Short Communication

BAT-26 and BAT-40 Instability in Colorectal Adenomas and Carcinomas and Germline Polymorphisms

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Analysis of the mononucleotide repeats BAT-26 and BAT-40 has reportedly revealed significant microsatellite instability in sporadic colorectal adenomas, whereas studies with dinucleotide and tetranucleotide repeats have not. In addition, BAT-26 has been reported to be “quasimonomorphic” in the germline. We evaluated BAT-26 and BAT-40 in a series of colorectal tumors previously analyzed with a panel of tetranucleotide repeats. Instability in BAT-26 or BAT-40 was significantly associated with tetranucleotide repeat instability in sporadic adenomas and carcinomas ($P < 0.0001$) and was similarly much less common in adenomas than in carcinomas. Germline polymorphisms in both BAT-40 and BAT-26 were identified, and the frequency of BAT-26 polymorphisms was significantly higher in African Americans than in Caucasians (7.7% versus 0.08%, $P < 0.001$). BAT-26 and BAT-40 may be very useful in evaluating instability in small tumors, as sufficient DNA to be amplified by large panels of microsatellites is not always available from such lesions. Polymorphisms in these microsatellites, however, limit their utility in determinations of microsatellite instability without corresponding normal DNA. (*Am J Pathol* 1999, 154:1637–1641)

BAT-26 and BAT-40 are poly(A) mononucleotide repeats located in introns of the *hMSH2* and 3- β -hydroxysteroid dehydrogenase genes, respectively.¹ Instability in BAT-26 and BAT-40 is a highly sensitive indicator of generalized microsatellite instability.^{1,2} In addition, BAT-26 has been reported to exhibit very little variation in germline DNA; this apparently “quasimonomorphic” na-

ture, especially when compared to the relatively large deletions in this repeat seen in unstable tumors, has been predicted to allow for the determination of tumoral microsatellite instability without reference to germline DNA.^{2,3} We and others^{4–8} have shown that instability is rare in sporadic colorectal adenomas, but these studies were performed with panels of dinucleotide and tetranucleotide repeats. A recent study⁹ using BAT-26 and BAT-40 reported microsatellite instability in approximately 25% of right-sided colorectal adenomas, a proportion very similar to that seen in right-sided colon cancers.^{10,11} In contrast to previous studies, this result implied that microsatellite instability is an early phenomenon in the sporadic adenoma/carcinoma sequence. That study⁹ did assume, however, that germline BAT-26 and BAT-40 were “quasimonomorphic” and therefore did not compare tumor results with those obtained from matched germline DNA. In the current study, we compare instability in BAT-26 and BAT-40 in colorectal adenomas and carcinomas to our previous results with tetranucleotide repeats. We also determine whether BAT-26 or BAT-40 can be used to evaluate instability without reference to the respective germline DNA.

Materials and Methods

Changes in length of the poly(A) mononucleotide repeats BAT-26 and BAT-40 were evaluated in a set of 164 sporadic colonic adenomas and 127 sporadic colonic adenocarcinomas previously characterized with respect to microsatellite instability with a panel of 10 tetranucleotide repeats and with a mononucleotide repeat in TGF- β RII.^{8,11,12} BAT-26 and BAT-40 were polymerase chain

Supported by grant numbers CA01755, CA48998, and CA61757 from the National Cancer Institute (NCI) and M01-RR064 (National Center Research Resources). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NCI.

Accepted for publication March 10, 1999.

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Table 1. Relationship between Microsatellite Instability Determined by Instability in BAT-26 or BAT-40 and Microsatellite Instability Determined by a Panel of 10 Tetranucleotide Repeats in 127 Colorectal Cancers (A) and 164 Colorectal Adenomas (B)

	Tetranucleotides	
	Stable (<30% instability)	Unstable (≥30% instability)
(A) Carcinomas		
BAT-26 or BAT-40		
Stable	108	2
Unstable	3	14
(B) Adenomas		
BAT-26 or BAT-40		
Stable	161	0
Unstable	1	2

There is a highly significant association between these two measures of instability in carcinomas and adenomas ($P < 0.0001$, χ^2 test).

reaction (PCR) amplified from normal (blood or normal colonic mucosa) and tumoral DNA for each adenoma or adenocarcinoma. PCR was performed as described previously,¹ except that primers were labeled with fluorescent dye, and the sizes of PCR products were evaluated on an ABI machine (Applied Biosystems Inc., Foster City, CA).

