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The Expression of Common Fragile Sites in Peripheral Blood Lymphocytes of Breast and Colorectal Cancer Patients with Aphidicolin

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BALCI, A., EKMEKCİ, A. and ÇETİN, R. *The Expression of Common Fragile Sites in Peripheral Blood Lymphocytes of Breast and Colorectal Cancer Patients with Aphidicolin.* Tohoku J. Exp. Med., 1999, **189** (2), 107-116 —— The frequency and distribution of aphidicolin induced common fragile sites was evaluated on chromosomes of peripheral blood lymphocytes in 10 breast and 10 colorectal cancer patients, and 10 healthy controls to determine correlation between specific fragile sites and cancer breakpoints. Fifty complete metaphases were screened from each culture and the results were evaluated by Student's *t*-test. The total number of fragile sites was found as 933 in breast cancer patients, 950 in colorectal cancer patients and 501 in control group. Both the number of aberrations per cell and number of aberrations per damaged cell were significantly higher in the patient groups. These findings indicate that genetic instability in the breast and colorectal cancer patients increased and fragile sites may play a critical role in the pathogenesis of breast and colorectal cancer. ———— fragile sites; colorectal cancer; breast cancer and aphidicolin

A fragile site is expressed as a non-staining gap, usually involving both chromatids (especially those on the common fragile sites), and is always at exactly the same chromosome. Also, it is inherited in a Mendelian fashion and shows fragility under appropriate in vitro conditions (Sutherland 1979). Common fragile sites are nonrandomly expressed in the genome and were previously induced by aphidicolin (APC) which is a specific inhibitor of eucaryotic DNA polymerase α and δ. Therefore, APC induces the most common fragile sites by blocking replication fork progression and inhibiting DNA repair processes (Glover et al. 1984; Baumstark 1992; Paz-y-Miño et al. 1997). Moreover, common fragile sites are also induced by low folate level, bromodeoxyuridine, 5-azacytidine or caffeine (Fundia and Larripa 1989).

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The role and molecular basis of common fragile sites are not well known. The previous reports have shown that there might be a correlation between location of fragile sites on chromosomes and recurrent chromosomal breakpoints found in cancer (Yunis and Hoffman 1989; Popescu et al. 1990; Ardisia et al. 1993; Egeli et al. 1997). However, some investigators consider that there isn't a relationship between fragile sites and cancer because these sites can be seen in every individual genome's (Puspurs et al. 1988; Porfirio et al. 1989; Mitchell et al. 1993).

Virtually every human cancer can occur in genetically predisposed individuals. The cytogenetic findings can sometimes lead to the discovery of specific genes. For example, it was recently found that a gene, called FHIT (Ohta et al. 1996), is located at 3p14 which is one of the most common fragile sites and it is either completely or partially missing in a wide variety of common cancers including colon, breast, and lung tumors (Negrini et al. 1996; Thiagalingam et al. 1996; Huebner et al. 1997).

Although efforts were made to understand the relationship between fragility and genetic alterations in some carcinomas, there is still no definitive conclusion about it. However, it has been suggested, there could be two distinct potential roles for fragile site in oncogenesis. The first would be specific, either with the fragile site being able to inactivate a gene, or as a region of genome instability to generate deletions resulting in abnormal transcripts. The second role of fragile sites would be to allow chromosomal rearrangement by breakage in response to clastogens (Sutherland et al. 1998). In this study we aimed to determine whether there is a possible relationship between fragile sites and breast and colorectal cancer or not.

Materials and Methods

Chromosome fragility was studied from the peripheral blood lymphocytes of ten female patients with breast cancer and ten colorectal cancer patients and ten normal healthy volunteers were studied as the control group. None of the individuals had a family history of cancer. These patients had no history of previous radiotherapy or chemotherapy, and none of them had smoking habits. Also there were no smoking habits in the control group. Pathological examination of all tumor specimens resulted in carcinoma. The age range was 23 to 67 in the breast cancer patients, 23 to 61 in the colorectal cancer patients and 27 to 58 in the controls (Table 1). Peripheral blood lymphocyte cultures were set up in medium 199 (M-7528, Sigma, Deisenhofen, Germany) which was supplemented with 10% fetal calf serum (S-00016, Seromed, Berlin, Germany), 1.5 % phytohemagglutinin (M 5030, Seromed) and 1% penicillin streptomycin (A 2212, Seromed) for 72 hours at 37°C. For each patient and control two cultures were set up. For spontaneous expression, one set was used without inducer. To the second set of culture 24 hours before harvesting, 0.2 μM APC (A-0781, Sigma) which was dissolved in

