The Evolution of Loss of Heterozygosity on Chromosome 17 during the Progression to Barrett’s Adenocarcinoma Involves a Unique Combination of Target Sites in Individual Specimens


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ABSTRACT

We have previously identified thirteen common minimally deleted regions (MRs) on chromosome 17 in twelve Barrett’s esophageal adenocarcinoma (BOA) specimens using 41 precisely mapped microsatellite markers (Dunn et al., Oncogene, 17: 987–993, 1999). The aim of the present study has been to identify the earliest sites of loss on this chromosome that arise and persist during the progression to BOA. This has been undertaken by the analysis of multiple carefully microdissected tissue samples from each of five esophagectomy specimens, several of which contained identifiable premalignant tissue. Our data demonstrate a stepwise accumulation of loss in each analyzed specimen, consistent with a single clonal pathway in four specimens and several coexisting pathways in one specimen. Several clonal anomalies (loss preceding heterozygosity and variable intrasample degrees of loss at different markers) were also observed. Within extensively deleted regions of the tumor (seen in three specimens), small deletions were detected in premalignant tissue, predominantly at the site of our identified MRs, and these losses were seen to expand and merge during the progression to BOA. Clonal losses at MRs were first detected in tissue showing early changes histologically, including Barrett’s intestinal metaplasia and intermediate-grade dysplasia. Our results provide further support for many of the MRs we have previously identified, thereby adding to evidence for the existence of multiple novel cancer-associated genes on chromosome 17 involved in the development of BOA.

INTRODUCTION

Esophageal cancer now ranks among the ten most prevalent human cancers attributable, in part, to an alarming recent increase in the incidence of adenocarcinoma of the esophagus in North America and Europe (1–3). In most cases, this cancer is preceded by the metaplastic abnormality of Barrett’s esophagus, in which the normal squamous epithelium of the esophagus is replaced by specialized columnar epithelium (4, 5). The etiological factors that promote this change are unknown. However, Barrett’s esophagus develops in ~10–12% of patients with chronic gastroesophageal reflux disease, and this predisposes to the development of adenocarcinoma of the esophagus and gastric cardia (5, 6).

The development of adenocarcinoma in Barrett’s esophagus follows a histologically defined sequence recognized as BIM-dysplasia-carcinoma (7). Barrett’s esophagus patients who suffer from gastroesophageal reflux often seek medical attention before they develop cancer (4). In these patients, the Barrett’s epithelium can be visualized and biopsied safely during gastrointestinal endoscopy (8). The appearance of dysplasia in such biopsies is presently used as a marker of malignant potential, but its usefulness is limited. Some patients with dysplasia will never develop cancer, whereas for others, dysplasia will represent a late stage of carcinogenesis (9). There is, moreover, a wide variation among pathologists in the definition of dysplasia (10, 11). The morbidity and mortality associated with Barrett’s adenocarcinoma is very high (12). If the opportunity for the surveillance of Barrett’s esophagus patients could be exploited more efficiently by the use of informative molecular markers, the prognosis for this disease might be significantly improved.

Numerous genetic abnormalities have been identified in BOA, several of which have been shown to occur in association with the clonal progression of this disease. Inactivation of the p53 and p16 TSGs and the development of aneuploid cell populations are the most characteristic abnormalities thus far identified, and these have generally been found in the majority of Barrett’s adenocarcinomas (13–21), as well as in some dysplastic (22–24) and metaplastic tissue (25–30). p53 mutations

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The abbreviations used are: BOA, Barrett’s esophageal adenocarcinoma; LOH, loss of heterozygosity; AI, allelic imbalance; MR, minimal region/minimally deleted region; GOI, gastroesophageal junction; Tc, tumor core; Tp, (tissue/tumor) peripheral to Tc; HGD, high-grade dysplasia; IGD, intermediate-grade dysplasia; LGD, low-grade dysplasia; BIM, Barrett’s intestinal metaplasia without dysplasia; IBM, BIM beneath the mucosal surface; OBIM, BIM at the mucosal surface; TSG, tumor suppressor gene; C17, chromosome 17.
are prevalent in the diploid and 4N cell populations that precede the development of aneuploidy (17), and identical p53 mutations found in dysplasia and adjacent carcinoma have served to reinforce the accepted concept of clonal expansion underlying the neoplastic transformation of Barrett’s esophagus (22, 24, 28, 31).

