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Application of mutagen sensitivity assay in a glioma case-control study

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ABSTRACT

Few risk factors for glioma have been identified other than ionizing radiation. The alkylating agent acrylamide is a compound found in both occupational and the general environment and identified as one of the forty known or suspected neurocarcinogens in animal models. The mutagen sensitivity assay (MSA) has been used to indirectly show reduced DNA repair capacity upon exposure to ionizing radiation in those with glioma compared to controls. In this study, MSA was used to assess its applicability to a glioma case-control study and to test the hypothesis that subjects with glioma may have lower DNA repair capacity after exposure to selected potential human neurocarcinogens (i.e. acrylamide), compared to controls. Approximately 50 case and 50 control subjects were identified from a clinic-based study that investigated environmental risk factors for glioma, who completed an exposure survey, and had frozen immortalized lymphocytes available. A total of 50 metaphase spreads were read and reported for each participant. The association of case-control status with MSA for acrylamide, i.e. breaks per spread, was examined by multivariable logistic regression models. The mean number of breaks per slide was similar between hospital-based controls and cases. In addition, case-control status or exposure categories were not associated with the number of breaks per spread. Although the MSA has been shown as a useful molecular epidemiology tool for identifying individuals at higher risk for cancer, our data do not support the hypothesis that glioma patients have reduced DNA repair capacity in response to exposure to acrylamide. Further research is needed before the MSA is utilized in large-scale epidemiological investigations of alkylating agents.

1. Introduction

Gliomas are the most common subgroup of malignant brain tumors [1]. However, scant information is available about the causes of these tumors apart from the known effects of ionizing radiation [2–4]. This study was designed to test the hypothesis that patients with glioma have a reduced capacity to repair DNA damage caused by exposure to selected alkylating neurocarcinogens.

As an alkylating agent, acrylamide is a compound that has been identified both in occupational exposure and in the general environment and has been identified as one of the forty known or suspected neurocarcinogens in animal models [5,6]. Exposure to alkylating agents with the potential to cause brain cancer may result from both endogenous and environmental exposures. An example for endogenous

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exposure includes formation of carcinogenic N-nitroso compounds in the stomach by the chemical reaction between certain nitrogen-containing compounds and nitrite under acidic conditions [7,8]. Bleomycin was used as a positive control to indicate DNA repair capacity. It is a known radiomimetic agent which creates free radicals that induce chromosomal breakage [9]. Bleomycin as the mutagen challenge and an alkylating animal neurocarcinogen, acrylamide are the focus of the current study.

In general, alkylating agents may attach a methyl group to DNA bases preventing DNA synthesis and RNA transcription, the formation of cross-links between bases, and the induction of mispaired bases resulting in permanent mutations [10]. If not repaired, adducts created via exposure to alkylating agents can directly result in changes or mutations in the DNA sequence. The neurocarcinogenicity of acrylamide has been suggested in two studies of rats, but was inconclusive in a third [11–14]. Epidemiological studies of the possible relationship between brain tumors and acrylamide in humans have been inconclusive [15,16].

Variability in the genes of the DNA repair pathways may alter an individual's capacity to repair DNA damage incurred by environmental exposures, resulting in the inability to completely repair DNA and an eventual accumulation of genetic mutations [10]. The bleomycin-induced mutagen sensitivity assay (MSA) is an in vitro measure of DNA repair capacity which indirectly measures both DNA damage and DNA repair expressed as "breaks per cell" (b/c) in short-term cultured lymphocytes [17,18]. A higher number of bleomycin-induced chromatid breaks compared to control cells indicates higher "mutagen sensitivity" and lower DNA repair capacity. Although the assay does not distinguish between DNA repair mechanisms nor does it identify the specific genes or DNA gene pathways involved [19,20], the assay is a very useful approach to delineating those populations that have a generalized deficit in DNA repair capacity and has been shown to distinguish between individuals with cancer and healthy controls, between individuals with cancer who will develop a second malignancy and those who will not, between those with a family history of cancer and those without and can predict cancer occurrence among a cancer-free cohort 15 years later [21–33]. Mutagen sensitivity is considered to be an expression of a toxicological response indicating increased risk of chronic health effects. Au [34] stated that the expression of mutagen sensitivity is predominantly related to prolonged exposure of the populations to certain environmental mutagens and has been used to measure individual susceptibility to environmental exposure [34–37]. The MSA has been used to investigate both the etiology [35,38,39] of, as well as survival with, glioma [40]. A reduced DNA repair capacity as measured by the MSA in lymphocytes of subjects with glioma compared to controls has been demonstrated with gamma radiation as the challenge agent [35,38,39] and polymorphisms in the RAD51L1 gene have been found to modulate mutagen sensitivity induced by gamma radiation [41].

