Hum Genet (1987) 76:134–137

Human Genetics

Variation in the expression of aphidicolin-induced fragile sites in human lymphocyte cultures

Ann P. Craig-Holmes, Louise C. Strong, Angela Goodacre, and Sen Pathak

Department of Pediatrics and Department of Genetics, University of Texas, M.D. Anderson Hospital and Tumor Institute at Houston, P.O. Box 209, Houston, TX 77030, USA

Summary. A correlation between specific fragile sites and cancer breakpoints has been suggested raising the question of fragile site expression as a predisposing factor in the occurrence of cancer in some persons. Before addressing the question of increased fragility among patients at high risk for cancer, we analyzed the variability of aphidicolin-induced fragile sites among nine normal persons and also among repeated samples from three of these individuals. Considerable variation in both the frequency and location of these fragile sites was observed and the data strongly suggest the significant variation of 6 of the 16 selected sites to be primarily due to sampling differences. These findings indicate that the use of fragile sites as a screening tool for patients at high risk of cancer should be carefully monitored relative to the variation inherent in both culture and individual expression.

Introduction

The biologic significance of fragile sites on human chromosomes has drawn considerable attention during the last few years (Daniel 1986; Le Beau 1986; Michels 1985; Shabtai et al. 1985), and although there appears to be no consistent phenotypic or clinical abnormality associated with the presence of fragile sites [with the exception of the fra(X)(q27)], some interesting correlations have been made. For example, fragile sites are frequently located at or near breakpoints of chromosomal rearrangements consistently found in tumor karyotypes (Le Beau 1986; Hecht and Glover 1984; Yunis and Soreng 1984; De Braekeleer et al. 1985; Hecht and Hecht 1986; Glover et al. 1986). In addition, recent reports have suggested a relationship between constitutional fragile sites (viz, observed in PHA-stimulated peripheral blood lymphocytes) and tumor-specific chromosomal rearrangements involving the same regions in leukemic patients (Le Beau and Rowley 1984; Yunis 1984) and in the tumor of a Ewing's sarcoma patient (Gollin et al. 1986). Thus, persons with a specific constitutional fragile site might be at greater risk for a neoplasm that is associated with a genetic alteration involving the same region.

Associating increased fragility with a clinical entity such as cancer is difficult, especially if fragile-site expression varies among normal people. Although population-based data on fragile sites are limited, a high degree of variability can be inferred from previous reports. For example, the frequency of fra(10)(q23) varied considerably among individuals (8%–52%) in one population study of rare fragile sites (Sutherland 1985) and, in those studies involving common fragile sites, the range of breaks scored for any one region was noticeably large (Glover et al. 1984; Yunis and Soreng 1984; Sutherland et al. 1985). Even less information is available concerning variability of expression within a single individual (Sutherland and Hecht 1985).

We addressed the question of variation among normal persons for the aphidicolin-induced fragile sites (Glover et al. 1984). Nine subjects were sampled in the initial study; three of these were subsequently sampled on three separate occasions to determine the extent of temporal variation within an individual.

Offprint requests to: L. C. Strong

Materials and methods

Peripheral blood was cultured for four days in RPMI 1640 medium (with folic acid) supplemented with 1.3% phytohemagglutinin (Wellcome), 100 units/ml of penicillin and streptomycin, and 20% fetal calf serum. Aphidicolin was added during the last 24 h of culture in a total concentration of 0.2 μ*M*. The cultures were exposed to 0.04 μg/ml colcemid for 25 min, treated with 0.075 *M* KCl for 20 min at room temperature, and rinsed three times in a 3:1 methanol-acetic acid fix. Air-dried slides were prepared and stained with quinacrine dihydrochloride fluorescent stain for Q-banding.

Breaks were scored from 50 cells from each individual and from each of the three repeat samples. Cells were randomly selected from coded slides, using as the only criteria in cell selection adequate spreading and morphology for identifying break sites. Cell scoring, culture, and slide preparation were conducted by the same researcher throughout these experiments. Each of the repeat samples was collected at the same time from all three subjects; the cultures were set up immediately and were subsequently treated in exactly the same manner to eliminate as many laboratory variables as possible.