LOH studies performed on clonal populations of premalignant and malignant cells serve to identify and delineate sites in the genome that are commonly deleted during the neoplastic progression (32, 33). Such target sites are presumed to contain TSGs and may be informative for the prediction of the future development of cancer. Shared novel microsatellite alleles in adjacent premalignant and malignant Barrett’s have been detected on chromosomes 9p (LGD, HGD, tumor), 4p+q, 5q, 9p+q, 12p+q, 17p+q 18q, 20p and 22q (HGD, tumor; Ref. 33). In Barrett’s adenocarcinoma the highest LOH rates reported (90–100%) have been on the p arm of C17, which contains the p53 TSG (13, 18, 33, 34). In our most recent study, we have shown that there may be multiple target sites of deletion on C17 (35). The other chromosomes that have most frequently demonstrated allelic loss in BOA include 1p, 3p, 3q, 6q, 11p, and 13q (7–38).

In premalignant tissue LOH has been detected on 5q, 4(p+q), 9(p+q), 12(p+q), 17(p+q), 18q, 20p, and 22q (13, 16, 18). Recent studies have been focused on the identification of possible patterns of AI during the progression to cancer (32, 33, 37, 38). In summary, certain tumors appear to follow a linear model of clonal evolution from premalignant tissue to tumor, whereas others involve a more complex evolutionary model. There have, however, been no detailed studies tracing the evolution of defined regions of LOH through the progressive stages of premalignancy to adenocarcinoma, and this is what we aim to do in this detailed study of C17.

**MATERIALS AND METHODS**

**Specimen Details and Tissue Preparation.** Five Barrett’s adenocarcinoma esophagectomy specimens were collected from the Mersey Regional Cardiothoracic Center Liverpool (specimens A–E). Specimens B and D contained highly differentiated tumors, each ~4 × 3 cm in size, arising at the GOJ; specimen A contained a moderately differentiated adenocarcinoma arising ~3 cm above the GOJ; specimen E contained a moderately differentiated tubulo-acinar tumor, ~8 cm wide, deeply invading the GOJ and almost completely encircling the esophagus, with evidence of lymph node invasion and metastases; and specimen C contained a poorly differentiated adenocarcinoma, ~8 cm wide with a focus ~2.5 × 2.5 cm in size, arising in the midesophagus and spreading. Within 1 h of collection, each specimen was cut open longitudinally and pinned out with the mucosal layer uppermost for sampling. Tissue blocks (12–15), each ~1 cm³ in size, were dissected from each specimen, from above, within, and below the tumor (Fig. 1). The blocks were immediately snap-frozen in isopentane precooled to ~80°C and then immersed in liquid nitrogen before storage at ~80°C.

**Microdissection and DNA Extraction.** Sections were cut from each block, H&E-stained, and examined histologically by an experienced consultant thoracic pathologist (J. R. G.). The tissue was classified as follows: tumor (Tc); tumor periphery (Tp); HGD; IGD; LGD; BIM; OBIM; IBIM; gastric columnar epithelium (c); and squamous epithelium (sq). Stages of dysplasia were graded as described by Hamilton and Smith (39). Regions of tissue were selected for microdissection from those H&E-stained sections containing the purest epithelial tissue of one of the above histotypes. Four 10-µm sections were cut immediately beneath the diagnosed section and a fifth section was subsequently H&E-stained to check that the histology had not changed. The microdissection involved the staining of each section for 10 s in 0.01% toluidine blue and the manipulation of moist tissue from each section under the microscope using an ultrafine drawn-out capillary. To avoid possible PCR contamination, the cryostat blade was cleaned between the cutting of each tissue block, and a new sterile capillary was used for each set of sections cut. DNA was then extracted from each microdissected sample using the Nucleon II DNA extraction kit for hard tissue (Scotlab).

