

*O*⁶-Alkylguanine-DNA Alkyltransferase in Cutaneous T-Cell Lymphoma: Implications for Treatment with Alkylating Agents¹

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ABSTRACT

Mycosis fungoides is a low-grade cutaneous T-cell lymphoma. Early treatment often involves the use of topical chemotherapy such as mechlorethamine or carmustine although single-agent oral chemotherapy with alkylators is common for advanced disease. Recently, in a Phase I study of the new alkylating agent temozolomide, two mycosis fungoides patients experienced a complete response. The mechanism of resistance to alkylating drugs such as temozolomide is thought to be due to the presence in tumor cells of the DNA repair protein, *O*⁶-alkylguanine-DNA alkyltransferase (AGT). The protein mediates a reaction with the *O*⁶-position of guanine in DNA, removing the lesion and leaving guanine intact. We, therefore, examined the levels of AGT in CD4+ T lymphocytes obtained by negative antibody selection from the blood of noncancerous individuals and mycosis fungoides patients, and in paraffin-embedded sections from mycosis fungoides patch, plaque, or tumor lesions and cells from involved lymph nodes. AGT protein levels were measured by quantitative immunofluorescence microscopy using a monoclonal antibody against human AGT. Using this approach, the mean level of our positive control (AGT-expressing cells) was 84,807 molecules/nucleus; values below 5,000 molecules/nucleus are considered very low. The mean AGT level in CD4+ T lymphocytes from noncancer-

ous and cancerous individuals was 18,618 (*n* = 12) and 8,593 (*n* = 5), respectively. The mean fraction of outliers in circulating CD4+ T lymphocytes from mycosis fungoides patients was statistically significantly lower than T cells in lymph nodes. AGT molecules/nucleus from lymph node biopsies from 8 of 10 patients showed low (<10,000 molecules/nucleus) or undetectable levels (*n* = 5) of AGT. The mean AGT level from samples of mycosis fungoides patch/plaque and tumor was also low at 221 (*n* = 4) and 2,363 (*n* = 6), respectively. Surprisingly, Hut78, a mycosis fungoides T-cell lymphoma cell line, was positive for AGT activity (median: 77,700 molecules/nucleus), and Hut102—another mycosis fungoides cell line—was low (median: 5,990 molecules/nucleus). Because AGT is a primary means of cell resistance to alkylating agents, the low level of AGT in neoplastic T lymphocytes from patients with mycosis fungoides suggests that treatment with alkylating agents producing *O*⁶-alkylguanine adducts, such as carmustine or temozolomide, may produce improved clinical outcomes.

INTRODUCTION

Mycosis fungoides is an uncommon form of lymphoma that is characterized by epidermotropic infiltrates of clonal malignant T lymphocytes. Although the disease always involves the skin, minimal patch/plaque involvement may be followed by the development of cutaneous tumors or spread to visceral organs and lymph nodes (1). The Sezary syndrome is an erythrodermic variant associated with a leukemic phase of the disease. The cell of origin in mycosis fungoides and Sezary syndrome has been characterized as a mature helper T lymphocyte (CD4+); however, the etiology and pathogenesis remains unclear (1–3). The nuclei of these cells are hyperconvoluted, and often the malignant cells do not express some common mature T-lymphocyte antigens, such as CD2-, CD5-, or CD7- (antigen deletion). In large cohorts of patients with long-term follow-up, the median survival after a diagnosis of mycosis fungoides is almost 10 years (4). However, for patients who present with tumor-stage disease, the Sezary syndrome or extracutaneous spread, the prognosis is more dismal.

For patients thought to have primarily cutaneous involvement, therapies have included electron beam irradiation to the skin (5–7), topical chemotherapeutics (8, 9), or PUVA³ [psoralens (a family of photoactivated compounds) combined with UV-A irradiation; Refs. 10–14]. In patients with nodal involve-

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³ The abbreviations used are: PUVA, psoralen and UV A light; BCNU, carmustine [name for 1,3-bis(2-chloroethyl)-3-nitrosourea]; AGT, *O*⁶-methylguanine-DNA methyltransferase; PMA, phorbol 12-myristate 13-acetate; PBMC, peripheral blood mononuclear cell.

ment, total nodal irradiation may be given. Systemic chemotherapy has been reserved mostly for patients with advanced stages of the disease. Complete response rates in early stages are high, typically 60–80%, but late stages rarely achieve a complete response (15).

