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Prognostic value of H-MLH1 after adjusting for RPA class in GBM patients

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TABLE OF CONTENTS

1. ABSTRACT

Repair of DNA adducts appears to be an important mechanism in chemotherapy responsiveness in glioblastoma multiforme (GBM). Meta-analyses have suggested that the addition of chemotherapy increases the percentage of long-term survivors. Because GBM is characterized by multiplicity of pathways that characterize growth and treatment resistance, we hypothesized probing a multiplicity of repair factors may be able to identify more than one prognostic factor that may be utilized in molecularly targeted therapy that might improve survival and QOL. Seven DNA repair factors showed statistical significance when added to the initial logistic model of RPA class on length of survival status. After adjusting for RPA class the only statistically significant result of the multivariable logistic regressions for these 7 DNA repair factors was that as hMLH1-MF1 increased, the odds of being a short-term survivor versus a long-term survivor decreased (OR: 0.913, 95% CI: 0.838-0.995**,** p=0.0385), multivariable analysis showed no associations between survival status and MGMT and p53 status, and the only statistically significant prognostic DNA repair factor was human Mut L Homologue 1 (hMLH1).

2. INTRODUCTION

Annually in the United States there are 22,500 cases of newly diagnosed malignant primary brain tumors in the adults, of which 70% are malignant gliomas. Approximately two thirds of malignant gliomas are GBMs that are characterized by devastating morbidity and a limited median survival with combined treatment of surgery, radiation and chemotherapy of 12-15 months. (1-2)

Meta-analyses have suggested that the addition of chemotherapy to surgery and radiation therapy may increase the one and two year survivals by 5-10% (3-4). A recent randomized trial by the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) has established radiotherapy with concomitant and adjuvant temozolomide as the current standard of care for newly diagnosed GBM (5). In this trial the median and 2-year survival rate for radiation alone versus radiation plus temozolomide was 12.1 months versus 14.6 month (p less than 0.001) and 10.4% versus 26.5%, respectively. It has long been clear that considerable variation in tumor response and survival

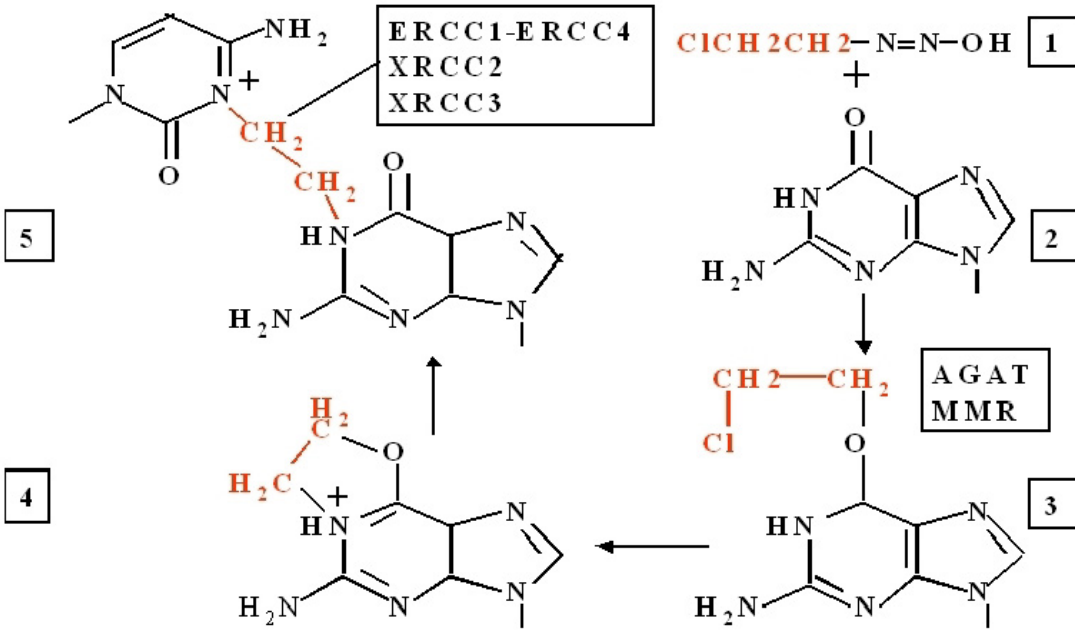

Figure 1. Chemistry of nitrosourea DNA alkylation. BCNU decomposes into chloroethylnitrosourea [1 (upper right)] which reacts with guanine [2 (middle right)] placing a chloroethyl adduct on the O-6 position [3 (lower right)]. This then likely proceeds through a ring intermediate [4 (lower left)] to form a Guanine-Cytosine interstrand cross-link [5 (upper left)]. The repair factors are indicated in boxes near the specific DNA lesions with which they most likely interact.

are observed among patients with glioblastoma multiforme (GBM) receiving similar therapy. The Radiation Therapy Oncology Group (RTOG) has previously combined several prognostic factors (age, Karnofsky performance status [KPS], and extent of surgical resection) in formulating prognostic categories utilizing recursive partitioning analysis (RPA) classes (6). While such clinical observations are valuable, there is clearly a need for further work aimed at elucidating the molecular mechanisms (molecular heterogeneity, invasive behavior, and multiplicity of growth pathways) underlying the differences among patients by identifying molecular factors with prognostic and predictive value. It is anticipated that such research may lead to increased refinement in prognostication and tailoring of therapy with resulting improvement in the outcomes of survival, toxicity, and quality of life. Methylating agents produce cytotoxic product O6 methylguanine DNA adduct, which initiates mismatch repair (MMR) pathway cycling, resulting in apoptotic cell death. Intact MMR is required for tumor cell kill by the two most commonly utilized classes of chemotherapy in the treatment of GBM; namely methylating (e.g. temozolomide and procarbazine) and alkylating (e.g. nitrosurea) agents.

