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Reaching for the Star: A Tumor Suppressor or an Oncogene?

By

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Reaching for the Star: Tumor Suppressor or Oncogene?

Sally B. El Sitt

Abstract

Astrocytomas are tumors occurring in young adulthood. Astrocytic tumors can be classified into four grades according to histologic features: grade I, grade II, grade III and grade IV. Malignant tumors, those of grade III and IV, are characterized by uncontrolled proliferation, which is known to be regulated by the family of Rho GTPases. StarD13, a GAP for Rho GTPases, has been described as a tumor suppressor in hepatocellular carcinoma. In the present study, IHC analysis on Grade I-IV brain tissues from patients showed StarD13 to be overexpressed in grade III and IV astrocytoma tumors when compared to grade I and II. However, when we mined the REMBRANDT data, we found that the mRNA levels of StarD13 are indeed higher in the higher grades but much lower than the normal tissues. The overexpression of a GFP-StarD13 construct in astrocytoma cells led to the increase in cell death and a decrease of cell viability. Knocking down StarD13 using siRNA led to a decrease in cell death and an increase in cell viability. When looking at the mechanism, we found that the tumor suppressor effect of StarD13 is through the inhibition of the cell cycle and not through the activation of apoptosis. When knocking down StarD13, we also saw an increase in p-ERK, uncovering a potential link between Rho GTPases and ERK activation. Our future interests would be to determine which Rho GTPase is responsible for this effect and to elucidate the direct link between the Rho GTPase and ERK.

Keywords: Astrocytomas, RhoGTPases, StarD13, GAP, Tumor suppressor, GFP-StarD13, StarD13-siRNA.

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GLOSSARY

CNS: Central nervous system

CP: Cerebellopontine

WHO: The World Health Organization

EGFR: Epidermal growth factor receptor

PTEN: Gene for phosphatase and tensin

PDGFR: Platelet-derived growth factor receptor

GTP: Guanosine-5'-triphosphate

GDP: Guanosine diphosphate

N-WASP: Wiskott-aldrich syndrome protein

WAVE: WASP-family verprolin-homologous protein

PAK1–6: p21-activated protein kinase

MRCKα/β: myotonic dystrophy kinase-related Cdc42 binding kinase

ROKα/β: RhoA-binding kinase

IQGAP1/2: IQ-motif containing with homology to Ras GAPs

Par6: cell polarity protein

Rac 1: Ras-related C3 botulinum toxin substrate 1

Cdc42: Cell division cycle 42

Rho A: Ras homologous member A

CRIB: Cdc42/Rac interactive binding

GAP: GTPase activating proteins

GEF: Guanine nucleotide exchange factors

GDI: Guanine nucleotide dissociation inhibitors

Dock: dedicator of cytokinesis

DH: Db1 homology

PH: Pleckstrin homology

PIP3: Phosphatidylinositol (3, 4, 5)-triphosphate

PI3K: Phosphatidylinositol 3-kinase

PDZ: PSD-95/ Discs large/zO-1

SH2: Src homology domain 2

SH3: Src homology domain 3

START: STAR-related lipid transfer

DLC1: Deleted in liver cancer 1

DLC2: Deleted in liver cancer 2

StarD13: StAR-related lipid transfer (START) domain

SAM: A sterile α motif

FAT: Focal adhesion targeting domain

ERK: Extracellular signal-regulated kinase

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal bovine serum

siRNA: Small interfering RNA

GFP: Green fluorescent protein

WST-1: Water-soluble tetrazolim salt

GST: Glutathione S-transferase

RBD: Rhotekin binding domain

PBS: Phosphate buffered saline

FACScan: Fluorescence Activated Cell Sorter

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

FITC: Fluorescein Isothiocyanate

Chapter One

INTRODUCTION

1.1. Brain tumors:

1.1.1. Occurrence:

Primary malignant central nervous system (CNS) tumors account for 2% of all cancers with an inconsistent rate of morbidity and mortality. Malignant CNS tumors constitute the leading cause of death from solid tumors in children and the third leading cause of cancer-related death for adolescents and adults aged 15 to 34 years (Buckner, et al., 2007). More than 18000 new diagnoses of brain and nervous system cancers are estimated by the American Cancer Society in the United States each year leading to more than 12,000 deaths (Chandana, Movva, Arora, & Singh, 2008).

1.1.2. Definition, Symptoms and Risk Factors:

Brain tumors are defined as a collection of neoplasms and are better known as "intracranial neoplasms". Most of these tumors have similar clinical presentation, diagnostic approach, and initial treatment (DeAngelis, 2001). Headache, nausea, vomiting, seizures, and altered mental functions represent the common generalized symptoms of a brain tumor with headache being the most common presenting symptom (Buckner, et al., 2007; Chandana, et al., 2008). Diet, alcohol, smoking, occupation and industry, infections, allergies, exposure to ionizing or nonionizing radiation, head trauma, family history, and inherited polymorphisms in genes related to carcinogen

metabolism, oxidative metabolism, and DNA repair constitute the possible risk factors that have been associated with brain tumors (Wrensch, Minn, Chew, Bondy, & Berger, 2002).

1.1.3. Treatment:

Treatment of primary brain tumors includes surgery, radiation therapy, and chemotherapy (Chandana, et al., 2008). Surgery represents the goal standard treatment of benign or potentially anaplastic brain tumors providing not only histological diagnosis but also radical surgical removal when feasible (Chandana, et al., 2008; Cornu, Dormont, Marsault, & Philippon, 1996). For patients with metastatic brain tumors, epidural spinal cord compression, and leptomeningeal metastases, radiotherapy is of great importance prolonging the survival of most patients (Buckner, et al., 2007). Several trials highlighted the importance of adjuvant chemotherapy, -using nitrosoureas, as having the ability to cross the blood brain barrier and being lipid soluble (Stewart, 2002).

1.1.4. Non gliomas:

CNS tumors can be either gliomas or nongliomas (Buckner, et al., 2007) (Table 1). Nongliomas can be benign tumors, such as meningiomas and pituitary adenomas, or malignant tumors, such as primitive neuroectodermal tumors (medulloblastomas), primary CNS lymphomas (Buckner, et al., 2007). Meningiomas, originated in the dura that covers the brain and spinal cord, represent the second most common benign CP (cerebellopontine) angle tumor arising from neoplastic meningothelial cells (Buckner, et al., 2007; Wiemels, Wrensch, & Claus, 2010). Pituitary adenomas are defined as nonmetastasizing neoplasms consisting of adenohypophysial cells. Some adenomas exhibit a slow rate of growth while some are rapidly growing tumors (Asa & Ezzat,

1998). Medulloblastomas, occurring in the posterior fossa, are very common in children representing 15–25% of all childhood primary central nervous system (CNS) neoplasms (Brandes, Paris, & Basso, 2003; Franceschi, et al., 2007). Primary CNS lymphomas account for 1% or less of all primary brain tumors (DeAngelis, 2001). The only two established causes of primary CNS tumors are heritable syndromes and ionizing radiation (Preston-Martin, 1996). Patients with a compromised immune system are at a higher risk for CNS lymphomas (Buckner, et al., 2007).

Histology	Percentage
Lymphoma	3.1
Nerve sheath	8.0
Craniopharyngioma	0.7
Pituitary	6.3
Glioblastoma†	20.3
Astrocytomas	9.8
Ependymomas	2.3
Oligodendrogliomas	3.7
Embryonal, including medulloblastoma	1.7
Meningioma	30.1
All other	13.9

Table 1: Distribution of all primary brain and CNS (central nervous system) tumors by histology, CBTRUS (Central Brain Tumor Registry of the United States) 1998-2002 (N=63,698). Gliomas account for 40% of all tumors and 78% of malignant tumors. From CBTRUS (2005). Statistical Report: Brain Tumors in the United States, 1998-2002. (Buckner et al. 2007).

1.1.5. Gliomas:

The diffuse infiltration of white matter tracts constitutes the primary characteristic of most gliomas (Buckner, et al., 2007). Gliomas comprise two major categories: astrocytic and oligodendroglial tumors, which can either, be low grade or high grade

(DeAngelis, 2001) (Table 2). Gliomas are genetically, histologically, and therapeutically heterogeneous and can take place anywhere in the brain but specifically affect the cerebral hemispheres (DeAngelis, 2001). The most common glial tumors are the malignant astrocytomas, the anaplastic astrocytoma and glioblastoma multiforme (DeAngelis, 2001).

Histology	Percentage
Ependymomas	5.6
Oligodendrogliomas	9.2
Pilocytic astrocytomas†	5.7
Diffuse astrocytomas	1.7
Anaplastic astrocytomas	7.9
All other astrocytomas	9.1
Glioblastomas	50.7
All other gliomas	10.1

Table 2: Distribution of all primary brain and CNS (central nervous system) gliomas by histology subtypes, CBTRUS (Central Brain Tumor Registry of the United States) 1998-2002 (N=25,539). Astrocytomas and glioblastomas account for 75% of all gliomas. From CBTRUS (2005). Statistical Report: Brain Tumors in the United States, 1998-2002. (Buckner et al. 2007).

1.2. Astrocytomas:

1.2.1. Definition:

Astrocytomas are tumors occurring in young adulthood defined as CNS neoplasms originated in astrocytes, star-shaped brain cells (Kennedy 2009). The brain consists of two types of cells: neurons, the basic structural unit and astrocytes, the supportive cells. Astrocytes provide the neurons with trophic, metabolic and structural

supports. The communication between the neurons and their supportive cells is mediated through Ca^{2+} signaling (Nag, 2011).

1.2.2. Classification:

The World Health Organization (WHO) system classifies the astrocytic tumors into four grades: grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma) with cytological atypia alone, grade III (anaplastic astrocytoma) showing anaplasia and mitotic activity in addition and grade IV (glioblastoma) presenting microvascular proliferation and/or necrosis (Louis, Ohgaki, Wiestler, & Cavenee, 2007). Malignant gliomas are those of grade III and IV (Wen & Kesari, 2008). Glioblastomas account for at least 80 percent of malignant gliomas (DeAngelis, 2001).

1.2.3. Glioblastoma:

Glioblastoma, the most deadly primary brain tumor, has been identified as a complex disease, in which many signaling pathways are disrupted mainly the epidermal growth factor receptor (EGFR) pathway (Bredel, et al., 2011) (Figure 1). Glioblastomas are divided into two subtypes based on biologic and genetic differences: primary glioblastomas occurring de novo in patients older than 50 years and secondary glioblastomas developing in younger patients through the progression from low-grade or anaplastic astrocytomas (Watanabe, et al., 1996; Wen & Kesari, 2008). High rate of overexpression or mutation of the epidermal growth factor receptor, *p16* deletions, and mutations in the gene for phosphatase and tensin homologues (*PTEN*) characterize primary glioblastomas whereas secondary glioblastomas involve mutations in the *p53* tumor-suppressor gene and overexpression of the platelet-derived growth factor receptor (PDGFR), abnormalities in the p16 and retinoblastoma (Rb) pathways (DeAngelis, 2001; Wen & Kesari, 2008).

