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Mutagenicity and Potential Carcinogenicity of Thiopurine Treatment in Patients with Inflammatory Bowel Disease

Truc Nguyen[1], **Pamela M. Vacek**[2,3], **Patrick O'Neill**[1,2], **Richard B. Colletti**[1], and **Barry A. Finette**[1,2,4]

[1]Department of Pediatrics, University of Vermont, Burlington, 89 Beaumont Avenue, VT 05405

[2]Vermont Cancer Center, University of Vermont, Burlington, 89 Beaumont Avenue, VT 05405

[3]Department of Medical Biostatistics, University of Vermont, Burlington, 89 Beaumont Avenue, VT 05405

[4]Department of Microbiology and Molecular Genetics, University of Vermont, Burlington, 89 Beaumont Avenue, VT 05405

Abstract

The thiopurines, azathioprine and 6-mercaptopurine, are effective immune-modulators and cytotoxic agents extensively used in the treatment of autoimmune diseases, graft rejection, and cancer. There is compelling epidemiologic evidence that thiopurine treatment increases the risk for a variety of tumors by mechanisms that are unclear. We investigated the *in vivo* mutagenicity of long-term thiopurine treatment by determining the frequency and spectra of somatic mutation events at the *HPRT* locus in peripheral T lymphocytes as well as the prevalence of mutant clonal proliferation in a cross-sectional analysis of data from 119 children and adults with inflammatory bowel disease (IBD). Analyses of variance and regression were performed to assess relationships among the frequency and spectra of *HPRT* mutations with disease, duration of illness, duration of treatment and total therapeutic dose of azathioprine and 6-mercaptopurine. We observed a significant increase in the frequency of somatic mutations in 56 subjects treated with thiopurines for IBD compared to 63 subjects not treated with thiopurines. This increase was related to both total dose ($p<0.001$) and duration of treatment ($p<0.001$). Comparative mutation spectra analysis of 1,020 mutant isolates revealed a significant increase in the proportion of all transitions ($p <0.001$), in particular G:C to A:T transitions ($p<0.001$). Combined analyses of two signatures for mutant clonality, *HPRT* mutation and *TCRβ* CDR3 region unique gene sequence also demonstrated a significant thiopurine-dependent increase in mutant cell clonal proliferation ($p<0.001$). These findings provide *in vivo* evidence for mutation induction as a potential carcinogenic mechanism associated with chronic thiopurine intervention.

Keywords

Thiopurine; mutagenicity; cancer risk; inflammatory bowel disease; *HPRT*; proliferation

Corresponding Author: Barry A. Finette M.D., Ph.D., University of Vermont, Department of Pediatrics, E203 Given Building, 89 Beaumont Ave, Burlington VT 05445-0068, Tel: 802-656-2296, Fax: 802-656-2077, Email: barry.finette@uvm.edu.

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INTRODUCTION

Azathioprine and 6-mercaptopurine (6-MP) are effective immune modulators and cytotoxic agents used extensively for the treatment of autoimmune diseases (1), transplant graft rejection (2), and cancer (3). Their pharmacologic effects are complex and involve both inhibition of purine biosynthesis and incorporation of 2-deoxy-6-thioguanine nucleotides into DNA (4,5). Cytotoxicity following thiopurine incorporation into DNA appears to require methylation of the thiol group, mispairing of S-methylthioguanine with thymine, with subsequent recognition and signaling by the mismatch repair system (4).

A major continued concern is the broad-based epidemiologic evidence for duration (6) and dose-dependent (7-9) increases in the incidences of mesenchymal and solid tumors following thiopurine treatment (6-17). This associated cancer risk has resulted in azathioprine being classified as genotoxic and a human carcinogen (18,19). While the carcinogenicity of thiopurines has been attributed to their immunosuppressive effects, comparative epidemiologic studies with other immune modulators also suggest a thiopurine-specific contribution to cancer risk (9,14,15,17). In particular, *in vitro* and animal investigations indicate these agents are directly mutagenic (4,20-25). In this regard, human biomonitoring investigations have observed a significant increase in the frequency of mutant T cells following thiopurine treatment for transplant rejection (26), systemic lupus erythematosus (27) and diabetes mellitus (28), but the etiology of these increases was not discerned. To date, the specific mechanism(s) responsible for the *in vivo* carcinogenicity of thiopurine treatment has not been elucidated.

In this study, we investigated potential *in vivo* carcinogenic mechanisms associated with thiopurine treatment by conducting a cross-sectional analysis of its mutagenicity by examining the frequency and spectra of somatic mutations at the hypoxanthine phosphoribosyltransferase (*HPRT*) locus in peripheral T cells, as well as determining the prevalence of *in vivo* clonal expansion of mutant isolates in subjects with inflammatory bowel disease (IBD) in relation to disease type, duration of illness, duration of treatment, and total thiopurine dose.

Material and Methods

Study Population

The overall research design employed is illustrated in Figure 1. *In vivo* mutagenicity of thiopurine treatment was examined in 119 children and adults with either ulcerative colitis (UC) or Crohn's disease (CD). Both of these chronic inflammatory bowel syndromes are associated with increased cancer risk at affected sites of inflammation (29) and lymphoma following thiopurine intervention (13). We elected to study the potential mutagenic effects of thiopurine treatment in subjects with IBD since only a portion of subjects receive thiopurine intervention, and in comparison to subjects with cancer or status post transplantation, these subjects do not receive long-term treatment with other established mutagenic agents. Subjects with IBD were recruited from the pediatric and adult gastroenterology clinics at Fletcher Allen Health Care, the tertiary care facility at the University of Vermont, and consented with protocols approved by the Committee on Human Research. Diagnosis and therapeutic intervention were confirmed by participating gastroenterologists and a review of medical records. Heparinized blood samples were collected from 39 children (≤18 years old) and 80 adults (>18 years old). Fifty-six subjects comprised a group of thiopurine treated (TP) subjects who received azathioprine (n=40), 6-MP (n=11), or both (n=5), including 19 children and 17 adults with CD and 7 children and 13 adults with UC (Table 1). Sixty-three subjects comprise a group of non-thiopurine treated (non-TP) subjects, including 4 children and 13 adults with CD and 9 children and 37 adults with UC. All subjects previously or concurrently received other treatment modalities that included: 5-aminosalicylic acid derivatives (n=98), corticosteroids (n=72), infliximab (n=21), metronidazole (n=30) and/or ciprofloxacin (n=7).