Results

All break sites were scored regardless of location or frequency. A total of 73 chromosomal regions was found to have at least one break in two or more of the nine persons examined. These are listed in Table 1. Of these 73 fragile sites, only seven regions had a break in all nine subjects, and the frequency with which the breaks occurred varied considerably. Twenty chromosomal regions appeared to be the most "common" in that breaks at those locations were observed in at

Table 1. Aphidicolin-induced breaks among nine subjects

Bands	A	B	C	D	E	F	G	H	I	Total
1p34	0	0	0	0	1	0	0	0	3	4
1p32	0	1	3	8	7	4	3	3	3	32
1p22	0	4	3	0	3	1	0	0	2	13
1q21	2	0	3	0	0	1	0	3	0	9
1q25	0	0	1	1	0	0	3	3	1	9
1q31	0	0	0	1	0	0	0	2	1	4
1q44	0	0	4	2	3	1	3	1	2	16
2p23	1	0	4	3	3	2	0	3	2	18
2p13[a]	1	1	3	1	2	3	2	3	2	18
2q14	2	1	1	0	2	0	2	0	1	9
2q31	0	0	1	3	0	0	1	0	0	5
2q33[a]	1	4	4	3	3	3	6	3	3	30
2q35	0	1	0	1	0	0	1	0	0	3
3p26	0	3	1	0	1	0	1	0	0	6
3p14[a]	19	26	34	29	36	26	22	17	30	239
3p12	0	0	0	2	0	0	1	0	0	3
3q13	0	0	1	2	1	0	0	0	1	5
3q26	2	0	2	1	0	1	1	2	2	11
3q27	0	0	2	0	0	1	1	0	3	7
4p16	1	1	0	2	1	1	0	3	1	10
4q21	0	0	1	0	0	1	2	0	0	4
4q25	0	2	0	0	0	0	0	0	2	4
4q31	3	1	3	3	1	0	4	4	4	23
5p14	1	0	0	1	0	1	1	0	2	6
5q15	0	1	0	0	2	0	0	0	0	3
5q22	0	0	0	0	0	0	1	2	0	3
5q31	2	2	1	2	0	0	0	2	1	10
5q35	1	0	0	0	0	0	0	0	1	2
6p23	0	0	0	1	1	1	1	1	1	6
6p21	0	0	1	1	0	2	0	4	2	10
6q21	4	2	3	2	3	1	0	1	3	19
6q26[a]	5	1	11	6	5	9	3	2	4	46
7p22	0	0	2	1	1	0	1	2	0	7
7p13	0	0	2	3	3	0	1	2	1	12
7q11	0	0	1	1	1	1	0	2	2	8
7q22	0	5	2	4	6	2	3	10	2	34
7q32	0	1	1	5	3	2	1	2	1	16
8q22	0	1	0	0	1	0	0	1	0	3
8q24	1	1	0	0	2	1	0	6	0	11
9p23	1	0	0	1	0	0	0	0	1	3
9q33	1	1	0	7	2	1	0	7	2	21
10p13	0	0	0	0	1	0	1	0	0	2
10q21	1	0	1	0	1	1	0	0	2	6
10q25	1	0	0	2	1	1	0	1	1	7
11p15	1	0	0	1	0	0	3	0	2	7
11p14	5	1	2	1	2	4	1	0	1	17
11p12	0	0	1	0	0	0	0	0	1	2
11q14	0	1	2	2	1	2	0	1	3	12
11q23	0	1	1	0	2	0	0	1	1	6
12p12	1	0	1	0	0	0	0	1	2	5
12q15	0	0	0	2	0	1	0	0	1	4
12q23	1	1	1	2	0	0	0	0	1	6
13q12	1	0	1	3	1	0	1	1	1	9
13q14	1	0	4	0	0	0	0	1	0	6
13q22	5	0	0	1	0	0	0	0	0	6
14q13	0	0	0	0	2	0	0	3	0	5
14q24[a]	1	1	5	4	8	3	6	5	3	36
15q15	0	3	1	0	2	0	1	0	0	7
16p12	0	0	1	0	1	0	0	1	0	3
16q22	0	2	6	10	0	0	3	0	1	22
16q23[a]	11	15	20	19	22	12	12	4	7	122
18p21	0	0	0	1	2	0	1	1	0	5
18q21	0	0	0	1	1	0	0	0	0	2
18q23	1	0	0	1	0	0	0	0	0	2
19p13	0	1	1	0	0	1	0	0	1	4
19q13	0	0	2	0	1	0	0	1	0	4
20q13	1	0	1	0	1	0	1	0	0	4
22q12	0	0	0	0	1	0	0	1	1	3
Xp22[a]	4	2	5	11	10	12	7	13	8	72
Xq12	0	1	2	0	0	0	0	0	1	4
Xq22	5	0	4	4	5	0	1	2	0	21
Xq24	3	0	0	1	0	0	0	1	1	6
Xq27	0	0	1	2	1	2	1	0	1	8
Total	91	89	161	168	160	105	106	130	129	1139[b]
Fra/Cell	1.8	1.8	3.2	3.4	3.2	2.1	2.1	2.6	2.6	2.53