In this study, we have employed the MSA to evaluate the applicability and utility of this assay in a glioma case-control study as a molecular epidemiology tool and to test whether patients with glioma have a reduced capacity to repair DNA damage caused by exposure to acrylamide.

2. Materials and methods

2.1. Study subjects, control population, and available samples for analysis

Table 1 summarizes the demographic profile of the sample population with an age range of 18–75 with a similar mean age for cases (55.5 years) and controls (56.1 years). The sample population was predominantly white (95%) and had about twice as many males than females in both case and control groups.

Lymphocytes from case and control subjects who consented to participate in a glioma case-control study conducted at Duke University and University of Illinois at Chicago were previously collected, immortalized with Epstein Barr Virus and stored frozen at vapor phase liquid nitrogen. Details of subject recruitment and data collection have previously been described [3,6,42,43] Briefly, study subjects were recruited during the period February 2006–April 2008 from Duke University Medical Center (DUMC) in North Carolina and North Shore University Health System (NSUHS; formerly Evanston Northwestern Healthcare) in Illinois. Survey data were stored and analyzed at University of Illinois at Chicago and Institutional Review Board approvals were obtained from all three institutions. Subjects with a histologically confirmed diagnosis (ICDO-3 sites C70.0–C72.9 and C75.1–C75.3) of a primary glioma [glioblastoma (ICDO-3 histology codes 9440–9442), astrocytoma (9400–9411 and 9420–9421), mixed glioma (9382) or oligodendroglioma (9450–9460)], who were 18 or older, English speaking, and residents of the United States were eligible for participation in the study. High-grade glioma was defined as WHO grade III or IV tumors, including glioblastoma, anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic mixed glioma, etc. Low-grade glioma was defined by WHO grade I or II, including astrocytoma, NOS, fibrillary astrocytoma, oligodendroglioma, mixed glioma, etc. Control subjects were enrolled from an orthopedic clinic where treatment was not likely to affect DNA repair capacity.

Table 1
Characteristics of the sample for glioma cases by treatment status and controls.

Characteristic	Cases n = 50	Controls n = 47
Age in years (mean, SD)	55.5 (10.4)	56.1 (11.0)
Gender (#, %)		
Male	34 (68.0)	30 (63.8)
Female	16 (32.0)	17 (36.2)
Race (#, %)		
White	45 (90.0)	38 (80.9)
Non-White	5 (10.0)	9 (19.2)

All associations are not significant at $p < 0.10$.

2.2. Lymphocyte preparation and treatment

For the current analysis, taking place at the University of New Mexico, sixty-seven cases and controls frequency matched by age (± 5 years) and gender were selected which had both survey data available and lymphocytes which had been frozen and stored. Upon initial cell culturing, there were 2 cell line failures with fewer than 50 metaphase spreads and 2 subjects were selected for replacement for a total of 67 cases. Of those 67 samples, metaphase spreads for 52 cases (78%) were read and reported; 13 cases (19%) had unreadable spreads for all assays due to thin spreads and 2 cases (3%) had failed cell lines (as noted above). Of the 52 cases, 20 (38%) reported that they had not received any chemotherapy or radiation treatment within the 6 months prior to their blood draw, while 31 (60%) were treated with radiation or chemotherapy within the previous 6 months of the blood draw; for one case (2%), treatment status was unknown. Upon initial cell culturing of the control sample spreads, 47 (59%) were read and reported. The remaining samples had cell line failures as previously described and were not analyzed.

