THE HYPOXIA INDUCIBLE CELL DEATH GENE BNIP3 IS MUTATED IN GLIOBLASTOMA MULTIFORME (GBM)

By

Fen Xue

A thesis submitted to the Faculty of Graduate studies
In partial fulfillment of the requirement
For the degree of

Master of Science

Department of Pathology
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Many thank go to my family and friends
for your never-ending support and love
especially my parents.

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LIST OF ABBREVIATIONS

AIF	apoptosis-inducing factor
Apaf-1	Apoptotic Protease Activating Factor1
Bax	Bcl-2 associated X protein
Bcl-2	B-cell leukemia/lymphoma 2
BNIP3	the Bcl-2 /adenovirus E1B 19Kda interacting protein-3
CD	conserved domain
cDNA	complementary DNA
CNS	central nervous system
ddNTP	dideoxy nucleotide triphosphate
EM	electron microscopy
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
HIF-1α	hypoxia-inducible factor 1α
HRE	hypoxia response element
HSP60	Heat Shock Protein-60
PBS	Phosphate-buffered saline
PCD	programmed cell death
PT	permeability transition
ROS	radical oxygen species
PT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SSCP	single-strand conformation polymorphism
TM	transmembrane
$\Delta\psi m$	transmembrane potential

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ABSTRACT

Glioblastoma multiforme, WHO grade IV astrocytoma is the most common and aggressive form of brain tumor with a median survival outcome of less than one year and is resistant to radiation and chemotherapy. BNIP3 (the Bcl-2 /adenovirus E1B 19Kda interacting protein) protein expression is increased in GBM and is a hypoxia-inducible pro-apoptotic Bcl-2 (B-cell leukemia/lymphoma 2) family member activated by hypoxia-inducible factor 1α (HIF-1α). It binds to mitochondria through its transmembrane (TM) domain, causes mitochondrial damage initiated by rapid opening of the mitochondrial permeability transition (PT) pore, and then induces cell death. We double immunostained paraffin embedded GBM tissue sections using BNIP3 and glial fibrillary acidic protein (GFAP) or cytochrome *c* (mitochondrial marker) antibodies. BNIP3 of primary GBM tumors in part was localized predominantly in cytoplasm with mitochondria and BNIP3 was co-expressed with GFAP in malignant astrocytes of primary GBM tumors.

Although BNIP3 protein is over-expressed in GBM, it paradoxically fails to induce cell death. GBM has extensive regions of hypoxia that is associated with a poor prognosis. Under chronic hypoxic stress, DNA instability occurs leading to selection of mutations. We detected mutations in the PEST sequence of BNIP3 of 9/55 (17%) primary GBM by RT-PCR that predict a truncated protein without a functional TM domain. These cDNA mutations were confirmed by single-strand conformation polymorphism (SSCP) analysis and DNA sequencing.

Our results suggest that BNIP3–induced cell death is reduced by the mutations of BNIP3 in primary GBM. This prevents BNIP3's integration with mitochondria. BNIP3 mutations may represent a common mechanism for GBM tumor cells to survive under hypoxia. This research will be helpful for a better understanding of GBM, identification of aggressive and treatment resistant forms, and may lead to the development of targeted therapies mitigating the effects of mutant BNIP3 on malignant glioma tumorigenesis.

INTRODUCTION

1. Molecular Mechanisms of Hypoxia Responsiveness

1.1. Hypoxia

Oxygen homeostasis is essential for our life. Hypoxia is a state of oxygen deficiency in inspired gases or in arterial blood and/or in the tissues that is sufficient to cause an impairment of function. Hypoxic tissues experience a very low availability of oxygen (less than 0.1-1 mmHg), compared with normal physiological levels/normoxia (arterial blood 95 mmHg, venous blood 40 mmHg, tissues 40 mmHg) or the complete absence of oxygen (anoxia), which is not seen clinically. Hypoxia is caused by the reduction in partial pressure of oxygen, inadequate oxygen transport, or the inability of the tissues to use oxygen.

Hypoxia is divided into four types:

Hypoxic hypoxia is a reduction in the amount of oxygen passing into the blood and results from defective oxygenation of the blood in the lungs.

Hypemic hypoxia refers to a reduction in the oxygen carrying capacity of the blood and results from a decreased concentration of hemoglobin in the blood or a reduced number of red blood cells.

Histotoxic hypoxia is characterized as the inability of the tissues to use oxygen in the presence of sufficient blood flow. This may occur due to the poisoning, for example

carbon monoxide, certain narcotics, chewing tobacco, and alcohol, thereby preventing oxygen use by the tissues.

Stagnant hypoxia (ischemic) is defined as an oxygen deficiency due to insufficient blood flow and results from slow peripheral circulation (Semenza *et al.*, 2000).

1.2. Apoptosis and Necrosis

Cell death involves two general processes: necrosis and apoptosis.

Apoptosis, or programmed cell death (PCD), is a process of deliberate suicide by an unwanted cell in a multicellular organism. Cells die in response to a variety of stimuli. Apoptosis is an important process during normal development. It is also involved in aging and various diseases such as cancer, AIDS, Alzheimer's disease and Parkinson's disease.

Necrosis means unprogrammed death of cells/living tissue (compare with apoptosis - programmed cell death). This can happen when not enough blood is supplied to the tissue, whether from injury, infection, infarction, cancer, radiation, or chemicals. Once necrosis occurs, it is not reversible.

During apoptosis, cells die in a controlled, regulated fashion. Apoptosis is distinguished from necrosis in which uncontrolled passive cell death leads to lysis of cells, inflammatory responses and serious health problems. Apoptosis, by contrast, is carried out in an ordered process that generally confers advantages during an organism's life cycle.

Apoptosis is characterized by chromatin condensation and cell shrinkage in the early stage. Then the nucleus and cytoplasm fragment, forming membrane-bound apoptotic bodies, which are surrounded by a piece of cell membrane and contain intact, functional cell components. In contrast, cells undergoing necrosis swell and rupture. The released intracellular contents can damage surrounding cells and often cause inflammation (Formigli *et al*, 2000; Brunelle *et al*, 2002).

1.3. Hypoxia and Solid Tumors

Solid tumors are the tumors of body tissues other than blood, bone marrow or the lymphatic system. The blood vessels supplying tumours are relatively poorly organised and disordered compared with those of normal tissues, leading to inefficient delivery of oxygen and other nutrients to many of the cells in tumours.

Two characteristics—hypoxia and necrosis—represent clear differences between tumors and normal tissues. Solid tumors are dependent on adequate oxygen delivery. Angiogenesis, the formation of new blood vessels, is an important step in the progression of these tumors. But neovascularization often lags behind the expanding tumor mass. Prolonged hypoxia of the tumor tissue also leads to necrosis, and necrotic regions are also characteristic of solid tumors (Kunz *et al*, 2003; Dewhirst, 2003).

Human solid tumors are invariably less well oxygenated than normal tissues. This leads to resistance to radiotherapy due to the low level of free oxygen radicals produced by the radiation in hypoxic cells compared to oxygenated cells (Marples *et al*, 2003; Sannazzari *et al*, 2002). There is also resistance to anticancer chemotherapy because the

delivery of chemotherapeutic drugs is impaired in hypoxic regions of tumors (Kunz *et al*, 2003; Knisely *et al*, 2002). Hypoxia may also contribute to processes that directly favor malignant progression and aggressiveness through effects on the expression and activity of tumor suppressor proteins (Zagzag *et al*, 2000; Kunz *et al*, 2003; Pennacchietti *et al*, 2003).

Hypoxic regions within solid tumors such as breast cancer express high levels of BNIP3 (Guo et al, 2001; Sowter et al, 2001).

1.4. Hypoxia and Mutation

A fundamental characteristic of cancer is the generation of tumor cell heterogeneity, i.e. cells with multiple mutated phenotypes, through a mechanism of genetic instability (Pienta et al, 1994; Nowak et al, 2002). Two possible contributors of genetic instability are increased mutation rate and impaired DNA repair activity. The tumor microenvironment is characterized by regions of fluctuating hypoxia, low pH, and nutrient deprivation. Under chronic hypoxic stress, DNA instability occurs leading to selection of mutations. The microenvironment of a solid tumor is itself mutagenic and an important source of genetic instability (Reynolds et al, 1996).

2. Glioblastoma Multiforme Tumors (GBM)

Brain tumors belong to the solid tumor category. They can arise from all cells of the central nervous system (CNS) including glial (astrocytic, oligodendroglial,

ependymal) and neuronal cells. In adults, the majority of brain tumors originate in glial cell lineages (predominantly astrocytomas) (Kleihues *et al*, 2002; DeAngelis *et al*, 2001).

Glioblastoma multiforme (GBM) is the most common and most malignant of the glial tumors. Composed of poorly differentiated neoplastic astrocytes, glioblastomas primarily affect adults, and they are located mainly in the cerebral hemispheres. Much less commonly, GBMs can affect the brain stem in children and the spinal cord. These tumors are World Health Organization (WHO) grade IV astrocytomas that make up about 40%-50% of adult brain tumors (Kleihues *et al*, 2002). Glioblastomas can be classified as primary or secondary. Primary GBMs usually arise *de novo* (e.g. without clinical or histopathological evidence of a pre-existing, less malignant precursor lesion) in adults older than 50 years and have usually a less than 3-month clinical history. Secondary GBMs typically develop in younger patients (<45 y) through malignant progression from a low-grade astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III). The time required for this progression varies considerably, ranging from less than 1 year to more than 10 years. The treatment of glioblastomas is palliative and includes surgery, radiotherapy, and chemotherapy.

GBM tumors show that BNIP3 expression is increased when compared to normal brain (Figure 3, Dr. D.D. Eisenstat)

3. Role of BNIP3 in Hypoxia-Induced Cell Death in GBM

3.1. BNIP3

Bcl-2 (B-cell leukemia/lymphoma 2), an oncogene that in follicular lymphoma is frequently linked to an immunoglobulin locus by the chromosome translocation t(14:18), was the first example of an oncogene that inhibits cell death rather than promoting proliferation. The Bcl-2 proteins are a family of proteins involved in the response to apoptosis and divided into two groups: anti-apoptotic proteins such as Bcl-2 and Bcl-X_L and pro-apoptotic proteins such as Bad or Bax (Bcl-2 associated X protein). Bcl-2 family members regulate both necrosis and apoptosis during hypoxia (MacCarthy-Morrogh *et al*, 1999). Over-expression of the anti-apoptotic proteins inhibits both apoptosis and necrosis induced by hypoxia. In contrast, the pro-apoptotic proteins promote hypoxia-induced apoptosis, but not necrosis (Cuisnier *et al*, 2003; Tanaka *et al*, 2003). Typically, the BH3 domain of pro-apoptotic Bcl-2 homologues mediates Bcl-2/Bcl-X_L heterodimerization and confers pro-apoptotic activity by association with the mitochondria (Gross *et al*, 1998).

