Mutagenesis after Cancer Therapy

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A subset of Hodgkin's disease (HD) and breast cancer patients have been reported to have elevated hprt mutant frequencies in peripheral blood lymphocytes after cessation of therapy. A subset of these patients are also known to develop second therapy-related malignancies. Therefore, it is clearly important to determine if these elevations in mutant frequency represent true, persistently elevated mutation frequencies. As a follow-up to our study of patients previously treated for HD, we recruited for a prospective study six previously treated HD patients and five patients who had been treated for squamous cell carcinoma of the head and neck. These individuals were studied several times over a 6–7 months. The results confirmed that a subset of patients have persistently high mutant frequencies when compared to 71 previously studied controls. The study was designed to determine if the elevated mutant frequencies of treated patients represented independent mutations or resulted from the in vivo expansion of single mutant cells. We used the polymerase chain reaction to examine DNA single strand conformation polymorphisms at the T-cell receptor-γ locus of individual mutant clones. This analysis showed that 20.1% of the mutants from Hodgkin's disease patients and 17.5% of the mutants from squamous cell carcinoma patients were siblings. The sibling mutants generally did not persist over time. However, one patient had one mutant clone that persisted, but slowly decreased in prevalence over a 7 month sampling period. The data demonstrate that treatments for cancer result in persistently elevated mutation frequencies at the hprt locus in some, but not all, patients. In addition, our results confirm previous studies indicating that it is not always appropriate to assume equality between mutant and mutation frequencies when studying individuals exposed to significant doses of known mutagens or carcinogens.

Introduction

Hodgkin's disease (HD) is among the most curable of cancers in that approximately 90% of patients with early-stage disease and 68% of patients with late stage disease are cured, as defined by remission of 5 years or more. Unfortunately, studies by a number of investigators have shown that patients in successful remission have a higher than expected risk for secondary cancers, including leukemia and solid tumors (I–9). The emergence of solid tumors, including those of the lung and breast, occurs as the time after successful radiotherapy or chemotherapy for HD increases beyond 10 years (I,8). The second cancer risk correlates with the type of treatment the patient received, with those experiencing the most intensive treatment at highest risk. Relative risk estimates up to 117 have been reported (8) for the development of leukemias in some long-term survivors who were treated with combination therapy of X-rays and chemotherapy. In those receiving radiation alone, a relative risk of 11 for leukemia has been reported in long-term survivors (8). At 15 years of follow-up, the solid tumor risk continues to increase; however, this risk has not been associated with a particular treatment type.

It has become clear that many neoplasms arise as the result of mutational events. Some cancers are heritable, including retinoblastoma (9,10), Wilms' tumor (11,12), and familial polyposis/colon cancer (13). Interestingly, the gene that is lost during the formation of retinoblastoma is also associated with the development of osteosarcomas (14), breast (15,16) and bladder cancer (17), and small carcinoma of the lung (18). These findings suggest that a genetic alteration, inherited at birth or occurring in somatic cells during life, increases the chance of developing cancer.

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Therefore, administration of a mutagenic treatment for a primary cancer may further increase the chances of development of second cancer by inducing heritable alterations of tumor-associated genes, such as seen with osteosarcoma after radiation treatment for retinoblastoma.

One of the most convincing studies associating cytotoxic therapy with secondary leukemia has been reported by LeBeau et al. (19). They showed that 17/63 patients with therapy-related acute nonlymphocytic leukemia and myelodysplastic syndrome had interstitial deletions on the long arm (q) of chromosome 5. Furthermore, the deletion encompassed the chromosome region 5q23-5q32. This region is known to include a number of hematopoietic growth factors, which may play a role in leukemia formation. Similar consistent alterations have been demonstrated on chromosome 7.

Laboratory techniques to detect the emergence of cell clones that carry specific changes associated with second cancers are continuously being developed. The goal of this research is to make these techniques applicable in a routine clinical setting; this can occur once the methods are validated, cost effective, and widely available. Most of the assays have not yet evolved to this stage.

