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Genetics

Clonal ordering of 17p and 5q allelic losses in Barrett dysplasia and adenocarcinoma

(DNA-content cell sorting/tumor suppressor genes)

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ABSTRACT **Both 17p and 5q allelic losses appear to be involved in the pathogenesis or progression of many human solid tumors. In colon carcinogenesis, there is strong evidence that the targets of the 17p and 5q allelic losses are *TP53*, the gene encoding p53, and *APC*, respectively. It is widely accepted that 5q allelic losses precede 17p allelic losses in the progression to colonic carcinoma. The data, however, supporting this proposed order are largely based on the prevalence of 17p and 5q allelic losses in adenomas and unrelated adenocarcinomas from different patients. We investigated the order in which 17p and 5q allelic losses developed during neoplastic progression in Barrett esophagus by evaluating multiple aneuploid cell populations from the same patient. Using DNA content flow cytometric cell sorting and polymerase chain reaction, 38 aneuploid cell populations from 14 patients with Barrett esophagus who had high grade dysplasia, cancer or both were evaluated for 17p and 5q allelic losses. 17p allelic losses preceded 5q allelic losses in 7 patients, both 17p and 5q allelic losses were present in all aneuploid populations of 4 patients, and only 17p (without 5q) allelic losses were present in the aneuploid populations of 3 patients. In no patient did we find that a 5q allelic loss preceded a 17p allelic loss. Our data suggest that 17p allelic losses typically occur before 5q allelic losses during neoplastic progression in Barrett esophagus.**

In 1976, Nowell hypothesized that cancer develops in association with an acquired genetic instability that predisposes to the development of genetically abnormal clones of cells (1). Some of these clones gain selective proliferative advantages, and subclones evolve that have accumulated genetic errors. There is now substantial evidence to support Nowell's hypothesis and the clonal evolution of many human tumors (2–6). However, it has been difficult to investigate the order in which genetic events occur in individual patients during neoplastic progression in humans for several reasons. First, premalignant lesions may be completely removed at the time of detection. For example, colonic adenomas are removed at colonoscopy; thus, their progression to cancer cannot be investigated. Second, histologic techniques such as cryostat sectioning that have been used to purify neoplastic cells from premalignant and malignant lesions cannot separate genetically different cell populations that may have a similar histologic phenotype. Third, normal epithelial and stromal cells that can obscure the detection of allelic losses may be relatively more abundant in small dysplastic samples than in large tumors, making purification of neoplastic cells by cryostat sectioning difficult at these early stages of neoplastic progression.

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Barrett esophagus is a condition in which the normal squamous epithelium of the esophagus is replaced by a metaplastic columnar epithelium. It develops as a complication in 10–12% of patients with chronic gastroesophageal reflux disease and predisposes to the development of esophageal adenocarcinoma (7). We and others (3–5, 8, 9) have demonstrated that neoplastic progression in Barrett esophagus is associated with a process of genomic instability and clonal evolution similar to that postulated by Nowell. For example, the evolution from a normal cell to a malignant cell in Barrett esophagus is frequently associated with the development of large changes in the DNA content, or ploidy, of the metaplastic epithelial cells (3, 4). In patients who have been prospectively evaluated by DNA-content flow cytometry, the evolution from diploid biopsies to single aneuploid cell populations and then to multiple aneuploid cell populations is a common occurrence (3, 4). In some patients, the clonal evolution has been documented by cytogenetic analysis of mucosal biopsies (5). In addition, it has also been shown that the development of Barrett adenocarcinoma is associated with allelic losses that include the tumor suppressor genes *TP53* (encoding p53) and *APC* (adenomatosis polyposis coli) (8–11). However, the order in which these allelic losses develop during neoplastic progression in Barrett esophagus has not been investigated.