Active single agents include alkylating agents such as BCNU (16, 17) and more recently, temozolomide (18, 19). The Phase I study of temozolomide included two patients with mycosis fungoides, and both patients had a complete response (19). BCNU has been used topically and complete response rates range from 30 to 60%, depending on the initial extent of disease (16). The duration of BCNU therapy is limited by the potential for hematological complications.

The DNA repair protein, O^6 -alkylguanine-DNA alkyltransferase (AGT, EC 2.1.1.63) has been implicated in the recognition and repair of alkylator-induced DNA damage introduced by chloroethylnitrosoureas (*i.e.*, BCNU) and methylating agents (*i.e.*, temozolomide; Refs. 20, 21). The presence of the AGT protein imparts resistance by removing toxic lesions formed at the O^6 -position of guanine (20). Chloroethylnitrosourea cross-links are prevented from forming either by the removal of the chloroethyl lesion from the O^6 -position before rearrangement, or by the reaction with the intermediate, 1, O^6 -ethanoguanine to form a cross-link between DNA and the repair protein (22, 23). The AGT protein is inactivated in the repair process. Several preclinical studies in tumor cells and in tumor xenograft studies suggest that the AGT concentration inversely correlates with sensitivity to alkylating agents (21, 22, 24, 25).

Studies evaluating AGT levels in patients receiving BCNU therapy support the role of AGT in resistance to chloroethylating and methylating agents. Both retrospective and prospective human studies have demonstrated a correlation between AGT concentration and clinical outcome after treatment with BCNU (26, 27). After an evaluation of 226 high-grade astrocytoma patients receiving BCNU therapy, Belanich *et al.*, (26) reported that low AGT content in tumors correlates with a better response to treatment and a greater chance for survival. Conversely, the presence of a subpopulation of cells in a tumor with elevated AGT is correlated with a poor prognosis. More recently, Jaeckle *et al.* (27) demonstrated that the AGT level in tumor tissue specimens may be a predictive marker of survival in patients with malignant astrocytoma that is independent of other previously described prognostic variables. The study was designed with adequate power to detect a tripling of survival hazards ratio for patients with high *versus* low AGT levels. Approximately 27% of human gliomas are low in AGT (28, 29).

There are examples of primary tumors with undetectable AGT activity, albeit at a very low frequency (20). This may explain, at least in part, the limited usefulness of BCNU and other alkylnitrosoureas in the effective treatment of cancer. Because BCNU and temozolomide have demonstrated activity in mycosis fungoides, we examined the AGT levels in CD4+ T lymphocytes from peripheral blood, sections from paraffin blocks of patch, plaque, and tumor stage mycosis fungoides, and involved lymph nodes, using a monoclonal antibody against AGT.

MATERIALS AND METHODS

Materials. Histopaque 1077 and Ionomycin were purchased from Sigma Chemical Co. (St. Louis, MO). PMA was bought from Life Technologies, Inc.. Advanced magnetic beads are from PerSeptive Biosystems (Framingham, MA). All of the antibodies except 3B8 are available through American Type Tissue Collection (Manassas, VA).

Patient Samples and Lymph Node Staging. Whole blood samples (40 ml) were collected in sodium-heparinized vacutainer tubes from noncancerous volunteers and mycosis fungoides patients. In addition to fresh samples, sections of paraffin-embedded tissue blocks from archived samples were also evaluated. The diagnoses were verified by pathological examination. Lymph node staging was done according to the National Cancer Institute lymph node classification system (30).

PBMC Isolation. Immediately after the collection of blood (40 ml), an equal volume of RPMI was added, and the diluted blood was layered on Ficoll-Paque (Histopaque 1077). After centrifugation at $400 \times g$ for 30 min at room temperature, the lymphocyte layer was removed and resuspended in 15 ml PBS and centrifuged at $250 \times g$ for 10 min at room temperature. Final pellets were resuspended in bead separation medium (HBSS with Ca^{2+} or Mg^{2+} , 4% human serum albumin, penicillin/streptomycin, and 20 mM HEPES).