Because only a subpopulation of the patients receiving a given therapy for HD develops a second cancer, we felt that it was important to determine whether certain individuals are hypersensitive to the induction of secondary genetic effects by the treatment for their HD and whether or not such differential sensitivity to therapy may be indicative of risk for second cancers. To investigate this, we have used an assay developed by several investigators (20–28) to measure mutations induced in peripheral blood lymphocytes. Mutation at the hypoxanthine-guanine phosphoribosyltransferase (hppt) locus, an enzyme that serves as a salvage pathway for nucleotide biosynthesis, is assayed by growth of the cells in a selective media containing 6-thioguanine. In the presence of this guanine analog, cells deficient in HPRT can survive. If cells from a treated individual are sampled and grown in 6-thioguanine, the frequency of mutants can be determined; this mutant frequency is an indication of overall somatic mutation induced by therapy.

### Somatic Mutation in Controls

Table 1 summarizes data obtained from controls. The mean mutant frequency (MF) in current smokers was 5.6 \( \times 10^{-6} \). In ex-smokers the mean MF was 3.8 \( \times 10^{-6} \), and in never-smokers it was 4.4 \( \times 10^{-6} \). The mean MF in the whole control group was 4.6 \( \times 10^{-6} \). The MFs ranges from 0.5 to 27.7 \( \times 10^{-6} \), with an upper 95% confidence limit of 12.6 \( \times 10^{-6} \). Current smokers had a mean MF that was 1.3-fold greater than nonsmokers (combined ex-smokers and never-smokers).

The effects of smoking, age, and gender on MF were examined using ANOVA. Current smoking was modeled using a "dose" term, which was weighted by the number of pack-years smoked; that is, smoking = (age at venipuncture - age smoking started) × number of packs smoked per day. When smoking status was included in the model in terms of pack-years, age did not significantly affect MF (slope = 0.035, \( R^2 = 0.139 \)). However, gender (\( p = 0.039 \)) and smoking (\( p = 0.007 \)) significantly affected MF. In nonsmokers (never-smokers and ex-smokers) alone, age had a borderline significant effect on MF (\( p = 0.059 \)), whereas gender was not significantly associated with MF. In current smokers alone, neither age nor gender significantly affected MF.

The MF's of three controls were repeatedly studied over a 1.5-year period. These data are shown in Table 2. The within individual variation in MF (Table 2) was less than the between individual variation among controls (Table 1). The variance of control 1 and control 3 were significantly different when compared to the variance of the whole control group (\( p < 0.05 \), \( F \)-test).

Therefore, our data from control MFs were generally in the range of 0.5–10.0 \( \times 10^{-6} \). Smoking was associated with an elevation in MF when a pack-year term quantitat-

### Table 1. Cloning efficiency (CE) and mutant frequency (MF) in smoking and nonsmoking controls.

| Group                        | Age                  | CE     | MF \( \times 10^{-6} \) |
|------------------------------|----------------------|--------|-------------------------|
|                              | Mean ± SD            | Range  | Mean ± SD               | Range                    |
| Current smokers (n = 18)     | 40.9 ± 15.2          | 18-65  | 28.8 ± 19.9              | 5.0-68.2                 | 5.6 ± 6.4                | 1.5-27.7               |
| Ex-smokers (n = 30)          | 54.8 ± 14.2          | 23-80  | 23.2 ± 20.0              | 2.7-94.1                 | 3.8 ± 3.2                | 0.5-16.5               |
| Never-smokers (n = 23)       | 39.4 ± 16.7          | 18-73  | 13.2 ± 10.8              | 1.3-37.0                 | 4.4 ± 3.2                | 0.7-15.4               |
| Nonsmokers (never-smokers and ex-smokers) (n = 53) | 48.2 ± 17.0          | 18-80  | 18.9 ± 17.2              | 1.3-94.1                 | 4.1 ± 3.2                | 0.6-16.5               |
| All controls (n = 71)        | 46.3 ± 16.7          | 18-80  | 21.1 ± 18.2              | 1.3-94.1                 | 4.6 ± 4.3                | 0.5-27.7               |

*Mean cloning efficiency is the average of duplicate plates seeded at 2, 5, and 10 cells per well.