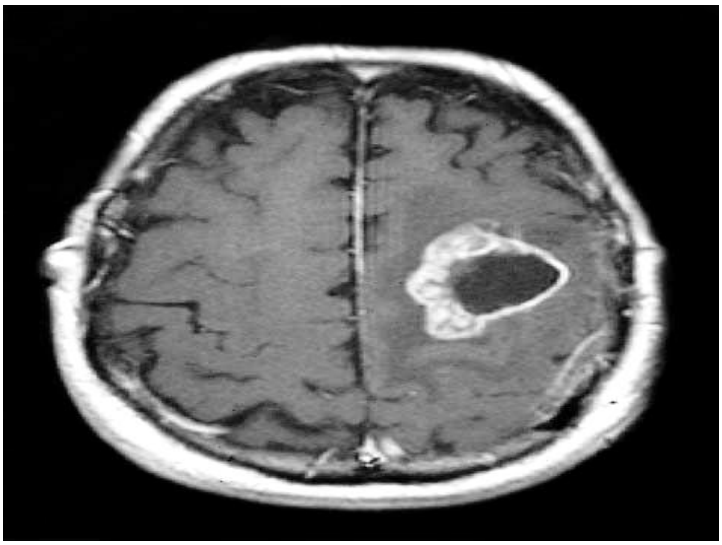


Fig 1: MRI of a Glioblastoma Multiforme in the Left Frontal Lobe, Obtained after the Administration of Gadolinium. The tumor is characterized by the irregular enhancing margin with central necrosis (DeAngelis 2001).

1.3. Rho GTPAses:

1.3.1. Definition:

Rho GTPases are known to be involved in the stimulation of cell cycle progression. The family of Rho GTPases contains 20 small G proteins playing important roles in the regulation of the cytoskeleton, the cell cycle, the cell migration and the cell polarity (Karlsson, Pedersen, Wang, & Brakebusch, 2009) (Figure 2). Rho GTPases are guanine nucleotide binding proteins existing in two forms: the active form which is GTP bound and the inactive one that being GDP bound and it is important to note that only in the active form, Rho GTPases can interact with other effectors mediating their cellular functions (Boettner & Van Aelst, 2002). Rho GTPases act as molecular switches in

many important mechanisms: actin polymerization, cell adhesion, and gene transcription (Ridley, 2001). The family of these proteins comprises many types of effectors including actin nucleation promoting molecules such as N-WASP and WAVE**,** kinases e.g. PAK1–6, MRCKα/β, ROKα/β, adaptors e.g. IQGAP1/2, Par6 and other protein families. The most important studied members of the Rho family are Rac 1(Ras-related C3 botulinum toxin substrate 1), Cdc42 (cell division cycle 42) and Rho A (Ras homologous member A) (Boettner & Van Aelst, 2002).

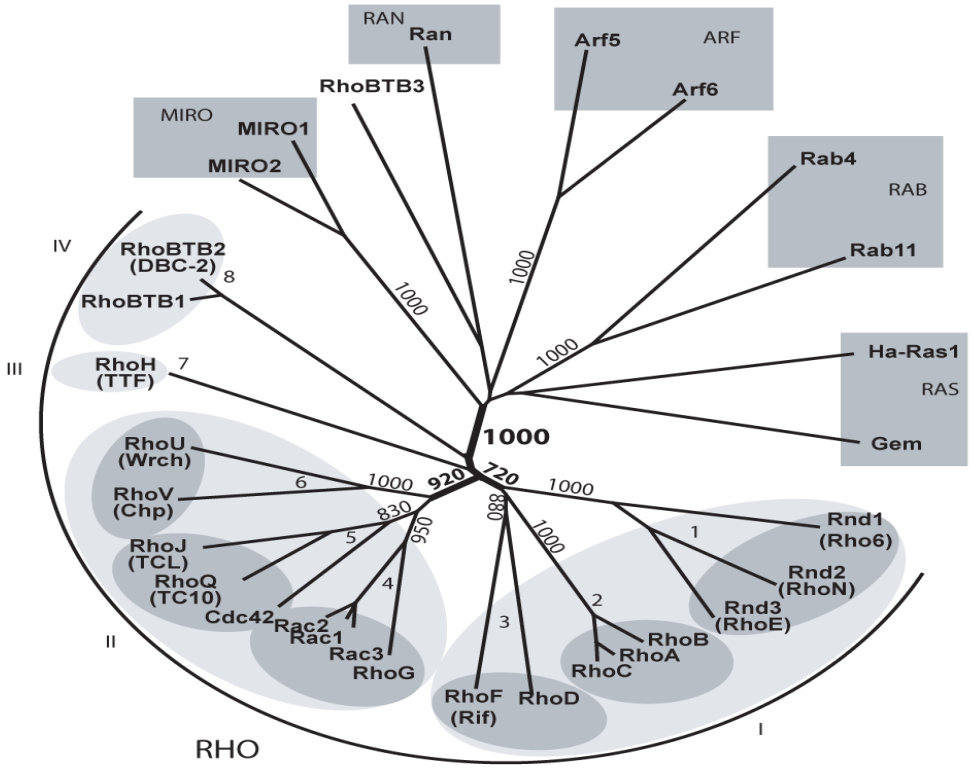


Figure 2: Delineation and structure of the Rho family. Proteins considered so far as Rho members were aligned with GTPases of other Ras-like families, and the unrooted tree was obtained by NJ (ClustalX). Only the Rho domains, corresponding to amino acids 5–173 of Rac1, were used for the alignment. Structuration into 4 clusters and 8 subfamilies is figured by light and dark gray ellipses, respectively. (Boureux et al. 2007).

1.3.2. Rac1:

Rac1 is a small GTP-binding protein belonging to the Rho subfamily of Ras-related protein involved in the regulation of cell morphology and growth. Rac1 acts also as a regulator of many cellular activities such as regulation of gene expression, actin cytoskeketal organization, regulation of cell proliferation and stimulation of cellular transformation and it plays a role in tumorigenesis and metastatic potential (Hwang, et al., 2004).

1.3.3. Cdc42:

Cdc42 is involved in the regulation of eukaryotic cell polarity important for cell migration and cell division because of its impact on the cytoskeleton (Johnson, 1999). Overexpression of Cdc42 in breast tumor and testicular cancer shows the correlation between the Cdc42 gene expression and tumor progression (Karlsson, et al., 2009). An important feature of Cdc42 is the well defined binding domain CRIB (Cdc42/Rac interactive domain) found in many effectors downstream of Cdc42 and Rac (Burbelo, Drechsel, & Hall, 1995). Cdc42 can be found in the cytoplasm or bound to the plasma membrane (Ziman et al. 1993).

1.3.4. RhoA:

RhoA can exist either in the cytoplasm or at the plasma membrane (Adamson, Paterson, & Hall, 1992). Overexpression of RhoA is found in many human tumors such as skin, liver and colon cancer (Karlsson, et al., 2009). Rho A is required to assemble stress fibers and to preserve cell adhesion. Nowadays it is evident that RhoA is tightly associated to the attainment of migratory and metastatic phenotypes and it is involved in an α6β4 integrin-mediated pathway leading to the formation of lamellipodia and migration in colon carcinoma cells and in the invasion of epithelial or hepatoma cell

lines. Moreover, RhoA is a major component of focal complexes, actin-rich cell surface structures located at the leading edge of lamellipodia (Abecassis, Olofsson, Schmid, Zalcman, & Karniguian, 2003).

1.3.5. Rho GTPases in cancer:

In cancer cells, alterations in signaling pathways render the cells defective in recognizing the normal environmental signals responsible for controlling cell proliferation, apoptosis, and differentiation (Alberts, et al., 2008). The rate of cell proliferation and the rate of cell apoptosis determine the ability of tumor cell populations to expand in number (Hanahan & Weinberg, 2000). Malignant tumors show several features including uncontrolled proliferation, enhanced migration properties, loss of epithelial cell polarity, and altered interactions with neighboring cells and the surrounding extracellular matrix. Genetic screens of many human cancers have revealed altered expression of various Rho family GTPases (Ridley, 2004). *RhoC* mRNA level is increased in metastatic melanomas and Rac3 activity is increased in highly proliferative breast cancer cell lines (Ridley, 2004). Regulators of Rho GTPases also show aberrant expression in human tumors, including Vav1 in neuroblastomas (Hornstein et al. 2003). A comparison of the gene expression pattern in a metastatic breast cancer cell line compared to its non-metastatic counterpart revealed that many genes encoding actin regulatory proteins are more highly expressed in metastatic cells (Wang, et al., 2002). All of these processes are regulated by the proteins of the Rho GTPase family making them with their regulators and effectors important in controlling tumor formation and progression in humans (Karlsson, et al., 2009).

The amount of Rho GTPases, in particular RhoA, has been shown to be frequently increased in different types of cancers suggesting the role of this family of proteins in human carcinogenesis (Fritz, Gnad, & Kaina, 1999). The Rho proteins implicated in many biological processes such as cell adhesion, migration, transcriptional activation,

cell-cycle progression are known for their important role in the formation and progression of tumors in vivo (Malliri & Collard, 2003).

1.3.6. Regulation of Rho GTPases:

The regulation of Rho GTPases is governed by three classes of regulatory proteins: GAP (GTPase activating proteins), GEF (guanine nucleotide exchange factors), which accelerate the very slow intrinsic guanine nucleotide exchange and GTP hydrolysis activity, and GDI (guanine nucleotide dissociation inhibitors) (Kawai, et al., 2009; Kim, Vigil, Der, & Juliano, 2009) (Figure 4). In order to accomplish several cellular functions, Rho GTPases cycle between an active GTP-bound form and an inactive GDP-bound form (Kawai, et al., 2009). GEF activates Rho GTPases by exchanging GDP for GTP while GAP inhibits these proteins by hydrolyzing GTP to GDP. GDI negatively regulates the Rho family members by binding to a prenylated GDP-bound Rho proteins removing Rho GTPases from the plasma membrane (Buchsbaum, 2007; Kawai, et al., 2009). GEFs seemed to be more important in the context of GTPase regulation. In response to various stimuli, GEFs gets activated and consequently lead to the activation of GTPases which undergo a conformational change enabling them to interact with downstream effectors, producing a biological response. GAPs, to which a "secondary" role has been attributed, can now interfere to complete the cycle (Bernards & Settleman, 2004). The family of RhoGEFs consists of approximately 80 molecules in the human genome. These proteins are a part of either the Dbl family or the Dock family (Salhia, et al., 2008). GEFs are characterized by the presence of DH (Db1 homology) domain, a core catalytic domain stabilizing GTP-free Rho intermediates. Another domain, the PH (pleckstrin homology) domain is found in most DH-containing Rho family GEFs (Buchsbaum, 2007). The catalytic activity of DH domain is affected by the PH domain which can bind to phosphorylated phosphoinositides as well as proteins (Rebecchi, Crivori, Sarra, & Cocconcelli, 1998). The PH domain promotes the localization of GEFs by interacting with PIP3 (Zheng, et

al., 1996). PI3K (phosphatidylinositol 3-kinase) activation leads to the production of PIP3 that binds to the PH domain of GEFs and contributes to their localization (Raftopoulou & Hall, 2004).

