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Bcl-2 and p53 protein expression in all grades of astrocytomas

By: Robyn L. Castleberg

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INTRODUCTION

An astrocyte is a brain cell which produces processes that attach to blood vessels and act as supporter cells to the brain parenchyma. However, if these cells undergo transformation they are no longer referred to as astrocytes, but instead are labeled astrocytomas. These tumor cells are classified as Primary Intercranial Tumors (origin is the brain) and are diagnosed according to their severity. The diagnoses range from low-grade astrocytomas (World Health Organization or WHO, grade I or II), which grow slowly and may have a latent period, to fast-growing anaplastic astrocytomas (WHO grade III) and glioblastoma multiforme (GBM, WHO grade IV), which produce fatal effects much faster (Henson, in press). A principle feature of astrocytomas is progression toward more malignant forms. Therefore, though grade II astrocytomas seem to have the best prognosis they too will eventually produce rapidly enlarging lesions with anaplastic histological features. This proclivity of all grades of astrocytomas to move towards anaplasia is the reason for the poor clinical outcome of patients with astrocytomas (Henson, in press).

Due to the poor prognosis of all types of astrocytomas, treatments such as radiation and chemotherapy have been used to significantly detour the final result of this primary intracranial tumor. However, sometimes these treatments fail to elicit the desired results and the astrocytomas either do not respond to treatment or produce a resistant cell population upon relapse of the originally responsive astrocytoma (Lowe 1993). Consequently, many experimental studies have begun to look at the underlying basis of cellular resistance to anticancer agents. For example, the recent discovery of the bcl-2 (B-cell lymphoma type II) gene and its derivative protein is helping to answer the question of why some cells with DNA damage or deregulated proliferations are not following the expected apoptotic (programmed cell death) pathway.
The bcl-2 gene, discovered in follicular B-cell lymphomas and found in cell lines which are characterized by apoptotic cell turnover, produces an inner mitochondrial membrane protein (Hockenbury 1991). Moreover, when the bcl-2 protein is overexpressed it counteracts the wild-type (normal) p53 protein, which initiates apoptosis (Louis 1994). The resulting suppression of the p53 protein allows the cell to become immortalized, anaplastic, or genomically instable, which produces prime conditions for oncogenesis (Figure 1) (Louis 1994). These results indicate the importance of bcl-2 in the survival of tumor cells and suggest one reason why some cells are resistant even after anticancer agents have damaged cellular DNA or other apoptotic initiators. Accordingly, understanding the mechanism or catalyst which oncogenic cells use to initiate overproduction of bcl-2 and inactivation of p53 may determine the success of cancer therapy.

In light of the above understanding of the interaction between bcl-2, p53, and apoptosis, scientists have examined many tumor tissues (breast, skin, prostate, etc.) looking for the expression of bcl-2 (Hockenbury 1991). However, one oncogenic tissue on which bcl-2 expression has not been researched is the astrocytoma. Therefore, in order to see if bcl-2 is overexpressed in astrocytomas foregoing apoptosis and if bcl-2's expression is related to the type of p53 present (wild type or mutant), both bcl-2 and p53 immunohistochemistry were performed on all grades of astrocytomas. Also, single stranded conformational polymorphism (SSCP) was performed on the p53 gene from each case to determine if it was mutant or wild type.

METHODS AND MATERIALS

Cases. All tumors were cerebral hemispheric astrocytomas from adults that were classified, according to the WHO criteria, by a neuropathologist, David N. Louis. One case was WHO grade I, five cases were WHO grade II, twelve cases were WHO grade III, and eleven cases were
WHO grade IV or GBM. All cases were from primary resection specimens. Paraffin embedded tissue was available for immunohistochemistry on all cases and tumor DNA was available for single stranded conformational polymorphism (SSCP).