### Table 2. Cloning efficiency (CE) and mutant frequency (MF) in controls repeated over 1.5 years.

| Subject          | n\(^a\) | CE, % Mean ± SD\(^b\) | Range | MF \( \times 10^{-6} \) Mean ± SD | Range |
|------------------|---------|-----------------------|-------|----------------------------------|-------|
| Control 1        | 26      | 31.0 ± 23.2           | 1.4-104.0 | 3.4 ± 2.8                       | 0.5-9.3|
| Control 2        | 9       | 17.4 ± 9.1            | 7.5-36.1 | 5.5 ± 2.9                       | 1.1-9.2|
| Control 3        | 7       | 41.7 ± 15.2           | 13.6-62.8 | 2.4 ± 1.3                       | 0.5-3.9|

\(^a\)Number of individual experiments.

\(^b\)Mean cloning efficiency is the average of duplicate plates seeded at 2, 5, and 10 cells per well.
ing cigarette "dose" was used in a model testing factors affecting MF in the controls. Smoking also was shown to have a significant effect on MF in a study reported by Cole et al. (23). In a study that included a small number of smokers (n = 4), Henderson et al. (25) reported a nonsignificant increase in MF associated with smoking. Among all controls, we found gender to significantly affect MF. In nonsmokers alone, however, the effect of age on MF approached significance, and gender had no effect. In smokers, neither age nor gender affected MF. Vijayalaksmi and Evans (29) and Cole et al. (23) reported no significant effect of gender on MF; however, they found a small increase in MF associated with age. Henderson et al. (25) also reported a small nonsignificant effect of age on MF. Trainor et al. (30) have also reported increase in age to be associated with increase in MF at hprt. Large numbers of study subjects at the extremes of age may be required for the effect of age on MF to become detectable. For instance, when newborn cord blood samples were included in the model testing for age effects, age significantly affected MF in the study by Henderson et al. (25).

Data from these controls, studied throughout a 1.5-year period, indicate that the MF remains stable over time in healthy controls. In addition, the variance within multiple samples on the same subject is less than samples between different individuals. These data are consistent with the findings of others (24,25,28). The mean control MF, 4.6 × 10^-6, is also similar to that previously reported (20–26,28).

**Untreated HD Patients**

Eighteen individuals with HD were studied before receiving any treatment (Table 3). The mean age of this cohort was 29.6 ± 10.3 years. The untreated patients had a significantly lower cloning efficiency (CE) than controls (11.3% versus 21.1%; p = 0.005, Wilcoxon test) and a higher MF (7.6 × 10^-6 versus 4.6 × 10^-6). The MF of the new patients did not differ significantly from the controls. ANOVAs showed that age, smoking, stage at diagnosis, and gender did not affect MF in this patient group. The subjects in the untreated HD cohort (new patients) had lower CEs when compared to controls. There are some reports of decreased responsiveness of lymphocytes to mitogenic stimulation in vitro from HD patients (31-35), which may explain the consistently lower CEs obtained in our study. A similar decrease in CE in cancer patients has been observed in an untreated breast cancer cohort (36). The MF in the untreated HD cohort (new patients) was not different from that in the control group. However, the presence of 3 of 18 persons with an elevated MF (above 12.6 × 10^-6) suggests that a fraction of HD patients begin therapy with MFs higher than the control level.

**Radiation Therapy Treatment of HD Patients**

Forty-five patients received only radiotherapy (Table 3). These patients had completed therapy 2-183 months before sampling. Their mean age was 35.1 ± 12.8 years. The mean CE for this group was 10.1% ± 7.6%, and the mean MF was 15.6 ± 24.4 × 10^-6 (Table 3). The CE was significantly different from the controls (p = 0.002, Wilcoxon).

The MFs in the radiotherapy and control groups differed significantly (p = 0.0001, Wilcoxon). In the radiation cohort, the majority of patients (69%) had MFs below the 95% confidence level for MF of controls, while 31% of these patients had MFs above this value (14 of 45).