Isolation of CD4+ T Lymphocytes from PBMCs. CD4+ T lymphocytes were isolated from PBMCs by negative selection using a cocktail of monoclonal antibodies to extract unwanted cells with goat antimouse IgG-coated magnetic beads and selection on a rare earth magnet (31). Briefly, to select for CD4+ T lymphocytes, total PBMCs were resuspended in bead separation medium (HBSS with Ca^{2+} or Mg^{2+} , 4% human serum albumin, penicillin/streptomycin, and 20 mM HEPES) and centrifuged, and pellets were resuspended in 50 ml of bead separation medium three times to remove platelets. Cell pellets were mixed with a cocktail of monoclonal antibodies specific for the following antigens: CD19, HLA class II, CD16, CD14, CD11b, and CD8, glycophorin. After a 1-h incubation at 4°C (20×10^6 cells/ml) on a rotator, the cells were then washed three times to remove free antibodies. Cells were then incubated with Advanced magnetic beads that had been precoated with goat antimouse IgG and rotated for 1 h at 4°C. The beads were washed before use with bead separation media. The cells coated with magnetic beads (*i.e.*, all of the PBMCs that were not CD4+ T lymphocytes) were removed by placing the cell suspension on a rare earth magnet three times for 5–10 min each time. The cells not pulled out of suspension were washed once with bead separation media. More than 95% were CD4+ as measured by flow cytometry (flow-activated cell sorter).

Additional Selection of Tumor Cells. In most cases, antigen deletion occurred (*e.g.*, CD2–, CD5–, or CD7–). Antigen-deleted CD4+ T lymphocytes were identified by flow cytometry using a fluorescein-labeled CD2, CD5, or CD7 monoclonal antibody. After antibody labeling, cells were fixed with 4% paraformaldehyde and were sorted based on fluorescence to obtain pure populations. Cells were then cytospun on polylysine-treated plates for immunohistochemistry of AGT protein.

Table 1 Characteristics of individuals at time of specimen acquisition

| | |
|------------------------------------|------------|
| Mycosis fungoides patients | |
| No. sample analyzed | 27 |
| No. unique patients | 26 |
| Male | 15 |
| Female | 11 |
| Median Age (range) | 68 (24–83) |
| Site specimen obtained from | |
| Skin patch | 3 |
| Skin plaque | 3 |
| Skin tumor | 5 |
| Lymph node (LN-2–4) | 10 |
| Sezary cells | 6 |
| Stage at specimen acquisition | |
| IA | 1 |
| IB | 4 |
| IIB | 2 |
| III | 5 |
| IVA | 15 |
| Median no. prior therapies (range) | |
| Topical | 1 (0–4) |
| Systemic | 2 (0–9) |
| Reactive Dermatitis Controls | |
| Specimen/Patient No. | 4 |
| Male | 4 |
| Female | 0 |
| Median Age (range) | 59 (39–79) |
| Normal Controls | |
| Specimen/Patient No. | 13 |

Determination of AGT Protein Levels by Quantitative Immunofluorescence.

All of the histological and cytological slide preparations were stored frozen until processed for quantitative immunofluorescence analysis. Paraffin-embedded tissue sections were stored at room temperature. Thawed tissue sections or cells were fixed with 4% paraformaldehyde/PBS at 25°C for 10 min, permeabilized with 0.1% Triton X-100/PBS, and blocked with 5% nonfat milk/PBS. Slides were then incubated for 1 h at room temperature with primary antibody (34 µg/ml Mab 3B8 in block), washed with 0.1% Triton X-100/PBS, incubated for 1 h with FITC-conjugated goat antimouse antibody (Sigma, St. Louis, MO) in block, and washed with 0.1% Triton X-100/PBS. DAPI was added at 0.5 µg/ml for 3 min followed by three washes in deionized water. Slides were mounted in 50% glycerol with Slowfade antifade agent (Molecular Probes, Eugene OR). Image acquisition was described previously (26). Data collection was restricted to infiltrating T cells identified by nuclear morphology. ANOVA was used to compare different groups for statistical significance.

RESULTS

Patient Characteristics. Patient and control samples for this study were collected from two institutions, the University of Chicago ($n = 30$) and Northwestern University Medical School ($n = 14$). See Table 1 for details on the patients and controls used for the experiments reported. The specimens of mycosis fungoides analyzed represented all of the sites and types of disease commonly encountered, namely skin patch, plaque, and tumor disease, as well as advanced nodal disease and circulating Sezary cells. To assure adequate neoplastic cell sampling, spec-