2.3. Mutagen sensitivity assay

Buffy coats from approximately 10 ml of blood from each subject collected in ACD (*Acid Citrate Dextrose*) tubes, were carefully layered over 5 ml of Percoll at room temperature and centrifuged at 2000 rpm for 45 min. The lymphocyte layer was removed and washed with phosphate-buffered saline (Gibco-BRL) and suspended in a medium containing Epstein Barr Virus and Cyclosporin A and cultured until the cells were transformed. For cryopreservation, the cells were suspended in 1 ml of freezing medium containing 10% DMSO *(Dimethyl Sulfoxide),*

transferred to a cryotube, cooled from 4 °C to −80 °C at a rate of −1 °C/min in a Nalgene container and stored at vapor phase liquid nitrogen until transported on dry ice to the University of New Mexico for conduct of the mutagen sensitivity assay. Samples were labeled with study ID number only and were grouped so that case and control samples were evaluated under identical conditions.

Lymphocyte cultures were set up following a protocol described previously [44]. Briefly, 1 vial of frozen lymphocytes was rapidly thawed and washed with RPMI 1640 medium (Invitrogen) in a 37 °C water bath and adjusted to a concentration of 1×10^6 cells/ml. The cells were then re-suspended in RPMI 1640 supplemented with 20% fetal bovine serum, 1.5% phytohaemagglutinin (Invitrogen, Rockville, MD), 2 mM L-glutamine and 100 U/ml each of penicillin and streptomycin. After the cells were cultured for 72 h at 37 °C, 1.0 mM of Acrylamide (Sigma–Aldrich Corp.) was added to the appropriate flask. Separately in another flask and an additional 19 h of incubation, 3 unit/ml of bleomycin (Sigma–Aldrich Corp.) was added into the culture and the culture was incubated at 37 °C for an additional 5 h.

To arrest the cells at metaphase, 10 μg/ml colcemid was added to the culture 1 h before harvest. The cells were treated in hypotonic solution (0.075 KCl; Sigma–Aldrich Corp.) and fixed in fixative (methanol:acetic acid = 3:1). The cells were then dropped onto clean microscopic slides, air-dried and stained with 4% Giemsa solution (Sigma–Aldrich Corp.) in Gurr Buffer (Invitrogen). Fifty well-spread metaphase cells per subject were examined to visually score the chromosomal breaks. Details of the criteria for the scoring of chromosomal breaks were described previously by Zheng et al. [32]. The slides were coded and scored without knowledge of the case–control status.

In order to establish the optimal conditions for bleomycin treatment and for testing DNA repair capacity, lymphocytes were isolated from four healthy non-smoking volunteers (four females aged 20–50 years). Cells were incubated at a concentration of 1×10^6 cells/mL for every experiment. From dose-response experiments conducted, the optimal concentrations of bleomycin (data not shown) and acrylamide were established for the assay. See Supplemental Table 1.

2.4. Retroactive exposure assessment for acrylamide; an alkylating animal neurocarcinogen

Potential occupational exposure of study subjects to acrylamide was retroactively assessed through a survey containing three major components (i.e., industry, product, job titles/tasks). The survey was designed to capture those specific industries, specific jobs/job titles and/or manufacturing of specific products that have been reported to be associated with exposure to acrylamide in the scientific literature and/or via review of USEPA, ATSDR and NIOSH publications. Through a comprehensive literature survey, all potential exposure scenarios were captured as shown in Supplemental Table II. In this broad assessment, change in product composition and/or product substitution over time due to changing technology and/or occupational health and safety regulatory environment was not considered.