There was no significant correlation (R = -0.141) between MF and time after completion of treatment. The effects of stage at diagnosis, age, gender, and smoking on MF were analyzed using ANOVA. When the model included the staging classification "A" or "B" (which indicates the absence or presence of systemic symptoms—fevers, night sweats, weight loss, etc.), age (p = 0.03) and stage (p = 0.03) were associated with MF. Gender and smoking had no effect on MF. However, when the stage classification was based only on nodal involvement (i.e., IIa and IIb are classified together), age, gender, smoking, and stage were no longer associated with MF.

**HD Patients Treated with Chemotherapy**

Among all patients studied, two received only chemotherapy. One (CHT1) was 40 years old, had a CE of 1.1%, an estimated maximal MF of 8.0 × 10^-6 (no mutants observed/576 wells plated), and had completed therapy 30 months before this study. The other patient (CHT2) was 39 years old, had a CE of 5.1%, and an elevated MF of 183.9 × 10^-6 (66 mutant clones/383 wells plated). CHT2 had completed chemotherapy several days before the study.

Twenty-three of the patients studied were treated with both radiation and chemotherapy (Table 3). The mean age for this group was 34.3 ± 14.1 years. They were studied 9-174 months after their last treatment, except for one patient who was currently undergoing radiotherapy but had finished chemotherapy. The mean CE, 10.7% ± 13.6%,
was statistically different from controls ($p = 0.007$, Wilcoxon); the MF, $15.7 \pm 12.4 \times 10^{-6}$, was significantly elevated over the control mean ($p = 0.0001$, Wilcoxon). ANOVA showed that there was no significant association between MF and age, stage at diagnosis, or smoking history.

Of the 23 patients receiving combined chemotherapy and radiotherapy, 44% (10 of 22) had MFs exceeding the upper 95% confidence level for controls. Thus, a greater proportion of these patients had elevated MFs than those receiving radiation therapy only (31%). A subpopulation of patient MFs were within control values of $0.5-10.0 \times 10^{-6}$. There also was no significant downward trend in MF with time after therapy in these treated patients ($R = 0.288$).

A study by Ammenheuser et al. (37) using an autoradiographic assay has shown that the MF transiently increases during and immediately after patient treatment with cyclophosphamide. However, the MF decreases to baseline levels shortly after cessation of treatment (1–2 months), suggesting selection against hprt mutants or cell turnover and repopulation. Recent studies by Nicklas et al. (38) on patients who were currently receiving radioimmunotherapy also reported elevated MFs, which were shown to be due to independent mutations by T-cell receptor studies. In neither of these studies, however, were patients studied years after cessation of treatment. Studies by Sala-Trepat et al. (39) show elevated MFs in patients who were sampled at various times (months) after treatment with radiation and chemotherapy for breast cancer. It is possible that in a subset of patients, independent mutations will persist in long-lived lymphocytes. The extent of persistence may be related to the exposure (radiation and/or chemotherapy) or to an inherent sensitivity of some lymphocytes to the exposure. It is clearly important to investigate the nature of the elevated MFs that are evident years after therapy ends.

**Possible Explanations for Persistently Elevated Mutant Frequencies in HD Patients**

Some radiotherapy patients have elevated MFs when compared to the controls; two distinct subpopulations are evident. One of these had MFs that were comparable to controls, irrespective of treatment. The fraction of patients with elevated MFs in the radiation-only cohort (31%) is higher than that observed in the untreated cohort (17%). Thus, it is unlikely that those radiotherapy patients with high MFs after therapy all began treatment with previously elevated MFs. In fact, after radiotherapy, the elevated fraction had just about doubled.

The emergence of a subpopulation with elevated MFs is even more striking when the MFs of those receiving both chemotherapy and radiotherapy were examined. A higher fraction of this cohort had elevated MFs (44%). The variation in MF in both of these groups (radiation and combination) was greater than the variation in the controls, further suggesting that the lymphocytes from different patients respond directly to such therapies. Furthermore, combined modality therapy is more intensive than radiotherapy. During radiotherapy, some lymphocytes are likely to escape exposure entirely. However, in combined modality therapy, these cells are exposed to systemic chemotherapy, which affects all lymphocytes, some of which may already have been damaged by radiation.

