Factors Influencing Mutation at the *hp rt* Locus in T-Lymphocytes: Women Treated for Breast Cancer


Genetics Laboratory, Department of Medicine, and the Vermont Regional Cancer Center, University of Vermont, Burlington, Vermont 05401

ABSTRACT

Forty-nine women with breast cancer were enrolled in a prospective, longitudinal study of the genetic damage caused by treatment. Assays of mutant frequency at the *hp rt* locus in peripheral blood lymphocytes were performed at approximately 6-month intervals for 2 years. Treatment consisted of surgery alone or additional tamoxifen, radiotherapy, or chemotherapy in various combinations. At 6 months, there was an elevation of mean mutant frequency compared to initial values (*P* = 0.004) which persisted for as many as 2 years. A significant elevation at 6 months occurred only in the group of women who received combination chemotherapy (*P* = 0.005). Within this group, 5 of 15 patients had striking elevations of mutant frequency following chemotherapy (>3 SD). Three of these 5 women had serum folate levels in the deficient range, while only one of 9 patients with lesser responses to chemotherapy were folate deficient. The change in mutant frequency after chemotherapy was inversely related to serum folate levels (*P* = 0.05) and to the number of years of smoking cigarettes (*P* = 0.01). We conclude that of the various modalities used to treat breast cancer, only chemotherapy was accompanied by a high risk of somatic mutation. A subset of patients manifested substantial increases in mutant frequency, often in association with low serum folate levels.

INTRODUCTION

Cancer of the breast is diagnosed in about 100,000 American women each year (1). Most of these patients will undergo surgical treatment, followed often by radiotherapy, hormonal manipulation, or chemotherapy in various combinations (1). The long-term health consequences of treating this large number of women with genotoxic agents have concerned patients and their physicians. Several studies have indicated that patients treated for primary cancer of the breast have an increased risk of second malignancies, particularly leukemia and preleukemia (2-4).

Three general approaches are being explored to decrease the risk of developing this devastating complication; namely, limiting genotoxic treatment to those patients who are most likely to benefit from therapy, using treatment modalities that have relatively little mutagenic/carcinogenic effects, and identifying patients who are particularly sensitive to the genotoxic effects of these treatment agents and decreasing, if possible, this sensitivity. A major research thrust of the large cooperative group trials is focused on the first of these approaches, i.e., defining the characteristics of treatment-responsive patients and their probability of response to assess risk:benefit ratios (5). Epidemiological studies and laboratory investigations utilizing animal and *in vitro* assays have been used to measure the mutagenic/carcinogenic effects of treatment modalities. Less information is available to help identify particularly sensitive individuals and understand the biological basis of their susceptibility to genetic damage by cancer treatment. The latter approach is attractive because a better understanding of mutagenesis/carcinogenesis in these individuals might lead to the design of preventive strategies. Consequently, we performed a prospective, longitudinal study of changes in the frequency of somatic mutation *in vivo* at the *hp rt* locus following treatment of cancer of the breast to delineate the characteristics of patients who are likely to develop genotoxic effects as a result of treatment.

MATERIALS AND METHODS

Population Studied. Women with histologically proven breast cancer were asked to participate in this study. After informed consent was obtained, following procedures approved by the University of Vermont Committee on Human Research, blood samples were collected, and the women completed an environmental questionnaire and a food frequency questionnaire, Nutrition Evaluator (N-squared Computing Analytic Software, Silverton, OR).

Following surgical removal of the primary tumor by segmental or total mastectomy and axillary dissection, subsequent treatment was chosen by the patient's physician. Postoperative radiation, when administered, consisted of approximately 5000 cGy (range, 4400-5220 cGy) to the involved pectoral region, utilizing tangential ports to minimize exposure of mediastinum, lungs, and bone marrow. Radiation was delivered at the rate of 200 cGy/day and usually was completed in 5 weeks. No attempt was made to include axillary, supraclavicular, and interpectoral and internal mammary lymph nodes. Some patients were entered on a NSABP protocol (B-14) which randomized patients to receive either tamoxifen, 10 mg twice/day, or placebo for 5 years. Other patients were entered on protocols which included tamoxifen, 10 mg twice/day (NSABP B-16 or B-20) for 5 years.