In addition to the occupational exposure module, this survey instrument had separate modules for capturing environmental and dietary exposures to acrylamide. The environmental module of the survey contained questions pertaining to living within one mile of specific industrial plants (e.g., rubber, plastic, petrochemical, chemical, coal or oil-powered power plant) or of hazardous waste sites/landfills; living within 1 mile of high traffic area or an airport or a gas station or a medical incinerator; types of heating employed in home; hobbies engaged at home (e.g., dyeing textile or paper, treating or preserving wood products, volunteering as a firefighter, painting rooms or homes, placing roofing tar on a roof); use of specific products at home (e.g., paints, adhesives, glues, varnishes, thinners, dyes, pigments, inks, kerosene, fuel oil, herbicides, insecticides, fungicides, hydraulic fluids, deicing fluids, anti-freeze fluids). The dietary module of the survey included questions pertaining to consumption of broiled or smoked or grilled/blackened food; consumption of French fries, fried potatoes, potato chips, pretzels, corn snacks, crackers, toasted or burned or crispy food; consuming foods or drinks that have been contained in rubber or plastic containers or plastic wrap or consuming foods microwaved in plastic container or plastic wrap; and consuming coffee or hot tea; to assess potential acrylamide exposures with dietary origin. This inquiry into occupational, environmental and dietary exposures cumulatively allows assessment of potential total body burden associated with the chemicals studied.

Finally, participants were asked whether they had ever had a CT scan to their head, face, neck or upper spine. They were instructed to only report CT scans from more than two years ago.

2.5. Statistical analysis

The resulting sample was examined and the proportion of unreadable slides for bleomycin, and acrylamide was compared by age, smoking status and gender for the whole sample and for controls only using chi-square tests. For readable slides, means and 95% confidence intervals of breaks per spread for bleomycin and acrylamide were estimated for radiation-treated and untreated cases as well as controls. *T*-tests were conducted to compare means for treated vs. untreated cases, and each type of case to controls.

The median values for bleomycin and acrylamide breaks per spread among controls were determined and dichotomous variables were created for each to differentiate values equal to or above the median versus below the median. Multivariable logistic regression models were performed to examine whether the risk of glioma was different for those with breaks per spread above versus below the median value for different categories. Separate models were employed for treated cases versus controls and untreated cases versus controls. Age and sex adjusted odds ratios (OR) and 95% confidence intervals were reported from each model. There were too few smokers in the sample to allow adjustment for smoking.

In an attempt to validate the survey, a sub-analysis of hospital-based controls was used to examine the association between reported occupational exposure to acrylamide and mean breaks per spread for bleomycin and acrylamide. We performed a similar sub-analysis to investigate the association between reported environmental exposures to acrylamide and mean breaks per spread. However, we could not perform a separate analysis for potential dietary exposures to acrylamide because every subject in the study was potentially exposed to acrylamide through food consumption based on survey responses. Similar analyses were not performed for our population of cases as they are not representative of the general population. Insufficient power was available to evaluate interactions between survey data and MSA data in cases and controls.In addition, the associations between self-reported exposure to CT scans to the head, neck, face or upper spine and mean breaks per spread were examined. *T*-tests were used to test for differences in means between those exposed and not exposed to acrylamide, and CT scans.

All analyses were performed using SAS Version 9.2 (SAS Institute: Cary, North Carolina).

3. Results

Lymphocytes for 65 glioma cases and 65 hospital controls were cultured. Spreads were unreadable for all conditions (bleomycin, and acrylamide) in 13 (20%) cases and 18 (28%) controls. One case who had not received any radiation or chemotherapy in the 6 months prior to the blood draw had unreadable spreads for all conditions except the negative control so this sample was also excluded. For spreads treated with bleomycin (positive control), 30 (46%) cases who received therapy (radiation or chemotherapy in the 6 months prior to the blood draw), 20 (31%) cases who did not receive therapy, and 47 (72%) controls were evaluated. For spreads treated with acrylamide, 29 (45%) cases

Table 2
Mean breaks per spread in glioma cases who have received therapy within the 6 months prior to the blood draw (treated), glioma cases who had not received therapy within the 6 months prior to the blood draw (untreated) and hospital-based controls.