Previous work has suggested that the ability of tumor cells to repair nitrosourea induced DNA adducts (see Figure 1) may be an important mechanism in predicting resistance to these agents. There are six potentially important repair factors for nitrosurea-induced DNA damage (7). Clinical studies have correlated tumor cell levels of MGMT, a repair factor that can remove the chloroethyl adduct on the O-6 position of guanine, with response to nitrosurea and temozolmide and survival in patients with malignant gliomas (8-11). *In vitro* studies utilizing a variety of cell lines have suggested that deficiencies in any of several repair pathways render cells more sensitive to nitrosoureas. These pathways include MGMT, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and p53 (12-16). Unfortunately, the repair pathways involved in the nitrosourea induced guanine-cytosine DNA interstrand cross-link have not been elucidated. However, studies of other types of interstrand cross-links have suggested that homologous recombination repair (HHR) may be important (17).

In this study, we have examined the predictive value for survival of four factors that have been associated with nitrosourea sensitivity [MGMT; the DNA mismatch repair factors, human MutS homolog (hMSH2) and human MutL homolog (hMLH1); and p53] and four factors that have been associated with sensitivity to DNA interstrand cross-linking agents [excision repair cross-complimenting 1 and 4 (ERCC1, ERCC4 (XPF)), x-ray repair cross-complimenting 2 and 3 (XRCC2, XRCC3)] by comparing their expression in the tumor cells of 28 long term survivors (greater than 18 months) with those of 35 short term survivors (less than 6 months) (16).

3. MATERIALS AND METHODS

We retrieved from the RTOG tumor repository 63 pretreatment tumor samples from patients with newly diagnosed GBM who were enrolled on five Radiation Therapy Oncology Group (RTOG) protocols and had been

Table 1. Immunohistochemistry materials and methods

ANTIBODY	VENDOR	CAT #	PRE-TREATMENT	CONCENTRATI0N	Kit
ERCC 1 Clone 8F1	NeoMarkers	MS671-R7	CB	PD	Dako LSAB2
ERCC4 Clone 219 Ab-1	NeoMarkers	MS-1381-P	CB	1:100 30 min	Dako LSAB2
XRCC2 Ab-1	NeoMarkers	MS-1592-R7	CB	PD / 20 min	Dako LSAB2
XRCC3 Ab-1	NeoMarkers	MS-1593-R7	CB	PD / 15 min	Dako LSAB2
MGMT	Chemicon	MAB16200	CB	1:100 15min	Dako LSAB2
MSH2	PharMingen	65051A	CB	1:100 15min	Dako LSAB2
MLH1	PharMingen	13271A	CB	1:20 20 min	Dako LSAB2
P53	Dako	M7001	CB	1:200 10 min	Dako LSAB2

Abbreviations: ERCC1, ERCC4: excision repair cross-compliment; XRCC2, XRCC3: x-ray repair cross-complimenting 2, 3. MGMT: O-6-Methylguanine-DNA Methyltransferase; h-MLH1: human MutL homolog; hMSH2: human MutS homolog

treated with combined radiation and nitrosurea chemotherapy. Survival time was calculated from the date of study registration to the time of death for short-term survivors, and to the time of death or last follow-up for long-term survivors. The tissue microarray was conducted on the basis of statistical review of protocols from which they were derived to identify patients who had survived either longer than 18 months (n=28) or less than 6 month (n=35). Data on patient prognostic factors and treatment were available from the RTOG clinical database but were not provided to the investigators during the study. Tumor microarrays were prepared from random areas of the tumor block selected by a pathologist for inclusion. Pathologist selected and marked all areas of block with viable tumor of greater than 75% without significant necrosis or inflammation. Three random areas from each block were selected to construct three replicate arrays, based on our previously published work on appropriate sampling techniques (18).

3.1. Immnohistochemistry (IHC)

Sections of the paraffin-embedded tumor tissue array were immunostained using an immunoperoxidase method with 3,3 diaminobenzidine as the substrate. Staining was performed on a DAKO Autostainer. Mouse monoclonal antibodies against human MGMT (Chemicon); MSH2, MLH1 (PharMingen); p53 (Dako); ERCC1, ERCC4, XRCC2, and XRCC3 (NeoMarkers) were used as the primary antibody (Table 1). The secondary antibody was a biotinylated goat antimouse antibody (DAKO; prediluted). Antibody binding was detected using horseradish peroxidase (LSAB2 System; DAKO). Appropriate controls were stained simultaneously. The slides were counterstained with hematoxylin (DAKO), dehydrated, and mounted. The percentage of positively stained cells and the mean intensity of staining were determined using an automated cell imaging system (ACIS; Chroma Vision). We have previously published several studies using this system (19-20). The tissue array stained with each antibody is digitally captured and presented to the pathologist. Area of tumor is selected for counting based on morphology. The threshold to optimize scoring was previously defined using digitally captured positive control materials. Negative controls for each assay were also evaluated. Table 1 shows the antibody clones, pretreatments, dilutions and controls used. The tissue microarray was enriched to include patients who had survived either longer than 18 months (long-term survival [LTS], n=28) or less than 6 months (short-term survival [STS], n = 35). Clinical data were available from the RTOG database. Samples were evaluated for expression of MGMT, hMLH1, hMSH2, p53, ERCC1, ERCC4, XRCC3 as determined by quantitative IHC.

Immunostaining was carried out as follows: Heat slides for 10 minutes in an 85-90 degrees C oven, Deparaffinize (rehydrate) through two 5 minute changes xylene, 2 rinses absolute ethanol, several dips in 95% ethanol, then rinse well in water. Place slides into citrate buffer for antigen retrieval into the pressure cooker with for 50 minutes. Rinse slides with tap water. Tap off excess water and carefully wipe around specimen and apply enough 3% hydrogen peroxide to cover specimen for 5 minutes at room temperature. Rinse slides in Tris Buffer. Wipe excess buffer from slides and set slides onto humidity chamber. Overlay PRIMARY antibody (See Table 1). Rinse in Tris Buffer. Overlay LINK (Biotin) for 10 minutes. (Dako LSAB II Kit) Rinse slides in Tris Buffer. Overlay LABEL (Streptavidin) and incubate for 10 minutes. (Dako LSAB II Kit) Rinse with Tris. Apply DAB (chromogen) for 5 minutes. (Research Genetics) Cover slides with commercially prepared hematoxylin (Dako) for approximately 30 seconds. Dehydrate and cover slip with Permount. All stains were performed on the Dako Autostainer using the same method as stated above.