The frequency of germline polymorphisms in BAT-26 and BAT-40 was evaluated in a population-based set of germline DNAs (prepared from lymphoblastoid cells) from 225 individuals with colon cancer and 307 age- and sex-matched controls without colon cancer. Because of the relative rarity of BAT-26 polymorphisms, this microsatellite was also evaluated in a population-based set¹³ of germline DNAs (blood or normal colonic mucosa) from 1664 individuals with colon cancer. For these germline determinations, a 5' tail (GTTTCT) was added to the reverse primer to complete the addition of an extra adenosine to PCR products.¹⁴ Reported PCR product sizes include a subtraction of 6 bp for comparison with PCRs performed without a 5' tail.

Results

One hundred twenty-seven colorectal adenocarcinomas were successfully amplified by six or more tetranucleotide repeats and either BAT-26 or BAT-40. Nineteen of 127 cancers (15%) were detected as unstable: 14 with the tetranucleotide repeats (30% or more instability)¹¹ and either BAT-26 or BAT-40, two with only the tetranucleotide repeats, and three with BAT-26 or BAT-40 alone (Table 1A). The relationship between microsatellite instability determined by tetranucleotide repeats and microsatellite instability determined by BAT-26 or BAT-40 instability is highly significant ($P < 0.0001$, χ^2 test). Sixteen of the 19 unstable cancers (84%) were right-sided, and these 16 tumors comprised 26.7% of the 60 right-sided cancers that were evaluated. Of the 67 left-sided cancers that were evaluated, three (4.5%) were unstable. The difference between the proportion of right-sided can-

cers that were unstable and left-sided cancers that were unstable (26.7% *versus* 4.5%) is statistically significant ($P < 0.01$, χ^2 test).

One hundred sixty-four colorectal adenomas were successfully amplified by six or more tetranucleotide repeats and either BAT-26 or BAT-40. This included 91 small adenomas, 35 large (≥ 1 cm) right-sided adenomas, and 38 large left-sided adenomas. Microsatellite instability was identified in three of the 164 adenomas (1.8%, Table 1B). Instability was detected in two adenomas by both the tetranucleotide repeat panel and BAT-26 and BAT-40; both were located in the proximal colon, and one was smaller than 1 cm, and one was larger. Instability with BAT-40 alone was detected in one large left-sided adenoma. The relationship between instability in adenomas as determined by the tetranucleotide repeat panel and as determined by BAT-26 or BAT-40 is highly significant ($P < 0.0001$, χ^2 test). Two of the 79 right-sided adenomas (2.5%) were unstable, *versus* one of 85 (1.2%) left-sided adenomas. The proportion of unstable right-sided adenomas is significantly less than the proportion of unstable right-sided carcinomas (2.5% *versus* 26.7%; $P < 0.001$, χ^2 test).

BAT-40 polymorphisms were identified in the germline of the original set of 289 individuals with colorectal adenomas and carcinomas, with allele sizes ranging from 87 to 111. The population frequency of these polymorphisms was then determined in a set of 225 individuals with colon cancer and 307 age- and sex-matched controls. Table 2 shows the allele sizes and the number of times each allele was observed. BAT-40 allele sizes in unstable adenomas and carcinomas are also shown in Table 2. There is nearly complete overlap in the sizes of BAT-40 alleles in the germline and those observed in unstable tumors. This is true even if one considers only the shortened BAT-40 tumor alleles (as demonstrated by comparison with the respective germline DNA); those sizes range from 88 to 102 and are completely encompassed by the germline range of 81–111. Only one tumor allele occurs outside of the range of germline alleles. This 118-bp allele is actually an insertion mutation, as the associated germline alleles were 96 and 107.

Representative examples of BAT-40 germline polymorphisms and BAT-40 deletions in unstable tumors are shown in Figure 1. BAT-40 alleles in tumors from individuals with germline polymorphisms appear very similar to BAT-40 alleles in unstable tumors; the polymorphisms are only identifiable as such by the observation of identical alleles in the germline.

Most BAT-26 germline alleles in the 289 individuals with colorectal adenomas and carcinomas were 122 bp in size, although minor deviations with allele sizes of 120 or 121 bp were occasionally observed. In two individuals, however, there was a more significant size deviation, with an allele of 110 bp in addition to the larger allele. The frequency of BAT-26 germline polymorphisms less than 120 bp was then determined in a population-based set of 1664 individuals with colon cancer. Table 2 shows the allele sizes and the number of times each allele was observed. Twelve polymorphisms smaller than 120 bp were identified, with allele sizes of 111, 112, and 114; the

Table 2. Comparison of BAT-40 and BAT-26 Germline Alleles with BAT-40 and BAT-26 Alleles in Unstable Tumors