An elevated MF may result from two causes. It may arise after expansion of a particular mutant clone, or it may be due to an increase in the number of independent mutants. In the first case, elevation of MF may be caused by antigenic stimulation of a preexisting hprt mutant or a cell carrying pre-mutagenic damage in the hprt gene. In treated patients, long-lived G$_0$ lymphocytes with such an altered hprt gene may undergo blastogenesis as part of a normal immune response, resulting in a quantifiable increase in MF. The MF might then decrease after the immunological response is complete.

This type of expansion of a mutant clone has been previously observed in an otherwise healthy nurse (40). Similarly, a study on atomic bomb survivors showed that when the clonal mutants are enumerated and the number of actual independent mutational events determined, those with elevated MFs had normal mutation frequencies when compared to controls (41). Thus, it is possible that all patients have a similar level of mutagenic damage induced in the hprt gene, but that the damage is visible as hprt mutants in only a subset, based on the probability of a particular T-cell responding to an antigenic stimulus later. Alternatively, there may be inherent variation among people in susceptibility to radiation or chemical-induced mutations. If that were true, then new mutations would be induced in only a subset of patients. These possibilities could be distinguished by T-cell receptor studies. If the first alternative were correct, then most of the observed mutants would have the same T-cell receptor pattern. If the second were correct, then most of the mutants would have different patterns.

**Prospective Study to Determine the Incidence of Sibling Mutants in Treated HD Patients**

We conducted a prospective study to further characterize the prevalence of independent and sibling (clonal) mutant lymphocytes in treated cancer patients. We applied a technique using the polymerase chain reaction (amplifying a variable region within the T-cell receptor-γ gene locus) combined with DNA single strand conformation polymorphism gel electrophoresis (PCR/SSCP) to determine the proportions of unique and sibling mutants that appeared in the cloning wells (42,43). We used this PCR/SSCP technique to examine prospectively sets of mutants isolated from patients treated for HD and squamous cell carcinoma (SCC) of the head and neck after repeated samplings.

Six HD patients (patients A–F) and five SCC patients (patients G–K) who had completed therapy were studied prospectively over 6–7 months (Table 4). The majority of
Table 4. Demographic data from treated cancer patients enrolled in the prospective study.

| Subject ID | Diagnosis | Age | Treatment | Current smoker (pack-years) |
|------------|-----------|-----|-----------|----------------------------|
| A          | HD        | 36  | IRR, MOPP | NO                         |
| B          | HD        | 24  | IRR, MOPP | YES (1.5)                 |
| C          | HD        | 39  | IRR, MOPP | NO                         |
| D          | HD        | 30  | IRR, MOPP | YES (7)                   |
| E          | HD        | 29  | IRR        | NO                         |
| F          | HD        | 48  | IRR        | YES (23)                  |
| G          | SCC       | 61  | IRR, PFL  | YES (60)                  |
| H          | SCC       | 37  | IRR, PFL  | NO                        |
| I          | SCC       | 60  | IRR, PFL  | YES (40)                  |
| J          | SCC       | 66  | IRR, PFL  | NO                        |
| K          | SCC       | 51  | IRR        | NO                        |

Abbreviations: HD, Hodgkin's disease; SCC, squamous cell carcinoma; MOPP, mechlorethamine, oncovin, prednisone, procarbazine; PFL, cisplatin, 5-fluorouracil, leucovorin; IRR, radiotherapy.

Cigarette smoking history in pack years: years smoked × packs smoked per day.