3.2. Statistical methods

Frequency tables with counts and percentages and summary statistics (median and range) were used to describe pretreatment characteristics and the DNA repair factors for each group. As shown in Table 2, there are six potentially important repair factors for nitrosourea-induced DNA damage. Logistic regression models were used to identify associations of DNA repair factors with length of survival status (short-term vs. long-term). Since RPA class is a combination of several independent prognostic factors (age, KPS, neurological function, extent of resection, RT dose, and mental status), all multivariate models were adjusted for RPA class. The initial model is the logistic regression of RPA class on length of survival status. To select potentially important variables, the DNA repair factors were tested one at a time in the logistic model with

Table 2. Potentially important repair pathways for DNA lesions

Repair Pathway	Factor(s)	Lesions Recognized and Repaired
1. Direct Reversal	MGMT	0-6 Guanine chloroethyl adduct
2. BER	DNAG, APE, DNAP, beta, ERCC1	Base damage and SSBs
3. Homologous repair (HR)	ERCC1, ERCC4	DSBs
Non-homologous repair	XRCC2, XRCC3	
Endjoining		
SS annealing		
4. NER	ERCC1, ERCC4	Bulky DNA adducts
5. DNA, ICL Repair	NER and HR are essential	Cross links (CLs)
6. DNA Mismatch and insertion/deletion loops	hMLH1, hMSH2, hMSH3, hMSH6	0-6 Guanine adduct-thymine mismatch

Abbreviations: MGMT: O-6-Methylguanine-DNA Methyltransferase; BER: Base excision repair; DNAG: DNA Glycosylase; APE: AP endonuclease; DNAP beta: DNA Polymerase beta; SSBs: single strand breaks; ERCC1, ERCC4: excision repair cross-compliment; XRCC2, XRCC3: x-ray repair cross-complimenting 2, 3. DSBs: double strand breaks. NER: nucleotide excision repair. ICL: interstrand cross links. h-MLH1: human MutL homolog; hMSH2: human MutS homolog

Table 3. Patient pretreatment characteristics

		Short-term Survivors (n=35)		Long-term Survivors (n=28)		
Age	Median	61		53		
	Range	29-79		25-73		
		n	%	n	%	Chi-square p-value
Age	Less than 60	16	46	21	75	0.0190
	Greater or equal to 60	19	54	7	25	
KPS	30-70	20	57	7	25	0.0104
	80-100	15	43	21	75	
Neurological Function	None/Minor	14	40	20	71	0.0129
	Moderate/Severe	21	60	8	29	
Mental Status	Normal	17	49	23	82	0.0006
	Minor/Gross confusion [1]	18	51	5	18	
Extent of Resection	Biopsy/Partial Resection	25	71	16	57	0.2372
	Total Resection	10	29	12	43	
Radiation Therapy Dose	Less than 60 Gy	5	14	1	4	.2295
	60 Gy	27	77	26	93	
	Greater than 60 Gy	3	9	1	4	
RPA Class[2]	III/IV	12	34	23	82	0.0001
	V/VI	23	66	5	18	
RTOG Protocol (CT)	7401 (BCNU vs. MeCCNU+DTIC)[3]	16	46	9	32	n/a
	7918 (BCNU)	3	9	3	11	
	8312 (BCNU)	8	22	2	7	
	9006 (BCNU)	7	20	9	32	
	9305 (BCNU)	1	3	5	18	

Abbreviations: KPS, Karnofsky Performance Status; RPA, Recursive Partitioning Analysis; CT, chemotherapy; RTOG, Radiation Therapy Oncology Group; n/a, not applicable [1]Only 1 patient had gross confusion [2]This is calculated using age, KPS, extent of resection, mental status, neurological function, and RT dose. [3]BCNU 1,3-bis (2-chloroethyl)-1-nitrosourea; MeCCNU 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea; DTIC 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide

RPA class. The difference between the initial model and the expanded model with the DNA repair factor has an approximate Chi-square distribution with one degree of freedom. Using a criterion of 0.10 for variable selection, those DNA repair factors that were found to be statistically significant were investigated. The odds ratios (OR) for each variable in the final multivariable logistic regression model along with their 95% confidence intervals (C.I.) and p-values are reported.

4. RESULTS

Patient pretreatment characteristics for the short-term (STS) and long-term survivors (LTS) are shown in Table 3. All patients received a nitrosourea, mostly BCNU [1,3-bis (2-chloroethyl)-1-nitrosourea] as part of a multidisciplinary therapeutic approach for their GBM. The descriptive statistics of tumor cells expressing DNA repair factors and the intensity of expression for the positive cells between the two groups are shown in Table 4. There was a wide range for each factor among the individual tumor specimens. We hypothesized that because nitosurea DNA alkylation is a sequential process, which begins at guanine O-6 chlorethyl alkylation and concludes with DNA interstrand cross-linking (see Figure 1), the effects of downstream repair factors may be more difficult to detect in the presence of high levels of upstream repair factors.