Variable	Treated Cases		Untreated Cases		Controls		p*Treated vs Untreated	p*Treated vs Controls	p*Untreated vs Controls
	n	Mean Breaks/Spread (95% CI)	n	Mean Breaks/Spread (95% CI)	n	Mean Breaks/Spread (95% CI)			
Bleomycin	30	0.71 (0.59, 0.83)	20	0.84 (0.67, 1.02)	47	0.81 (0.72, 0.90)	0.17	0.15	0.60
Acrylamide	29	0.03 (0.02, 0.04)	19	0.04 (0.02, 0.05)	46	0.04 (0.03, 0.05)	0.08	0.10	0.82

*t-test for means

who received therapy, 19 (29%) cases who did not receive therapy, and 46 (71%) controls were evaluated. For all subjects combined and for controls only, there were no statistically significant differences in the frequency of unreadable spreads by age, gender, or smoking status for bleomycin, or acrylamide. In addition, there were no statistical differences between cases and controls by age, gender, or race (Table 1). Of the 50 cases included in the study, 42 (84%) had glioblastoma, NOS/giant cell glioblastoma, while the remainder (16%) had anaplastic astrocytoma/astrocytoma, NOS. No association between treatment status (untreated versus treated) and histology was found.

Mean breaks per slide were similar among cases receiving therapy within the 6 months prior to the blood draw, cases not receiving therapy within the 6 months prior to the blood draw, and hospital-based controls (Table 2). Mean breaks per slide for cell lines treated with acrylamide were very low (0.03, 0.04, and 0.04, respectively) compared to bleomycin treated cell lines (0.71, 0.84, and 0.81, respectively). With mean breaks per slide split at the median value for controls, those with a 'mean breaks per slide' value higher than the median did not have an increased risk for glioma (Table 3).

To determine if the mean breaks per slide was associated with possible exposure to acrylamide in the workplace, in the environment or with reported exposure to CT scans, data were stratified by exposure category for controls (Table 4). For acrylamide, the data allowed a more refined categorization of those exposed to acrylamide occupationally (n = 41), those who were not (n = 6) and those exposed to acrylamide in the environment (n = 41) and those who were not (n = 6). A more refined assessment focusing on those only exposed in the occupational environment or only in the general environment could not be performed due to small sample size in each category (n = 5). Mean breaks per cell of those treated with acrylamide were consistently low across all exposure categories and no differences by exposure category were noted for possible exposure to acrylamide in the workplace or in the environment.

There were also no differences observed between exposed and non-exposed categories for exposure to CT scans.

Table 3
Logistic regression analysis of glioma cases who have received therapy within the 6 months prior to the blood draw (treated), glioma cases who had not received therapy within the 6 months prior to the blood draw (untreated) and hospital-based controls, with cell line values split at the median value among controls.

Breaks Per Spread	Treated Cases No (%)	Untreated Cases No (%)	Controls No (%)	Adjusted[a] OR Treated vs Control (95% CI)	Adjusted[a] OR Untreated vs Control (95% CI)
Bleomycin					
< 0.72	18 (60.0)	8 (40.0)	22 (46.8)	1.00 (ref)	1.00 (ref)
≥0.72	12 (40.0)	12 (60.0)	25 (53.2)	0.61 (0.24, 1.56)	1.12 (0.36, 3.43)
Acrylamide					
< 0.04	18 (62.1)	9 (47.4)	21 (45.7)	1.00 (ref)	1.00 (ref)
≥0.04	11 (37.9)	10 (52.6)	25 (54.3)	0.49 (0.19, 1.29)	1.27 (0.40, 3.98)

[a] Adjusted for age and sex (Note: There were not enough cases who were smokers to adjust for smoking in the model).

Table 4
Mean breaks per spread in hospital-based controls only, by exposure to acrylamide and exposure to computerized tomography (CT) scans.