The Bcl-2 /adenovirus E1B 19Kda interacting protein, BNIP3 (formerly NIP3), is a pro-apoptotic Bcl-2 (B-cell leukemia/lymphoma 2) family member that was originally identified in a yeast two-hybrid screen using the potential binding partners of the adenoviral Bcl-2 functional homologue, the E1B 19K protein. E1B 19K and Bcl-2 bind to BNIP3 via regions of shared homology (Boyd *et al*, 1994).

BNIP3 was subsequently cloned and predicted to be 194 amino acids (Boyd *et al*, 1994). The level of BNIP3 mRNA in mouse tissues was examined and two transcripts were detected. The 1.7 kb transcript is found in all tissues and the 2.5 kb transcript in only certain tissues such as brain, heart, kidney, liver, and submaxillary gland. In human breast tissues, BNIP3 mRNA is expressed in human breast carcinoma MCF-7 as a major transcript of 1.7 kb and two minor transcripts of 1.5 and 1.3 kb (Chen *et al*, 1997). In other human tissues, the two transcripts were apparent and the larger was prominently expressed in brain and heart (Yasuda *et al*, 1998b).

3.2. BNIP3 Structure and Function (Figure 1)

The structure of BNIP3 is similar to other Bcl-2 family members. It contains a NH_2 (amino)-terminal PEST sequence, a BH3-like domain (BH3), a COOH (carboxyl)-terminal transmembrane (TM) domain and a unique conserved domain (CD) of 16 amino acids of unknown function.

The PEST sequences are rich in proline (P), glutamate (E), serine (S) and threonine (T). It is involved in rapid turnover of protein whose degradation is controlled by the proteosome (Rogers *et al.* 1986). Cells transfected by BNIP3 show decreasing amount of protein over an extended time course. Treatment with lactacystin, a proteasome inhibitor, permits an accumulation of protein (Chen *et al.*, 1999; Cizeau *et al.*, 2000).

The BNIP3 protein also contains a BH3-like domain, with limited sequence homology to the Bcl-2 homology 3 (BH3) domain. Typically, the BH3 domain of pro-apoptotic Bcl-2 homologues mediates Bcl-2/Bcl-X_L heterodimerization and confers pro-apoptotic activity. However, in contrast to other Bcl-2 family members, the removal of the BH3-like domain in BNIP3 does not affect its killing activity (Chen *et al.*, 1999; Cizeau *et al.*, 2000). The BH3 domain is a nine amino acid sequence that contains a conserved leucine and aspartate at positions 1 and 6, respectively. These two residues are involved in heterodimerzation with Bcl-2 proteins and the critical component of pro-apoptotic activity (Kelekar and Thompson, 1998). Residues 110-118 are thought to reveal the limited sequence homology to other BH3-only pro-apoptotic proteins (Yasuda et al., 1998; Ray et al., 2000).

The TM domain of BNIP3 at residues 164-184 is essential for BNIP3 homodimerization, mitochondrial localization and BNIP3-induced cell death. The homodimerization has been confirmed by two-dimensional electrophoresis of trypsin-digested fragments and the yeast two-hybrid assay (Chen *et al.*, 1997). Co-localization with the mitochondrial protein Heat Shock Protein-60 (HSP60) and the mitochondrial-specific stain Mitotracker demonstrated that the domain targets BNIP3 to the outer mitochondrial membrane (Chen *et al.*, 1997; Chen *et al.*, 1999; Cizeau *et al.*, 2000). Overexpression of BNIP3 induces apoptosis in Rat-1 cells at 12 hours post-transfection that was measured by chromatin condensation (Chen *et al.*, 1997). Expression of BNIP3 is not able to induce cell death when the TM domain was deleted (Chen *et al.*, 1999; Cizeau *et al.*, 2000). The endogenous BNIP3 is loosely associated with the mitochondria, and when it is overexpressed it is integrated into the mitochondrial membrane. BNIP3

over-expression induces plasma membrane permeability and mitochondrial damage initiated by rapid opening of the mitochondrial permeability transition (PT) pore, loss of transmembrane potential ($\Delta\psi m$) and production of radical oxygen species (ROS) (Yasuda *et al.*, 1999; Vande Velde *et al.*, 2000; Kim *et al.*, 2002). Following BNIP3 expression, electron microscopy (EM) images show extensive cytoplasmic vacuolization, autophagosomes, and mitochondrial deformation in which the cisternae were destroyed, but minimal nuclear damage (electron dense bodies) characteristic of necrosis. Furthermore, BNIP3-induced cell death is independent of caspase activation, cytochrome *c* release from mitochondria and cell shrinkage. Only during the later stages of BNIP3-induced cell death are features characteristic of apoptosis present, specifically chromatin condensation and DNA fragmentation (Vande Velde *et al.*, 2000).

The conserved domain (CD) of 16 amino acids in BNIP3 overlapping with the BH3-like domain (BH3) is conserved in BNIP3 throughout evolution but is not found in other Bcl-2 family members (Cizeau *et al.*, 2000). Similar to the BH-like domain, the deletion of the CD domain does not affect BNIP3's killing activity (Chen *et al.*, 1999; Cizeau *et al.*, 2000).

3.3. Role of Mitochondria

Mitochondria, which are comprised of two lipid membranes, are the primary energy producers of the cell and are essential to cell survival. They play an important role in the regulation of cell death by (1) release of cytochrome C, which together with Apaf-1 and ATP forms a complex with pro-caspase 9, leading to activation of caspase 9 and the caspase cascade, (2) disruption of ATP production, electron transport, and oxidative

phosphorylation, and (3) alteration of the redox potential, resulting in increased cellular oxidative stress (Green *et al*, 1998).

PT pore opening, $\Delta\psi m$ suppression and ROS production are all observed in apoptosis (Yasuda *et al.*, 1999; Vande Velde *et al.*, 2000; Kim *et al.*, 2002). The pro-apoptotic Bcl-2 proteins are often found in the cytosol where the act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic Bcl-2 proteins and can lead to the formation of pores in the mitochondria and the release of cytochrome *c* and other pro-apoptotic molecules from the intermembrane space. This leads to the formation of the apoptosome and the activation of the caspase cascade (Gross *et al.*, 1998; Gross *et al.*, 1999). One mitochondrial apoptogenic protein is apoptosis-inducing factor (AIF), a flavoprotein released in response to apoptotic signals that translocates to the nucleus to induce DNA fragmentation and chromatin condensation (Susin *et al*, 1999).

3.4. Cytochrome *c* Release

Cytochrome *c* is an essential component of the mitochondrial respiratory chain. It is a soluble protein, localized in the intermembrane space and loosely attached to the surface of the inner mitochondrial membrane. Cytochrome *c* is important to the process of creating cellular energy, the main function of mitochondria. When mitochondria are damaged, cytochrome *c* is released into the main body of the cell through the PT pore (Shimizu et al., 1999), and if the cell itself is damaged, into the surrounding tissue.

Cytochrome *c* was suggested to mediate (together with dATP) the formation of the Apaf-1 (Apoptotic Protease Activating Factor1)/Caspase-9 complex that results in activated Caspase-9. Caspase-9 is thought to trigger a caspase-cascade leading to apoptosis, so cytochrome *c* release from mitochondria is indicative of apoptosis (Reed, 1997b).

3.5. Glial Fibrillary Acidic Protein (GFAP)

Glial fibrillary acidic protein (GFAP) is one of the major structural (intermediate filament) proteins in astrocytes, and its expression is markedly up-regulated following injury. If the central nervous system is injured through trauma or disease, astrocytes react by rapidly producing more glial fibrillary acidic protein. GFAP is probably involved in controlling the shape and movement of astrocytes and assists in maintaining the protective barrier that allows only certain substances to pass between blood vessels and the brain (blood-brain barrier). The protein probably also plays a significant role in the interactions of astrocytes with other cells. BNIP3 co-localizes with GFAP in malignant astrocytes (Burton *et al*, 2003, submitted).

3.6. Hypoxia-inducible factor 1 (HIF-1)

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric protein composed of two subunits – HIF-1α and amino terminal HIF-1ß. Under oxygenated conditions (normoxia), HIF-1α is rapidly degraded, but HIF-1α degradation is blocked under low oxygen conditions. HIF-1 mediates tumor cell responses to changes in oxygen concentration and belongs to a class of molecules termed transcription factors. HIF-1ß is constitutively expressed, but when the levels of HIF-1α are high, then HIF-1 transcription activity is

initiated. HIF –1 binds to the hypoxia response element (HRE, with the core sequence of 5' CGTG-3'). By binding to the HRE, present in the promoter/enhancer regions of target genes, HIF1 increases the expression of these target genes in response to hypoxia. In human cancers, HIF-1α is overexpressed as a result of intratumoral hypoxia and genetic alterations affecting key oncogenes and tumor suppressor genes (Ijer *et al.,* 1998; Semenza *et al.,* 1997).

The biological activity of transcription factor HIF-1 is determined by the expression and activity of the HIF-1α subunit. HIF-1α contributes significantly to the pathophysiology of major categories of human disease, including myocardial and cerebral ischemia, cancer, pulmonary hypertension, congenital heart disease and chronic obstructive pulmonary disease. HIF-1 is a nuclear protein that activates gene transcription in response to reduced cellular O_2 concentration and has been shown to directly induce the transcription of the related pro-apoptotic BNIP3 (Kothari *et al*, 2004). Expression of these genes has been associated with induction of VEGF, BNIP3 and NIX (Mu *et al*, 2003; Sowter *et al*, 2003).

3.7. Role of BNIP3 in Hypoxia-Induced Cell Death in GBM (Figure 2)

Astrocytomas, a class of malignant brain tumors, are very oxygen dependent (Brat *et al*, 2003). The low-grade astrocytomas first acquire their blood supply by coopting existing normal brain blood vessels without initiating angiogenesis. However, when grade III astrocytomas progress to grade IV astrocytomas/glioblastomas or GBM, they have the features of hypoxic and necrotic areas. The evolution of these areas results from vessel regression (Holash *et al*, 1999a) and increased tumor cell proliferation (Brat *et al*, 2002).