Various chemotherapy regimens were used. Eight patients received 600 mg/m² cytoxan and 40 mg/m² Adriamycin, i.v. every 21 days for 4 cycles (CA). Three patients received 100 mg/m² cytoxan for 14 days orally or 600 mg/m² i.v., 40 mg/m² methotrexate i.v., and 600 mg/m² 5-fluorouracil i.v., on day 1, repeated each 3-4 weeks for 6-8 cycles (CMF). Two patients received 100 mg/m² methotrexate and 600 mg/m² 5-fluorouracil i.v. on days 1 and 8, with leucovorin "rescue," repeated each 4 weeks for 13 weeks (MF). One patient received 500 mg/m² cytoxan, 50 mg/m² Adriamycin, and 50 mg/m² 5-fluorouracil i.v. once each 3 weeks for 6 cycles. Another patient received 8 mg l-phenylalanine mustard orally for 5 days, 58 mg Adriamycin, and 600 mg 5-fluorouracil i.v. every 6 weeks for 2 years. All but the last patient completed her chemotherapy within the first 6 months following diagnosis.

Sample Collection and Cryopreservation. Peripheral blood was collected by venipuncture. Serum was separated from clotted blood and frozen for subsequent analysis of vitamin levels. The remaining heparinized blood was separated on a gradient and the mononuclear cell fraction collected, washed, and frozen as previously described (6).

T-Lymphocyte-cloning Assay. Cryopreserved cell samples were rapidly thawed and washed by centrifugation, and the cell number was...
determined. The cloning assay was performed as described previously (7). In brief, the cells were incubated at a density of 1 x 10^6 cells/ml in medium RPMI 1640 containing 20% medium HL-1 (Ventrex), 5% defined, supplemented bovine calf serum (Sterile Systems), and 1 µg/ml PHA (HA17, Wellcome Diagnostics) for 36–40 h to achieve mitogen stimulation. The cell number was determined, and the cells were plated at limiting dilution in 96-well (round bottom) microtiter dishes (Nunc) at 1, 2, 5, and 10 cells/well in the absence of TG and at 2 x 10^5 cells/well in the presence of 10 µM TG in 200 µl of medium RPMI containing 20% HL-1, 5% bovine calf serum, 20% growth factor supplement, 0.125 µg/ml PHA, and 1 x 10^7 irradiated (90 Gy) human lymphoblastoid accessory cells. The growth factor supplement was the cell-free supernatant medium used in a lymphokine activated killer cell therapy. This procedure used the incubation of the mononuclear cell fraction from patients at 1.5 x 10^6 cells/ml in medium Aim V (Gibco) containing 2% autologous plasma and 1500 units/ml recombinant interleukin-2 for 3–4 days. The cell-free supernatant was collected, filtered, and frozen. Growth-promoting activity was assayed as described previously by a cell-cloning assay (7). The batches used in this study were used at a final concentration of 20%. The optimal amount of PHA (0.125 µg/ml) was also defined by the cell-cloning assay.

The microtiter dishes were incubated for 10–14 days and growing colonies determined by use of an inverted phase contrast microscope. Cloning efficiencies (CE) were calculated by use of the Poisson relationship

\[ P_0 = e^{-x} \]

where \( P_0 \) is the fraction of wells without colony growth. The CE = \(-\ln(P_0/x)\), where \( x \) = the average number of cells/well (i.e., 1, 2, 5, 10, or 2 x 10^5). The mutant frequency is the ratio of the mean CE in the presence (selected) and absence (unselected) of TG.

Assays of Vitamin Levels. Measurements of serum folate and vitamin B12 levels were performed by radioassay (SimulTRAC-S Solid Phase Radioassay Kit, Becton Dickinson Immunodiagnostics, Orangeburg, NY).

Statistical Analysis. Except where otherwise stated, all means are geometric mean values, and statistical analyses were based on log transformation (8). Comparisons between groups were made using Student’s t test or the paired t test and the Mann-Whitney U test.

RESULTS

Forty-nine women with breast cancer were enrolled at the time of presentation to their surgeons with a breast mass. Initial peripheral blood samples were obtained from 48 women; the mutant frequency at the hprt locus in lymphocytes isolated from these samples was 12.0 ± 1.7 x 10^-6 (Table 1). This value was not statistically different from mutant frequencies in normal women or in women with benign breast masses. All women then underwent surgery to remove the cancerous growth. Assays of mutant frequency were repeated at approximately 6-month intervals for 2 years in all women who survived their disease and consented to continue in the study. Of these 49 women, one patient died after 6 months in the study, and 4 women withdrew (2 after initial enrollment, 1 after 1 year, and 1 after 18 months of study). Some assays were repeated to test reproducibility. All available data from every enrolled patient were included in these analyses, except when the cloning efficiency was <0.01 (9).