**Immunohistochemistry.** 6-μm sections of paraffin embedded tumors were cut with a microtome and were heat-fixed unto Poly-L-lysine coated slides. The paraffin was removed using xylene and the tissue was rehydrated with ethanol. Sections used for p53 staining were blocked with normal horse serum and incubated with p53 antibodies (PAb 1801 Oncogene Sci), diluted 1:1000, overnight at 4°C. The sections were then exposed to biotinylated horse anti-mouse IgG and Streptavidin-horseradish peroxidase (ABC reagent) followed by diaminobenzidine (Vectastain DAB Kit), which stains antibodies brown, and methyl green (a green counterstain to diaminobenzidine). Sections for bcl-2 staining were boiled in sodium-citrate (pH 6) for 15 minutes, blocked with normal horse serum, and incubated with bcl-2 antibodies (Dako), diluted 1:250, overnight at 4°C. Biotinylated horse anti-mouse IgG and ABC reagent were then applied to the sections, and diaminobenzidine solution and methyl green staining followed, respectively. Controls were performed for both p53 and bcl-2 by omitting the primary antibody and by staining tissue sections of oncogenic human lymphnode as positive and negative controls. The slides were evaluated for the presence and proportion of positively stained cells and for the subcellular localization of the reaction product by a neuropathologist, David N. Louis.

**SSCP Analysis.** SSCP was performed as described (Louis et. al. 1993), and exons 5 through 8 of the p53 gene were amplified from tumor DNA using PCR conditions and oligonucleotides according to protocol (Louis et. al. 1993). The amplification products were separated on 6% non-denaturing polyacrylamide gels with 10% glycerol overnight at 4 Watts. The gels were dried and exposed to autoradiography film.
RESULTS:

bcl-2 and p53 protein expression in astrocytomas.

To determine whether the 29 astrocytomas express the protein bcl-2 and p53, these tumors were studied with immunohistochemistry using two antibodies, anti-bcl-2 (Dako) and anti-p53 (PAb 1801 Oncogene Sci). Anti-bcl-2 (Dako) is a monoclonal mouse antibody which reacts specifically with the integral membrane bcl-2 oncoprotein located in the mitochondria (DAKO Specification Sheet). Positive reaction was observed only in cytoplasm and varied in intensity and population, with some cytoplasm staining stronger and/or more frequently than others (Fig. 2). Furthermore, all but one of the 29 cases stained positive for the bcl-2 protein (Table 1).

To detect the p53 protein, the monoclonal antibody for p53 (PAb 1801 Oncogene Sci), which is human-specific for the NH₂ terminus of both wild type and mutant p53 protein, was used (Rubio 1993). Immunohistochemistry with Pab 1801 showed variable positive nuclear staining, with some nuclei staining darker than others (Fig. 3). Also, many of the astrocytomas did not show any staining and were therefore labeled negative (Table 1).

The positive control lymphoma sections for both bcl-2 and p53 showed moderate to strong staining in the cytoplasm and nucleus, respectively (Fig. 4). Furthermore, the negative control lymphoma sections in which primary antibody was omitted or an irrelevant antibody was used, resulted in no immunohistochemical reaction for either bcl-2 or p53 (Fig. 4).

SSCP on p53 genes from all astrocytoma cases.

SSCP analysis showed three cases to have mutations in an area between exon 5 and exon 8 (Table 1). One case had a mutation in exon 6, a second case showed a mutation in exon 5, and
a third case expressed a frameshift mutation at codon 262 (this frameshift was sequenced at an
earlier time). The other 26 cases expressed wild-type amino acid sequences for exons 5-8.

DISCUSSION

Due to the significance of apoptosis to developmental processes, major interest has arisen
in genes capable of regulating programmed cell death. One of the most important strides in our
understanding of apoptotic cell death regulation in vertebrates has come from studies of the
oncogene bcl-2 (Boise et. al. 1993). Overexpression of bcl-2 protein blocks the programmed cell
death, which means it also takes precedence over the p53 protein that plays a role in initiating
apoptosis (Williams and Smith 1993). Furthermore, many different cell tissue types have
expressed the bcl-2 protein, however, research on bcl-2 expression in astrocytomas had not
transpired before now.

In our study, we showed that bcl-2 protein is expressed in all grades of astrocytomas,
whether p53 was or was not present. To begin our research, we performed
immunohistochemistry on lymphoma sections which were known to positively express p53 and
bcl-2 protein. This allowed for fine-tuning of both immunohistochemical procedures before
astrocytomas sections were used. In the procedural testing on p53, it was found that the best
staining occurred when the diaminobenzidine from the Vectastain DAB Kit was used in place of
the Working DAB solution, and when the biotinylated horse-anti-mouse antibody was diluted
1:400 instead of 1:500 (which the original procedure required). After the protocol for p53 was
determined, work on the bcl-2 procedure began.