Table 5. Mutant frequency (MF), incidence of sibling mutants (Sibs), and mutation frequency in a prospective study of Hodgkin's disease patients.

| Subject ID | Months since last treatment | % CE | No. of positive wells | No. of wells plated | MF × 10⁻⁵ | SIBS | No. of independent mutations | Mutation frequency × 10⁻⁵ |
|------------|----------------------------|------|-----------------------|---------------------|------------|------|-------------------------------|--------------------------|
| A-1        | 30                         | 13.9 | 5                      | 960                 | 1.9        | 0    | 5                            | 1.9                      |
| A-2        | 32                         | 7.0  | 6                      | 788                 | 5.6        | 0    | 6                            | 5.6                      |
| A-3        | 34                         | 3.7  | 4                      | 948                 | 5.7        | 0    | 4                            | 5.7                      |
| A-4        | 37                         | 15.1 | 25                     | 798                 | 11.0       | 1:8 | 19                           | 8.3                      |
| B-1        | 28                         | 12.6 | 10                     | 768                 | 5.2        | 0    | 10                           | 5.2                      |
| B-2        | 29                         | 21.7 | 17                     | 384                 | 10.6       | 0    | 17                           | 10.4                     |
| B-3        | 34                         | 26.6 | 31                     | 768                 | 7.5        | 1:7 | 25                           | 6.2                      |
| B-4        | 36                         | 11.2 | 27                     | 576                 | 21.4       | 1:2 | 26                           | 26.6                     |
| C-1        | 31                         | 15.6 | 85                     | 960                 | 29.7       | 1:4 | 41                           | 16.5                     |
| C-2        | 32                         | 10.6 | 43                     | 768                 | 28.8       | 2:3 | 39                           | 24.6                     |
| C-3        | 36                         | 9.9  | 60                     | 960                 | 32.6       | 2:2 | 52                           | 28.1                     |
| C-4        | 38                         | 13.2 | 44                     | 960                 | 17.8       | 1:4 | 41                           | 16.5                     |
| D-1        | 34                         | 5.9  | 8                      | 576                 | 11.8       | 0    | 8                            | 11.8                     |
| D-2        | 35                         | 11.5 | 38                     | 576                 | 29.7       | 1:4 | 33                           | 25.7                     |
| D-3        | 37                         | 11.6 | 10                     | 768                 | 5.6        | 0    | 10                           | 15.6                     |
| D-4        | 40                         | 34.3 | 35                     | 384                 | 13.9       | 1:2 | 32                           | 12.7                     |
| E-1        | 41                         | 55.0 | 11                     | 960                 | 1.1        | 1:3 | 8                            | 0.8                      |
| E-2        | 43                         | 21.0 | 65                     | 960                 | 16.7       | 1:5 | 50                           | 12.7                     |
| E-3        | 48                         | 20.4 | 20                     | 768                 | 6.5        | 1:3 | 18                           | 5.8                      |
| F-1        | 35                         | 16.3 | 35                     | 576                 | 19.2       | 1:19| 17                           | 9.2                      |
| F-2        | 36                         | 7.3  | 36                     | 960                 | 26.2       | 1:17| 18                           | 14.4                     |
| F-3        | 37                         | 12.9 | 21                     | 576                 | 14.4       | 1:4 | 13                           | 12.3                     |
| F-4        | 39                         | 5.2  | 6                      | 480                 | 12.1       | 1:2 | 5                            | 10.1                     |
| F-5        | 42                         | 5.1  | 12                     | 768                 | 15.4       | 1:10| 12                           | 15.4                     |

**Bolded IDs indicate combination therapy patients.**

**Clonally derived mutants = no. of different sibling groups (banding patterns): no. of clones in group.**

**Seven clones with one identical banding pattern.**

**Patients E and F were radiotherapy patients.**

**Sibling identical to others from patient F was identified.**

Each patient was sampled three to six different times. After determining the hprt MF, all mutant clones and 5–10 nonselected clones (grown in the absence of 6-thioguanine) were removed from the microtiter wells and subjected to PCR/SSCP analysis. These data were analyzed for the incidence of sibling mutants by examining the banding patterns on the autoradiograms (Tables 5 and 6). Several patients were noted to have sibling clones.