Table 5 shows the results of the model fitting when each DNA repair factor was added one at a time to the initial logistic model of RPA class on length of survival status. Seven DNA repair factors showed statistical significance at the 0.10 level when added to the initial model. Table 6 contains the results of the multivariable logistic regression models for these 7 DNA repair factors. The only statistically significant result was that as hMLH1-MF1 increased, the odds of being a short-term survivor versus a long-term survivor decreased after adjusting for

Table 4. Descriptive statistics of tumor cells expressing DNA repair factors

	Short-Term Survivors (n=35)		**Long-Term Survivors(n=28)**	
MGMT	Mean	SE	Mean	SE
	10.23	1.57	14.07	2.69
	Median	Range	Median	Range
	7	1-31	9	1-70
MGMT-MFI	Mean	SE	Mean	SE
	56.97	0.45	57.54	0.56
	Median	Range	Median	Range
	57	53-62	57.5	52-64
P53	Mean	SE	Mean	SE
	30.97	4.52	33.57	4.30
	Median	Range	Median	Range
	25	1-100	26.5	5-96
P53-MFI	Mean	SE	Mean	SE
	67.34	2.30	66.68	2.18
	Median	Range	Median	Range
	63	51-122	61.5	56-96
hMLH1	Mean	SE	Mean	SE
	40.20	5.08	49.75	3.93
	Median	Range	Median	Range
	33	0-90	53	3-90
hMLH1-MFI[1]	Mean	SE	Mean	SE
	40.20	5.08	49.75	3.93
	Median	Range	Median	Range
	63.97	1.29	67.39	1.12
hMSH2	Mean	SE	Mean	SE
	17.00	2.46	19.36	2.14
	Median	Range	Median	Range
	12	0-60	16	5-40
hMSH2-MFI[1]	Mean	SE	Mean	SE
	58.74	1.29	58.89	0.69
	Median	Range	Median	Range
	57	52-95	58	53-68
ERCC1	Mean	SE	Mean	SE
	64-03	4.39	60.25	4.19
	Median	Range	Median	Range
	70	4-97	62.5	6-91
ERCC1-MFI	Mean	SE	Mean	SE
	65.17	1.90	67.11	0.93
	Median	Range	Median	Range
	66	11-84	66	60-82
ERCC4[2]	Mean	SE	Mean	SE
	51.05	5.48	55.37	3.23
	Median	Range	Median	Range
	54.2	8.7-99.5	58	22.9-85.6
ERCC4-MFI[2]	Mean	SE	Mean	SE
	87.44	2.06	83.54	1.46
	Median	Range	Median	Range
	85	69-128	85.5	64-96
XRCC2[2]	Mean	SE	Mean	SE
	85.33	4.05	80.53	4.30
	Median	Range	Median	Range
	94.6	25.7-99.6	91.7	24.7-98.9
XRCC2-MFI[2]	Mean	SE	Mean	SE
	81.66	1.90	78.71	1.68
	Median	Range	Median	Range
	79.5	64-106	77.5	63-95
XRCC3[2]	Mean	SE	Mean	SE
	96.15	1.59	94.32	2.41
	Median	Range	Median	Range
	98.8	54.6-99.8	99	46.6-99.6
XRCC3-MFI[2]	Mean	SE	Mean	SE
	116.63	3.17	120.96	3.30
	Median	Range	Median	Range
	116	84-157	120	86-161

Abbreviations: SE, standard error; MGMT: O-6-Methylguanine-DNA Methyltransferase; h-MLH1: human MutL homolog; hMSH2: human MutS homolog; ERCC1, ERCC4: excision repair cross-compliment; XRCC2, XRCC3: x-ray repair cross-complimenting 2, 3. [1]One short-term survivor patient is missing these values. [2]Three short-term survivor patients are missing these values.

RPA class (OR: 0.913, 95% CI: 0.838-0.995**,** p=0.0385). Specifically, an increase in the hMLH1-MF1 by 1 unit meant a decrease in the odds of being in the STS group by 9% after adjusting for RPA class (Table 6). Multivariable

Table 5. Model fitting values with continuous DNA repair factors

Variables in Model	-2 Log L	Change[1]	p-value[2]
RPA class	71.280		
RPA class, MGMT	69.896	1.384	0.2394
RPA class, MGMG-MFI	69.809	1.471	0.2252
RPA class, p53	70.812	0.468	0.4939
RPA class, p53-MFI	71.254	0.026	0.8719
RPA class, hMLH1	70.671	0.609	0.4352
RPA class, hMLH1-MFI	66.321	4.959	0.0260
RPA class, hMSH2	71.232	0.048	0.8266
RPA class, hMSH2-MFI	70.825	0.455	0.5000
RPA class, ERCC1	70.763	0.517	0.4721
RPA class, ERCC1-MFI	70.847	0.433	0.5105
RPA class, ERCC4	68.075	3.205	0.0734
RPA class, ERCC4-MFI	66.579	4.701	0.0301
RPA class, XRCC2	67.921	3.359	0.0668
RPA class, XRCC2-MFI	68.261	3.019	0.0823
RPA class, XRCC3	67.520	3.76	0.0525
RPA class, XRCC3-MFI	67.062	4.218	0.0400

Abbreviations: RPA, Recursive Partitioning Analysis; h-MLH1: human MutL homolog; hMSH2: human MutS homolog; ERCC1, ERCC4: excision repair cross-compliment; XRCC2, XRCC3: x-ray repair cross-complimenting 2, 3. [1]Change = (log-likelihood [-2 Log L] of two-variable model) – (log-likelihood of RPA class only model) [2]This is from the Chi-square test statistic with 1 degree of freedom.

Table 6. Multivariable Logistic Regression Models for the Results from Table 5

Variables	Comparison	OR	95% C.L.	p-value[1]
RPA Class	III/IV vs.	RL	--	
	V/VI	10.004	(2.756-36.308)	0.0005
hMLH1-MFI (n=62)	Continuous	0.913[2]	(0.838-0.995)	0.0385
RPA Class	III/IV vs.	RL	--	
	V/VI	8.678	(2.579-29.201)	0.0005
ERCC4 (n=60)	Continuous	0.995	(0.972-1.018)	0.6655
RPA Class	III/IV vs.	RL	--	
	V/VI	8.692	(2.528-29.886)	0.0006
ERCC4-MFI (n=60)	Continuous	1.045	(0.974-1.121)	0.2180
RPA Class	III/IV vs.	RL	--	
	V/VI	8.663	(2.571-29.191)	0.0005
XRCC2 (n=60)	Continuous	1.008	(0.982-1.034)	0.5607
RPA Class	III/IV vs.	RL	--	
	V/VI	8.704	(2.459-30.810)	0.0008
XRCC2-MFI (n=60)	Continuous	1.002	(0.941-1.066)	0.9619
RPA Class	III/IV vs.	RL	--	
	V/VI	9.211	(2.679-31.666)	0.0004
XRCC3 (n=60)	Continuous	1.025	(0.966-1.088)	0.4098
RPA Class	III/IV vs.	RL	--	
	V/VI	9.248	(2.684-31.859)	0.0004
XRCC3-MFI (n=60)	Continuous	0.981	(0.948-1.016)	0.2819

