromosomal areas, 2p and 10q, which have not been described previously in BA. Gains and high-level amplifications on 2p have been detected by CGH in several other neoplasms, including gastric adenocarcinoma,[30] in which the amplification of the proto-oncogene *NMYC* located on 2p23–24 was described. *NMYC* amplification has been observed in multiple studies of neuroblastoma, in which *NMYC* amplification remains the most widely accepted predictive parameter of long-term, disease-free survival.[31] However, we found no statistically significant correlation between DNA gain on 2p23–24 and survival ($P > 0.05$). The gain on 10q25–26 might indicate involvement of the *K-SAM* gene, which was previously identified in a gastric cancer cell line[32,33] and is known to encode one of the heparin-binding growth factor receptors or fibroblast growth factor receptors. Recently, using immunohistochemistry, Hattori et al demonstrated that 20 of 38 undifferentiated type advanced gastric cancers overexpressed the K-SAM protein.[34] The most frequent DNA gain we observed was on 8q. This amplification involves the minimal common chromosomal region 8q23–24, which contains the proto-oncogene *CMYC*, previously shown to be amplified by interphase FISH in 15.5% of gastric adenocarcinomas.[35] In our investigation we demonstrated a higher frequency of 8q amplification than had been reported in the previous cytogenetic studies of

Table 5. Comparison of FISH and CGH Analyses of 13 Adenocarcinomas

Case	FISH analysis		CGH analysis	
	Her-2/*neu*	20q13.2	17q	20q
2	amplification*	NA	**amp17q11-q23**	+20
3	NA	amplification	no change	+20q
6	amplification	NA	+17q	+20
7	NA	amplification	no change	+20
8	no gain	no gain	no change	no change
11	NA	amplification	no change	+20
12	amplification*	amplification	+17q	+20q
14	amplification	NA	+17	+20
15	amplification*	amplification	+17q11-q23	+20q
18	NA	amplification	no change	+20q
19	amplification*	no gain	**amp17q11-q24**	no change
23	NA	amplification	+17q	+20q
25	no gain	NA	no change	no change

All cases are BA.
*Tight clusters of signals in more than 30% of tumor cell nuclei.
NA, not analyzed; high-level amplification detected by CGH is presented in boldface.