Ten women received no treatment after surgery, and three patients were treated with tamoxifen. Thirty patients received postoperative irradiation to the involved breast; of these, 10 subsequently also were given tamoxifen, while 9 received chemotherapy. Six patients received postoperative chemotherapy alone.

The changes in mutant frequency during a 2-year observation period in women with breast cancer treated by a variety of modalities are shown in Table 1. At 6 months, there was a statistically significant elevation (\( P = 0.004 \)) in mutant frequency. By 12 months, the mutant frequency had decreased toward initial levels, and the difference was no longer significant (\( P = 0.13 \)). During the subsequent year, there was a further gradual decline in the mean mutant frequency, approaching the level found in the initial blood samples. There was no statistically significant change in mean lymphocyte unselected cloning efficiency throughout the observation period (Table 1).

The changes in mutant frequency over time were then analyzed according to the postoperative treatment that the patients received. For this purpose, the patients were divided into three treatment groups: (a) noncytolytic therapy, women who received no further treatment or tamoxifen alone; (b) radiation therapy, patients who were treated with radiation alone or with additional tamoxifen; and (c) chemotherapy, patients who received chemotherapy with or without additional radiotherapy or tamoxifen.

As shown in Table 2, there was a modest increase at 6 months in mutant frequency at the hprt locus in the lymphocytes from women who received no postsurgical treatment or tamoxifen alone, but this difference was not statistically significant, and the level declined to slightly below that found initially at enrollment by 1 year. The women who received radiation treatment with or without tamoxifen had a higher initial mean mutant frequency than was found in the other two groups. This may have been due, at least in part, to the fact that these women were older, on average, than those in the other groups. The measured mutant frequency was age dependent in these patients as has been reported previously (6, 10, 11). The mutant fre-

| Table 1 Mutant frequencies at the hprt locus and unselected cloning efficiencies in peripheral blood lymphocytes from women with breast cancer
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mutant frequency ((x10^-6))</th>
<th>n</th>
<th>Unselected cloning efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>12.0 ± 1.7*</td>
<td>48</td>
<td>0.37 ± 0.23</td>
</tr>
<tr>
<td>6 mo</td>
<td>16.7 ± 1.7*</td>
<td>40</td>
<td>0.36 ± 0.17</td>
</tr>
<tr>
<td>12 mo</td>
<td>14.4 ± 1.7*</td>
<td>33</td>
<td>0.40 ± 0.25</td>
</tr>
<tr>
<td>18 mo</td>
<td>14.1 ± 1.6</td>
<td>22</td>
<td>0.38 ± 0.21</td>
</tr>
<tr>
<td>24 mo</td>
<td>13.5 ± 1.8</td>
<td>11</td>
<td>0.40 ± 0.23</td>
</tr>
</tbody>
</table>

* Mean ± SD.

| Table 2 Mutant frequencies and unselected cloning efficiencies in peripheral blood lymphocytes from women with breast cancer treated by various modalities
<table>
<thead>
<tr>
<th>Sample</th>
<th>Noncytolytic</th>
<th>Radiation</th>
<th>Chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant frequency ((x10^-6))</td>
<td>Initial</td>
<td>9.9 ± 1.6*</td>
<td>13.2 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>6 mo</td>
<td>12.5 ± 1.3</td>
<td>18.1 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>12 mo</td>
<td>9.5 ± 1.2</td>
<td>16.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>18 mo</td>
<td>9.5 ± 1.1</td>
<td>15.6 ± 1.8</td>
</tr>
</tbody>
</table>

Unselected cloning efficiency

| Initial | 0.52 ± 0.31 | 0.32 ± 0.21 | 0.31 ± 0.14 | 19 |
| 6 mo    | 0.48 ± 0.11 | 0.34 ± 0.19 | 0.32 ± 0.17 | 15 |
| 12 mo   | 0.56 ± 0.34 | 0.39 ± 0.24 | 0.29 ± 0.14 | 11 |
| 18 mo   | 0.54 ± 0.11 | 0.32 ± 0.22 | 0.35 ± 0.20 | 6  |

* Mean ± SD.

frequency increased at 6 months, but this difference was not statistically significant, and then it returned toward initial levels. In contrast, the patients who received chemotherapy had a statistically significant elevation of mutant frequency at 6 months ($P = 0.005$) which then declined. Although only 15 patients received chemotherapy, lymphocytes from some individuals were studied twice, and all values are included in the data presented. There was not a statistically significant change in the unselected cloning efficiencies with time in any of the three groups (Table 2).