Barrett esophagus is an excellent model system in which to investigate the order of genetic events during clonal evolution in neoplastic progression. Because of the high morbidity and mortality associated with esophagectomy, patients with dysplasia frequently undergo endoscopic biopsy surveillance until they develop cancer (4, 7). In addition, esophagectomy specimens from patients with Barrett adenocarcinoma frequently contain multiple stages of neoplastic progression that can be evaluated to determine the order of genetic events (3, 12). Furthermore, 95% of Barrett adenocarcinomas are aneuploid, and multiple aneuploid cell populations are commonly present in both premalignant epithelium and adenocarcinoma (3, 4, 12). Therefore, DNA-content cell sorting can be used to separate aneuploid cell populations from diploid cells and from each other to investigate genetic abnormalities that develop during neoplastic progression (9).

Allelic losses of both 17p and 5q have been detected in many human solid tumors, including those of the esophagus and colon (8, 10, 11, 13). Chromosome 17p is known to harbor *TP53* (for tumor protein p53), a tumor-suppressor gene found to be lost or mutated in many tumors (14, 15). Chromosome 5q harbors two closely linked genes, *APC* and *MCC* (mutated in colorectal cancers), both of which have abnormalities that have been detected in colon and esophageal cancers (2, 8, 10, 16–18). In colon cancer, which is one of the most extensively studied malignancies, it has been suggested that 5q allelic losses precede 17p allelic losses. This order was largely

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inferred from the prevalence of 5q and 17p allelic losses in different classes of adenomatous polyps and unrelated cancers from different patients (13).

We have detected a high prevalence of 17p (92%) and 5q (77%) allelic losses in aneuploid cell populations from Barrett adenocarcinoma (9, 10). Our goal was to investigate the order in which allelic losses of 17p and 5q developed during clonal evolution in the same patient with Barrett esophagus. Therefore, we investigated 17p and 5q allelic losses in 38 aneuploid cell populations from 14 informative patients with Barrett esophagus who had high-grade dysplasia, adenocarcinoma, or both.

MATERIALS AND METHODS

Tissue samples were obtained from esophagectomy specimens and endoscopic biopsies by using previously published "mapping" techniques that permit the localization of abnormalities within the esophageal mucosa (3, 4). Esophagectomy or endoscopic biopsy specimens from 29 patients who had high-grade dysplasia or adenocarcinoma or both were screened by DNA-content flow cytometry to detect those that had two or more aneuploid cell populations. Twenty-one of 29 patients (72%) had two or more aneuploid cell populations. Fourteen of these 21 patients were informative for DNA polymorphisms on 17p and on 5q and were investigated for allelic losses. Two or more aneuploid populations and the corresponding diploid population from the same tissue samples were isolated from each patient by using DNA-content cell sorting as described (9). DNA was extracted from each sorted sample as described (9). The DNA from each sorted sample as well as the patient's normal gastric tissue were evaluated for 17p and 5q allelic losses by using the polymerase chain reaction and, in some cases, restriction digests of amplified products of sequences containing DNA polymorphisms on 17p and 5q in or closely linked to *TP53* and *APC*, respectively. The DNA from 3000–6000 sorted nuclei was used per reaction. The DNA primer sequences and amplification conditions used have been published (8, 10, 11, 19–21).

Table 1. Pattern of 17p and 5q allelic losses in aneuploid cell populations from Barrett dysplasia and adenocarcinoma