	Germline		Unstable tumors	
	Allele size (bp)	# observed	Allele size (bp)	# observed
BAT-40*	111	5	118	1
	110	3	110	2
	109	7	109	1
	108	90	108	4
	107	186	107	3
	106	34	106	1
	105	46	105	5
	104	189	104	1
	103	8	103	1
	102	1	102	1
	98	1	101	2
	97	3	100	1
	95	12	99	1
	94	4	98	1
	93	6	97	1
	92	1	96	3
	91	1	95	2
	90	1	94	2
	87	1	92	3
	86	1	91	1
	85	1	88	1
	81	1		
BAT-26†	120 or more (major allele is 122)	1652	120 or more	12
	114	1	118	1
	112	1	117	1
	111	10	113	2
			112	1
			111	1
			110	4
			109	4
			108	2
			107	2

Substantial overlap exists between the range of germline alleles and the range of alleles in unstable tumors for both microsatellites.

*Germline results from 225 individuals with colon cancer and 307 controls; tumor results from 16 tumors unstable with BAT-40.

†Germline results from 1664 individuals with colon cancer; tumor results from 15 tumors unstable with BAT-26.

frequency of these polymorphisms was 0.7% (12 of 1664). BAT-26 allele sizes in unstable tumors are also shown in Table 2. As can be seen, there is substantial overlap between the range of shortened BAT-26 germline alleles (111–114) and the range of shortened alleles in unstable tumors (107–118).

Representative examples of BAT-26 germline polymorphisms and BAT-26 deletions in unstable tumors are shown in Figure 2. BAT-26 alleles in tumors from individuals with germline polymorphisms appear very similar to BAT-26 alleles in unstable tumors; as in the case with BAT-40 (see above), the polymorphisms are only identifiable as such by the observation of identical alleles in the germline.

Information with respect to race was available on 1300 of the 1664 individuals and in six of the 12 individuals with BAT-26 polymorphisms. Five of the six individuals identified themselves as African American (all with 111-bp

Figure 1. Paired germline and tumor results demonstrating (A) BAT-40 germline polymorphisms and (B) BAT-40 deletion mutations in unstable tumors. (NI is normal germline DNA, Ad is adenoma, and Ca is carcinoma; size in base pairs is indicated by the scale at the top of the figure. Signal amplitude is indicated by the scale on the right.) BAT-40 alleles in tumors from individuals with germline polymorphisms appear very similar to BAT-40 alleles in unstable tumors; the polymorphisms are only identifiable as such by the observation of identical alleles in the germline.

alleles), and one as White/Hispanic. The frequency of BAT-26 germline polymorphisms in African Americans was 7.7% (5 of 65); this was significantly greater than the frequency of polymorphisms in Caucasians of 0.08% (1 of 1235, $P < 0.001$, χ^2 test).

In the population-based set of individuals with colon cancer and age- and sex-matched controls, there were two BAT-26 germline polymorphisms smaller than 120 (114-bp alleles) in 204 individuals with colon cancer and one such polymorphism (118-bp allele) in 287 controls. None of the tumors from individuals with BAT-26 polymorphisms in any sample showed generalized microsatellite instability. No difference was observed in the frequency or type of BAT-40 germline polymorphisms between individuals with colon cancer and age- and sex-matched controls (data not shown).

Figure 2. Paired germline and tumor results demonstrating (A) BAT-26 germline polymorphisms and (B) BAT-26 deletion mutations in unstable tumors. (NI is normal germline DNA, Ad is adenoma, and Ca is carcinoma; size in base pairs is indicated by the scale at the top of the figure; signal amplitude is indicated by the scale on the right.) BAT-26 alleles in tumors from individuals with germline polymorphisms appear very similar to BAT-26 alleles in unstable tumors; the polymorphisms are only identifiable as such by the observation of identical alleles in the germline.

Discussion

This study confirms that the mononucleotide repeat microsatellites BAT-26 and BAT-40 are highly sensitive and specific indicators of generalized microsatellite instability, as results with these repeats on colorectal tumors were significantly associated with results obtained with a panel of 10 tetranucleotide repeats. BAT-26 instability is also reported to be highly correlated with instability determined by a large panel of dinucleotide repeats.² The high correlation of BAT-26 and BAT-40 instability with other measures of instability suggests that these microsatellites may be very useful in evaluating instability in small tumors, as sufficient DNA to be amplified by large panels of microsatellites is not always available from such lesions.