Since the most important changes in mutant frequency occurred during the first 6 months, these data were analyzed further (Fig. 1). In the “noncytolytic therapy” group, 7 of 8 mutant frequencies measured at 6 months were within one standard deviation of the arithmetic mean of the initial values for this group, and all were within 2 standard deviations. Similarly, in the radiation therapy group, the majority of patients (13 out of 16) had mutant frequencies at 6 months which were within one standard deviation of the mean of initial levels, and 14 of 16 were within 2 standard deviations. However, the mutant frequencies in lymphocytes from 2 of these women were more than 3 standard deviations from the mean. Only 5 of 15 women treated with chemotherapy had mutant frequencies within one standard deviation of the mean of initial levels, and only 9 of 15 within 2 standard deviations. Five of these 15 patients had mutant frequencies that were more than 3 standard deviations from the mean. When the mutant frequencies at 6 months in the “chemotherapy” group were analyzed without these five women, there no longer was a statistically significant difference from initial levels. Therefore, the statistically significant elevation in mean mutant frequency at 6 months observed in the chemotherapy group was due primarily to the marked increase in mutant frequency in these 5 patients.

The background information and laboratory data relevant to these 5 women were studied to identify, if possible, factors which might contribute to their increased sensitivity to mutagenic drugs. These patients did not have unusually high initial mutant frequencies: mean, $10.6 \times 10^{-6}$; range, $7.4-16 \times 10^{-6}$. On average, they were younger (mean age, 39.6 years; range, 32–47 years) than women who did not have a marked change in mutant frequency with chemotherapy (mean age, 47.2 years; range, 39–68 years). Three of the 5 had received radiation in addition to chemotherapy, but this proportion was not strikingly higher than in the group as a whole. Only one of the 5 had received tamoxifen in addition to chemotherapy. All 5 patients had received cyclophosphamide in combination with other drugs, but 7 of 10 patients who did not have exaggerated elevations in mutant frequency also received combination chemotherapy which included cyclophosphamide. Only one of the 5 had a maternal history of breast cancer. Three of these patients had never smoked, and the other 2 were current smokers, for a duration of 10 and 12 years.

None of the 5 patients reported dietary deficiencies of folates, vitamins A, B12, C, or E, selenium, or calcium. Caffeine ingestion by all 5 was modest: mean, 72.5 mg/day (range, 13.2–129.1 mg/day). None of the patients had serum vitamin B12 levels in the deficient range. Of interest, however, was the fact that 3 of the 5 patients who manifested unusually high mutant frequencies in response to chemotherapy had serum folate levels in the deficient range (<3 ng/ml), while only one of 9 patients whose responses were within 3 SD of the mean had a low serum folate level. Measurement of the $\chi^2$ after division of the mutant frequency of patients who received chemotherapy into two groups, ≥3 SD of the mean of the initial values, and serum folate levels into 2 groups, ≥3 ng/ml, indicated borderline statistical significance, $P = 0.052$ ($\chi^2 = 3.76$). The relationship between the change in mutant frequency at the hprt locus during the first 6 months of chemotherapy and initial serum folate levels is shown in Fig. 2. At serum folate levels in the normal range (>6 ng/ml), there is little change in the mutant frequency. However, within the “indeterminate” range, mutant frequency is more likely to increase following 6 months of chemotherapy, and this effect is more striking in women with serum folate levels in the deficient range (<3 ng/ml). The relationship between the data and the curve as drawn is significant at the $P = 0.05$ level.

Within the group of 15 women who received chemotherapy, there was no correlation between their age and the magnitude of change in mutant frequency at the hprt locus after 6 months of treatment (data not shown). However, there was an inverse correlation between the change in mutant frequency during this time period and the number of years of smoking cigarettes, $r = -0.64$, $P = 0.01$ (Fig. 3).