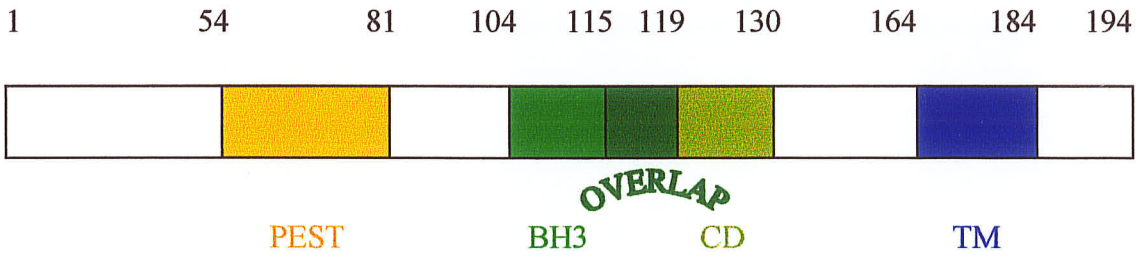
Mitochondria play a central role in the induction of cell death. HIF-1 is overexpressed in human glioblastoma biopsies and the level of expression is correlated with GBM progression (Zagzag *et al*, 2000). BNIP3 is induced in hypoxic regions of many solid tumors and is activated by the transcription factor HIF-1α. BNIP3 may cause necrosis-like cell death through the mitochondrial permeability transition pores. The BNIP3 promoter contains hypoxia response elements (HREs) regulated by HIF-1α and, when transfected under hypoxic conditions, the promoter is activated (Kothari *et al*, 2003). BNIP3 mediates cell death in a caspase-independent manner through its interaction with the mitochondria.

Under hypoxia, the overexpressed nuclear protein HIF-1 in GBM tumor cells binds to HREs on the BNIP3 promoter and activates BNIP3 by HIF- 1α. The endogenous BNIP3 is loosely associated with the outer mitochondrial membrane through its transmembrane (TM) domain, and when it is overexpressed it is integrated. BNIP3 induces rapid opening of mitochondrial PT pore accompanied by $\Delta\Psi$m suppression and increased ROS production (Yasuda *et al.*, 1999; Vande Velde *et al.*, 2000; Kim *et al.*, 2002). This causes mitochondrial damage and the release of cytochrome *c*, which functions as a cofactor with dATP for Apaf-1 binding and activation of caspase 9 and downstream effector caspases, and other pro-apoptotic molecules from the intermembrane space, then leads to the formation of the apoptosome and the activation of the caspase cascade leading to apoptosis (Gross *et al.*, 1998; Gross *et al.*, 1999). The mitochondrial apoptogenic protein is apoptosis-inducing factor (AIF), a flavoprotein

released in response to apoptotic signals that translocates to the nucleus to induce DNA fragmentation and chromatin condensation in a caspase-independent manner (Susin *et al*, 1999). Finally, BNIP3 induces cell death.

In conclusion, overexpression of BNIP3 initiates a cell death pathway activated by protein integration into the outer mitochondrial membrane. This requires PT pore opening. Cell death manifests as mitochondrial dysfunction, plasma membrane damage, and the morphology of necrosis. BNIP3-induce cell death is independent of caspases activation, cytochrome *c* release from mitochondria and cell shrinkage (Vande Velde *et al.*, 2000).

Figure 1: Schematic diagram of the BNIP3 Protein



PEST – PEST sequences are involved in rapid turnover of BNIP3 protein whose degradation is controlled by the proteosome

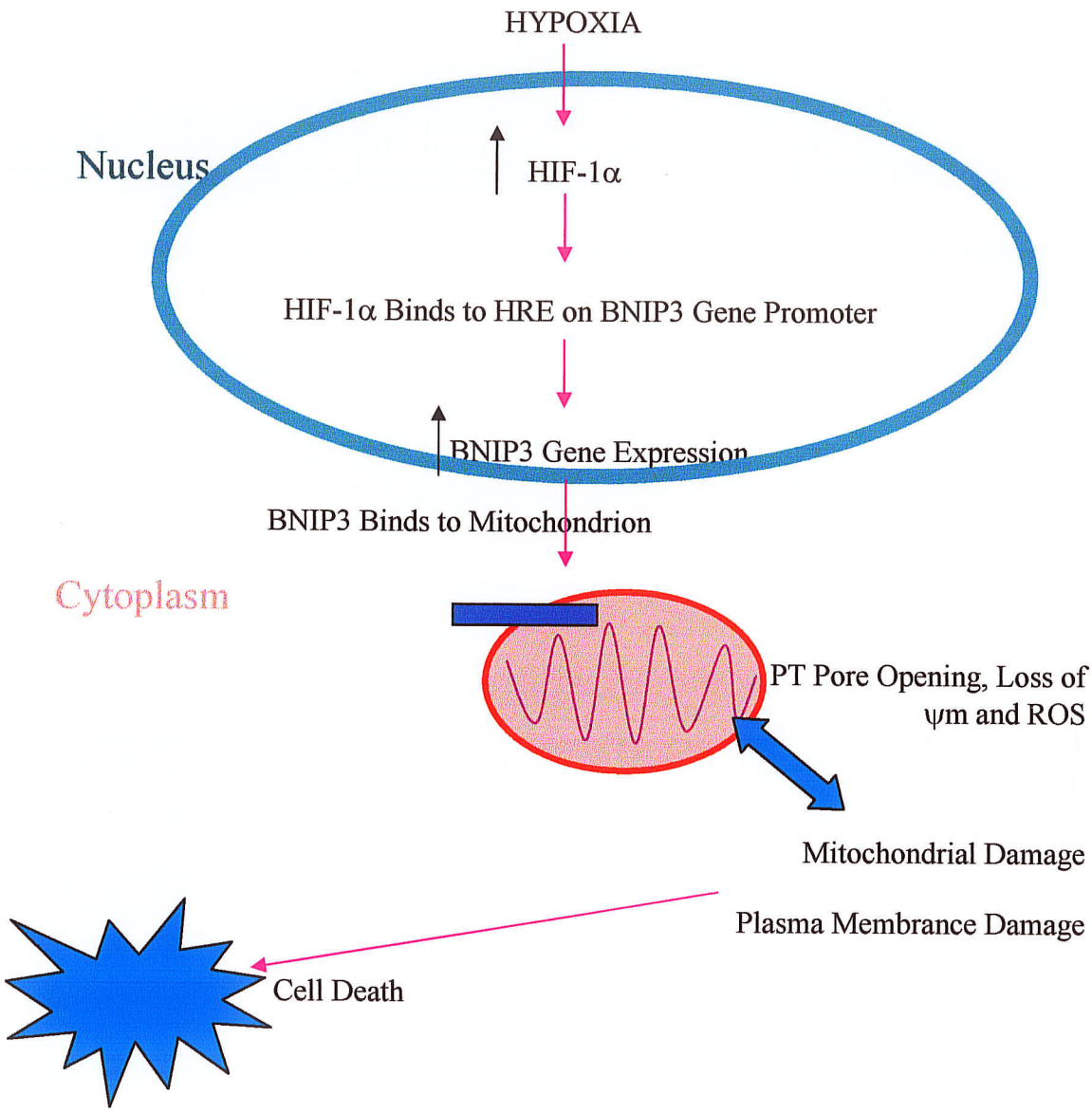
BH3 – BH3 domain facilitates heterodimerization with antagonists Bcl2/Bcl-xL and promotes cell death

CD – Conserved domain stretch of 16 amino acids that are evolutionary conserved from C.*elegans* to mammals

TD – Transmembrane domain targets protein to the outer mitochondrial membrane and is required to induce cell death

OVERLAP - The overlap of BH3 and CD domains

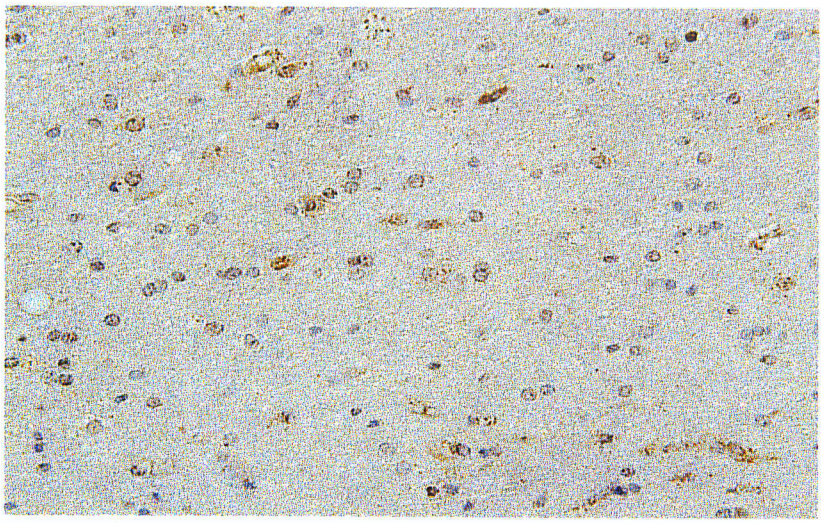
Figure 2: Role of BNIP3 in Hypoxia-Induced Cell Death



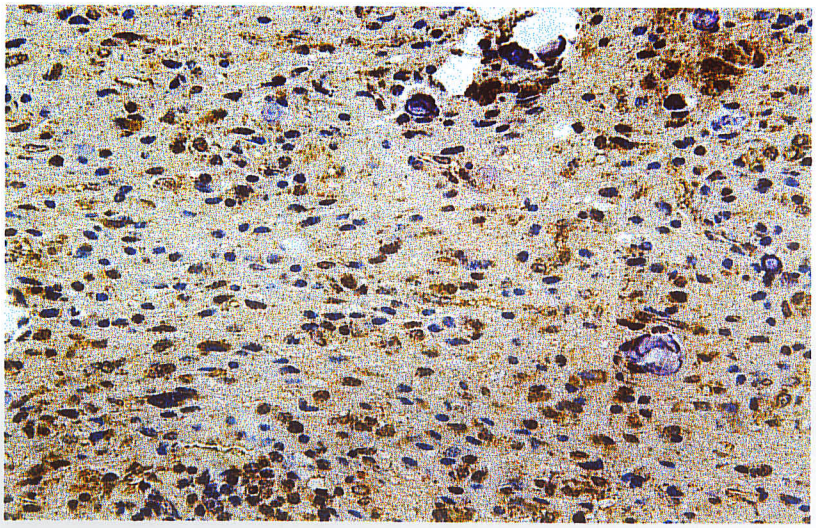
The endogenous BNIP3 is loosely associated with the mitochondria. Under hypoxia, the overexpressed nuclear protein HIF-1 in GBM tumor cells binds to HREs on the BNIP3 promoter and activates BNIP3 by HIF- 1α. When BNIP3 is overexpressed it is integrated. BNIP3 over-expression induces plasma membrane permeability and mitochondrial damage initiated by rapid opening of the mitochondrial permeability transition (PT) pore, loss of transmembrane potential (Δψm), and production of radical oxygen species (ROS). Finally, BNIP3 induces cell death.