Abbreviations: OR, odds ratio; RL, reference level; C.L., confidence level; RPA, Recursive Partitioning Analysis; h-MLH1: human MutL homolog; ERCC4: excision repair cross-compliment; XRCC2, XRCC3: x-ray repair cross-complimenting 2, 3. [1]This is from the Wald Chi-square test statistic [2]Example: This odds ratio estimates how much less likely it is to be in the short-term survivor group versus the long-term survivor group among patients with increasing hMLH1-MFI values compared to those patients with lower hMLH1-MFI values after adjusting for RPA class.

analysis showed no associations between survival status and MGMT and p53 status. The small number of patients in each survivor group made it neither possible nor meaningful to look at the DNA repair factors jointly.

5. DISCUSSION

In this study we have examined the possible roles of several DNA repair proteins as predictive factors for survival among patients with GBM receiving post-operative irradiation and nitrosoureas. Nitrosoureas are felt to produce their tumor cell cytotoxic effects by DNA damage, which leads to cell death through necrosis or induction of apoptosis. There are several normal cellular responses to DNA alkylation which include cell cycle arrest, attempts at repair, and an upregulation or heightened sensitization of the cellular apoptotic apparatus. Important mechanisms of tumor cell drug resistance include alterations in these normal pathways such as an increased efficiency of lesion repair or a decreased ability to signal apoptosis. DNA repair factors that have higher expression in short term GBM survivors are most likely acting through enhanced repair while those higher in the long survivors may be primarily inducing tumor cell death. Thus, if inclusion of chemotherapy increases the chances of long term survival for patients with GBM, it is reasonable to investigate chemotherapy resistance mechanisms as possible predictive factors for survival.

One strategy for selective and efficient tumor therapy is for DNA repair modulation to be targeted against tumor cells with suboptimal DNA repair. The multiplicity

of GBM's molecular pathways necessitates the evaluation of more than one predictive or molecular marker. A feature that is common to human cell DNA repair is the redundancy it has for the removal of many lesions. Loss of one or more repair pathway does not fully disable the repair process but makes the tumor more dependent upon the remaining pathways for its growth. This could lead to exploiting the "Achilles' heel" of certain tumors: targeting the remaining pathways, upon which the tumor's growth is dependent, should improve the chances for better response (21).

5.1. MMR

One of the mechanisms of resistance to alkylating agents involves DNA mismatch repair pathways. Deficiency in MMR pathways can endow tumor cells with resistance to the cytotoxic effects of alkylating agents: This cytotoxic resistance can be explained as follows: The O-6-methylgunanine lesion can initiate apoptosis through sensing by the DNA MMR apparatus and signaling through the mitochondrial apoptosis pathway in a p53-dependent and independent manner (22). The MMR pathway is critical in mediating the cytotoxic effect of O-6-methylguanine. The MMR pathway, which is composed of several proteins (hMLH1, hMSH2, hMSH3, hMSH6, and hPMS2 (see Table 2) is programmed to correct errors in DNA base pairing that arise during DNA replication. During DNA replication, DNA polymerase mispairs O-6-methylguanine with thymine. This mispairing triggers MMR-dependent removal of the mispaired thymine leaving the O-6-methylguanine, which causes subsequent mispairing with yet another thymine leading to futile cycling of the MMR system, which causes accumulation of DNS double-strand breaks, which trigger p53-dependent cell cycle arrest and apoptosis (23). Thus tumors that are deficient in MMR are relatively resistant to alkylating agents such as nitrosureas, temozolomide, and procarbazine. MMR deficiency can be caused by mutations in the hMLH1 or hMSH2 genes (hereditary nonpolyposis colon cancer) or due to methylation of the hMLH1 gene promoter, which is not known to occur in malignant gliomas and is known to occur in only five of the 60 cell lines in the National Cancer Institute tumor panel (24).

A paradoxical relationship between MMR and drug sensitivity has been observed in studies in MMR-deficient cell lines. Some tumor cell lines defective in MMR have been reported to show greater sensitivity to nitrosoureas. These findings have led to the conclusion that MMR may be active in nitrosourea lesion repair, although other explanations such as effects of MMR on the G2/M cell cycle checkpoint are also possible (15-25-26). In our study, conversely, we observed no subsets where MMR expression was higher in the short term survivors. Rather, our observations that MMR expression is lower in the short term GBM survivors irrespective of MGMT and p53 expression suggest that MMR is functioning through another mechanism, most likely, as explained above, through DNA lesion sensing and initiation of the apoptosis signaling pathways. Although MGMT can repair the cytotxic damage caused by alkylating agents, yet resistance to these agents can still be shown in tumors with low level of MGMT and MMR-deficiency (see discussion under MGMT) (27).

Our results confirmed the prognostic value of hMLH1 previously reported by other investigators. The MMR gene hMLH1 maintains genomic integrity by mediating the activation of cell cycle checkpoints and apoptosis. It may be active in nitrosurea lesion repair, predicts the clinical response of malignant astrocytomas to nitrosureas, recognizes the O-6-methylguanine DNA adducts formed by methylation agents (procarbazine and temozolomide), and potently triggers the apoptotic pathway (15-25-28-29). In its absence, neither temozolomide nor BCNU can activate apoptosis (23). Potential models, with emphasis upon hMLH1, need to be further investigated for possible interactions between DNA repair factors, their pathways, the lesions that are recognized and repaired by them, and their relevance to the choice of chemotherapy.