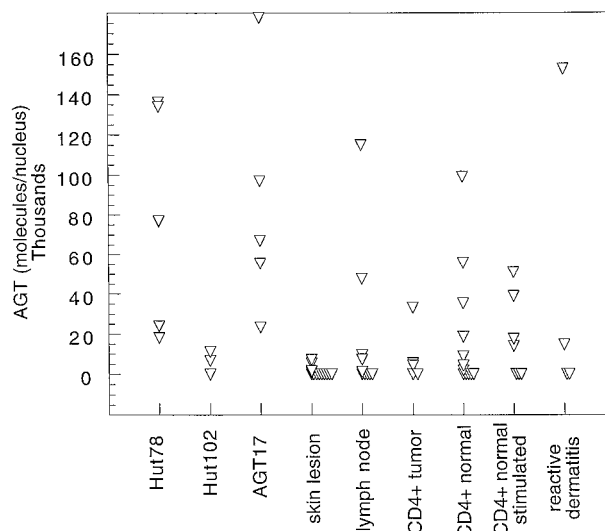


Fig. 1 AGT (molecules/nucleus) of individual samples and cell lines. The average AGT level in each tissue sample is plotted as a single open triangle, separated into tissue groups. Cell lines: Hut78, Hut 102, AGT17. Mycosis fungoides patient samples: skin lesions (patch, plaque, and tumor), lymph node samples (stage 2–4), circulating CD4+ tumor cells. Noncancerous individuals: CD4+ normal, CD4+ normal stimulated with PHA/ionomycin, and reactive dermatitis.

imens with significant tumor burden were preferable, and indeed, approximately 80% of samples came from patients with advanced stage of disease (stage IIB, III, or IVA). The median age of patients, 68 years, was typical for this disease. Patients were not heavily pretreated before study sample acquisition. The median number of topical treatments before entry was one, and the median number of systemic prior therapies was only two. The most common prior systemic therapies were oral corticosteroids, and IFN- α . For patients with prior chemotherapy exposure, single-agent alkylators and antimetabolites were most common. Samples of reactive dermatoses were obtained from University of Chicago exclusively and were confirmed by their clinical dermatologists not to represent nonspecific lesions that could evolve into mycosis fungoides.

Levels of AGT. The AGT levels of samples were measured by quantitative immunofluorescence microscopy using a monoclonal antibody against human AGT (Fig. 1). Quantitative immunofluorescence using this antibody has been shown to correlate with AGT activity as measured by the standard biochemical analysis using a [3 H]methylated DNA substrate (32). AGT levels in tumors were reported as the mean \pm SE and median AGT level in cell nuclei and as the fraction of outliers, which measures the fraction of cells with high levels of nuclear AGT (Table 2). AGT17, a cell line produced by transfection of the AGT-deficient astrocytoma cell line A1235 with human AGT gene and expressing high AGT levels, was used as a positive control (84,807 \pm 26,184 molecules/nucleus). Pure populations of CD4+ T lymphocytes were isolated from blood samples collected from noncancerous individuals and mycosis fungoides patients using negative antibody selection. Flow cytometry indicated the purity of CD4+ cells in the range of 92–98% for all of the samples. A set of CD4+ T lymphocytes

Table 2 AGT (molecules/nucleus) of cell lines, paraffin-embedded dermatitis and MF^a patient material, and CD4+ cells from noncancerous and cancerous MF patients

| Origin of material | Sample type | No. of samples | Molecules/nucleus | | | % outliers | | |
|-------------------------------|---------------------|----------------|-------------------|--------|--------|------------------|-----|------------------|
| | | | Mean | SE | Median | Mean | SEM | Median |
| MF patients | Skin lesions | 11 | 1,389 | 756 | 0 | 1.7 ^b | 1.2 | 0 |
| | Patch/Plaque | 5 | 221 | 221 | 0 | 0.7 | 0.7 | 0 |
| | Tumor | 6 | 2,363 | 1,283 | 811 | 2.6 | 2.2 | 0.3 |
| | Lymph nodes | 10 | 18,094 | 11,728 | 661 | 7.8 ^b | 3.8 | 2.1 |
| | LN-2 | 2 | 4,793 | 4,793 | 4,793 | 2.0 | 0.1 | 2.0 |
| | LN-3 | 5 | 24,483 | 22,689 | 0 | 8.8 | 7.0 | 2.0 |
| | LN-4 | 3 | 16,312 | 15,656 | 1,321 | 9.8 | 6.4 | 5.0 |
| Antigen-positive ^c | CD4+ | 4 | 0 | 0 | 0 | 2.8 | 1.4 | 2.6 |
| Antigen-deleted ^c | CD4+ | 5 | 8,593 | 6,233 | 4,273 | 2.2 ^b | 1.1 | 1.2 ^b |
| Healthy individuals | | | | | | | | |
| Unstimulated | CD4+ | 12 | 18,618 | 8,895 | 3,069 | 5.5 | 2.5 | 1.4 |
| Stimulated | CD4+ | 7 | 17,298 | 7,718 | 13,839 | 3.5 | 1.1 | 3.3 |
| | Reactive dermatitis | 5 | 33,426 | 29,950 | 0 | 3.7 | 2.7 | 1.5 |
| Cell lines | | | | | | | | |
| | Hut78 | 6 | 77,700 | 20,846 | 76,900 | 6.5 | 1.8 | 7.4 |
| | Hut102 | 3 | 5,990 | 3,260 | 6,758 | 2.7 | 0.7 | 3.1 |
| | AGT17 | 4 | 84,807 | 26,184 | 66,977 | 7.8 | 1.6 | 6.5 |
| | Keratinocytes | 1 | 125,124 | | | | | |