The total equivalent dose (moles, converted to mmoles) of thiopurine exposure for subjects was determined by summing the total amount of thiopurines prescribed (grams) up to sample acquisition, divided by the molecular weight of each drug.

HPRT T cell cloning assay for determining somatic mutant frequency

The *HPRT* T cell cloning assay has been extensively used as a mutagenicity biomarker for determining the frequency and spectra of preexisting and exogenously induced somatic mutation events in peripheral human T lymphocytes (30,31). Briefly, since *HPRT* encodes a phosphoribosylation enzyme in the purine salvage pathway, analysis of *in vivo* mutation events is accomplished by isolating *HPRT* deficient T cells from peripheral blood through *in vitro* selection with 6-thioguanine (6-TG). This approach allows only cells with inactivating gene mutations to proliferate in the presence of 6-TG. The *HPRT* T cell cloning assay has a number of additional characteristics that has made it a valuable human mutation biomarker system. *HPRT* mutant T cells arise in a normal T cell milieu, uncomplicated by metabolic and genetic derangements inherent in diseased or malignant cells, allowing for the evaluation of mutagenic signatures related to exposure. Since the *HPRT* locus is located on the X- chromosome, a single mutation event can result in a selectable mutant phenotype, and is thus more sensitive than selectable autosomal gene loci. In addition, *HPRT* T cell mutant isolates can be expanded *in vitro* and used for a both mutation spectra and clonality analyses. The determination and statistical analysis for *in vivo* somatic *HPRT* mutant frequency (Mf) in peripheral T cells by cloning *HPRT* mutants following limiting dilution in the absence and presence of 6-TG have been previously described (32,33).

Molecular analysis of *HPRT* mutations and determination of mutant cell proliferation

The *HPRT* biomarker system also allows for the analysis of mutation spectra changes associated with exogenous/iatrogenic exposures, as well as the identification of independent *in vivo* sequential mutation events (34) and mutant clonal proliferations by simultaneously analyzing two independent clonality indicators, *HPRT* mutations and unique T cell receptor β (*TCR β*) CDR3 variable gene region sequences (35,36). Experimental approaches, methods and interpretations of mutation spectra, and comparison of *HPRT* mutations and *TCRβ* CDR3 variable gene regions of mutant isolates for determining mutant proliferation have been previously described (34-36). *HPRT* mutation spectra were determined by analyzing up to 10 mutants per TP treated subject and up to 5 mutants per non-TP treated subject. *HPRT* mutations were characterized by performing reverse transcriptase-PCR amplification (RT-PCR) from approximately 1×10^4 cells to identify mutations within coding regions, exon exclusions or intron inclusions. Single exon or multiplex genomic PCR of *HPRT* gene regions (37) was performed to identify splice mutations, deletions or insertions. *HPRT* mutants that showed exclusion of exons 2-3 in cDNA were screened for V(D)J recombinase-mediated deletions of *HPRT* exons 2-3 with specific primers spanning that region (38). PCR products were either separated by gel electrophoresis, extracted and purified via Qiagen's QIAquick gel extraction kit, or treated with 3.75 units of exonuclease I and 1.25 units of shrimp alkaline phosphatase, prior to DNA sequencing.

The characterization of the *TCRβ* CDR3 variable gene regions and *HPRT* mutations in mutant isolates provide two independent measures for clonal proliferation that can be followed longitudinally (34,35) (supplemental Figure 1). For *TCRβ* variable gene sequence analysis, RT-PCR generated cDNA was amplified using a primer to the *TCRβ* constant region and a mix of 26 *TCRβ* V region primers (39). The PCR products were then processed and sequenced as described above. The prevalence of *in vivo* clonal expansion was determined by analyzing 621 *HPRT* mutations and 611 *TCRβ* CDR3 variable gene region sequences from 114 subjects from whom two or more mutants were available (supplemental Figure 1; supplemental Table 2). The *in vivo* frequency of independent mutation events (MutFreq) was ascertained by

multiplying each subject's *HPRT* Mf by the ratio of independent mutation events to the total analyzed mutants.

