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Laboratory Investigation

Genomic copy number changes of DNA repair genes *ERCC1* and *ERCC2* in human gliomas

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Abstract

Abnormalities of the genomic region of chromosome 19q13.2–13.4 are a common occurrence in brain malignancies and contain a possible tumor suppressor gene involved in gliomas. Since abnormalities of DNA repair are associated with malignancy, we assessed DNA status of the nucleotide excision repair genes located in this area, viz. ERCC1 and ERCC2.

Radiodensitometry was used to assess gene copy number in samples obtained from brain tumor specimens from 24 patients. Nine tumors were of lower grade histology (3 pilocytic astrocytomas, 2 gangliogliomas, 4 astrocytomas); 15 tumors were pathologiclly higher grade (4 anaplastic astrocytomas, 11 glioblastomas). Tumor samples were obtained prior to radiation or chemotherapy. Abnormalities of gene copy number of ERCC1 and ERCC2 were observed in 11/24 specimens (46%). Whereas increased and decreased copy numbers were observed for ERCC1, only decreases in copy number of ERCC2 were seen. Three tumors (all lower grade) showed concurrent allelic loss of ERCC1 and ERCC2. Abnormalities of copy number for these genes were not associated with response to subsequent therapy nor survival. However, allelic loss of ERCC2 was associated with younger age at diagnosis when compared to those specimens which did not show loss. There were no significant differences between lower grade and higher grade tumors with respect to these investigations.

Abnormalities in copy number of ERCC1 and ERCC2 are common in glial tumors. Further study of this genomic region is necessary to define the importance of these observations in tumor pathophysiology and treatment.

Introduction

Genetic changes in tumors are frequent, and most likely represent a multistep pathway to the development of malignancy. In human gliomas, a number of abnormalities have been noted in a variety of different chromosomal areas on both a cytogenetic and molecular scale, and may possibly reflect the inherent genomic instability of these tumors. Included in these recurrently noted changes include abnormalities of chromosome 19q [1–4]. Allelic losses in the 19q13.2–13.4 region have been noted in a range of glioma subtypes, including astrocytomas, anaplastic astrocytomas, glioblastomas, oligodendrogliomas, anaplastic oligodendrogliomas, and mixed gliomas [1–4]. Recent reports [4] have narrowed the common area of DNA loss to a region of about 8 centimorgans (5 megabases), between the microsatellite markers APOC2 and HRC, including genes ERCC1 and ERCC2 [5].

ERCC1 and ERCC2 are essential genes in the nucleotide excision repair process, and participate in the repair of DNA lesions such as pyrimidine dimers induced by ultraviolet light [6, 7] or interstrand cross links and intrastrand adducts created by chemotherapeutic agents such as cisplatin [8, 9]. Nucleotide excision repair is considered the major pathway for DNA repair in mammalian cells [10]. Recently, DNA repair genes have been found to be associated with the suppression of the tumor phenotype [11, 12] and hence, ERCC1 and ERCC2 are candidate tumor suppressor genes for the area of deletion from 19q [1, 3]. Dabholkar *et al.* [13, 14] have shown however that increases in mRNA expression of ERCC1 are associated with poor response to platinum based chemotherapy in ovarian tumors. As well, *in vitro* studies in Chinese hamster ovary cells have revealed increased resistance to alkylating agents such as melphalan, when ERCC1 is overexpressed in a transfected plasmid construct [15].

Human glial tumors are known to be resistant to a variety of DNA targeted cytotoxic chemotherapeutic drugs, including nitrosureas and platinum compounds [16]. The reasons for resistance to these chemotherapeutic compounds is not definitively known, but may be related to a variety of different mechanisms which include DNA repair, detoxification of BCNU (glutathione-S-transferase), or a multidrug resistant phenotype (either MDR or MRP) [13, 16, 17]. Since human gliomas show both a frequent loss of genetic material from chromosome 19q13.2–13.4, and because they are resistant to a host of different DNA damaging therapeutic interventions (radiation, nitrosureas, platinum based compounds, etc.), we felt DNA repair genes located in this region may be associated with tumorigenesis or drug resistance in these tumors. We therefore investigated the question of gene copy number of the ERCC DNA repair genes in the 19q region in human gliomas.