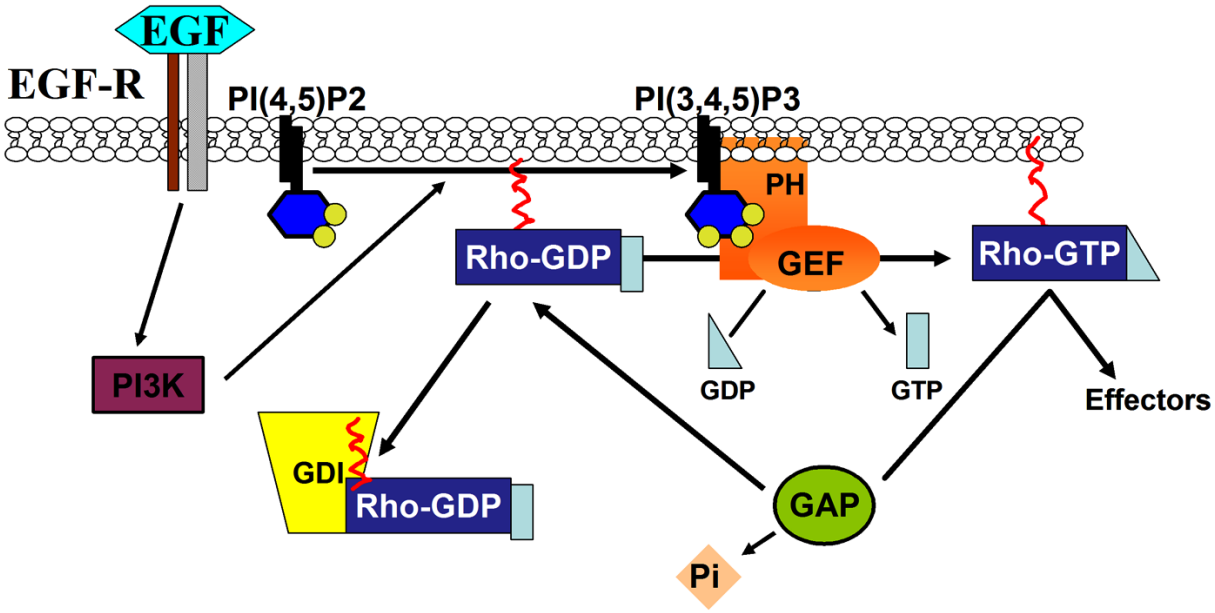


Figure 3: The regulation of Rho GTPases by GAPs, GEFs and GDIs. RhoGEFs activate the RhoGTPases by promoting the GTP nucleotide exchange. GAPs inactivate Rho GTPases by stimulating the intrinsic GTPase activity leading to the GDP-bound inactive form of Rho. Rho GTPases are kept inactive by being sequestered by GDIs in the cytosol. (El-Sibai).

1.4. RhoGAPs:

GAPs belong to a specific family of GTPases that accelerate the rate of GTP hydrolysis by up to 10^5times (Rittinger, et al., 1997). Hence, a tumor suppressor role has been suggested for GAPs counteracting the oncogenic potential of Rho proteins (Yau, et al., 2009). All the members of the RhoGAP family have a conserved RhoGAP

domain consisting of 200 amino acids sequences with more than 20 % of these sequences being identical to other family members (Ahmadian, Wiesmuller, Lautwein, Bischoff, & Wittinghofer, 1996; Moon & Zheng, 2003). Rittinger et al. examined the crystal structures of few RhoGAP domains with their Rho GTPase substrates, revealing the topology and critical residues in the active site which participate in catalysis. Nine helices and a highly conserved arginine residue located in a loop structure constitute the structure of the RhoGAP domain (Gamblin & Smerdon, 1998). This critical arginine residue of the GAP is responsible for the enhancement of GTP hydrolysis by stabilizing the transition state of the substrate binding site of the small GTPase (Moskwa, Paclet, Dagher, & Ligeti, 2005). The C-terminus of RhoGAP includes a GAP domain of more than 140 amino acids (Lamarche & Hall, 1994). Rho/RacGAPs include a wide variety of domains that possibly interact with proteins and lipids in addition to the GAP domain. This interaction can change the activity, the localization, or the substrate specificity of the relevant GAP (Moskwa, et al., 2005). Examples of such domains are: PDZ, SH2, SH3, and SEC14 which are involved in protein-protein interactions, membrane targeting, and cellular localization. RhoGAPs can contain from one to nine domains (Kandpal, 2006). GAPs have captured the attention because about 0.5 % of all predicted human genes encode likely GAPs making them important in GTPase regulation (Bernards & Settleman, 2004). RhoGAPs are overabundant; their number exceeds the number of the Rho GTPases by 2- to 3-fold (Kim, et al., 2009; Lamarche & Hall, 1994; Tcherkezian & Lamarche-Vane, 2007) (Figure 3). Several possible explanations have been proposed to answer this issue including the fact that some GAPs are specific for a single Rho-GTPase or a specific Rho-GTPase pathway, while others are expressed preferentially in certain types of tissues and show tissue-specific functions. Moreover, Rho-GAPs have been shown to act as effectors or scaffold proteins through their GAP domain which functions as a recognition site in this case (Tcherkezian & Lamarche-Vane, 2007). In addition to their role as negative regulators of the Rho GTPases, RhoGAPs are involved in specific biological functions such as exocytosis, endocytosis, cell migration and cytokinesis (Grogg & Zheng 2010). Several mechanisms, including protein–protein interactions, phospholipid interactions,

phosphorylation, subcellular translocation and proteolytic degradation are responsible for the regulation of GAP activity (Bernards & Settleman, 2004).

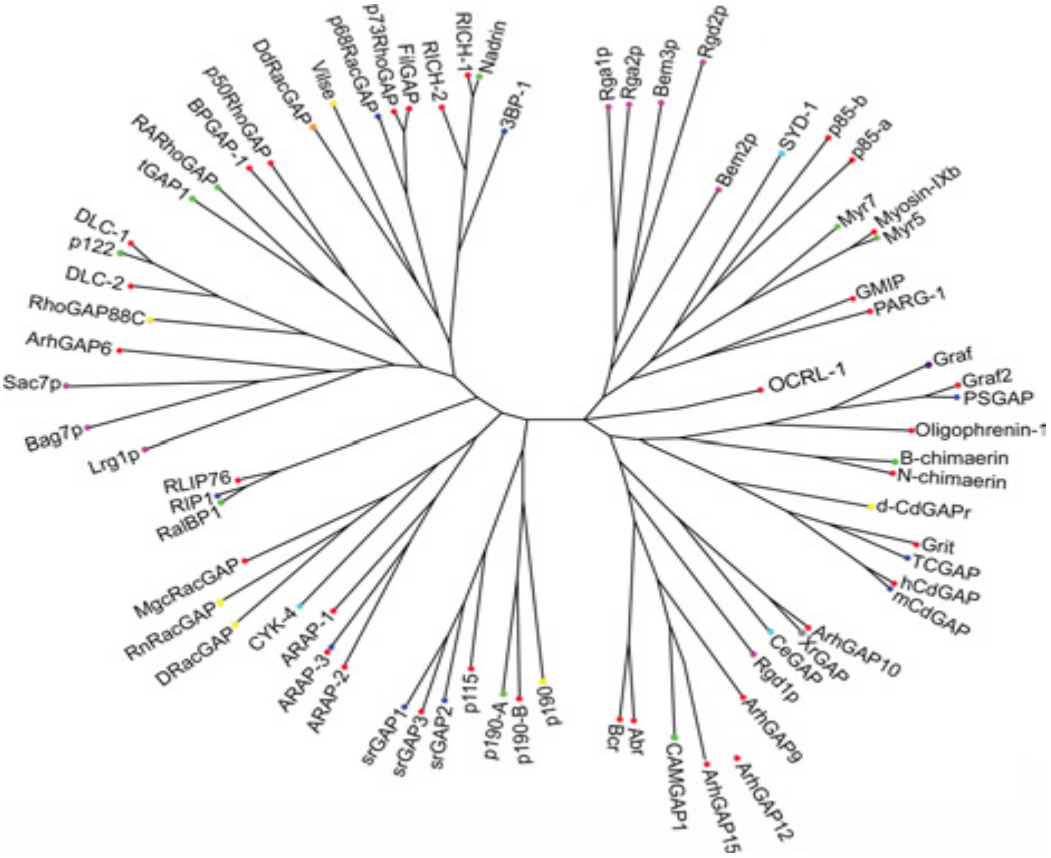


Figure 4: Phylogenic tree of the RhoGAP family from yeast to human. An unrooted tree based on sequence homology of the conserved RhoGAP domain. The RhoGAP domains of 73 characterized GAPs were aligned with the ClustalW program and the phylogenic tree was generated with the Phylodraw program. (Tcherkezian & Lamarche-Vane, 2007).

1.4.1. The DLC family:

Garrett et al. identified the first RhoGAP (Tcherkezian & Lamarche-Vane, 2007). Since that time more than 50 RhoGAPs in the human genome were characterized, three of which contain START domain: DLC1, DLC2 known also as START-GAP2 or StarD13 and DLC3 (Soccio & Breslow, 2003). The three DLC proteins are characterized by the presence of three motifs: a sterile α motif (SAM), a RhoGAP catalytic domain, and a START (Star-related lipid transfer) domain (Qian, et al., 2007). The SAM domain consists of about 70 residues and has been shown to play several roles particularly as protein interaction modules because of its ability to interact with other SAM domains (Li, et al., 2007). SAM domains are located on the N-terminus and may bind to DNA or RNA (Liao & Lo, 2008). DLC2-SAM shows only 15-30% homology with other SAM domains and is considered as the prototype in the family of the DLC2-related proteins (Li, et al., 2007). The structure of DLC2-SAM domain has been revealed by nuclear magnetic resonance (NMR) methods that showed an anti-parallel four-helix bundle (Kwan & Donaldson, 2007). RhoGAP domains switch off Rho GTPases by converting the active GTP-bound Rho proteins to the inactive GDP-bound state (Liao & Lo, 2008). Star-related lipid transfer (START) domains consist of about 210 amino acid lipid binding domains involved in lipid metabolism, lipid transport, and cell signaling events. The DLC family has been established as tumor suppressor genes although little is known about their mechanism of action. The RhoGAP activity is needed for DLC-dependent tumor suppressor activity (Qian, et al., 2007).

1.4.2. StarD13:

When searching for additional candidate tumor suppressor loci critical in hepatocellular carcinoma after the well-known and established tumor suppressor genes *p53, c-myc, p16*^{ink4} and *β-catenin,* Ching et al. identifies a novel gene *DLC2* on chromosome *13q12* which was found to be underexpressed in hepatocellular carcinoma (Ching, et al., 2003; Ng, et al., 2006). *DLC2* which is also known as steroidogenic acute regulatory protein-related lipid transfer (START) domain containing protein 13 (StarD13), display high level of homology with DLC1 (deleted in liver cancer 1), a gene coding for a Rho GTPase activating protein. These two proteins have 51% identity and 64% similarity at the level of their amino acid sequences sharing the same SAM-RhoGAP-START domain organization (Ng, et al., 2006; Ullmannova & Popescu, 2006) (Figure 5A). DLC1 has been described as a tumor suppressor acting as a GTPase activating protein (GAP) for members of the Rho GTPase family, mainly RhoA-C and Cdc42 involved in cell migration and regulation of the cytoskeleton (Kim, et al., 2009). DLC1 is down-regulated in many types of cancers including lung, breast, prostate, kidney, colon, uterus, ovary, and stomach due to two major causes: genomic deletion and promoter hypermethylation. DLC1 is involved in the regulation of actin cytoskeleton, cell shape, attachment, migration, proliferation, focal adhesion formation, cell survival, and induction of apoptosis (Liao & Lo, 2008; Lin, et al., 2010).