	Aneuploid populations			
		Allelic losses		
Patient	DNA content	17p	5q	Histology
1. VH	2.3N	−	−	HGD
	3.1N	+	−	HGD
2. BT	3.3N	+	−	CA
	6.0N	+	−	CA
3. RR	3.2N	+	−	HGD
	3.7N	+	−	CA
4. DS	3.2N	+	−	HGD
	3.5N	+	−	HGD
	4.0N	+	−	HGD
	2.8N	+	+	CA
	3.8N	+	+	CA
5. WW	4.2N	+	−	CA
	2.7N	+	−	CA
	2.4N	+	+	CA
6. WH	3.8N	+	−	HGD
	2.5N	+	+	HGD
	2.8N	+	+	HGD
	4.2N	+	+	HGD
7. HB	3.4N	+	−	CA
	3.6N	+	−	CA
	3.8N	+	+	CA
8. HH	3.1N	+	−	CA
	2.8N	+	+	CA
9. CM	2.4N	+	−	CA
	3.8N	+	−	CA
	4.0N	+	+	CA
10. CJ	3.6N	+	−	HGD
	3.0N	+	+	HGD
	2.8N	+	+	CA
11. DW	2.3N	+	+	CA
	2.7N	+	+	CA
	5.0N	+	+	CA
12. WJ	3.6N	+	+	HGD
	4.0N	+	+	HGD
13. JI	3.1N	+	+	CA
	3.4N	+	+	CA
14. HJH	2.9N	+	+	CA
	3.1N	+	+	CA

N, ploidy; HGD, high-grade dysplasia; CA, cancer.

RESULTS AND DISCUSSION

In 7 of 14 patients, all of the aneuploid populations had 17p allelic losses, but only a subset of aneuploid populations had 5q allelic losses (Table 1, patients 4–10; see also Figs. 3 and 4). In the "mapped" specimens, populations of cells with 17p allelic losses always occupied discrete regional areas in the

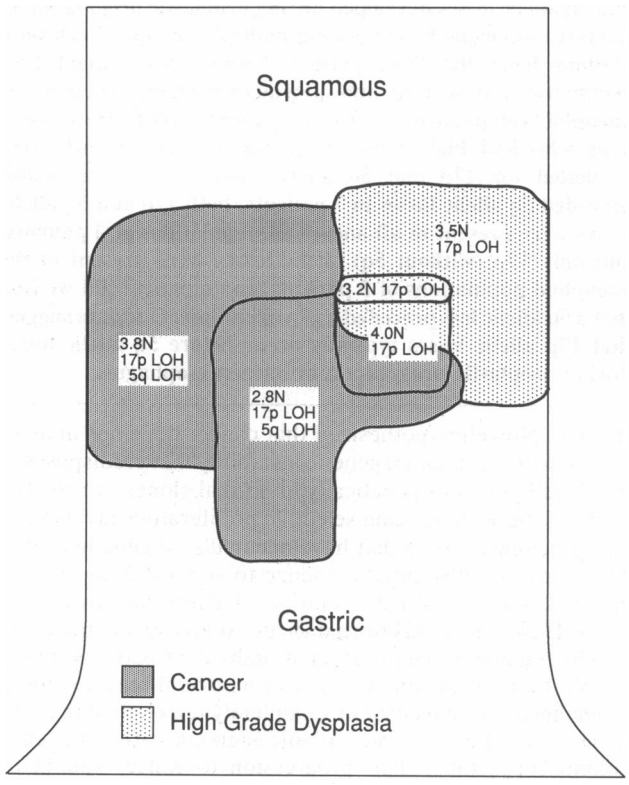

FIG. 1. An esophagectomy specimen (patient 4 in Table 1) illustrating the principles of clonal ordering in Barrett esophagus. The areas occupied by each aneuploid cell population are outlined by heavy black lines. LOH = loss of heterozygosity = allelic loss. In this patient, the cancer had two aneuploid populations with DNA contents of 2.8N and 3.8N, where N is ploidy. The 2.8N and 3.8N aneuploid populations in the cancer had both 17p and 5q allelic losses. There was an adjacent area of high-grade dysplasia that had three aneuploid populations with DNA contents of 3.2N, 3.5N, and 4.0N that contained 17p allelic losses but no 5q allelic losses. The adjacent normal mucosa was diploid and contained neither 17p nor 5q allelic losses. In this patient, therefore, a 17p allelic loss developed as an early event that was passed on to three different aneuploid subclones in a large region of high-grade dysplasia. The transition to cancer was associated with the development of a 5q allelic loss.