**LOH Analysis by Silver Staining.** Each DNA sample was serially diluted and titrated by PCR with a standard marker (D17S1864) so that the minimum amount of DNA could be used for subsequent LOH analysis. The Tc of each specimen was screened for LOH along the entire length of C17 using 41 precisely ordered, physically mapped microsatellite markers (23 on 17p and 18 on 17q). The physical location of the markers was confirmed by the Unified Database (UDB) for C17 at the Weizmann Institute website. The location of the markers is derived from integrating several maps; any discrepancies between existing maps are highlighted by multiple appearances in the data tables given. The results of this analysis for specimens A–D have been published previously (35); Table 1 contains the LOH data for the 41 markers for specimens A–E. In the next step, the multiple remaining samples from each specimen were screened for LOH, by using all of the markers that displayed loss in the Tc sample, plus several markers that had not displayed loss in the tumor. By repeat testing with some of the previously heterozygous markers, we intended: (a) to avoid bias in the results (by testing only samples previously showing LOH); (b) to test the reproducibility of the results found in the previous study (35); and (c) to identify any clonal anomalies, e.g., loss preceding heterozygosity. The PCR reactions were performed in 25-µl volumes in standard buffer (NH₄ buffer; Bioline) containing 1.5 mm MgCl₂, 200 μM dNTP, 5 pmol of each primer, and 0.5 units of Taq polymerase. The PCR parameters used for each marker were: 94°C for 5 min, then 25 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and finally 72°C for 2 min. The MS PCR products were electrophoresed on a 10% polyacrylamide gel and were viewed by silver staining. Because we observed considerable variation in the degree of LOH detected with different markers within the same sample, a scoring system for LOH was used. Scores of 0, 3, 5, and 9 were given (0 representing no loss, 3 representing the smallest detectable marginal loss (~30%)).
and 9 representing very clear loss (~90%); and for practical purposes, scores of ≥5 were assigned as loss.

AI was assessed both by visual analysis by two independent researchers (J. R. D. and J. G.) and by image analysis. Image analysis was carried out using the UV Band gel-scanning program (UVTech).

Lasko and Cavanee (40, 41) have suggested a number of chromosomal mechanisms to explain the loss of an allele in tumor. These include: (a) deletion of the wild-type chromosome resulting in hemizygosity at all loci near the TSG; (b) loss followed by duplication resulting in two copies of one allele and loss of the other; (c) mitotic recombination between homologues resulting in heterozygosity at loci in the proximal region and homozygosity throughout the rest of the chromosome, including the TSG locus; and (d) localized events such as point mutations, small deletions, and gene conversions. In this investigation, we have no way of determining which mechanism is playing a major role.

**LOH Analysis of p53 and Surrounding Microsatellite Markers.** To demonstrate that p53 is distinct from MRs I and II (which flank this gene), we carried out LOH analysis by silver staining on normal and tumor tissue from the five esophagectomy specimens (A–E), using p53 and the 14 microsatellite markers closest to p53. The physical position of the markers was obtained from the Unified Database for C17.

**RESULTS**

In this study we have generated a detailed map of the evolution of LOH on C17 in five Barrett’s adenocarcinoma esophagectomy specimens (A–E).

In various tissue blocks obtained from esophagectomy (specimens A–E), the following tissue histotypes were identified: squamous epithelium (sq), BIM, gastric columnar epithelium (c), LGD, IGD, HGD, adenocarcinoma peripheral to the visible Tc, and adenocarcinoma from the visible Tc. Fig. 2A shows H&E-stained sections of IBIM; Fig. 2B shows IGD; Fig. 2C shows HGD; and Fig. 2D shows Tc tissue.

DNA extracted from the various tissue histotypes was then used for LOH analysis by silver staining, using those markers that displayed loss within tumor tissue in the previous study (35) and using the same LOH scoring system. The LOH results from each specimen are shown in Table 2, which also illustrates the physical location of each marker tested and the position of the MRs identified in the previous study. The results in Table 2 illustrate a stepwise accumulation of LOH within some tissue samples for each specimen, consistent with a clonal evolution of loss during the progression to adenocarcinoma. In four specimens the data are consistent with a single clonal pathway leading to tumor formation (specimens A, B, D, and E). This is most clearly demonstrated in specimen B, in which there are
five identifiable steps in the clonal pathway. The retention of heterozygosity at \(NF1\) in sample 4 OBIM is the only clonal irregularity apparent for this specimen.