Similar to DLC1, DLC2 is down-regulated in several types of cancer including lung, ovarian, renal, breast, uterine, gastric, colon and rectal tumors (Ullmannova & Popescu, 2006). Through its RhoGAP activity, the DLC2 protein acts on RhoA-C and Cdc42 but not on Rac1 (Ching, et al., 2003; Kawai, et al., 2009; Leung, et al., 2005). An established effect of DLC2 GAP domain overexpression is the inhibition of the Rho-mediated formation of actin stress fibers (Ching, et al., 2003). The StarD13 also inhibits Ras signaling and Ras-induced cellular transformation in a GAP-dependent manner (Ching, et al., 2003; Kawai, et al., 2009; Ng, et al., 2006). START-GAP2 is found to be located in focal adhesions. The interaction between FAT (focal adhesion targeting)

domain, which consists of amino acid residues present on the 318–472 position in the N-terminal region, and tensin2, one of focal adhesion components, is responsible for the localization of DLC2 in focal adhesions (Kawai, et al., 2009). Xiaorong et al. reported significant correlations between underexpression of DLC2 and cell differentiation. In addition, a negative correlation was established between DLC2 and RhoA. DLC2 seemed to inhibit hepatocarcinogenesis by suppressing RhoA activity (Xiaorong, Wei, Liyuan, & Kaiyan, 2008). On the other hand; a study conducted by Yau et al. investigated the role of DLC2 by generating DLC2-deficient mice. The mice that were defective in DLC2 were able to survive to adulthood unlike the knockout of DLC1 which led to embryonic lethality. These findings suggest that DLC1 might be able to compensate the functions of DLC2 in embryonic development. The DLC2-deficient mouse has emerged as an important tool to study the function of DLC2 in other types of cancer (Yau, et al., 2009).

In addition to the RhoGAP domain, the START domain plays an important role by targeting the tumor suppressor DLC2 to mitochondria in tight association with lipid droplets, which are defined as a reservoir of lipids implicated in the synthesis and maintenance of membranes. This indicates a possible role of DLC2 in lipid transport and in the regulation of the mitochondrial pathway of apoptosis and mitochondrial membrane permeability (Ng, et al., 2006).

The tumor suppressor StarD13 possesses four isoforms: DLC2α, DLC2β, DLC2γ, and DLC2δ (Figure 5B). DLC2α and DLC2β, containing the full SAM-RhoGAP-START domain organization, showed only differences in few amino acids at the N-terminus. DLC2γ has two domains instead of three: RhoGAP and START domains while DLC2δ has only the SAM domain (Leung, et al., 2005; Li, et al., 2007). The study conducted by Leung et al. characterized two amino acids K618E and R622E that are critical for the RhoGAP activity of DLC2.

Another study conducted by Leung reported that the deleted in liver cancer 2 suppresses cell growth via the regulation of the Raf-1-ERK1/2-p70S6K signalling pathway through its RhoGAP function. ERK1/2 pathway is known to be required for the development of cancer cell characteristics from uncontrolled cell growth to increased cell motility and cell invasion (Leung, et al., 2010).

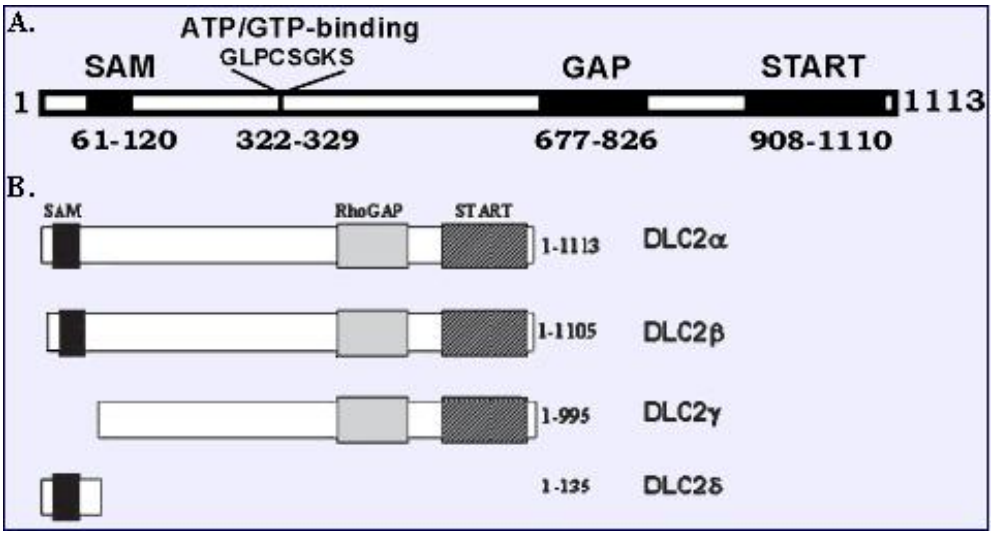


Figure 5: A. The StarD13 protein domains:

a) A sterile α motif (SAM)

b) A RhoGAP catalytic domain

c) A START (Star-related lipid transfer) domain

B. The four isoforms of StarD13 (DLC2α,β,γ,δ).

(Atlas of Genetics and Cytogenetics in Oncology and Haematology)

1.5. The ERK1/2 signalling pathway:

Growth factors are known for their ability to promote cell survival and proliferation (Boucher et al., 2000). Many of these growth factors once they stimulate their receptors, activate extracellular signal-regulated kinases, ERKs, also known as

MAP kinases (De Vries-Smits et al., 1992). MAPK (Mitogen-activated protein kinase) pathways are defined as evolutionarily conserved kinase modules linking extracellular signals to the machinery that controls fundamental cellular processes such as proliferation,growth, differentiation, apoptosis and migration (Dhillon et al., 2007). Among the six distinct groups of MAPKs that have been characterized in mammals; extracellular signal-regulated kinase (ERK)1/2, ERK3/4, ERK5, ERK7/8, Jun N-terminal kinase (JNK)1/2/3 and the p38 isoforms α/β/γ(ERK6)/γ, the ERK pathway, which is deregulated in one-third of all human cancers, is the best studied (Dhillon et al., 2007). ERKs (MAP kinases) get activated by phosphorylation by MAPKKs (MAP kinase kinases) which are activated by MAPKKKs (MAP kinase kinase kinases) (Peyssonnaux & Eychène 2001). Several studies reported the implication of Rho proteins in the regulation of ERK1/2; Rho proteins act by determining the magnitude or duration of ERK1/2 activation in response to RAS activation induced by either growth-factor stimulation or oncogenic mutation (Sahai & Marshall 2002).

1.6. Purpose of our study:

Our laboratory has an interest in brain tumors, particularly astrocytomas. Based on the newly described role of StarD13 as a tumor suppressor for hepatocellular carcinoma, we had an interest to test this role in astrocytomas. For this purpose, two approaches were used: the first one was the overexpression of StarD13 using the GFP-StarD13 construct and the second one, the knockdown of StarD13 using siRNA. Following the altered expression of StarD13, the cell viability was assessed using two different methods: the trypan blue exclusion method and the cell proliferation reagent WST-1. In addition, the effect of StarD13 overexpression and knockdown on cell cycle and apoptosis was determined using flow cytometry and annexin V binding assays. In order to understand the mechanism of action of StarD13 as a tumor suppressor, this study highlighted the link between StarD13 and p-ERK. This will confirm StarD13 as a general potent tumor suppressor and will propose a therapeutic role in many types of

cancers through the overexpression of this tumor suppressor, which would lead to a decrease in malignancy.

Chapter Two

MATERIALS & METHODS

2.1. Cell line and cell culture:

The human glioma cell line T98G was maintained in DMEM containing 10% fetal bovine serum (FBS) and 100U penicillin/streptomycin at 37°C, 5% CO_2 in a humidified chamber.

2.2. Transfection with siRNA:

Cells were transfected with StarD13 siRNA or with control siRNA sequences targeting GL2 luciferase for 72 hours prior to the day of the experiment using Hiperfect (Qiagen) as described by the manufacturer. StarD13 suppression of protein levels was analyzed by Western blotting using antibodies against total StarD13.

2.3. Overexpression with GFP:

Cells were transfected with GFP-StarD13 or with GFP-JB662 (control) for 24 hours prior to the day of the experiment using Lipofectamine (Invitrogen) as described by the manufacturer. StarD13 overexpression was analyzed by Western blotting using antibodies against total StarD13.

2.4. Proliferation:

Depending on the type of experiment, cells were seeded either in 12 well plate or in 96 well plate. After 24 h of seeding, cells were transfected with StarD13 siRNA or overexpressed with GFP-StarD13. At the end of each treatment period, cell viability was determined using two methods:

Trypan blue exclusion method: 10 μL of cells were mixed with 10 μL of trypan blue. Dead cells appear blue, whereas living cells exclude trypan blue and appear bright. The percent viability was determined by visually counting the dark cells compared with the live bright cells.

Cell Proliferation Reagent (WST-1; Roche, Mannheim, Germany). At the end of the treatment period, water-soluble tetrazolim salt (WST-1 was added to the cells and kept in a humidified incubator (37°C) at 95% air and 5% CO2 for 4 hours. WST-1 is a tetrazolium salt that, when in contact with metabolically active cells, gets cleaved to formazan by mitochondrial dehydrogenases. The formazan dye was then measured colorimetrically at 450 nm. The results were expressed as percent of control.

2.5. Pull down assays:

Cells were lysed and incubated with GST-CRIB or GST-RBD and the pull-down assay performed using the RhoA/Rac1/Cdc42 Activation Assay Combo Kit (Cell BioLabs) following the manufacturer's instructions. GTP-RhoA, GTP-Rac1 or GTP-Cdc42 was detected by western blotting using the anti-RhoA, anti-Rac1 or anti-Cdc42 antibodies provided in the kit.

2.6. Flow cytometry:

Cells were seeded in 6 well plate and after 24 hours transfected with StarD13 siRNA or with GFP-Star. Treated cells were placed into 15 mL Falcon tubes and centrifuged at 1500 rpm for 5 minutes. The pellet was then washed by resuspending it in 1 mL of ice-cold 1x phosphate buffered saline (PBS). Cells were centrifuged again under the same conditions as before and resuspended in 1 mL ice cold 1x PBS followed by 4 mL of 70% ethanol and stored at -20°C for a few days. Later, cells were thawed and centrifuged at 1500 rpm for 5 minutes, washed with 1 mL ice-cold 1x PBS, centrifuged again, and then treated with 100 µL of RNase and incubated for an hour at 37°C. The cells were then pelleted at 2000 rpm for 5 minutes, and the pellets were washed with 500 µL of 1x PBS, transferred to labeled 6 mL polystyrene round bottom falcon tube, and stained with 30 µL propidium iodide for 10 minutes in the dark. Cells were analyzed using a FACScan, which indicated the distribution of the cells into their respective cell cycle phases based on their DNA content. G0/G1 cells were 2n; S-phase cells were >2n but <4n while G2/M were 4n. Cell DNA content was determined by CellQuest software. An increase in cells in the pre-G1 phase is indicative of an increase in apoptosis. The ratio of cells in the pre-G1 phase was compared with those of the control.

2.7. Western Blotting:

Protein lysates were prepared by lysing cells in lysis buffer. Protein samples were separated by SDS-PAGE on 8% or 12% gels and transferred to nitrocellulose membranes. After they were blocked with 5% non fat dry milk in PBS containing 0.1% Tween-20 for 1 h at room temperature, the membranes were incubated with primary antibody (Goat polyclonal anti-StarD13 antibody obtained from Santa Cruz). After washing, the membranes were incubated with secondary antibody. Then the membranes

were washed and treated with western blotting chemiluminescent reagent to visualize the bands, the results were obtained on Kodak film.

2.8. Immunohistochemistry:

Frozen human astrocytoma tissues of grades I and IV were sectioned to 8 μm sections using a refrigerated microtome. Tissues were fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.5% Triton-X100 for 10 minutes. To decrease background fluorescence, tissues were rinsed with 0.1M glycine then incubated with 0.1M glycine for 10 minutes. For blocking, tissues were incubated 4 times with 1% BSA, 1% FBS in PBS for 5 minutes. Samples were stained with StarD13 primary antibody for 2 hours and with a fluorophore-conjugated secondary antibody for 2 hours. Tissue fluorescent images were taken using a 10X objective on a confocal microscope. For image analysis, all digital images were imported in image J software (National Institutes of Health, MA). The total fluorescence intensity of a fixed area from at least 10 different frames from each tissue was determined.