esophageal mucosa and were immediately adjacent to populations of cells that showed both 17p and 5q allelic losses (Fig. 1). In addition, in every patient who had 17p or 5q allelic losses in multiple aneuploid populations, the same allele was always lost, suggesting the sequential evolution of clones with accumulated genetic abnormalities (Fig. 2–5). In the remaining 7 patients, each aneuploid population had either no allelic losses, 17p allelic losses alone, or both 17p and 5q allelic losses (Table 1, Figs. 2 and 5). In no case were 5q allelic losses detected in aneuploid populations that retained two 17p alleles. In one case (patient 12), one of four diploid cell populations from dysplasia that were evaluated for allelic losses had allelic losses of both 17p and 5q. In all other cases, diploid populations from dysplasia as well as cancer retained both 17p and 5q alleles. In biopsies from adenocarcinomas, we detected 17p allelic losses in 24 of 24 aneuploid cell populations (100%) and 5q allelic losses in 14 of 24 aneuploid cell populations (58%). We also detected 17p allelic losses in 13 of 14 aneuploid populations (93%) and 5q allelic losses in 6 of 14 aneuploid populations (43%) from high-grade dysplasia without adenocarcinoma, indicating that both 17p and 5q allelic losses can occur before invasion during neoplastic progression in Barrett esophagus (Table 1). Thus, 17p and 5q allelic losses cannot be equated with the development of carcinoma in Barrett esophagus. Instead, they appear to be a part of the process of clonal evolution that culminates in carcinoma.

The formalism for investigating the order of genetic events in a dependent pathway was established as part of the early investigations of the yeast cell cycle (22). Basically, any two events, A and B, can be related to each other in one of four ways: A can always precede B (dependent events), B can always precede A (dependent events), A and B can always occur together (interdependent events), or A and B can occur independently of each other (independent events). According to this formalism, our experiments strongly support the possibility that 17p allelic losses precede 5q allelic losses during neoplastic progression in Barrett esophagus. In addition, the data conclusively rule out two of the other three possibilities: 5q allelic losses do not always precede 17p allelic losses, and 17p and 5q allelic losses do not always occur together. Furthermore, our results favor a 17p → 5q order of dependent events over the fourth possibility, that 17p and 5q allelic losses occur independently of each other. However, our results cannot conclusively rule out the possibility that, with the evaluation of additional cases, a subset of tumors might be found in which 5q allelic losses precede 17p allelic losses.

The 17p → 5q order of allelic losses that we report in Barrett esophagus differs from that proposed in colon cancer, in which it is believed that 5q allelic losses precede 17p allelic losses (13). Our results may differ from those in colon cancer for several reasons. The gene targets of the 17p and 5q allelic losses may be different in colonic and Barrett adenocarcinomas. Further work will need to be done to determine with certainty the genes on chromosomes 17p and 5q that are involved in the pathogenesis or progression of Barrett adenocarcinoma. However, in Barrett esophagus, there is evidence that the target of 17p allelic losses includes the *TP53* gene encoding p53 (8, 9, 11, 24, 25). In the present study using polymorphisms within the *TP53* gene, we were able to demonstrate that the allelic losses included *TP53* in at least 26 of the 38 aneuploid populations with 17p allelic losses. The target of 5q allelic losses in Barrett esophagus has not yet been determined. We have previously demonstrated that 5q allelic losses in Barrett's adenocarcinoma involve both *APC* and *MCC* in the majority of tumors in which they have been sought (10), suggesting that the gene on 5q involved in carcinogenesis in Barrett esophagus may be *APC*, *MCC*, or a closely linked gene. It is also possible that the same genes are involved in neoplastic progression in both organs but that