^a MF, mycosis fungoides.

^b Significant difference at $P < 0.05$; nonparametric ANOVA of outliers among lymph nodes, skin lesions, and MF CD4+ T cells.

^c CD4+ cells from MF patient samples were separated into CD7 or CD2 antigen-deleted (depending on the immunophenotype) and antigen-positive.

from the same group of noncancerous individuals were activated with PMA/ionomycin because activated CD4+ T lymphocytes may more closely resemble CD4+ T lymphocytes found in tissue. However, activation with PMA/ionomycin did not significantly alter levels, which suggests that AGT levels are not activation-dependent. There was a large interindividual variation but no statistical difference among the three groups: CD4+ T lymphocytes, $18,618 \pm 8,895$ molecules/nucleus; stimulated CD4+ T lymphocytes, $17,298 \pm 7,718$ molecules/nucleus; CD4+ T lymphocytes from mycosis fungoides patients, $8,593 \pm 6,233$ molecules/nucleus (ANOVA, $P = 0.77$). Further selection of antigen-deleted malignant cells used a fluorescein-labeled antibody and cell sorting revealed a mean of $8,593 \pm 6,233$ molecules/nucleus. Interestingly, the mean levels were quite low relative to other tumors for which BCNU is considered front-line chemotherapy. For example, using this same technique in measuring AGT in 167 primary brain tumors, we found that the mean number of molecules/nucleus was $132,000 \pm 15,000$, and the mean percent of outliers for all of the tumors was 19 ± 1 (26).

We extended our studies to evaluate AGT levels in mycosis fungoides patch, plaque, and tumor samples as well as in lymph node samples that were staged according to the National Cancer Institute lymph node classification system. AGT was virtually undetectable in T cells in patch/plaque stage disease, and AGT in skin tumor biopsies measured less than 7,200 molecules/nucleus. On the other hand, AGT in lymph node biopsies averaged over 18,000 molecules/nucleus, and this value was statistically significantly higher than AGT in skin lesions ($P < 0.05$ by ANOVA; Table 2).

Reactive dermatitis samples from patients without mycosis fungoides were evaluated as a model of noncancerous inflammatory skin disease. Four of five samples had no detectable

AGT activity, yet one had a value of 153,000 molecules/nucleus.

Hut78 and Hut102 are CD4+ T-cell lymphocytes derived from peripheral blood of patients with mycosis fungoides. Surprisingly, Hut78 was positive for AGT activity ($77,700 \pm 20,846$ molecules/nucleus). In contrast, Hut102, a mycosis fungoides line) had low activity ($5,990 \pm 3,260$ molecules/nucleus) similar to levels measured from our patients with mycosis fungoides or Sezary syndrome. Hut 102 cells are HTLV-I positive and Hut78 are HTLV-I negative. The level of AGT in keratinocytes was 125,124 molecules/nucleus.

DISCUSSION

The aim of this study was to evaluate the cellular capacity of neoplastic T lymphocytes to repair lesions induced at the O⁶-position of guanine in mycosis fungoides patients. Our results indicate that a significant portion of patients have low to undetectable levels of AGT in patch/plaque, tumor, lymph node, and circulating CD4+ malignant cells. This is the only malignancy reported to date in which a significant portion of patients have low to undetectable AGT protein levels. Although it is well known that the level of AGT differs among tissue types and there is wide interindividual variation in the levels of AGT protein, the vast majority of primary tumors have moderate-to-high AGT levels (20). Two recent reports demonstrate a correlation between low tumor AGT as measured by quantitative immunohistochemistry and survival of malignant brain tumor patients treated with BCNU (26, 27). Therefore, AGT level in tumor tissue may be a predictive marker of survival in patients treated with alkylating agents. Our results encourage the use of alkylating agents such as BCNU and temozolomide in the treatment of mycosis fungoides.