Statistical Analysis

Because *HPRT* Mf has a lognormal distribution, all statistical analyses were based on its natural logarithm (lnMf), which has been shown to be related to age and non-selected cloning efficiency (CE) (32). We therefore adjusted for age and CE by including them as covariates in all analyses of lnMf (40). A summary of each subject's gender, age, duration of illness, type and treatment duration, total dose of thiopurine, CE, Mf, lnMf, and MutFreq is provided (supplemental Table 1). Analysis of covariance was used to compare differences in lnMf among controls, non-TP and TP treated subjects, as well as between children and adults with UC and CD. The Student-Newman-Keuls test was used to adjust for multiple pair-wise comparisons. In TP treated subjects, the effects of treatment duration and total dose on Mf and MutFreq were assessed by regression analyses. A Chi-square test was used to compare the percents of TP and non-TP treated subjects with mutant cell proliferation, and a Mantel-Haenzel test for linear association was used to assess the dose-dependence of increases in the percent of subjects with mutant cell expansions. Mutation spectra data were analyzed using logistic regression to assess the statistical significance of differences in the proportions of specific mutations among controls (supplemental Table 3), non-TP and TP treated subjects. Subjects under the age of 12 were excluded from mutation spectra analyses because differences in the age distributions of normal controls and IBD subjects precludes adjustment for age-related spectra changes in this group. Age group (12-18 or ≥19 years) and sex were included in the regression models as covariates. Although mutations from the same subjects may be correlated, we could not use a hierarchical regression model in these analyses because the spectra data for adult controls did not contain unique subject identifiers. The effects of treatment were determined by comparing data only from the TP and non-TP treated subjects. Hierarchical logistic regression models with age group (12-18 or ≥ 19 years), thiopurine treatment (yes or no) and treatment by age group interaction were fitted to determine if the effect of treatment differed in adults and children. Similar models were fitted using thiopurine treatment categories based on the total dose (0, 1-133, 134-289, 290-727 and ≥ 728 mmoles) to determine if the proportion of a particular class of mutation displayed a dose-response relationship. The non-zero categories correspond to dose quartiles for all TP treated subjects. All of these models included study subject as a random effect to account for potential correlation between mutations from the same individual.

Results

In vivo HPRT mutant frequency in subjects with IBD

Unadjusted *HPRT* Mf and lnMf data with respect to type of IBD and thiopurine treatment for the 119 children and adults studied as well as previously reported data utilized for controls (32,40,41) are summarized in Table 1. The effects of IBD and non-TP treatment on the *in vivo* background *HPRT* Mf were initially assessed by comparing lnMf in non-TP treated subjects to controls after adjustment for age and CE. A statistically higher lnMf was observed in one sub-group, non-TP treated children with UC ($p<0.05$), while lnMf in all the other non-TP treated IBD groups were not significantly elevated. Analysis of lnMf for the non-TP treated IBD group as a whole showed no statistical difference from controls, indicating that IBD itself and non-thiopurine interventions did not significantly affect the background *HPRT* Mf in non-TP treated IBD subjects.

For the 56 TP treated subjects, total thiopurine dose ranged between 3.8 and 2,379 mmoles taken over 0.04 to14.1 years. We observed a significantly higher mean lnMf in TP treated subjects compared to controls and non-TP treated subjects (Table 1, $p < 0.05$). Importantly our

sub-analysis revealed no significant difference in lnMf between TP treated IBD children or adults or between TP treated IBD subjects with UC or CD. Regression analysis revealed no significant increases in lnMf associated with duration of illness for non-TP treated subjects, Figure 2 (Panel A), (supplemental Table 1). In contrast, we observed a significant increase in lnMf in TP treated subjects associated with duration of illness ($p=0.004$), Figure 2 (Panel A), but of importance, this association was not significant after adjustment for either duration of treatment or total thiopurine dose. Specifically, the significant increases in lnMf in TP treated subjects were related to both duration of treatment ($p<0.001$), Figure 2 (Panel B)(supplemental Table 1) and total thiopurine dose ($p<0.001$), Figure 2 (Panel C), independent of duration of illness. The estimated rate of increase in lnMf in TP treated subjects was 0.776 per year of treatment, corresponding to a doubling in Mf. The estimated increase in lnMf with each 100 mmole increase in total dose was 0.467, corresponding to a 67% increase in Mf. The rates of increase in lnMf in TP treated subjects did not differ significantly between treated children and adults, or treated UC and CD subjects. The rates of increase were also not drug specific since there were no significant differences in lnMf among subjects treated with only azathioprine, 6-MP or both agents, Figure 2 (Panel B), (supplemental Table 1).

Prevalence of *in vivo* mutant cell clonal proliferation and frequency of independent somatic *HPRT* mutations (MutFreq)

To gain insight into the potential effects of *in vivo* mutant clonal expansion and selection for *HPRT* mutants on the treatment-specific increases in mutant frequencies (Mf), we investigated the relationship between the prevalence of clonal mutant proliferation and the calculated MutFreq in TP and non-TP treated IBD subjects.

We determined whether thiopurine treatment results in an increase in the prevalence of clonal proliferation of *HPRT* mutants by comparing 621 characterized *HPRT* mutations and 611 *TCRβ* regions from 876 *HPRT* mutants from 114 non-TP and TP treated IBD subjects (supplemental Table 2 and supplemental Figure 1). The percents of non-TP treated children and adults in which there were two or more *HPRT* mutant isolates associated with mutant clonal expansions were 0% and 14.6% respectively, Figure 3 (Panel A). In contrast, there were significant increases in the percents of TP treated children (72.2%) and adults (63.3%) with mutant clonal expansions compared to non-TP treated subjects ($p< 0.001$) Figure 3 (Panel A). There were also significant dose-dependent increases in the proportion of subjects with mutant clonal expansions ($p<0.001$), Figure 3 (Panel B).

The prevalence of *in vivo* mutant clonal expansion in these subjects was then used to correct for the possible contribution of *in vivo* mutant proliferation on the treatment-specific increases in lnMf to estimate the frequency of independent *in vivo* somatic mutation events (MutFreq). For TP treated subjects, the MutFreq was significantly lower than Mf ($p<0.001$) as a consequence of mutant clonal expansions associated with treatment. But of importance, we also observed significant increases in MutFreq in TP treated subjects as seen with Mf which correlated specifically with treatment duration and total dose ($p<0.001$) Figure 2 (Panel C), (supplemental Table 1). This strongly suggests that the significant increases in MutFreq following thiopurine treatment are not the result of selective *in vivo* expansion of preexisting *HPRT* mutants.