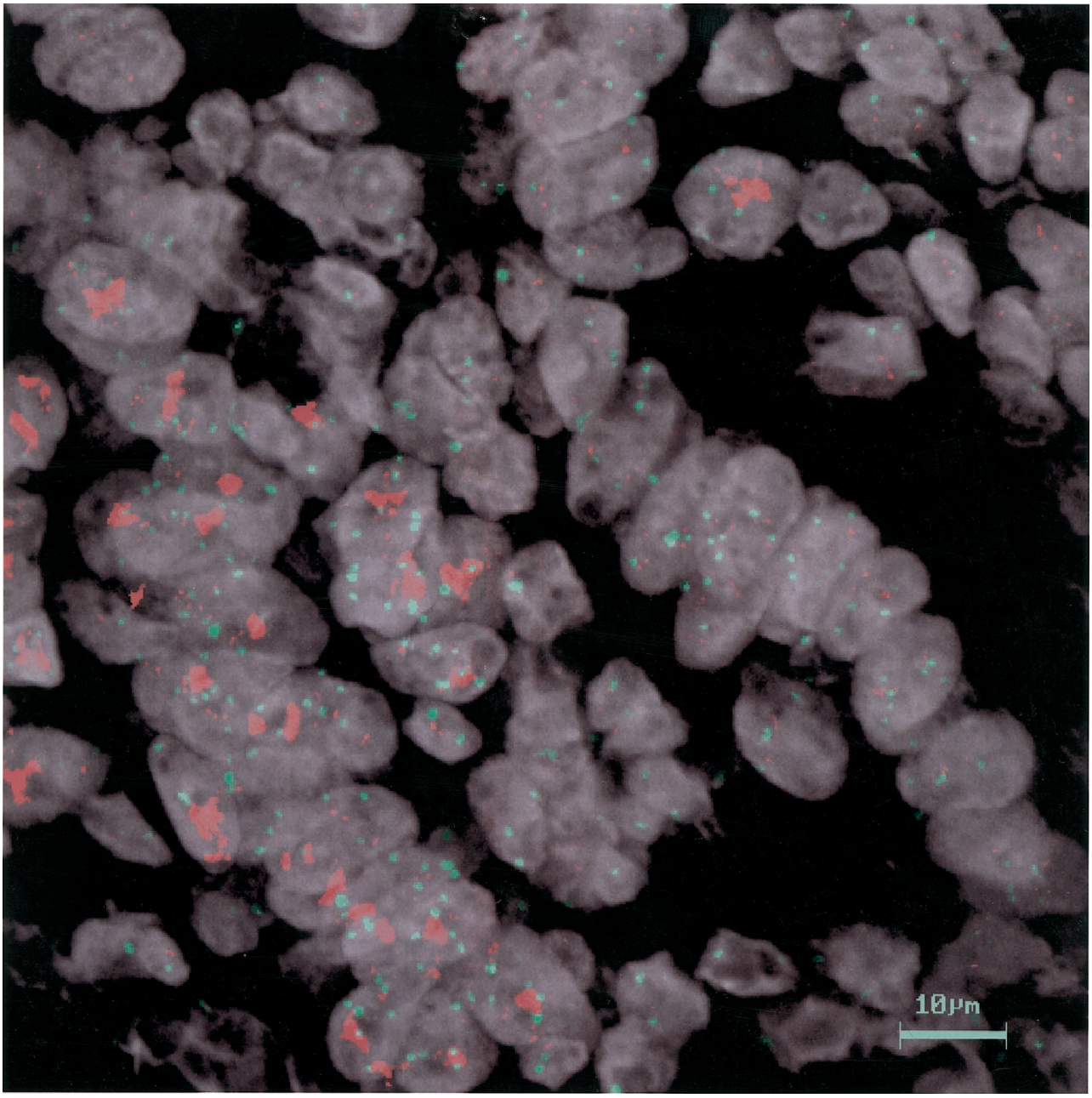

Figure 3. Heterogeneity of Her-2/neu gene amplification in Barrett's adenocarcinoma (case 15, BA). Dual-color FISH with Her-2/*neu*-specific probe (red signals) and chromosome 17 centromeric probe (green signals) counterstained with DAPI. Note tumor cells on the left BA areas with high-level Her-2/*neu* amplification (clusters), whereas tumor cells on the right site show no or low-level Her-2/*neu* amplification.

BA[11–13] or gastric carcinoma.[30,32] Furthermore, amplification of 8q23–24 was already detectable in IM areas without dysplasia, ie, in morphological preneoplastic mucosa. Other frequent gains and losses detected in our study point to further gene alterations including the *EGFR* gene (chromosome 7p12[36]), *AIB1*, *BTAK*, *SRC* (chromosome 20q),[37,38] *CDKN2A* (p16), (chromosome 9p21-pter[39]), *DPC4* (chromosome 18q[40]) and, so far unknown, candidate genes on three discrete areas on chromosome 4q.[41] LOH studies of several of these gene loci indicate that they are involved in esophageal adenocarcinoma. However, CGH is limited in its ability to detect DNA losses and amplifications, because the use of metaphase chromosomes limits detection of events involving small genomic regions (of <20 Mbp) and resolution of closely spaced aberrations. This may explain why our analysis failed to find some of the alterations, such as loss of heterozygosity on 17p, which are known to be very frequent in BA.[2,42] It has been proposed that hybridization to high resolution arrays of mapped sequences rather than the metaphase spreads used in conventional CGH might overcome this problem.[43] A further methodological problem may occur in the interpretation of telomeric regions by CGH.[44] In our study some of the observed changes are located near telomeric regions, but for the most part they cover more than a single chromosomal

band. Moreover, telomeric regions did not reveal chromosomal imbalances in our control hybridizations (DOP-PCR-amplified DNA obtained from histomorphologically normal esophageal squamous epithelium was hybridized with non-amplified male or female reference DNA). Thus, it seems unlikely that chromosomal imbalances near the telomeric regions have been misinterpreted.