Figure 3: BNIP3 Expression Is Increased In Primary GBM Tumors Compare To Normal Brain (Dr. D.D. Eisenstat)

Normal brain or primary GBM paraffin-embedded tumor tissues were immuno-stained with antibodies against BNIP3 (brown). The DNA was counterstained with hematoxylin (blue). BNIP3 is expressed at high levels in primary GBM tumors.



Normal Brain



Primary GBM

RATIONALE AND HYPOTHESIS

BNIP3 in hypoxic regions of GBM is activated by the transcription factor HIF-1α. BNIP3 mediates cell death in a caspase –independent manner through its interaction with mitochondria. This is facilitated by binding of BNIP3 to the mitochondria membrane through its transmembrane (TM) domain. BNIP3 causes necrosis-like cell death through rapid opening of the mitochondrial PT pores. Hypoxia upregulates BNIP3 expression. The elevated level of BNIP3 within GBM should correlate with increased cell death. Paradoxically, GBM tumors show that BNIP3 expression is increased compare to normal brain (Figure 3, Dr. D.D. Eisenstat), but tumor cells remain viable. Why is a cell death gene highly expressed in these viable tumors?

We hypothesized that mutations in BNIP3 are prevalent in both primary GBM tumors and are associated with a poor prognosis.

OBJECTIVE

To identify the prevalence and significance of mutations in BNIP3 within primary and secondary GBM tumors.

MATERIALS AND METHODS

1. Single-Strand Conformation Polymorphism (SSCP) Protocol

Single strand conformation polymorphism (SSCP) is a sensitive method of detecting variations in the sequence of a gene. SSCP is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequence (often a single base pair), which results in a different secondary structure, and a measurable difference in mobility through a gel. SSCP was first announced in 1989 as a new means of detecting DNA polymorphisms, or sequence variations (Sunnucks et al., 2000). SSCP analysis takes advantage of this quality of single-stranded DNA and is generally considered to be most suitable for the detection of mutations in short stretches of DNA. In this method, the region of interest in the genome or cDNA is amplified by polymerase chain reaction (PCR), denatured to form single strands, and analyzed by non-denaturing polyacrylamide gel electrophoresis (Orita. et al.,1989). Mutations are detected as differences of mobility of the DNA bands between controls and test samples. The test sample has a different conformation if there is a single sequence change; it migrates differently by electrophoresis.

DNA & Primers:

Genomic DNA was extracted from brain tumor (primary glioblastoma multiforme) tissues and peripheral blood of patients (Brain Tumor Tissue Bank, London, Ontario). The PCR primers were designed for the flanking site of exons 3 of the BNIP3 gene, in

which point mutations have been confirmed by sequencing. The size of expected PCR products was about 128 base pairs.

The sequences of primers were as follows:

Exon 3 Sense: CAT TCA CCT TCC AGC TTA CCT GTG (forward)

Antisense: CCC ATT CTA TTC ACA TCG CCA AG (reverse)

PCR Reaction:

PCR reaction mixtures contained 3ul of high fidelity Taq polymerase (Roche), 100ng of DNA, 10pmol of each primer, 0.4ul of dNTP (5mM dATP, 25mM dCTP, 25mM dGTP, 25mM dTTP) mixtures and 0.5ul of 10mci/ml α-^{32}p-dATP, 3ul of 25mM MgCL2, 5ul of 10X PCR buffer in a total 50ul volume.

PCR amplification consisting of one cycle of 94 °C for 5min, 35 cycles of (94 °C for 1min, 63 °C for 45sec., 72 °C for 1 min), one cycle of 72 °C for 10min. in a thermal cycler (Eppendorf).

Gel Electrophoresis:

The labelled PCR products (5ul) were mixed with 10ul of formamide dye (95% formamide, 20mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and denatured for 5min at 95 °C, and immediately chilled on ice. Control samples were treated as both denatured and non-denatured. 3-5ul of each sample was applied onto a

nondenaturing polyacrylamide gel (size: 40 x 20 x 0.03cm). We used 10% polyacrylamide gel (29: 1 acrylamide to bisacrylamide) containing 10% glycerol in 0.5 X TBE buffer. The electrophoresis apparatus was from BIO-RAD (Sequence unit) and gel running was performed at room temperature (22-24 °C) at 5mA overnight. The electrophoresis running buffer was 0.5 X TBE (44.5mM Tris, 44.5mM Boric acid, 1mM EDTA, PH 8.3).

To make 10% glycerol gel 50ml

10 ml	50% glycerol
16.6ml	30% (29:1) acrylamide
2.5ml	10X TBE
20.9ml	ddH_2O
150ul	20% APS
30ul	TEMED

The gel was dried and exposed to X-ray film overnight at room temperature.

2. Isolation Protocol of Genomic DNA from Peripheral Blood of GBM Patients (QIAGEN DNeasy Mini Kit)

Genomic DNA was extracted from brain tumor (primary glioblastoma multiforme) tissues and peripheral blood leukocytes of patients (Brain Tumor Tissue Bank, London, Ontario).

(1) Pipet 20 µl proteinase K into the bottom of a 1.5 ml microcentrifuge tube.

(2) Add 10 µl anticoagulated blood.

(3) Adjust the volume to 220 µl with PBS.

(4) Add 200 µl Buffer AL. Mix thoroughly by vortexing.

(5) Incubate for 10 min at 70°C.

(6) Add 200µl ethanol (96–100%) to the sample and mix thoroughly by vortexing.

(7) Pipet the mixture from step 3 into the Dneasy spin column placed in a new 2 ml collection tube. Centrifuge at ≥ 6000 x*g* (8000 rpm) for 1 min. Discard flow-through and collection tube.

(8) Place the DNeasy spin column in a new 2 ml collection tube, add 500 µl Buffer AW1, and centrifuge at ≥ 6000 x*g* (8000 rpm) for 1 min. Discard flow-through and collection tube.

(9) Place the DNeasy spin column in a 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at full speed to dry the DNeasy membrane. Discard flow-through and collection tube.

(10) Place the DNeasy spin column in a clean 1.5 ml or 2 ml collection tube, and pipet 100 µl dd H_2O directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥ x*g* (8000 rpm) to elute.

(11) Repeat elution once as described in step 10.

3. Isolation Protocol of Genomic DNA from Tumor Tissues of GBM Patients (QIAGEN DNeasy Mini kit)

(1) Cut up to 25 mg tissue into small pieces, place in a 1.5 ml microcentrifuge tube, and add 180 ul Buffer ATL.

(2) Preheat a full 1.5 mL tube of Buffer ATL to 55°C.

(3) Add 20 ul of Proteinase K, vortex and incubate at 55°C. Vortex occasionally during incubation. Add 4 ul of Rnase A to the animal tissue and vortex.

(4) Incubate mixture for 2 min at room temperature.

(5) Vortex for 15 sec. and add 200 ul of Buffer AL to the same tube. Vortex. Incubate for 10 min on at 70°C.

(6) Add 200 ul ethanol (96-100%) to sample, vortex.

(7) Pipet mixture from step 6 into the DNeasy mini column sitting in a 2 ml collection tube. Centrifuge at ≥ 6000xg (8000 rpm) for 1 min. Discard flow-through and collection tube.

(8) Place the DNeasy mini column in a new 2 ml collection tube, and add 500 ul Buffer AW1. Centrifuge for 1 min. at ≥ 6000xg (8000 rpm). Discard flow-through and collection tube.

(9) Place DNeasy column into a 2 ml collection tube, and add 500 ul Buffer AW2, and centrifuge at full speed for 3 min. Discard flow-through and collection tube.

(10) Place the DNeasy mini column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 100 ul ddH_2O directly onto the DNeasy membrane. Incubate at room temperature for 1 min. Then centrifuge at ≥ 6000 xg (8000 rpm) to elute.

(11) Repeat elution in step 10 once.

Proteinase K, Buffer AL, ALT, AW1, AW2, and the DNeasy mini column are obtained from QIAGEN.

Phosphate-buffered saline (PBS) buffer: 0.02 M sodium phosphate buffer with 0.15 M sodium chloride, pH adjusted to 7.4, containing (a) 5% bovine serum albumin and 0.05 to 0.1 % sodium azide; and (b) 1% goat serum and 0.1% NaN_3.

4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Protocol

RT-PCR is short-form for reverse transcriptase-polymerase chain reaction. It is a technique in which an RNA strand is transcribed into a DNA complement to be able to subject it to amplification by polymerase chain reactions (PCR). Transcribing an RNA strand into a DNA complement is termed reverse transcription (RT) and is done by the enzyme reverse transcriptase. Afterwards, the original RNA is digested enzymatically, and a second strand of DNA is synthesized. The complementary DNA (cDNA) and its antisense counterpart are then amplified by PCR.

It is an important technique in the diagnosis of genetic diseases and in the determination of the abundance of specific RNA species as a measure of gene expression.

RT-PCR sometimes refers to real-time PCR, a recently developed variation of reverse transcriptase-polymerase chain reaction designed for quantitative purposes. Real-

time refers to the fact that some of the PCR machines can display and monitor the progress in each reaction as the reaction proceeds. To avoid confusion with the 2 "RT", the term quantitative PCR or quantitative RT-PCR is preferred (Freeman 1999; Reaymaekers, 2000).

The technique consists of two parts:

1) The synthesis of cDNA (complementary DNA) from RNA by reverse transcription (RT).
2) The amplification of a specific cDNA by the polymerase chain reaction (PCR).

Protocol (Invitrogen)

1. Program the thermal cycler so that cDNA synthesis is followed immediately with PCR amplification automatically.

A) cDNA synthesis and pre-denaturation:

Perform 1 cycle of: 45-55°C for 15-30 min, 94°C for 2 min.

B) PCR amplification

Perform 35-40 cycles of: denature 94°C for 15 s, anneal 49.2°C for 30 s, extend 72°C for 1 min/kb.

2. Add the following to the microcentrifuge tubes placed on ice. Reaction cocktails can be made when multiple reactions are being assembled.

Components	Volume/50 µl	Final Concentration
2X Reaction Mix	25 µl	1 X
Template RNA	x µl	1 µg
Sense Primer (10 µM)	1 µl	0.2–0.4 µM
Anti-sense Primer (10 µM)	1 µl	0.2–0.4 µM
RT/Platinum® *Taq* HiFi Mix	1– 2 µl	
Autoclaved distilled water	to 50 µl	

3. Gently mix and make sure that all the components are at the bottom of the amplification tube.

4. Analyze the amplification product.

5. DNA sequencing:

(1) Cleaning up RT-PCR product

(2) Sequencing from PCR product

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. The sequencing reaction used in ABI 310 sequencing is a PCR elongation based on the Sanger dideoxy nucleotide triphosphate (ddNTP) terminator method. In our case, we will use a reaction mix containing the four ddNTPs each conjugated with a unique fluorescent dye. As shown in the following table, each dye

when excited by a laser emits a specific fluorescence. This fluorescence is detected by a CCD and processed into the electropherogram.