Materials and methods

DNA preparation

Tumors were obtained at craniotomy and frozen in liquid nitrogen, and transferred to a – 80° C freezer for storage. DNA from primary tumor tissue or normal brain was extracted by lysis, using buffer containing 20 mM Tris HCl pH 8/10 mM EDTA/0.5% SDS/100 mM NaCl/10 mg proteinase K at 50–55° C overnight. The lysate was extracted with phenol and chloroform and ethanol precipitated. The DNA pellet was washed with 70% ethanol, lyophilized, and resuspended in TE (10 mM Tris HCl pH 8/1 mM EDTA). All tumors were pathologically verified using a three tiered system [18]. Five μg of either tumor or normal brain genomic DNA was transferred to nylon membranes using a slot blot apparatus (Stratagene Inc., La Jolla, CA) according to the manufacturer's instructions, and crosslinked at 1200 Joules in a UV crosslinker (Stratagene Inc., La Jolla, CA). All tumor specimens were acquired prior to any treatment interventions.

Probes/hybridization

Hybridization was performed with cDNA probes for ERCC1 [19] and ERCC2 [20]. Control single copy probe pHHH163 (localizing to chromosome 6p21) was obtained from the American Type Culture Collection (Rockville, MD). In each case, 30–60 ng were labelled with α-^{32}P dCTP to a specificity of $> 10^8$ cpm/mg. Membranes were prehybridized with 0.6 M NaCl/8 mM EDTA/120 mM Tris HCl pH 7.4/0.1% sodium pyrophosphate/1% SDS/10% dextran sulfate/100 mg/ml salmon sperm DNA for 6 hours, with probe subsequently being added. After 18 hours at 42° C, membranes were washed in 0.1 × SSC/0.1% SDS, and exposed to film for 24–48 hours. Blots were stripped and hybridized with each probe two or three times and exposed to film prior to densitometry analysis.

Densitometry analysis

Resultant autoradiograms were digitized using a Sony SC-77 CCD camera (Cypress, CA) linked to a Scion LG-3 video frame grabber (Fredrick, MD) in a Macintosh II series computer (Cupertino, CA) as well as on a Molecular Dynamics Densitometer (Sunnyvale, CA). The former images were captured with the program NIH-Image (version 1.55) as 8 bit data (256 grey scale values) and utilized as uncompressed TIFF files. Bands were analyzed by obtaining integrated density measurements with standardized pixel areas, and normalized to control calculations of single copy probes of normal human brain DNA. For each sample, copy number for ERCC1 and ERCC2 was defined as [(normalized integrated density value ERCC)/(normalized integrated density value pHHH163)]. Ratios ≥ 2 for each tumor specimen were considered an increase in copy number; decreases in copy number were defined similarly with a value ≤ 0.5.

Patient data

Patient data was obtained from the medical records of the treating hospitals. Information regarding age, sex, pathologic grade of tumor, number and type of chemotherapeutic interventions and time to death were obtained.

Statistics

Single pairwise comparisons were performed using a Student's t-test function, with multiple pairwise comparisons corrected with either the Bonferroni or Tukey modifications. Significant difference was assumed with a $p \leq 0.05$. Two sided p values are reported.

Results

Tumor histology/patient characteristics

Table 1 shows the histology and patient characteristics of the unselected study population. Pilocytic astrocytomas [3] and gangliogliomas [2] were combined and labelled low grade [5]. Four tumors were graded as astrocytoma, four as anaplastic astrocytoma, and 11 as glioblastoma. One tumor in the glioblastoma group was initially diagnosed as an oligodendroglioma, but upon re-resection (and whence tissue DNA was obtained) was found to be pathologically a glioblastoma. This patient received no interval treatment except surgery.