[a] Seen in all subjects

[b] Seven regions had a break in only one of the nine subjects. These represented a total of 12 breaks

least seven of the subjects; however, these sites did not consistently represent sites with the highest frequency of breaks. For example, 4p16 and 13q12 each had breaks in seven of the nine individuals but accounted for only 2% of the total breaks scored. More total breaks were observed at 16q22 and Xp22, but most of these occurred in only four or five of the people studied; and nearly half of the 32 breaks at band 1p32 were scored in only two persons.

Even more variation was observed when the results of the repeated samples were compared to those of the nine subjects. Table 2 shows the results from the repeated samples for 19 of the most frequently observed break sites. These include all sites with $>2\%$ of the total breaks for each of the three individuals. When the two sets of data were compared (i.e., nine subjects versus repeat samples), we found it increasingly difficult to ascertain what might be considered a "common" or constitutive fragile site. Theoretically, seven sites were the most common in that they were observed in all nine subjects; however, three of these could not be confirmed in the repeated samples. Band 5q31 was especially exemplary of the variation in sampling. In the original series (Table 1), no breaks at 5q31 were seen in subjects E, F, or G. For these individuals, the subsequent series of repeated samples (Table 2) revealed breaks at 5q31 in all but two samples and represented at least 2% of the total breaks in each of the three subjects. In addition, breaks were scored at band 3p26 in all samples of the repeated set, yet represented less than 1% of the total breaks scored from the nine individuals.

Not only was sampling variation apparent but there appeared to be some differences among subjects as well. This was particularly suggestive for bands 3p26, 7q32, 14q24, and Xp22. To determine if the observed variation was significant,

Table 2. Chromosome break sites from repeated samples in three subjects representing >2% of their total breaks

Bands	Subject E				Subject F				Subject G			
	1	2	3	Total (%)	1	2	3	Total (%)	1	2	3	Total (%)
2p23	2	4	1	2.4	2	0	0	0.6	3	1	0	1.0
2p21	0	0	0	0.0	0	1	6	2.0	1	4	4	2.1
2q31	0	0	2	0.7	0	4	5	2.6	0	4	0	1.0
2q33	1	2	1	1.4	4	0	2	1.7	5	0	5	2.4
3p26	1	1	3	1.7	2	3	7	3.4	6	7	5	4.3
3p14	21	35	28	29.1	22	30	36	25.1	21	36	29	20.4
3q26	2	0	4	2.1	1	3	3	2.0	0	2	6	1.9
4q31	2	2	5	3.1	5	3	0	2.3	4	0	1	1.2
5q31	0	5	4	3.1	1	5	2	2.3	0	7	3	2.4
6p21	3	0	6	3.1	3	2	2	2.0	0	2	3	1.2
6q21	1	3	2	2.1	1	1	4	1.7	1	1	3	1.2
6q26	2	4	7	4.5	1	8	4	3.7	4	0	0	1.0
7q22	2	5	4	3.8	3	6	5	4.0	2	9	4	3.6
7q32	1	2	0	1.0	1	0	2	0.9	7	6	2	3.6
9q33	2	0	4	2.1	1	3	1	1.4	4	1	2	1.7
14q24	3	4	0	2.4	2	4	1	2.0	12	8	6	6.2
16q23	5	2	4	3.8	0	4	8	3.4	5	4	5	3.3
Xp22	2	9	3	4.8	4	7	12	6.6	13	9	8	7.1
Xq22	1	3	4	2.8	0	3	0	0.9	6	2	5	1.9
Total breaks in 50 cells	75	109	105		88	124	139		134	145	143	
Total				231				273				328
Total (%)				69				61				55

Table 3. Chromosome breakage by selected site in repeated samples from three subjects