In specimen C, the patterns of loss detected are inconsistent with a single clonal pathway, although several samples do share some of the same losses. Several different clonal pathways must be considered to account for the LOH data for this esophagectomy specimen.

A further important feature displayed by specimens A and B is that the large deleted regions that are present in the core tumor develop as a number of smaller regions of loss that increase in size and finally merge, and that the majority of these earlier clonal losses all occur within one or more of the MRs we have previously identified on C17 (35).

Overall, those target sites that displayed loss in histologically defined premalignant tissue within an inferred clonal pathway leading to tumor formation include: (a) MR VI (specimen D), which displayed loss in squamous epithelium; (b) MRs II, III, IV, VII, VIII, and XII/XIII (specimen B) and MRs III and VI (specimen D and E) and MR XI (specimen E), which displayed loss in BIM or IGD; and MR IV (specimen A), which displayed loss in HGD. The most frequently deleted region of C17 in premalignant tissue was MRIII which was deleted in specimens A, B, D, and E. Table 3 contains a summary of the MRs lost in histotypes from each specimen and shows the accumulation of losses from premalignant tissue to cancer.

A microsatellite marker within the \(p53\) gene and 14 surrounding MS markers were tested for LOH in the normal and tumor tissue samples originally selected from the five esophagectomy specimens (A–E; Table 4). LOH within \(p53\) was seen in specimens A, B, and D; there was no LOH at \(p53\) in specimens C or E. In specimens A and D, \(p53\) LOH is distinct, being flanked by heterozygous sites at MS markers D17S1881 and D17S1844, and D17S1353 and D17S1805, respectively. LOH within the \(p53\) gene in specimen B, however, does not appear to be distinct, rather a small part of an extensive deletion covering the area between D17S1854 and D17S804 (MRII). Further analysis of \(p53\) LOH in Tp from specimen B (4p) showed no LOH at \(p53\); LOH in 4p was seen at MRs III, IV, and VII (Table 2).

**DISCUSSION**

C17 is one of the most important targets of loss in many tumors (1–52). AI in C17 in BOA is well-established (16–31). Furthermore, in a previous study, we demonstrated the presence of interstitial deletions covering many different regions of C17 and varying in size from 1–50 cM. Overall, using 41 microsatellite markers, we identified 13 MRs of LOH (6 on 17p and 7 on 17q), which suggests the presence of more than one novel TSG on C17 involved in BOA (35).

The three TSGs, \(p53\) at 17p13.1, \(NF1\) at 17q11.2, and \(BRCA1\) at 17q21.1, are already known to exist on C17, and \(p53\) has been shown to play an important role in the development of BOA (15, 17, 18, 25–27, 30). These TSGs occur within, or in the vicinity of, 3 of the 13 MRs that we have identified: MR II (\(p53\)), MR VII (\(NF1\)), and MR VIII (\(BRCA1\)). Two additional MRs occur at the site of candidate TSGs: MR I (HIC1 OVCA1/ OVCA2; 42, 43) and MR XI (TOC; 44–47). Our previous study, therefore, suggests the presence on C17 of additional novel TSG(s) that are involved in BOA.

In the present study, we have sought additional evidence that the 13 MRs identified in the 12 BOA tumors represent true target sites by investigating the clonal evolution of loss within and around these regions in premalignant and adjacent tumor tissue from five Barrett’s esophagectomy specimens.

A stepwise accumulation of loss occurring at different genetic markers in tissue samples that histologically mark the progression to adenocarcinoma is indicative of a clonal pathway of evolving loss (48). Such clonal pathways, involving two to five steps of accumulating losses, could be discerned in all five of our examined esophagectomy specimens.