Mutation spectra analysis

The *in vivo* mutagenicity of thiopurine treatment was further examined by comparing 648 independent mutations from a composite of controls and 372 independent mutations from 105 IBD subjects 12 years and older (supplemental Tables 2 and 3). Nineteen percent of the randomly selected *HPRT* mutants from IBD subjects remained uncharacterized, which is consistent with previous human biomonitoring *HPRT* spectra studies (36,42). Reasons for the

uncharacterized mutants include: 1) exon exclusions in mutants from female subjects in which splice alterations and deletions could not be confirmed or detected; 2) no cDNA detected, with genomic PCR products of exons 1 and 9 showing no mutations; 3) cDNA revealed no mutation, and 4) incomplete analysis.

We observed no significant differences in the spectra of mutations between non-TP treated subjects compared to controls, Figure 4 (Panel A), (supplemental Tables 2 and 3). This establishes that there is no detectable IBD disease or non-TP treatment specific differences in the proportions of mutations. In contrast, there was a significant increase in the proportion of all transitions in TP treated subjects (52.9%) compared to non-TP treated subjects (39.8%; $p=0.016$), and controls (34.4%; $p<0.001$) after adjustment for age and multiple observations per subject. In particular there was a significant increase in the proportion of G:C to A:T transitions at non-CpG dinucleotides in TP treated subjects (38.3%) compared to non-TP treated subjects (27.7%; $p=0.037$), and controls (20.7%; $p<0.001$), Figure 4 (Panel A), (supplemental Tables 2 and 3). The increase in transitions was also accompanied by a concomitant significant decrease in the proportion of all transversions among TP treated subjects (22.8%) compared to controls (34.6%), $p=0.021$.

We also observed a significant dose-dependent increase in the proportion of all transitions ($p=0.013$), in particular G:C to A:T transitions ($p=0.045$) at non-CpG dinucleotides after adjustment for age and multiple observations per subject, which supports our overall spectra findings (Figure 5; Supplemental Table 2). This increase in G:C to A:T transitions is consistent with the *in vitro* mutagenic signature observed for these drugs (4,20,22).

Thiopurine treatment also resulted in a distinct distribution of unique G:C to A:T transitions at non-CpG dinucleotides at both coding and non-coding/splice-site base pair positions in TP treated subjects compared to non-TP treated subjects (Supplemental Table 4, Figure 5). There was an increase in the occurrence of unique G:C to A:T transitions at non-CpG dinucleotides ($n=38$ compared to $n=32$) at both coding and non-coding sites and in the number of unique sites ($n=18$ compared to $n=12$) in TP treated subjects compared to non-TP treated subjects. There were 20 common sites (coding and non-coding) in which these transitions occurred in both non-TP treated and TP treated subjects. The most frequent mutation coding sites (≥ 3 events) for G:C to A:T transitions at non-CpG dinucleotides in non-TP treated subjects were at positions 538, 551, and 617. For TP subjects the most frequent coding sites were at positions 3, 197, 463, 485, 539, and 617. Of interest is that G:C to A:T transitions at non-CpG dinucleotides at positions 463 and 539 in TP treated subjects was not observed in non-TP treated subjects. Analogous findings were also observed at splice-site sequences. In addition, the distribution of these transitions at coding and non-coding regions is consistent in each treatment group and distribution subcategory. These data suggest that the distribution of G:C to A:T transitions at non-CpG dinucleotides in TP subjects represents both thiopurine-specific and preexisting mutations and not simply an increase in spontaneous or hotspot mutation events.

Discussion

Investigations attempting to link treatment-specific mutations to carcinogenic risk in humans are extraordinarily difficult because of the necessity to control for a multitude of complex variables including the genetic and cellular effects of the underlying disease(s), duration and total dose of each therapeutic intervention as well as other environmental exposures. Furthermore, tumors themselves are genomically unstable, making potential primary and secondary cancer-relevant mutations difficult to discern. With respect to this study, definitively linking thiopurine mutation induction in IBD subjects to the formation of specific tumors is currently not possible since data for cancer specific mutations in tumors from subjects who

only received thiopurine treatment does not exist. Hence, identifying the *in vivo* induction of treatment-specific mutations in non-transformed cells is an extremely important approach for elucidating potential carcinogenic mutagenic events directly in subjects receiving this treatment.

In this study we provide evidence that chronic thiopurine treatment results in a dose-dependent increase in the *in vivo* frequency of somatic mutations, in particular a treatment-specific increase in G:C to A:T transitions at non-CpG dinucleotides, which is consistent with the *in vitro* mutation signature of these drugs (4,22). These data strongly suggest that thiopurine intervention is mutagenic in humans.

These findings were ascertained by determining the frequency and spectra of somatic mutations at the *HPRT* locus in peripheral human T cells, and an examination of *in vivo* preexisting or induced mutant cell clonal proliferation by combining the analysis of two distinct clonality signatures (35). Since the mutation events observed were investigated in subjects receiving chronic thiopurine treatment, the possibility exists for the *in vivo* selection and enrichment of preexisting *HPRT* mutants with potential growth advantages that may influence the relative contribution of thiopurine therapy on the observed Mf. This effect is unlikely since we observed a significant dose-dependent increase in MutFreq, that have been corrected for both preexisting and treatment induced clonal expansion of mutant cells. The potential *in vivo* cytotoxic effects of thiopurines on proliferating non-*HPRT* mutants with the concomitant survival of *HPRT* mutants could also theoretically have influenced our results. This appears unlikely as well since we concurrently observed significant dose-dependent mutation spectra changes. The latter observations establish the *in vivo* mutagenicity of thiopurine intervention, since significant spectra changes would not occur if this treatment simply enriched for the expansion of preexisting *HPRT* mutants. It is also important to note that all of these findings were independent of the underlying disease, duration of disease, and other therapeutic interventions.