5.2. MGMT

The most important initial DNA lesion induced by BCNU appears to be a chloroethyl adduct on the 0-6 position of guanine. This lesion can be removed by MGMT, the DNA repair factor that has received the most attention in GBM patients to date. The O-6 adduct is capable of activating apoptosis, and this signaling is initiated by MMR, possibly through recognition of a mismatched base incorporated into the complimentary DNA strand (23). Thus, it is of interest that our study failed to show a difference in expression of MGMT between the short and long surviving groups (Table 4). While this may have been due, at least in part, to statistical power, it is possible that the effect of MGMT is complex and related to the functioning of other factors. It is known that tumors with low levels MGMT can still show resistance to the cytotoxic effects of alkylating agents suggesting other mechanisms of resistance (30). Some of those mechanisms include: MMR deficiency, p53 mutations, overexpression of anti-apoptotic proteins (Bcl-2 or Bcl-X1), or active BER pathway (27-31). As has been reported by others an important interaction between MGMT and MMR does exist in patients receiving temozolomide (30). Several prior studies have suggested that MGMT expression does correlate with response and survival in patients with gliomas (8-9-10). However, these studies have included patients with grade 3 and 4 gliomas and sometimes low grade tumors. Among these studies, only Jaeckle, *et al.* has presented a subset analysis by tumor grade in patients receiving nitrosoureas. These authors found that MGMT levels appeared to be considerably more predictive of survival in patients with anaplastic astrocytoma (median survivals 8 vs. 29 months for high and low MGMT) than GBM (7 vs. 12 months).

Studies examining MGMT promoter methylation also present some difficulties in interpretation. A negative correlation between methylation (presumably associated with lower MGMT levels) and survival was found in a population of patients with grade 3 and 4 tumors, and in patients with low grade astrocytomas (9-32). However, in patients with GBM receiving predominantly nitrosoureas as chemotherapy, one study found no correlation (33).

Conversely, in GBM patients receiving temozolomide, a strong relationship was shown (11-34). In sum, these results suggest that pre-treatment MGMT status may have a predictive value for patients with GBM receiving nitrosoureas and maybe more important in patients receiving O-6 guanine methylating agents (34). The recently completed international GBM trial (EORTC/RTOG 0525) will help in further clarifying this issue.

5.3. p53

Inactivation of p53 has been associated with both increased and decreased sensitivity to nitrosoureas *in vitro* (16-25). Likewise, p53 has been evaluated in a number of previous studies as an individual prognostic factor for patients with GBM with inconsistent results (35). These complex variations may indicate that p53 is important but only in the context of intact relevant signaling pathways. p53 expression is usually difficult to detect by IHC unless the molecule has been stabilized by mutation or cellular stress such as chemotherapy exposure. In these instances, p53 binding by the E-3 ubiquitin ligase, MDM2, is prevented. Therefore, high p53 expression by IHC in unstressed cells has been suggested as a marker for missense mutated, and presumably non-functional, p53. In gliomas, as in other tumors, the concordance between high IHC expression and mutation is about 70-80% (36-37).

Although not a significant prognostic factor individually in our study, p53 appears to have important interactions with MMR and ERCC1. As with other DNA repair factors, p53 has a complex and incompletely understood relationship with MMR, likely involving other molecules such as ATM/ATR (38-39). MMR and p53 participate in nitrosourea sensitivity pathways in complex ways that are dependent and independent of each other and yet to be fully elucidated (23). MMR/DNA interactions can result in phosphorylation of p53 on serine residues 15 and 392 with resultant stabilization (40). In addition, at least one pathway of MMR related apoptosis signaling goes through p53 (41). And recent data from our group and others have presented evidence that p53 may be a transcription factor for hMSH2, possibly participating in a feedback loop (42).

5.4. NER

NER factors ERCC1 and ERCC4 recognize and repair bulky DNA adducts (see Table 2). ERCC1 functions in the 5'-DNA nicking step of NER and may serve a similar role in HRR. Deficiencies of ERCC1 and ERCC4 seem to be most predictive of increased cyclophosphamide sensitivity in a panel of CHO excision repair mutant cell lines (43). Unfortunately, there is little information regarding these two repair pathways in the repair of nitrosourea induced DNA adducts. One group has suggested that expression of ERCC2, a component of NER, correlates *in vitro* with resistance to BCNU (14). Alternatively, one group proposed a model of DNA interstrand cross-link repair that involves the $ERCC_1$ and $ERCC_4$ driver through a mechanism of recombinational repair (44). The relationship of $ERCC_1$/$ERCC_4$ to survival will need to be investigated in series larger than ours. The question of the relative contribution of HRR to nitrosourea sensitivity is also complicated by the observation that HRR is also important in the repair of double-strand DNA breaks induced by radiation a treatment also received by our patients (45).

Limitations in this study include its retrospective nature and its small sample size. Retrospective studies have the flaw of not being able to adequately offer a satisfactory accounting or explanation for selection bias. The small sample is partly inherent in the fact that patients with GBMs have a short median survival time so it is a challenge to obtain pathology specimens on an adequate number of long-term survivors. This study was a retrospective analysis and, therefore, was not designed to take statistical power into consideration. It needs to be emphasized that just because some associations were not found between the DNA repair factors and length of survival does not mean that these associations do not exist. A prospective study needs to be done to insure enough statistical power to find these associations if they truly exist.