Because the patients were treated with different drug combinations and varying schedules, the contribution of a particular drug to the observed change in mutant frequency was difficult to evaluate from this small series of patients. Both CMF and CA were associated with substantial elevations in mutant frequency.
cell yield and lymphocyte unselected cloning efficiency. Others within 2 SD, and one third had elevations at 6 months that this reflects the influence of low unselected cloning efficiencies were at pretreatment levels. It is possible that, if we had assayed of the radiotherapy, when cell counts and cloning efficiency were performed approximately 4 months after the completion a greater mass of tissue (estimated to be 60% more) than the radiotherapy but not after chemotherapy for cancer patients. They also observed a decrease in mononuclear combination chemotherapy had mutant frequencies which were >3 SD above initial levels.

increase may have been found. However, our results suggest that there is little persistent evidence of somatic mutation after radiation, whereas those treated with surgery alone or radiotherapy were associated with a nonsignificant risk. Duration of alkylating agent therapy appeared to influence the risk, in that the relative risk associated with <18 months of therapy was 8.8 but increased to 14.7 in patients treated >18 months. Fisher et al. (5) reviewed the NSABP experience and found an increased risk of leukemia following both chemotherapy and radiotherapy. However, in the latter group, 2 of 6 cases of leukemia were chronic lymphatic leukemia. Since this type of leukemia is not usually associated with genotoxic exposure, the conclusion should be viewed with caution. Our results are also consistent with in vitro investigations which suggest that alkylating agents are more potent carcinogens than ionizing radiation (17).

Within the group of women treated with chemotherapy, there appeared to be a subgroup that was particularly susceptible to genetic damage. In our investigation of 107 women, including the 49 reported here who were studied prior to treatment, we iden
tified age, cigarette smoking, and serum folate levels as factors that influenced the frequency of genetic damage at the hprt locus. When the possible role of these factors in chemotherapy-induced mutation was analyzed, we found no effect of age but we did find an unexpected inverse relationship with duration of cigarette smoking. In contrast, low serum folate levels were associated with higher mutant frequencies. Consistent with this, folate levels in the deficient range were particu
tially common in the women who had the most marked elevations of mutant frequency after chemotherapy.

Folic acid deficiency has been associated with chromosomal damage and with an increased risk of malignancy. Patients with megaloblastic anemia caused by folic acid or vitamin B12 deficiency (which blocks folate metabolism) have numerous karyotypic abnormalities including gaps, breaks, despiralization, and increased sister chromatid exchanges (18–20). Similar
chromosomal aberrations have been induced in Chinese hamster ovary cells by incubation in folate-deficient medium (21). An increased frequency of micronucleated erythrocytes was found in folate-deficient humans and mice (22, 23). This cyto- genetic damage was enhanced by caffeine in both species (23, 24). Incubation of human lymphocytes in low folate medium promotes expression of fragile sites and increases the frequency of sister chromatid exchanges (25, 26).

The risk of cancers of the mouth and pharynx, stomach carcinoma, melanoma, multiple myeloma, and myeloid leukemia is increased in pernicious anemia patients (27). Folate supplementation of ulcerative colitis patients was associated with a 62% lower incidence of cancer compared to patients not receiving supplementation (28). Uterine cervical dysplasia was found to progress to carcinoma in situ in folate-deficient women (29). Folate plus hydroxocobalamin supplementation significantly improved bronchial squamous metaplasia in smokers compared to placebo (30). Finally, the incidence of mammary tumors in rats induced by procarbazine was increased 50–70% by coadministration of the folate antagonist, methotrexate (31). Taken together, these observations indicate that folate deficiency promotes chromosome damage and may contribute to carcinogenesis. Our current findings extend these observations by suggesting that folic acid deficiency may enhance the frequency of somatic mutations induced by chemotherapy.

Given the relatively small number of patients and short observation period, it is not possible to determine from this study whether an increased susceptibility to somatic mutation at the hprt locus is associated with a higher risk of second malignancies in breast cancer patients. Our results indicate that a study designed to answer this question should focus on women treated with chemotherapy which includes an alkylating agent and that the modulating influence of nutritional factors, particularly folic acid, should be considered.

REFERENCES
Factors Influencing Mutation at the *hprt* Locus in T-Lymphocytes: Women Treated for Breast Cancer


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