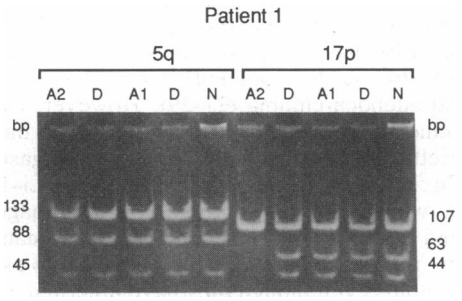

FIG. 2. 17p allelic loss without 5q allelic loss in one aneuploid cell population (patient 1 in Table 1). Lanes: N, normal tissue; D, diploid sorted cells from dysplasia; A1, 2.3N aneuploid cell population; A2, 3.1N aneuploid cell population. To detect 17p allelic losses, DNA was amplified by using polymerase chain reaction primer sequences flanking an *Msp* I restriction site in intron 6 of *TP53*. A 107-base-pair (bp) amplicon was created and digested with *Msp* I. The cut allele resulted in 63- and 44-bp bands. To detect 5q allelic losses, DNA was amplified by using polymerase chain reaction primer sequences flanking an *Rsa* I restriction site in exon 11 of *APC*. A 133-bp "amplicon" was created and digested with *Rsa* I. The cut allele resulted in 88- and 45-bp bands. All products were electrophoresed on a 7% polyacrylamide gel and stained with ethidium bromide. Lanes from right to left show the following results. The patient's normal tissue, diploid sorted cells from dysplasia, and aneuploid population A1 retained both 17p alleles. In aneuploid population A2, there was a 17p allelic loss of the cut allele. There were no allelic losses of 5q in the patient's normal tissue, diploid sorted cells from dysplasia, or in either aneuploid cell population.

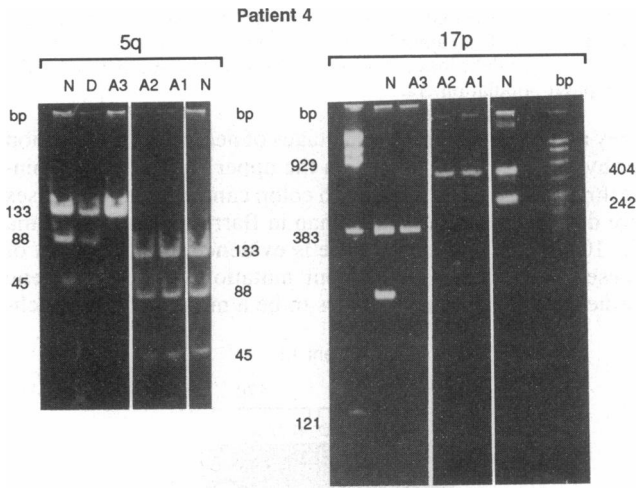

FIG. 3. 17p allelic losses in three aneuploid cell populations with only one of these populations having a 5q allelic loss in addition to the 17p allelic loss (patient 4 in Table 1). Lanes: N, normal tissue; D, diploid sorted cells from the cancer; A1, 4.0N aneuploid cell population; A2, 3.5N aneuploid cell population; A3, 3.8N aneuploid cell population. To detect 17p allelic losses, DNA was amplified by using DNA sequences within the VNTR (variable number tandem repeat) locus *D17S5* at 17p13.3 detected by probe pYNZ22. Alleles range in size from 170 to 870 base pairs (bp). 17p products from A1 and A2 were electrophoresed on a 10% polyacrylamide gel, and from A3, on a 6% polyacrylamide gel. To detect 5q allelic losses, polymerase chain reaction sequences flanking an *Rsa* I restriction site in exon 11 of *APC* were used. The cut allele resulted in 88- and 45-bp bands. The digested products of A1 and A2 were electrophoresed on a 7% gel, and of A3, on a 10% gel. Lanes from right to left show the following results. The patient's normal tissue retained both 17p alleles. In all three aneuploid cell populations, 17p allelic loss of the smaller allele could be demonstrated. The patient's normal tissue and aneuploid populations A1 and A2 retained both 5q alleles. However, in aneuploid population A3, a 5q allelic loss of the cut allele could be demonstrated.