Chromosome band	Subject E			Subject F			Subject G		
	1	2	3	1	2	3	1	2	3
1p32	0	0	0	0	0	0	0	2	1
2q31	0	0	2	0	4	5	0	4	0
2q33	1	2	1	4	0	2	5	0	5
3p14[a]	21	35	28	22	30	36	21	36	29
4q31	2	2	5	5	3	0	4	0	1
6q26	2	4	7	1	8	4	4	0	0
7q22[b]	2	5	4	3	6	5	2	9	4
7q32	1	2	0	1	0	2	7	6	2
9q33	2	0	4	1	3	1	4	1	2
11p13	0	0	1	1	0	0	2	0	1
13q14[b]	1	2	0	0	1	0	2	2	1
13q22[a]	1	1	0	2	0	0	3	1	1
14q24[c]	3	4	0	2	4	1	12	8	6
16q22[b]	1	0	1	1	0	0	3	1	1
16q23	5	2	4	0	4	8	5	4	5
Xp22	2	9	3	4	7	12	13	9	8
Total cells	50	50	50	50	50	50	50	50	50
Breaks per cell	1.50	2.18	2.10	1.60	2.48	2.78	2.68	2.90	2.86

[a] $p < 0.05$ for samples only
[b] $p < 0.05$ for both samples and subjects
[c] $p < 0.05$ for subjects only

a statistical analysis was made on 16 specifically selected chromosomal regions. These included: (1) sites previously described by Glover et al. (1984) as the most frequent fragile sites (i.e., 2q31, 3p14, 6q26, 7q32, 16q23, and Xp22); (2) two chromosomal regions known to be associated with childhood cancer and thus of interest as a "high-risk" screening tool (11p13 and 13q14); (3) regions less sensitive to aphidicolin but in close proximity to some of the other selected sites (e.g., 13q22 and 16q22).

Table 3 presents the data from these 16 sites with the results of the statistical analysis. A two-way analysis of variance was made to evaluate both differences among subjects and differences among the multiple samples from each person. Six chromosomal regions were found to vary significantly ($p < 0.05$); however, the reasons for the variation were not uniform. Two of the breakpoints showed a significant variation in frequency among the samples only (within person differences), three showed both within and among person variation, and one region (band 14q24) revealed a significant difference among subjects only. Therefore, although 6 of the 16 sites demonstrated significant variation in the frequency of breakage induced by aphidicolin, this variation appeared to be primarily the effect of sample differences.

Discussion

The results of this study support the findings of Glover et al. (1984) in that the effect of aphidicolin on chromosome breaks is nonrandom and ubiquitous in nature. The average number

of breaks in 50 cells for the nine subjects of this report was 126 (2.53 breaks per cell), and over 75% of those breaks were located in only 23 chromosomal regions. Clearly, the most common of these fragile sites are bands 3p14, 16q23, and Xp22. Collectively, these three regions represent at least 30% of all breaks induced with aphidicolin (Glover et al. 1984; present data) and with FUdR plus caffeine (Yunis and Soreng 1984). Other chromosomal fragile sites, though also nonrandom, are expressed with greater variability. In this study the order of fragile site frequency among the original nine subjects was different (with the exception of 3p14) from that of the repeat samples from three of these individuals. In addition, not all of the six most frequently observed regions previously reported by Glover et al. (1984) were equally represented in this study; the 2q31 fragile site was involved in less than 0.5% of the total breaks in our series of nine subjects.

Such discrepancies, however, are not surprising in view of the variation found among the different samples of subjects E, F, and G. Although only a portion of these data was analyzed statistically, the results suggest that the frequency of fragile site expression varies as much among samples from the same individual as it varies among subjects. This finding probably represents the tip of the iceberg, since only 16 regions were chosen for analysis and at least two of these bands (11p13 and 13q14) do not represent a recognized common or rare fragile site. In fact, band 13q14 which was of special interest because of its association with retinoblastoma, was one of the six sites demonstrating significant variation among multiple samples as well as among the different subjects. Therefore, a correlation of an aphidicolin-induced fragile site at 13q14 and a predisposition to somatic rearrangement involving this region would be highly questionable if data were obtained from only one sample.

Reasons for the variation in fragile site expression observed in this analysis are difficult to ascertain but several possibilities may be considered. The variation may simply reflect laboratory or technical artifacts. A decreased frequency of fragile sites has been reported to be associated with a delay in processing blood samples (Jacobs et al. 1980; Brookwell et al. 1982; Daniel et al. 1984). In addition, the effect of using different stocks of medium, aphidicolin, colchicine, etc., as well as different criteria in scoring breaks would contribute to varying results obtained from different samples. In the present study, however, considerable care was taken to keep all known technical variables constant, so that this explanation seems unlikely.