2.9. Annexin Staining:

Cells were seeded at a density of 1x 10^6 cells/mL. After 24 h of seeding, cells were transfected with either control siRNA or StarD13 siRNA and incubated for 72h. At the end of the incubation period, cells were trypsinized and centrifuged at 1500 rpm for 5 minutes. The pellet was then washed by resuspending it in 1 mL of ice-phosphate buffered saline (PBS). Cells were centrifuged again under the same conditions as before. Cells were then stained with 5μL of Annexin V FITC and 10 μL of Propidium

Iodide cells and incubated at room temperature for 10 minutes and protected from light. The fluorescence of the cells was determined immediately with a flow cytometer. Cells, which are early in the apoptotic process, will stain with the Annexin V FITC Conjugate alone. Live cells will show no staining by either the Propidium Iodide Solution or Annexin V FITC Conjugate. Necrotic cells will be stained by both the Propidium Iodide Solution and Annexin V FITC Conjugate.

2.10. REMBRANDT database:

To determine the expression of StarD13 in human gliomas, we mined the publicly available *Re*pository for *M*olecular *Bra*in *N*eoplasia *D*ata (REMBRANDT) gene expression microarray database containing 452 clinically annotated brain tumor specimens (National Cancer Institute, 2005; REMBRANDT home page: <http://rembrandt.nci.nih.gov>; accessed December 20, 2010). We specifically examined the gene expression data from nonneoplastic brain (NB, n = 28), low-grade astrocytomas (LGGs, n = 148), and glioblastoma multiformes (GBMs, n = 226).

Chapter Three

RESULTS

3.1. StarD13 is a GAP for RhoA and Cdc42 not for Rac1:

In order to study the role of StarD13 in astrocytoma malignancy, we started by verifying that StarD13 is a GAP for RhoA and Cdc42 and not for Rac1. This was achieved by studying the activation of the three Rho GTPases in T98 cells following the transfection by GFP-StarD13. Using a GST-CRIB pull down assay, we found that the levels of the active RhoA and the active Cdc42 were lower in cells transfected with GFP-StarD13 (Figure 6 A and B) as compared to the controls. On the other hand, the overexpression of StarD13 did not affect the active Rac1 (Figure 6C). This confirmed that StarD13 is a specific GAP for Rho and Cdc42.

3.2. StarD13 is underexpressed in astrocytoma:

Then we wanted to investigate the expression levels of StarD13 in astrocytoma malignancy using immunohistochemistry. Tissues of grade I and IV were stained with anti-StarD13 antibody (Figure 7A) and the intensity of the signal was measured using image J software. Our results showed that there was around 30% increase in the expression of StarD13 in grade IV tumors as compared to grade I (Figure 7B).

3.3. StarD13 overexpression reduces cell viability:

In order to determine the role of StarD13 in cell viability, we transfected T98 cells with GFP-StarD13 and studied the effect of this overexpression on cell viability using two methods: the trypan blue exclusion method and the Cell Proliferation Reagent (WST-1).

The overexpression was apparent looking at the GFP channel and through the effect of over expressing GFP-StaD13 on stress fiber formation (due to Rho inhibition) as revealed by Rhodamine Phalloidin staining (Figure 8D). This is compared to cells transfected with GFP alone (Figure 8D, upper panels) where stress fibers were not affected. The overexpression of StarD13 decreased the percentage of live cells from 97% to 92% (Figure 8A). This was consistent with the results of the WST-1 which showed a decrease of approximately 20% in cell viability (Figure 8C).

3.4. StarD13 knockdown increases cell viability:

To confirm the previous results, we knocked down expression of StarD13 with siRNA. StarD13 expression was reduced by 50% as compared to cells transfected with control siRNA duplexes (Figure 9D). The percentage of live cells in StarD13-siRNA treated cells was increased as compared to control-siRNA treated cells (Figure 9A). These results were consistent with those of the WST-1 which showed an increase of about 30% in cell viability (Figure 9C).

3.5. StarD13 is overexpressed in glioblastoma according to Rembrandt results:

Our immunochemistry results suggested StarD13 might be an oncogene and not a tumor suppressor, as the literature suggested, since StarD13 looked to be overexpressed in grade IV astrocytoma, as compared to grade I. However over expressing and knocking down StarD13 led to a decrease and increase in cell proliferation, respectively, as would be expected of a tumor suppressor. In order to reconcile our results, we mined the Rembrandt (Repository of Molecular Brain Neoplasia Data) database which hosts diverse types of molecular research and clinical trials data related to brain cancers, including gliomas. The results showed StarD13 is underexpressed in tumor tissues as compared to non-tumor tissues. However, if we compare the expression level in GBM (glioblastoma) which is grade IV astrocytoma to lower grade tumors, we find that the levels of StarD13 mRNA in grade IV is higher (Figure 10). This was consistent with our IHC results.

3.6. StarD13 does not affect the apoptosis:

The effect of StarD13 on cell viability could be through inducing apoptosis. To study the effect of StarD13 on apoptosis, cells were stained with Annexin V FITC. The fluorescence was immediately measured by a flow cytometry. Cells, which are early in the apoptotic process, will stain with the Annexin V FITC Conjugate alone. Live cells will show no staining by either the Propidium Iodide solution or Annexin V FITC conjugate. Necrotic cells will be stained by both the Propidium Iodide solution and Annexin V FITC conjugate. Our results showed that the knockdown of StarD13 did not affect the apoptosis. The percentage of apoptotic cells was not significantly different between the controls and the transfected cells (Figure 11 and Table).

3.7. StarD13 affects the cell cycle:

In order to understand the mechanism through which StarD13 is affecting cell viability, we went on analyzing the effect of StarD13 knockdown on the cell cycle. This was achieved using flow cytometry. Our results showed an increase in the percentage of cells in the S/G2 phase as compared to the cells in the G0 phase (Figure 12). This showed that knocking down StarD13 led to an increase in cycling cells, meaning StarD13 plays the role of a tumor suppressor inhibiting the cell cycle.

3.8. ERK is downstream effector from StarD13:

In order to determine the mechanism of action of StarD13, we knocked it down and looked at its effect on p-ERK, the extracellular signal-regulated kinase. The western blot showed an increase in pERK levels in siRNA-StarD13 transfected cells as compared to control non transfected cells (Figure 13).

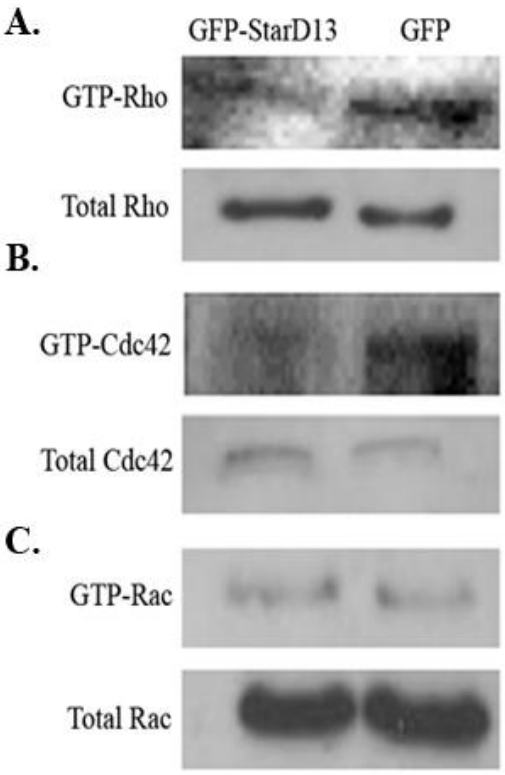


Figure 6. StardD13 is a specific GAP for Rho and Cdc42. T98G cells were transfected with either GFP alone (right lanes) or with GFP-StarD13 (left lanes). The cells were then lyzed and incubated with **A**) GST-RBD (Rhotekin binding domain), or with **B**) GST-CRIB (Cdc42 and Rac interactive binding domain) to pull down active Rho or Cdc42 and Rac, respectively. The samples were then blotted with Rho, Cdc42 and Rac antibodies. The lower gels in each panel are western blots for the total cell lysates for loading control.

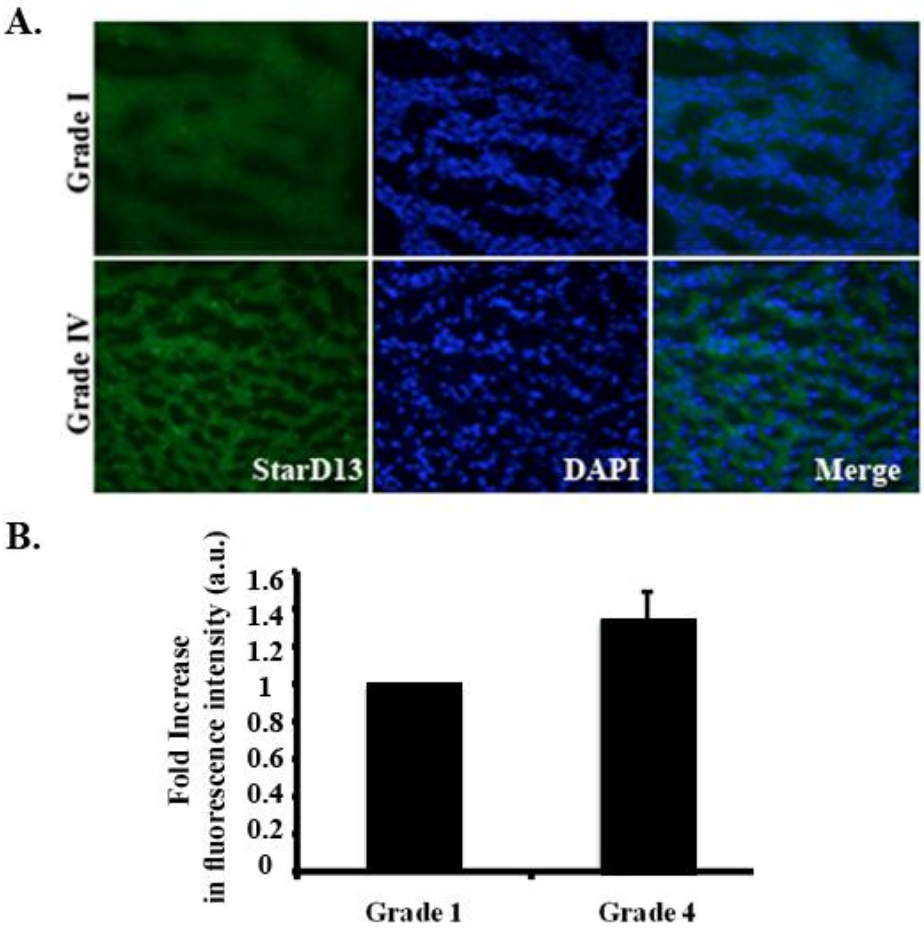


Figure 7: StarD13 is overexpressed in Grade IV astrocytoma compared to grade I. Immunohistochemistry for tumor tissues of grades I, and IV that were obtained from patients diagnosed with glioblastoma. **A**) representative micrographs of tissues that were stained with DAPI (middle panels) or with anti-StarD13 (left panels). **B**) The intensity of the signal was measured using ImageJ software and expressed as fold increase to grade I. the data is the mean -/+ SEM.