Copy number changes

Figure 1 depicts the results of the slot blot autoradiograms of ERCC1 and ERCC2 grouped by tumor histology, as compared to the single copy probe pHHH163. Table 2 shows the results of the copy number changes by histologic tumor grade. Densitometric measurements revealed allelic losses of ERCC1 in four cases (4/24, 17%). Losses were noted in 1/5 (20%) low grade tumors, 2/4 (50%) astrocytomas, and 1/11 (9%) glioblastomas. None of the anaplastic astrocytomas showed evidence of allelic loss. Increase in copy number of ERCC1 was seen in four tumors (4/24, 17%). The range of copy number increase by integrated density measure-

Table 1. Patient characteristics

Histology	Median age (range)	Male	Female
Low grade	17 (7–31)	3	2
Astrocytoma	35 (24–42)	1	3
Anaplastic astrocytoma	34 (28–53)	2	2
Glioblastoma	57 (22–75)	7	4
Total		13	11

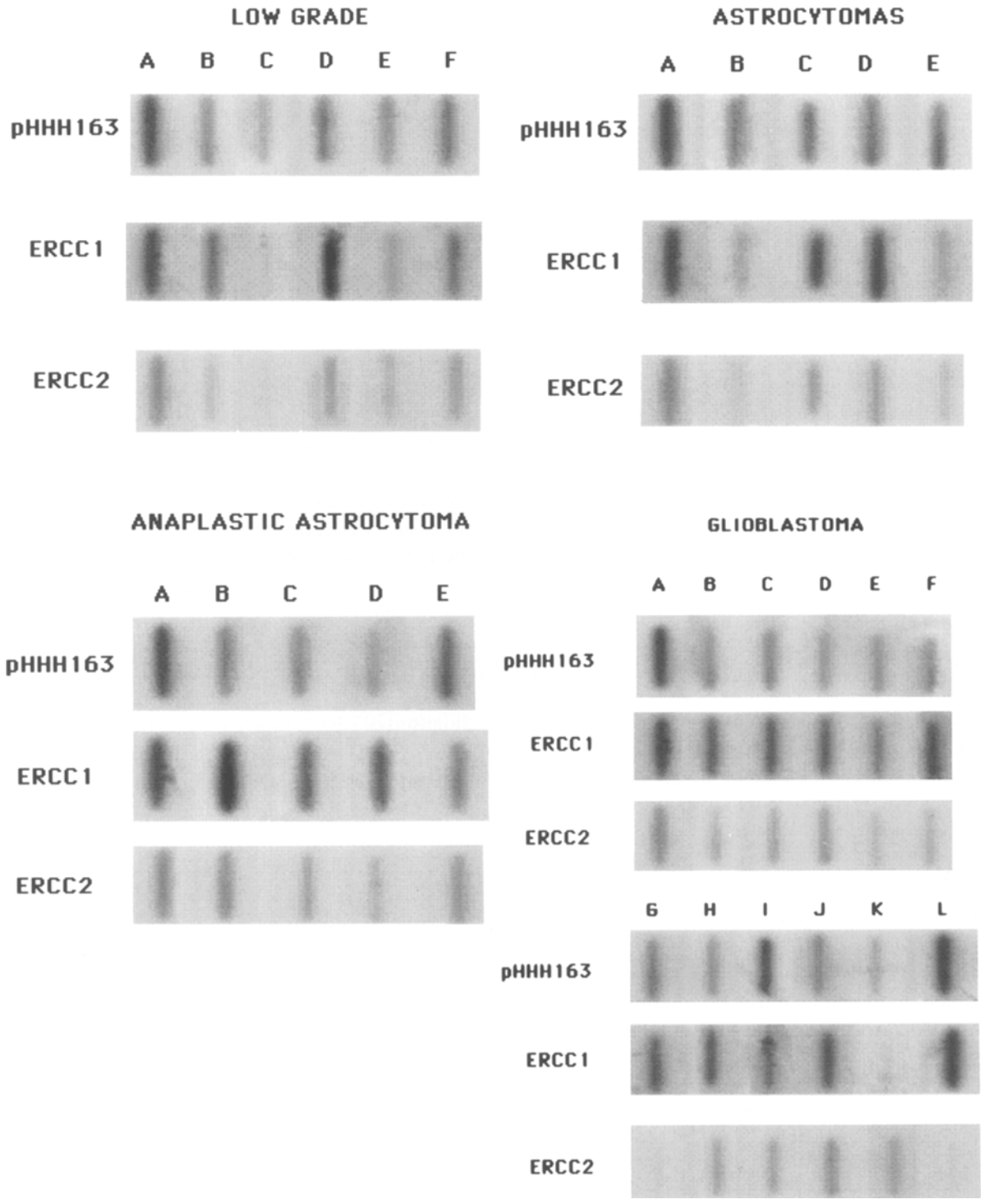
LOW GRADE
A B C D E F
pHHH163
ERCC1
ERCC2
ASTROCYTOMAS
A B C D E
pHHH163
ERCC1
ERCC2
ANAPLASTIC ASTROCYTOMA
A B C D E
pHHH163
ERCC1
ERCC2
GLIOBLASTOMA
A B C D E F
pHHH163
ERCC1
ERCC2
G H I J K L
pHHH163
ERCC1
ERCC2