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7. REFERENCES

1. DN Louis, H Ohgaki, OD Wiestler, WK Cavenee, PC Burger, A Jouvet, B W Scheithauer, P Kleihues : The 2007 WHO classification of tumors of the central nervous system. Lyon, France, *IARC Press*, (2007)

2. CBTRUS 2008 statistical report: primary brain tumors in the United States, 1998-2002: Central Brain Tumor Registry of the United States, http://www.cbtrus.org (2000-2004)

3. HA Fine, KB Dear, JS Loeffler, PM Black, GP Canellos: Meta-analysis of radiation therapy with and without adjuvant chemotherapy for malignant gliomas in adults. *Cancer* 71, 2585-2597 (1993)

4. LA Stewart: Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomized trials. *Lancet* 359, 1011-1018 (2002)

5. R Stupp, WP Mason, MJ van den Bent, M Weller, B Fisher, MJ Taphoorn, K Belanger, AA Brandes, C Marosi, U Bogdahn, J Curschmann, RC Janzer, SK Ludwin, T

Gorlia, A Allgeier, D Lacombe, JG Cairncross, E Eisenhauer, RO Mirimanoff: Eirpoean Organization for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Groups. Radiotherapy plus concomitant and ajuvant temozolomide for glioblastoma. *N Engl J Med* 352:987-96 (2005)

6. WJ Curran Jr, CB Scott, J Horton, JS Nelson, AS Weinstein, AJ Fischbach, CH Chnag, M Rotman, SO Asbell, RE Krisch: Recursive partitioning analysis of prognostic factors in three Radiation Therapy Oncology Group malignant glioma trials. *J. Natl. Cancer Inst* 85, 704-710 (1993)

7. S Madhusudan, MR Middleton : The emerging role of DNA repair proteins a predictive, prognostic and therapeutic targets in cancer. *Cancer Treat.Rev* 31:603-617 (2005)

8. M Belanich, M Pastor, T Randall, D Guerra, J Kibitel, L Alas, B Li, M Citron, P Wasserman, A White, H Eyre, K Jaeckle, S Schulman, D Rector, M Prados, S Coons, W Shapiro, D Yarosh: Retrospective study of the correlation between the DNA repair protein alkyltransferase and survival of brain tumor patients treated with carmustine. *Cancer Res* 56, 783-788 (1996)

9. M Esteller, J Garcia-Foncillas, E Andion, SN Goodman,OF Hidalgo, V Vanaclocha, SB Baylin, JG Herman: Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *New Eng. J. Med.* 343, 1350-1354 (2000)

10. KA Jaeckle, HJ Eyre, JJ Townsend, S Schulman, HM Knudson, M Belanich, DB Yarosh, SI Bearman, DJ Giroux, SC Schold: Correlation of tumor O^6 methylguanine-DNA methyltransferase levels with survival of malignant astrocytoma patients treated with bis-chloroethylnitrosourea: A Southwest Oncology Group Study. *J. Clin. Oncol.* 16, 3310-3315 (1998)

11. ME Hegi, AC Diserens, S Godard, PY Dietrich, L Regli, S Ostermann, P Otten, G Van Melle, N de Tribolet, R Stupp *et al:* Clinical trial substantiates the predictive value of O-6-*methylguanine-DNA methyltransferase* promoter methylation in glioblastoma patients treated with temozolomide. *Clin. Cancer Res*. 10:1871-1874 (2004)

12. WJ Bodell, K Tokuda, DB Ludlum: Differences in DNA alkylation products formed in sensitive and resistant human glioma cells treated with N-(2-chloroethyl)-N-nitrosourea. *Cancer Res.*48:4489-4492 (1998)

13. BP Engelward, A Dreslin, J Christensen, D Huszar, C Kurahara, L Samson: Repair-deficient 3-methyladenine-DNA glycosylase homozygous mutant mouse cells have increased sensitivity to alkylation-induced chromosome damage and cell killing. *EMBO. J.* 15:945-952 (1996)

14. ZP Chen, A McQuillan, G Mohr, LC Panasci: Excision repair cross-complementing rodent repair deficiency gene 2 expression and chloroethylnitrosourea resistance in human glioma cell lines. *Neurosurg*. 42:1112-1119 (1998)

15. G Aquilina, S Ceccotti, S Martinelli, R Hampson, M Bignami: M. N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea sensitivity in mismatch repair-defective human cells. *Cancer Res*. 58:135-141 (1998)

16. GW Xu, CL Nutt, MC Zlatescu, M Keeney, I Chin-Yee, JG Cairncross: Inactivation of p53 sensitizes U87MG glioma cells to 1,3-bis(2-chloroethyl)-1-nitrosourea. *Cancer Res.* 61:4155-4159 (2001)

17. IU De Silva, PJ McHugh, PH Clingen, JA Hartley: Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. *Mol. Cell. Biol*. 20:7980-7990 (2000)

18. M Milanes-Yearsley, ME Hammond, TF Pajak, JS Cooper, C Chang, T Griffin, D Nelson, G Laramore, M Pilepich: Tissue micro-array: a cost and time-effective method for correlative studies by regional and national cancer study groups. *Mod Pathol* 15(12):1366-73 (2002)

19. A Pollack, M DeSilvio, LY Khor, R Li, TI Al-Saleem, ME Hammond, V Venkatesan, CA Lawton, M Roach 3rd, WU Shipley, GE Hanks, HM Sandler: Ki-67 staining is a strong predictor of distant metastasis and mortality for men with prostate cancer treated with radiotherapy plus androgen deprivation: Radiation Therapy Oncology Group Trial 92-02.*J Clin Oncol*. 1; 22(11):2133-40 (Jun 2004)

20. A Chakravarti, K Heydon, CL Wu, E Hammond, A Pollack, M Roach, H Wolkov, P Okunieff, J Cox, J Fontanesi, R Abrams, M Pilepich, W Shipley: Radiation Therapy Oncology Group. Loss of p16 expression is of prognostic significance in locally advanced prostate cancer: an analysis from the Radiation Therapy Oncology Group protocol.86-10. *J Clin Oncol*. 1;21(17):3328-34 (Sep 2003)

21. S Madhusudan, ID Hickson: DNA repair inhibition: a selective tumor targeting strategy. *Trends in Molecular Medicine* 11(11):503-511 (2005)

22. S D'Atri, L Tentori, PM Lacal, G Graziani, E Pagani, E Benincasa, G Zambruno, E Bonmassar, J Jiricny: Involvement of the mismatch repair system in temozolomide-induced apoptosis. *Mol Pharmacol* 54:334-341 (1998)