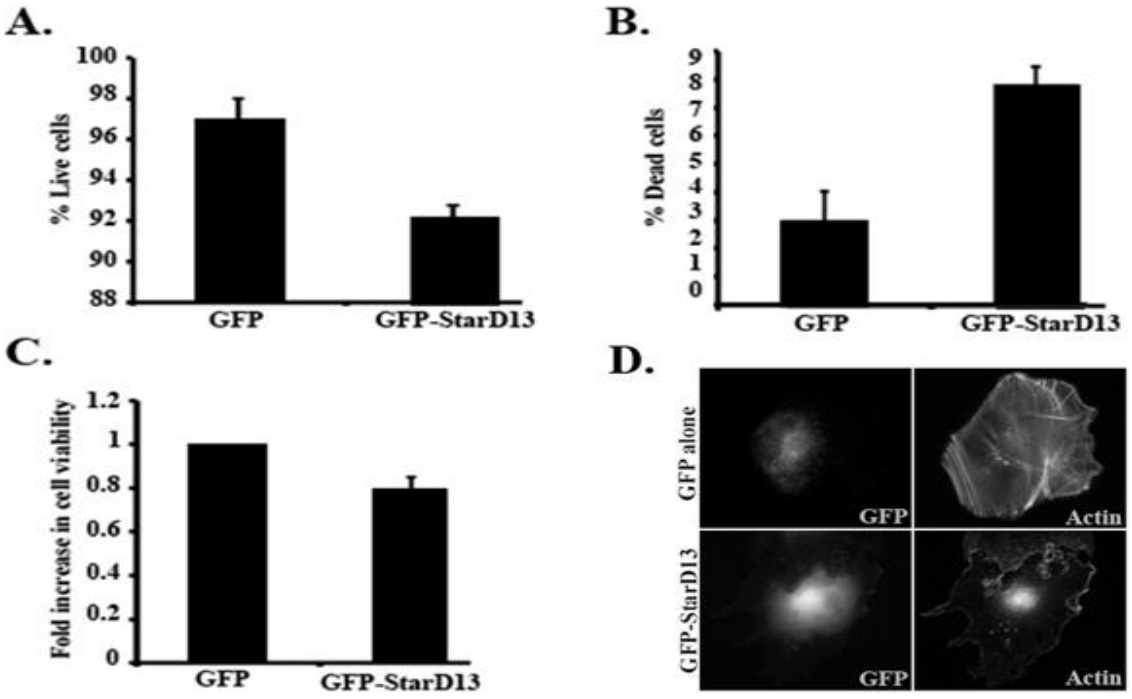


Figure 8: StarD13 overexpression decreased cell viability. Cells were either transfected with GFP alone or with GFP-StarD13. Cell viability was then determined using the Trypan blue exclusion method (A and B) or the WST-1 cell proliferation reagent (C). **A and B)** The results were expressed as percent of total number of cells. **C)** Results were expressed as fold increase from control (GFP alone). Data is the mean -/+ SEM. D) Cell were transfected with GFP alone (upper panel) or GFP-StarD13 (lower panel) and stained with Rhodamine Phalloidin.

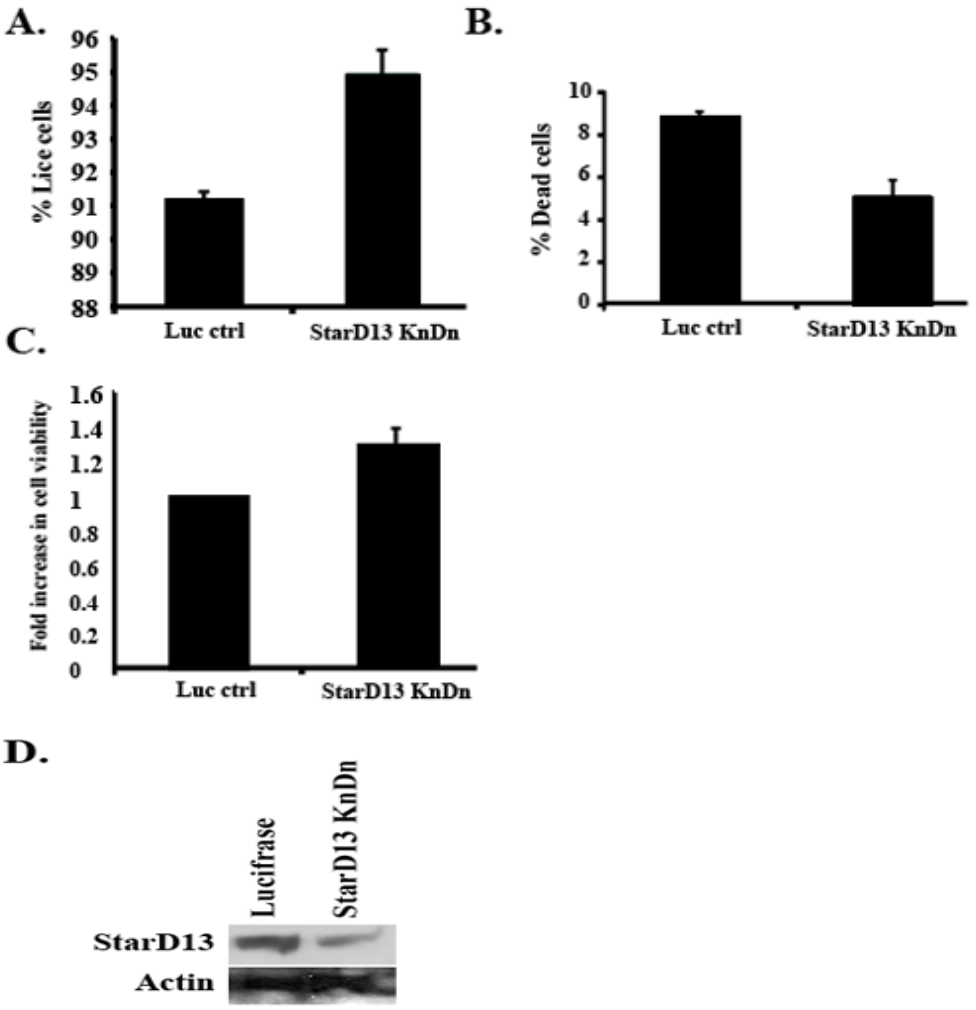


Figure 9: StarD13 underexpression increases cell viability. Cells were either transfected with Luciferase control siRNA or with StarD13 siRNA. Cell viability was then determined using the Trypan blue exclusion method A) and B) or the WST-1 cell proliferation reagent (C). **A and B)** The results were expressed as percent of total number of cells. **C)** Results were expressed as fold increase from control (luciferase). Data is the mean -/+ SEM. **D)** western blot showing the decrease in StarD13 expression compared to actin, lower panel) in cells transfected with StarD13 siRNA compared to luciferase control (left lane).

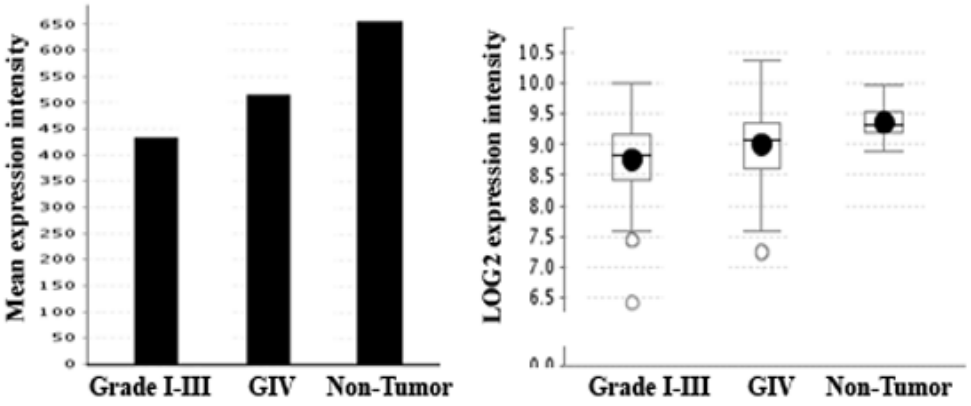
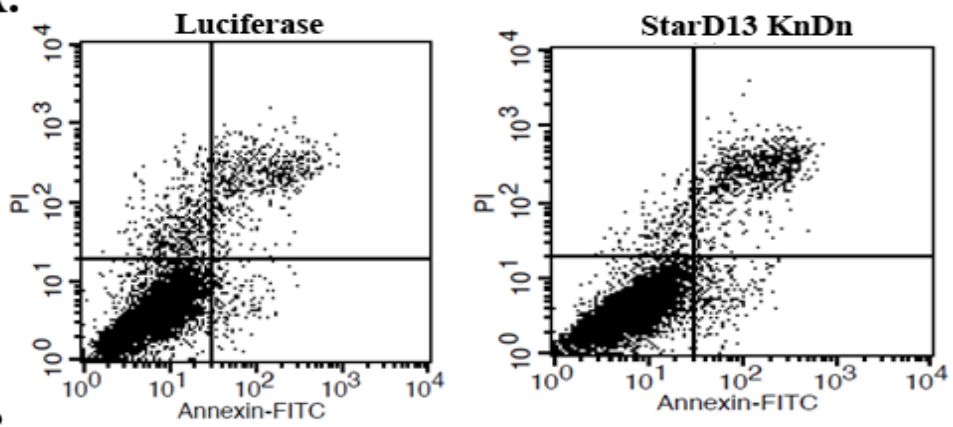


Figure 10. StarD13 is underexpressed in glioma as compared to normal tissues. Data analyzed from Rembrandt website. mRNA from 28 non-tumor patient tissues, 148 astrocytoma tissues (grade I-III) and 226 grade IV astrocytomas were quantified for expression levels.

A.



B.

	Luciferase control	StarD13 KnDn
Dead cells	2.265	1.23
Necrotic cells	3.58	5.72
Apoptotic cells	1.21	2.42
Live cells	92.945	90.63

Figure 11: StarD13 had no significant effect on apoptosis. Cells were transfected with either control luciferase siRNA or with StarD13 siRNA. The cells were then trypsinized and stained with 5µL of Annexin V FITC and 10 µL of Propidium Iodide. **A)** The fluorescence of the cells was determined immediately with a flow cytometer. Cells, which are early in the apoptotic process, will stain with the Annexin V FITC Conjugate alone. Live cells will show no staining by either the Propidium Iodide solution or Annexin V FITC conjugate. Necrotic cells will be stained by both the Propidium Iodide solution and Annexin V FITC conjugate. **B)** Table summarizing the results shown in A.