Exposure	Bleomycin		Acrylamide	
	n	Mean Breaks/ Spread (95% CI)	n	Mean Breaks/ Spread (95% CI)
Work Exposure to Acrylamide	41	0.78 (0.68, 0.87)	40	0.03 (0.02, 0.04)
No Work Exposure	6	1.06 (0.80, 1.31)	6	0.09 (0.003, 0.17)
p-value		**0.04**		0.15
Environmental Exposure to Acrylamide	41	0.83 (0.73, 0.93)	40	0.04 (0.03, 0.05)
No Environmental Exposure	6	0.68 (0.57, 0.80)	6	0.03 (−0.006, 0.07)
p-value		**0.04**		0.72
Exposed to CT scans	24	0.83 (0.69,0.97)	24	0.04 (0.03, 0.05)
Not Exposed	23	0.80 (0.68,0.92)	22	0.04 (0.02, 0.06)
p-value		0.7		0.84

4. Discussion

While gliomas are the most common subgroup of malignant brain tumors, very little is known about risk factors for these tumors apart from ionizing radiation [2–4]. While neurocarcinogenicity of acrylamide has been suggested in rat studies, human studies have been inconclusive. New evidence indicates that the primary target of acrylamide is the nerve terminal and that the subsequent inhibition of corresponding membrane-fusion processes impairs neurotransmitter release promoting degeneration [45] but not necessarily cancer. However, the electrophilic nature of acrylamide may adduct nucleophilic sulfhydryl groups on proteins involved in membrane fusion and that the adduction of thiol groups might be associated with acrylamide's carcinogenic effects [45].

In order to address the effects of acrylamide on glioma cells, we selected a molecular epidemiology tool, the mutagen sensitivity assay, for its adaptability for a variety of mutagenic agents and for its' measurable outcomes, which can provide information about DNA damage and repair pathways [27,46]. Other chemical mutagens which have been utilized in published studies include BPDE (i.e., a chemical associated with tobacco-related cancers), UV radiation and 4-nitroquinoline-1-oxide (both of which have been used in skin cancer studies) [46]. Summaries of mutation sensitivity assay studies using these chemicals are reviewed in Berwick and Vineis [47] and Li et al. [46]. One concern about phenotypic assays, such as the mutagen sensitivity assay, has been the typical wide variation in results that may depend on the timing of the assay (within individual variation), the individual performing the assay (within observer variation), and the laboratory where the assay has been performed (inter-laboratory variation). Erdei et al. [27]evaluated the reliability of MSA by assessing intra-individual, intra-observer and inter-laboratory variability. They showed high correlation for all tests and good concordance between the data from test laboratories (i.e., Memorial Sloan–Kettering Cancer Center in New York, NY and University of Texas M.D. Anderson Cancer Center in Houston, TX). The authors concluded that their results support continued use of the MSA by different laboratories and provide evidence for the assay's

potential to differentiate cases with cancer compared to controls and to identify at-risk subgroups among cancer patients and healthy individuals [27]. These results further provided the rationale for employing MSA in our study.

Previously few studies have examined mutagen sensitivity in glioma, and in those, gamma radiation has been the factor used to induce chromosomal breakage [35,38–40,48]. In these studies, a higher frequency of chromatid breaks per cell induced by radiation was observed in those with glioma when compared to controls. This suggests a reduced capacity to repair DNA damage from radiation in individuals with glioma. Other chemicals known to cause DNA damage including alkylating agents, and acrylamide are suspected animal neurocarcinogens [5,6,11–14]. However, in this study, DNA repair did not differ significantly between glioma cases and controls in response to bleomycin or acrylamide treatment.

Cases who received chemotherapy and/or radiation in the previous six months before the blood draw had mean breaks per slide more similar to the controls than cases who had not received chemotherapy and/or radiation in the six months prior to the blood draw. As cases who had received chemotherapy and/or radiation in the six months prior to the blood draw had slightly, but not statistically significantly, lower mean breaks per cell for bleomycin and acrylamide, it is possible that receiving treatment may have "primed" their DNA repair capabilities, especially if the tumors have become drug-resistant, such that the cells in persons being treated for glioma were quicker to repair the damage caused by these induced insults [47,49]. As suggested by the similar p-value in Table 3, the exposure to bleomycin appeared to trend toward being protective against breaks, providing some support for this theory. Alternatively, those undergoing recent chemotherapy or radiation may have reduced capacity to repair DNA damage in the lymphocytes [47,50]. It is possible that results for cases receiving treatment may be biased, misrepresenting the underlying DNA repair capacity of these individuals. However, neither results for cases receiving treatment nor those who did not receive treatment differed significantly from controls.