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Fig. 1. Results of DNA slot blots of glial tumors probed with pHHH163, ERCC1 and ERCC2. Column A shows the normal brain control used for each blot. Panel A. Low grade tumors. ERCC1 showed allelic loss in one tumor (sample C) and increased copy number in one tumor (samples D). ERCC2 revealed allelic loss in two tumors (samples B and C); note concurrent loss of ERCC1 and ERCC2 in sample C. Panel B. Astrocytoma. ERCC1 and ERCC2 were noted to be lost concurrently in two samples (B and E). No increases in copy number of either gene were noted. Panel C. Anaplastic astrocytoma. Increases in copy number of ERCC1 were seen in samples B amd D. No allelic loss was noted of either gene in anaplastic astrocytoma. Panel D. Glioblastoma. Increased copy number of ERCC1 was noted in one specimen (sample F) with loss noted in one (sample K). ERCC2 showed loss in samples G and L.

ments in these tumors was 2–4.1. In contrast to the ERCC1 allelic loss results, 2/4 (50%) of anaplastic astrocytomas showed increases in copy number. In addition, 1/5 (20%) low grades revealed an increase in copy number, with 1/11 (9%) glioblastomas showing a copy number increase.

No tumors revealed increases in copy number of ERCC2. No concomitant loss of ERCC2 was noted with the presence of increase in copy number of ERCC1. Deletions of ERCC2 were noted in six tumors (6/24, 25%). Of the low grade tumors, 2/5 (40%) showed allelic loss; one of these also revealed loss of ERCC1. Both astrocytoma tumors which showed loss of ERCC1 also had allelic loss of ERCC2 (2/4, 50%). As noted for ERCC1, no anaplastic astrocytoma was found to harbor loss of ERCC2. Finally, 2/11 (18%) glioblastomas showed ERCC2 loss; none of these was associated with ERCC1 loss.

Patient correlations

Age

As a group, median age at diagnosis was not different between the patients with increases in copy number of ERCC1 (median 37.5 years, n = 4) and those who did not (median 32 years, n = 20, p > 0.90). Similarly, age differences were not noted between the subgroups which had allelic loss of ERCC1 (median 35 years, n = 4) and those who did not (median 35 years, n = 2, p > 0.80). Finally, a non-significant trend with respect to age was seen in those patients with loss of ERCC2 (median 29.5 years, n = 6) compared to those without loss (median 40, n = 18, p = 0.10).

Chemotherapy/survival

Table 3 shows the data correlating ERCC status and clinical outcome in the 15 patients with higher grade gliomas (anaplastic astrocytoma and glioblastoma). The primary chemotherapeutic agent administered to these patients were the nitrosoureas, although procarbazine and vincristine were also given, either alone or separately. No significant difference in survival or number of courses of chemotherapy administered was noted between the patients who had no change in ERCC1 and ERCC2 status versus those who showed increases in copy number of allelic loss. Stratifying the results with and without the anaplastic astrocytoma patient data did not alter the results.