A second explanation is that the variations may reflect the asynchronous nature of the cells in culture and the effects of sampling from this heterogeneous population. All compounds so far tested which induce fragile sites are involved with some phase of DNA synthesis (Sutherland and Hecht 1985; Glover et al. 1986; Le Beau 1986). Aphidicolin, for example, inhibits a DNA polymerase involved with DNA synthesis but not with DNA repair. During the 24 hours of culture that aphidicolin is present, the cells are at various stages of the cell cycle, some progressing through one S-phase, others through more than one S. The proportion of cells undergoing DNA synthesis during aphidicolin exposure may affect the expression of fragile sites at many of the chromosome regions.

The molecular nature of the fragile site may contribute even more greatly to this variation. Certainly, the microscopic expression of these "fragile sites" is only the cytologic manifestation of molecularly different chromatin, and additional studies are needed at the molecular level to allow these biologically distinct regions to become better understood.

The variability observed in this study does not negate the validity or significance of common fragile sites nor question their potential biologic significance. It does, however, raise obvious questions concerning the criteria to be used in ascertaining the presence or absence of fragile sites. Variation associated with the expression of these regions should be addressed and caution taken when this cytogenetic phenomenon is used to assess a predisposition to any cancer-related event.

Acknowledgements. We thank Dr. Barry Brown and Dr. David Ramsey for their advice and help in the statistical evaluation of these data. This work was supported in part by PHS grant CA34936 awarded by the National Cancer Institute, U.S. Department of Health and Human Services, and the Retina Research Foundation.

References

Brookwell R, Daniel A, Turner G, Fishburn J (1982) The fragile X(q27) form of X-linked mental retardation: FUdR as an inducing agent for fra(X)(q27) expression in lymphocytes, fibroblasts, and amniocytes. Am J Med Genet 13:139–148

Daniel A (1986) Clinical implications and classification of the constitutive fragile sites. Am J Med Genet 23:419–427

Daniel A, Ekblom L, Phillips S (1984) Constitutive fragile sites 1p31, 3p14, 6q26, and 16q23 and their use as controls for false-negative results with the fragile(X). Am J Med Genet 18:483–491

De Braekeleer M, Smith B, Lin CC (1985) Fragile sites and structural rearrangements in cancer. Hum Genet 69:112–116

Glover TW, Berger C, Coyle J, Echo B (1984) DNA polymerase a-inhibition by aphidicolin induces gaps and breaks at common fragile sites in human chromosomes. Hum Genet 67:136–142

Glover TW, Coyle-Morris J, Morgan R (1986) Fragile sites: overview, occurrence in acute nonlymphocytic leukemia and effects of caffeine on expression. Cancer Genet Cytogenet 19:141–150

Gollin SM, Perrot LJ, Gray BA, Kletzel M (1986) Spontaneous expression of fra(11)(q23) in a patient with Ewing's sarcoma and t(11;22)(q23;q11). Cancer Genet Cytogenet 20:331–339

Hecht F, Glover TW (1984) Cancer chromosome breakpoints and common fragile sites induced by aphidicolin. Cancer Genet Cytogenet 13:185–188

Hecht F, Hecht BK (1986) Chromosome 9 in acute lymphoblastic leukemia: breaks in band 9p21–22 and a fragile site. Cancer Genet Cytogenet 21:1–3

Jacobs PA, Glover TW, Mayer M, Fox P, Gerrard JW, Dunn HG, Herbst DS (1980) X-linked mental retardation: a study of 7 families. Am J Med Genet 7:471–489

Le Beau MM (1986) Chromosomal fragile sites and cancer-specific rearrangements. Blood 67:849–858

Le Beau MM, Rowley JD (1984) Heritable fragile sites and cancer. Nature 308:607–608

Michels VV (1985) Fragile sites on human chromosomes: description and clinical significance. Mayo Clin Proc 60:690–696

Shabtai F, Klar D, Hart J, Halbrecht I (1985) On the meaning of fragile sites in cancer risk and development. Cancer Genet Cytogenet 18:81–85

Sutherland GR (1985) Heritable fragile sites on human chromosomes. XII. Population cytogenetics. Ann Hum Genet 49:153–161

Sutherland GR, Hecht F (1985) Fragile sites on human chromosomes. Oxford University Press, New York Oxford

Sutherland GR, Parslow MI, Baker E (1985) New classes of common fragile sites induced by 5-azacytidine and bromodeoxyuridine. Hum Genet 69:233–237

Yunis JJ (1984) Recurrent chromosomal defects are found in most patients with acute nonlymphocytic leukemia. Cancer Genet Cytogenet 11:125–137

Yunis JJ, Soreng AL (1984) Constitutive fragile sites and cancer. Science 226:1199–1204

Received November 3, 1986 / Revised January 15, 1987