Terminator	Acceptor Dye	Emission Peak (nm)	Electropherogram Color
ddATP	dichloroR6G	565	green
ddCTP	dichloroROX	630	blue
ddGTP	dichloroR110	535	black
ddTTP	dichloroTAMRA	600	red

forward primer: atgtcgcagaacggagca

reverse primer: tcaaaaggtgctggtggag

primer 1AR: atgagtctggagggagtag

primer 2AF: ccaatcccatatccaatc

5. RNeasy Mini Protocol for Isolation of Total RNA from GBM Tumor Tissues (QIAGEN)

(1) Disrupt tissue and homogenize lysate

(2) Centrifuge lysate for 3 min at maximum speed in a microcentrifuge and use only the supernatant in subsequent steps.

(3) Add 1 volume (usually 350 µl or 600 µl) of 70% ethanol to the cleared lysate, and mix well by pipetting. Do not centrifuge.

(4) Apply 700µl of the sample, including any precipitate which may have formed, to an RNeasy mini spin column sitting in a 2-ml collection tube. Centrifuge for 15 sec at ≥ 8000 xg (10,000 rpm).

(5) Pipet 700µl Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at ≥ 8000 xg (10,000 rpm) to wash.

(6) Transfer RNeasy column to a new 2-ml collection tube. Pipet 500µl Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at ≥ 8000 xg (10,000 rpm) to wash.

(7) Pipet 500µl Buffer RPE onto RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane.

(8) Transfer RNeasy column into a new 1.5-ml collection tube and pipet 30–50 µl of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at ≥ 8000xg (10,000 rpm) to elute. Repeat if the expected RNA yield is >30 µg.

Buffer RW1, Buffer RPE, and RNeasy columns are obtained from QIAGEN.

6. Immunofluorescence Staining on Paraffin Embedded GBM Tissue Section

Immunostaining is the laboratory process of detecting a specific protein in tissues using antibodies. These antibodies are labeled with a compound that is seen as a colored

deposit when viewed microscopically. Since 1941, when Coons and colleagues revolutionized the identification of tissue antigens using a direct fluorescence method (Coons et al. 1941), immunostaining has been used as an important method of investigation in both diagnostic histopathology and research.

Paraffin-embedded primary GBM tumor section slides were obtained from the Brain Tumor Tissue Bank (London, Ontario) and baked in an oven at 70°C for 40 minutes. After deparaffinizing (10 minutes in Xylene) and rehydation (2 minutes each in Xylene, 100%, 95%, 85%, 75%, 50% Ethanol), the slides were transferred to H_2O and placed in Coplin jars with antigen retrieval solution (Dako Corporation) at 95°C for 40 minutes. Then the slides were removed from the jars, cooled to room temperature, washed three times for 5 minutes in PBS (0.5% Triton X100) and were placed in a humidity chamber. Immunohistochemistry blocking buffer (1X PBS, 0.2% Triton X100, 0.02% sodium azide, 5% goat serum, 0.1% bovine serum albumin) was added to each slide to block them for 2 hours at room temperature.

Staining for First Antigen:

(1) Primary Antibody.

After the blocking step, the primary antibody, polyclonal anti-BNIP3 was diluted in blocking solution (1:200) and added to slides. The slides were incubated at 4°C overnight.

(2) Secondary Antibody.

The slides were subsequently washed three times for 5 minutes in PBS (0.5% Triton X100). Then the appropriate anti-rabbit biotinylated secondary antibody (1:200 dilution in blocking solution, Jackson) was added to slides for 2 hours at room temperature.

(3) Tertiary Reagent.

The slides were subsequently washed three times for 5 minutes in PBS (0.5% Triton X100). Then the third reagent, strepavidin conjugated to the appropriate fluorochrome Oregon Green 488 (1:200 dilution in blocking solution, Molecular Probes) was added to slides for 2 hours at room temperature.

Staining for Secondary Antigen:

(1) Second Primary Antibody.

After the slides were subsequently washed three times for 5 minutes in PBS (0.5% Triton X100), the secondary primary antibody, anti-GFAP (1:200 dilution in blocking solution, Dako) or anti-cytochrome c (1:300 dilution in blocking solution, Molecular Probes) was added to the slides. The slides were incubated 4°C overnight.

(2) Secondary Antibody.

The slides were subsequently washed three times for 5 minutes in PBS (0.5% Triton X100). Then the secondary antibody, strepavidin conjugated to the appropriate fluorochrome Texas-Red (1:300 dilution in blocking solution, Jackson) was added to slides for 2 hours at room temperature.

Vectashield with DAPI stain (staining DNA, Vector Inc.) was added to each slide and a coverslip was placed and sealed. The sections were examined and photographed under an Olympus Fluoview Confocal Microscope with ImagePro Plus 5.0 software.

RESULTS

1. BNIP3 is Mutated in Primary GBM Tumors.

(1) We determined the presence of BNIP3 mutations in primary GBM tumors (Brain Tumor Tissue Bank, London, Ontario). RT-PCR was performed on RNA isolated from these primary GBM tumor tissue samples and the resulting cDNA encoding BNIP3 was obtained using oligonucleotide primers for the open reading frame of BNIP3 (forward primer atgtcgcagaacggagca, and reverse primer tcaaaaggtgctggtggag).

We used part of these tumor samples and found that 5 GBM samples demonstrated a mutation in the cDNA encoding the PEST domain (insertion of A at nucleotide 236) of BNIP3 (Table 1, Figure 4). In normal brain tissues, no mutations were found. These mutations all result in a frameshift in the open reading frame of BNIP3 eliminating the TM domain and predict a truncated protein.

(2) To determine the prevalence of BNIP3 mutation in exon3 (containing the PEST domain sequence) in primary GBM tumors, single strand conformation polymorphism (SSCP) analysis was performed. Genomic DNA was extracted from primary glioblastoma tumor tissue (Brain Tumor Tissue Bank, London, Ontario). A specific pair of PCR primers (forward and reverse) was used to amplify the desired DNA fragments from individuals. The PCR primers flanking the intron/exon junction of exons

3 of the BNIP3 gene were designed, in which point mutations have been confirmed by sequencing. The sequences of primers were cattcaccttccagcttacctgtg (forward) and cccattctattcacatcgccaag (reverse). Single-stranded DNA is produced by asymmetric PCR: the primer on one side of the fragment is greatly in excess over the other primer. After the low-concentration primer supply is exhausted, continued PCR produces only the target single strand. Genomic DNA was digested with restriction endonucleases. The mobilities of the single stranded fragments were compared by electrophoresis on a neutral polyacrylamide gel. Electrophoresis was carried out for about 24 hours at room temperature. The labelled PCR products (5ul) were mixed with 10ul of formamide dye and denatured for 5min at 95°C. Gel was dried and exposed to X-ray film overnight at room temperature. Bands were detected by radioactive labeling.

Exon 3 of the BNIP3 gene showed an additional upper band (mutation) in GBM DNA that was not present in normal brain tissues. Using SSCP, we discovered mutations in BNIP3 of 9 of 55 (17%) primary GBM tumors (Figure 5).

2. Immunofluorescence Staining on Paraffin Embedded GBM Tissue Sections

We used double immunofluorescence staining on paraffin embedded tissue sections to label BNIP3 with GFAP or cytochrome *c* on normal brain tissue section and GBM tissue sections.

(1) BNIP3 of 55 primary GBM tumors in part (16/44) showed predominantly a cytoplasmic co-localization with mitochondria (Table 2, Figure 7).

After deparaffinizing, rehydration, and washing, paraffin-embedded primary GBM tumor section slides were immunostained with antibodies against BNIP3 (green) and cytochrome *c* (marker for mitochondria, red). The DNA was stained by DAPI stain (blue). The images were captured on a fluorescence microscope with deconvolution software. The BNIP3-fluorescence is bright green and the cytochrome *c*-fluorescence is bright red.

BNIP3 in primary GBM tumors in part (16/44) predominantly showed a cytoplasmic localization and BNIP3 is primarily expressed with mitochondria. But BNIP3 in normal brain tissue primarily showed a nuclear localization and did not localize to mitochondria.

(2) BNIP3 was co-localized with GFAP in primary GBM tumors (Figure 6).

After deparaffinized, rehydated, and washed, paraffin-embeddd primary GBM tumor section slides were immunostained with antibodies against BNIP3 (green) and GFAP (marker for intermediate filaments in astrocytes, red). The DNA was stained by DAPI stain (blue). The images were captured on a fluorescent microscope with deconvolution software. The BNIP3-fluorescence is bright green and the GFAP-fluorescence is bright red.

BNIP3 in primary GBM tumors in part predominantly showed a cytoplasmic localization and BNIP3 co-localized with GFAP in primary GBM tumors. But BNIP3 in normal brain tissue primary showed a nuclear localization and rarely co-localized with GFAP in normal brain.