Like p53, the bcl-2 protocol also required some changes in order to improve staining.
The first lymphomas stained for bcl-2 produced high background staining and therefore the
dilutions of primary antibody were adjusted. It was found that the primary antibody (anti-bcl-2) diluted 1:250 worked much better than the original dilution of 1:40. After this adjustment was made, the background staining was virtually eliminated. However, because some cases persisted in showing slight background staining, a primary antibody was added to the control tissues in order to show anti-bcl-2 (Dako) was actually staining bcl-2 protein and not some other element. The primary antibody used on the controls was anti-Large T antibody. Its use did not change the staining pattern and therefore supported the effectiveness of anti-bcl-2 (Dako) in recognizing the bcl-2 protein.

Though both of the procedures were modified to give the best results, there were still two cases which were unidentifiable after the procedural changes were implemented. Both cases had an excessive amount of background staining and therefore made it impossible to accurately estimate either p53 or bcl-2 expression. Because immunohistochemistry did not yield any results on these two cases, it was decided not to perform SSCP on their p53 genes.

After the immunohistochemistry results were known for both p53 and bcl-2 on all twenty-nine cases, SSCP was performed on the p53 gene from the twenty-seven identifiable cases. If the p53 gene was wild type, then it would be able to perform apoptosis. However, if it were mutant it would not be able to initiate apoptosis (Louis 1994). Therefore, in cases expressing mutant p53, bcl-2 overexpression would not be expected because the cell can not initiate programmed cell death with mutant p53.

The SSCP results on exons 5 through 8 showed the p53 gene was mutant in three cases. When this information was compared to the bcl-2 results it was found that when p53 was wild type, bcl-2 was usually overexpressed in more than two-thirds of all the astrocytomas in that case. However, it was found that the three mutant p53 cases expressed bcl-2 in less than one-third of
all the astrocytomas in each specific section (Table 1). Therefore, these results help support the conclusion that bcl-2 is not greatly overexpressed when p53 is mutant.

In this research project, new information on astrocytomas was obtained. Our findings added astrocytomas to the list of tissues expressing bcl-2 and they suggest that bcl-2 expression may be controlled by the type of p53 present. Bcl-2 seems to be overexpressed when p53 is wild type, but when p53 is mutant, bcl-2 is minimally expressed in the astrocytoma section. Though this research has furthered scientists' understanding of bcl-2, p53, and astrocytomas, more studies need to be performed to better establish our findings.

One such study, which I feel would be beneficial, would be to conduct research on what types of cancer treatments induce the most bcl-2 protein. To do this for astrocytomas, one would need to evaluate different tissue sections which are similar in most aspects but differed in the type of treatment (radiation, chemotherapy, etc.). Along with this study, it would be advantageous to evaluate other tumor tissues to see if there is any difference between astrocytomas and other cancer types. When completed, this study would allow physicians to chose the best treatment plan for their patient. It would also further scientist's knowledge about cancer which would move the medical world one step closer to finding a cure for cancer.
WORKS CITED


Henson, J. W., B. Schnitker, K Correa, A. vonDeimling, F. Fassbender, Hong-Ji Xu, W. Benedict, D. Yandell and D. Louis, The retinoblastoma gene is involved in malignant progression of astorcytomas. in press.


TABLE 1. Classification, Expression and mutation analysis

<table>
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+ less than 1/3 expression  
++ between 1/3 and 2/3 expression  
+++ More than 2/3 expression
Figure 1. Normal functions of wild-type p53 (top) and probable abnormal functions of mutant p53 (bottom) (Louis 1994).
Figure 2. Immunohistochemistry on paraffin-embedded astrocytomas for bcl-2. Astrocytomas (WHO grade III) demonstrating abundant bcl-2 staining of the cytoplasm in tumor cells (top) and glioblastoma multiforme showing moderate bcl-2 staining in the cytoplasm (bottom) (All 400X).
Figure 3. Immunohistochemistry on paraffin-embedded astrocytomas for p53. A moderate amount of p53 nuclear staining is expressed in the tumor cells of this astrocytoma (WHO grade III) (400X).
Figure 4. Immunohistochemistry on paraffin-embedded lymphomas and astrocytomas for bcl-2. Lymphomas incubated with an irrelevant antibody demonstrates no cytoplasmic staining (top left). Lymphoma showing positive bcl-2 staining in the cytoplasm of tumor cells (top right). 4C. bcl-2 positive astrocytoma (WHO grade III) incubated with an irrelevant antibody expresses no cytoplasmic staining (bottom) (All 400X).