We were unable to specifically evaluate the effects of other potential environmental exposures because of limited data. The most important exogenous exposure likely to influence our observations would be environmental tobacco smoke (ETS), which has previously been shown to result in an increase in V(D)J recombinase mediated deletions in newborns (43), and no spectra changes or an increase in the proportion of G:C to T:A transversions in adults (44, 45). This is in contrast to the significant proportional decrease in transversions, and an increase in G:C to A:T transitions at non-CpG dinucleotides observed in TP treated subjects.

The induction of G:C to A:T transition mutations by thiopurine treatment is likely the result of incorporation of 6-thioguanine nucleotides into DNA and subsequent mispairing with thymine in replicating cells (4,20,22,25). Support for the carcinogenicity of a thiopurine specific mutagenic mechanism can be inferred from epidemiologic evidence correlating azathioprine treatment with significantly higher incidences of solid tumors, lymphoproliferative disorders, and skin cancers when compared to other immune modulators, including mycophenolate mofetil which closely resembles thiopurines in its mechanism of action (9,14,15,17,25,46). Mycophenolate mofetil primarily inhibits inosine monophosphate dehydrogenase resulting in the inhibition of purine synthesis, while azathioprine and 6-mercaptopurine exert their cytotoxicity by inhibiting purine synthesis and by incorporation into DNA. This suggests the increased cancer risk associated with thiopurine treatment is associated with the mutagenicity of these agents following DNA incorporation.

The significant increase in the prevalence of treatment-specific cell proliferation may represent an additional mechanism for accumulating G:T mismatches from unrepaired replication errors (47), as well as by enhancing incorporation of 6-thioguanine nucleotides during DNA replication. Clonal proliferation among mutants, with the associated failure to find similar

patterns among non-mutant cells, further implicates cell proliferation as a significant *in vivo* mutagenic mechanism.

Cellular resistance to thiopurines has been shown *in vitro* to select for cells defective in mismatch repair (MMR), while microsatellite instability has been observed in acute myelogenous leukemia from transplant patients following azathioprine treatment (8). It is not known whether our findings could also be attributed to the *in vivo* selection of MMR defective cells and the *in vivo* emergence of cells with genomic instability.

Our data indicate that thiopurine treatment could potentially lead to somatic mutations at disease-specific loci in actively replicating tissue cell populations including: 1) the bone marrow, which has been shown to incorporate higher levels of 6-thioguanine nucleotides (48), and 2) activated immune cells and epithelial tissues undergoing repeated cycles of repair and cell regeneration, especially in subjects with IBD and chronic graft versus host disease (29,49). Lymphocytes and skin epithelium of patients who received thiopurine treatment have also been shown to incorporate 6-thioguanine nucleotides (24,50). In this study, we also observed an increase in G:C to A:T transitions at non-CpG dinucleotides in clonally proliferating activated T cells, some of which may have a recent precursor origin (data not shown). Altogether, these observations provide an important connection between the mutagenicity of thiopurine intervention in these cell populations and the increased risk of leukemia (8), lymphoma (7,12,13) and skin cancer (9,10,17).

In this cross-sectional study, we provide evidence that suggest thiopurine intervention is mutagenic, resulting in a dose-dependent increase in the frequency of somatic mutations and treatment-specific mutation induction of G:C to A:T transitions at non-CpG dinucleotides. These findings provide direct *in vivo* evidence for mutation induction as a potential carcinogenic mechanism associated with chronic thiopurine intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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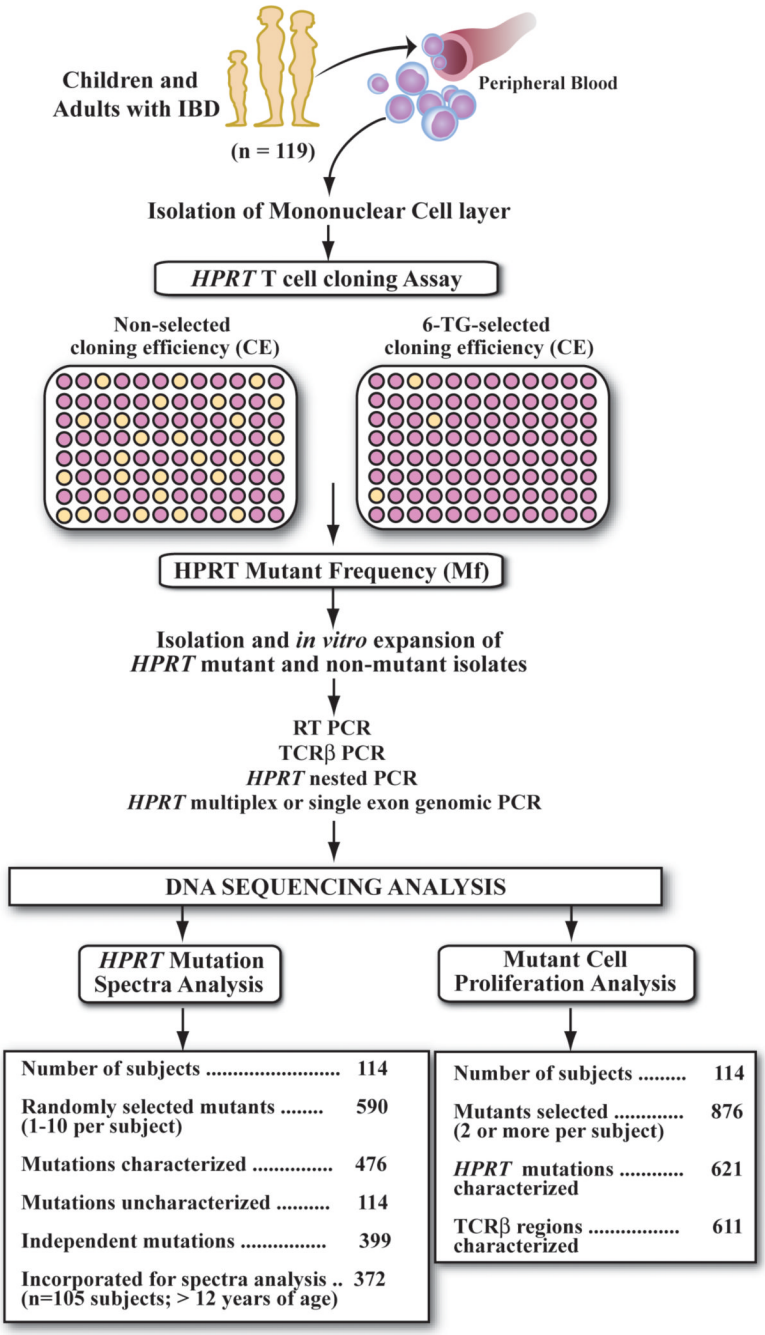