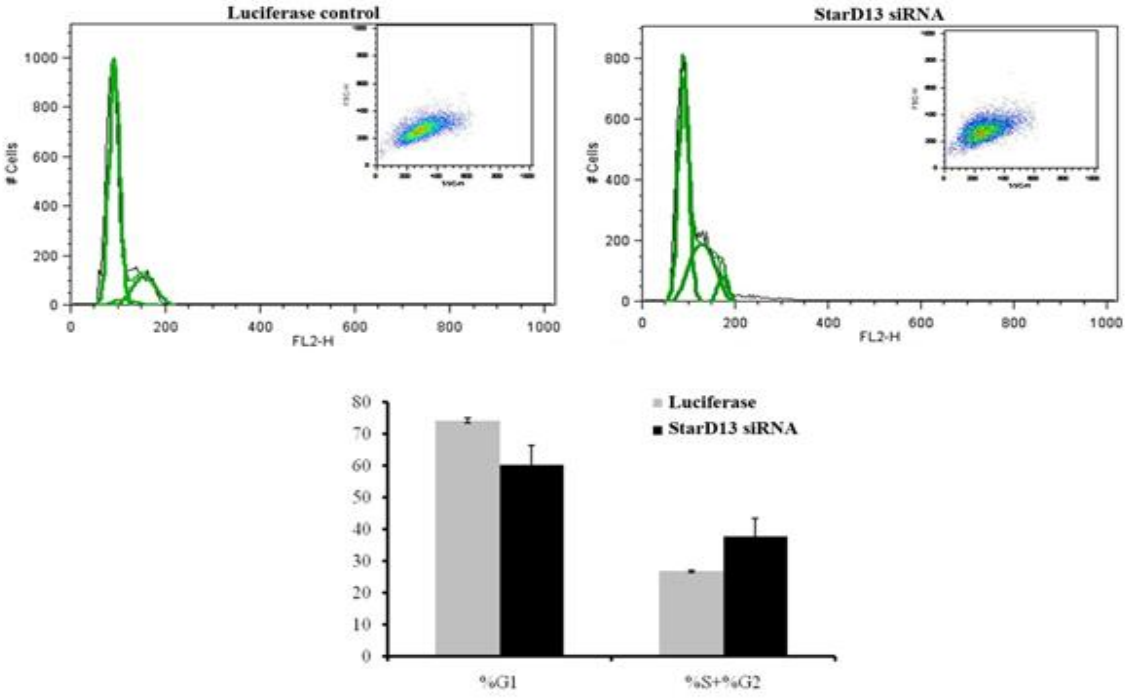


Figure 12: StarD13 represses the cell cycle. Cells were transfected with luciferase or StarD13 siRNA and stained with 30 μL propidium iodide for 10 minutes. Cells were analyzed using a FACScan, which indicated the distribution of the cells into their respective cell cycle phases based on their DNA content. G0/G1 cells were 2n; S-phase cells were >2n but <4n while G2/M were 4n. Cell DNA content was determined by CellQuest software.

Figure 13: StarD13 affects the cell cycle through an effect on ERK. Cells were transfected with luciferase (left lane) or StarD13 (right lane) siRNA, lyzed and blotted for p-ERK (upper gel) or actin for loading control (lower gel).

Chapter Four

DISCUSSION

In this study, we examined the role of StarD13 in astrocytoma malignancy. We confirmed that StarD13 is a specific GAP for Rho and Cdc42. IHC analysis showed that StarD13 is overexpressed in grade IV astrocytoma compared to grade I. Mining online databases explained this observation by StarD13 being indeed overexpressed in grade IV astrocytoma as compared to grade I, however StarD13 is underexpressed in astrocytoma (grade I and IV) as compared to normal tissues. This confirmed StarD13 as a potential tumor suppressor in astrocytoma. In order to directly prove that, we overexpressed or knocked down StarD13 and looked at the effect of cell viability, apoptosis and proliferation in a cell culture model. In astrocytoma cell lines, overexpressing StarD13 led to no effect on apoptosis but to a decrease in cell viability and cell proliferation as reflected by a decrease in cells in S and G2 phase. Knocking down StarD13 with StarD13 siRNA led to no effect on cell apoptosis but to an increase in cell viability and cell proliferation. Knocking down StarD13 also showed an increase n phosphorylated ERK.

Ching et al. was the first to identify and characterize StarD13 in hepatocellular carcinoma (HCC). We wanted to determine the role of this RhoGAP in another tumor model which is astrocytoma. Ching et al. reported the function of StarD13 as a GAP for RhoA and Cdc42 not for Rac1. Our results demonstrated that cells overexpressing StarD13 possessed reduced levels of RhoA and Cdc42 activation; however, the activation of Rac1 was not affected. Therefore, similar to its role in HCC, StarD13 has a RhoGAP activity for RhoA and Cdc42 in astrocytoma.

The IHC analysis on Grade I-IV brain tissues from patients showed StarD13 to be overexpressed in grade III and IV astrocytoma tumors when compared to grade I and II.

This was contradictory to other studies where the StarD13 gene was found to be down-regulated in several types of cancer including lung, ovarian, renal, breast, uterine, gastric, colon and rectal tumors (Ullmannova & Popescu, 2006). These results led us to formulate the following hypothesis: Contrary to other tumor models, in brain astrocytomas, StarD13 seems to be an oncogene and not a tumor suppressor.

To verify this hypothesis, we wanted to determine the effect of StarD13 altered expression on cell viability. The overexpression of GFP-StarD13 construct in astrocytoma T98 cells led to the increase in cell death and a decrease of cell viability. This was verified using two methods: counting with trypan blue and adding the water soluble tetrazolium salt WST-1. On the other hand, knocking down StarD13 using siRNA led to a decrease in cell death and an increase in cell viability. These data established the growth suppressor role of StarD13 in astrocytoma. This was consistent with previous results which reported the role of StarD13 as a tumor suppressor in several types of cancer. However, these data refuted our hypothesis and did not explain our IHC results. StarD13 seems to be a tumor suppressor consistently with the literature.

To answer this issue, we mined the REMBRANDT data; we found that the mRNA levels of StarD13 are indeed higher in the higher grades but much lower than the normal tissues. Hence, our IHC results were consistent with the REMBRANDT results.

It would remain of interest to establish the significance of the overexpression of StarD13 as the malignancy of astrocytoma increases. Another study in our lab showed that StarD13 is needed for astrocytoma cells to undergo motility. This might explain why StarD13 is overexpressed in grade IV astrocytoma compared to grade I.

The effect of cell viability could be either due to decreased proliferation or increased apoptosis. To test which underlying mechanism was responsible for the tumor suppressor function of StarD13, we studied the effect of StarD13 knockdown on cell

cycle using flow cytometry. In StarD13 siRNA transfected cells, the percentage of cells at the S/G2 phase was higher than that of the control cells. And the percentage of cells at the G1 phase was lower. These results indicated that the deleted in liver cancer 2 blocks the cells in the G1 phase inhibiting the cell cycle progression. This was consistent with a study conducted by Leung et al. which demonstrated that a stable expression of StarD13 caused accumulation of cells in the G1 phase leading to the inhibition of cell growth.

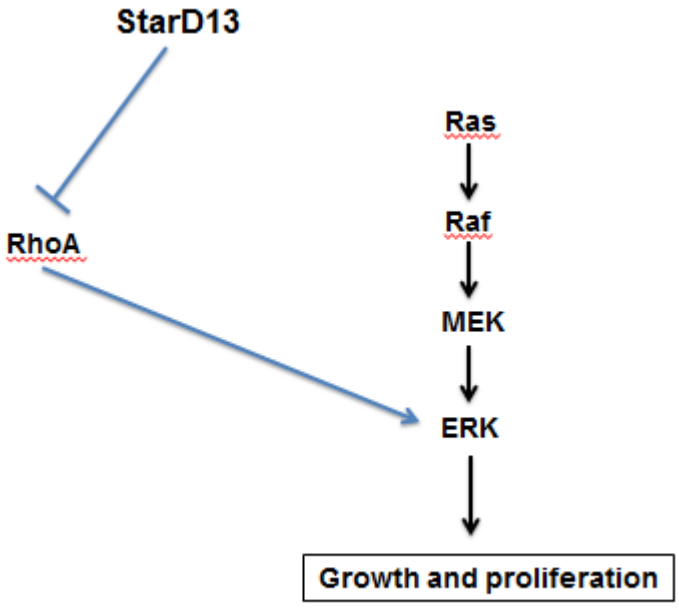
In addition, our results showed that StarD13 did not induce apoptosis. This was reflected by the percentage of apoptotic cells which was approximately similar in the StarD13 siRNA transfected cells and the control cells.

To examine the underlying mechanism behind the tumor suppressor role of StarD13, we have investigated the level of ERK phosphorylation after the silencing of StarD13. The results showed an increase in the level of p-ERK in the cells transfected with StarD13 siRNA as compared to non transfected cells. These findings were in accordance with the study conducted by Leung which showed that StarD13 suppresses cell growth via the regulation of the Raf1-ERK1/2-p70S6K signaling pathway. Since p-ERK, the extracellular signal-regulated kinase, is involved in the regulation of cellular growth and proliferation of several tumor types (Zheng et al. 2003), we suggested that maybe our StarD13 is affecting the cell growth of tumor cells via p-ERK pathway. This is quite interesting since it directly link Rho or Cdc42 to the inhibition of a MAPK pathway.

Chapter 5

CONCLUSION

In this study, we describe for the first time the tumor suppressor effect of StarD13 in astrocytoma. StarD13 has increased cell death and decreased cell viability. When looking at the mechanism, the tumor suppressor effect of StarD13 was found to be through the inhibition of the cell cycle and not through the activation of apoptosis .This protein is underexpressed in tumors but, very interestingly, its expression level increases as the tumors gain more malignancy. This phenomenon requires further investigation. We also revealed that the tumor suppressor effect of StarD13 in astrocytoma is through the inhibition of the ERK pathway. Future interest would be to elucidate the link between this Rho GAP and the MAPK pathway.



Chapter Six

BIBLIOGRAPHY

Abecassis, I., Olofsson, B., Schmid, M., Zalcman, G., & Karniguian, A. (2003). RhoA induces MMP-9 expression at CD44 lamellipodial focal complexes and promotes HMEC-1 cell invasion. *Exp Cell Res, 291(2),* 363-376.

Adamson, P., Paterson, H. F., & Hall, A. (1992). Intracellular localization of the P21rho proteins. *J Cell Biol, 119(3),* 617-627.

Ahmadian, M. R., Wiesmuller, L., Lautwein, A., Bischoff, F. R., & Wittinghofer, A. (1996). Structural differences in the minimal catalytic domains of the GTPase-activating proteins p120GAP and neurofibromin. *J Biol Chem, 271(27),* 16409-16415.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2008). *Molecular Biology of the Cell* (4th ed). New York: Garland Science.

Asa, S. L., & Ezzat, S. (1998). The cytogenesis and pathogenesis of pituitary adenomas. *Endocr Rev, 19(6),* 798-827.

Bernards, A., & Settleman, J. (2004). GAP control: Regulating the regulators of small GTPases. *Trends Cell Biol, 14(7),* 377-385.

Boettner, B., & Van Aelst, L. (2002). The role of Rho GTPases in disease development. *Gene, 286(2),* 155-174.

Boucher, M.J., Morisset, J., Vachon, P., Reed, J., Laine, J., & Rivard, N. (2000). MEK/ERK Signaling Pathway Regulates the Expression of Bcl-2, Bcl-XL, and Mcl-1 and Promotes Survival of Human Pancreatic Cancer Cells. *J. Cell. Biochem. 79,* 355–369.

Boureux, A., Vignal, E., Faure, S., & Fort, P. (2007). Evolution of the Rho Family of Ras-Like GTPases in Eukaryotes. *Mol Biol Evol, 24 (1),* 203-216.

Brandes, A. A., Paris, M. K., & Basso, U. (2003). Medulloblastomas: Do molecular and biologic markers indicate different prognoses and treatments? *Expert Rev Anticancer Ther, 3(5),* 615-620.

Bredel, M., Scholtens, D. M., Yadav, A. K., Alvarez, A. A., Renfrow, J. J., Chandler, J. P., …Harsh, G.R. (2011). NFKBIA deletion in glioblastomas. *N Engl J Med, 364(7),* 627-637.

Buchsbaum, R. J. (2007). Rho activation at a glance. *J Cell Sci, 120(7),* 1149-1152.