TABLE 1. *Information about patients with breast and colorectal cancer, and controls*

Subject No.	Breast cancer patients			Colorectal cancer patients			Controls	
	Age	Sex	Histopathology	Age	Sex	Histopathology	Age	Sex
1	67	F	Infiltrative ductal ca.	61	F	Adeno ca.	58	M
2	63	F	Epidermoid ca.	57	F	Adeno ca.	45	M
3	48	F	Infiltrative ductal ca.	45	F	Adeno ca.	43	F
4	45	F	Invasive papillary ca.	42	M	Adeno ca.	38	F
5	45	F	Infiltrative ductal ca.	42	M	Adeno ca.	38	M
6	43	F	Infiltrative ductal ca.	40	F	Adeno ca.	33	F
7	43	F	Infiltrative ductal ca.	37	F	Adeno ca.	30	M
8	39	F	Infiltrative ductal ca.	32	M	Adeno ca.	29	F
9	30	F	Medullar ca.	26	F	Adeno ca.	28	F
10	23	F	Infiltrative ductal ca.	23	M	Adeno ca.	27	F

No., number; ca., carcinoma; F, female; M, male.

medium 199, was added. To both set of cultures for the last 75 minutes before harvesting, 0.1 mg/ml of colchicine (77 120, Serva, Heidelberg, Germany) was added. Chromosome spreads were made according to routine procedures. All cell cultures were coded before slide preparation.

Thus, cells were scored blindly for chromosome aberrations without knowledge of treatment and patient type. Slides were then digested with trypsin and stained with giemsa (GTG banding). Fifty complete metaphases from each culture were screened for chromosome gaps, breaks, free fragments or triradial figures and recorded. Each chromatid break was recorded as one break, and exchange figure was recorded as two breaks. The classification of fragile sites was done according to the First Human Gene Mapping Interim Meeting (HGM9.5) (Hecht et al. 1990).

Statistical analysis

Student's t-test was used for statistical evaluation of mean number of aberrations per cell in three groups and to compare these data between patients and corresponding controls. Differences were considered significant at $p < 0.05$.

RESULTS

The expression of APC induced common fragile sites was studied in cultured peripheral blood lymphocytes of 10 breast cancer patients, 10 colorectal cancer patients, and 10 normal healthy individuals. An adequate number of metaphases were obtained from APC treated cultures of three groups. Thus, APC did not affect cell growth greatly or diminish the mitotic index significantly at the 0.2 μM concentration.

Therefore many APC induced aberrations were seen in all groups. These

aberrations were usually scored as chromosome gaps, chromosome breaks, chromatid gaps or chromatid breaks, whereas deletions, chromosome fragments, triradial configurations and rearrangements were also seen at low frequencies.

The statistical analysis of our results showed that both breast and colorectal

TABLE 2. *Expression frequencies of fragile sites in the breast cancer patients*

Subject No.	No. of cells	No. of dam. cells	Total fragile sites	Abr. rate per cell	Abr. rate per dam. cell
1	50	44	136	2.72	3.09
2	50	32	72	1.44	2.25
3	50	34	76	1.52	2.23
4	50	23	66	1.32	2.87
5	50	39	91	1.82	2.53
6	50	29	69	1.38	2.38
7	50	36	163	3.26	4.53
8	50	18	53	1.06	2.94
9	50	38	87	1.74	2.30
10	50	39	120	2.40	3.07
Total	500	332	933		
Mean±S.D.			93.3±35.2*	1.86±0.70*	2.81±0.69*

*$p<0.05$ vs. control (Table 4).
No., number; dam., damage; Abr., aberration; S.D., standard deviation.