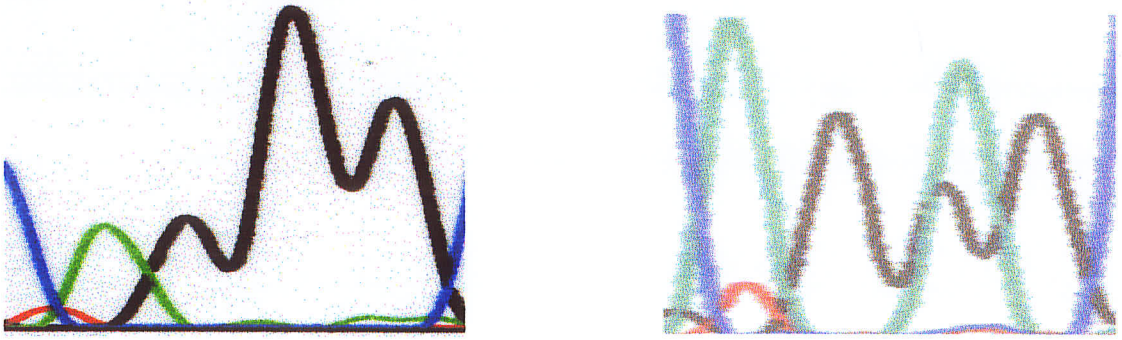
Table 1: BNIP3 Is Mutated In Primary GBM

GBM Tumor Number	Mutation In cDNA	Location Of The Mutation In The Protein	Functional Singnificance
894GBM	Insertion A at nt 236(235_236insA)	PEST Domain	Elimination of the TM Domain
819GBM	Insertion A at nt 236(235_236insA)	PEST Domain	Elimination of the TM Domain
813GBM	Insertion A at nt 236(235_236insA)	PEST Domain	Elimination of the TM Domain
456GBM	Insertion A at nt 236(235_236insA)	PEST Domain	Elimination of the TM Domain
346GBM	Insertion A at nt 236(235_236insA)	PEST Domain	Elimination of the TM Domain

Table 2: The Location of BNIP3 of 44 Primary GBM Tumors

Cytoplasm (36.36%)	Cytoplasm/Nucleus (25%)	Nucleus (38.64%)
BTTB# 997	BTTB# 532	BTTB# 903
869	1022	782
346	813	803
894	1059	984
677	661	450
471	769	762
650	853	570
839	987	539
1046	1037	750
1044	999	1034
872	967	1041
596		975
1032		744
292		766
719		437
1020		745
		1023

Figure 4: DNA Sequencing



AGGG

Wild Type

AGGAG

PEST Mutation
236_ins A

Figure 5: SSCP (GBM Tumor Tissue)

Normal Brain Tissue = Lane 6 GBM = Rest (Lane 3, 4, 7, and 8 = Mutant BNIP3)

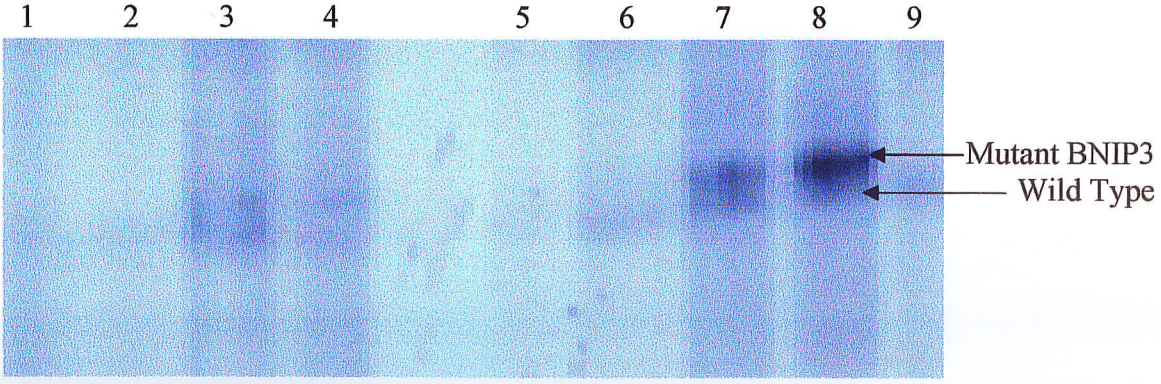
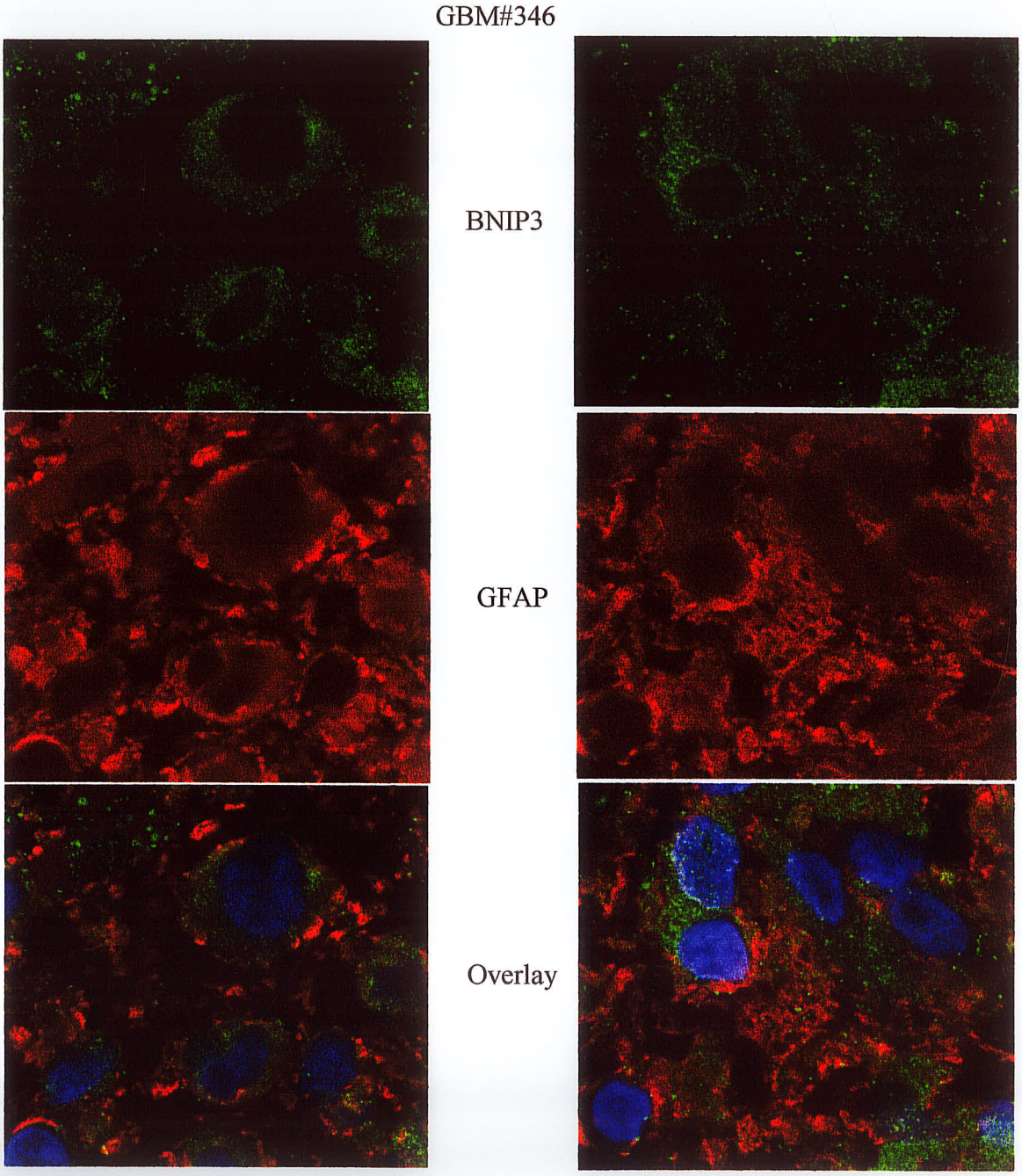


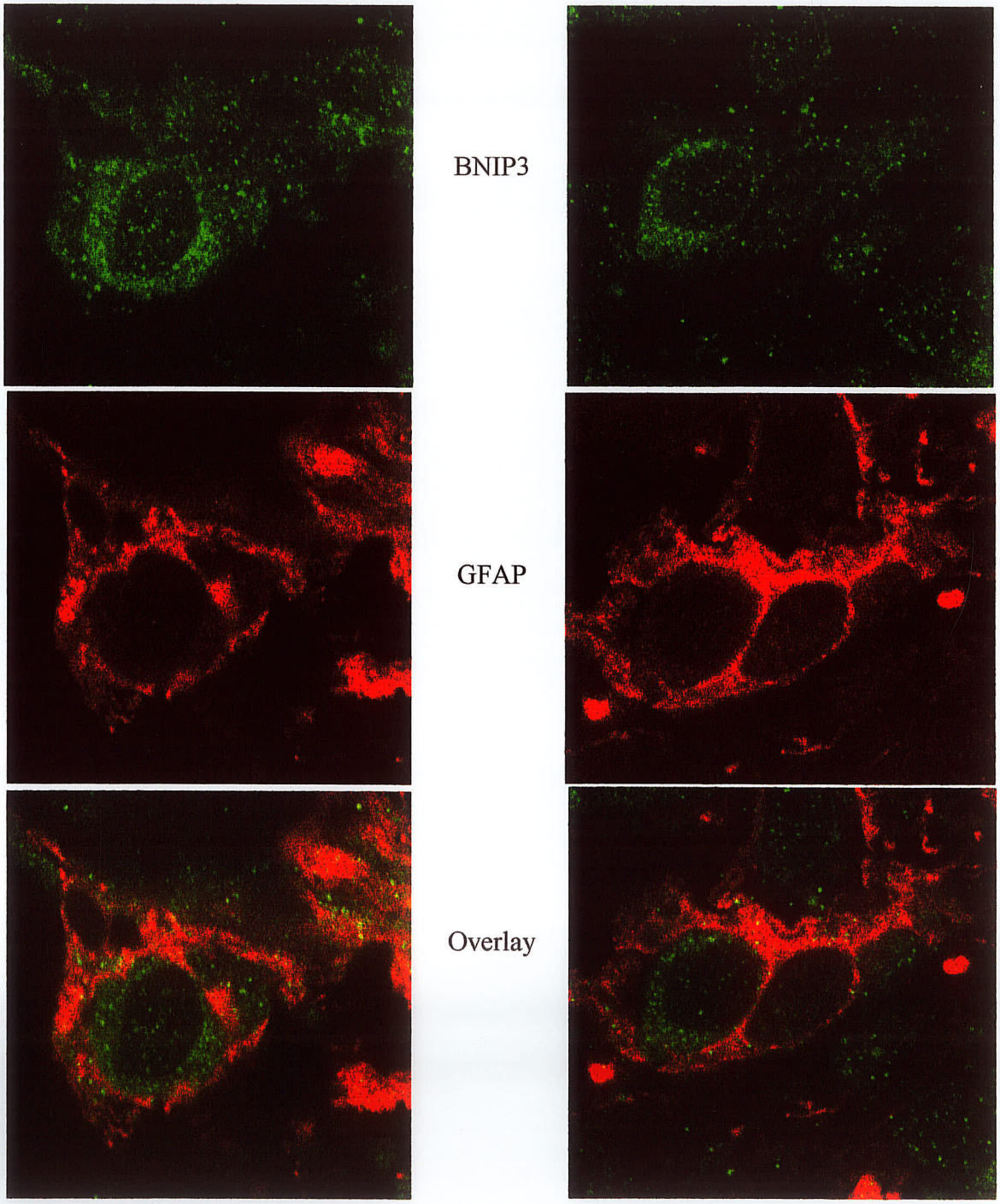
Figure 6: Double Immunofluorescence Staining on Paraffin Embedded Normal Brain and GBM Tissue Sections

A) BNIP3 (Green) Is Co-Localized With GFAP (Red) in Primary GBM Tumors.