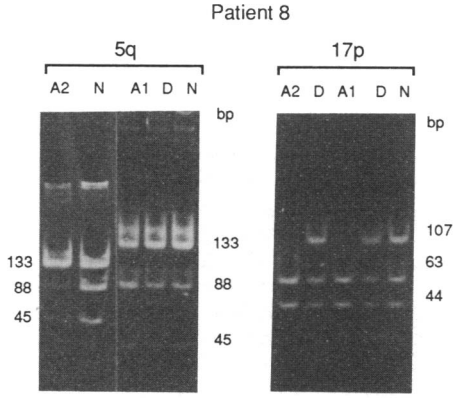

FIG. 4. 17p allelic losses in two aneuploid cell populations with only one of these populations having a 5q allelic loss in addition to the 17p allelic loss (patient 8 in Table 1). Lanes: N, normal tissue; D, diploid sorted cells from the cancer; A1, 3.1N aneuploid cell population; A2, 2.8N aneuploid cell population. To detect 17p allelic losses, DNA was amplified by using polymerase chain reaction primer sequences flanking an *Msp* I restriction site in intron 6 of *TP53*. A 107-bp amplicon was created and digested with *Msp* I. The cut allele resulted in 63- and 44-bp bands. To detect 5q allelic losses, primer sequences flanking an *Rsa* I restriction site in exon 11 of *TP53* were used. A 133-bp amplicon was created and digested with *Rsa* I. The cut allele resulted in 88- and 45-bp bands. All 17p products were electrophoresed on a 7% polyacrylamide gel. The 5q products from the aneuploid population A1 were electrophoresed on a 7% polyacrylamide gel, and the aneuploid population A2, on a 10% polyacrylamide gel. Lanes from right to left show the following results. Normal tissue and diploid sorted cells retained both 17p alleles. In both aneuploid cell populations, 17p allelic loss of the uncut band could be demonstrated. The normal tissue, diploid sorted cells from the cancer, and aneuploid population A1 retained both 5q alleles. However, 5q allelic loss of the cut allele could be demonstrated in the aneuploid population A2.

they are selected at different stages of neoplastic progression or by different mechanisms in the upper and lower gastrointestinal tracts. For example, in colon cancer, 5q allelic losses are detected less frequently than in Barrett adenocarcinoma (2, 10, 13). In the colon, there is evidence that the target of these allelic losses is *APC* but mutation of the *APC* gene rather than allelic loss appears to be a more common mechanism of *APC* alteration in the initiation of colon cancer (2). In contrast, we have detected 5q allelic losses in 77% of Barrett adenocarcinomas (10). Therefore, if *APC* is the target of these 5q allelic losses in Barrett adenocarcinoma, the mechanism by which *APC* alterations occur in the esophagus may be different from that in the colon.

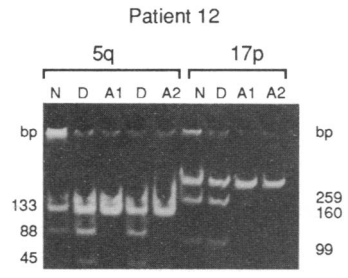

FIG. 5. Both 17p and 5q allelic losses demonstrated in two aneuploid cell populations (patient 12 in Table 1). Lanes: N, normal tissue; D, diploid sorted cells from the dysplasia; A1, 3.6N aneuploid cell population; A2, 4.0N aneuploid cell population. To detect 17p allelic losses, DNA was amplified by using DNA sequences flanking a *Bst*UI restriction site in exon 4 of *TP53*. A 259-base-pair (bp) amplicon was created and digested with *Bst*UI. The cut allele resulted in 160- and 99-bp bands. To detect 5q allelic losses, primer sequences flanking an *Rsa* I restriction site in exon 11 of *APC* was used. A 133-bp amplicon was created and digested with *Rsa* I. The cut allele resulted in 88- and 45-bp bands. All products were electrophoresed on a 10% polyacrylamide gel. Lanes from right to left show the following results. Normal tissue from the patient and diploid sorted cells from the dysplasia retained both 17p and 5q alleles; however, 17p and 5q allelic losses of the cut alleles could be demonstrated in aneuploid cell populations A1 and A2.