Table 2. Tumor histology and ERCC status

Histology	Increase ERCC1 (%)	Decrease ERCC1 (%)	Decrease ERCC1 (%)	Total# changes (%)
Low grade	1/5 (20)	1/5 (20)	2/5 (40)	3/5 (60)[1]
Astrocytoma	0/4 (0)	2/4 (50)	2/4 (50)	2/4 (50)[2]
Anaplastic astrocytoma	2/4 (50)	0/4 (0)	0/4 (0)	2/4 (50)
Glioblastoma	1/11 (9)	1/11 (9)	2/11 (18)	4/11 (37)
Total	4/24 (17)	4/24 (17)	6/24 (25)	11/24 (46)

[1] A ganglioglioma showed allelic loss of both ERCC1 and ERCC2.
[2] Both tumors showed allelic loss of ERCC1 and ERCC2.

Discussion

DNA repair is important in the genesis of a number of malignancies, including those associated with Xeroderma pigmentosum [21, 22], ataxia telangiectasia [23, 24], human non-polyposis colon cancer [11] and possibly other human cancers [25]. Whereas O6-alkylguanine DNA methyltransferase has been associated with chemotherapeutic resistance in gliomas [16], the association of specific abnormalities of DNA repair genes has not been previously associated with this disease. In this report, we document abnormalities of DNA gene copy number for the human nucleotide excision repair genes ERCC1 and ERCC2, which occur in up to 46% of tumor samples prior to either radiation or chemotherapeutic intervention.

Abnormalities of the region of chromosome 19q13 have been associated with a variety of brain tumors of glial origin in several recent reports [1–4]. Loss of heterozygosity has been noted in the 19q13.2–13.4 region using minisatellite and other polymorphic probes [1–4]. The common area of deletion spans a region of about 8 centimorgans, and includes genes APOC2, ERCC2, ERCC1, DNA ligase I, DM and HRC [4, 5]. The changes reported in brain tumor specimens are variable, and appear to differ in different tumor subtypes. However, this is the first report of increases as well as decreases in gene copy number, for one or more genes in this region. The changes noted in our specimens may reflect the inherent genomic instability of the tumors, or may be related to this region being particularly affected subsequent to tumor development. Further studies will be necessary to define the role (if any) of these copy number changes on glial tumorigenesis.

mRNA expression of ERCC1 and ERCC2 has been studied in human glioma and ovarian tumor specimens by this group [13, 14, 26]. In patients with gliomas, matched malignant and non-malignant tissues were studied for mRNA expression of these two genes. Excellent concordance of expression of these two genes was seen in normal non-malignant tissues; however, disordered expression of the two genes was noted in malignant tissues [26]. We believe a reason for disordered expression at the mRNA level in malignant brain tissues may be related to copy number abnormalities of genomic DNA for these two genes. In human ovarian cancer, disordered expression of ERCC1 and ERCC2 has also been observed [13, 14], and studies of DNA copy number are in progress. Further studies will be needed correlating copy number changes and mRNA and protein expression to substantiate this hypothesis.

Increased mRNA expression of ERCC1 is associated with clinical resistance to DNA damaging chemotherapy in human ovarian cancer [13, 14], and such studies are in progress in human malignant gliomas in this laboratory. The current study did not show a direct relationship between DNA copy number for ERCC1 and clinical outcome in this disease. This may possibly reflect a lesser role of these genes in resistance to the nitrosoureas such as BCNU, or the result of the small sample size studied. Nevertheless, we believe additional studies of DNA repair genes are warranted in neoplasms of the brain and ovary, as well as other malignancies where DNA damaging therapy is frequently employed.

Table 3. ERCC status and clinical outcome in anaplastic astrocytoma and glioblastoma

	n	Median # courses of chemotherapy (range)	Median survival (range) (months)
Abnormal ERCC1/2 copy number	6	4 (0–8)	17 (6–33)
Normal ERCC1/2 copy number	9	3 (0–9)	15 (1–34)

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