Figure 1. Research overview for investigating *in vivo* mutagenicity and mutant cell clonal expansion in subjects treated with thiopurines for IBD

Mononuclear cells from peripheral blood are plated at limiting dilutions in the absence (non-selection) and higher density in the presence of 6-thioguanine (selection). *HPRT* Mf is determined by calculating the ratio of mean cloning efficiency (CE) of selected mutant isolates over mean non-selected CE of T cells in the absence of selection. Both *HPRT* mutations and rearranged *TCRβ* CDR3 gene regions are utilized to determine *in vivo* mutant cell expansion, while only unique *HPRT* mutations are utilized to determine mutation frequency (MutFreq) and mutation spectra changes.

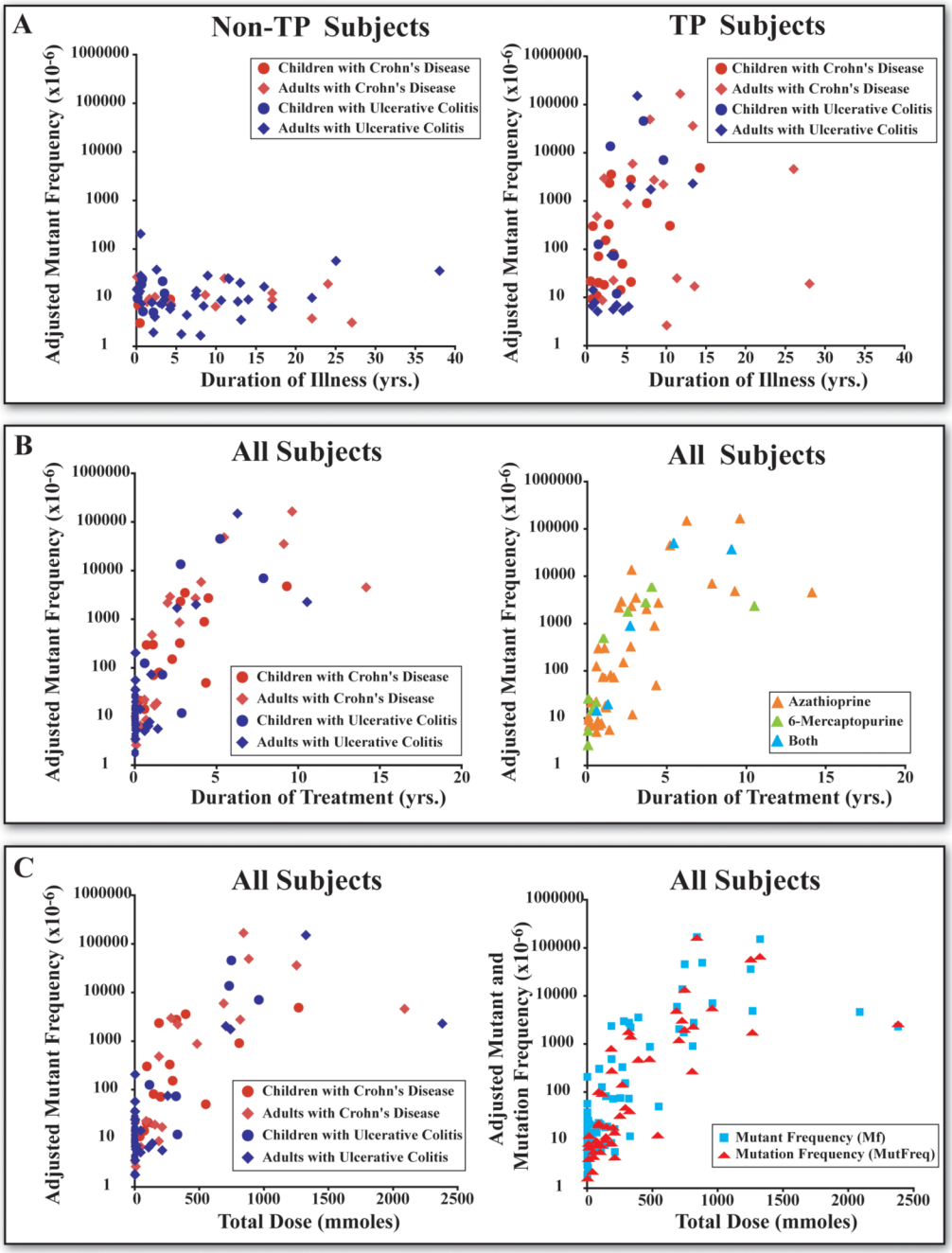

Figure 2. Comparative analysis of adjusted *HPRT* mutant frequency (Mf) with respect to duration of illness, duration of treatment, and total dose of thiopurines