Treatment for cancer and/or the cancer itself may influence the results of the MSA [47] and it has been suggested that measures of DNA repair capacity be performed in persons unaffected by cancer. Therefore, the control study population was used to investigate the possible association between the mutagen sensitivity results and the survey results of potential workplace, environmental and dietary exposures to acrylamide. The controls were primarily recruited from an orthopedic clinic, for which treatment was not thought to influence DNA repair capacity. In the control population, there were no significant differences that were observed in mean breaks per spread between those who reported exposure to acrylamide in occupational or general environment and those who did not. These findings may be due to a number of factors related to toxicokinetics of acrylamide, recall bias associated with past exposures and/or artifact of laboratory protocol for mutagen sensitivity assay. In terms of constructing exposures retrospectively, the survey results may not have enough accuracy and sensitivity to adequately assess exposures in terms of duration, timing or concentration and may suffer from subject recall bias. Furthermore, sample size was a limiting factor leading to reduced power in our analysis. In addition to the limitations aforementioned, the mean breaks per spread results for acrylamide yield consistently very low levels (Table 2) could also be a result of acrylamide toxicokinetics in humans, which is shown to have a terminal elimination half-life of 2.4–7.0 h [51]. Therefore, further research is needed for acrylamide and other alkylating agents, including alkylating animal neurocarcinogens.

Several limitations are noteworthy with regard to this study. The number of study subjects was small, especially for the cases when stratified by those cases receiving chemotherapy and/or radiation in the previous 6 months and those cases that did not. The same limitation existed in the study of different exposure categories pertaining to acrylamide. The study focused on select metabolites of the primary compounds, i.e., specifically acrylamide, rather than a more complete array of biomarkers. Furthermore, fresh lymphocytes were not available for this study. Instead, frozen lymphocytes had to be used for the mutagen sensitivity assay. In this study, 2 case and 15 control cell lines failed to grow, while 13 cases and 18 controls had cell lines where all spreads (including the negative controls) were unreadable. Viable lymphocytes for use in the MSA have been obtained using cryopreserved whole blood [52] or from cryopreserved lymphocytes [44] stored for up to 12 months. Although frozen lymphocytes had a higher number of mean breaks per cell than fresh lymphocytes [52], all lymphocyte samples for cases and controls in our study were frozen before culturing and use in the mutagen sensitivity assay.

In summary, while our study advances knowledge in the application of MSA as a molecular epidemiology tool in a case-control glioma study, it does not offer support for the hypothesis that alkylating agents are associated with a reduced DNA repair capacity among glioma patients. Due to the limitations in our study, further research into the impact of alkylating agents on DNA repair capacity in patients who have undergone chemotherapy should be conducted to validate our conclusion.

Authors contribution

Drs Berwick, McCarthy designed the study and applied for Research Ethics Board approval. Drs. Shimek, Rasheed, Il'yasova, Ali-Osman, Bigner and Davis recruited the patients, and Drs. Flores Byrd as well Natalia Gurule, Emily Gonzales and Kirsten White collected the data. Drs. Berwick, McCarthy, Flores. Byrd, Shimek, Rasheed, Il'yasova, Ali-Osman, Bigner, Erdal, Flores, Davis as well Natalia Gurule, Emily Gonzales Alexis Leyba and Kirsten White analyzed the data and prepared draft figures and tables. Dr. Erdal prepared the manuscript draft with important intellectual input from Drs Berwick, McCarthy, Flores, White and Alexis Leyba. All authors approved the final manuscript. Drs McCarthy and Erdal had complete access to the study data.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxrep.2017.12.010.

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