TABLE 3. *Expression frequencies of fragile sites in the colorectal cancer patients*

Subject No.	No. of cells	No. of dam. cells	Total fragile sites	Abr. rate per cell	Abr. rate per dam. cell
1	50	33	69	1.38	2.09
2	50	38	103	2.06	2.71
3	50	22	55	1.10	2.50
4	50	26	49	1.02	1.88
5	50	33	100	2.00	3.03
6	50	42	93	1.86	2.21
7	50	35	104	2.08	2.97
8	50	33	82	1.64	2.48
9	50	45	153	3.06	3.40
10	50	40	142	2.84	3.55
Total	500	347	950		
Mean±S.D.			95±33.84*	1.9±0.67*	2.68±0.55*

*$p<0.05$ vs. control (Table 4).
No., number; dam., damage; Abr., aberration; S.D., standard deviation.

TABLE 4. *Expression frequencies of fragile sites in the healty control subjects*

Subject No.	No. of cells	No. of dam. cells	Total fragile sites	Abr. rate per cell	Abr. rate per dam. cell
1	50	31	58	1.16	1.87
2	50	29	51	1.02	1.76
3	50	20	39	0.78	1.95
4	50	27	44	0.88	1.63
5	50	24	46	0.92	1.92
6	50	29	44	0.88	1.52
7	50	30	69	1.38	2.30
8	50	13	30	0.60	2.30
9	50	34	67	1.34	1.97
10	50	28	53	1.06	1.89
Total	500	265	501		
Mean±S.D.			50.1±12.16*	1.0±0.23*	1.91±0.24*

No., number; dam., damage; Abr., aberration; S.D., standard deviation.

cancer patients expressed significantly increased number of APC induced aberrations as compared to controls ($p<0.05$) (Tables 2, 3 and 4). A total of 933 aberrations were seen in the 500 metaphases from breast cancer patients, 950 aberrations in the 500 metaphases from colorectal cancer patients and 501 in the 500 metaphases from control metaphases. The fragile sites at 3p14, 3p21.3, 7p13, 7q31, 7q32, 16q23 and Xp22.3 in the colorectal cancer patients, and 3p14, 3q13, 6q26, 7q22, 7q31, 7q32, 16q23, Xp22.3 and Xq22 in the breast cancer patients showed an increased expression when compared with the control group ($p<0.05$). There is a considerable overlap in the distribution of fragile sites between all groups. One hundred seventeen bands, which showed fragile sites in at least one or more individuals, were recorded in the breast cancer patients. Only 72 of the 117 bands were seen in the controls. In addition, 4 different sites from patients were seen in the controls. One hundred four bands, which showed fragile sites, were recorded in the colorectal cancer patients. Sixty seven of the 104 patients were seen in the controls, and three different sites from patients were seen in the controls.

The most frequently expressed of these APC-induced fragile sites are bands 3p14, 16q23, Xp22.3, 7q32 and 7q31. They were the same for all groups (Table 5). In addition, a number of fragile sites were expressed only in the colorectal cancer patients (3p12, 5q21, 6p21.1, 6q25, 8q13, 9p34, 10p13 and 12q14) or only in the breast cancer patients (1p34, 3q22, 4q13, 6q22, 7p21, 9q12, 10p11.2, 13q21, 14q31, 15q21, 15q26.1, 17q12, 19q13.4 and Xq13) or only in the controls (12p12, 14q32, 16q11.2 and Xp11.23). However, the expression of most of these fragile sites was only in one or two individuals in each case. No fragile sites were seen

TABLE 5. *The most frequently expressed common fragile sites in analyzed groups (n = 10)*

Fragile site	Breast		Colorectal		Control	
	T. No. FS	Mean±S.D.	T. No. FS	Mean±S.D.	T. No. FS	Mean±S.D.
1q44	13	1.3±1.05	12	1.2±1.39	6	0.6±0.84
3p14	182	18.2±5.94*	176	17.6±4.88*	125	12.5±2.46
4q31.1	13	1.3±1.19	16	1.6±1.07	8	0.8±1.03
6p25	10	1±2.49	11	1.1±1.1	4	0.4±0.69
6q26	25	2.5±2.01	20	2±2.7	10	1±1.05
7p13	9	0.9±1.91	16	1.6±1.17*	4	0.4±0.51
7q22	10	1±0.8*	9	0.9±1.19	1	0.2±0.42
7q31	27	2.7±1.8*	35	3.5±1.4*	8	0.8±0.6
7q32	33	3.3±1.3*	32	3.2±1.9*	10	1±1.05
14q24.1	12	1.2±1.5	15	1.5±1.6	5	0.5±0.7
16q23	146	14.6±2.59*	134	13.4±4.78*	95	9.5±3.8
Xp22.3	93	9.3±2.5*	64	6.4±2.5*	35	3.5±1.2
Xq22	24	2.4±0.96*	19	1.9±1.1	10	1±0.94

*$p < 0.05$ vs. control.
T. No. FS, total number of fragile sites; S.D., standard deviation.

on chromosomes 21 and Y.