Mf values have been adjusted for the effects of age and CE by normalizing to the average age and CE for each subject (35). **Panel A)** Relationships between adjusted Mf and duration of illness in non-TP and TP treated subjects. **Panel B)** Relationships between adjusted Mf and duration of thiopurine treatment in IBD subjects, as well as the relationships between adjusted Mf and treatment duration of different thiopurine interventions. **Panel C)** Relationships between adjusted Mf and MutFreq and total thiopurine dose in IBD subjects.

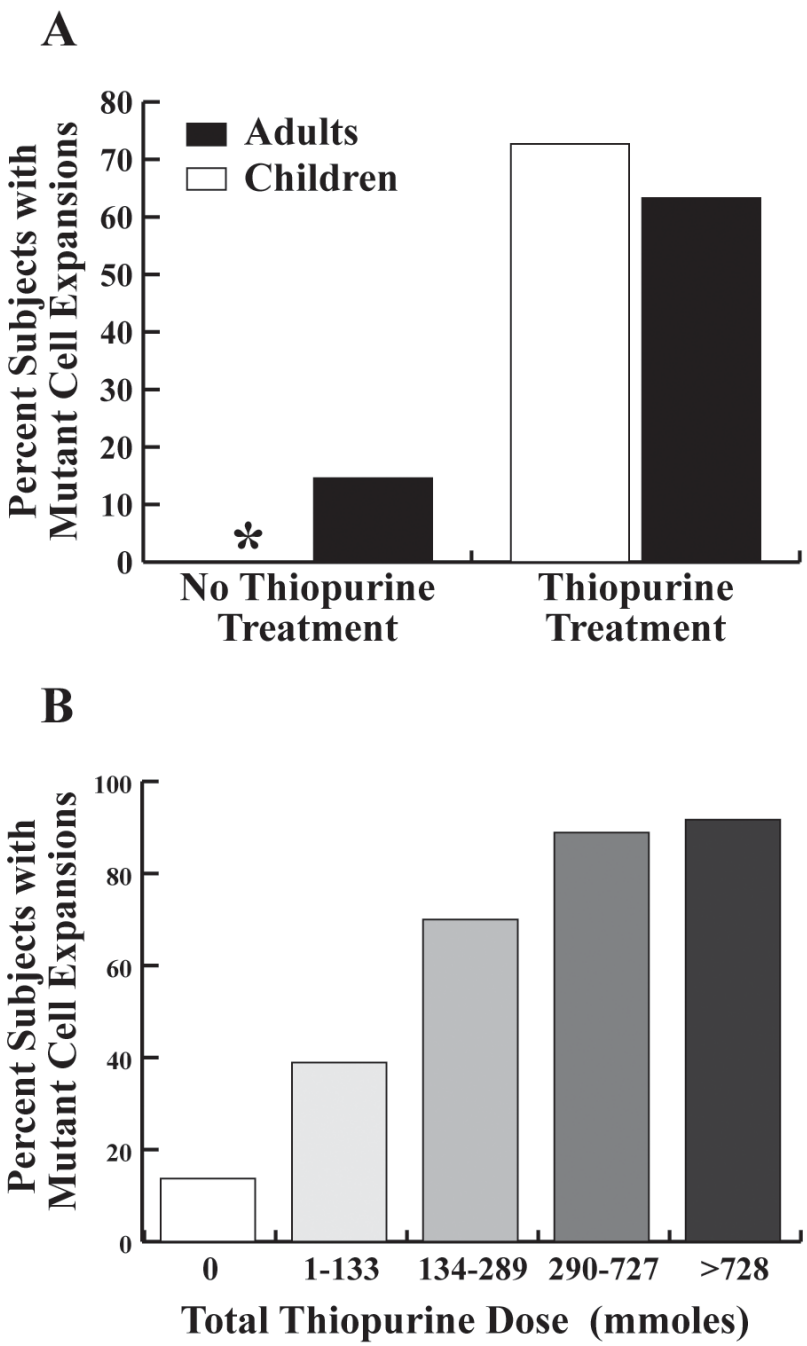

Figure 3. Prevalence of *in vivo* mutant cell proliferation in TP and non-TP IBD treated subjects
Panel A) The proportion of subjects with evidence of *in vivo* expansion of mutant cells in TP and non-TP treated subjects. (*) 0% of non-TP treated children had evidence of mutant cell expansions. **Panel B)** The proportion of subjects with evidence of *in vivo* mutant cell expansion compared to total thiopurine dose.