A particularly important aspect of our study was the ability to study areas precisely corresponding to the metaplasia-dysplasia-carcinoma sequence of Barrett's esophagus using laser microdissection. The detection of chromosomal changes in these areas allowed us to assess clonal evolution in the precursor lesions of BA. In several samples we found a high concordance between specific aberration patterns and the steps of the metaplasia-dysplasia-carcinoma sequence (Table 2). The observation of common molecular abnormalities in premalignant lesions and adjacent carcinomas suggests that the process of clonal expansion underlies the proposed histopathological tumorigenetic pathway in Barrett's esophagus. This is also supported by recent studies, one of which reported two cases with a consistent pattern of X chromosome inactivation, indicating clonal expansion from metaplasia to dysplasia and carcinoma.[45] This other study demonstrated in three cases that dysplastic Barrett's epithelium and adjacent BA displayed the same pattern of microsatellite alleles at multiple loci.[15] Very recently, a case report of a multifocal BA using CGH indicated that monoclonal evolution of the cancer cells was a more likely mechanism than the concept of field cancerization.[46] The latter study also reported shared DNA gains and losses of HGD adjacent to BA, indicating that the HGD was a precursor of the BA. In our series of samples no substantial difference in the pattern and frequency of chromosomal changes between HGD and BA was observed. Thus, our data generally support the paradigm of the clonal derivation of invasive cancer from HGD, with the BA exhibiting the same genetic alterations seen in the HGD, as well as, in some cases, additional changes not yet present in the HGD lesion. On the other hand, a review of our data also shows that some HGD lesions had genetic abnormalities that were not present in the corresponding adjacent invasive cancer. This indicates that genetic divergence during the clonal evolution of cancer, particularly at the time when HGD progresses to invasive cancer, is also an important factor.

This phenomenon of genetic divergence is also illustrated by the fact that a number of areas of IM and dysplasia exhibited chromosomal changes that were not detected in the BA from the same resection specimen. For example, the IM from cases 10 and 16 shared only a few chromosomal changes with the corresponding BA sample. Either the carcinoma in such cases did not arise from the clone of premalignant tissue that was examined, or, during the process of clonal expansion (an ongoing process in both the carcinoma and premalignant lesions), new abnormalities arose in the premalignant areas that were not present when the carcinoma first developed. This may be illustrated by gains on chromosome 3p, which are more frequently found in precursor lesions than in invasive carcinoma (Figures 1 and 2), reflecting the clonal diversity and genetic complexity which cannot sufficiently be explained by linear accumulation of alterations in Barrett's esophagus.

The development of divergent clones during the process of clonal expansion is proposed be the explanation for tumor cell heterogeneity.[47] Such intratumoral heterogeneity was demonstrated in our cases by the results of our FISH analysis. Each of the investigated carcinomas was composed of several areas with different centromeric 17/Her2-*neu* signals, indicating a high prevalence of intratumor heterogeneity (Figure 3). Even if there were limited concordance between areas of premalignancy and adjacent BA, this does not necessarily conflict with the model of histological progression. Barrett et al demonstrated, using Barrett's esophagus cell lines, that clonal evolution is more complex than predicted by linear models.[42] In addition, most premalignant human tissues do not appear to progress to cancer, even when multiple somatic genetic abnormalities are present.[48–51] This is probably a reflection of the existence of many divergent neoplastic subpopulations and highlights one of the main problems associated with surveillance of Barrett's patients, namely sampling error.

Despite these aspects, we were able to detect recurrent copy number changes in IM, LGD, and HGD that had not been described previously. The most frequent early cytogenetic events seen in premalignant lesions (IM and LGD) were 8q amplification and Y chromosome loss. However, in the four corresponding pairs there was only one concordance for 8q. Loss of the Y chromosome was already present in three from four cases with loss of the Y chromosome in LGD (Table 3 and Table 4). Particularly interesting was a remarkable increase in gains on chromosome 7q and loss on chromosome 4q between precursor lesions (IM, LGD) and BA (Table 4), indicating that there is an important genetic event at this regions during progression. At the other end of the sequence, ie, between BA and LN, losses on 4q and 5q and gains of chromosomal regions 2p, 8q, 10q, and 20q were more frequent in LN than in the primary BA. These late events are consistent with the model of clonal expansion of tumor cell populations during tumor progression and may indicate chromosomal areas involved in the development of metastatic potential.

In conclusion, tumor development in Barrett's esophagus is proposed to occur via a multistep pathway which is recognized histologically as a metaplasia-dysplasia-carcinoma sequence. Using an approach combining laser-assisted microdissection and CGH, we detected recurrent chromosomal changes in this proposed sequence. Furthermore, we identified novel amplified chromosomal regions on chromosomes 2p and 10q in both BA and premalignant lesions. Although the detection of common chromosomal alterations in premalignant lesions and adjacent BA suggests a process of clonal expansion, the occurrence of several chromosomal changes without specific order was also apparent and provides striking evidence that clonal evolution is more complex than predicted by linear models.

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