The importance of AGT in determining responses to chemotherapy is clearly demonstrated (20, 33); however, how these levels are regulated in human cells is not well understood (34). Whereas the vast majority of normal human tissues and cultured cell lines constitutively express AGT, a subset of tumor cell lines seem to be totally AGT-deficient. Because AGT varies according to cell type and tissue, mechanisms must exist to strictly regulate these levels in AGT-expressing cells and to suppress AGT in certain lines. The *AGT* gene is present in cells reported to have undetectable AGT activity (as measured by a biochemical assay) with no gross rearrangements or deletions; however, both the protein and mRNA levels are essentially undetectable.

Studies by Wani and D'Ambrosio (35) indicate that in skin dermis, 93% of the fibroblasts express high levels of AGT mRNA. Within the epidermis, approximately 64% and 65% of the basal, and granular epithelial cells, respectively, express AGT mRNA. This is confirmed by our measurement of AGT in actively dividing (basal-like) keratinocytes of 125,000 molecules/nucleus. Expression of AGT in the epithelial cells of the suprabasal layer of the epidermis was undetectable. The skin infiltrates of mycosis fungoides localize to the upper dermis and display epidermotropism. Although intraepidermal lymphocytes can be seen in benign dermatosis (exocytosis), in general, benign infiltrates are located in the dermis. A more thorough investigation by Tong *et al.*, (36) indicated a difference in the expression of AGT as a function of cell type and level of cellular differentiation. In normal human skin, fibroblasts consistently express high levels of AGT, whereas epithelial cells in the suprabasal cell layer of the epidermis express low-to-undetectable levels of AGT. The skin has a strong gradient of differentiation that appears to include suppression of AGT expression. Therefore, malignant T lymphocytes that migrate to the skin may also down-regulate their AGT in response to paracrine secretion of cytokines. There may be a factor in the epithelial cells of the suprabasal layer of epidermis that suppresses expression of AGT. On the other hand, in our study AGT in lymph nodes was significantly higher on average than in skin. This may explain why patients with early stage skin disease respond well to topical administration of alkylating agents but later regress because of the reservoir of resistant T cells in the lymph node. More aggressive therapy directed to these resistant lymph node cells or inactivation of AGT by pretreatment with *O*⁶-benzyl-guanine (33) may be needed.

A second possibility is that AGT expression is suppressed by a mechanism involving cytosine methylation in AGT promoter sequences or through posttranscriptional events affecting RNA processing, stability, or transport. Because loss of expression is rarely due to deletion, mutation, or rearrangement of the *AGT* gene, and methylation of discrete regions of the CpG island of AGT has been associated with the silencing of the gene in cell lines, methylation is likely to play a role. In cells in culture with undetectable AGT, the AGT promoter contains specific CpG methylation hot spots that are tightly linked to and are potential markers of gene silencing (37, 38). More recent data suggest that epigenetic inactivation of AGT by methylation of CpG sequences within the *AGT* gene plays an important role in primary human neoplasia (39–41). The differential expression of AGT in skin and T lymphocytes within the skin may indeed

be the result of differences in the methylation patterns in the promoter region of this gene. It is possible that the methylation status of T cells within the dermis is under the control of the surrounding skin cells.

In addition to AGT playing a role in resistance to chemotherapy, it is well known that this protein protects against carcinogenesis. Cell types low in AGT are more susceptible to damage by *O*⁶-alkylating carcinogens. It is conceivable that mycosis fungoides originates from carcinogenic exposure of skin-associated T lymphocytes to carcinogens that alkylate the *O*⁶-position of guanine in DNA. Point mutations arising in cells unable to repair the damage might ultimately lead to tumor initiation. It is known that one of the altered oncogenes in this disease is *ras* (42, 43), which may arise from persistent *O*⁶-methylguanine adducts (44, 45).

In summary, this is the first demonstration of a primary malignancy with low-to-undetectable AGT protein levels. We are currently evaluating temozolomide in a Phase II trial for patients with relapsed mycosis fungoides and Sezary syndrome.

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