Mutation frequencies for each sampling were calculated by counting each independent mutant and sibling group as one mutation. In most cases, the MF closely approximated the mutation frequency (Tables 5 and 6). There were no significant trends in MF or mutation frequency over time, with the exception of patient F (Table 5). This patient had one sibling clone that was found in each peripheral blood
Table 6. Mutant frequency (MF), incidence of sibling mutants (Sibs), and mutation frequency in a prospective study of squamous cell carcinoma patients.

| Subject ID* | Months since last treatment | % CE | No. of positive wells | No. of wells plated | MF × 10⁻⁶ | SIBSb | No. of independent mutations | Mutation frequency × 10⁻⁶ |
|-------------|-----------------------------|------|-----------------------|---------------------|-----------|-------|--------------------------|-------------------------|
| G-1         | 9                           | 7.1  | 4                     | 192                 | 14.8      | 0     | 4                        | 14.8                    |
| G-2         | 10                          | 2.7  | 4                     | 288                 | 25.9      | 0     | 4                        | 25.9                    |
| G-3         | 11                          | 4.8  | 14                    | 384                 | 38.7      | 0     | 14                       | 38.7                    |
| G-4         | 13                          | 3.7  | 8                     | 192                 | 57.5      | 1:2* | 6                        | 42.9                    |
| G-5         | 14                          | 6.9  | 7                     | 384                 | 13.3      | 0     | 7                        | 13.3                    |
| H-1         | 4                           | 21.0 | 11                    | 192                 | 14.0      | 0     | 11                       | 14.0                    |
| H-2         | 5                           | 19.7 | 8                     | 476                 | 4.3       | 0     | 8                        | 4.3                     |
| H-3         | 6                           | 27.4 | 9                     | 192                 | 8.8       | 0     | 9                        | 8.8                     |
| H-4         | 8                           | 18.7 | 18                    | 288                 | 17.3      | 1:5  | 13                       | 12.4                    |
| H-5         | 10                          | 25.1 | 29                    | 384                 | 15.6      | 0     | 29                       | 15.6                    |
| I-1         | 6                           | 10.8 | 3                     | 384                 | 3.6       | 0     | 3                        | 3.6                     |
| I-2         | 7                           | 11.1 | 2                     | 384                 | 2.4       | 0     | 2                        | 2.4                     |
| I-3         | 8                           | 14.3 | 2                     | 288                 | 2.4       | 0     | 2                        | 2.4                     |
| I-4         | 9                           | 8.8  | 14                    | 768                 | 10.5      | 1:3  | 12                       | 8.9                     |
| I-5         | 10                          | 12.4 | 13                    | 576                 | 9.2       | 1:3  | 9                        | 6.4                     |
| I-6         | 11                          | 17.1 | 10                    | 192                 | 15.6      | 0     | 10                       | 15.6                    |
| J-1         | 4                           | 34.6 | 22                    | 384                 | 8.5       | 1:3  | 19                       | 7.3                     |
| J-2         | 5                           | 9.1  | 6                     | 384                 | 8.7       | 0     | 6                        | 8.7                     |
| J-3         | 8                           | 17.2 | 9                     | 288                 | 9.2       | 0     | 9                        | 9.2                     |
| J-4         | 9                           | 15.1 | 14                    | 384                 | 12.3      | 0     | 14                       | 12.3                    |
| K-1         | 3                           | 1.8  | 1                     | 480                 | 5.8       | 0     | 1                        | 5.8                     |
| K-2         | 4                           | 34.0 | 8                     | 384                 | 3.1       | 0     | 8                        | 3.1                     |
| K-3         | 5                           | 20.8 | 12                    | 288                 | 7.5       | 0     | 12                       | 7.0                     |

*Bolded IDs are combination therapy patients.
**Clonally derived mutants = no. of different sibling groups (banding patterns): no. of clones in group.
†Two clones with one identical banding pattern.
‡Patient K is radiotherapy patient.