B) BNIP3 (Green) Is Co-Localized With GFAP (Red) in Primary GBM Tumors.

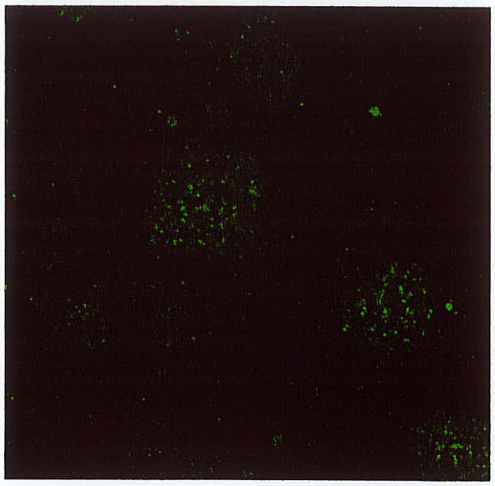
GBM#1022



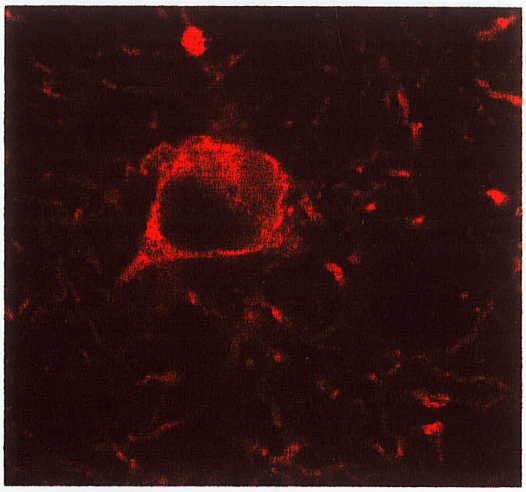
B) Normal Brain

Normal Brain Tissue #638

BNIP3 (green)



GFAP (red)



Overlay

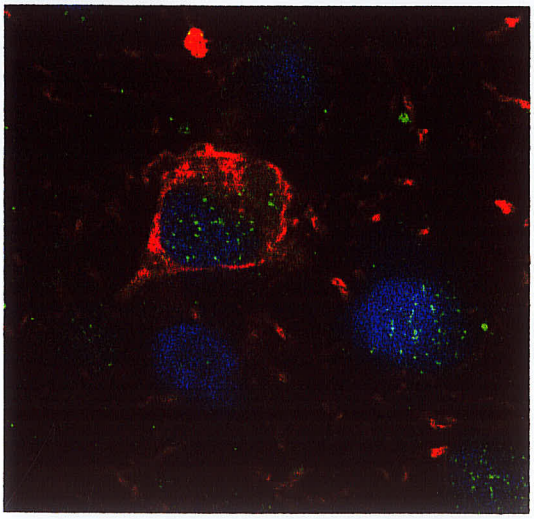
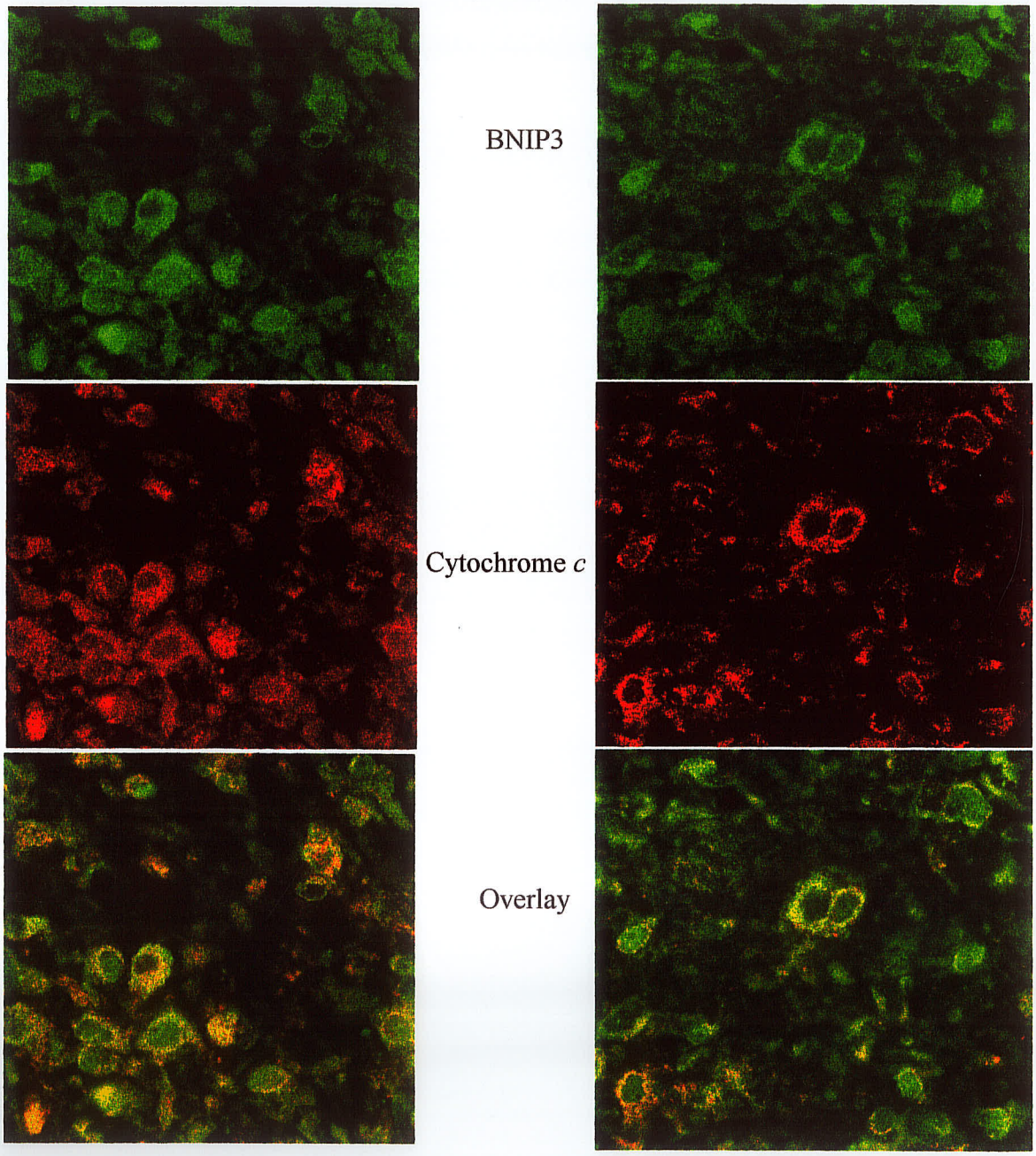
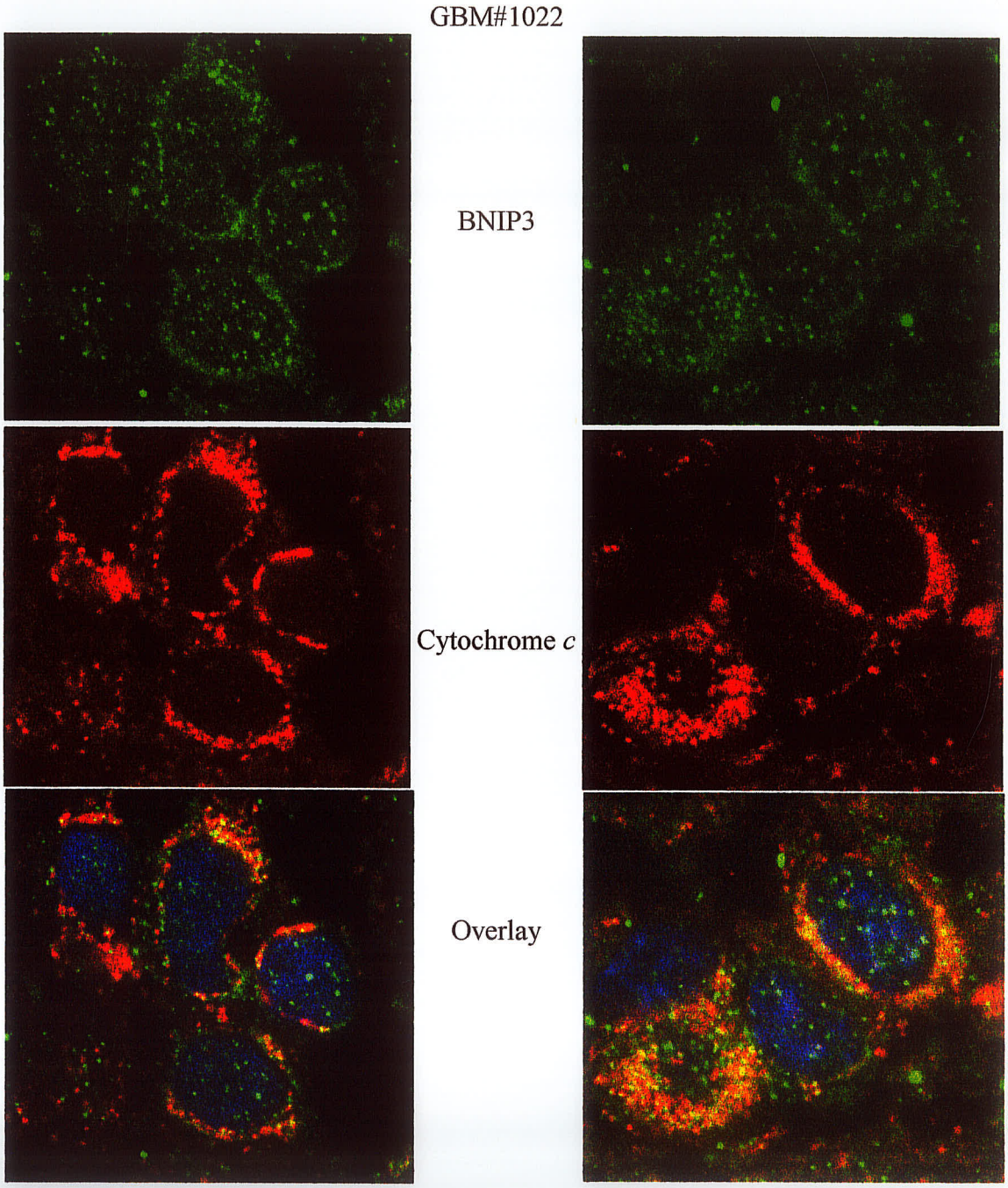


Figure 7: Double Immunofluorescence Staining on Paraffin Embedded Normal Brain and GBM Tissue Sections

A) BNIP3 (Green) of GBM Tumors Is Predominantly Cytoplasmic and Co-Localized With Mitochondria (Red).