In four specimens (A, B, D, and E), the patterns of loss
appear to confirm a single clonal pathway leading to tumor formation, although in three of these specimens (A, B, and E), clonal anomalies involving one or two markers that displayed heterozygosity subsequent to loss were observed. In specimen A, steps 1 and 2 of the clonal pathway show loss at D17S520; there is subsequent retention of heterozygosity at D17S520 in step 3; finally, D17S520 is lost in steps 4 and 5. In specimen B, LOH at NF1 was seen in step 1, retention of heterozygosity at NF1 was then seen in step 2, and, finally, LOH at NF1 was seen in steps 3–5. It should be noted that in specimens A, B, and E, there are possible alternatives to the pathways of loss suggested by the authors; furthermore, it is not known which pathway is correct in any one specimen. The pathways suggested in Table 2 are those considered by the authors to be the most likely pathways of loss. For example, in specimen A, an alternative pathway could be: step 1, 11LGD→step 2, 8BIM→step 3, 8HGD→step 4, 6Tp→step 5, 4Tc. In specimen C, an alternative pathway could be: step 1, 4OBIM→step 2, 5Tc→step 3, 2Tc.

There have been several studies documenting shared molecular abnormalities in Barrett’s premalignant tissue and adenocarcinoma, consistent with a process of clonal expansion underlying the histological pathway of tumor development. These include the detection of identical mutations in the p53 and p16 TSGs (25–27, 29) and identical losses and novel alleles on multiple chromosomal arms (32, 33). In other studies (32, 33), similar to our findings, numerous clonal anomalies were detected (LOH and novel alleles were present in premalignant tissue but not in coexisting carcinoma). Several possibilities may account for such anomalies. It may be argued that, during early tumorigenesis, numerous divergent clones harboring different genetic abnormalities may exist, and, of these, only one may acquire dominance and undergo further clonal expansion leading to tumor formation. Thus, if the dominant progenitor clone that gave rise to the carcinoma is not sampled, shared abnormalities will not be detected. It is also possible that early losses that are not present in the tumor sample are random losses of no significance to the development of the tumor.

An additional clonal anomaly identified in this study has been the detection of considerable variation in the degree of loss at different sites on C17 within the same sample; and to reflect this variation, we have used a specific scoring system. Because numerous, very clear losses were observed in many samples, and careful tissue microdissection was routinely performed, the best explanation for this observation is intrasample clonal heterogeneity.

Fig. 2  H&E-stained tissue sections showing some of the tissue histotypes obtained for analysis. A, IBIM from specimen D; B, IGD from specimen B; C, HGD from specimen B; D, Tc from specimen A.
We observed LOH in specimen D in histologically defined normal squamous epithelium. Other researchers in breast tissue (52) have also reported LOH in histologically normal tissue. This highlights the importance of testing as many tissue samples as it is possible to microdissect, inasmuch as the histologically normal tissue samples may carry the earliest clonal losses involved in a genetic pathway.

In three specimens that carried extensive deletions within Table 2:

| Spec A | Evolution of the extensive region of LOH on C17 in esophagectomy specimen A. Losses occur in an approximate stepwise fashion, steps 1–5 are shown. LOH occurs first in step 1 at D17S520 in BIM from block 8. Step 2 shows loss at D17S786, D17S952, D17S1852, D17S954, D17S955, NFI, and D17S784 in peripheral tumor taken from block 6. Finally, step 5 shows loss at D17S952 extending to the centromere, then loss at NFI & D17S784 in Tc from block 4. Spec B: Evolution of the extensive region of LOH on C17 in esophagectomy specimen B. Losses occur in an approximate stepwise fashion; steps 1–5 are shown. Loss occurs first in step 1 at D17S954, D17S955, and NFI in IGD from block 5. Step 2 shows loss at D17S954, D17S955, D17S800, and D17S802–D17S784 loss, with retention of heterozygosity at NFI in OBIM from block 4. Step 3 shows loss at D17S786, D17S952, D17S1852, D17S954, D17S955, NFI, D17S800, D17S809, and D17S802–D17S784 in IBIM from block 4. Step 4 shows loss at D17S786, D17S952, D17S1852, D17S954, D17S799, D17S921, D17S955, and NFI–D17S784 in Tp from block 4. Finally, step 5 shows loss at all of the markers tested in Tc from block 4. The disappearance of loss at NFI in sample 4OBIM is the only clonal irregularity apparent for this specimen. Spec C: Patterns of LOH on C17 in esophagectomy specimen C. The patterns of LOH are inconsistent with a single clonal pathway, although some samples do share the same losses. Several clonal pathways must be considered to account for the LOH data for this specimen (e.g., (a) 8C–6T–2T–6C–2T; and (b) 4T–6T–2T). Spec D: Evolution of LOH on C17 in esophagectomy specimen D. The most extensive regions of LOH in this specimen involve two markers: D17S954 and TCF2. The suggested pathway of LOH consists of two steps: step 1, LOH at TCF2 in normal squamous epithelium (sq) and Tp from block 2; step 2, LOH at TCF2 and D17S954 in IBIM and Tc from block 5. In IGD and Tp from block 6, and Tc from blocks 3 and 4. Spec E: Evolution of the extensive region of LOH on C17 in esophagectomy specimen E. Losses occur in an approximate stepwise fashion, steps 1–4 are shown. LOH occurs first in step 1 at D17S520 and D17S785 in IBIM from block 3. In step 2 there is loss at D17S520, D17S805, D17S785, and D17S800 in OBIM from block 3. Loss then occurs in step 3 at D17S786–D17S925 (inclusive) and D17S804 in Tc from block 5. Finally, there is an extensive region of loss that occurs in all of the markers tested in Tc from block 6. Other losses that occur outside the proposed clonal pathway include LOH at D17S804 and D17S955 in OBIM from block 4; in LGD from block 2, there are extended regions of loss between D17S520 and D17S959 (inclusive), a single loss at TCF2, and then LOH between D17S230 and D17S784 inclusive. No physical distance is given for MS marker SSTR2; it has been placed within the table according to the cytogenetic banding location. p53 is at 9.1 Mb; BRCA1 is at 52.7 Mb. \( \square \), LOH; \( \bigcirc \), noninformative; \( \bigotimes \), heterozygous; \( \mathbb{G} \), centromere.
the Tc (specimens A, B, and E), isolated losses within these regions were found to arise in preceding tissue, and to expand and merge during the tumorigenic progression. Moreover, the majority of these clonal losses were located within 1 of the 13 MRs that we previously identified. These comprise MRs II, III, IV, VI, VII, VIII, XI, XII, and XIII. Furthermore, the Tc in specimen E displayed loss at all of the informative markers tested, which is suggestive of a numerical chromosomal alteration. This raises the possibility that this alteration may have arisen not by chromosomal mis-segregation but rather as the end result of the accumulation of several expanding deletions. Large deletions, which are prevalent in many tumors, confound attempts to define small commonly deleted regions, because the targets of extensive loss are unknown. If, as a general mechanism of tumorigenesis, transient loss at target sites is followed by expansion of small, commonly deleted regions and the deletions merge, then positional information on the target sites within large deletions and aneuploid chromosomes could be gained from studying the hierarchy of loss in premalignant tissue. This could present a powerful approach for more detailed LOH mapping, especially if—as our and other (52) data suggests—differential loss can be found by sampling histologically normal tissue and different sites within, and surrounding, the tumor.

Overall, the losses we have detected in this study within an inferred clonal pathway, preceding the final step within the Tc, have occurred at MR II (specimen A and B), MR III (specimens B, D, and E), MR IV (specimens A and B), MR V (specimen A), MR VI (specimen D), MR VII (specimens A and B), MR VIII (specimen B), MR XI (specimen E), and MR XII/XIII (specimen E).
Table 3  Summary of earliest sites of LOH identified within each tissue histotype taken from each esophagectomy specimen (A–E) showing LOH identified at minimal regions (MRs) I–XIII

Tissue taken from above the GOJ of each esophagectomy specimen is shown.

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<th>Tissue histotypes</th>
<th>Sq*</th>
<th>BIM</th>
<th>OBIM</th>
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<th>IGD</th>
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* Sq. squamous epithelium; ns, not sampled; →, the direction of progression from premalignant tissue to cancer.

Table 4  Results from LOH analysis of p53 and 14 surrounding microsatellite (MS) markers in the original tumor samples from the esophagectomy specimens A–E

No physical distance given for MS marker SSTR2; it has been placed within the Table according to the cytogenetic position. ■, LOH; □, noninformative; ▶, heterozygous; ▲, centromere.
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