Table 7. Mean mutation frequency in treated cancer patients compared to 71 healthy historical controls.

| Group                              | Patient | n¹ | Mean mutation frequency (× 10⁻⁶) ± SD |
|------------------------------------|---------|----|-------------------------------------|
| Hodgkin's disease                  | A       | 4  | 5.4 ± 2.6                           |
|                                   | B       | 4  | 10.6 ± 7.0                          |
|                                   | C       | 4  | 24.1 ± 5.3b                         |
|                                   | D       | 4  | 16.5 ± 8.4b                         |
|                                   | E       | 3  | 6.4 ± 6.0                           |
|                                   | F       | 5  | 12.3 ± 2.7*                         |
| Squamous cell carcinoma            | G       | 5  | 27.1 ± 13.5b                        |
|                                   | H       | 5  | 11.0 ± 4.5*                         |
|                                   | I       | 6  | 6.6 ± 5.1                           |
|                                   | J       | 4  | 9.4 ± 2.1*                          |
|                                   | K       | 3  | 5.6 ± 2.4                           |
| Historical controls (14)           | (71 healthy individuals) | 4.6 ± 4.3e |

¹Number of independent determinations of mutation frequency.
²Above 95% confidence interval from historical controls.
³Historical data is mutant frequency.
⁴Significantly different from controls (p < 0.05, Student's t-test).

Sample and persisted for 7 months. The prevalence of this clone declined with each time point (54.3%, 47.2%, 19.1%, 33.3%, 8.3% of total mutant clones observed). When these clones were removed from the analysis and counted as only one mutation, the patient's mutation frequency was generally within the normal range, even though the MF appeared elevated. When each individual's mutation frequency was estimated as the mean of the repeat measurements, and compared to the historical mean for healthy controls, it was clear that several patients had significantly elevated mutation frequencies, while others were within the normal range (Table 7). Thus, this prospective study confirmed our earlier observation that there is a persistent elevation in hprt mutation frequency in a subset of treated cancer patients (44).

Conclusions

We have shown that a subset of patients treated successfully for HD with either radiotherapy alone or combined modality (radiotherapy and chemotherapy) months to years before the study have MFs that are within the range of controls. However, other patients treated similarly have elevated MFs (44). This finding is consistent with recent
work of Branda et al. (45). Therefore, we initiated this prospective study to determine if those patients with elevated MFs have an increased prevalence of sibling mutants or a higher frequency of independent mutations. The results generally were consistent with the latter explanation. For both groups of treated cancer patients, the MF was approximately equal to the mutation frequency. The contribution to MF from sibling mutants did not appear to alter the analysis when examined overall, and some patients had persistently elevated mutation frequencies years after cessation of therapy. Thus, the alterations in MF which we observed in the treated HD patients may indicate a type of persistent genetic damage resulting from treatment. This is consistent with the work of O’Neill et al. (28) and Nicklas et al. (46). These investigators detected small numbers of sibling clones in control populations (approximately 10% of total mutants collected) when they used Southern blotting to analyze rearrangements occurring at the T-cell receptor-β gene locus; in these experiments, the MF was also a good estimator of the mutation frequency.

The MFs in these treated HD and small-cell carcinoma patients were generally stable over the multiple sampling times. The patients tended to have either consistently high (>12 × 10^{-6}) or consistently low (within the normal range of 1–12 × 10^{-6}) MFs, although some variation did occur. One HD patient underwent a significant clonal expansion, which greatly affected the MF. This also has been observed previously in two other studies (40,41). Therefore, we believe it is likely that the treatments induced persistently elevated mutation frequencies in only a subset of patients.

Previous work in primates suggests that in vivo exposure to DNA alkylating agents can induce persistent genetic damage, detectable long after initial exposure. Zimmer et al. (47) recently reported that a single intra-peritoneal injection of ethyl nitrosourea (ENU) induced an elevated hprt mutant frequency in peripheral blood lymphocytes of two cynomolgus monkeys that persisted for at least 2 years. Kelsey et al. (48,49) also reported that ethylene oxide induces sister chromatid exchanges in the lymphocytes of cynomolgus monkeys that are detectable 6–7 years after cessation of chronic inhalation exposure. These observations are consistent with our current demonstration that cancer therapy induces persistent genetic damage, detectable as elevated hprt mutation frequencies in a subset of treated patients.

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