23. MJ Hickman, LD Samson: Role of DNA mismatch repair and p53 signaling induction of apoptosis by alkylating agents. *Proc. Natl. Acad. Sci. USA* 96:10764-10769 (1999)

24. P Taverna, L Liu, AJ Hanson, A Monks, SL Gerson, *et al:* Characterization of MLH1 and MSH2 DNA mismatch repair proteins in cell lines of the NCI anticancer drug screen. *Cancer Chemother Pharmacol* 46:507-16 (2000)

25. G Aquilina, S Ceccotti, S Martinelli, S Soddu, M Crescenzi, P Branch, P Karran, M Bignami: Mismatch repair and p53 independently affect sensitivity to N-(2-

chloroethyl)-N'-cyclohexyl-N-nitrosourea. *Clin. Cancer Res*. 6:671-680 (2000)

26. Y Hirose, M Katayama, D Stokoe, DA Haas-Kogan, MS Berger, RO Pieper: The p38 mitogen-activated protein kinase pathway links the DNA mismatch repair system to the G2 checkpoint and to resistance to chemotherapeutic DNA-methylating agents. *Mol. Cell Biol.* 23:8306-8315 (2003)

27. L Liu, SL Gerson : Targeted modulation of MGMT : clinical implications. *Clin Cancer Res* 12 :328-31(2006)

28. T Fukushima, Y Katayama, T Watanabe, A Yoshino, A Ogino, T Ohta, C Komine: Promotor hypermethylation of mismatch repair gene hMLH1 predicts the clinical response of malignant astrocytomas to nitrosourea. *Clin Cancer Res* 11:1539-1544 (2005)

29. D Fink, S Aebi, SB Howell: The role of DNA mismatch repair in drug resistance. *Clin Cancer Res* 4:1-6 (1998)

30. HS Friedman, RE McLendon, T Kerby, M Dugan, SH Bigner, AJ Henry, DM Ashley, J Krischer, S Lovell, K Rasheed, F Marchev, AJ Seman, I Cokgor, J Rich, E Stewart, OM Colvin, JM Provenzale, DD Bigner, MM Haglund, AH Friedman, PL Modrich: *J Clin Oncol.* 16(12):3851-3857 (1998 Dec)

31. JN Sarkaria, GJ Kitange, D James, R Plummer, H Calvert, M Weller, W Wick: Mechanisms of chemoresistance to alkylating agents in malignant glioma. *Clin Cancer Res.* 14:2900-2908 (2008)

32. C Komine, T Watanabe, Y Katayama, A Yoshino, T Yokoyama, T Fukushima: Promoter hypermethylation of the DNA repair gene 06-methylguanine-DNA methytransferase is an independent predictor of shortened progression free survival in patients with low-grade astrocytomas. *Brain Pathol*. 13:176-184 (2003)

33. H Kobayashi, N Ishii, J Ikeda, Y Sawamura, Y. Iwasaki: Methylation profiling of the DNA repair genes in glioblastoma multiforme and its clinical relevance. *Proc. Amer. Soc. Clin. Oncol*. 23, 125 (2004)

34. ME Hegi, AC Diserens, T Gorlia, MF Hamou, N de Tribolet, M Weller, JM Kros, JA Hainfellner, W Mason, L Mariani, JE Bromberg, P Hau, RO Mirimanoff, JG Cairncross, RC Janzer, R Stupp: MGMT gene silencing and benefit from temozolomide in glioblastoma. *N. Engl J Med* 352:997-1003 (2005)

35. C Nieder, S Petersen, C Petersen, H Thames: The challenge of p53 as prognostic and predictive factor in gliomas. *Cancer Treat. Rev*. 26:67-73 (2000)

36. EW Newcomb, WJ Madonia, S Pisharody, FF Lang, M Koslow, DC Miller: A correlative study of p53 protein alteration and p53 gene mutation in glioblastoma multiforme. *Brain Pathol*. 3:229-235 (1993)

37. H Koga, S Zhang, T Kumanishi, K Washiyama, T Ichikawa, R Tanaka, J Mukawa: Analysis of p53 gene mutations in low- and high-grade astrocytomas by polymerase chain reaction-assisted single-strand conformation polymorphism and immunohistochemistry. *Acta. Neuropathol* 87:225-232 (1994)

38. Y Luo, FT Lin, WC Lin: ATM-mediated stabilization of hMut L DNA mismatch repair proteins augments p53 activation during DNA damage. *Mol. Cell Biol*. 24:6430-6444 (2004)

39. S Caporali, S Falcinelli, G Starace: DNA damage induced by temozolomide signals to both ATM and ATR: role of the mismatch repair system. *Mol. Pharmacol*. 66:478-491 (2004)

40. DR Duckett, SM Bronstein, Y Taya, P Modrich: hMutSalpha-and hMutLalpha-dependent phosphorylation of p53 in response to DNA methylator damage. *Proc. Natl. Acad. Sci. USA* 96:12384-12388 (1999)

41. GM Li: The role of mismatch repair in DNA damage-induced apoptosis. *Oncol. Res*. 11:393-400 (1999)

42. CT Warnick, B Dabbas, CD Ford, KA Strait: Identification of a p53 response element in the promoter region of the *hMSH2* gene, required for expression in A2780 ovarian cancer cells. *J. Biol. Chem*. 276:27363-27270 (2001)

43. BS Andersson, T Sadeghi, MJ Siciliano, R Legerski, D Murray: Nucleotide excision repair genes as determinants of cellular sensitivity to cyclophosphamide analogs. *Cancer. Chemother. Pharmacol*. 38:406-416 (1996)

44. T Bessho, D Mu, A Sancar: Initiation of DNA interstrand cross-link repair in humans: the nucleotide excision repair system makes dual incisions 5" to the cross-linked base and removes a 22-28-nucleotide-long damage-free strand. *Mol. Cell Biol*. 17:6822-6830 (1997)

45. A Pastink, JCJ Eeken, PHM Lohman: Genomic integrity and the repair of double-strand DNA breaks. *Mut. Res*. 480:37-50 (2001)

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