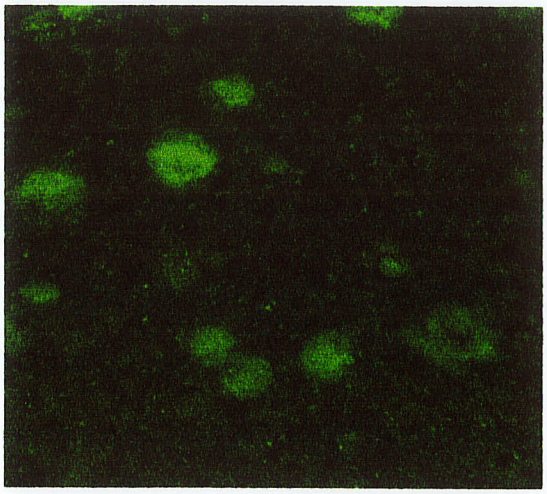
B) BNIP3 (Green) of GBM Tumors Is Co-Localized With Mitochondria (Red).



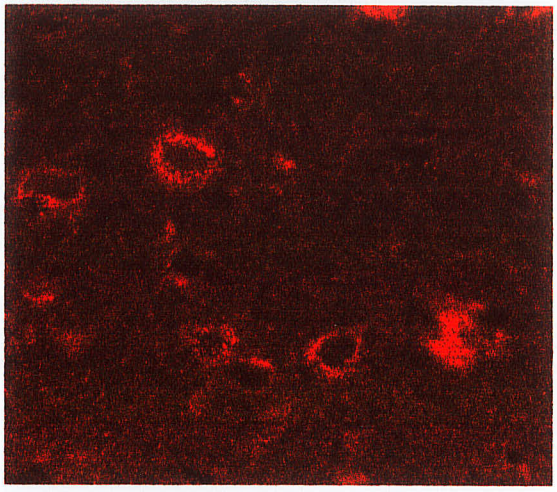
C) Normal Brain

Normal Brain Tissue #638

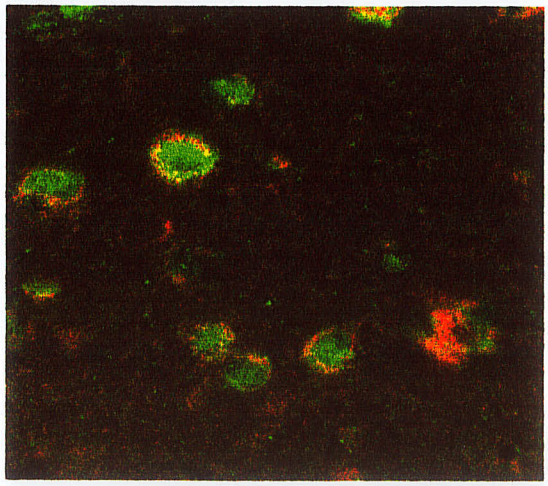
BNIP3



Cytochrome *c*



Overlay



DISCUSSION

Glioblastoma multiforme tumors (GBM) are World Health Organization (WHO) grade IV astrocytomas. GBMs are an aggressive form of brain cancer with a median survival of less than one year. This tumor is resistant to chemotherapy and radiation (Kleihues *et al*, 2002; DeAngelis *et al*, 2001). As solid tumors expand, they can rapidly outgrow the tumor vasculature. This leads to areas of hypoxia within the GBM tumor (Kunz *et al*, 2003; Dewhirst *et al*, 2003). Under prolonged oxygen deprivation, cells undergo cell death and form necrotic regions. Surrounding these necrotic regions are viable cells under constant low oxygen stress. The extent of hypoxic region in the tumor is associated with the poor prognosis and aggressiveness of GBM (Zagzag *et al*, 2000; (Kunz *et al*, 2003; Pennacchietti *et al*, 2003). Because of the low level of free oxygen radicals produced by the radiation compared to oxygenated cells, the hypoxic cells are able to resist radiation (Marples *et al*, 2003; Sannazzari *et al*, 2002) and the delivery of chemotherapeutic drugs is impaired in the poorly oxygenated regions of GBM tumors (Kunz *et al*, 2003; Knisely *et al*, 2002). Genomic instability occurs due to chronic hypoxic stress. This selects for mutations that prevent hypoxia-induced cell death (Kunz *et al*, 2003; Knisely *et al*, 2002).

Hypoxia-inducible factor 1 (HIF-1) is a nuclear protein that activates gene transcription in response to reduced cellular O_2 concentrations and has been shown to directly induce the transcription of VEGF and pro-apoptotic BNIP3 (Mu *et al*, 2003; Sowter *et al*, 2003). The biological activity of transcription factor HIF-1 is determined by

the expression and activity of the HIF-1α subunit. . HIF-1 is overexpressed in human glioblastoma biopsies and the level of expression is correlated with tumor progression (Zagzag *et al*, 2000).

Cell death involves two general processes: necrosis and apoptosis. Bcl-2 family members are divided into two groups: anti-cell death (survival) and pro-cell death proteins. Under low oxygen, they regulate apoptosis and necrosis. Over-expresion of the anti-cell death protein Bcl-2 inhibits apoptosis and necrosis induced by hypoxia (MacCarthy-Morrogh *et al*, 1999; Cuisnier *et al*, 2003). The Bcl-2 /adenovirus E1B 19Kda interacting protein (BNIP3) is a pro-apoptotic Bcl-2 (B-cell leukemia/lymphoma 2) family member. It contains 4 domains, a NH_2 (amino)-terminal PEST sequence, a BH3-like domain (BH3), a COOH (carboxyl)-terminal transmembrane (TM) domain and a conserved domain (CD) of unknown function. The PEST sequences are proposed to be involved in rapid turnover of BNIP3 protein. The TM domain is critical for homodimerization and is involved in BNIP3 induced cell death that targets BNIP3 to the mitochondria.

Hypoxia upregulates BNIP3 expression. Under hypoxia, the overexpressed nuclear protein HIF-1 in GBM tumor cells binds to HREs on the BNIP3 promoter and activates BNIP3 by HIF- 1α (Kothari *et al*, 2003). The elevated level of BNIP3 within GBM should correlate with increased cell death. Paradoxically, GBM tumors show that BNIP3 expression is increased compared to normal brain (Figure 3, Dr. D.D. Eisenstat), but tumor cells remain viable. Why is a cell death gene highly expressed in these viable tumors? We know that under chronic hypoxic stress, DNA instability occurs leading to

selection of mutations. The microenvironment of a solid tumor is itself mutagenic and an important source of genetic instability (Reynolds et al, 1996).

To determine whether BNIP3 cDNA is mutated in primary GBM tumors, we sequenced the cDNA encoding BNIP3 derived from samples of primary GBM tumors. We found mutations in the PEST domain (insertion of A at nucleotide 236) in 5 tumors (Table 1, Figure 4). In normal brain, no mutations were found. These mutations all result in a frameshift in the reading frame of BNIP3 eliminating the TM domain. To determine the presence of mutation in the BNIP3 gene, single strand conformation polymorphism (SSCP) analysis was also performed. Exon 3 of the BNIP3 gene (containing the PEST domain sequence) showed an additional (upper) band in 9 of 55 GBM DNA that was not present in DNA from normal brain (Figure 5). This indicates that a nucleotide insertion in BNIP3 cDNA was due to a mutation in exon 3 of the BNIP3 gene. The mutations result in the loss of functional TM domain so that BNIP3 cannot localize to the outer of membrane of mitochondria and induce rapid opening of the mitochondrial PT pore. From previous studies, we know that BNIP3 over-expression induces plasma membrane permeability and mitochondrial damage initiated by rapid opening of the mitochondrial permeability transition (PT) pore, loss of transmembrane potential ($\Delta\psi m$) and production of radical oxygen species (ROS). The mitochondrial damage causes the release of cytochrome *c*, disturbs ATP production and activates caspases. Finally, this results in cell death. Therefore, due of the mutations in BNIP3, the mutant BNIP3 protein is missing a TM domain, so it fails to induce cell death. Mutant BNIP3 may also interfere with the function of endogenous wild-type BNIP3 by forming heterodimers, thereby acting in a dominant negative manner (S. Zhang *et al*, in preparation).

To identify what kind of pattern of BNIP3 is expressed in the primary GBM tumors, if BNIP3 localizes to mitochondria, and whether BNIP3 co-localizes with GFAP in primary GBM tumors, we immunostained the primary GBM tumors with BNIP3 antibody, GFAP antibody, and cytochrome *c* antibody. BNIP3 in 16 of 44 primary GBM tumors was primarily cytoplasmic and BNIP3 localized to mitochondria. BNIP3 co-localized with GFAP in primary GBM tumors. However, BNIP3 in normal brain tissue primarily showed a nuclear localization and did not co-express with mitochondria and rarely co-localized with GFAP.

GFAP is an intermediate filament marker for astrocytes. BNIP3 co-expresses with GFAP in primary GBM tumors. This signifies that BNIP3 is expressed in malignant astrocytes. In many GBM tumors BNIP3 is predominantly cytoplasmically localized. BNIP3 PEST domain mutations cause a frameshift elimination of the functional TM domain of BNIP3 and cannot bind to mitochondria. Mitochondrial damage cannot be initiated and BNIP3 fails to induce cell death. This may explain, in part, why GBM cells survive despite BNIP3 expression. This is associated with a poor prognosis for GBM.

SIGNIFICANCE

Glioblastoma multiforme, WHO grade IV astrocytomas with a median survival of less than one year, is the most common and malignant form of adult brain tumor. Our research found that in 9 of 55 (17%) primary GBM tumors, the hypoxia-inducible pro-cell death gene BNIP3 was mutated in the PEST domain leading to a truncated protein with elimination of TM domain responsible for BNIP3's ability to induce cell death. The research will be helpful toward a better understanding of GBM as one of the most aggressive and treatment resistant forms of cancer and may lead to the development of targeted therapies mitigating the effects of mutant BNIP3 on malignant glioma tumorigenesis.

FUTURE DIRECTION

(1) To determine whether BNIP3 mutations also occur in matched blood samples of these GBM tumor patients (germline). RT-PCR will be performed on these blood samples and the resulting cDNA encoding BNIP3 will be sequenced. We will also perform SSCP analysis on the blood samples.

(2) To determine whether HIF-1α and glucose transporter protein (glut)-1 are co-expressed with BNIP3 in GBM turmors so that we can confirm that BNIP3 is expressed in hypoxic regions of GBM tumors. We will use the double immunofluorescence staining on paraffin-embedded tissue sections technique to label BNIP3 and HIF-1α or glut-1 on normal brain and GBM tissue sections.

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