Buckner, J. C., Brown, P. D., O'Neill, B. P., Meyer, F. B., Wetmore, C. J., & Uhm, J. H. (2007). Central nervous system tumors. *Mayo Clin Proc, 82(10),* 1271-1286.

Burbelo, P. D., Drechsel, D., & Hall, A. (1995). A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J Biol Chem, 270(49),* 29071-29074.

Chandana, S. R., Movva, S., Arora, M., & Singh, T. (2008). Primary brain tumors in adults. *Am Fam Physician, 77(10),* 1423-1430.

Ching, Y. P., Wong, C. M., Chan, S. F., Leung, T. H., Ng, D. C., Jin, D. Y., & Ng, I.O. (2003). Deleted in liver cancer (DLC) 2 encodes a RhoGAP protein with growth suppressor function and is underexpressed in hepatocellular carcinoma. *J Biol Chem, 278(12),* 10824-10830.

Cornu, P., Dormont, D., Marsault, C., & Philippon, J. (1996). Surgery of brain tumors. *Rev Prat, 46(4),* 432-438.

DeAngelis, L. M. (2001). Brain tumors. *N Engl J Med, 344(2),* 114-123.

De Vries-Smits, A., Burgering, B., Leevers, S., Marshall, C., & Bos, J. (1992). Involvement of p21^{ras} in activation of extracellular signal-regulated kinase 2. *Nature, 357,* 602 - 604.

Dhillon, AS., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene, 26,* 3279–3290.

Franceschi, E., Tosoni, A., Paioli, A., Cavallo, G., Spagnolli, F., & Brandes, A. A. (2007). Challenges and progress in the treatment of adult medulloblastomas. *Future Oncol, 3(2),* 115-117.

Fritz, G., Gnad, R., & Kaina, B. (1999). Cell and tissue-type specific expression of Ras-related GTPase RhoB. *Anticancer Res, 19(3),* 1681-1688.

Gamblin, S. J., & Smerdon, S. J. (1998). GTPase-activating proteins and their complexes. *Curr Opin Struct Biol, 8(2),* 195-201.

Grogg, M.W., & Zheng Y. (2010). *Rho GTPase-Activating Proteins in Cancer*. Retrieved from http://www.springerlink.com/content/?k=The+Rho+GTPases+in+Cancer

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100(1),* 57-70.

Hornstein, I., Pikarsky, E., Groysman, M., Amir, G., Peylan-Ramu, N., & Katzav, S. (2003). The haematopoietic specific signal transducer Vav1 is expressed in a subset of human neuroblastomas. *J Pathol, 199(4),* 526-533.

Hwang, S. L., Hong, Y. R., Sy, W. D., Lieu, A. S., Lin, C. L., Lee, K. S., & Howng, S.L. (2004). Rac1 gene mutations in human brain tumours. *Eur J Surg Oncol, 30(1),* 68-72.

Johnson, D. I. (1999). Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiol Mol Biol Rev, 63(1),* 54-105.

Kandpal, R. P. (2006). Rho GTPase activating proteins in cancer phenotypes. *Curr Protein Pept Sci, 7(4),* 355-365.

Karlsson, R., Pedersen, E. D., Wang, Z., & Brakebusch, C. (2009). Rho GTPase function in tumorigenesis. *Biochim Biophys Acta, 1796(2),* 91-98.

Kawai, K., Seike, J., Iino, T., Kiyota, M., Iwamae, Y., Nishitani, H., & Yagisawa, H. (2009). START-GAP2/DLC2 is localized in focal adhesions via its N-terminal region. *Biochem Biophys Res Commun, 380(4),* 736-741.

Kim, T. Y., Vigil, D., Der, C. J., & Juliano, R. L. (2009). Role of DLC-1, a tumor suppressor protein with RhoGAP activity, in regulation of the cytoskeleton and cell motility. *Cancer Metastasis Rev, 28,* 77-83.

Kwan, J. J., & Donaldson, L. W. (2007). The NMR structure of the murine DLC2 SAM domain reveals a variant fold that is similar to a four-helix bundle. *BMC Struct Biol, 7,* 34.

Lamarche, N., & Hall, A. (1994). GAPs for rho-related GTPases. *Trends Genet, 10(12),* 436-440.

Leung, T. H., Ching, Y. P., Yam, J. W., Wong, C. M., Yau, T. O., Jin, D. Y., & Ng, I.O. (2005). Deleted in liver cancer 2 (DLC2) suppresses cell transformation by means of inhibition of RhoA activity. *Proc Natl Acad Sci U S A, 102(42),* 15207-15212.

Leung, T.H., Yam, J.W., Chan, L.K., Ching, Y.P., & Ng, I.O. (2010). Deleted in liver cancer 2 suppresses cell growth via the regulation of the Raf-1–ERK1/2–p70S6K signalling pathway. *Liver International, 30,* 1315-1323.

Li, H., Fung, K. L., Jin, D. Y., Chung, S. S., Ching, Y. P., Ng, I. O., …Sun, H. (2007). Solution structures, dynamics, and lipid-binding of the sterile alpha-motif domain of the deleted in liver cancer 2. *Proteins, 67(4),* 1154-1166.

Liao, Y. C., & Lo, S. H. (2008). Deleted in liver cancer-1 (DLC-1): A tumor suppressor not just for liver. *Int J Biochem Cell Biol, 40(5),* 843-847.

Lin, Y., Chen, N. T., Shih, Y. P., Liao, Y. C., Xue, L., & Lo, S. H. (2010). DLC2 modulates angiogenic responses in vascular endothelial cells by regulating cell attachment and migration. *Oncogene, 29(20),* 3010-3016.

Louis, D. N., Ohgaki, H., Wiestler, O. D., & Cavenee, W. K. (2007). *WHO Classification of Tumours of the Central Nervous System* (4th ed.). United Kingdom: World Health Organization

Malliri, A., & Collard, J. G. (2003). Role of Rho-family proteins in cell adhesion and cancer. *Curr Opin Cell Biol, 15(5),* 583-589.

Moon, S. Y., & Zheng, Y. (2003). Rho GTPase-activating proteins in cell regulation. *Trends Cell Biol, 13(1),* 13-22.

Moskwa, P., Paclet, M. H., Dagher, M. C., & Ligeti, E. (2005). Autoinhibition of p50 Rho GTPase-activating protein (GAP) is released by prenylated small GTPases. *J Biol Chem, 280(8),* 6716-6720.

Nag, S. (2011). Morphology and properties of astrocytes. *Methods Mol Biol, 686,* 69-100.

Ng, D. C., Chan, S. F., Kok, K. H., Yam, J. W., Ching, Y. P., Ng, I. O., & Jin, D.Y. (2006). Mitochondrial targeting of growth suppressor protein DLC2 through the START domain. *FEBS Lett, 580(1),* 191-198.

Peyssonnaux, C., & Eychène, A. (2001). The Raf/MEK/ERK pathway: New concepts of activation. *Biology of the Cell, 93,* 53−62.

Preston-Martin, S. (1996). Epidemiology of primary CNS neoplasms. *Neurol Clin, 14(2),* 273-290.

Qian, X., Li, G., Asmussen, H. K., Asnaghi, L., Vass, W. C., Braverman, R., …Lowy, D.R. (2007). Oncogenic inhibition by a deleted in liver cancer gene requires cooperation between tensin binding and Rho-specific GTPase-activating protein activities. *Proc Natl Acad Sci U S A, 104(21),* 9012-9017.

Raftopoulou, M., & Hall, A. (2004). Cell migration: Rho GTPases lead the way. *Dev Biol, 265(1),* 23-32.

Rebecchi, A., Crivori, S., Sarra, P. G., & Cocconcelli, P. S. (1998). Physiological and molecular techniques for the study of bacterial community development in sausage fermentation. *J Appl Microbiol, 84(6),* 1043-1049.

Ridley, A. J. (2001). Rho GTPases and cell migration. *J Cell Sci, 114(15),* 2713-2722.

Ridley, A. J. (2004). Rho proteins and cancer. *Breast Cancer Res Treat, 84(1),* 13-19.

Rittinger, K., Walker, P. A., Eccleston, J. F., Nurmahomed, K., Owen, D., Laue, E., …Smerdon, S.J. (1997). Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP. *Nature, 388(6643),* 693-697.

Sahai, E., & Marshall, C. (2002). RHO–GTPases and cancer. *Nature Reviews Cancer, 2,* 133-142.

Salhia, B., Tran, N. L., Chan, A., Wolf, A., Nakada, M., Rutka, F., …Rutka, J.T. (2008). The guanine nucleotide exchange factors trio, Ect2, and Vav3 mediate the invasive behavior of glioblastoma. *Am J Pathol, 173(6),* 1828-1838.

Soccio, R. E., & Breslow, J. L. (2003). StAR-related lipid transfer (START) proteins: mediators of intracellular lipid metabolism. *J Biol Chem, 278(25),* 22183-22186.

Stewart, L. A. (2002). Chemotherapy in adult high-grade glioma: A systematic review and meta-analysis of individual patient data from 12 randomised trials. *Lancet, 359(9311),* 1011-1018.

Tcherkezian, J., & Lamarche-Vane, N. (2007). Current knowledge of the large RhoGAP family of proteins. *Biol Cell, 99(2),* 67-86.

Ullmannova, V., & Popescu, N. C. (2006). Expression profile of the tumor suppressor genes DLC-1 and DLC-2 in solid tumors. *Int J Oncol, 29(5),* 1127-1132.

Wang, W., Wyckoff, J. B., Frohlich, V. C., Oleynikov, Y., Huttelmaier, S., Zavadil, J., …Condeelis, J.S. (2002). Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. *Cancer Res, 62(21),* 6278-6288.

Watanabe, K., Tachibana, O., Sata, K., Yonekawa, Y., Kleihues, P., & Ohgaki, H. (1996). Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. *Brain Pathol, 6(3),* 217-223; discussion 223-214.

Wen, P. Y., & Kesari, S. (2008). Malignant gliomas in adults. *N Engl J Med, 359(5),* 492-507.

Wiemels, J., Wrensch, M., & Claus, E. B. (2010). Epidemiology and etiology of meningioma. *J Neurooncol, 99(3),* 307-314.

Wrensch, M., Minn, Y., Chew, T., Bondy, M., & Berger, M. S. (2002). Epidemiology of primary brain tumors: current concepts and review of the literature. *Neuro Oncol, 4(4),* 278-299.

Xiaorong, L., Wei, W., Liyuan, Q., & Kaiyan, Y. (2008). Underexpression of deleted in liver cancer 2 (DLC2) is associated with overexpression of RhoA and poor prognosis in hepatocellular carcinoma. *BMC Cancer, 8,* 205.

Yau, T. O., Leung, T. H., Lam, S., Cheung, O. F., Tung, E. K., Khong, P. L., …Ng, I.O. (2009). Deleted in liver cancer 2 (DLC2) was dispensable for development and its deficiency did not aggravate hepatocarcinogenesis. *PLoS One, 4(8),* e6566.

Zheng, J., Cahill, S. M., Lemmon, M. A., Fushman, D., Schlessinger, J., & Cowburn, D. (1996). Identification of the binding site for acidic phospholipids on the pH domain of dynamin: implications for stimulation of GTPase activity. *J Mol Biol, 255(1),* 14-21.

Ziman, M., Preuss, D., Mulholland, J., O'Brien, J. M., Botstein, D., & Johnson, D. I. (1993). Subcellular localization of Cdc42p, a Saccharomyces cerevisiae GTP-binding protein involved in the control of cell polarity. *Mol Biol Cell, 4(12),* 1307-1316.