For the APC induced cultures there was large variability between individuals within each group for the number of aberrations observed per cell but number of aberrations were always higher in the patient group.

DISCUSSION

According to previous studies there are two suggestions, one of which consider the relationship between fragile sites and cancer break points (Ochi et al. 1988; Ardisia et al. 1993; Richard et al. 1994; Egeli et al. 1997). The second group of reports have a disagreement with the first group suggesting that it is impossible to relate the common fragile site with the cancer breakpoints (Puspurs et al. 1988; Porfirio et al. 1989; Mitchell et al. 1993). The result of our study supports the first suggestion, that in the breast and colorectal cancer patients expression frequency of APC-induced fragile sites were significantly higher than in the controls, both in terms of number of aberrations per cell ($p < 0.05$) and number of aberrations per damaged cell ($p < 0.05$). Although there are some pessimistic view of the association between fragile sites and cancer breakpoints, the statistically significant association remains between fragile sites and cancer breakpoints, even taking into account the nonrandom occurrence of both fragile sites and cancer breakpoints.

There is some individual variability in the frequency and distribution of fragile sites in all groups. The variations may reflect the asynchronous nature of

the cells in culture and the effects of sampling from this heterogeneous population. APC inhibits DNA polymerases, and during APC exposure the cells can be at various stage of cell cycle. Thus, the proportion of cells undergoing DNA synthesis may affect the expression of fragile sites at many of the chromosome regions.

In agreement with previous reports, we found the site 3p14 was expressed most frequently, followed by 16q23, Xp22.3, 7q32 and 7q31 respectively after first two sites, the expression order of fragile sites were variable in the previous published reports (Craig-Holmes et al. 1987; Sokova et al. 1992). In spite of this variation, the most frequently expressed sites in the present study were also highly expressed in other studies. The general hierarchy of site expression appears, therefore, to be rather consistent between laboratories.

The clinical significance of the 3p14 fragile site, which is the most frequently expressed of these APC-induced fragile sites, is its potential involvement in several malignancies. Studies of small-cell, nonsmall-cell, and squamous-cell lung tumors (Daly et al. 1991; Yokoyama et al. 1992; Egeli et al. 1997; Fong et al. 1997), renal carcinomas (Tajara et al. 1988; Li et al. 1993), pancreatic tumors (Shridhar et al. 1996), breast carcinomas (Man et al. 1996; Negrini et al. 1996) and esophageal, stomach, and colon carcinomas (Ohta et al. 1996) have indicated that chromosomal breakages, deletions and balanced reciprocal translocations involving 3p14 are originated through breaks in 3p14. The 3p14 fragile site may contribute to the formation of these deletions and rearrangements and to the interruption of flanking gene sequences as a result of predisposition to breakage.

The second most frequently expressed fragile site in our study is 16q23 followed by Xp22.3. The MAF oncogene is located on 16q23 (Verma and Triantafillou 1998). An active gene cluster localized to Xp22.3, has been demonstrated on the inactivated X chromosome (Mandel et al. 1989; Austin 1991). Moreover, in this present study 7q31 is the other most frequently observed fragile site and its incidence in our patients was significantly higher than in the control group ($p<0.05$). It has been suggested that the 7q31 interval is the most commonly deleted region in breast cancer (Zenklusen et al. 1994). Our results also show that some of the fragile sites overlap the important gene regions for breast cancer, as estrogen receptor gene is located in 6q24-q27 fragile site (McKusick and Amberger 1993). In this present study, the incidence of 6q26 fragile site in the breast cancer patients was higher than in the control group.

Although the precise relationship between fragile site and cancer remains an enigma, our results support the suggestion that fragile sites may predispose to specific chromosomal breakages and rearrangements for breast and colorectal cancers. In conclusion, fragile sites may not be directly related to malignancy, but it is plausible that the malignant process utilizes these areas of common break points, eventually resulting in chromosome and gene rearrangements.

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