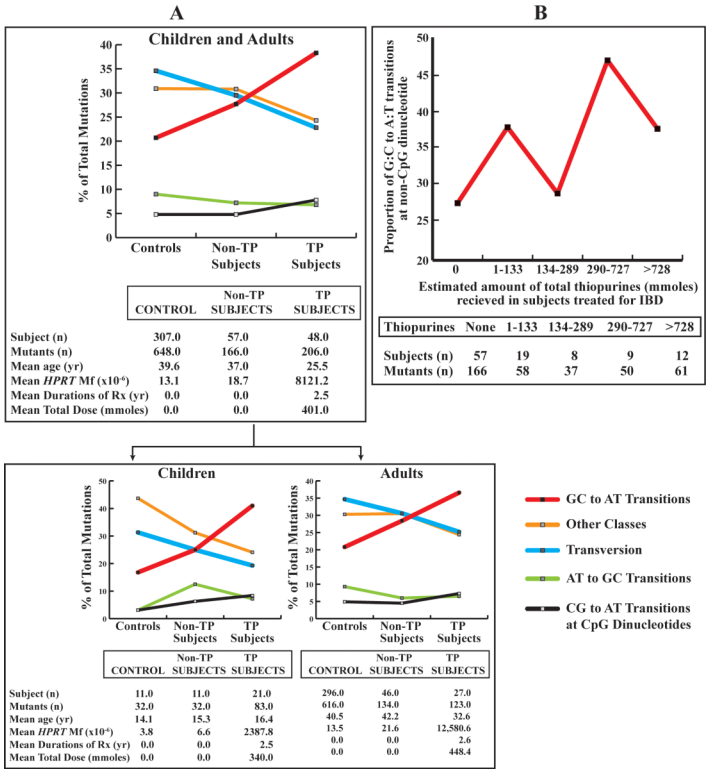

Figure 4. Mutation spectra in Non-TP and TP treated IBD subjects (≥ 12 years of age) and normal controls

Panel A) Mutation spectra distribution in non-TP and TP treated children and adults combined and separate compared to normal controls. The "Other Classes" of mutations include: exon deletions, micro-deletions, nucleotide duplications or losses, insertions, and complex insertion/deletion mutations. **Panel B)** The relationship between the proportion of G:C to A:T transitions at non-CpG dinucleotides and total dose of thiopurine treatment.

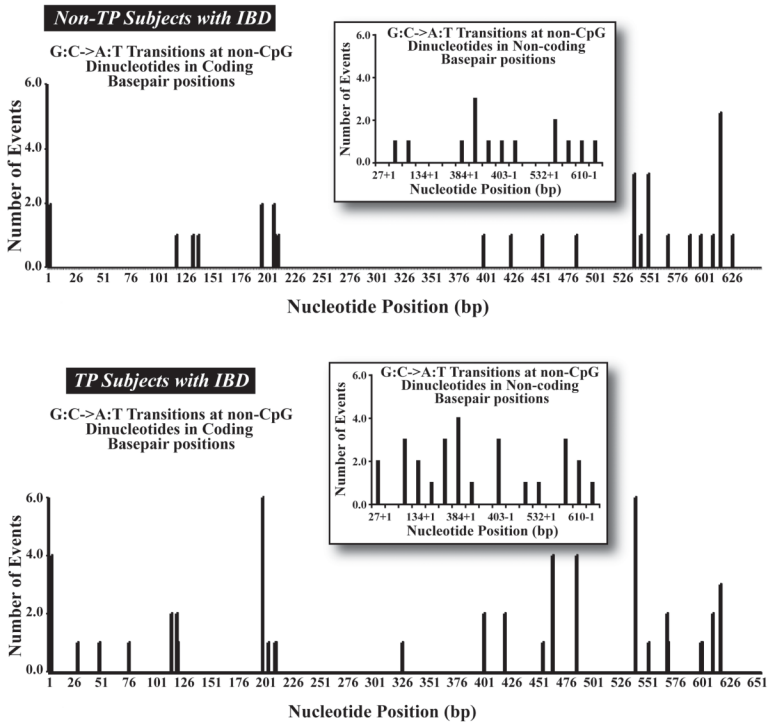

Figure 5. Distribution and frequency of G:C to A:T transitions at non-CpG dinucleotides mutations in TP and non-TP treated subjects at both coding and non-coding (splice-site) base pair positions in the *HPRT* locus

Table 1

Duration of illness, total dose and frequency of *HPRT* T cell mutants in subjects with IBD

Subjects	Number	Age (yrs)*	Duration of Illness (yrs)*	Total thiopurine dose (mmoles)*	Mf^{+} (x 10^{-6})*	$lnMf^{+}$ (x 10^{-6})*
Normal Controls	**280**					
Children	**49**	**5.7** (4.6)			**2.3** (2.2)	**0.4** (1.0)
Adults	**231**	**52.8** (16.0)			**13.7** (9.0)	**2.4** (0.7)
No Thiopurine Treatment						
Ulcerative colitis	**46**					
Children	**9**	**14.4** (2.1)	**1.3** (1.3)	**0.0**	**6.8** (4.2)	**1.7** (0.7)
Adults	**37**	**39.7** (15.9)	**7.8** (8.2)	**0.0**	**23.7** (38.3)	**2.5** (1.1)
Crohn's Disease	**17**					
Children	**4**	**15.8** (1.2)	**1.2** (2/0)	**0.0**	**3.9** (1.4)	**1.3** (0.4)
Adults	**13**	**42.9** (10.4)	**11.2** (9.3)	**0.0**	**12.6** (7.3)	**2.4** (0.5)
Thiopurine Treatment						
Ulcerative colitis	**20**					
Children	**7**	**14.9** (4.4)	**4.1** (3.1)	**458.1** (354.9)	**6340.4** (12287)	**5.5** (3.7)
Adults	**13**	**30.7** (11.1)	**4.3** (3.5)	**459.7** (697.8)	**6246.6** (20612.6)	**4.0** (3.3)
Crohn's Disease	**36**					
Children	**19**	**15.0** (3.3)	**3.9** (3.6)	**264.4** (314.7)	**368.2** (617.2)	**4.1** (2.1)
Adults	**17**	**33.6** (13.4)	**9.4** (7.9)	**501.2** (548.7)	**15768.2** (39664.8)	**6.0** (3.6)

*Mean (standard deviation)

+Mf and lnMf data summarized here has not been adjusted for CE and age. Mean lnMf is computed from the individual log-transformed Mf value