We identified substantial germline polymorphism in BAT-40, with nearly complete overlap of BAT-40 alleles in the germline with those in unstable tumors. As demonstrated in Figure 1, polymorphisms in BAT-40 could be misinterpreted as instability-related mutations if the corresponding germline DNA is not evaluated. Consistent with a recent study,¹⁵ the polymorphic nature of BAT-40 indicates that this microsatellite cannot be used to determine instability accurately in a tumor without reference to the respective normal DNA. One unstable tumor con-

tained a BAT-40 insertion mutation of at least 11 bases. This appears to be the first identification of an insertion mutation, as to our knowledge all previously reported mutations were deletions.¹⁵

Germline polymorphisms were also rarely seen in BAT-26, with an overall rate of 0.7%, and a frequency in African Americans of 7.7%. This study represents the first identification of such polymorphisms. Of interest is a recent study of BAT-26 instability in over 500 tumors from several primary sites, which identified three false positives; that is, these were tumors in which BAT-26 was shortened, but the tumor was classified as stable by other mononucleotide repeats and by a large panel of dinucleotide repeats.³ The BAT-26 alleles in these "false positives" were 7 and 12 bp shorter than the most common allele. This would translate into allele sizes of 115 and 110 under our experimental conditions, and these values are very close to the germline polymorphisms of 114 and 110–112 bp that we identified. Indeed, the substantial ladder associated with BAT-26 could lead to difficulties in distinguishing a 115-bp allele from a 114-bp allele, or a 111-bp allele from a 110-bp or 112-bp. Moreover, the rate of "false positivity" of 0.5% is similar to the overall rate of BAT-26 polymorphism of 0.7% that we observed. It is possible that evaluation of germline DNA in the previous study would have revealed germline polymorphisms as the explanation for these "false positives."

Substantial overlap between shortened BAT-26 germline alleles and shortened BAT-26 alleles in unstable tumors was present. As shown in Figure 2, this overlap indicates that BAT-26 instability cannot be evaluated with 100% accuracy without reference to normal DNA. Although mistakes in assignment of instability status without reference to normal DNA would be relatively rare, such mistakes could have a profound impact on a study in which instability itself was relatively rare, a relatively large percentage of African Americans were studied, and/or in which several lesions from an individual with a germline polymorphism were evaluated.

The addition of BAT-26 and BAT-40 to our panel of microsatellites did not result in a substantial increase in the detection of sporadic adenoma instability. Such instability was still quite rare, and the proportion of right-sided unstable adenomas (2.5%) was significantly less than the proportion of right-sided unstable carcinomas (26.7%), implying that instability is a relatively late change in the adenoma/carcinoma sequence. This is in contrast to a previous study of BAT-26 and BAT-40, which found that 25% of right-sided adenomas, even relatively early adenomas, exhibit microsatellite instability, implying that instability is an early change.⁹ That study assumed that BAT-40 and BAT-26 were "quasimonomorphic" in the germline, however, and compared tumor results with germline data from a set of 10 unrelated individuals rather than with the respective normal DNAs. Given the germline polymorphisms in BAT-40, as we (see above) and others¹⁵ have identified, and in BAT-26 (see above), it is clear that accurate evaluation of the instability status of these microsatellites requires comparison of tumor results with those obtained from the respective germline DNA. Thus it is possible that some of the ap-

parent adenoma instability identified in BAT-26 and BAT-40 by this previous study was actually the result of germline polymorphisms, and that instability in sporadic adenomas, as we and others have demonstrated,⁴⁻⁸ is quite rare.

Significant differences in the frequency of BAT-26 or BAT-40 polymorphisms were not observed in colon cancer cases *versus* controls. Because of the rarity of BAT-26 polymorphisms, however, analysis of a larger series of individuals, especially African Americans, would be necessary to exclude an increased risk of colorectal neoplasia associated with these polymorphisms.

In conclusion, microsatellite instability determined by BAT-26 and BAT-40 correlates with microsatellite instability determined by a panel of tetranucleotide repeats and is similarly much less common in sporadic colorectal adenomas than in carcinomas. These microsatellites may be very useful in evaluating instability in small tumors, as sufficient DNA to be amplified by large panels of microsatellites is not always available from such lesions. Nonetheless, germline polymorphisms in both BAT-26 (especially in African Americans) and BAT-40 limit their utility in determinations of microsatellite instability without corresponding normal DNA.

Acknowledgments

We acknowledge the contributions of Drs. Bette Caan and Kristin Anderson to the supporting research grants. We also acknowledge Ms. Sandie Edwards, Ms. Karen Curtin, and Ms. Linda Ballard for data collection and analyses.

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