Our results may also differ from those in the colon because different methods were used to determine the order of events. In the colon, order was based on the prevalence of allelic losses in different classes of adenomatous polyps and unrelated cancers from different patients, whereas we have investigated the order of events in multiple aneuploid cell populations from the same patient. In addition, we investigated the development of 17p and 5q allelic losses in aneuploid cell populations. If 17p allelic losses lead to a greater likelihood of subsequently developing aneuploid cell populations than do allelic losses of 5q, 17p allelic losses might be preferentially involved in the tumors that we evaluated. It should be noted, however, that aneuploidy develops as an early event in neoplastic progression in Barrett esophagus, that 95% of Barrett's adenocarcinomas are aneuploid, and that multiple aneuploid cell populations are common in both high-grade dysplasia and cancer in Barrett esophagus (3, 4, 12). Therefore, we believe that the tumors we have studied are representative of neoplastic progression in Barrett esophagus and that we have not selected a subset with different biological characteristics. Finally, it might be argued that, in the seven patients in whom we demonstrated a 17p → 5q order of allelic losses, the aneuploid populations showing both allelic losses evolved independently from those showing only a 17p allelic loss. However, we believe this is unlikely because in any given patient, the same 17p allele was lost in all aneuploid populations.

Our results suggest that 17p allelic losses develop as early events in the progression to adenocarcinoma in Barrett esophagus. We and others have also shown that *TP53* mutations and p53 protein overexpression develop as early events during neoplastic progression in Barrett esophagus (24, 25). There is also evidence that p53 abnormalities develop at early stages in the progression to breast cancer (26). In this regard, it is interesting that patients with Li–Fraumeni syndrome, who have inherited a mutation in *TP53*, are predisposed to the development of breast cancer but not esophageal adenocarcinoma (27–29). However, esophageal adenocarcinoma typically arises in patients who have developed Barrett esophagus as a result of chronic gastroesophageal reflux disease (7). Thus, patients with Li–Fraumeni syndrome, who do not have chronic gastroesophageal reflux disease and therefore do not develop premalignant Barrett metaplasia, may not be at risk for progression to esophageal adenocarcinoma even though they have inherited a mutation in *TP53*.

There are at least two mechanisms that could explain a strongly preferential 17p → 5q order of allelic losses during neoplastic progression in Barrett esophagus. The target genes on 17p and 5q might be part of a dependent pathway in which it is essential to lose the 17p gene before 5q allelic losses can be selected. Alternatively, 17p allelic losses might increase the rate of genomic instability and the probability of subsequent allelic losses of other chromosomal regions. Available data do not permit us to conclusively distinguish these two possibilities, but we favor the latter possibility for several reasons. First, there is emerging evidence that p53 functions as a "checkpoint" protein that permits the repair of DNA damage in the G_1 interval of the cell cycle (30). Second, inactivation of p53, with resulting inappropriate entry into S phase is believed to be associated with the development of genomic instability and aneuploidy (31–33). Finally, there is abundant evidence that the target of 17p allelic losses in

Barrett esophagus includes the p53 gene, *TP53* (8, 9, 11, 24, 25).

In summary, we have used DNA-content flow cytometric cell sorting and the polymerase chain reaction to investigate the order of 17p and 5q allelic losses in different aneuploid cell populations from the same patient. Using these techniques, we report a 17p → 5q order of allelic losses in neoplastic progression in Barrett esophagus. Therefore, cell-sorting based on DNA content can not only provide purified neoplastic cells for genetic investigations but also can be used to investigate the order of genetic events during clonal evolution within a single patient. By using this approach, the order in which genetic events occur during clonal evolution can be investigated in human neoplastic progression *in vivo*. This methodology can be used on small tissue samples, including endoscopic biopsies, making possible the prospective evaluation of genetic abnormalities in patients who are in surveillance for premalignant conditions such as Barrett esophagus.

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